Chemical ecology of azoxyglycosides: Plant-insect interactions between Neotropical cycads (Zamiaceae) and leaf herbivores.

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Abstract

Cycad plants protect all their tissues with a group of toxic metabolites, called azoxyglycosides (AZGs). Thus, only specialist herbivores can feed on the cycad foliage. We describe phenological changes in leaf structure and toxicity in the Panamanian cycad *Zamia stevensonii* which correlate with patterns of activity and herbivory by specialist insects. More than half of *Z. stevensonii* individuals produced a synchronous flush of new leaves during the first two months of the wet season. Leaf development is characterized by quick leaf expansion and delayed greening. Youngest leaves are densely covered with trichomes and have the highest AZG concentrations. These defenses rapidly decrease as leaves expand. Leaves older than 100 days are effectively protected by toughness. A vulnerable period exists as AZG concentrations drop and before leaves have completely toughened during which leaves are susceptible to herbivory by specialist insects. Damage by specialist herbivores, *Eumaeus godartii* butterflies and *Aulacoscelis appendiculata* beetles, closely follow the leaf flush and account for the loss of approximately 37% of all leaflets produced. Adult beetles are mobile and, despite having a wide range of leaf ages to choose from, they only feed on leaves between 10 and 100 days old, mainly on leaves between 20 and 60 days old. The defensive strategy of cycads parallels that of other plant lineages in exhibiting a delayed greening and a switch from chemical to mechanical protection.

These observations beg the question of whether intense herbivory induces and/or selects for higher AZG levels. To address this question, leaf AZG content was measured in damaged and undamaged leaves of two-year-old *Zamia furfuracea* plants. AZG levels remain stable both in damaged and systemic leaves 3, 7 and 15 days after wounding. These results support other lines of evidence indicating that AZGs are allocated to the leaf primordia and not replenished during the life of a leaf. However, it remains unknown if there is a delayed induction in AZGs that would be perceivable in the subsequent leaf flush.

To better understand the defensive strategy of cycads, AZG levels were analyzed in different ontogenetic stages of distinct *Dioon edule* populations in

Mexico. Individuals were divided into three ontogenetic stages: seedlings, juveniles and adults. The sex of adult individuals was determined based on the presence of reproductive structures. The genetic structure of these populations was assessed using 14 microsatellite markers. Plant genotype and individual heterozygocity do not correlate with AZG levels. AZG levels in fully developed leaves decrease with plant age with highest AZG concentrations found in seedlings, followed by juveniles and the lowest concentrations in adults. Our results suggest that plant priorities change during ontogeny with older plants investing less in chemical defense. As well, adult female plants have higher AZG levels than males, suggesting that the cycad defensive strategy could be linked to the costs of reproduction. Male individuals, that produce new leaves more often than females, may be able to withstand higher herbivory levels than females and, hence, invest less in defense.

The brilliant red aposematic beetles in the subfamily Aulacoscelidinae have an intimate association with Neotropical Zamiaceae. By feeding on the cycad foliage, these beetles sequester AZGs. When perturbed, they reflexively bleed from the joint of the tibia and the femur. Cycasin and macrozamin AZGs, were identified by NMR and accurate mass spectra in these reflexive secretions. We also propose that the larvae of some aulacoscelidine species develop inside cycad seeds.

Résumé

Les cycadées protègent leurs tissus avec un groupe de métabolites toxiques, appelés les azoxyglycosides (AZGs). Les herbivores spécialisés sont les seuls à pouvoir se nourrir de leur feuillage. Nous allons tout d'abord décrire les changements phonologiques dans la structure de leur feuille puis, ensuite, déterminer la teneur toxique des cycadées panaméenne *Zamia stevensonii,* et enfin, corréler ces changements avec les incidences de insectes herbivores. Plus de la moitié de tous les individus *Z. stevensonii* produisent une série de nouvelles feuilles durant les deux premiers mois de la saison des pluies. Le développement des feuilles est caractérisé par une expansion foliaire rapide et par un verdissement retardé. Les jeunes feuilles sont couvertes de trichomes et ont la plus haute teneur en AZGs mais leurs défenses diminuent à mesure que leurs feuilles se développent.

La dureté des feuilles âgées de plus de 100 jours leur assure une protection. Comme la concentration de AZGs diminue avant que les feuilles ne deviennent suffisamment dures, il y a une période vulnérable durant laquelle les feuilles sont sensibles aux insectes herbivores spécialisés. Les dommages effectuées par les herbivores spécialisés - le papillon *Eumaeus godartii* et le coléoptère *Aulacoscelis appendiculata* - représentaient environ 37% de toutes les folioles produites. Les coléoptères adultes sont mobiles et, en dépit d'un large éventail d'âge de feuilles desquelles choisir, ils se nourrissent des feuilles âgées entre 10 et 100 jours, et préfèrent celles âgées entre 20 et 60 jours. Il existe des parallèles entre la stratégie défensive des cycadées et celle d'autres lignées de plantes qui présentent un retard de verdissement et un changement d'un protection chimique à mécanique.

Ces observations nous poussent à nous demander si ces herbivores intenses provoquent et/ou sélectionnent des taux plus élevés de AZGs. La teneur en AZG a donc été mesurée à la fois dans des feuilles abimées et dans des feuilles en bon état de plusieurs plantes *Zamia furfuracea* âgées de deux ans. Les taux de AZG sont restés stables au niveau local et systémique 3, 7 et 15 jours après l'apparition d'une blessure. Ces résultats soutiennent d'autres sources de données selon lesquelles

AZGs sont attribués à les primordia et pas renouvelés au cours de la vie d'une feuille. Cependant, on ne sait toujours pas s'il existe un retard en la induction des AZGs qui ce serait visible dans les feuilles suivantes.

Afin de mieux comprendre la stratégie défensive des cycadées, les taux de AZG ont été analysés à différents stades ontogéniques des populations *Dioon edule* au Mexique. Les individus ont été répartis en 3 stades ontogéniques: semis, jeunes et adultes. Le sexe de l'adulte était déterminé en fonction de la présence d'une structure reproductive. La structure génétique de ces populations a été déterminée à l'aide de 14 marqueurs microsatellites. Le génotype de la plante et l'hétérozygotie de l'individu ne sont pas corrélés avec les taux de AZG. Les taux de AZG dans les feuilles totalement développées diminuent avec l'âge de la plante: les taux les plus élevés de AZG sont trouvés dans les semis, puis dans les jeunes plants et les plus faibles concentrations se retrouvent auprès des adultes. Nos résultats suggèrent que les priorités de la plante changent au cours de l'ontogénèse et que les plantes âgées investissent moins dans leur défense chimique. Aussi, il est apparu que les plantes adultes femelles ont des taux plus élevés de AZG que chez les mâles, ce qui suggère que la stratégie défensive des cycadées pourrait être liée aux coûts de la reproduction. Les individus mâles, qui produisent de nouvelles feuilles plus souvent que les femelles, peuvent être en mesure de résister à des niveaux d'herbivores plus élevés que les femelles et, par conséquent, devraient investir moins dans leur défense.

Les coléoptères aposématique dans la sous-famille Aulacoscelidinae ont une relation intime avec les Zamiaceae Neotropical. En se nourrissant du feuillage des cycadées, ces coléoptères séquestrent les AZGs. Lorsqu'ils sont perturbés, ils ont le réflexe de saigner par l'articulation du tibia et du fémur.

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Contribution of authors to manuscripts

This thesis is written in the form of five separate manuscripts, three of which have already been published. I am the first author for all five manuscripts. The following section details the contribution of the authors to the manuscripts.

Chapter 2 is published in the American Journal of Botany (2014, vol. 101: 437-447). It is co-authored by Adriel Sierra and Donald Windsor (Smithsonian Tropical Research Institute), Jacqueline C. Bede is the senior author. Adriel Sierra performed 40% of the fieldwork. Donald Windsor and Jacqueline C. Bede contributed intellectually to the design of the study, data analysis and revision of the manuscript. I designed the study, performed 60% of the fieldwork, all the lab-work, analyzed the data and wrote the manuscript.

In Chapter 3, Jacqueline C. Bede and I designed the experiment. I conducted the experiment, analyzed the data and wrote the manuscript. Jacqueline C. Bede edited the text.

In Chapter 4, the study was designed with the assistance of Jacqueline C. Bede and Laura Yañez-Espinosa (Universidad Autónoma de San Luis Potosí). Gabriel Rubio-Mendéz (Universidad Autónoma de San Luis Potosí) assisted me with some of the fieldwork. I conducted the lab-work, analyzed the data and wrote the manuscript. The text was edited by Laura Yañez-Espinosa and Jacqueline C. Bede.

Chapter 5 was published in the Journal of Chemical Ecology (2011, vol 37: 736-740). The manuscript is co-authored by Julieta Ledezma from the Museo de Historia Natural Noel Kempff Mercado, at Santa Cruz, Bolivia, Luis Cubilla-Rios from the Faculty of Natural and Exact Sciences and Technology of the University of Panama, Jacqueline C. Bede and Don Windsor. Julieta Ledezma helped with the collecting and exportation permits from Bolivia. Luis Cubilla-Rios helped analyze and interpret the Nuclear Magnetic Resonance (NMR) and accurate mass spectra. Jacqueline C. Bede helped with the HPLC methodology, supervised the study and edited the manuscript. Don Windsor helped with the collection of specimens and

their secretions, supervised the study and edited the text. I collected the insect specimens and their secretions, performed the HPLC analysis, prepared the samples for NMR and accurate mass analyses and wrote the manuscript.

Finally, Chapter 6 has been published in the Journal of Systematic Entomology (2012, vol. 37: 747-757). The paper is co-authored by Duane McKenna (University of Memphis) and Donald Windsor as the senior author. Donald Windsor obtained the larval specimen and performed a preliminary description, supplied DNA sequences from several beetle species and revised the text. Duane McKenna commented on the phylogenetic reconstructions and the text. I performed a detailed description of the larva, reconstructed the phylogeny and wrote the manuscript.

Contributions to Knowledge

Chapter 2:

1) This is the first report that documents the strong parallels in the defensive strategies between a tropical gymnosperm and angiosperms that switch from a chemical to a mechanical defense and exhibit a delayed greening of the leaves.

2) This study shows that the highest herbivory levels of cycad leaves can be explained by phenology and changes in leaf traits.

3) My data suggests that high AZGs concentrations limit specialist herbivory.

4) Leaf toughness was identified as an effective defense against cycad specialist herbivores.

5) The amount of photosynthetic tissue lost to specialist herbivores was recorded for two years.

Chapter 3:

1) This is the first study on the induction of the cycad defenses. We observed that AZGs are not induced in response to mechanical wounding. These results support other studies, including Chapter 2, that suggest that AZGs are not replenished during the life of a leaf.

Chapter 4:

1) AZGs levels were evaluated in terms of plant sex and ontogeny. I found that chemical defenses decrease with plant age and that males invest less in chemical defense than females. The patterns observed in chemical defense are of interest to a broad range of ecologists and evolutionary biologists. Our study demonstrates that cycads are an interesting model to study the tradeoffs that exist between growth,

reproduction and chemical defense. Understanding these trade-offs in this ancient lineage of gymnosperms will provide insights into the evolution of plant defenses.

2) I identified an incipient genetic differentiation between lowland and highland *D. edule* individuals. Researcher Laura Yañez-Espinosa and Mexican authorities are taking these results into consideration for the conservation strategy for this cycad species.

Chapter 5:

1) I have showed that both genera of Aulacoscelidinae beetles sequester cycad toxins and use them for their own defense.

2) Due to the phylogenetic placement of the Aulacoscelidinae as part of the Orsodacnidae and sister group to all other chrysomelids, several authors lamented the lack of knowledge on the basic biology of this group of beetles. This is the first report on the defensive secretions of the Aulacoscelidinae beetles. I have documented their ability to reflexively bleed, as well as identified some of the toxic components that are part of their secretions.

Chapter 6:

1) This is the first record of the feeding habit of an immature Aulcoscelidinae.

List of Abbreviations

18S	gene coding 18S ribosomal RNA
28S	gene coding 28S ribosomal RNA
AGNES	agglomerative nesting (hierarchical clustering)
AZG	azoxyglycoside
BMAA	β-*N*-methylamino-L-alanine
BOAA	β-oxalylamino-L-alanine
CO1	gene encoding cytochrome oxidase 1
DNA	deoxyribonucleic acid
F	fixation index
GLM	generalized linear model
He	expected heterozygocity
Ho	observed hetrozygocity
I	Shannon information index
IBD	isolation by distance
MAM	methylazoxymethanol
ML	maximum likelihood
MS	mass spectrometry
Na	number of alleles per locus
Ne	effective number of alleles
NMR	nuclear magnetic resonance
OD	optimal defense theory
PCA	principal component analysis
PHt	proportion of heterozygous loci
RNA	ribonucleic acid
SEM	scanning electron microscope
uHe	unbiased expected heterozygocity

Introduction

Plants can be considered the "ultimate chemists" as more than 200,000 plant secondary metabolites have been described (Chinou 2008; Harborne 1993). Primary metabolites are defined as those that maintain the main functions and are needed for growth and reproduction (Chinou 2008). In comparison, the overwhelming diversity of phytochemicals are secondary metabolites that play a multitude of distinct roles, many of these conferring ecological advantages (Jones 1979; Ryan 2002; Schoonhoven et al. 2005; Seigler 1998). In particular, these chemical compounds are often produced by the plants to deter insect herbivory (Bennett and Wallsgrove 1994; Hartmann 1996; Hartmann 2004). Therefore, insect herbivory has been a key driver in the evolution of diverse plant secondary metabolites (Becerra et al. 2009; Hartmann 1999; Pichersky and Gang 2000).

In return, insects have evolved tolerance to these noxious phytochemicals, and sometimes are even attracted to the toxins and utilize them for their own defense (Strong et al. 1984; van Emden 1978). In many cases, the evolutionary relationship is believed to begin with tolerance of plant defenses that enables the insect to exploit a resource (the plant) that may not be available to other herbivores (Schoonhoven et al. 2005). Once the defense is breached, then some insect species use the defensive chemical as a cue to locate their host (Hartmann 1999). Hence, the same chemical compound that is a deterrent to most insect species may serve as a feeding stimulant to a restricted subset of insects (van Emden 1978).

A central goal in understanding plant-herbivore interactions is to understand phenotypic, genotypic and geographic variation in plant defense and predict its influence on insect communities (Stamp 2003; Strong et al. 1984). Questions like "*how does intra-annual variation in phytochemistry affect insect communities*?" has captured the interest of plant-insect ecologists. This thesis focuses on the chemical ecology of azoxyglycosides (AZGs), a group of defensive compounds that are exclusive to cycads (Order Cycadales). The thesis evaluates phenological, ontogenetic and induced changes in AZGs and the role that these compounds play in

mediating the association with leaf herbivores associated with cycads. Special attention is given to a group of cycad herbivores that has been neglected in most of the ecological literature on cycads, the beetles of the subfamily Aulacoscelidinae. The overall objectives of my thesis were 1) describing spatial and temporal variations in AZGs content in cycad leaves and 2) linking the variations observed to the incidence of insect herbivores. With this information in hand we can gain a comprehensive understanding of the defensive strategy of cycads that can then be compared to that of other plant lineages.

1. Literature Review

1.1. Optimal Defense Theory

Allocation to chemical protection is not a trivial dilemma for plants; limited resources are diverged from growth and reproduction into the biosynthesis of defensive secondary metabolites (Rhoads 1979, Bryant et al. 1991). The effects that growth habit (tree vs. herbaceous) and growth rate can have on the defensive startegy of plants has been elegantly put into different axioms that allow botanists to formulate testable hypothesis (Mackey 1974, Coley et al. 1985). By analysing the occurrence and distribution of alkaloids in different plant tissues and at different periods during plant development, Doyle McKey (1974) proposed what today is recognize as the Optimal Defense Theory (OD). The OD stipulates that 1) individuals will allocate resources to defense in ways that will maximize their fitness and 2) defenses are costly to the organism. From these corollary hypothesis more precise and testable hypothesis follow. For example, a) within a plant, tissues that are more vulnerable to predation will be more heavily protected, b) defenses will be deployed when the threat of predation is high and c) under high risk of predation, slow-growing species will invest heavily on constitutive defenses (Resource Availability Hypothesis, Coley et al. 1985, for a comprehensive review on the plant defense hypotheses see Stamp 2003). Evidence to support OD comes from the trade-off between chemical defense versus growth and/or reproduction which is well documented in several plants families (Bryant et al. 1991, Mckey 1979). Studies on morphs with high and low chemical defenses performed on a wide range of species producing alkaloids, cyanogenic glucosides, glucosinolates or monoterpenes, have found a negative correlation between the concentration of the defensive metabolites with vegetative growth and reproduction (Rhoads, 1979; Purrington 2000; Strauss et al. 2002). In the case of cycad plants and their toxic azoxyglycoside (AZG) metabolites, no studies had evaluated their defensive strategy in the light of the OD prior to this thesis. Furthermore, as a monophyletic lineage of gymnosperms that predates the appearance of flowering plants, the parallels that may exist between

defensive strategy of cycads and that of other lineages is of interest to evolutionary biologists.

1.2. The Zamiaceae

Cycads (order Cycadales) are woody plants that produce seeds in reduced fertile leaves called sporophylls. Their unique morphological features distinguishes them from the other gymnosperms (conifers, ginkgo, and gnetales); these include motile sperm cells, pinnately compound leaves and coralloid roots that house symbiotic cyanobacteria (Jones 2002; Pant and Das 1990; Stevenson 1990). Cycad trunks lack axillary buds but possess unique girdling leaf traces that create a criss-cross pattern (Stevenson 1990). The cycads fossil record spans at least 270 million years and indicates these ancient gymnosperms may have reached their peak abundance and diversity in the Mesozoic Era (200-150 mya) when they were a major component of forests worldwide (Mamay 1969; Norstog and Nicholls 1997; Zhifeng and Thomas 1989).

Today, growing mostly in tropical and subtropical forests, cycads are a small plant group considered to be monophyletic and whose phylogenetic position, in relation to other gymnosperms, is unclear (Chaw et al. 2005; Hill et al. 2003). The recognized number of extant species and genera ranges from 185-300 and 10-12, respectively (Hill et al. 2003; Jones 2002; Stevenson 1992). The largest of the two extant cycad families is the Zamiaceae. The Zamiaceae contains ten genera of which *Ceratozamia, Chigua, Dioon, Microcycas* and *Zamia* are endemic to the Neotropics, *Encephalartos* and *Stangeria* (some authors support the family Stangeriaceae) endemic to Africa, and *Bowenia, Lepidozamia* and *Macrozamia* endemic to Australia (Hill et al. 2003; Hill et al. 2005; Stevenson 1990; Stevenson 1992). The genus *Cycas* belongs to the Cycadaceae family and is distributed throughout China, Japan, Southeast Asia (including Australia and Papua New Guinea), India and Madagascar (Jones 2002).

The Zamiaceae family was first described by Heinrich G. Reichenbach in 1837 and is characterized by having cataphylls (stipules absent or present but always

growing from the vascular tissue) in the vegetative shoots, cones originating laterally or terminally on the main shoot, rounded seeds with a red, orange or yellow sarcotesta (Yañez-Espinosa 2006). Systematic relationships between most Zamiaceae genera are well understood and supported. However, this is not the case with *Dioon* and *Stangeria*, genera occurring on separate continents that could eventually be circumscribed to their own respective families (Hill et al. 2003; Zgurski et al. 2008). Many of the cycad species distributed worldwide are threatened by habitat destruction, poaching or invasive alien pests (Hill and Stevenson 2004; Marler and Moore 2010). Some cycad species are now extinct in the wild and many others have been reduced to the point where natural reproduction is no longer possible (Jones 2002). As a consequence, all species of the Order Cycadales are listed as endangered and protected under CITES (2008) as well, 289 species appear in the IUCN (2009) "red list".

Cycads are dioecious, each individual being strictly male or female. Pollen on males and ovules on females, are produced on sporophylls. While sporophylls are loose in the Cycadaceae, they are compacted into cones (also referred to as strobili) in the Zamiaceae (Norstog and Nicholls 1997). Pollination in the Zamiaceae is achieved by host-specific insects (Donaldson 1997; Downie et al. 2008; Jolivet 2005; Norstog and Nicholls 1997; Stevenson et al. 1998; Terry 2001; Wilson 2002). *Dioon* species are mainly pollinated by weevils (Belidae: Curculionoidea) (Vovides 1991b) while *Zamia* species rely on beetles belonging to the Erotylidae for fertilization (Tang 1987). Other beetle species that pollinate *Encephalartos, Macrozamia* and *Lepidozamia* species are found among the families Erotylidea, Tenebrionidae, Anthribidae, Boganiidae and Nitidulidae (Donaldson 1997; Donaldson et al. 1995; Jones 2002). Thrips (Cycadothrips: Thysanoptera) also are important pollinators of Australian *Macrozamia* species (Terry et al. 2007). While some pollinator assemblages are considered to be relatively recent associations (Downie et al. 2008), other associations could, in fact, be relict (Crowson 1991; Farrell 1998; Jolivet 2005; Labandeira 2000). Biogeographic, phylogenetic and fossil data indicate that the association between Boganiidae beetles and their cycad hosts in Africa and

Australia date to the Lower Cretaceous Period (about 100 mya) when Africa and Australia were still joined (Donaldson et al. 1995; Labandeira 2000).

In the cycad-pollinator paradigm, plant volatiles attract pollinators that congregate, mate and oviposit on the plant's reproductive structure, mainly in male cones (Jones 2002; Vovides et al. 1993). In some genera like *Dioon* and *Macrozamia*, male and female cones produce heat during pollination (up to 12°C above ambient temperature) increasing volatile emission and engaging insects in a "push-pull" pollination strategy, where pollinators are repulsed from hot male cones and attracted to female cones (Terry et al. 2007).

Female cones of the Zamiaceae can achieve huge sizes of up to 40 kg (Norstog and Nicholls 1997). Seeds have a bright yellow, orange or red fleshy coat (sarcotesta), a stony seed case (sclerotesta) and a starchy kernel (endosperm). The flesh and kernel both contain toxic AZG metabolites (De Luca et al. 1980). Regardless of their toxicity, cycads have historically been used as a food source by humans throughout the world. Archaeological excavations in Australia have dated the use of *Macrozamia riedlei* (Zamiaceae) back to the late Pleistocene (13,200 years bp) (Smith 1982). Archaeological evidence for the human use of *Dioon edule* in the Americas dates to at least 6,300 years bp (MacNeish 1958). The traditional detoxification processes employed for cycad consumption involve leaching, fermenting, roasting and/or aging (Beck 1992; Bonta 2010; Smith 1982; Thieret 1958; Whiting 1963). While cycad consumption is less common today, some cultures still harvest cycad seeds and use the same preparation methods, particularly in times of food scarcity (Thieret 1958). For example, *Dioon edule* seeds are still ground into flour to make tamales in northeastern Mexico (Bonta 2010). Contemporary use of cycads as food or medicine has been documented in Mexico, South Africa, Andaman Islands, Guam, Comoro Islands, Ryukyu Islands and northern Australia (Thieret 1958; Watt and Breyer-Brandwijk 1962; Whiting 1963). Although ancestral cooking techniques are generally thought to remove toxicity, a disease endemic to the island of Guam in Micronesia, colloquially called "lytigo-bodig", has been linked to cycad consumption. Cycad toxicity is thought to produce a pathology

that is described as an “amyotrophic lateral sclerosis” and/or “Parkinson-dementia complex”; however, the responsible compounds and mechanism of toxicity is still being heavily debated (Borenstein et al. 2007; Kisby et al. 1992). Cattle are also commonly affected by cycad toxicity (Whiting 1963). Cows and sheep grazing on cycad leaves and seeds suffer strong gastrointestinal disturbances and loose the ability to move their hind limbs. This condition regularly leads to the death of the animal (Thieret 1958).

1.3. Azoxyglycosides

Cycads contain several kinds of rare chemical compounds, mainly unusual carotenoids and toxins that have long intrigued botanists and chemists (Cardini and Bonzi 2005; Nishida et al. 1955; Watt and Breyer-Brandwijk 1962; Whiting 1963). The possible implication of cycad toxins in the etiology of a neurodegenerative disease afflicting the population of Guam promoted scientific attention including a series of six conferences sponsored by the National Institute of Health between 1962-1972 (Pan 1997; Spencer et al. 1991). The two most likely suspects implicated in toxicity the related disease complexes are either azoxyglycosides (AZGs) or non-protein amino acids (Pan 1997; Schneider et al. 2002).

AZGs are composed of an azoxy moiety ($RN=N^{+}(O^{-})R$) and a sugar. The azoxy moiety is a dipolar *N*-oxide of an azo compound, molecules that are extremely rare in nature (Engel et al. 2003). Azoxy compounds are found only in cycads, a few species of Actinobacteria in the genus *Streptomyces* and the entomophagous zygomycete *Entomopthora virulenta* (Claydon 1978; Ding et al. 2012). Azoxy compounds may function as virulence factors, deterrents or means to defend habitat (Claydon 1978; Ding et al. 2012). In cycads, two common cycad AZGs, cycasin and macrozamin, protect all tissues (De Luca et al. 1980; Jones 2002; Yagi 2004; Yagi and Tadera 1996). AZGs are composed of the azoxy compound, methyl azoxymethanol (MAM), with only variation in the attached sugar moieties. Cycasin and macrozamin are the glycosylated or primeverosated (glucose + xylose) forms of MAM, respectively (Fig. 1.1). Other AZGs that can be found in cycads are collectively known as Neocycasins (Nagahama et al. 1959; Yagi et al. 1985).

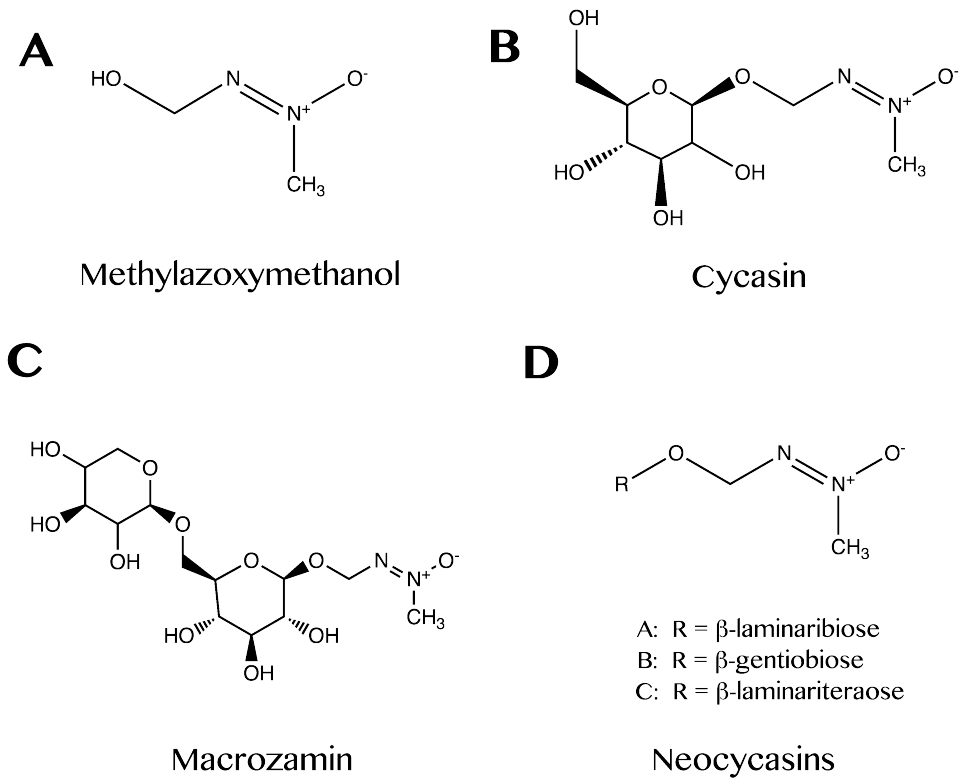

Fig. 1.1. Chemical structures of different azoxyglycosides.

All AZGs isolated thus far have a β-glucosidic linkage to MAM (Yagi et al. 1985). The glycosylated storage forms are non-reactive (Laqueur and Spatz 1968). AZGs are toxic when ingested but not when injected subcutaneously into animal models (Laqueur et al. 1967). Thus, the bacterial flora present in the gut plays an important role in modulating AZG toxicity (Laqueur and Spatz 1968). During herbivory, plant- or insect gut microbe-associated glycosidases cleave AZGs to free MAM, the toxic compound (Kobayashi and Matsumoto 1965; Laqueur et al. 1967). MAM is highly reactive and will alkylate proteins and nucleic acids, such as DNA, leading to potent mutagenic properties (Laqueur et al. 1967; Laqueur and Spatz 1968; Spencer et al. 2012). MAM-associated neurotoxic and carcinogenic properties have been well documented (Laqueur and Spatz 1968; Matsushima et al. 1979; Seawright et al. 1990; Smith 1966; Teas and Dyson 1967). MAM-associated DNA lesions can be

repaired by a methyltransferase; however, in mice, when these lesions are not repaired, brain development is perturbed and malignant tumors are induced in peripheral organs, such as the liver and pancreas (Spencer et al. 2012). While the toxicology of AZGs has received considerable study, other aspects remain unknown. For example, the biosynthetic pathway of AZGs and the site of synthesis have not been elucidated. Before the studies presented in this thesis, the temporal and spatial variation of AZG in cycads was not known.

In other organisms, the biosynthesis of related azoxy-compounds have been elucidated. The aliphatic nonsymmetrical azoxide, valanimycin is an antibiotic produced by the actinobacteria *Streptomyces viridifaciens* (Garg et al., 2008). Valanimycin is derived from L-valine and L-serine, with isobutylamine and isobutylhydroxylamine as intermediates (Yamato et al., 1986; Parry et al., 1992; Garg et al., 2008). MAM is also an aliphatic nonsymmetrical azoxide and could potentially have related precursors and intermediates.

AZGs are nitrogen-rich compounds and a potential source of this nitrogen is through nitrogen fixation by cyanobacterial symbionts associated with cycad coralloid roots (Lindblad and Costa, 2002). Cyanobacteria fix atmospheric nitrogen (N_2) to forms that can be transported and utilized by the plant, such as glutamine, citrulline and glutamic acid (Pate et al., 1988). It is strongly suspected that the symbiosis might influence AZG biosynthesis (Lindblad and Costa, 2002).

Although there is coherent evidence for the defensive role of AZGs, most of it is anecdotal. As previously described, accounts of intoxicated cows and sheep that have consumed cycad foliage are numerous (Thieret 1958; Whiting 1963; Whiting 1989). The severity of the intoxication in these accounts varies, some animals dying hours after the ingestion, other dying in the following days and some even recovering (Whiting 1963). As similar symptoms can be observed in experimental animals exposed to AZGs these metabolites are believed to be responsible for the intoxication of cattle (Laqueur and Spatz 1968). Additional evidence of the defensive role of AZGs comes from experiments showing that cycasin can protect the insect specialist insects that sequester cycasin by deterring potential predators (Bowers

and Larin 1989; Castillo-Guevara and Rico-Gray 2002; Castillo-Guevara and Rico-Gray 2003). While these studies showed that cycasin alone can deter ant and bird predators, they did not demonstrate the direct toxic effects on the predators (Bowers and Larin 1989; Castillo-Guevara and Rico-Gray 2002). The deleterious effect of AZGs on generalist insect herbivores has not been studied, probably because generalist are seldom encountered on cycads. It is important to note that azoxybenzene, one of the few naturally occurring azoxy compounds which is produced by the fungus *Entomophthora virulenta,* is toxic to an array of insects, including mosquitoes, the European corn borer, cockroaches, houseflies and the fruit tree red spider mite (Claydon 1978; Eaton and Davies 1948; Haring 1946). It is currently unknown if AZGs, that posses the same dipolar *N*-oxide as azoxybenzene, are also toxic to generalist insects. However the apparent lack of generalist herbivores on the cycad foliage seems to suggest this may be the case (Clark and Clark 1991).

1.4. The non-protein amino acids

Another group of secondary metabolites found in cycads that has received considerable attention are non-protein amino acids, such as β-*N*-methylamino-L-alanine (BMAA). BMAA was originally ascribed to symbiotic cyanobacteria living in the cycad coralloid roots (Cox et al. 2005; Cox et al. 2003; Kisby et al. 1992). BMAA acts on the nervous system when ingested (Brownson et al. 2002; Kurland 1988; Seawright et al. 1990). The biomagnification of BMAA through the food chain involving cycad, bats and humans was proposed to be the causative factor in the neurodegenerative disease in Guam (Banack and Cox 2003; Cox et al. 2003). BMMA was thought to pass and biomagnify from the cyanobacteria symbionts to cycads, from the cycad seeds to humans and to fruit eating bats, and from the bats meat again to chiropterophagous humans (Banack and Cox 2003; Cox et al. 2003). However, recent research has challenged these ideas and argued in favor of a plant origin for BMAA (Marler et al. 2010). Further, the toxicity of BMAA has recently been questioned by a study that claims that the amounts required to produce a toxic

effect are impossible to find in nature (Lee and McGeer 2012). These discrepancies derive from the difficulty in correctly differentiating BMAA from its isomer as well as the variation in the amounts measured when using different analytical methods (Faassen 2014; Kruger et al. 2010; Kubo et al. 2008). While the toxicity of BMAA has been questioned and, maybe, even exaggerated, the high toxicity of AZGs is well documented.

β-Oxalylamino-L-alanine (BOAA) is another unusual amino acid found in cycads (Pan 1997). BOAA isolated from chikling peas, *Lathyrus sativus* (Fabaceae), has been shown to cause neurolathyrism (Spencer et al. 1986). Other toxins present in cycads include domoic acid, kainic acid and β-sitosterol β-D-glucoside (BSSG) (Pan 1997; Wilson et al. 2002). The function of these toxic secondary metabolites is not understood (Snyder and Marler 2011). Insect performance and preference studies could help clarify the roles of these secondary metabolites in cycads.

1.5. Cycad insect herbivores

Many plant secondary metabolites, such as AZGs, are believed to play a role in controlling insect behavior (Schoonhoven et al. 2005). Although cycads appear to be largely devoid of generalist insect herbivores, a restricted group of insects have evolved tolerance to AZGs and even use them for their own defense. Several butterfly species belonging to the *Taenaris* (Nymphalidae), *Luthrodes* and *Eumaeus* (Lycaenidae) genera, as well as the pollinating weevil *Rhopalotoria mollis* (Belidae), feed on cycads and sequester AZGs (Castillo-Guevara and Rico-Gray 2002; Nash et al. 1992; Rothschild 1992; Schneider et al. 2002). The mechanisms involved in AZG sequestration are not understood. The only experiment to date involve caterpillars of the tiger moth, *Seirarctia echo*, that occasionally feed on *Zamia integrifolia*. When reared on MAM-enriched food, the caterpillars catalyzed the conversion to the less-toxic cycasin form, probably using a gut-associated glycosyltransferase, and stored cycasin in the haemolymph (Teas 1967). Limiting AZG toxicity could also be achieved by inhibiting glycosidases and transporting cycasin from the gut into the haemolymph. In insects, such a transporter has not been identified; however, cycasin can be transported by a Na^+/glucose co-transporter in rabbit intestinal cells

(Hirayama et al. 1994). On the other hand, MAM may be able to freely diffuse across membranes into the haemolymph and be modified in specialized tissues, such as the fatbody (Hirayama et al. 1994).

Lycaenid butterflies in the genus *Eumaeus* are obligate cycad herbivores of Neotropical Zamiaceae (Bowers and Larin 1989; Martínez-Lendech et al. 2007; Rothschild 1992; Rothschild et al. 1986). The females lay a batch of 8-20 eggs on leaf bases, raquis, trunks or cones of *Ceratozamia, Dioon* and *Zamia* species. The caterpillars are gregarious and feed predominantly on the leaves, but occasionally consume cone and seed tissue (Schneider et al. 2002). Caterpillars sequester AZGs from their host mainly in the form of cycasin (Bowers and Larin 1989; Nash et al. 1992; Rothschild 1992; Rothschild et al. 1986). The caterpillars exhibit a red and white aposematic coloration that contrasts strongly with the bright green cycad foliage. The sequestered AZGs are passed to adult via the pupa. AZGs in the adult butterflies can be found in the wing scales, haemolymph and spermatophores. Females *of E. atala* decorate their eggs with scales possibly as a warning of their chemical protection (Schneider et al. 2002). Other cycad specialists butterflies in the Lycaenidae are *Chilades pandava* Horsfield that feeds on the genus *Cycas* in southern Asia (Marler et al. 2012) and *Theclinesthes onycha* Hewitson that feeds on Australian cycads (Forster and Machin 1994; Nash et al. 1992). Whether these two species sequester cycasin remains unknown.

Another group of herbivorous insects closely associated with Neotropical cycads that tolerates AZGs are erotylid beetles in the genus *Pharaxonotha* (previously ascribed to Languriidae: the Languriidae family has been merged with Erotylidae). The association of these beetles with cycads range from pollinators to herbivores (Mora et al. 2013; Tang 1987). They are reported to be the main pollinator of some of the *Ceratozamia, Dioon, Mycrocycas* and *Zamia* species (Chavez and Genaro 2005). In contrast, they are the main pest for *Dioon edule* by boring into the seed and feeding on the embryo.

Another erotylid beetle that is reported to be a cycad specialist is a species of *Nomotus* Gorham (Windsor et al. 1999). These small black beetles are often seen

feeding along with *Eumaeus godartii* Biosduval caterpillars and *Aulacoscelis appendiculta* Cox & Windsor (see Chapter 2) on the leaves of several *Zamia* species in Central Panama. Both beetle species are attracted to the frass of *E. godartii* caterpillars.

Part of this thesis focuses on the association between another group of specialist insects with their cycad hosts: the association between the Aulacoscelidinae and the Neotropical Zamiaceae. The Aulacoscelidinae (Orsodacnidae: Chrysomeloidea) have been neglected in most of the ecological literature possibly due to their ephemeral appearances on the cycad foliage. Their association with the this ancient lineage of gymnosperms, their phylogenetic position within the Orsodacnidae as sister clade to all other chrysomelids make them an interesting group to study.

1.6. Insect diet breadth

Throughout this thesis the terms "specialist" and "generalist" are used to describe the diet breadth of insect herbivores. These terms are used inconsistently throughout the scientific literature; I will make explicit how they are used here. For the purpose of this thesis, a cycad specialist insect is an insect that either completes its live-cycle on a cycad plant and feed exclusively on cycad tissues (i.e. *Eumaeus* spp., *Rhopalotria mollis*) or has a seasonal association with a cycad plant from which it acquires its toxins (i.e. Aulacoscelidinae beetles). The fact that all members of an extant taxonomic group are associated to cycads makes the case stronger for a specialized association. All *Eumaeus* species feed as caterpillars exclusively on Neotropical cycads, they complete their live cycle on cycads. In the case of the Aulacoscelidinae beetles, their is a general lack of information on the feeding habits of both adults and immature stages. However, considering the aulacoscelidine species for which we do have information on their feeding habit, there is good support for a close association between the beetle subfamily and the Neotropical cycads (see Chapter 6).

Two factors are thought to be important in host specialization, 1) plant chemistry and 2) nutrient balance. Plant chemicals are not just defenses insects have to overcome but can also be strong cues that are useful for making the best host choice (Bernays 2001). When there are more items to choose from, making the right decision is harder and takes longer. It seems that there is a trade-off between the efficiency in choosing a suitable host and the amplitude of the diet (Bernays 2001). Specialists have less information to process in regards of finding their host while generalist have to deal with competing signals. A few studies show that when presented with hosts of low and high quality, specialists are always able to choose the high quality host, while generalists often make bad decisions (Bernays 2001). On the other hand, generalists seem to be better at coping with an imbalanced diet and compensating for the lack of a specific nutrient (Raubenheimer & Simpson 2003). In the case of the cycad specialist herbivores, *Eumaeus* spp. have to acquire all their nutrients from the cycad tissues, while the adult Aulacoscelidinae beetles are known to feed indiscriminately on pollen from several angiosperm species, and could compensate any nutrient imbalance this way. Lycaenidae genera closely related to *Eumaeus* feed mainly on monocots and the cycad feeding is thought to be secondary feeding habit with monocot feeding being the ancestral state (Ehrlich and Raven 1964). It is interesting to note that an Australian cycad specialist beetle *Lilioceris negripes* (Criocerinae: Chrysomelidae) is also thought to have switched from a monocot host to cycad feeding (Hawkeswood 1992). What, if any, biochemical pre-adaptions to AZG tolerance was achieved by feeding on monocots, is unknown. As for the Aulacoscelidinae, it has been speculated that the association with the cycads is ancestral and may date back to the Jurassic period (Crowson 1991, see Chapter 6). This hypothesis has been supported with fossil and phylogenetic data. Although this an intriguing possibility, the hypothesis is extremely hard to test due to 1) the lack of information on the basic biology of the Aulacoscelidinae and 2) the limited number of characters that can be used to compare extant species to the Mesozoic fossils.

Connecting statement

Chapter 1 provided an overview of cycads and their specialist herbivores. Chapter 2 discusses the leaf phenology of *Zamia stevensonii* and the defensive strategy of a cycad in the face of specialized herbivores. Attention is given to relate leaf phenology to the incidence of insect herbivores. In particular, the potential protective role of AZGs is evaluated and AZG concentration is put into context with leaf development.

The plant population studied in Chapter 2 is situated in the surroundings of Lago Alajuela inside the Chagres National Park in Central Panama. Its taxonomic status was revised and described as a new species in December 2012 with the new epithet *Zamia stevensonii*. This *Zamia* population is referred to as *Zamia* cf. *elegantissima* in Chapter 5 and 6 that were written and published before December 2012.

2. Leaf traits and herbivory levels in a tropical gymnosperm, *Zamia stevensonii* (Zamiaceae)

2.1. Abstract

Slow-growing understory cycads invest heavily in defenses to protect the few leaves they produce annually. The Neotropical cycad *Zamia stevensonii* has chemical and mechanical barriers against insect herbivores. Mechanical barriers, such as leaf toughness, can only be established once the leaf has expanded. Therefore, chemical defenses may be important during leaf expansion. How changes in leaf traits affect the feeding activity of cycad specialist insects is unknown. We investigated leaf defenses and specialist herbivore incidence on *Z. stevensonii* during the first year after leaf flush.

Herbivore incidence, leaf production and leaf traits that might affect herbivory, such as leaf age, lamina thickness, resistance-to-fracture, work-to-fracture, trichome density and chlorophyll, water and toxic azoxyglycoside (AZG) content were measured throughout *Z. stevensonii* leaf development. Principal Component Analysis and generalized linear models identified characteristics that may explain herbivore incidence.

Synchronized *Z. stevensonii* leaf development is characterized by quick leaf expansion and delayed greening. Specialist herbivores feed on leaves between 10 and 100 days after flush, and damage approximately 37% of all leaflets produced. Young leaves are protected by AZGs but these defenses rapidly decrease as leaves expand. Leaves older than 100 days are protected by toughness.

As AZG concentrations drop before leaves become sufficiently tough, there is a vulnerable period during which leaves are susceptible to herbivory by specialist insects. This slow growing gymnosperm invests heavily in constitutive defenses in

the face of highly specialized herbivores, underlining the convergence in defensive syndromes by major plant lineages.

2.2. Introduction

Plants have multiple strategies, both chemical and morphological, to protect their leaves. Mechanical or chemical defenses are metabolically expensive and plants must balance trade-offs between different strategies (Coley et al. 1985; Kikuzawa and Lechowicz 2011). Leaf toughness is an important means for reducing herbivory in tropical forest understories since it is costly for an insect herbivore to eat tough leaves (Coley 1983; Hochuli 1996; Kitajima et al. 2012; Lucas et al. 2000). However, young leaves require flexibility for expansion and therefore cannot use toughness as a defense at this stage. Therefore, young leaves must rely on other defensive traits, such as toxic secondary metabolites and trichomes (Coley and Aide 1991; Kursar and Coley 2003). During leaf expansion, a defensive syndrome that comprises a switch from chemical defenses to toughness has been described for several understory angiosperms (Kursar and Coley 2003). In contrast, little information is available on the defensive strategies of tropical understory gymnosperms, such as cycads.

Cycads (Cycadales) are dioecious gymnosperms with extremely slow growth rates; trunk elongation may be on the order of 2-3 cm/year for understory species (Norstog and Nicholls 1997; Vovides 1990). Cycads grow by pulses of leaf flushes from the stem apex. Like other understory species, their coriaceous leaves can remain alive and attached to a plant many years (Norstog and Nicholls 1997). Some cycad populations exhibit a synchronized leaf flush where most individuals of the population produce flushes of new leaves during a period of a few weeks. This was proposed to be a strategy to minimize herbivory by satiating low insect populations with an ephemeral resource (Clark and Clark 1988; Clark and Clark 1991; Clark et al. 1992). However, synchronized flushing of new leaves might be influenced by cone production. Marler (2010) reported that senescence of male cones affected the timing of subsequent leaf flushing events. Leaf production may cease after these expensive reproductive events. For example, *Zamia skinneri* Warscz. ex A. Dietr.

females do not flush new leaves for several years after producing cones (Clark and Clark 1988). The limited production of long-lived leaves by cycads highlights the benefits of protecting them from herbivores.

Highly toxic azoxyglycosides (AZGs) protect all cycad tissues (Norstog and Nicholls 1997). Azoxyglycosides are dipolar *N*-oxides with well-documented carcinogenic and mutagenic properties (Laqueur and Spatz 1968; Matsushima et al. 1979; Smith 1966; Teas and Dyson 1967). When the sugar moiety from the AZG is cleaved by either plant- or insect gut-glycosidases, the azoxy core (methyl-azoxymethanol (MAM)) becomes highly reactive leading to alkylation of DNA or proteins (Laqueur and Spatz 1968). While AZGs are a good defense against generalist herbivores, cycad specialists have adaptations that allow them to cope with plant toxicity. AZGs present in cycad leaves are metabolized rapidly and may even be sequestered and utilized by herbivore specialists (Castillo-Guevara and Rico-Gray 2002; Prado et al. 2011; Rothschild 1992; Rothschild et al. 1986; Schneider et al. 2002). In the Neotropics, the lepidopteran, Eumaeus spp. (Lycaenidae) and beetles in the subfamily Aulacoscelidinae (Orsodacnidae) incorporate cycad-derived AZGs for their own defense (Rothschild et al. 1986; Bowers and Larin 1989; Rothschild 1992; Norstog and Nicholls 1997; Schneider et al. 2002; Prado et al. 2011). How these two groups of cycad specialists avoid the deleterious effects of AZGs is unknown, although re-glycosylation of the free azoxy group has been suggested (Teas 1967).

Zamia stevensonii A.S. Taylor & G. Holzman (Zamiaceae: Cycadales) is a lowland neotropical cycad species distributed in central Panama whose tough leaves can persist for over 10 years (A.S. Taylor pers. comm.; Taylor and Holzman, 2012). Previously considered as part of *Z. elegantissima* Schutzman, Vovides & Adams (Schutzman et al. 1998), the recently described *Z. stevensonii* has thinner trunks, smaller leaves with fewer leaflets and a characteristic white flush of new leaves that has given it the common name of “blanco” among cycad growers (Taylor and Holzman 2012). Reaching lengths of 120 cm, *Z. stevensonii* leaves can take up to 4 months to reach full size (Taylor & Holzman 2012). *Zamia stevensonii* is host to a

suite of specialized leaf herbivores; *Eumaeus godartii* Boisduval (Lepidoptera: Lycaenidae), *Aulacoscelis appendiculata* Cox & Windsor (Coleoptera: Orsodacnidae), *Nomotus* sp. (Coleoptera: Erotylidae, previously Languriidae) plus unidentified Erotylidae species (pers. observation, Windsor et al. 1999). All these insect species may feed together on a single plant. These specialist insects are voracious feeders, and in some cases, may completely defoliate the cycad of its new foliage. Generalist insects are not observed to feed on this cycad species.

Field observations suggest that specialist insect activity is closely synchronized with the production of new foliage. Young leaves are particularly vulnerable; specialists cycad herbivores seldom eat coriaceous older leaves. How cycads limit herbivory on young leaves is not known. In this study, we evaluate how the occurrence of specialist herbivores varies in relation to changing leaf traits, including leaf age, lamina thickness, resistance-to-fracture, work-to-fracture, trichome density, AZGs, chlorophyll and water content. We also assess the overall impact of insect herbivores, by monitoring tissue loss during leaf development. The objectives of the study were to 1) identify leaf protective mechanisms that a slow-growing understory gymnosperm employs in the face of well-adapted herbivores and 2) quantify the overall loss of new foliage to insect herbivores.

2.3. Materials and methods

2.3.1. Study area

Zamia stevensonii (Holotype PMA104661-104668) grows in the understory of tropical deciduous forests on the phosphorus-rich, calcareous soils of the Chagres Valley in central Panama (Nottingham et al. 2012; Taylor and Holzman 2012). Our two study sites are situated in the semi-deciduous forest that surrounds Lago Alajuela inside the Chagres National Park (09°12.516N, 79°35.964W; 09°12.482N, 79°32.335W). The forest is characterized by a sharp 3-4 month dry season and an average annual rainfall of 2,400 mm (Candanedo et al., 2003). Large patches of *Z. stevensonii* occur on calcareous sedimentary rock outcrops.

Zamia stevensonii individuals produce pinnately compound leaves in discrete flushes. Herbivore incidence was recorded from March 2011 to August 2013, a period including three annual leaf flushes. Leaf trait (Table 2.1) data were collected for 103 plants during 2011. Plants were surveyed twice a week from March to July and revisited six months and one year later. Leaf production was recorded for an additional 117 plants in 2012 and 2013 (January 2012 – August 2013). Plants were surveyed once per month except in April, May and June when they were visited twice per week and in July 2013 when were they were not censused.

2.3.2. Leaf selection and herbivory evaluation

Plants exhibiting swollen crowns were inspected for new leaf production in March 2011. If leaf primordia were protruding from the cataphylls and visible, plants were flagged and assigned an identification number, the leaf flush was assigned as day 0 (N = 103). Plants were followed twice a week for 18 weeks and revisited after six months and one year. Leaf trait measurements were performed on non-damaged as well as damaged leaves. If the first leaf of the flush suffered herbivory, the insect causing the damage was recorded and the corresponding leaflet samples were collected for trait measurements (N = 28). Leaf damage was estimated as the proportion of leaflets damaged. Considering that damage to a young leaflet commonly led to its abscission, leaflets were considered damaged, regardless of the degree of damage. Usually, the insect causing damage was still present, but in the cases where they were absent (N = 13), the consumer's identity was deduced from its feeding pattern. Leaflets with a characteristic pattern of pierced holes could be confidently assigned to *A. appendiculata* (Windsor et al. 1999), while chewed leaves were assigned to *E. godartii*. The first leaf of undamaged flushes (N = 75) was collected for leaf trait measurement. Plants were randomly selected to obtain the greatest possible range of leaf ages. By selecting only a single leaf from a plant, sample independence was maintained. Leaf collections included leaves ranging in age from 7 to 365 days old. The collected leaves had 4 to 16 pairs

of leaflets, leaf trait measurements were performed consistently on the first, third or fourth pair of leaflets from the apex depending on the trait.

2.3.3. Leaf toughness

Resistance-to-fracture and lamina thickness were measured and used to calculate "work-to-fracture". Resistance-to-fracture was treated separately from lamina thickness as well as combined (work-to-fracture) since Westbrook et al. (2011) observed that mortality rates in tree seedlings were negatively correlated to resistance-to-fracture but independent of work-to-fracture. Resistance-to-fracture (Meganewtons·m^{-2}) was measured as the average force required for a penetrometer to pierce through the centre of each one of the fourth pair of leaflets from the apex (Aranwela et al. 1999; Lucas et al. 2000).

A Pesola penetrometer was used for young, soft leaves (0.02-3.7 Kg·cm^{-2}) and a Handpi GY-3 fruit penetrometer was used for tougher leaves (1-12 Kg·cm^{-2}). The overlapping measuring ranges allowed for calibration between the two systems. However, tender leaves (<5 days old) could not be quantified as they were too soft and below the detection threshold of both penetrometers. Lamina thickness (mm) was measured twice with hand calipers at the center of both 4th pair of leaflets from the apex and the values averaged. Work-to-fracture (J·m^{-2}) was calculated by multiplying the resistance-to-fracture by the lamina thickness (Lucas et al. 2000).

2.3.4. Trichomes

Non-glandular trichomes are present on *Zamia* leaflets (Stevenson 1981). Trichome density was measured under a light stereoscope by counting the trichomes on a 2 x 2 mm^2 area on the abaxial side of the first leaflet pair.

2.3.5. Chlorophyll and water content

The third pair of leaflets from the apex was frozen in liquid nitrogen. One leaflet was used to determine water content by weighing the thawed leaflet, then

drying it at 40°C for 48 hr and weighing it again. A subsample of the other leaflet (100-200 mg) was ground in liquid nitrogen to a fine powder and analyzed for chlorophyll *a* and chlorophyll *b* content (Wellburn 1994). After extraction in 80% acetone, samples were maintained on ice for 30 min then centrifuged at 6,000 rpm for 1 min. The supernatant was analyzed spectrophotometrically (Tecan Infinite M200 Pro) at 646 and 663 nm and chlorophyll *a* and *b* content calculated according to Wellburn (1994).

2.3.6. Azoxyglycoside (AZG) content

AZG content was measured in the remaining tissue of the third pair of leaflets. Small pieces (100-200 mg) were ground in liquid nitrogen, weighed and extracted in chilled 70% ethanol. After extraction on ice for 20 min, samples were centrifuged at 20,000 rpm for 10 min. The supernatant was transferred to a new vial, frozen in liquid nitrogen and lyophilized. Samples were re-suspended in 25 mM sodium acetate buffer, pH 5.0. In parallel, two aliquots of each sample were diluted 6.5 times in sodium acetate buffer; one sample was incubated overnight at room temperature with β-glucosidase (Sigma, 0.5 U) and the other sample left untouched. β-glucosidase converts AZGs to the methyl azoxymethanol (MAM) core, allowing the quantification of MAM and AZG equivalents. Sister samples were analyzed by high performance liquid chromatography on a reverse-phase HI-Plex Na (Octo) column (300 x 7.7 mm, Agilent) using an isocratic elution of 100% water at a flow rate of 0.45 mL/min at 65°C (Yagi 2004; Yagi et al. 1980; Yagi et al. 1983). Compounds were detected by a photodiode array detector at 215 nm. MAM acetate (National Cancer Institute) was converted into MAM by incubation with an esterase enzyme (Sigma) in 1M Tris-HCl buffer, pH 8.5 for one hour at room temperature and used to generate a 7-point standard curve. Levels of MAM were identified based on the retention time and confirmed by the removal of the AZG peak in the β-glycosidase-treated subsample (Prado et al. 2011).

Table 2.1. Leaf traits measured for *Zamia stevensonii*.

Leaf Trait	Abbreviation	Unit
Leaf age	Age	days
Leaf length	Size	cm
Lamina thickness	Thick	mm
Resistance to fracture	Res fract	$MN \cdot m^{-2}$
Work to fracture	Work F	$J \cdot m^{-2}$
Chlorophyll *a* content	Chl_A	$g \cdot mg^{-1}$ FW
Chlorophyll *b* content	Chl_B	$g \cdot mg^{-1}$ FW
Water content	H_2O	percentage of fresh weight
Azoxyglycoside content	AZGs	$\mu mol \cdot g^{-1}$ DW
Trichome density	Trich	number per mm^2

2.3.7. Leaf production and herbivore incidence

To determine leaf losses to herbivores in 2012 and 2013, two 10 x 10 m quadrats and a 170 x 2 m transect were established. The quadrats included 67 *Z. stevensonii* individuals, and the transect, 50. Leaf production and the proportion of leaflets damaged by insect herbivores was monitored for these 117 individuals over a 20 month period (January 2012 – August 2013).

2.3.8. Statistical Analysis

2.3.8.1. Leaf development

Leaf trait data were analyzed by fitting deterministic models using non-linear least squares (nls function in R)(Bolker 2008). The Michaelis-Menten model was fitted to leaf size, chlorophyll content and leaf toughness data which increased rapidly until an abrupt asymptote was reached. In this model, $F(x) = ax/(b+x)$, x is

the age of the leaf in days, a is the maximum value reached by the response variable and *b* is the value of the explanatory variable at which the response variable is half of its maximum. In this case, *b* is the number of days after flushing at which the response variable reaches half its maximum. In contrast, a negative exponential model, $F(x) = ae^{-bx}$, was fitted to the water content, AZG content and trichome density of leaves of age *x* as these traits decreased quickly but slowly reached an asymptote. In this model, the slope is given by parameter *b*; increases in this parameter produce a more pronounced slope. By fitting deterministic models to the data, we were able to compare the parameters of interest across different leaf traits. The parameters of interest were the days required to reach half maximum for leaf size, chlorophyll content, lamina thickness and leaf toughness and the rate of change (slope) for water content, AZG content and trichome density.

2.3.8.2. Herbivore incidence during 2011

To understand the relationship between leaf traits and insect damage, principal component analysis (PCA) was performed on a correlation matrix of the log-transformed leaf traits on the set of 103 observations. Then the observations were plotted in the two-dimensional leaf trait space (biplot) coding observations as either damaged or undamaged. To test if the proportion of leaflets damaged was actually constrained at both ends of the first principal component, the proportion of damage was regressed to principal component 1 and several different deterministic models were fitted to the data. The models tested included linear, exponential, biexponential and Gaussian. The best fit was assessed by the Akaike information criterion (AICc) and R-squared values.

2.3.8.3. Protection of the young leaves

Preliminary analysis of the three-year survey showed a constraint on herbivory during the first 10 days of leaf development. This restriction on herbivory was supported by PCA results. Therefore, we tested if the 2011 leaf trait data could explain the lack of herbivore damage during the first few days after leaf flush. One

possible explanation is that the youngest leaves were not eaten because they are not present long enough for the herbivore to find and attack them. Another explanation is that defense mechanisms deployed by the plant during early leaf development (trichomes and AZGs) deter herbivores. To test these two hypotheses, we used a binomial generalized linear model (GLM) approach to model the proportion of leaflets damaged as a linear function of five variables: leaf age, exposure time, leaf toughness, trichome density and AZG concentration (*glm* function in R with *logit* as the link function)(Crawley 2012; Zuur et al. 2009). The initial model looked like this:

proportion of damaged leaflets = age + exposure time + toughness + trichomes + azgs

Exposure time was calculated as the number of days from the first herbivory observation to the day the leaf was collected. As the model exhibited overdispersion, a quasibinomial family was specified. We continued to simplify the model by stepwise exclusion of the terms, fitting the simplified models and assessing the changes in fit (*drop1* function in R); F tests, predictors with P values > 0.05 were excluded. To visualize the behavior of the simplified model, we performed and plotted a series of predictions using only the predictors supported by the F tests and controlling one variable at a time.

2.4. Results

2.4.1. Phenology

The total number of leaves on *Z. stevensonii* individuals ranged from 1 to 23 (8.8 ± 4.7; average ± SD) with 4-16 leaflet pairs per leaf (9.5 ± 3.0; average ± SD). As expected, older, larger plants tended to have longer leaves with more leaflets (data not shown). More than half (62 and 55 %) of all *Z. stevensonii* individuals produced a synchronous flush of new leaves during the first two months of the wet season in 2012 and 2013 (Fig. 2.1, A and B). Sparse leaf flushing events also occurred between September and November 2012. The leaf flushes of *Z. stevensonii* consisted of an average of 2-3 leaves with a maximum of 7 leaves and exhibited erect ptyxis (Stevenson 1981). The 117 *Z. stevensonii* individuals studied, produced similar

number of leaves, 217 and 223, during 2012 and 2013, respectively. However the peak flush during 2013 was more synchronous than the one in 2012 (Fig. 2.1, Table 2.3). The difference could reflect the smaller number of plants that contributed to the 2013 peak flush (Table 2.3).

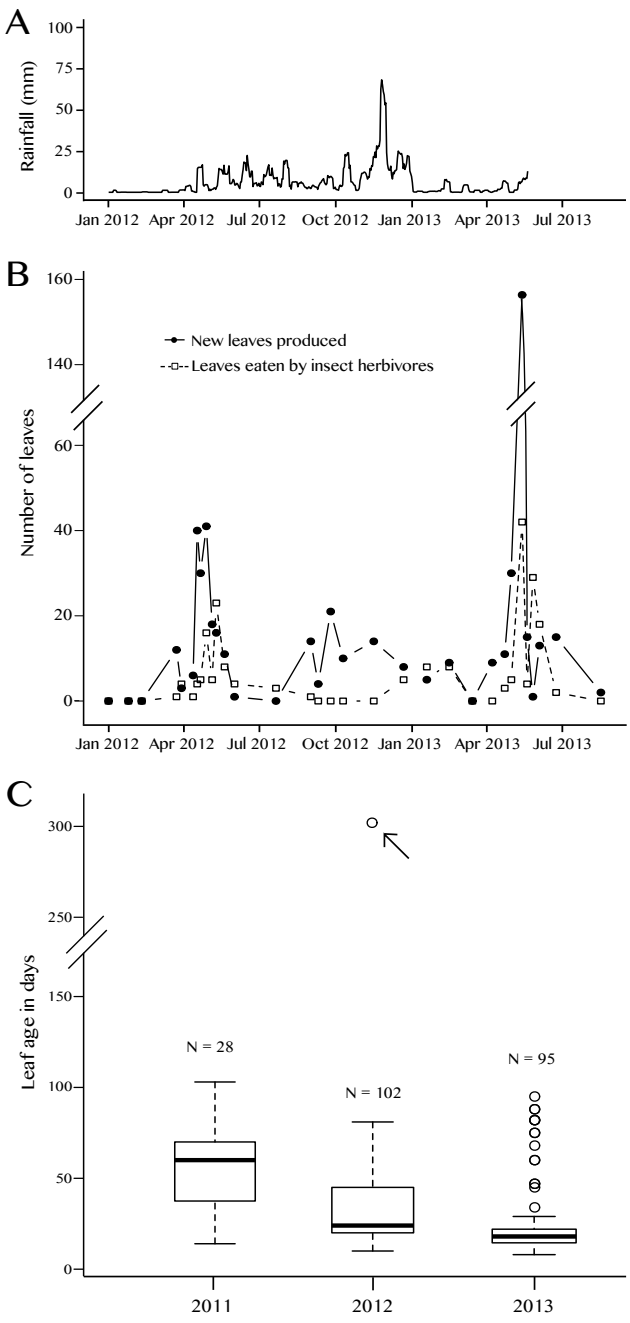

Fig. 2.1. Rainfall, phenology and herbivore incidence on *Zamia stevensonii*. A) Seven day running average of the daily rainfall (January 2012-May 2013). (B) New leaf production (•) and insect damage (□) during 2012 and 2013. (C) Age when leaves were attacked by herbivores (2011, 2012 and 2013). Open circles indicate outliers and the arrow indicates an herbivory event by *E. godartii* caterpillars on a 305 day old leaf.

2.4.2. Changes in leaf traits during leaf development

Pubescent leaflets were tightly folded against each other with the abaxial side exposed at the initiation of leaf flushing. These small, young leaves contained the highest AZG concentrations (>500 μmol·g^{-1} DW) and water content (≈ 85%). Within a week, the faintly pigmented leaves began to unfold. Soon thereafter, greening began, with chlorophyll content increasing over the subsequent year (Fig. 2.2E). Chlorophyll *a* concentrations reached half-maximal values at 80 days whereas chlorophyll *b* increased at a slightly lower rate taking up to a year to reach their half-maximal value. Leaf water content declined relatively slowly over the course of an entire year (Fig. 2.2F, minimum value = 60%), while both trichome density and AZG content decreased very rapidly and closely followed changes in leaf size (Fig. 2.2, G and H). Unbranched, non-glandular trichomes densely covered the youngest leaves (>20 per mm^2) but their density decreased rapidly as the leaf expanded. Within three weeks, all trichomes had fallen away and were not replaced (Fig. 2.2H). AZGs levels decreased at a slower pace going from >500 μmol·g^{-1} DW to approximately 100 μmol·g^{-1} DW levels in 2 months (Fig. 2.2G). AZG levels declined as leaf size increased (ρ= -0.528, $p<0.001$, Table 2.2) suggesting that plants protect young leaves with high AZG levels but these become diluted as leaflets expand. In leaves older than six months, AZG levels were below HPLC detection limit (≈ 1 μmol·g^{-1} DW).

Two weeks after leaf flush, *Z. stevensonii* leaves reached half of their full size, which was attained at approx. 100 days. Leaf size and lamina thickness increased quickly and reached an abrupt asymptote approximately at 80 and 110 days, respectively (Fig. 2.2, A and B). In contrast, resistance-to-fracture increased at a slower rate (Fig. 2.2C). Therefore, leaves first invested in growth with half-maximal size reached at 14 days and half-maximal lamina thickness reached at 31 days. Toughness, indicated by the half-maximal resistance-to-fracture, was only achieved 59 days after leaf flush.

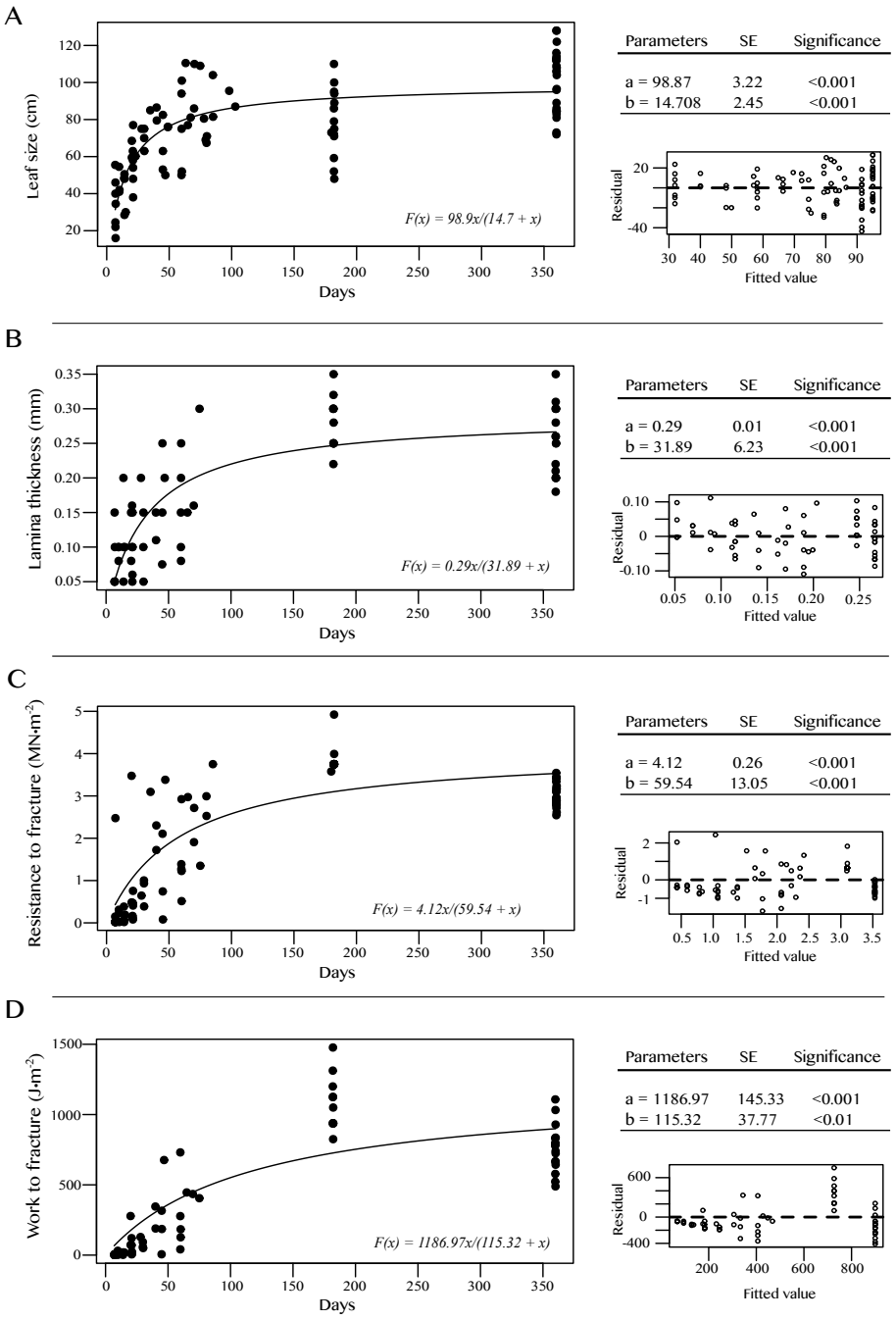

Fig. 2.2. Changes in the leaf traits of *Zamia stevensonii* over a one-year period. The phenology of leaf traits was measured during 2011. Day 0 equals the day leaf primordia protruded the cataphylls and were first visible. (A) Leaf expansion (B) Lamina thickness (C) Resistance-to-fracture (D) Work-to-fracture- (E) Chlorophyll a and chlorophyll b concentration (F) Water content (G) Azoxyglycoside (AZG) content (H) Trichome density. The Michaelis-Menten model ($F(x) = ax/(b+x)$) was fitted to data A-E. Negative exponential models, $F(x) = ae^{(-bx)+65}$, $F(x) = ae^{(-bx/20)+5}$,$F(x) = ae^{(-bx/10)}$, were fitted to F, G and H, respectively. Residual plots and a summary of the parameters for each model can be found at the right of the main graph.

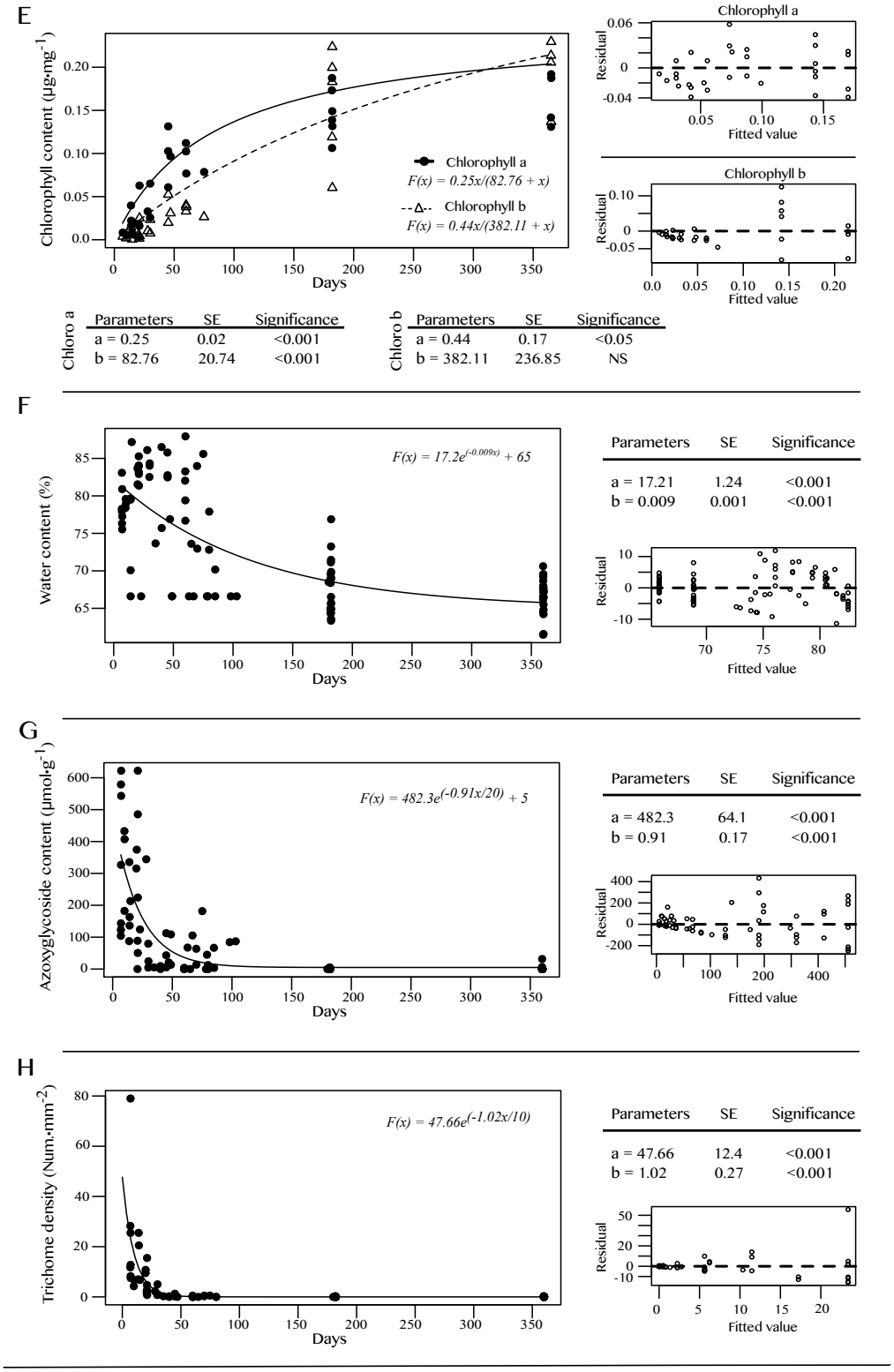

Fig. 2.2. Continued.

Table 2.2. Correlation coefficients (Spearman's ρ) between *Zamia stevensonii* leaf traits. All coefficients are significant to at least $P < 0.001$. Values above the diagonal are number of pairwise comparisons.

	Age	**Size**	**Thick**	**Res fract**	**Work F**	**Chl_A**	**Chl_B**	**H_2O**	**AZGs**	**Trich**
Age		93	85	90	65	50	50	101	100	83
Size	0.754		65	82	65	50	50	93	92	75
Thick	0.780	0.673		65	na	50	50	85	85	82
Res fract	0.676	0.574	0.754		na	50	50	90	89	83
Work F	0.837	0.759	na	na		49	49	65	65	63
Chl_A	0.915	0.696	0.663	0.830	0.845		50	50	50	49
Chl_B	0.927	0.744	0.669	0.842	0.854	0.983		50	50	49
H_2O	-0.687	-0.512	-0.679	-0.701	-0.714	-0.576	-0.621		100	83
AZGs	-0.788	-0.528	-0.597	-0.655	-0.720	-0.719	-0.744	0.551		83
Trich	-0.832	-0.636	-0.733	-0.761	-0.841	-0.804	-0.812	0.668	0.760	

na = not applicable

2.4.3. Herbivore incidence

Most leaf flushes occurred within a two-month period (April-May), with a few plants flushing earlier than April. Therefore, during April-May, within a small forest patch, roaming herbivores had a wide range of leaf ages from which to choose from. Insect herbivory occurred only on leaves between 10 and 103 days old, and herbivory was highest on leaves between 20 and 30 days of age (Fig. 2.1C). In the three years of this study only four exceptions were noted; three incidences at 9 days by *A. appendiculata* and one incidence at 305 days by *E. godartii* caterpillars (Fig. 2.1C).

Losses of leaflets of the newly produced leaves due to insect herbivores, was 41.01% in 2012 and 33% in 2013 (Table 2.3). These sizeable losses in photosynthetic potential resulted from damage by three species of insects: *E. godartii* caterpillars, and adult beetles, *A. appendiculata* and *Nomotus* sp.

Table 2.3. New leaf production and herbivore damage on *Zamia stevensonii* during three leaf flushing events.

Year	No. of plants surveyed	No. of plants producing new leaves	Total leaves produced	No. of plants with herbivore damage	Total production lost to herbivores (%)*
2012	117	73	217	39	41.01%
2013	117	64	223	53	33.00%

		Herbivore responsible for the damage (%)			
	No. of leaves damaged**	*A. appendiculata*	*E. godartii*	*A. appendiculata* + *E. godartii*	*Nomotus* sp.
2011	28	65%	27%	0%	8%
2012	102	54.2%	18.6%	8%	5%
2013	95	51.9%	12%	20%	12%

* No. of leaflets damaged divided by the total amount of leaflets produced during the flushing event of April-May.
** Including partially damaged leaves.

Groups of 20 or more *A. appendiculata* beetles on a single leaf were not uncommon during the last two weeks of April and the first two weeks of May in each of the three years of the study. Frequently, *A. appendiculata* feeding began at the tip of the outermost pair of leaflets with beetles working their way toward the leaf base, sometimes even ingesting the petiole. While some cycads were heavily attacked by large groups of both *E. godartii* caterpillars and *A. appendiculata* beetles, a close neighboring plant bearing a similarly aged flush of new leaves could remain undisturbed.

Adult *A. appendiculata* accounted for the majority of herbivore damage on *Z. stevensonii*, especially during the period of new leaf production in

April and May, whereas *E. godartii* caterpillars were present throughout the year. These two insect specialists sequester toxic plant-derived AZGs for their own protection (Rothschild et al. 1986; Rothschild 1992; Castillo-Guevara and Rico-Gray 2002; Schneider et al. 2002; Prado et al. 2011). In the three years of this study, *A. appendiculata* accounted for 51-65% of herbivore damage, and *E. godartii* for 12-27% of the herbivore damage (Table 2.3). An additional 8-20% damage was caused by both these species feeding together on the same leaf. Adult *Nomotus* sp. beetles accounted for the rest of the herbivory (5-12%).

2.4.4. Herbivory and leaf traits

Herbivory on the youngest and most tender leaves (<9 days) was not observed. Herbivory rarely occurred on fully toughened leaves (> 750 $J \cdot m^{-2}$): herbivory was restricted to leaves with toughness values between 10-697 $J \cdot m^{-2}$. Highest levels of herbivory were recorded on leaves containing 10-200 $\mu mol \cdot g^{-1}$ DW AZGs, few trichomes (<35 trichomes/mm^2) and high water content (66-86%).

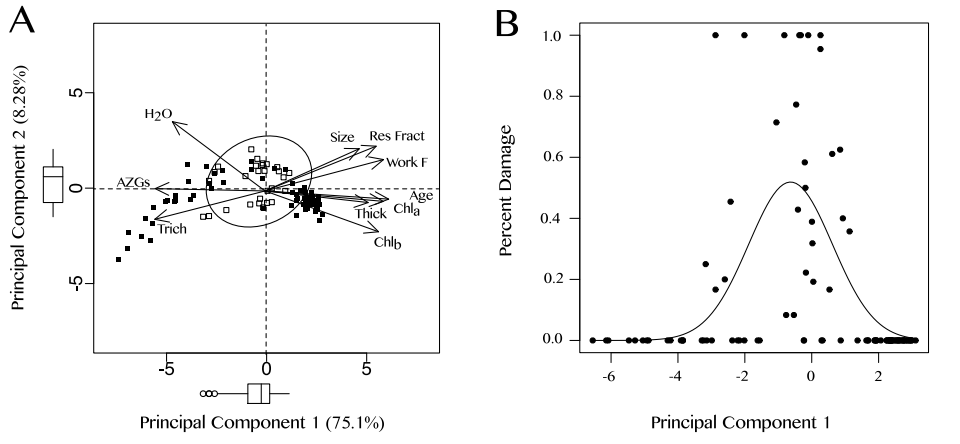

Fig. 2.3. Correlations between leaf traits and leaf damage. (A) Principal component analysis biplots of *Zamia stevensonii* leaf traits. Open squares represent damaged leaves and filled squares represent undamaged. A 0.95 confidence interval ellipse has been drawn around the damaged leaf data. Box-and-whiskers plots represent the distribution of the damaged data along the two principal components. (B) Relationship between principal component 1 and leaf damage, calculated as the number of leaflets damaged divided by the total number of leaflets. A Gaussian model was fit to the data ($F(x) = a*e(-0.5*((x-b)/c)^2)$).

Principal component 1 (PC1) explained >75% of the variation in *Z. stevensonii* leaf traits (Fig. 2.3A, Table 2.4). PC1 illustrates a trade-off between toxicity, trichomes and water content versus leaf chlorophyll content, lamina thickness, toughness and age. Leaf herbivory was restricted by the two extremes of PC1, presumably by AZG content and trichomes in younger leaves and by toughness in older leaves (Fig. 2.3B).

Table 2.4. First and second principal component loadings for leaf traits.

Leaf traits	PC1	PC2
Age	0.96665	-0.09108
Size	0.81989	0.23945
Thick	0.8177	0.07774
Res fract	0.90663	0.26115
Work F	0.94499	0.2228
Chl_A	0.91325	-0.1597
Chl_B	0.85378	-0.475
H_2O	-0.75097	0.55471
AZGs	-0.85165	-0.0993
Trich	-0.868	-0.30442

The proportion of leaflets eaten is a linear function of leaf age and AZG concentration ($p < 0.001$), while time of exposure, and surprisingly trichomes, had no significant effect ($P = 0.747$ and $P = 0.1058$ respectively; Table 2.5). Both, age and toxin concentration have a negative effect on the proportion of leaflets damaged. The simplified model (proportion of damaged leaflets = age + azgs) predicted that the chances of herbivory on leaves of ≥180 days is near zero and that high levels of toxins can suppress herbivory despite the age of the leaves (Fig. 2.4). These results suggest that older leaves are well protected because of their toughness while younger, tender leaves are protected by toxins, even against herbivores that sequester AZGs. However, as leaves expand and AZG levels drop, and before leaves reach maximum toughness, cycads are vulnerable to herbivory by specialist insects.

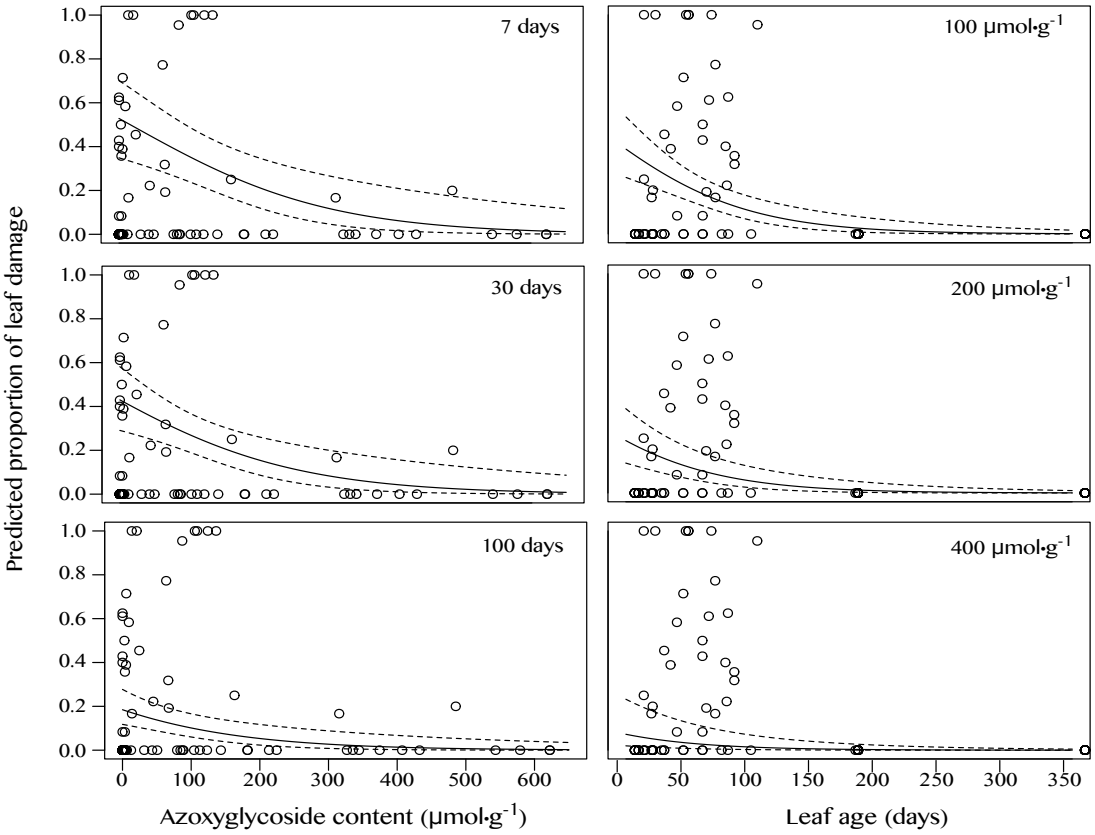

Fig. 2.4. Generalized linear model predictions for the proportion of leaf damage based on leaf age and AZG concentration. Fitted values (solid lines) and 95% confidence bands for the optimal binomial GLM. On the left panels, predictions were performed at specific leaf ages, 7, 30 and 100 days. On the right panels, predictions were performed at specific azoxyglycoside (AZG) toxin concentrations, 100, 200 and 400 µmol·g $^{-1}$. Open circles are the actual observations; the same data is used in all six panels.

Table 2.5. Analysis of deviance of binomial GLM predictors

Predictor	Residual Degrees of freedom	Residual Deviance	F	Pr(>F)
Leaf age	63	395.33	12.8926	<0.001
Exposure time	62	394.76	0.1054	0.7466
Work to fracture	60	275.59	3.0535	0.0858
Trichomes	59	260.82	2.6983	0.1058
AZGs	61	292.31	18.7172	<0.001

2.5. Discussion

Understory plants are limited by the amount of light that penetrates the canopy, therefore, they grow slowly and produce few leaves (Coley et al. 1985; Kikuzawa and Lechowicz 2011). In general, herbivory on tropical forest understory plants is highest on young leaves that have not toughened completely (reviewed in (Coley and Barone 1996; Coley and Kursar 1996). This study examined leaf traits of the slow-growing neotropical cycad, *Z. stevensonii,* under the pressure of several highly specialized herbivores. Although the time scale of leaf expansion is considerably longer, the defensive syndrome we observed in this gymnosperm parallels that of understory angiosperm trees that exhibit delayed greening of leaves (Kursar and Coley 1992a; Kursar and Coley 1992b; Kursar and Coley 2003; Poorter and Bongers 2006). Thus, our results are consistent with the resource availability hypothesis, that predicts that under high risk of predation, slow-growing species will invest heavily on constitutive defenses (Coley et al. 1985). This, therefore, highlights the convergence of defensive syndromes by major plant lineages.

The leaves of *Z. stevensonii* are fully stocked with chlorophyll after they reach full toughness and have passed through the period of intensive herbivory (Fig. 2.2E). Chlorophyll content reflects photosynthetic capacity and nitrogen status of the leaf. Delaying chlorophyll synthesis until after mechanical defenses are developed may allow the plant to minimize loss of resources (Kursar and Coley 1992a; Kursar and Coley 1992b; Kursar and Coley 2003).

The herbivory rates we observed are moderately high compared to those measured for tropical understory dicots by Kursar and Coley (2003) who defined herbivory levels of less than 20% as low and of more than 60% as high. In the two years of our study 33 - 41% of leaflets were damaged by herbivores. More than 40% of the leaves are at least partially damaged during leaf expansion. Herbivory by specialist insects begins approximately ten days after leaf flush and then drops drastically at approximately 100 days. Two important components of leaf toughness, lamina thickness and resistance-to-fracture, are close to their maximum by 100 days. Lamina thickness (mean = 0.25 mm, SD = 0.09) and resistance to fracture (mean =

3.28 $MN \cdot m^{-2}$, SD = 0.53) exhibited by mature *Z. stevensonii* leaves are higher than in shade leaves of 177 woody angiosperm trees meta-analyzed by Onoda et al. (2011; Lamina thickness mean = 0.187; Force to punch mean = 2.35 $MN \cdot m^{-2}$). *Zamia stevensonii* leaves are tough enough to limit herbivory: it takes too much energy to eat tough leaves, or to make mandibles that are not worn out by eating them (Hochuli 1996). Clark et al. (1992) found that first instar larvae of *Eumaeus minyas* Hübn. can only feed on *Zamia skinneri* if the leaves are younger than 65 days, while the later instars are able to eat mature leaves. Similarly, the first instar *E. godartii* cannot eat tough, mature leaves; when new foliage is not present, the caterpillars may engage in cannibalism until their mandibles are strong enough to pierce the toughened leaves (Castillo-Guevara and Rico-Gray 2002). Therefore, leaf toughness at 100 days of age may substantially reduce *E. godartii* populations and pose a formidable barrier to *A. appendiculata* herbivory (Clark and Clark 1991).

Specialist herbivores exhibited spatial heterogeneity in their occurrence, some plants were heavily attacked while a neighboring plant that had a similar flush of new leaves remained undamaged. Spatial heterogeneity of cycad herbivores has been reported for the armored scale, *Aulacaspis yasumatsui* Takagi, infesting *Cycas micronesica* K.D. Hill and *C. revoluta* Thunb. in Guam, where a healthy plant can be growing next to a heavily infested plant (Marler 2013). While this aggregation pattern can be expected for insects with reduced mobility, it is surprising it applies to mobile adult aulacoscelidine beetles. Several chrysomeloid beetle species are known to aggregate due to a blend of plant derived volatiles and pheromones released by the males (Beran et al. 2011; Dickens et al. 2002). The chemical cues involved in the aggregation of *A. appendiculata* are unknown.

Presumably because of the presence of highly toxic AZGs, most insect herbivores are unable to feed on *Zamia* species. Clark and Clark (1991) found that only cycad specialists feed on *Z. skinneri* in Costa Rica. In our study, we found three highly specialist insects use *Z. stevensonii* as their host plant. Specialist herbivores, such as adult *A. appendiculata* beetles and *E. godartii* caterpillars, are voracious and can damage an entire leaf flush of *Z. stenvensonii* in one day. These specialists

sequester and use cycad AZGs for their own defense (Prado et al. 2011; Prado et al. 2012; Rothschild 1992; Schneider et al. 2002). *A. appendiculata* sequesters AZGs from *Z. stevensonii* and then releases the compounds as a component of defensive secretions from the joints when the insect is disturbed (Prado et al. 2011, Chapter 6). So how do leaves less than ten days of age escape herbivory by these insects that sequester AZGs? Our data shows that specialist herbivores feed on cycad leaves containing few trichomes and AZG levels in the range of 10-480 $\mu mol \cdot g^{-1}$ DW, but do not feed on youngest leaves, that are covered with trichomes (>20 per mm^2) and contain AZG levels above 500 $\mu mol \cdot g^{-1}$ DW. This suggests, that specialist herbivores are unable to feed on young pubescent leaves with high AZG levels. However, an alternate hypothesis is that the absence of insect herbivory on the youngest leaves is a result of the brief amount of time they are exposed to herbivores. This may explain the pattern of *E. godartii* herbivory as the adult female butterfly anticipates new leaf production and lays its eggs on *Z. stevensonii* trunks, old leaf bases, and occasionally on the new emerging leaves. The delay until eggs hatch could explain the absence of herbivory on the youngest leaves. However, after defoliating a plant completely, *E. godartii* caterpillars can move to a neighboring plant that may have newly produced leaves (pers. observations; Clark and Clark 1991). Furthermore, a small forest plot has cycad leaf flushes of many ages, *E. godartii* caterpillars and *A. appendiculata* adults have a range of leaf ages from which to choose. Finally, our generalized linear model predicted that highest AZG levels are negatively correlated with herbivore damage whereas exposure time and trichomes did not significantly affect herbivory (Table 2.5, Fig. 2.4). In fact, most herbivore damage occurred on leaves 20-30 days old, when AZG levels were between 50-200 $\mu mol \cdot g^{-1}$ DW. Therefore, taken all together our data suggest that in the new flush of *Z. stevensonii* leaves, high toxin levels protect the plant against generalist and specialist insects. As the leaf expands, relative AZG concentrations decrease and specialist insects are now able to cope with AZG levels and may even be attracted by them. Similar observations have been shown in other plant-specialist insect systems where specialist herbivores are not completely immune to the plants' toxins, but rather are capable of tolerating

moderately high doses (Adler et al. 1995; Ali and Agrawal 2012; Baldwin 1988; Berenbaum et al. 1989). However, we cannot dismiss the possibility that specialist herbivores may be avoiding young leaves for reasons not accounted in our study. Cycads produce a plethora of unknown compounds that potentially could be mediating herbivore interactions (Snyder and Marler 2011). Furthermore, insects could be choosing leaves with higher nutritional quality. To differentiate between these hypotheses, behavioral choice assays comparing beetle and caterpillar responses to foods of different nutritional quality and AZG concentrations are needed.

If AZGs do deter specialist herbivores at high concentrations, why does the plant refrain from maintaining high levels throughout development? Are energetic costs associated with AZG biosynthesis limiting or are there physiological restrictions associated with biosynthesis or storage? Considering the extreme reactivity of the aglycone of AZGs, they may well represent a source of autotoxicity (Baldwin and Callahan 1993). Where AZGs are stored in the leaf remains unknown. AZGs are not stored in trichomes since the trichomes are nonglandular and even after they are lost (≈3 weeks), leaves still contain significant AZG levels (Fig. 2.2H). Norstog and Fawcett (1989) proposed that AZGs are compartmentalized in idioblasts in the male and female cones; it is unknown if these compounds are also present in leaf idioblasts. Compartmentalization of AZGs could limit their contact with endogenous glycosides that are needed to convert the compound to a reactive form but could also limit their accumulation. These observations also beg the question whether intense herbivory selects for higher AZG levels.

2.6. Acknowledgments

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Connecting statement

Chapter 2 evaluated changes in *Zamia stevensonii* leaf traits during leaf development and how traits correlated with herbivore incidence. The study provided us with insights into the potential role that AZGs play in the defensive strategy of cycads. However, we do not know if AZGs are induced upon wounding or if herbivore pressure is selecting for higher AZG levels. Chapter 3 addresses the question if AZGs are induced upon mechanical wounding. We performed this study using a commercially available Neotropical cycad *Zamia furfuracea*. This manuscript is under preparation for submission to the Journal of Chemical Ecology as a "Rapid Communication".

3. Mechanical wounding does not induce azoxyglycoside production in the cycad *Zamia furfuracea*

3.1. Abstract

Defenses against herbivores are costly; thus, plants must carefully manage the allocation of their resources. Deploying defenses only when needed is a common and often advantageous strategy. Mechanical damage of leaves results in the production of toxic metabolites, both locally and systemically in several plant species. Cycads, an ancient lineage of gymnosperms, are protected by azoxyglycosides (AZGs), an unusual class of nitrogen-based metabolites that are highly toxic. Due to these compounds, only a few specialist herbivores can cope with cycad toxicity. We measured leaf AZG content locally and systemically after mechanical wounding in two-year-old *Zamia furfuracea* plants. AZG levels remained stable locally and systemically 3, 7 and 15 days after wounding. Our results support other lines of evidence indicating that AZGs are allocated to the young leaf primordia and not replenished during the life of a leaf.

3.2. Introduction

Plants strongly invest in defenses against herbivores (Hochuli 1996; Kessler and Baldwin 2002). Their vulnerability arises from their sessile nature and, in most cases, constant exposure to pest and pathogens. However, defenses are costly and plants must manage the allocation of resources to growth and reproduction or defense (Bennett and Wallsgrove 1994; Coley 1988; Kursar and Coley 2003; Lan et al. 2014; Purrington 2000; Simms 1992). In addition, defensive compounds are often stored and concentrated in specialized structures to prevent autotoxicity (Baldwin and Callahan 1993; Purrington 2000; Vovides 1991a). Therefore, there are significant costs associated with the production and storage of defensive compounds, particularly if resources, such as nitrogen or light are limiting (Baldwin 1998; Coley et al. 1985; Loreau and de Mazancourt 1999; Purrington 2000; Simms 1992). The constitutive presence of toxic compounds puts a continual selection

pressure on insect herbivores to evolve adaptive strategies to avoid or detoxify them (Baldwin 1998; Kessler and Baldwin 2002). Therefore, in addition to constitutive defenses, plants also activate the biosynthesis of defensive compounds in response to herbivory (Baldwin 1988; Bartlet et al. 1999; Doughty et al. 1995). Induction of the defensive compounds can occur locally (i.e. on the same leaf or on the same branch) or also distally/systemically (Baldwin 1998; Moreira et al. 2009).

Induced defensive responses have been identified in many conifer species (Martin et al. 2003; Miller et al. 2005; Moreira et al. 2014; Phillips and Croteau 1999). Beetle and fungal infestation leads to a defensive response involving anatomical and biochemical changes that increase the biosynthesis of oleoresin in *Abies*, *Picea* and *Pinus* species (Christiansen and Krokene 1999; Franceschi et al. 2005; Lewinsohn et al. 1991). Similar defensive response can be observed after mechanical wounding or with the application of exogenous jasmonates (Franceschi et al. 2002; Lewinsohn et al. 1991; Moreira et al. 2014; Moreira et al. 2009). Although jasmonate treatment can have additional responses; jasmonate application and wounding are usually considered similar but not identical treatments (Miller et al. 2005; Purrington 2000).

Cycads (Order Cycadales) are dioecious gymnosperms that protect their tissues from herbivory with azoxyglycosides (AZGs) (De Luca et al. 1980; Engel et al. 2003; Yagi 2004). AZGs are dipolar *N*-oxides that readily alkylate DNA, RNA and proteins (Laqueur and Spatz 1968; Smith 1966; Spencer et al. 2012). The high toxicity of these compounds has been demonstrated in bacterial, animal and plant cells (Fiala and Stathopoulos 1984; Matsushima et al. 1979; Smith 1966). In the plant, these compounds presumably play a protective role; their toxicity is only overcome by a few specialist insect herbivores (Rothschild et al. 1986; Schneider et al. 2002). Therefore, AZGs are effective deterrents of generalist herbivores and at high concentrations (>400 $\mu mol \cdot g^{-1}$ DW) may even deter specialists (Prado et al. 2014). The highest concetrations are found in the young leaves (Prado et al. 2014; Yagi et al. 1983). In fact, AZG levels in young leaves are almost 300 times higher than in mature leaves (Prado et al. 2014). However, it remains unknown whether AZGs

are induced in cycads in response to wounding. Here we report that AZGs in the leaves of the cycad *Zamia furfuracea* are not locally or systemically induced 3, 7 and 15 days after mechanical wounding.

3.3. Methods

3.3.1. Plants

Zamia furfuracea L. fil. is a lowland costal species from the gulf of Mexico characterized by a short-arboresent or subterranean stem and scruffy leaves covered with rusty brown hairs (Jones 2002; Whitelock 2002). This tough and hardy species grows in sparse coastal scrub and savanna where it experiences strong seasonal periods of aridity and salt spray (Whitelock 2002). When mature, *Z. furfuracea* has compound leaves composed of 5-20 pairs of leaflets (Jones 1998). The main leaf herbivore of *Z. furfuracea* is the cycad specialist *Eumaeus toxea* Godart (Lepidoptera: Lycaenidae) (Martínez-Lendech et al. 2007).

One-year-old *Z. furfuracea* individuals were purchased from the Jurassic Plants Nursery, B.C., Canada. Seedlings were transplanted and kept in a growth chamber for one year in separate pots with the following conditions: 14 hr light, 10 hr dark, 250 $\mu moles \cdot m^{-2}S^{-1}$. The plants had been grown by the nursery from a batch of seeds originally obtained from the Montgomery Botanical Garden, Miami, Florida. The two-year-old plants had between 2-6 leaves consisting of 1-3 pairs of leaflets with an average rachis length of 12.4 cm. Six plants were randomly assigned to each of seven treatments; control (0, 3, 7 or 15 days) or wounded (3, 7, 15 days). Mechanically damaged plants were pierced three times with a paper punch on the second leaflet from the apex of the leaf. Approximately 5% of the leaf tissue was removed. Leaflets were harvested at 0, 3, 7 and 15 days after the beginning of the experiment. From each wounded plant, three samples were collected and analyzed separately: 1) the wounded leaflet (local), 2) its opposing leaflet (local-systemic), and 3) the second leaflet from the apex of a different leaf (systemic).

3.3.2. Azoxyglycoside extraction and quantification

Azoxyglycoside extraction was performed according to Prado et al. (2011) and Prado et al. (2014). Briefly, leaflets (100-200 mg) were ground in liquid nitrogen and extracted in chilled 70% ethanol. After incubation on ice for 20 min, samples were centrifuged at 20,000 rpm for 10 min and the supernatant was lyophilized. Samples were re-suspended in 25 mM sodium acetate buffer, pH 5.0 conatining a β-glucosidase (Sigma G4511, 0.5 U). β-glucosidase converts AZGs to the methylazoxymethanol (MAM) core. Samples were analyzed by HPLC on a reverse-phase Hi-Plex Na (Octo) column (300 x 7.7 mm, Agilent) using an isocratic elution of 100% water at a flow rate of 0.45 mL/min at 65°C (Yagi et al., 1980; Yagi et al., 1983; Yagi, 2004). Compounds were detected by a photodiode array detector at 215 nm. MAM was identified based on its retention time and spectra. MAM acetate (National Cancer Institute) was converted into MAM by incubation with an esterase enzyme (Sigma) in 1M Tris-HCl buffer, pH 8.5 for one hour at room temperature and used to generate a 7-point standard curve. MAM concentrations were convereted to AZG equivalents.

3.3.3. Data Analysis

A linear model was fitted for AZG content ($\mu mol \cdot g^{-1}$ FW) using treatment and leaflet (local, local-systemic or systemic) as the explanatory variables. A Shapiro-Wilk test was performed to assess normality of the residuals of the model. As the residuals were not normally distributed, pairwise comparisons between each treatment and controls were performed using both a Kruskall-Wallis test and a Wilcoxon-Mann-Whitney test under the alternative hypothesis that wounded leaflets would have a greater AZG content (one-sided). Local, local-systemic and systemic samples were analyzed independently.

3.4. Results

AZG levels in the *Z. furfuracea* leaflets range from 0.07-5.7 μmol·g^{-1} of fresh weight (Fig. 3.1). AZG concentrations in local, local-systemic and systemic samples after 3, 7 and 15 days are not different from the control groups. Pairwise comparisons analyzed by the Kruskal-Wallis test or the Wilcoxon-Mann-Whitney one-sided test do not show a significant difference in AZGs in the experimental groups compared to controls (Table 3.1).

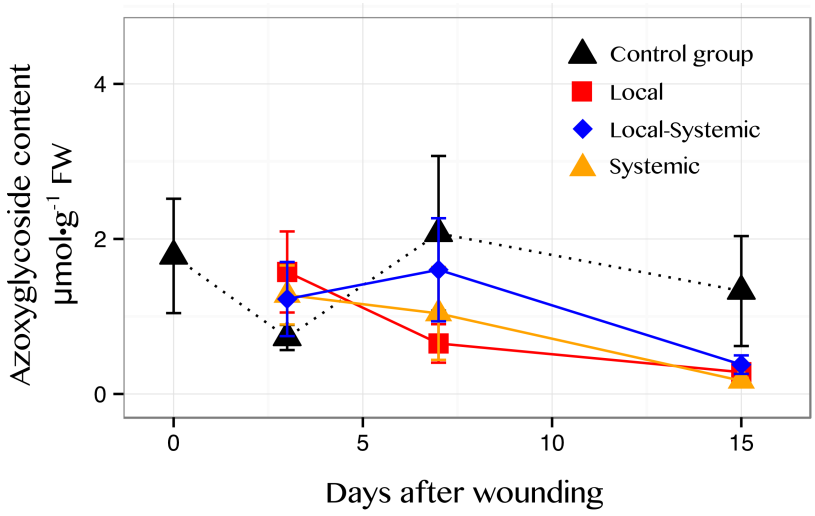

Fig. 3.1. Azoxyglycoside content in relation to wounding. Points represent the mean in AZG concentration +/- standard error.

Table 3.1. *P*-values of Kruskal-Wallis test and Wilcoxon-Mann-Whitney one-sided test (bold) of AZG content in controls versus wounded plants.

	Days after wounding		
	3	**7**	**15**
Local	*0.1229*	*0.1252*	*0.4233*
	0.07449	**0.9441**	**0.803**
Local-Systemic	*0.4062*	*0.3472*	*0.0707*
	0.2279	**0.8452**	**0.9704**
Systemic	*0.848*	*0.3718*	*0.715*
	0.4508	**0.8283**	**0.6688**

3.5. Discussion

AZG levels in the mature leaves of two-year old *Z. furfuracea* plants (1.33 ± 1.70 μmol·g^{-1} FW; mean ± SD) are higher than those in the mature leaves of adult plants in *Z. stevensonii* (0.45± 1.63 μmol·g^{-1} FW; mean ± SD)(Prado et al. 2014). This could possibly illustrate species-specific differences or the potential ontogenetic changes in the chemical defense of leaves. AZGs levels in the leaves of the cycad *Dioon edule* are higher in seedlings as compared to adults (Prado et al. unpublished). The levels reported for *D. edule* (Chapter 4) were obtained by sieving out leaf fibers and using a double extraction (Bernays 2001), hence they cannot be compared directly to those reported for *Z. stevensonii* and *Z. furfuracea*. Nontheless the levels recovered for seedlings of *D. edule* ranged form 7-12 μmol·g^{-1} FW. Yagi (2004) recovered levels ranging from 3.9 – 79.29 μmol·g^{-1} (transformed from percentage of fresh weight using the molecular weight of cycasin) from the leaves of 32 cycad species. All together these results suggest strong species-specific variations of the AZG content.

Fifteen days after mechanical damage, changes in AZG content are not observed in *Z. furfuracea* leaves. These results support those of Yagi et al. (1983) and Prado et al. (2014) that suggest AZGs are not replenished during the life of a leaf. Young leaves have high AZG concentrations that are diluted as the leaf expands and toughens. Toughness appears to be the main means of defense against both

specialist and generalist herbivores in mature leaves (Prado et al. 2014). Therefore, AZGs might only be important in the protection of the young, rapidly-expanding foliage.

However, the timeframe that was used to collect the samples might have not been the adequate for a slow growing gymnosperm, such as *Z. furfuracea*. Moreira et al. (2009) inspected changes in resin and phenolic concentrations two months after methyl jasmonate treatment in *Pinus pinaster*. Miller et al. (2005) inspected changes in terpenoids only 20 days after insect wounding or methyl jasmonate treatment in *Picea sitchensis*. Therefore, a longer timeframe after wounding might be needed to observe an induced response. Also, as cycads grow by pulses of leaf flushes, induction of AZGs could be delayed to the subsequent leaf flush. It is unknown if AZGs are produced by the leaf primordia or allocated to them at an early developmental stage. Delayed induction of AZGs in response to wounding should be assessed by recording changes in the subsequent leaf flush.

Connecting statement

Chapter 2 focused on adult *Zamia stevensonii* individuals in central Panama. However, the highest cycad mortality rates usually occur at the seedling stage. This is true for the Mexican cycad *Dioon edule,* which exhibits an inverse "J" population structure with high mortality during the first years of life. This implies that cycad seedlings are vulnerable and hence, might invest more heavily in chemical defenses. Therefore, in Chapter 4 we measured leaf AZG levels in different ontogenetic stages of the long-lived cycad *D. edule*. In this study, we considered plant ontogeny, sex and genetic background as possible predictors of AZG levels. The manuscript is co-authored by Laura Yañez-Espinosa and her student Gabriel Rubio-Méndez at the Instituto de Investigaciones de Zonas Desérticas (IIZD) of the Universidad Autónoma de San Luis Potosí; L. Yañez-Espinosa and her student G. Rubio-Méndez previously characterized these cycad populations.

4. Effects of plant ontogeny, gender and genotype on leaf azoxyglycoside content in *Dioon edule* Lindl.

4.1. Abstract

Throughout their life, plants use multiple strategies, including phytochemicals, to protect their most vulnerable tissues, often young leaves and reproductive structures, from pathogens and herbivores. *Dioon edule*, like all cycads, contain highly toxic azoxyglycoside (AZG) metabolites; however, ontogenetic variability of these compounds in this long-lived Mesoamerican cycad (ca. 2000 years) is unknown. Here, we investigate the effects of plant age, sex and genetic background to AZG levels in the mature leaves of wild *D. edule* populations from eastern Mexico. Individuals were divided into three ontogenetic stages: seedlings, juveniles and adults. The genetic structure of these populations was determined using microsatellite markers. AZG levels do not correlate with plant genotype and individual heterozygocity. The genetic analysis identified a distinction between lowland and highland individuals. Within these two groups, AZG levels were higher in the leaves of lowland individuals. AZG levels decrease with plant age: The highest foliar AZG levels are in the seedlings, followed by juveniles and then adults that contain the lowest AZG levels. These results suggest that plant priorities change with age. Cycad protection of leaves reflect the photosynthetic importance of leaves in younger plants, particularly given their potential long lifespan. Female adult plants have higher AZG levels compared to males, suggesting that the defense of leaves could be linked to the costs of reproduction.

4.2. Introduction

Plants use multiple strategies to protect themselves against insect herbivores, including secondary metabolites that act as deterrents or toxins (Rhoades 1979; Schoonhoven et al. 2005). Both the biosynthesis and storage (if necessary) of these phytochemicals, uses valuable resources that could otherwise be directed towards growth and reproduction (De Luca and St Pierre 2000; Stamp 2003). Therefore,

there are trade-offs between the metabolic flux into growth or defense, and the resource allocation to these compounds often changes throughout a plant's ontogeny reflecting shifts in developmental and reproductive priorities (Boege and Marquis 2005; Bowers and Stamp 1993; Van Dam et al. 2001). Two main processes associated with plant ontogeny have been identified that influence resource allocation: changes in plant size (i.e. increase in the resource acquisition organs like roots and leaves), and shifts from growth to reproductive priorities (Boege and Marquis 2005; Weiner 2004). Therefore, to understand a plant's chemical defensive strategy, one must take into account its ontogenetic trajectory.

Depending on the plant species, phytochemical levels often fluctuate reflecting their defensive strategy, reproductive status and life history traits (Boege and Marquis 2005; Stamp 2003). Contrasting studies have found that depending on the plant species, defensive secondary metabolites may increase or decrease as plants age (Boege 2005; Bowers and Stamp 1993; Fritz et al. 2001; Goodger et al. 2004; Macedo and Langenheim 1989; Reichardt et al. 1984; Schappert and Shore 2000; Van Dam et al. 2001; Wallace and Eigenbrode 2002; Wolfson and Murdock 1990). One theory to account for these apparent differences is that there are trade-offs between the costs associated with the specific secondary metabolite and the benefits in producing these compounds at particular ontogenetic stages. The Optimal Defense Hypothesis states that defenses are costly and plants must optimize resources allocated to defense in a way that will maximize their fitness (Rhoades 1979; Rhoades and Cates 1976). Furthermore, within an organism, the hypothesis predicts that defenses are allocated in direct proportion to the risk of the particular tissue and the value of that tissue in terms of fitness (Rhoades 1979). This is supported by evidence of the costs associated with secondary metabolite production and the tradeoffs between growth and reproduction *vs*. defense (Gershenzon 1994a; Gershenzon 1994b; Lan et al. 2014; Strauss et al. 2002; Vrieling and van Wijk 1994). Given their importance, the production of many defensive phytochemicals is a highly controlled process by which plants can limit the amount of resources that are allocated to chemical defense (De Luca and St Pierre 2000).

Therefore, as physiological requirements and priorities change with ontogeny, we expect changes in the investments to chemical defense (Boege and Marquis 2005).

As well, reproductive strategies may affect resource allocation into plant defenses; gender-related phytochemical differences are described for several dioecious species (Lloyd and Webb 1977; Obeso 1997; Obeso 2002). Due to the higher investments associated with female reproduction, males dioecious plants tend to grow faster than females (Ataroff and Schwarzkopf 1992; Lloyd and Webb 1977). For example, slow-growing females of the dioecious tree, *Acer nugundo*, invest more in the protection of their leaves and are more resistant to insect herbivory compared to faster-growing males (Jing and Coley 1990). Plant genotype can also impact phytochemical levels (Kliebenstein et al. 2001; Wimp et al. 2007). Spatial variations in phytochemical defense reflect genetic background as well as the genetic diversity (i.e. inbred vs outbred) (Kariyat et al. 2013; Strauss et al. 2002). Recombination levels and allele segregation, which are related to sexual reproduction, may influence a plant's susceptibility to insect herbivores (Ashman 2002; Johnson et al. 2009; Kariyat et al. 2013; Levin 1975). Several studies have shown that inbreed plants are more susceptible to herbivory than outbreeds (Bello-Bedoy and Núñez-Farfán 2011; Johnson et al. 2009; Kariyat et al. 2013). In addition, environmental factors such as altitude may influence secondary metabolite content in some plants species (Ganzera et al. 2008; Spitaler et al. 2006). Erelli et al. (1998) measured lower leaf tannins and a better insect performance on high elevation *Betula papyrifera* individuals. While Rasmann et al. (2014) measured higher flavonoid levels at high elevations but found no clear trend for other secondary metabolites.

Cycads are an interesting model plant to investigate ontogenetic changes in chemical defense; they are a dioecious, extremely long-lived species that periodically produce flushes of long-lived leaves (a leaf life time of up to 10 years) (Norstog and Nicholls 1997). As well, cycads pulses of leaf flushes are intercalated with coning events in both males and females (Norstog and Nicholls 1997). Different reproductive costs between males and females have been identified in a few cycad

species (Clark and Clark 1988; Ornduff 1987; Vovides 1990). Clark and Clark (1988) found that the costs associated with female reproduction heavily depressed leaf production (Clark and Clark 1988). These costs are also evident by the differences in coning frequencies between males and females.

Dioon edule Lindl. (family Zamiaceae) is a medium-sized cycad endemic to eastern Mexico. *D. edule* populations exhibit an inverse "J" structure with high mortality during the first years of life. Therefore, only a small proportion of individuals reach sexual maturity and only a small fraction of these adults live up to an outstanding estimate of 2000 years (Rubio-Méndez 2010; Vovides 1990). Gender differences associated with reproduction costs are reflected in coning frequencies (Vovoides, 1990); 2.8 – 8.8 years in males compared to 10.0 – 52.0 years in females. Major environmental stresses for this species are prolonged drought, fire and insect herbivory (Mora et al. 2013; Vovides 1990; Yáñez-Espinosa et al. 2014). Generalist insect herbivores are not observed on cycads (Clark and Clark 1991; Prado et al. 2014). However, specialist herbivore insects that feed on *D. edule* include seed-boring erotylid beetles (previously ascribed to Languriidae) in the genus *Pharaxonotha* the leaf-feeding lycaenid caterpillars *Eumaeus debora* Hbn. and *E. childrenae* G. Gray and occasionally the aulacoscelidine beetle *Aulacoscelis vogti* Monrós (Mora et al. 2013; Vovides 1990; Prado et al. 2014; Yáñez-Espinosa et al. 2014). Cycad leaves are believed to be protected from generalist herbivores by highly toxic azoxyglycosides (AZGs) (Norstog and Nicholls 1997). Upon herbivory, plant or insect-gut glycosidases cleave AZGs to free the toxic aglycone, methylazoxymethanol (MAM) that alkylates DNA, RNA and proteins (Kobayashi and Matsumoto 1965; Laqueur and Spatz 1968; Spencer et al. 2012). The mutagenic and carcinogenic properties of AZGs are well documented and validated in bacterial, animal and plant models (Laqueur and Spatz 1968; Matsushima et al. 1979; Seawright et al. 1990; Smith 1966; Teas and Dyson 1967). However, this defense can be overcome by specialist insects that not only are able to survive on cycad leaves but, in some cases, sequester host-derived AZGs for their own defense (Bowers and Larin 1989; Castillo-Guevara and Rico-Gray 2003; Clark and Clark 1991; Nash et al.

1992; Prado et al. 2011; Prado et al. 2014). Even though, specialist herbivores have strategies to detoxify these compounds, it is proposed that in young leaves (<10 days) high AZG levels limit herbivory by these specialist insects (Prado et al. 2014).

Given that the Optimal Defense Hypothesis predicts that their are trade-offs in resource allocation between growth and chemical defense and that ontogeny, gender and genotype might have an effect on such trade-offs, the objectives of this research is to investigate AZG levels in the slow-growing, dioecious cycad, *D. edule*. Specifically, we focused on 1) determining if plant genotype and individual heterozygocity correlate with spatial variation in AZG levels, 2) monitoring ontogenetic changes in AZG levels in fully developed cycad leaves, 3) identifying differences in AZG between adult female and male cycads and 4) identifying other factors that could contribute to differences in AZG levels. We speculate that plant genotype correlates strongly with AZG production and outbreed individuals, those exhibiting a higher level of heterozygocity, would have higher AZG levels. We also expect spatial variation in AZG content to be related to genetic diversity. Based on our current understanding of *D. edule* life history, we also hypothesize that AZG levels are highest in younger plants to ensure protection of the leaves that are so critical for these long-lived plants. In adults, due to differences in coning frequency between males and females that may reflect reproductive costs, we hypothesize that AZG investments differ between genders.

4.3. Materials and Methods

4.3.1. Distribution of Dioon edule Lindl.

Dioon edule has a north-south distribution along the Sierra Madre Oriental, ranging almost 1,600 km. Distinct populations can be found in the States of Tamaulipas, San Luis Potosí, Querétaro, Hidalgo and Veracruz (González-Astorga et al. 2003; Mora et al. 2013; Vovides 1990; Whitelock 2004). The altitudinal range for this species is from sea level to 1,525 meters above sea level (masl), growing mostly in tropical deciduous thorn forest and oak forest, and rarely, in grasslands.

The study area is located in the southern part of the State of San Luis Potosí, an area known as Huasteca Potosina. The area is characterized by calcareous rock formations, calcareous and clayey-soils and a semi-warm humid climate (Puig 1976). Large stands of *D. edule* are found on the slopes and tops of mountains as part of the understory of tropical deciduous forests and oak forests (*Quercus laeta* Liebm). Sugar cane plantations in the valleys have almost completely replaced the native vegetation.

4.3.2. Sample collection

Leaves were collected from cycads found in a range from 388-1058 masl; tropical deciduous thorn forest occurred at the lower sites and oak forest at the higher sites (Table 4.1). Leaf samples were collected from seven different sites (Fig. 4.1) previously characterized demographically by Rubio-Mendéz (2010). These sites are spread across an area of approximately 2,750 km^2 (Fig. 4.1). At each location, 21 individuals, including 7 seedlings, 7 juvenile and 7 adults (N = 147), were chosen based on the presence of healthy leaves and a minimum of 20 m between adjacent samples. Ontogenetic stages were defined according to protocols of Rubio-Méndez (2010) and Álvarez-Yépiz et al. (2014). Seedlings were defined as individuals lacking an above ground stem bearing only one leaf. Juveniles are individuals lacking an above ground stem but producing crowns (flushes) of leaves of more than two leaves. Adults were defined as individuals that have a visible above ground stem and produce flushes of leaves composed of more than three leaves. When present, live or senesced reproductive structures were used to assign the gender of adult plants.

A geographical reference consisting on longitude, latitude and elevation was recorded for each sample collected. The total number of leaves, the length of the longest leaf, stem height, stem diameter and number of previous crowns of leaves were recorded. The number of previous crowns, a proxy for plant age (Vovides 1990), were counted using the number of girdling leaf traces on the trunks. A healthy fully toughened mature leaf was selected from each plant. The leaf was cut

into segments, keeping leaflets attached to the rachis and placed on silica gel flakes on ice.

4.3.3. Chemical analysis

The thick, waxy cuticle that characterizes *Dioon* leaflets can hamper the extraction of metabolites and DNA. Leaflets were lyophilized and ground in liquid nitrogen. The powdered leaflets were sieved through a 0.5 mm mesh to remove cuticle pieces and most fibers. The remaining green powder, thus, contained largely leaf mesoderm.

As Prado et al. (2014) found low AZG levels (≈0.46 μmol·g^{-1} FW) in mature leaves of the cycad *Zamia stevensonii*, the extraction protocol was modified to include a second extraction step. One hundred mg of mesoderm tissue was extracted in 1.5 mL of 70 % ice-chilled ethanol (EtOH) and vigorously vortexed using a tissue disrupter for 4 min. Samples were incubated on ice for 20 min and then centrifuged at 13,200 rpm for 10 min. The supernatant was transferred to a new vial. The pellet was re-suspended in an additional 1 mL 70% EtOH, vortexed and centrifuged at 13,200 rpm for 10 min. Supernatants were combined and frozen at -80°C and then lyophilized. Lyophilates were re-suspended in 25 mM sodium acetate buffer solution, pH 5, containing 0.5 U/mL β-glycosidase (Sigma) and incubated at room temperature overnight. β-glycosidase cleaves the sugar off the AZGs to generate the methylazoxymethanol (MAM) core. AZGs were quantified by MAM equivalents by HPLC following Yagi (2004) and Prado et al. (2011).

4.3.4. Genotyping Dioon edule

Genomic DNA was extracted using the DNeasy kit (QIAGEN) following the manufacturer's protocols with minor modifications. Leaflets were ground in liquid nitrogen and sieved through a 0.5 mm mesh to remove cuticle and fiber particles. The protocol was adjusted for the extraction of 200 mg of tissue. Eleven microsatellite markers for genotyping (Table 4.2); 4 markers described by

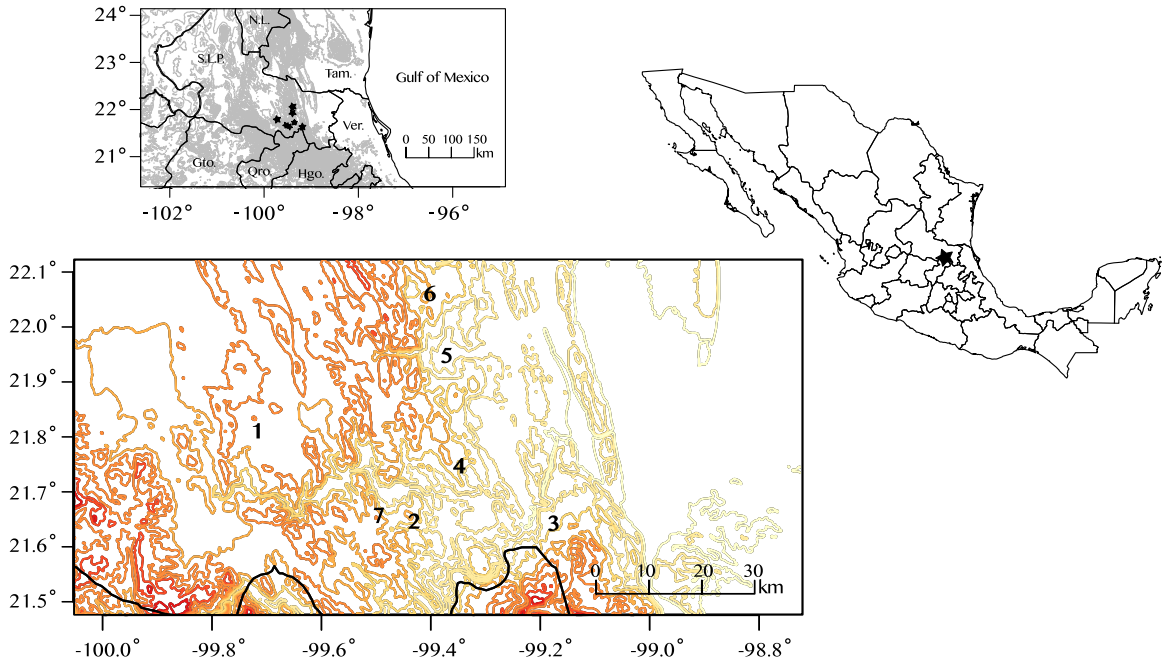

Fig. 4.1. Map of collection sites. Stars and numbers indicate locations where samples were collected. 1-Agua de Gamotes, 2-Los Anteojos, 3-El Chijol, 4-Los Pocitos, 5-Rincón del los Naranjos, 6-San Nicolas de los Montes, 7-Suacillos.

Table 4.1. Collection sites

Site	Forest type	Elevation range (masl)	Population density (ind/ha)*	Arborescent species associated*		
				Quercus laeta	*Brahea dulcis*	*Bursera simaruba*
Agua de Gamotes	Oak	999-1053	3325	x		
Los Anteojos	Oak	1039-1058	2700	x	x	
El Chijol	Oak	727-784	2150	x	x	
Los Pocitos	Oak	772-780	2100	x	x	
Rincón de los Naranjos	LTD	388-399	1075		x	x
San Nicolas de los Montes	LTD, Oak	834-944	-	x	x	x
Saucillos	Oak	889-893	3775	x		

Abbreviations: LTD, Low tropical deciduous forest.

* As described in Rubio-Méndez 2010.

Moynihan & Meerow (Moynihan et al. 2007) and 7 recently developed by Cibrain-Jaramillo at Mexico's National Laboratory for Genomics on Biodoversity (LANGEBIO, unpublished). Microsatellite amplicons were analyzed and scored on a Li-Cor 4300 DNA Analyzer using SAGA version 2.0.

Table 4.2. Microsatellite markers used to genotype *Dioon edule* individuals.

Locus	Primer sequence	Repeat motif	Allele range	Source*
2001304	Fwd: CTGGGCCTCGACATAACATT Rvs: TCAAAATCATTCCGGCTTTC	ATAA	179-203	LANGEBIO
2001597	Fwd: CTTACAAGCGGCACCATTG Rvs: AAGCAGGCCAGACTTCAGAC	TA	195-211	LANGEBIO
2001955	Fwd: CTGCCGAGGAGGGACA Rvs: CGCAGGGTTGGAGAGC	TGC	202-217	LANGEBIO
2002082	Fwd: TGACCTTGCCTTAGGTCAAAA Rvs: AGATGTGGGTGACACGTCCT	GT	230-266	LANGEBIO
2002349	Fwd: AGAGCTGCTTCCACGTTCAT Rvs: GCGGAAACTTCTTCAACAGC	TCT	368-386	LANGEBIO
2002757	Fwd: TGGGAAATGCACACCTAAAA Rvs: ACCTGGGCCACTTGAGG	CA	248-312	LANGEBIO
2003643	Fwd: CGAACTTGAAGACGATGACG Rvs: CGGGTAGCACCAAAGATTGT	GCA	222-246	LANGEBIO
Cap5	Fwd: CACTACCACCCCTATACCAC Rvs: GACTTGAGCTTGTCTTTGTTG	CT	225-241	Moynihan et al.
Ed3	Fwd: GCATGAGGAGCTTGTTCCGT Rvs: CTGTGAACTCCTGAAAGCATC	CT	123-127	Moynihan et al.
Ed9	Fwd: CCTTGTGTTACTTTGAGCACC Rvs: CAACAATGTAAGTGATGATGCC	CAT	244-268	Moynihan et al.
Tom5	Fwd: CGTTTCCATTGGAGAGACAAG Rvs: CCATCCAAGTGAGTGATACAAG	TC	224-226	Moynihan et al.

*Microsatellite markers were developed by Cibrián-Jaramillo (unpublished) at Mexico's National Laboratory of Genomics for Biodiversity (LANGEBIO) and by Moynihan et al. (2007) as specified.

4.3.5. Genetic analyses

Population genetic structure was assessed graphically using an agglomerative hierarchical clustering (AGNES) of the genetic distances of individuals using the package *Cluster* in R (Kaufman and Rousseeuw 2009), as well as a Principal Component Analysis (PCA) of a correlation matrix of the genotypes

using the package *adegenet* in R (Jombart 2008). To evaluate genetic isolation by distance (IBD), a Mantel test was performed between the geographic distance matrix and genetic distance matrixes using the package *vegan* (Dixon 2003).

4.3.6. Statistical analysis

As the distribution of AZG data was skewed (Shapiro-Wilk normality test, $p < 0.001$) and there was a heterogeneity of variance across age categories (Bartlett homogeneity of variance test, $p < 0.001$), a Kruskal-Wallis Rank Sum test was performed to test differences in AZG levels; first on the complete data set followed by multiple comparisons. The relationship between plant age and AZG content for males and females was analyzed by fitting linear models. Seedling and juvenile data were also included in both of these models.

Mantel tests were additionally used to reconcile genotype and leaf chemistry from genetic distance matrices and AZG distance matrices for levels from each ontogenetic stage. We also assessed the correlation between AZG content with individual heterozygocity (expressed as the proportion of heterozygous loci, PHt).

4.4. Results

4.4.1. Azoxyglycosides

AZG levels in mature *D. edule* leaves range from 1.52 to 26.51 $\mu mol \cdot g^{-1}$ fresh weight. These levels are comparable to other cycads. For example, *Z. stevensonii* mature leaves contain an average of 0.46 $\mu mol \cdot g^{-1}$ FW (Prado et al. 2014). Yagi (2004) found that leaves of 32 cycad species contain AZG levels ranging between 3.9–79.29 $\mu mol \cdot g^{-1}$ FW.

4.4.2. Population structure

The genetic structure of the populations is not related to the geographic distance. Eleven microsatellite markers and geographic coordinates were used to

assess isolation by distance. Mantel tests between the geographic distance matrix and a genetic distance matrix (by individuals or by site) did not indicate isolation by distance (Fig. 4.2). The maximum distance between the individuals sampled is ≈ 60 km. Overall, genetic diversity is relatively homogenous across study sites (Table 4.3). The effective number of alleles and observed heterozygocity (Ho) did not vary greatly ranging 2.644–3.783 and from 0.393–0.487, respectively. The fixation index (F) is highest in Saucillos (0.425) and lowest at the Rincón de los Naranjos site (0.160).

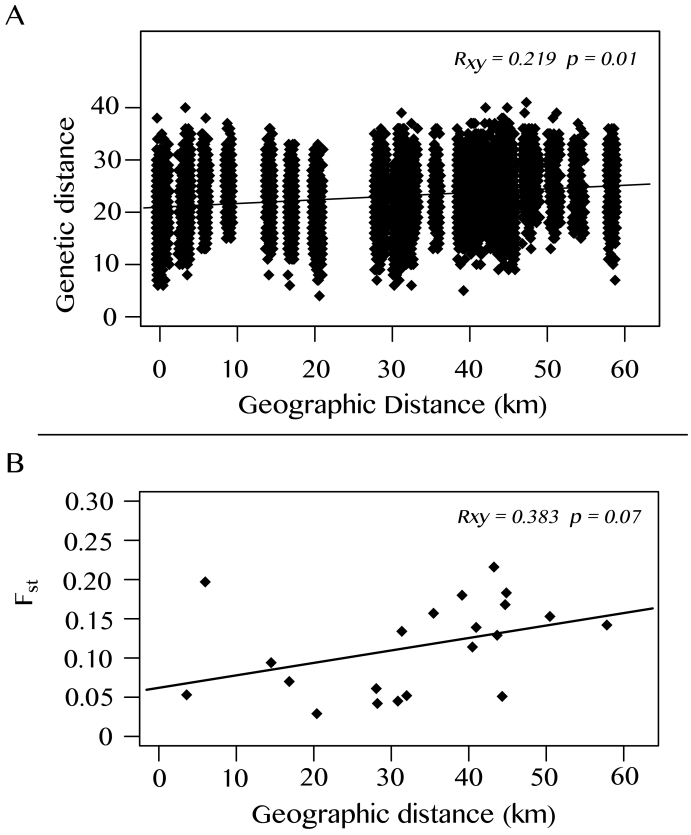

Fig. 4.2. Isolation by distance (IBD) plots. A) IBD by individuals, B) IBD by population sites looking at fixation index (Fst). Standardized Mantel correlation statistic (*Rxy*) and *p* values are shown.

Table 4.3. Genetic diversity of *Dioon edule* in the Huasteca Potosina.

Site	N	Na	Ne	I	Ho	He	uHe	F
Agua de Gamotes	21	4.636	2.644	1.022	0.393	0.514	0.555	0.306
Los Anteojos	21	5.182	3.783	1.329	0.487	0.656	0.687	0.281
El Chijol	21	5.182	3.491	1.280	0.423	0.644	0.667	0.307
Rincón de los Naranjos	21	5.182	3.466	1.218	0.481	0.595	0.613	0.160
Los Pocitos	21	4.818	2.945	1.143	0.464	0.582	0.602	0.223
San Nicolas de los Montes	21	5.455	3.371	1.139	0.457	0.560	0.597	0.205
Saucillos	21	4.727	3.126	1.182	0.382	0.612	0.632	0.425

Mean number of alleles per locus (Na), mean effective number of alleles (Ne), Shannon Information Index (I), observed heterozygocity (Ho), expected heterozygocity (He), unbiased expected heterozygocity (uHe) and fixation index (F).

Ontogeny and sex

A negative relationship is observed between plant age and AZG content with seedlings containing the highest AZG levels (Fig. 4.3A). AZG levels are lower in juveniles and were lower in adult plants. Even though there is no difference in the total number of leaves between female and male adult cycads (25.2 ± 2.3 *vs*. 22.7 ± 1.9, respectively), lower AZG levels are found in males compared to females (slopes of regression of -0.043 and -0.016 respectively, Fig. 4.3, B and C).

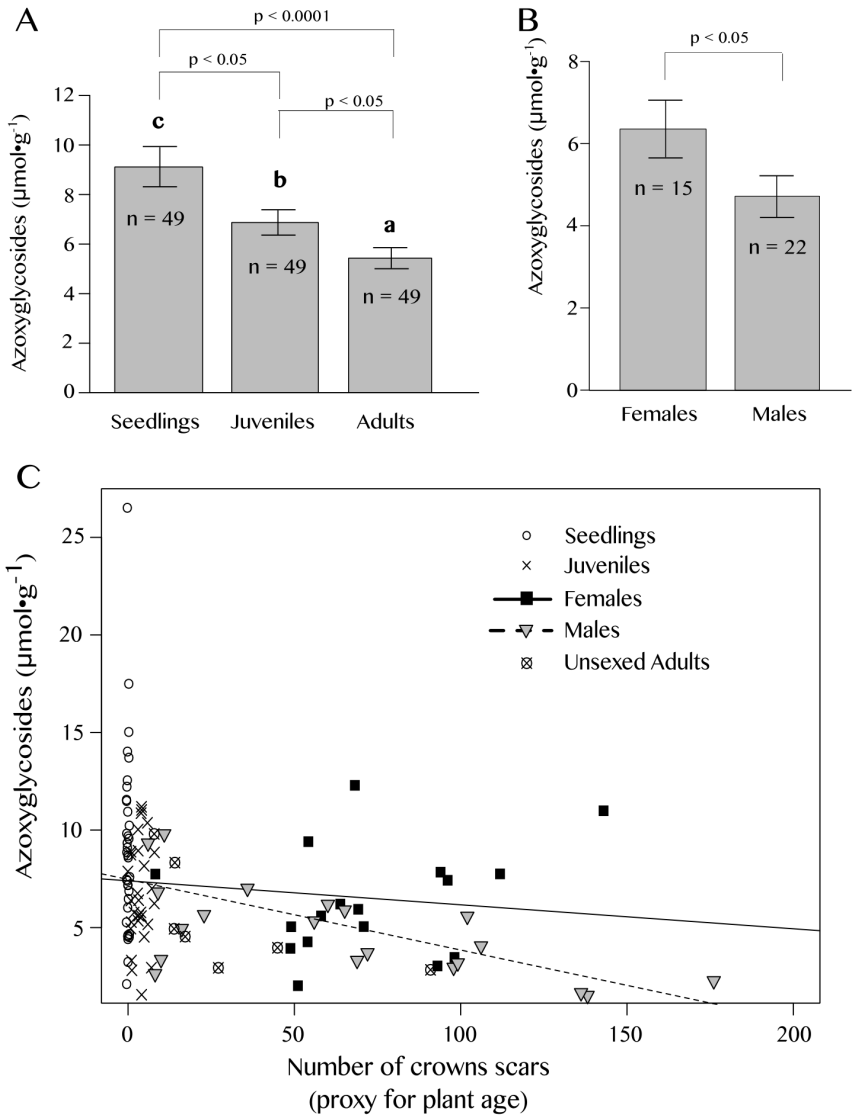

Fig 5.3. *Dioon edule* leaf azoxyglycoside levels. A) AZGs content at different ontogenic stages B) AZG content in adult females compared to males. Bars represent +/- standard error of the mean. In A, significant differences were determined using paired Kruskal-Wallis tests. In B, significant differences were determined using the Mann-Whitney-Wilcoxon test. C) Scatterplots of AZG levels and the number of cycad crowns that serves as a proxy for plant age. Linear models were fitted for adult female and male cycads.

The relationship between plant ontogeny and individual heterozygocity with AZG content was assessed using Mantel tests on the distance matrixes. Plant genotype correlates only weakly to AZG content in seedlings but not in adults or

juveniles (Table 4.4). The proportion of heterozygous loci did not correlate with AZG content in any of the ontogenetic stages.

Table 4.4. Mantel standardized *Rxy* values for the relationship between the genetics and azoxyglycoside levels.

	Genotype	Individual heterozygocity
All	0.1327**	-0.0121 NS
Seedlings	0.2167*	-0.0562 NS
Juveniles	0.1177 NS	-0.0238 NS
Adults	0.0029 NS	0.0898 NS

** $p < 0.01$; * $p < 0.05$; NS, not significant
Mantel *Rxy* values are based on 999 permutations

4.4.3. Altitude

The genetic structure of the population was further assessed through an agglomerative nesting (hierarchical clustering) analysis (AGNES), Principal Component Analysis (PCA) on the genotypes and a modeled-based clustering method using the software STRUCTURE 2.3.2 (Pritchard et al. 2000, Fig. 4.4, 4.5, Appendix 1). All three analyses suggest an incipient genetic differentiation between lowland (<800 masl) and highland sites (>800 masl). The AGNES and the PCA mostly grouped together El Chijol, Los Pocitos and El Rincon de los Naranjos sites, all below 800 masl. While the sites above 800 masl, Agua de Gamotes, Los Anteojos, San Nicolas de los Montes and Suacillos are grouped mostly together under both analyses. The output of the STRUCTURE analysis is included as Appendix 1.

Among the ontogenetic stages, the only difference in AZG content that reflect these altitude is found in adult cycads (Fig. 4.6); lowland adult cycads have 40% higher AZG content than the highland adults (6.5 ± 0.68 compared to 4.5 ± 0.36 $\mu mol \cdot g^{-1}$ respectively).

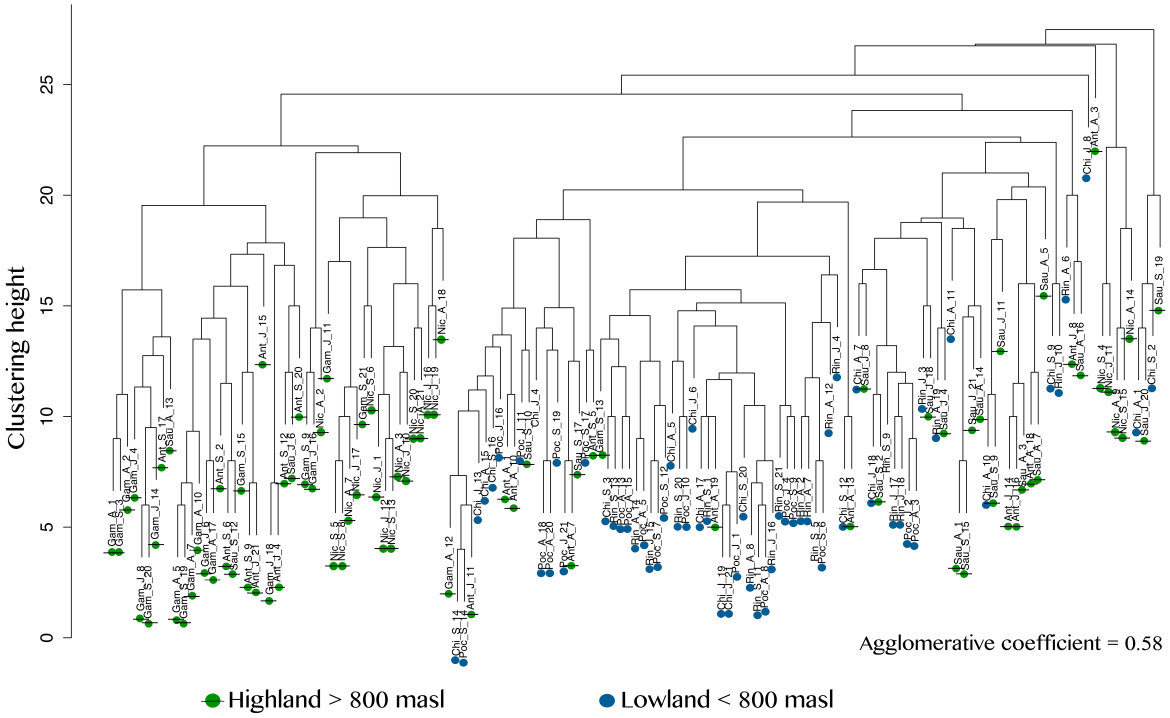

Fig. 4.4. Dendrogram of agglomerative hierarchical clustering (AGNES) of genetic distances.

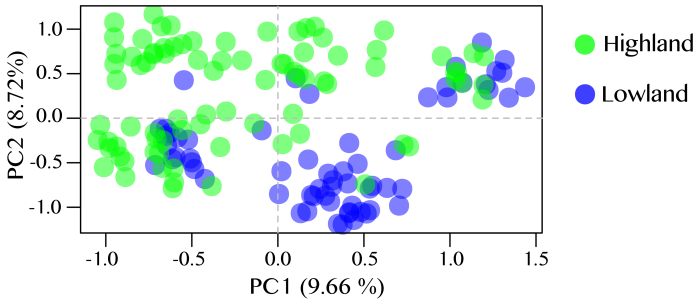

Fig. 4.5. Principal component analysis of *Dioon edule* cycad genotypes**.** Lowland sites are below 800 masl while highland are above.

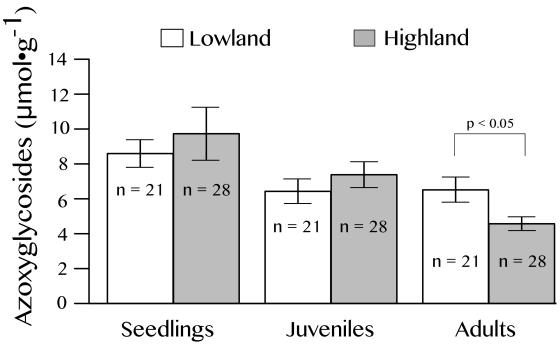

Fig. 4.6. Azoxyglycoside content in relation to ontogenetic stages in lowland and highland *Dioon edule* cycads. Bars represent the standard error of the mean. Lowland sites are below 800 masl while highland are above.

4.5. Discussion

Plant genetics did not explain the variation in foliar AZG levels observed in *D. edule*. AZG content is independent of individual heterozygocity at all ontogenetic stages. Plant genotype is only weakly correlated with AZG content of seedlings. Different results may be expected if specific genes related to AZG production (currently unknown) had been evaluated. The proximity of the sites is probably the reason why isolation by distance between sites is not observed. However, the limited number of markers used (11) as well as their neutral nature might also explain the lack of correlation between plant genetics and chemistry.

When studying phenotypic variability, Whitelock (2004) recognized two varieties and five distinct morphologies of *D. edule* (*i.e.* maximum size of stems, morphology and color of leaves, morphology of both female and male cones, color of sarcotesta, size and shape of sclerotesta). Our study examined individuals from the Huasteca Potosina, a small section of the large distribution of *D. edule*. Nonetheless, we found evidence of differentiation between lowland and highland individuals. This agrees to some extent with the distinct forms that Whitelock (2004) recognized as varying with altitude. The genotypic differentiation is matched by a difference in AZG content in adult plants, where lowland individuals produce higher levels of toxins compared to highland cycads. Since there is strong evidence of gene flow and consistent levels of genetic diversity across sites, the genetic and chemical variation

observed between highland and lowland sites is probably explained by different environmental pressures.

In *D. edule*, AZG foliar defenses decrease as plants age (Fig. 4.3, A and C); on average leaf AZG levels in seedlings have 68% higher levels than adults. As seedlings only have one or two leaves for the first 3-5 years, these results suggest that cycads invest more heavily in chemical protection at this stage compared to older plants.

In a study on leaf production and the cost of reproduction in the cycad *Zamia skinneri*, Clark and Clark (1988) found strong differences in reproduction costs between female and male cycads; leaf production in females is depressed for two years after coning implying higher costs associated with female reproduction (Clark and Clark 1988). As in *Z. skinneri*, the difference in the physiological costs of reproduction could be a determining factor in the frequency and quantity of leaf protection in *D. edule* (Clark and Clark 1988). Our results suggest that due to reproductive costs, females may also produce fewer leaves than males and invest more in the protection of those leaves. Male individuals that can produce leaves more frequently than females, could possibly withstand higher herbivory levels and, hence, invest less in defense. A few studies on dioecious woody angiosperms have found female individuals better defended against insect herbivores than male plants (Boecklen and Hoffman 1993; Danell et al. 1991; Jing and Coley 1990).

In our study, females and males showed no difference in the total number of leaves (25.2 ± 2.3 and 22.7 ±1.9, respectively). Compensatory mechanisms that alleviate the costs of reproduction have been identified in several dioecious species (Obeso 2002). Changes in assimilate demand can result in changes in photosynthetic rate (Obeso 2002; Wardlaw 1990). For example, Ginkgo females plants exhibit higher photosynthetic rates than males (Jing et al. 2008). It is unknown if a similar compensatory mechanism exists in cycads.

Our results suggest that *D. edule* regulates AZG production and that the benefits of their production changes as plant age and is different among sexes. Our results are compatible with the Optimal Defense Hypothesis; the consequences of

losing a leaf at the seedling stage represent a greater risk than losing a leaf at the adult stage, hence, seedlings are better defended. If costs associated with female reproduction, as opposed to male, are indeed higher, the same principle could apply to the observed gender differences in AZGs. The consequences of losing a leaf is higher for females as opposed to male plants. Future ecological studies of cycads should evaluate insect damage, preference and performance on different ontogenetic stages and sexes.

4.6. Acknowledgements

We thank Marc Hersh, Gabriel Gálvez, Andrés Everaert and Hugo "Lagarto" Castillo for assistance in the field and the laboratory. This research was funded through CONACYT (AP, GR-M and LY-E) and NSERC (JCB) grants.

Connecting statement

One of the protagonists of this thesis are the aulacoscelidine beetles that feed on the cycad foliage as adults. The following Chapter reports on plant AZGs sequestration by aulacoscelidine beetles and the possible role these compounds play in their defensive secretions. The name Aulacoscelinae was used rather than the correct name, Aulacoscelidinae. The reason for this inconsistency is that in 2010 when the manuscript was under preparation the debate on the correct name was remained unsettled.

5. Two genera of Aulacoscelinae beetles reflexively bleed azoxyglycosides found in their host cycads

5.1. 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 seques- tration of plant secondary metabolites appeared early in leaf beetle evolution.

5.2. 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.

5.3. Methods and materials

5.3.1. 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 vogti* 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.

5.3.2. Azoxyglycoside extraction and high performance liquid chromatography

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.

5.3.3. 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.

5.4. Results

5.4.1. 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.

5.4.2. 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 4.1). *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 5.1. Azoxyglycoside (AZG) content in complete adult specimens of three Aulacoscelinae species and their respective host cycad species.

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

[a]Whole body of adult insect. [b]Leaves.

5.4.3. 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 vogti*, 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. 5.1A). Mass spectroscopy identified a mixture of compounds with masses of 275.08, 286.09, 309.2, 337.06, and 437.14m/z, indicating that the major component of the sample is cycasin (275.08603m/z) with minor levels of macrozamin (437.14246m/z) (Fig. 5.1B).

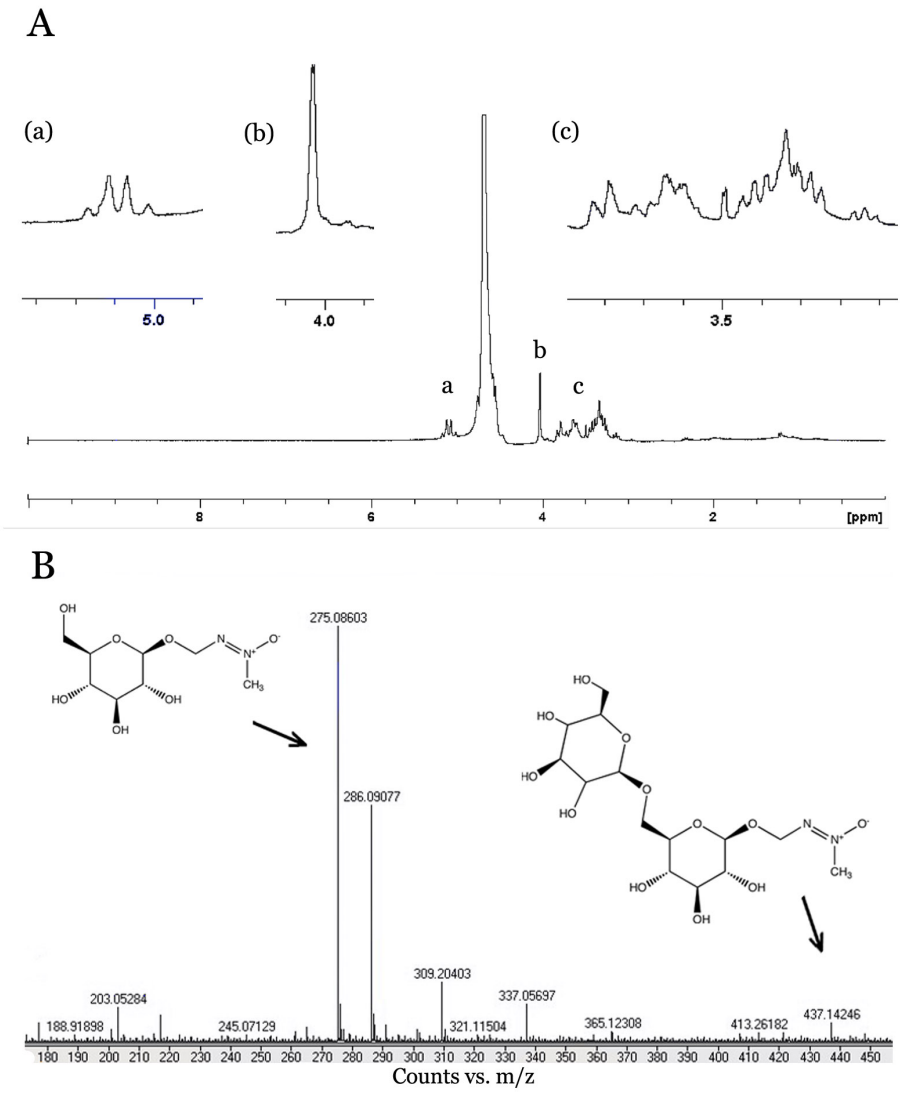

Fig. 5.1. Azoxyglycosides identified from the reflex bleeding of *Janbechynea paradoxa*. A) ^{1}H-NMR spectrum of cycasin in deuterated water; a) anomeric CH, b) CH_3 and c) other CH, CH_2 and OH. B) Accurate mass spectrometry of reflex bleeding; peaks corresponding to cycasin (275.09603 m/z) and macrozamin (437.14246 m/z) are identified.

5.5. 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. 5.1). 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 vogti*. 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 (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 2005). 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.

5.6. Acknowledgements

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Connecting statement

In Chapters 2 and 5, some information has been provided on the feeding habits of adult Aulacoscelidinae beetles and their relationship to the Zamiaceae. However, no information was available on the feeding habits of the immature stages. Chapter 6 reports on the occurrence of an aulacoscelidine larva found developing inside the female gametophyte of a cycad seed. The manuscript includes a morphological description, a phylogenetic reconstructions of the major lineages of the Chrysomeloidea and a discussion on the association of aulacoscelidines with cycads. This is the first report of the feeding habits of an immature aulacoscelidine.

6. Molecular evidence of cycad seed predation by immature Aulacoscelidinae (Coleoptera: Orsodacnidae)

6.1. Abstract

Adult beetles in the small subfamily Aulacoscelidinae (superfamily Chrysomeloidea) are known to feed on the foliage and juices of New World cycads (Order Cycadales; family Zamiaceae), but the habits of larvae have long remained a mystery. We provide the first direct evidence that Aulacoscelidinae larvae feed on and develop within the megagametophyte of the Mesoamerican cycad, *Dioon merolae* (Zamiaceae). Phylogenetic analyses based on partial DNA sequences from 3 genes recover a cycad seed-feeding larva proposed to belong to Aulacoscelidinae. These observations reveal a more intimate feeding relationship between Aulacoscelidinae and their New World cycad host plants than was previously recognized. Further, adult Aulacoscelidinae have long been noted to resemble Jurassic fossil chrysomeloids in the extinct subfamily Protoscelidinae. The molecular, morphological, ecological and fossil data reported herein are broadly compatible with an early association between Aulacoscelidinae and their gymnosperm hosts.

6.2. Introduction

The Phytophaga (130, 000 species), comprising the Chrysomeloidea and Curculionoidea, together contain close to 50% of phytophagous insect species and nearly 80% of phytophagous beetle species (McKenna and Farrell 2009). Most Phytophaga are associated with angiosperms, and their overwhelming taxonomic diversity may have resulted from colonization and radiation on the emerging flowering plants (angiosperms) during the Cretaceous (Crowson 1981; Duckett et al. 2004; Farrell 1998; Gómez-Zurita et al. 2007; McKenna et al. 2009). During the Jurassic, before the rise of flowering plants to ecological dominance, the ancestors of Phytophaga lived in forests composed of Pteridophyta, Ginkgoales, Coniferales,

Cycadales and †Bennettitales (Anderson et al. 2007; Labandeira et al. 2007; Pant 1987). It is therefore likely that one or more of these groups were the hosts of early Phytophaga, and that the complex plant–phytophage associations observed today may have originated well before the angiosperm radiation on one or more of these formerly important lineages of plants (Labandeira 2000; Labandeira et al. 2007). Some lineages within the Chrysomeloidea and Curculionoidea are today associated with gymnospermous plants, particularly conifers (Order Coniferales) and cycads (Order Cycadales) (Crowson 1991; Farrell 1998; Kuschel 1983). These observations have led researchers to speculate that modern associations between certain Phytophaga and gymnosperms could have originated in the early Mesozoic (Crowson 1991; Farrell 1998; Kuschel 1983). Although the 'early origin' hypothesis is intriguing, it has been difficult to evaluate because adult and larval host plants of certain key Phytophaga remain incompletely known. Chief among these are members of the 'pleisiomorphic' chrysomeloid subfamily Aulacoscelidinae. The interrelationships between Aulacoscelidinae (and other Orsodacnidae), Cerambycidae (s.l.), Chrysomelidae, and certain other Chrysomeloidea remain inadequately known, but their inclusion within the Chrysomeloidea is undisputed (Gómez-Zurita et al. 2008). Adult Aulacoscelidinae are associated with cycads from northern Mexico to central Bolivia (Windsor et al. 1999). Additionally, adults have been collected in areas of the Southwestern U.S.A. where there are no native cycad populations (Clark et al. 2004; Riley et al. 2003). Angiosperm pollen-feeding by adult Aulacoscelidinae has been reported (Monrós 1954; Santiago-Blay 2004). Despite the angiosperm pollen-feeding habits of adults, occurrence of adult Aulacoscelidinae far from native cycad populations, the lack of trophic data for larvae, and the absence of direct fossil evidence, cycad-feeding by Aulacoscelidinae has been speculated to have possible origins in the Lower Jurassic, nearly 200 Ma ago (Cox and Windsor 1999a; Crowson 1981; Jolivet 2005; Kuschel and May 1990; Medvedev 1968; Santiago-Blay 2004; Windsor and Jolivet 1997). Both the modern day association of adult Aulacoscelidinae with cycads and the position of Aulacoscelidinae within Chrysomeloidea are consistent with an early association of Aulacoscelidinae with cycads, but do not rule out other scenarios, including more

recent secondary associations with gymnosperms as are known among some extant phytophagous insects, e.g. Lepidoptera and criocerine beetles on cycads (Forster and Machin 1994; Hawkeswood 1992; Nash et al. 1992) as well as certain weevils on conifers and cycads (Downie et al. 2008; Oberprieler R 1999; Oberprieler et al. 2007).

Here we report the discovery of an aulacoscelidine larva associated with the megagametophyte of *Dioon meroale* De Luca, Sabato & Vázquez-Torres (Cycadales; Zamiaceae). We provide a description of the specimen and report on its phylogenetic placement among three species representing both known genera of Aulacoscelidinae and 15 additional species of Chrysomeloidea, based on analyses of DNA sequences from three genes. Our report helps fill a long-conspicuous void in our understanding of the evolution of host usage and morphology in Chrysomeloidea.

6.2.1. Subfamily Aulacoscelidinae

The Aulacoscelidinae comprise 28 species in two genera; *Aulacoscelis* Duponchel & Chevrolat and *Janbechynea* Monrós (Santiago-Blay, 2004), the latter being divided further into two provisional subgenera, *Janbechynea* and *Bothroscelis* Monrós. After being treated as part of the Sagrinae (Chapuis 1874; Crowson 1946; Jacoby 1877), Aulacoscelidinae were elevated to subfamily rank within the Chrysomelidae (Monrós 1953; Monrós 1954). For comprehensive reviews of the history of aulacoscelidine classification see Reid (1995) and Santiago-Blay (2004). The name Aulacoscelidinae derives from the Greek *aulax*, which refers to the furrow made by a plough on the ground, and *skelis*, which refers to the ribs of beef (Reid 1995; Santiago-Blay 2004). In the conjunction *Aulacoscelis* (furrowed rib), the authors probably were referring to the sulcate tibia of *Aulacoscelis melanocera*, a morphological feature shared by all Aulacoscelidinae. Features of hind wing venation, male external genitalia and the female spermatheca suggest that Aulacoscelidinae are related closely to Orsodacninae (Kuschel and May 1990; Reid 1995; Reid 2000; Suzuki and Windsor 1999). Morphology of the first instar larvae of

Orsodacne cerasi L., *O. lineola* Panzer (Cox 1981) and *Aulacoscelis appendiculata* Cox & Windsor (Cox and Windsor 1999a; Cox and Windsor 1999b) also suggest close affinities of the two taxa. Similarly, molecular phylogenetic analyses recover Aulacoscelidinae and Orsodacninae as sister taxa, although without strong nodal support (Duckett et al. 2004; Gómez-Zurita et al. 2007, 2008).

Cycad leaf-feeding by adults has been reported in both genera of Aulacoscelidinae; *Janbechynea paradoxa* Monrós on *Zamia boliviana* (Brongn.) A. DC. (Prado et al. 2011) and *A. appendiculata* on *Zamia* cf. *elegantissima* Schutzman, Vovides & Adams (Cox & Windsor 1999a). Adults of other species of Aulacoscelidinae also have been collected on *Dioon* (Santiago-Blay 2004; Prado et al. 2011). Adults in both genera of Aulacoscelidinae appear suddenly early in the rainy season and feed mainly on the newly-produced, immature leaves of cycads, although occasionally older leaves are attacked. The beetles puncture the epidermis of cycad leaves with their sharp mandibles and ingest the oozing fluids (Windsor et al. 1999) from which they sequester toxic azoxyglycosides (Prado et al. 2011). These cycad-specific secondary metabolites can be exposed to potential predators through a reflex bleeding mechanism (Prado et al. 2011). After feeding and mating on their cycad hosts for approximately 2 weeks, the beetles vanish until the beginning of the next year's rains. Aulacoscelidinae beetles also feed on exposed cycad root and stem tissue (A. Prado, unpublished data), as well as the fronds of ornamental plantings of Asian *Cycas* spp. Angiosperm pollen has been identified in the gut of adult *A. melanocera* (Crowson 1991) and also in the faeces of *A. appendiculata* (specifically, when they make their first appearance on cycads) in Central America (A. Prado, unpublished data). Adult beetles have been collected in southern North America on the flowers of *Croton* (Asteraceae) and *Hechtia* (Bromeliaceae) (Clark et al. 2004; Santiago-Blay 2004). *Aulacoscelis vogti* Monrós in northeastern Mexico has been observed feeding and mating on *Brahea dulcis* (Hum., Bonpl. & Kunth) Mart. (Arecaceae) flowers and has been observed in smaller numbers on the leaves and cones (both sexes) of *Dioon edule* Lindl. (Zamiaceae) (Prado et al. 2011). *Aulacoscelis appendiculata* has been kept in the laboratory for approximately 2 months feeding

on mango slices and/or palm flowers (Windsor et al. 1999). These observations suggest that aulacoscelidine adults are pollen generalists but have a close association with cycads, from which they sequester toxic compounds for chemical defense (Prado et al. 2011). A first instar larva of *A. appendiculata* was reared successfully from an egg deposited in the laboratory (Cox and Windsor 1999a), but the larval host plant(s) remained a mystery (Windsor and Jolivet 1997).

6.2.2. The case for an ancient association between Aulacoscelidinae and the Cycadales

The phylogenetic position of Aulacoscelidinae has long been of interest to systematists studying Chrysomeloidea due to plesiomorphic morphological features of adults (e.g. adult mandibles with a definite molar region) (Kuschel and May 1990; Mann and Crowson 1981; Reid 1995; Suzuki and Windsor 1999). The resemblance of adult Aulacoscelidinae to Jurassic fossils assigned to the extinct subfamily Protoscelidinae has generated debate about their temporal origin and phylogenetic placement (Crowson 1991; Kuschel and May 1990; Medvedev 1968; Zhang 2005). However, Reid (1995) and later Grimaldi and Engel (2005) doubted that fossils of †*Protoscelis* Medvedev, †*Protosceloides* Medvedev, †*Pseudomegamerus* Medvedev and †*Cerambyomima* Medvedev contain sufficient character data to conclude they are close relatives of Aulacoscelidinae, and suggest that the association of Aulacoscelidinae with cycads is recent. However, the recently discovered fossil protoscelidine, †*Tarsomegamerus mesozoicus* (Zhang 2005) from the Jurassic Daohugou biota in present-day China – an extremely well-preserved specimen – has a tibia with a longitudinal medial carina flanked by two longitudinal furrows (Zhang 2005). The furrows of this fossil are in the same position and orientation as in extant *Aulacoscelis* species and revision of the protoscelidine fossils could shed more light on their relationship to the aulacoscelidines (Santiago-Blay 2004). Controversially, *T. mesozoicus* was assigned subsequently to an extinct family of Elateriformia (Kirejtshuk et al. 2010), despite the apparent lack of such tibial ornamentation in elateriforms.

Whether the association of Aulacoscelidinae with cycads has a relatively recent or ancient origin is far from being resolved. Nonetheless, indirect support for the ancient association hypothesis comes from the alleged resemblance of aulacoscelidines to fossils (e.g. †*Protoscelis*,†*Tarsomegamerus*) that occur in gymnosperm-dominated strata in which cycads are common, their plesiomorphic morphological features, their phylogenetic position within Chrysomeloidea, their present day association with cycads (as adults), their ability to sequester cycad toxins, and the unusual mode of adult feeding (Crowson 1991; Windsor & Jolivet 1997; Santiago-Blay 2004; Jolivet 2005; Prado et al. 2011).

6.2.3. New insights, the Dioon seed

On July 2004 the Florida Department of Agriculture identified an infested *Dioon* seed (Cycadales: Zamiaceae) that had come from Mexico. The seed being shipped to the Montgomery Botanical Center contained a larva with possible resemblance to the Chrysomeloidea. The seed was collected originally in the province of Oaxaca in May 2004. From the locality information of the collection and seed morphology, the seed can be assigned with certainty to *Dioon merolae* De Luca, Sabato & Vázquez-Torres from the central valleys of Oaxaca (Fig. 6.1) (De Luca et al. 1981b). *Dioon merolae* grows in warm humid and sub-humid climates in the Mexican States of Oaxaca and Chiapas (Cabrera-Toledo et al. 2012; Cabrera-Toledo et al. 2010). In the State of Oaxaca it grows on the Sierra Madre del Sur where it is separated orographically from *D. rzedowskii* De Luca, Moretti, Sabato & Vázquez-Torres, *D. purpusii* Rose, *D. califanoi* De Luca & Sabato and *D. spinulosum* Dyer which grow on the inner mountains of the Sierra del Norte (De Luca et al. 1981). *Dioon merolae* grows at higher elevations (900–1700 m) than *D. holmgrenii* De Luca, Sabato & Vázquez-Torres which grows closer to the coast of Oaxaca (580–850 m) (De Luca et al. 1981a).

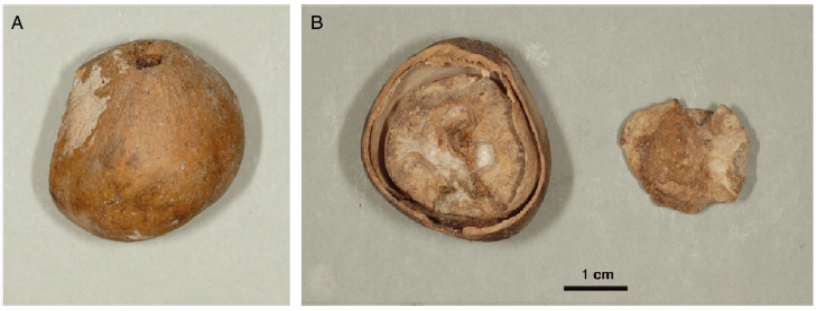

Fig. 6.1. Seed of *Dioon merolae* De Luca, Sabato & Vázquez-Torres showing feeding damage caused by the larva of an Aulacoscelidinae beetle: (A) hard sclerotesta; (B) interior of the seed, showing endotesta, and larval feeding damage to the embryo and endosperm.

The putative chrysomeloid larva found inside the *D. merolae* seed was sent to DW at the Smithsonian Tropical Research Institute (STRI) for identification (Fig. 6.2). Unfortunately, the mandibles that had been dissected from the head prior to shipment, were overlooked and lost during ethanol replenishment upon arrival at STRI. The larva was photographed under a light microscope (www.discoverlife.org as *Janbechynea* sp.) and later using a SEM. After removing a sample of tissue for DNA extraction, the specimen was shipped to M.L. Cox at the Natural History Museum in London. Unfortunately the specimen was destroyed by fungus in transit. Because this is the first known chrysomeloid larva to be found in cycad tissue we consider it important to report on what we learned about its morphology and phylogenetic affinities, despite the loss of this unique specimen.

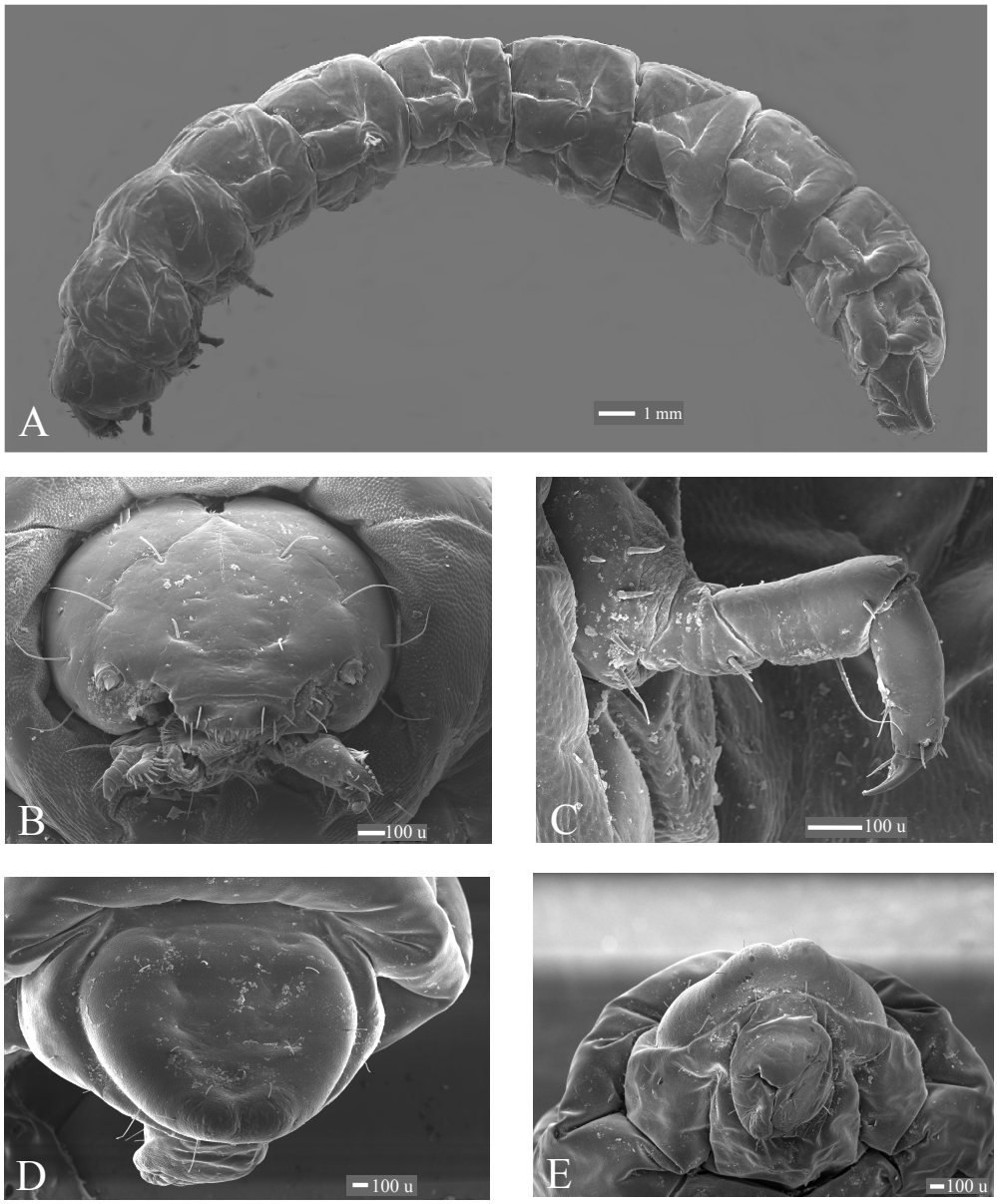

Fig. 6.2. Scanning electron microscope (SEM) images of Aulacoscelidinae larva: (A) General habitus, lateral view of complete larva; (B) anterior view of head; (C) lateral view of leg showing coxa, tronchanter, femur and tibiotarsus; (D) dorsal view of anal plate; (E) ventral view of abdominal segment 10.

6.3. Methods

The single larva found inside a seed of *Dioon merolae* was imaged using a Jeol 5300LV SEM (Fig. 6.3). A modest morphological description of the larva was prepared after the specimen was lost, based on the SEM images and notes. Genomic DNA was extracted from a 4 x 4 mm section removed from one side of the larva. Partial sequences from the CO1 and 28S and 18S genes were obtained from the resulting genomic DNA using the primer sets detailed in Appendix 2. To identify the taxonomic affinity of the unidentified chrysomeloid larva, these DNA sequences were compared both individually and in a concatenated alignment to orthologous sequences obtained previously from 10 species of Chrysomelidae in 6 subfamilies (Cassidinae, Chrysomelinae, Criocerinae, Donaciinae, Eumolpinae and Sagrinae), one Cerambycidae (subfamily Cerambycinae) and 6 other taxa representing known or presumed early divergent Chrysomeloidea (Aulacoscelidinae, Orsodacninae, Palophaginae and Oxypeltinae) (Table 6.1). Sequences for sixteen taxa were obtained by extracting genomic DNA from adult specimens collected and identified by DW as indicated in Table 6.1. For each specimen, DNA sequences from three genes were obtained using the primer sets detailed in Appendix 2. DNA sequences for three other species were obtained from Genbank (accession numbers: FJ867850.1, FJ867681.1, AJ850051.1 for *Ericmodes sylvaticus*; FJ859984.1, FJ867816.1, FJ867699.1 for *Oxypeltus quadrispinosus*; FJ000508.1, FJ000433.1 for *Palophagus bunyae*). Two sequences were missing from our matrix – CO1 for *Palophagus bunyae* and 18SA for *Lilioceris nigripes*.

Table 6.1. Species list.

ID Number	Species	Family/Subfamily
BTOLDDM0176	*Ericmodes sylvaticus*	Protocucujidae*+
695	*Aulacoscelis appendiculata*	Aulacoscelidinae
7721	*Aulacoscelis vogti*	Aulacoscelidinae
7598	*Janbechynea paradoxa*	Aulacoscelidinae
761 / 1153	*Acromis sparsa*	Cassidinae
1062	*Spaethiella marginata*	Cassidinae
1038	*Xenochalepus hespenheidi*	Cassidinae
1355	Unidentified Cerambycinae	Cerambycinae
1145	*Platyphora flavoannulata*	Chrysomelinae
1131	*Lilioceris nigripes*	Criocerinae
1132	*Lilioceris* sp.	Criocerinae
845	*Donacia* sp.	Donaciinae
JJG321	*Callisina quadripustulata*	Eumolpinae
696	*Megascelis puella*	Eumolpinae
7692	*Orsodacne cerasi*	Orsodacninae
7691	*Orsodacne humeralis*	Orsodacninae
BTOLDDM0168	*Oxypeltus quadrispinosus*	Oxypeltinae+
FJ000433	*Palophagus bunyae*	Palophaginae+
9007	*Mecynodera coxalgica*	Sagrinae
1118	Putative aulacoscelidine larva	**?**

*Outgroup +Obtained from Genbank

All 20 sequences were aligned using Clustal W (Thompson et al. 1994) and the alignment then refined manually. Substitution models were selected for each gene using Modeltest v3.7 (Posada 2005) for maximum-likelihood (ML) analyses and MrModeltest v2.3 (Nylander 2004) for Bayesian analyses.

Phylogenetic analysis was performed via parsimony inference using a heuristic search with and without coding gaps in the alignments, via ML inference, and via Bayesian inference. Each analysis was performed for the three individual genes separately and for a concatenated matrix that included data from all three genes (2145 total bp). *Ericmodes sylvaticus* (Protocucujidae) was used as an outgroup in all analyses (Table 6.1) because it is a member of the superfamily Cucujoidea (close relatives of Phytophaga) and because DNA sequences were available for several genes from a previous study (McKenna et al. 2009).

A parsimony analysis was implemented in PAUP v4.0 beta (Swofford 1998) and a ML analysis was implemented in RAxML v7.2.8 (Stamatakis et al. 2008). For the concatenated ML analysis the model of evolution chosen was GTR + I + G. Bayesian inference was implemented in MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) using two separate runs, each of 10 000 000 generations, with four chains and parameters according to the models specified in Appendix 2.

For analyses of the combined dataset, partitions were set to allow analytical parameters to vary within each partition. The CIPRES Science Gateway was used to perform the Bayesian analyses (Miller et al. 2010). We assessed convergence and stationarity under Bayesian inference using the standard deviation of split frequencies between runs, trace plots of loglikelihood scores, and using graphical and statistical analyses implemented in Tracer v1.4 (Rambaut and Drummond 2007) and AWTY (Nylander et al. 2008). Post-burn in trees were pooled and used to generate a 50% majority-rule consensus tree.

6.4. Results

6.4.1. Larval morphology

Final instar larva. Head width: 1.37 mm; Body length: 33.2 mm; Body width: 3.17 mm.

General habitus. Elongate and cylindrical, approximately same width along entire length, greatest width at meso and metathorax; head and anal plate darker than the rest of body; mostly glabrous with well-developed setae on the head, prothorax, abdominal segments 9–10.

Head. Small, pro- to hypognathous, protracted, oval (Fig. 6.3A); epicranial suture with stem absent or very short; frontal sutures narrow and reaching antennae; endocarina reaching mid-frons; frons bearing two large setae and two sensilla along the frontal sutures, mid-frons bearing two sensilla and lower frons bearing one large seta; vertex bearing five sensilla; gena bearing three large setae; stemmata apparently absent (there is a slight possibility that a single highly reduced stemma is present posterodorsal to the antenna, but higher resolution scans will be needed to verify its presence); labrum bearing six moderately long setae dorsally (Fig. 6.3F), more than eight curved setae along anterior margin on each side, anteromedially bearing four setae and numerous elongate spicules; antennae very short and embedded in antennal fossa (Fig. 6.3D), 3-segmented, anteriorly-directed, First segment at least three times broader than long, second segment with apex truncate bearing two small setae dorsally, one larger seta ventrally, a basiconic sensillum twice as long as segment two and segment three, segment three cigar-shaped bearing three small setae; maxillary palpi 3-segmented (Fig. 6.3C), basal two segments sub-equal in length, apical segment conical, apex truncated with ring of small setae apically; galea club shaped (Fig. 6.3C), bearing two setae ventrally and more than 14 thick setae on its lateral margin extending to apical margin.

Legs. Three pairs well developed, chaetotaxy similar in each pair (Fig. 6.3B); coxa bearing 6 large and 2 very small setae ventrally plus 2 small setae posteriorly; trochanter bearing 2 large setae ventrally and 1 short seta dorsally plus 1 short seta posteriorly; femur bearing 1 very long seta ventrally, plus 2 long and 3 short setae apically plus 1 short seta dorsally; tibiotarsi bearing 1 long seta ventrally and 3 setae apically; pretarsal claw elongate, slightly curved and bearing a long seta on inner margin.

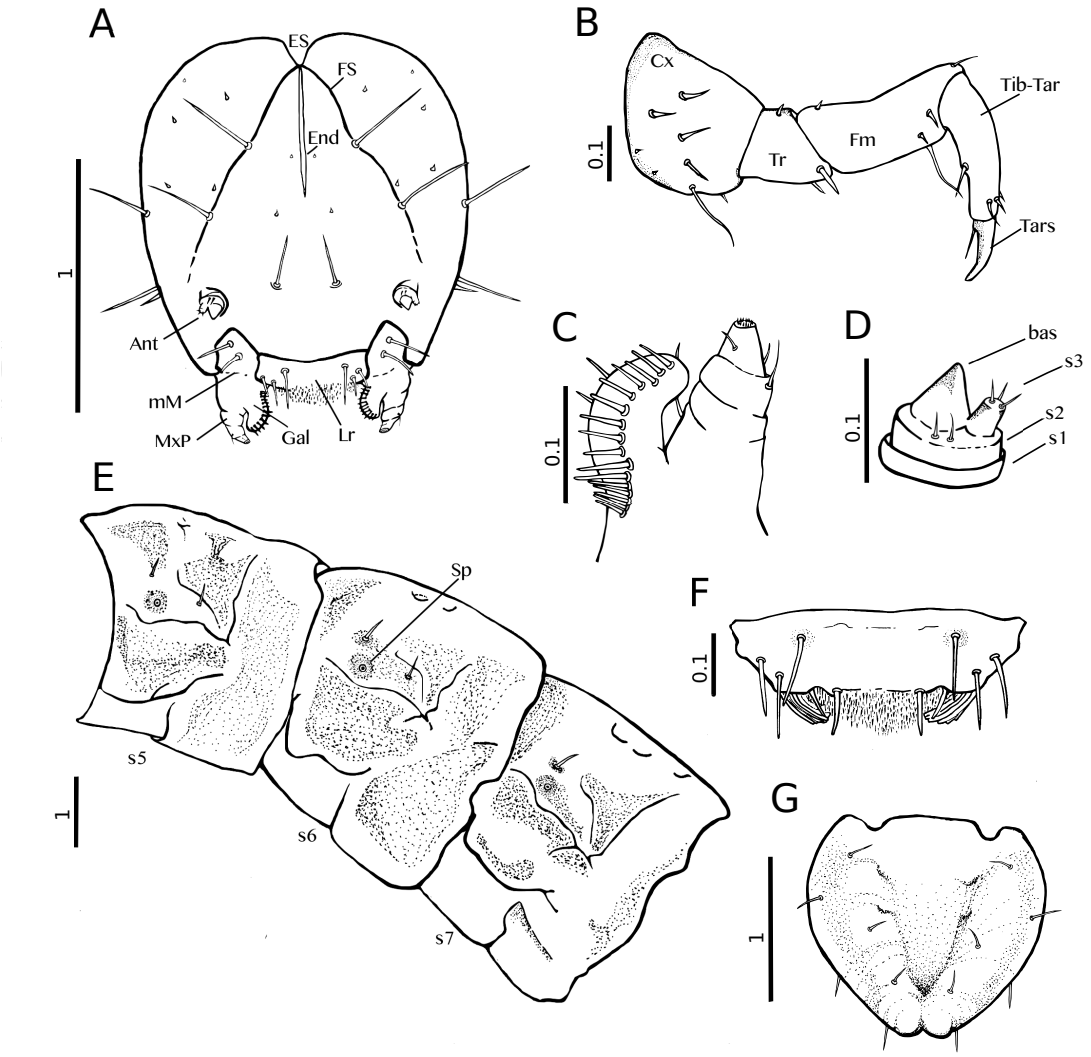

Fig. 6.3. Head and body of Aulacoscelidinae final instar larva: (A) frontal view of head; (B) ventral view of leg, (C) dorsal view of right galea and maxillary palpus; (D) dorsal view of right antenna; (E) left lateral view of abdominal segments 5–7; (F) frontal view of labrum; (G) dorsal view of anal plate. Abbreviations: Ant, antenna; bas, basiconic sensillum; Cx, coxa; End, endocarina; ES, epicranial suture; Fm, femur; FS, frontal suture; Gal, galea; Lr, labrum; mM, missing mandibles; MxP, maxillary palpus; Sp, spiracle; s1, s2, s3, antennal segments 1-3; s5, s6, s7, abdominal segments 5-7; Tars, pretarsal claw; Tib-Tar, tibiotarsus; Tr, trochanter.

Abdomen. Abdominal segments 1–8 mostly glabrous with annular spiracles disposed laterally, abdominal segments 1–6 bearing two small setae, one dorsal and one posterior to the spiracle (Fig. 6.3E); abdominal segments 7–8 bearing one setae dorsal to the spiracle; abdominal segment 9 bearing a smooth anal shield slightly

bifid in its posterior margin, bearing 8 long setae dorsally plus 4 long setae ventrally (Fig. 6.3G); abdominal segment 10 ventrally situated, circular, bearing 6 long setae along its margin.

6.4.2. DNA sequences

The final COI alignment was 527 characters long, of which 216 characters were parsimony informative (Appendix 2). No informative gaps were obtained from the COI alignment. The 28S alignment was 548 characters long, of which 129 characters were parsimony informative, 26 parsimony informative gaps were identified. The 18S alignment was 516 characters long, of which 46 characters were parsimony informative. There were seven informative gaps. Concatenation of the three alignments produced a matrix 2145 characters long that contained 391 parsimony informative characters and 33 parsimony informative gaps.

6.4.3. Phylogenetic analyses

The intention of our phylogenetic reconstructions was not to resolve the phylogenetic relationships of the subfamilies of the large superfamily Chrysomeloidea, but instead to evaluate the phylogenetic affinity of the putative aulacoscelidine larva. Therefore, our taxon sample was focused on Aulacoscelidinae, known and putative relatives, and selected outgroups. We analysed the concatenated 3-gene matrix under ML and Bayesian inference (Figs. 6.4, 6.5). Based on our evaluation (see Methods) the Bayesian analyses reached convergence quickly, at approximately 12 000 generations.

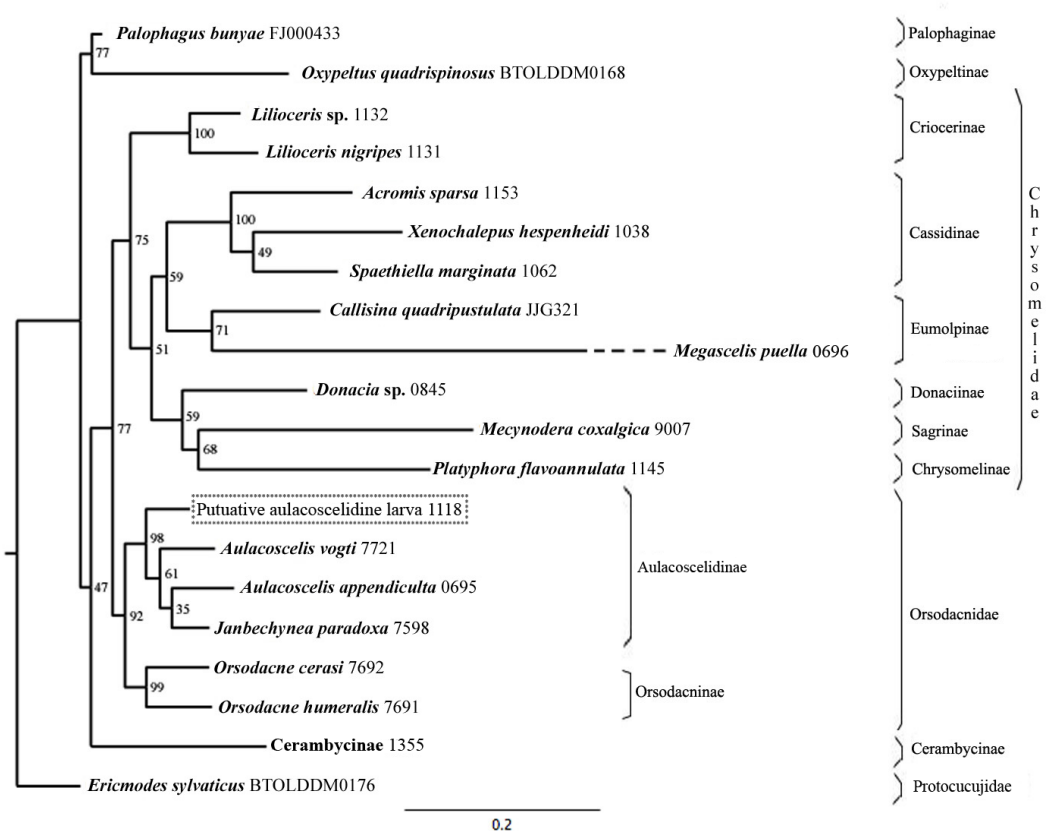

Fig. 6.4. Maximum likelihood phylogenetic tree (best tree logL = −10904.57) based on a combined matrix of *CO1, 28S* and *18S* DNA sequences from 19 Chrysomeloidea and one cucujoid outgroup. Numbers alongside each node are estimates of ML bootstrap support.

Interrelationships among the early diverging branches of Chrysomeloidea were mostly unresolved, consistent with previous molecular phylogenetic studies involving Chrysomeloidea (Gómez-Zurita et al. 2007). The resulting 50% majority rule ML consensus tree recovers the putative aulacoscelidine larva in a position sister to the other three sampled Aulacoscelidinae, with strong nodal support (Figs. 6.4, 6.5; 98% BS, 1.0 PP), consistent with its identification as a member of the subfamily Aulacoscelidinae. Additionally, *Aulacoscelis appendiculata* was found to be more closely related to *Janbechynea paradoxa* than to *A. vogti*, but with weak support. Aulacoscelidinae was recovered as a sister clade to Orsodacninae (Table 6.2).

Table 6.2. Graphical summary of the phylogenetic affinity of the putative chrysomeloid larva, and the phylogenetic position of the Aulacoscelidinae, based on analysis of partial sequences of 18S, 28S and CO1.

Tree building method	Markers			
	18S	28S	CO1	All
Parsimony best tree		A	O	A
Parsimony best tree with gaps coded		A	*	A
Maximum Likelihood Majority Rule		A	O	A
Maximum Likelihood best tree		A	A	A
Bayesian Majority Rule		A	O	A
Bayesian best tree	O	A	O	A

An **O** or an **A** indicate that the sequences from the larva in the *Dioon merolae* seed were recovered within a monophyletic Orsodacnidae (Orsodacninae & Aulacoscelidinae sequences mixed) or in a monophyletic Aulacoscelidinae, respectively. Cross-hatching indicates a monophyletic Aulacoscelidinae not sister to Orsodacninae, a light gray square represents a polyphyletic Orsodacnidae (Aulacoscelidinae + Orsodacninae); an intermediate grey represents a monophyletic Orsodacnidae with Aulacoscelidinae and Orsodacninae paraphyletic, and a dark grey square represents a monophyletic Orsodacnidae with Aulacoscelidinae and Orsodacninae as sister groups. *No informative gaps were obtained from the CO1 sequences.

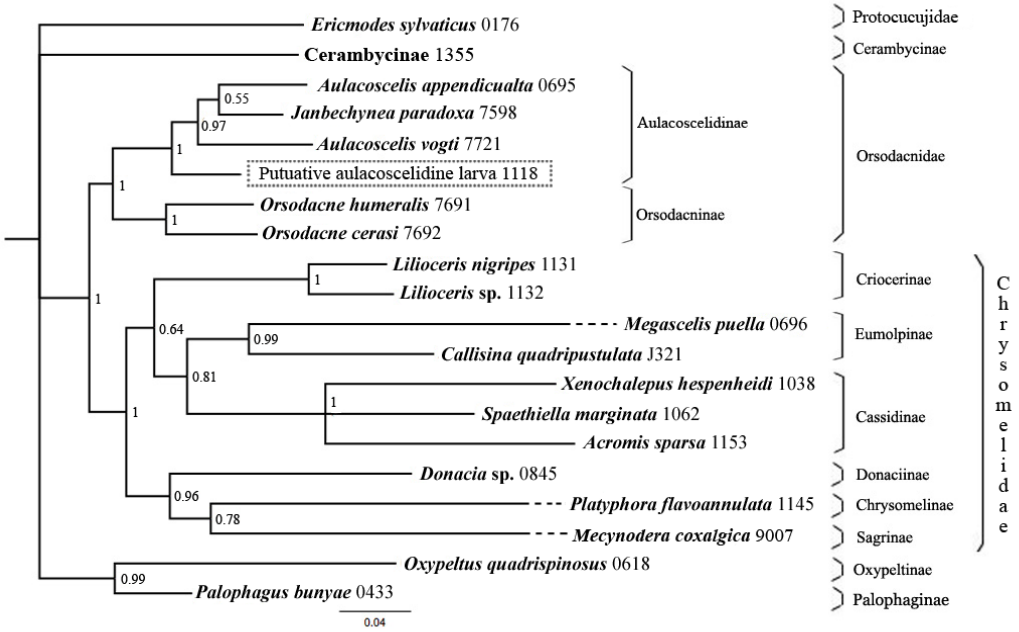

Fig. 6.5. Bayesian 50% majority rule consensus tree resulting from analysis of the combined data (CO1, 28S and 18S). Numbers alongside each node are posterior probabilities.

6.5. Discussion

6.5.1. Larval morphology

The cycad-feeding larva described herein differs from the larvae of Chrysomelinae (Reid 1991) by lacking a paronychial appendix in the tibio-tarsi. It differs from the shape of Cassidinae larvae (Lawrence 1991) by being cylindrical and elongate, and from the larvae of the Eumolpinae (Reid and Storey 1993) by the reduced length of the stem of the epicranial suture. The larvae of Palophaginae differ from the cycad-feeding larva by having six stemmata and the anal shield on abdominal segment nine not sclerotized (Kuschel and May 1990).

All known larvae of Orsodacnidae (*Orsodacne* species and *Aulacoscelis appendiculata*) are first instars. The size of our larva indicates it is final instar. However, according to Cox (1981) the fundamental morphology of chrysomeloid first instar larvae changes little during development, permitting some comparisons

to be made between instars. The Aulacoscelidinae larva found in the *Dioon merolae* seed strongly resembles the first-instar larva of *A. appendiculata* described by Cox and Windsor (1999a), as well as the *Orsodacne* larvae described by Cox (1981) and Mann and Crowson (1981). All four descriptions (including ours) indicate larvae have an epicranial suture with a very short stem, a reduced number of stemmata and an anal plate on abdominal segment nine. The aulacoscelidine larva described herein bears three pairs of large setae in the frons, as does the first-instar larva of *A. appendiculata* (Cox & Windsor, 1999a), but not the *Orsodacne* species (which have five pairs of setae in the frons) (Cox 1981; Mann and Crowson 1981). The shape of the anal shield is distinct in the larvae of all three orsodacnids, with *A. appendiculata* having a smooth posterior margin, the *Orsodacne* species bearing two urogomphi, and the aulacoscelidine from the *Dioon* seed having a slightly bifid margin. The anal shield differs from *A. appendiculata* and the *Orsodacne* spp. by having a smooth surface. The larva described herein bears little resemblance to the *Orsodacne* sp. larva described by Böving & Craighead (1931) in the prothorax, presence of legs, shape of head, epicranial suture stem, stemmata and shape of second antennal segment. Based on the reduced number of stemmata, Cox & Windsor (1999a) speculated an internal feeding behavior for *A. appendiculata* in Panama. This is supported by the apparent absence or extreme reduction of stemmata in the SEM of the Mexican specimen and its habit of internal feeding on cycad seeds. Whereas one pair of stemmata has been found in other orsodacnids, our evidence is insufficient to distinguish if there is one extremely reduced stemma posterodorsal to the antenna, or if it is completely absent.

6.5.2. The Aulacoscelidinae and the Cycadophyta

Cycads have been regarded as the most ancient extant lineage of seed-bearing land plants. Although evidence of recent radiations in cycads (e.g. Zamiaceae) show that many cycad species are relatively young (Nagalingum et al. 2011), extant cycad lineages (including several genera) were well established by the Late Cretaceous (Anderson et al. 2007; Nagalingum et al. 2011; Norstog and Nicholls

1997; Pant 1987). The absence of cycadophilous insect taxa common to both old and new world cycads (Crowson 1991), could result from early divergence of cycad genera. Herbivory of aulacoscelidines on cycads might well be as ancient as speculated by Crowson (1981) and Medvedev (1968), but a proper evaluation of this idea requires a better understanding of the Mesozoic fauna as well as documentation of current associations. If the Aulacoscelidinae have been associated with cycads for the last 160 Ma, their restriction to the Neotropics might represent an ancestral relationship to one particular lineage of Neotropical Zamiaceae (i.e. *Ceratozamia, Dioon* or *Zamia*) and a later expansion to other cycad genera/species. *Aulacoscelis appendiculata* in Panama and *Janbechynea paradoxa* in Bolivia are strongly attracted to the leaves of the exotic *Cycas revoluta* (Cycadaceae) and will feed on these introduced plants [L. Werding (Bolivia), A. Prado, personal observations]. This behavior demonstrates an ability to feed on the leaves of distantly-related cycad species. A majority of known cycad–insect associations appear to be secondary. For example, all weevil genera associated with cycad gametophytes in Africa and Australia have close relatives in genera associated with angiosperms (Oberprieler 1999; Oberprieler et al. 2007; Downie et al. 2008). Therefore in the majority of cases the colonization of cycad gametophytes by weevils may be a relatively recent phenomenon. Studies on the African cycad-weevils (Amorphocerini) (Downie et al. 2008) support a recent colonization of *Encephalartos* (Zamiaceae) species, which radiated during the Pliocene/Pleistocene, according to Treutlein et al. (2005) or the Miocene, according to Nagalingum et al. (2011). Reid (1995) suggested that the association of certain Criocerinae with Cycadaceae (e.g. Lilioceris nigripes in Australia) is likely to be of recent origin because only two species feed on cycads and most criocerines are associated with monocotyledonous angiosperms.

We present molecular and morphological evidence that the immature chrysomeloid found feeding on a Mexican *Dioon* cycad seed belongs to Aulacoscelidinae. This is the first account of a chrysomeloid larva developing in a cycad gametophyte. The species of Aulacoscelidinae involved remains unknown

until a greater number of species of adult Mexican Aulacoscelidinae are sequenced. A phylogenetic analysis including more members of the subfamily will improve our ability to attribute the larva to a genus. Based on collecting locality information from specimens of Aulacoscelidinae in collections, the larva could belong to either *Aulacoscelis candezi* Chapuis, *A. confusa* Monrós or *A. melanocera* Duponchel & Chevrolat which have been collected in the Tehuantepec region of Mexico or in the central valleys of Oaxaca (Jacoby 1891; Monrós 1954; Santiago-Blay 2004).

The rarity of these beetles and their brief period of activity in nature may explain why the feeding habit has been observed so seldom. During mating congregations, female Aulacoscelidinae may oviposit on only a small number of individual cycad plants, making observation unlikely. Eggs might be deposited near the developing seeds, possibly inside the female cone with the young larvae borrowing their way into the seed, eventually being well protected by a toxic sarcotesta and a hard scelerotesta (Norstog & Nicholls 1997). Larval behaviour might be similar to that of some bruchid beetles that oviposit on the outside of the pod or seed of their hosts (Southgate 1979). Whether pupation of the aulacoscelidine described herein occurs inside the seed (which is most likely based on the advanced state of the larva) or whether the larva exits the seed to pupate in the soil remains unknown. The phenology of its host, *D. merolae* is poorly known, but from a study carried out on *D. edule* by Vovides (1990) we can expect the female coning intervals for *D. merolae* to be at least 5–10 years. This sparse coning behaviour could also account for apparent beetle rarity and for concentration of overposition on a few individual plants.

Despite their occasional visits to cycad cones, it is unlikely that aulacoscelidines are effective pollinators, as no pollen bearing structures have been described for the Aulacoscelidinae. Even though *Aulacoscelis vogti* visits both female and male cones of *D. edule*, a weevil in the genus *Rhopalotria* (Belidae: Curculionoidea), is considered the main pollinator (Vovides 1991b). When visiting *D. edule* cones, *A. vogti* probably feeds on pollen and pollination drops.

Our analyses support the placement by Kuschel & May (1990), Reid (1995) and Gómez-Zurita et al. (2007) of the Aulacoscelidinae as a clade within the Orsodacnidae and as a sister clade to the Orsodacninae. The Orsodacninae have been collected mainly on flowers where they can damage floral tissues (Cox 1981; Mann and Crowson 1981). *Orsodacne atra* (Ahrens) has been collected in North America on a variety of different plant families (more than 12), mostly angiosperms, but also on two genera of the Pinaceae (Clark et al. 2004). This could be indicative of a pollen generalist habit, as proposed for the Aulacoscelidinae.

Multiple lines of natural history evidence point toward a deep association between aulacoscelidines and cycads (i.e. adult feeding, cycad-toxin sequestration, and larval feeding habits). However, the recent association scenario cannot be ruled out. The ancient association hypothesis for the aulacoscelidines can be tested further only by documenting the feeding relationships for more species in the subfamily, including both immature stages and adults.

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7. General conclusions and future directions

7.1. Conclusions

In this thesis, I have described spatial and temporal variation in AZG levels in the leaves of cycads. At short time scales, changes occur during leaf development; AZGs are highest in the youngest leaves and then decrease to basal levels in the mature leaves. The levels observed in new flushes of young leaves might be high enough to still protect the cycad foliage from specialist herbivores. As AZGs levels fall in the maturing leaves, and before leaves are sufficiently tough, leaves are vulnerable to herbivory by specialist insects. Leaf toughness is an effective defense against cycad specialists. There are strong parallels between the defensive strategy of cycads and that of other plant lineages that exhibit a delayed greening and a switch form chemical to mechanical protection.

At a longer time scales, foliar AZG levels change with plant ontogeny; AZG content is higher in seedlings than in juvenile or adult plants. This pattern suggests that as growth and reproductive priorities change during plant development, the relationship between costs and benefits in producing these compounds changes. Sex differences in the investments in these defensive compounds were identified; AZG levels are higher in adult females than in males. This finding suggests that the costs of reproduction in cycads are disproportionate as previously speculated. Both the patterns of AZG levels observed during plant ontogeny and in relation to sex, suggest that the leaf chemical defense in cycads might be proportional to the consequences of losing the leaf.

Aulacoscelidinae beetles have an intimate association with the Zamiaceae. The beetles sequester AZGs and use them for their own defense. The associations between cycads and herbivore insects are fascinating and still present many unresolved questions. For example, *how broad or narrow are beetle and butterfly assemblages on the Neotropical cycads? What are the insect adaptations that help them cope with AZGs? Have butterfly and beetle converged on the same mechanisms to*

tolerate AZGs? These are all magnificent questions that could be evaluated in future research dissertations.

7.2. Future directions

Many aspects of AZG chemical ecology still remain a mystery. The biosynthetic pathway of these compounds and the site of synthesis have not been described. Advances in this regard will allow future research to focus on the costs of AZG production. As well, the role that the symbiotic nitrogen-fixing cyanobacteria, in the coralloid roots, play in AZG production should be evaluated. Due to the correlative nature of Chapter 3, I could not identify AZGs as the absolute cause of the lack of herbivory in the youngest foliage. However, these results raise the question of how tolerant are cycad specialist to AZGs? In terms of the induction of AZGs in response to wounding, the future direction is to assess if the application of exogenous jasmonates, a treatment that is usually considered similar to wounding, increases AZG levels in the subsequent leaf flush.

Another major component that is lacking is the systematic documentation of herbivory on seedlings and juvenile plants. The leaves of seedlings have higher AZG levels, but if this actually translates into less herbivory is unknown. Future ecological studies on cycads should evaluate insect damage, preference and performance on different ontogenetic stages and sexes. Long term monitoring programs of cycad populations could determine what are the actual costs of reproduction vs. chemical defense by relating cone and leaf production with AZG levels and herbivore incidence. The Optimal Defense Hypothesis could be rigorously evaluated in such a long-term study.

In terms of the insect mechanisms that allow MAM tolerance, future studies should evaluate the activity of insect gut UDP-glucosyltransferases. High activity of a UDP-glucosyltransferase capable of re-glycosilating MAM was observed in the leaves of *Cycas*, possibly to prevent autotoxicity. It is possible that cycad specialists use similar mechanisms to tolerate MAM.

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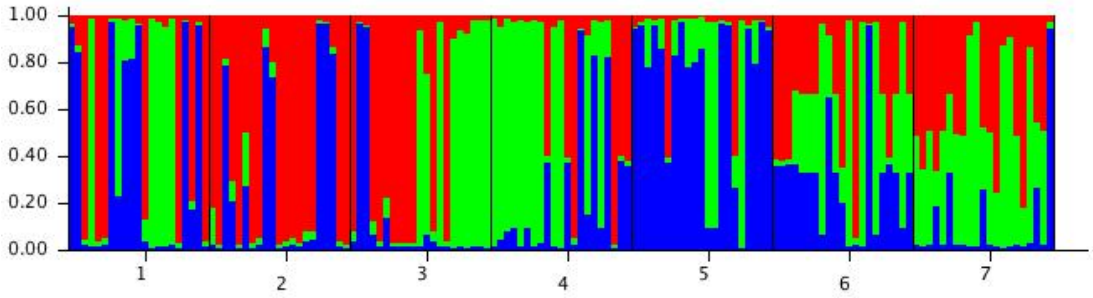
APPENDIX 1

Prado, Rubio, Yañez-Espinosa & Bede

Effects of plant ontogeny, gender and genotype on leaf azoxyglycoside content in *Dioon edule* Lindl.

Population structure analysis using STRUCTURE software (Pritchard et al. 2000)



Estimated population structure of 7 *Dioon edule* populations. Each individual is represented by a vertical line, wich is divided into *K* colors that represent the individual's estimated membership fractions in *K* clusters (*K*=3). 1- El Chijol, 2-Rincón de los Naranjos, 3-Los Pocitos, 4-Los Anteojos, 5-Saucillos, 6-Agua de Gamotes, 7- San Nicolas de los Naranjos.

APPENDIX 2

Prado, McKenna & Windsor

Molecular evidence of cycad seed predation by an immature aulacoscelidine beetle (Coleoptera: Orsodacnidae)

Genes, primers, and substitution models used in phylogeny reconstruction.

Regions	Primers	Primer sequence (5'-3')	Fragment Length (bp)	Model of Evolution ModelTest	Model of Evolution MrModelTest
CO1S	1718F	GGA GGA TTT GGA AAT TGA TTA GTT CC	527	GTR+I+G	GTR+I+G
	2191R	CCC GGT AAA ATT AAA ATA TAA ACT TC			
CO1L	2183F	CAA CAT TTA TTT TGA TTT TTT GG			
	3014R	TCC AAT GCA CTA ATC TGC CAT ATT			
28S	D2up4	GAG TTC AAG AGT ACG TGA AAC CG	548	GTR+G	GTR+G
	D2dnB5	CCT TGG TCC GTG TTT CAA GAC			
18SA	VARBESTUP	TCC GAT AAC GAA CGA GAC TC	516	TIM+I+G	GTR+I+G
	VARBESTDN	GTT ACG ACT TTT ACT TCC TC			
18SB	Sb25	TCT TTG GCA AAT GCT TTC GC	411		
	Sa07	ATT AAA GTT GTT GCG GTT			