

# Annotating PDFs using Adobe Reader XI

Version 1.4 January 14, 2014

## 1. Update to Adobe Reader XI

The screen images in this document were captured on a Windows PC running Adobe Reader XI. Editing of DJS proofs requires the use of Acrobat or Reader XI or higher. At the time of this writing, Adobe Reader XI is freely available and can be downloaded from <http://get.adobe.com/reader/>

## 2. What are eProofs?

eProof files are self-contained PDF documents for viewing on-screen and for printing. They contain all appropriate formatting and fonts to ensure correct rendering on-screen and when printing hardcopy. DJS sends eProofs that can be viewed, annotated, and printed using the free version of Acrobat Reader XI (or higher).

## 3. Comment & Markup toolbar functionality

### A. Show the Comment & Markup toolbar

The Comment & Markup toolbar doesn't appear by default. Do one of the following:

- Select View > Comment > Annotations.
- Click the Comment button in the Task toolbar.

*Note: If you've tried these steps and the Annotation Tools do not appear, make sure you have updated to version XI or higher.*

### B. Select a commenting or markup tool from the Annotations window.



*Note: After an initial comment is made, the tool changes back to the Select tool so that the comment can be moved, resized, or edited. (The Pencil, Highlight Text, and Line tools stay selected.)*

### C. Keep a commenting tool selected

Multiple comments can be added without reselecting the tool. Select the tool to use (but don't use it yet).

- Right Click on the tool.
- Select Keep Tool Selected.

## 4. Using the comment and markup tools

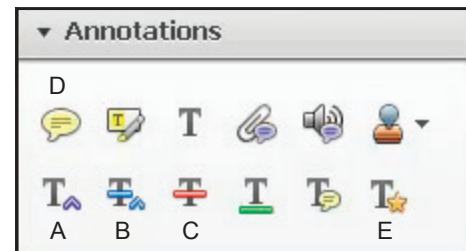
To *insert*, *delete*, or *replace* text, use the corresponding tool. Select the tool, then select the text with the cursor (or simply position it) and begin typing. A pop-up note will appear based upon the modification (e.g., inserted text, replacement text, etc.). Use the Properties bar to format text in pop-up notes. A pop-up note can be minimized by selecting the  button inside it. A color-coded  symbol will remain behind to indicate where your comment was inserted, and the comment will be visible in the Comments List.

## 5. The Properties bar

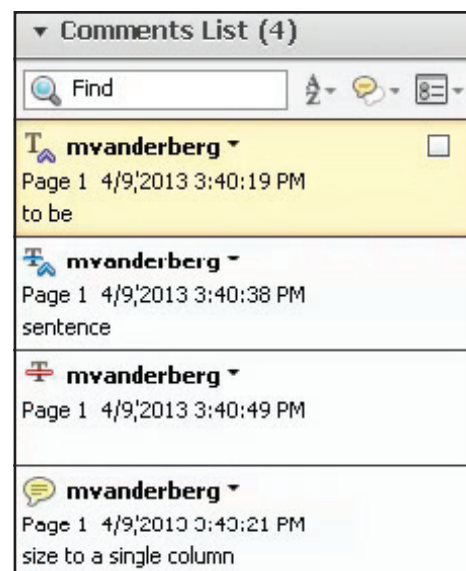
The Properties bar can be used to add formatting such as bold or italics to the text in your comments.

To view the Properties bar, do one of the following:

- Right-click the toolbar area; choose Properties Bar.
- Press [Ctrl-E]



- A. Insert Text tool
- B. Replace Text tool
- C. Delete Text tool
- D. Sticky Note tool
- E. Text Correction Markup tool



## 6. Inserting symbols or special characters

An 'insert symbol' feature is not available for annotations, and copying/pasting symbols or non-keyboard characters from Microsoft Word does not always work. Use angle brackets < > to indicate these special characters (e.g., <alpha>, <beta>).

## 7. Editing near watermarks and hyperlinked text

eProof documents often contain watermarks and/or hyperlinked text. Selecting characters near these items can be difficult using the mouse alone. To edit an eProof which contains text in these areas, do the following:

- Without selecting the watermark or hyperlink, place the cursor near the area for editing.
- Use the arrow keys to move the cursor beside the text to be edited.
- Hold down the shift key while simultaneously using arrow keys to select the block of text, if necessary.
- Insert, replace, or delete text, as needed.

## 8. Summary of main functions

- A. Insert text - Use Insert Text tool (position cursor and begin typing)
- B. Replace text - Use Replace Text tool (select text and begin typing)
- C. Delete text - Use Strikethrough Text tool (select text and press delete key)  
*Note: The Text Correction Markup tool combines the functions of all three tools.*
- D. Sticky Note - Use Sticky Note tool to add comments not related to text correction.

## 9. Reviewing changes

To review all changes, do the following:

- Click the Comments button to reveal the comment tools
- Click the triangle next to Comments List (if not already visible)

*Note: Selecting a correction in the list will highlight the corresponding item in the document, and vice versa.*

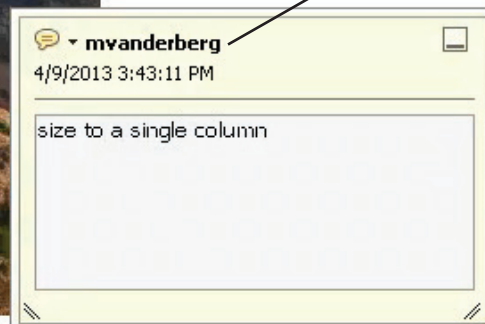
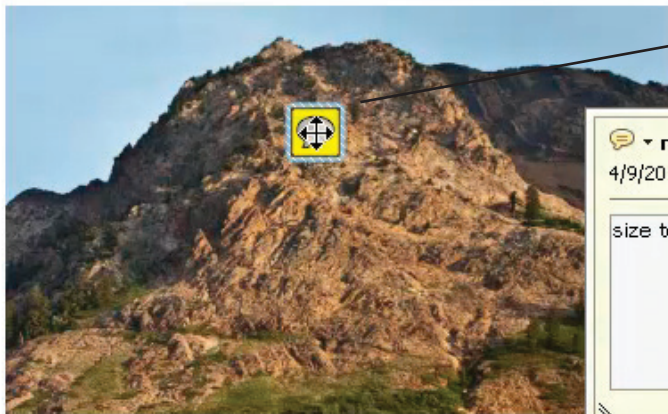
## 10. Still have questions?

Try viewing our brief training video at <https://authorcenter.dartmouthjournals.com/Article/PdfAnnotation>

### This PDF needs to be proofread and annotated.

*Note: these annotations will not actually change the content of the PDF – they just point out the areas where corrections are needed. The actual corrections will be made to the native article files.*

1. **Insert Text Tool:** Text needs inserted into this sentence.
2. **Replace Text Tool:** Some of the text in this paragraph needs to be replaced.
3. **Delete Text Tool:** Some of the text in this overly long sentence needs to be deleted.
4. **Sticky Note Tool:** This image needs to be reduced:



A

B

C

D

## LEAF TRAITS AND HERBIVORY LEVELS IN A TROPICAL GYMnosperm, *Zamia stevensonii* (Zamiaceae)<sup>1</sup>

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- **Premise of the study:** 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 be established only after 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 incidence of specialist herbivores on *Z. stevensonii* during the first year after leaf flush.
- **Methods:** Herbivore incidence, leaf production, and leaf traits that might affect herbivory—including leaf age, lamina thickness, resistance-to-fracture, work-to-fracture, trichome density, and chlorophyll, water, and toxic azoxyglycoside (AZG) content—were measured throughout leaf development. Principal component analysis and generalized linear models identified characteristics that may explain herbivore incidence.
- **Key results:** Synchronized leaf development in *Z. stevensonii* is characterized by quick leaf expansion and delayed greening. Specialist herbivores feed on leaves between 10 and 100 d after flush and damage ~37% of all leaflets produced. Young leaves are protected by AZGs, but these defenses rapidly decrease as leaves expand. Leaves older than 100 d are protected by toughness.
- **Conclusions:** Because 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 against highly specialized herbivores, underlining the convergence in defensive syndromes by major plant lineages.

**Key words:** *Aulacoscelis*; azoxyglycosides; delayed greening; *Eumaeus*; leaf toughness; specialist insects; trichomes; *Zamia*; Zamiaceae.

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, because it is costly for an insect herbivore to eat tough leaves (Coley, 1983; Hochuli, 1996; Lucas et al., 2000; Kitajima et al., 2012). 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). By 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<sup>-1</sup> for understory species (Vovides, 1990; Norstog and Nicholls, 1997). 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 for many years (Norstog and Nicholls, 1997). Some cycad populations exhibit a synchronized leaf flush in which most individuals of the population produce flushes of new leaves during a period of a few weeks. It has been proposed that this is a strategy to minimize herbivory by satiating low insect populations with an ephemeral resource (Clark and Clark, 1988, 1991; Clark et al., 1992). However, synchronized flushing of new leaves may 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, female *Zamia skinneri* Warscz. ex A. Dietr. 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 (Smith, 1966; Teas and Dyson, 1967; Laqueur and Spatz, 1968; Matsushima et al., 1979). When the sugar moiety from the AZG is cleaved by either plant or insect glycosidases, the azoxy core (methyl-azoxymethanol [MAM]) becomes highly reactive, leading to alkylation of DNA or proteins (Laqueur and Spatz, 1968). Although AZGs are a good defense against generalist herbivores, cycad specialists have adaptations that allow them to cope with plant toxicity. The AZGs present in cycad leaves are metabolized rapidly and

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TABLE 1. Leaf traits measured in *Zamia stevensonii*.

Leaf trait	Abbreviation	Unit
Leaf age	Age	Days
Leaf length	Size	cm
Lamina thickness	Thick	mm
Resistance-to-fracture	Res fract	MN m <sup>-2</sup>
Work-to-fracture	Work F	J m <sup>-2</sup>
Chlorophyll <i>a</i> content	Chl <sub>A</sub>	g mg <sup>-1</sup> FW
Chlorophyll <i>b</i> content	Chl <sub>B</sub>	g mg <sup>-1</sup> FW
Water content	H <sub>2</sub> O	Percentage of fresh weight
AZG content	AZGs	μmol g <sup>-1</sup> DW
Trichome density	Trich	Number mm <sup>-2</sup>

may even be sequestered and used by herbivore specialists (Rothschild et al., 1986; Rothschild, 1992; Castillo-Guevara and Rico-Gray, 2002; Schneider et al., 2002; Prado et al., 2011). 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 reglycosylation 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 >10 yr (Taylor and Holzman, 2012; A. S. Taylor, personal communication). Previously considered 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 “blanco” among cycad growers (Taylor and Holzman, 2012). Reaching lengths of 120 cm, leaves of *Z. stevensonii* can take up to 4 mo to reach full size (Taylor and Holzman, 2012). *Zamia stevensonii* is host to a suite of specialized leaf herbivores, including *Eumaeus godartii* Boisduval (Lepidoptera: Lycaenidae), *Aulacoscelis appendiculata* Cox & Windsor (Coleoptera: Orsodacnidae; Cox and Windsor, 1999), *Nomotus* sp. (Coleoptera: Languriidae), plus unidentified species in the family Languriidae (Windsor et al., 1999; A. Prado et al., personal observation). 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 have not been observed feeding 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; specialist cycad herbivores seldom eat coriaceous older leaves. How cycads limit herbivory on young leaves is not known. Here, 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, and AZG, 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 against well-adapted herbivores and (2) quantify the overall loss of new foliage to insect herbivores.

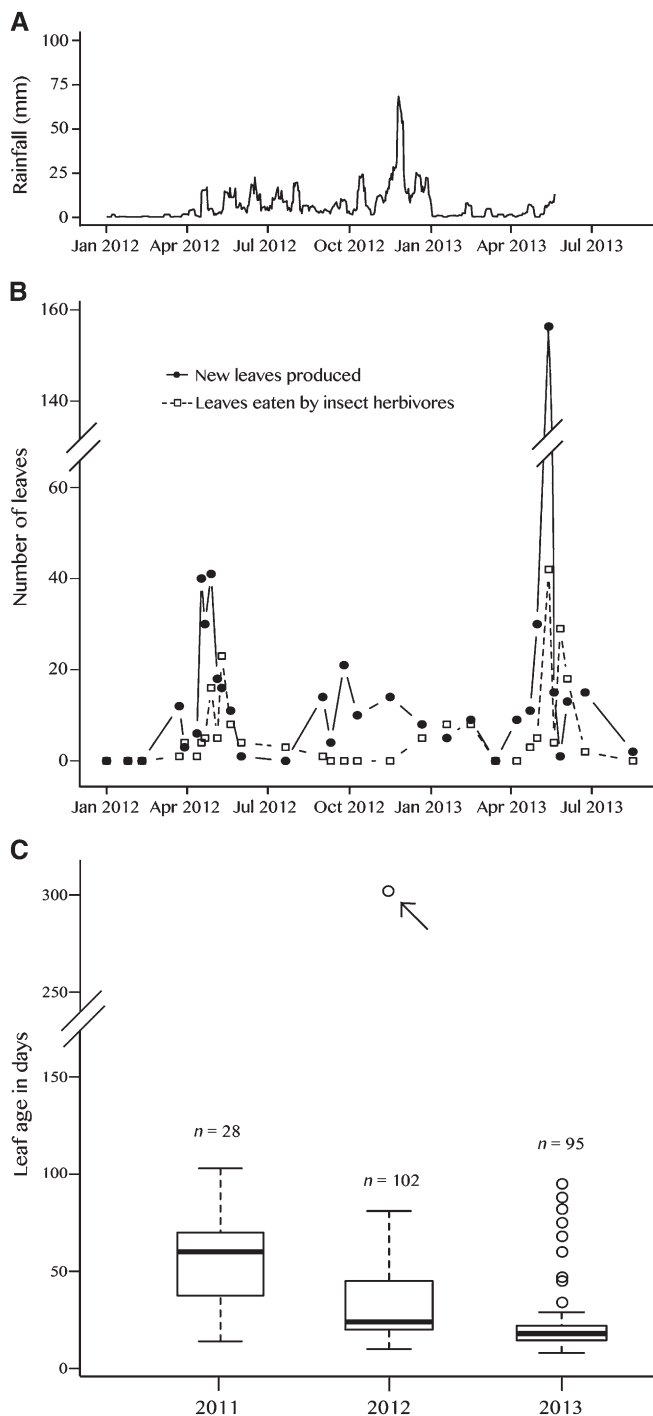


Fig. 1. Rainfall, phenology, and herbivore incidence on *Zamia stevensonii*. (A) The 7-d running average of daily rainfall (January 2012–May 2013). (B) New leaf production and insect damage during 2012 and 2013. (C) Age when leaves were attacked by herbivores (2011, 2012, and 2013). Open circles indicate outliers, and the arrow indicates an herbivory event by *E. godartii* caterpillars on a 305-d-old leaf.

## MATERIALS AND METHODS

**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 semideciduous 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 dry season of 3–4 mo and an average annual rainfall of 2400 mm (Candanedo et al., 2003). Large patches of *Z. stevensonii* occur on outcrops of calcareous sedimentary rock.

Individual *Z. stevensonii* 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 data (Table 1) were collected for 103 plants during 2011. Plants were surveyed twice a week from March to July and revisited 6 mo and 1 yr 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 they were not censused.

**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 wk and revisited after 6 mo and 1 yr. Leaf trait measurements were performed on nondamaged 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 the damage was still present; if the insect was absent ( $n = 13$ ), its 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), whereas 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 that ranged in age from 7 to 365 d. 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.

**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), given that Westbrook et al. (2011) observed that mortality rates in tree seedlings were negatively correlated with 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 center of each leaflet in the fourth leaflet pair 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 d old) could not be quantified because 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 leaflets in the fourth pair from the apex, and the values were averaged. Work-to-fracture ( $J m^{-2}$ ) was calculated by multiplying the resistance-to-fracture by the lamina thickness (Lucas et al., 2000).

**Trichomes**—Nonglandular trichomes are present on *Zamia* leaflets (Stevenson, 1981). Trichome density was measured under a light stereoscope by counting the trichomes on a  $2 \times 2$  mm<sup>2</sup> area on the abaxial side of the first leaflet pair.

**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 and then drying it at 40°C for 48 h 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 6000 rpm for 1 min. The supernatant was analyzed spectrophotometrically (Tecan Infinite M200 Pro) at 646 and 663 nm, and chlorophyll *a* and *b* content were calculated according to Wellburn (1994).

**Azoxyglycoside content**—Azoxyglycoside 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 20000 rpm for 10 min. The supernatant was transferred to a new vial, frozen in liquid nitrogen, and lyophilized. Samples were resuspended in 25 mM sodium acetate buffer, pH 5.0. In parallel, two aliquots of each sample were diluted 6.5× in sodium acetate buffer; one sample was incubated overnight at room temperature with  $\beta$ -glucosidase (0.5 U; Sigma-Aldrich, St. Louis, Missouri, USA), and the other sample was left untouched.  $\beta$ -glucosidase converts AZGs to the 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 ( $300 \times 7.7$  mm, Agilent) using an isocratic elution of 100% water at a flow rate of 0.45 mL  $min^{-1}$  at 65°C (Yagi et al., 1980, 1983; Yagi, 2004). Compounds were detected by a photodiode array detector at 215 nm. MAM acetate (National Cancer Institute, Washington, D.C., USA) was converted into MAM by incubation with an esterase enzyme (Sigma-Aldrich) in 1M Tris-HCl buffer, pH 8.5, for 1 h at room temperature and used to generate a 7-point standard curve. Levels of MAM were identified on the basis of retention time and confirmed by the removal of the AZG peak in the  $\beta$ -glycosidase-treated subsample (Prado et al., 2011).

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

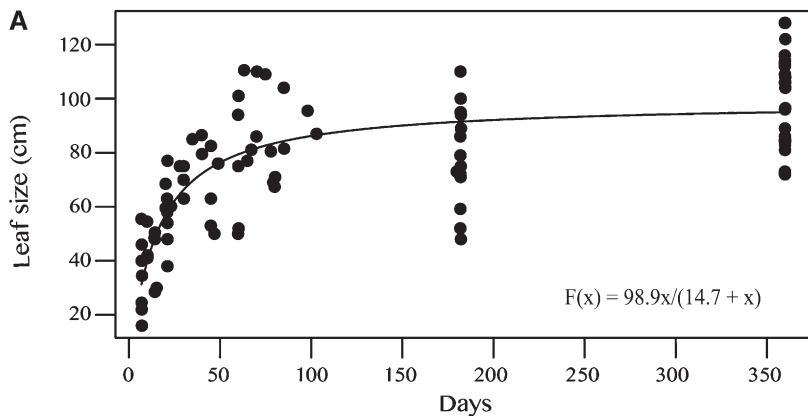
**Statistical analysis**—**Leaf development**—Leaf trait data were analyzed by fitting deterministic models using nonlinear least squares (nls function in R; Bolker, 2008). The Michaelis-Menten model was fitted to leaf size, chlorophyll content, and leaf toughness data, which increased rapidly until an abrupt

TABLE 2. New leaf production and herbivore damage on *Zamia stevensonii* during three leaf-flushing events.

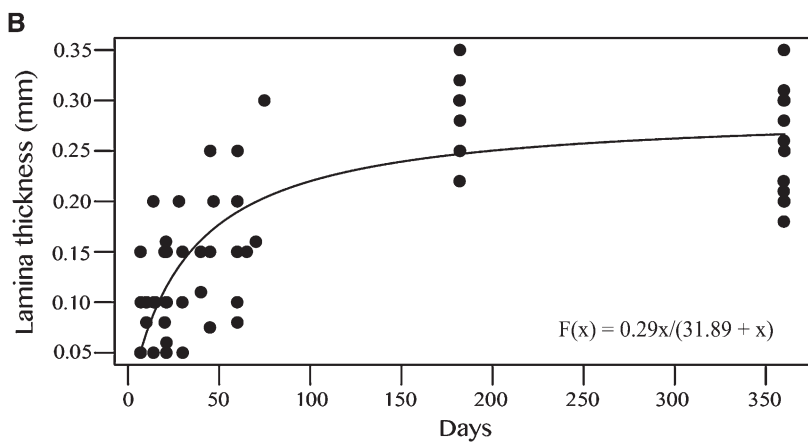
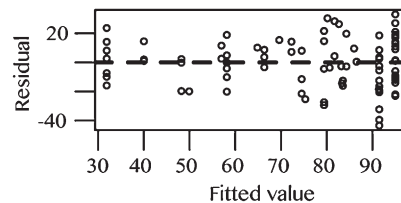
Year	Number of plants surveyed	Number of plants producing new leaves	Total number of leaves produced	Number of plants with herbivore damage	Total production lost to herbivores (%) <sup>a</sup>
2012	117	73	217	39	41.01%
2013	117	64	223	53	33.00%
Herbivore responsible for the damage (%)					
	Number of leaves damaged <sup>b</sup>	<i>Aulacoscelis appendiculata</i>	<i>Eumaeus godartii</i>	<i>A. appendiculata</i> + <i>E. godartii</i>	<i>Nomotus</i> sp.
2011	28	65%	27%	0%	8%
2012	102	54.2%	18.6%	8%	5%
2013	95	51.9%	12%	20%	12%

<sup>a</sup>Number of leaflets damaged divided by the total amount of leaflets produced during the flushing event of April–May.

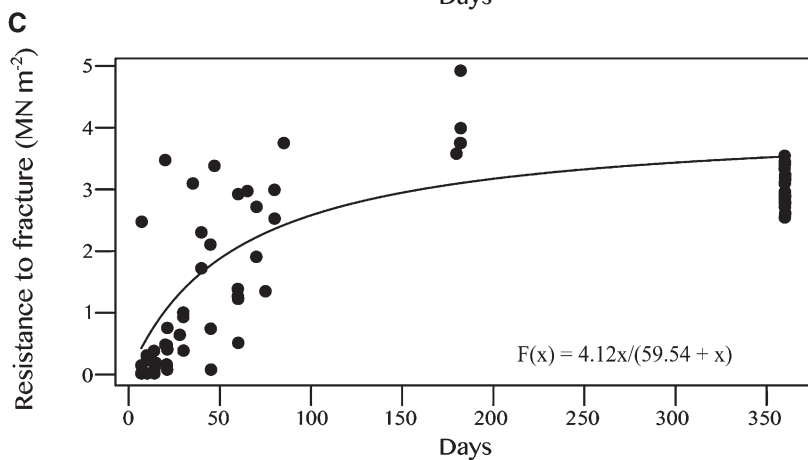
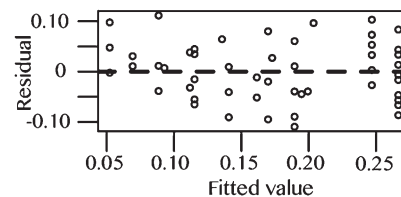
<sup>b</sup>Including partially damaged leaves.



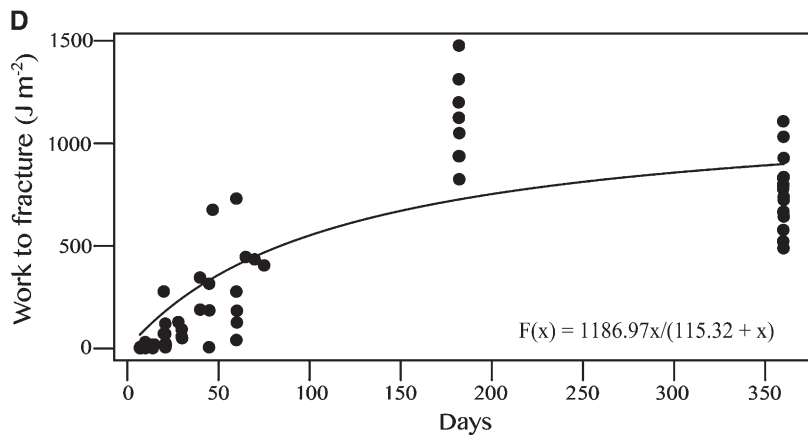
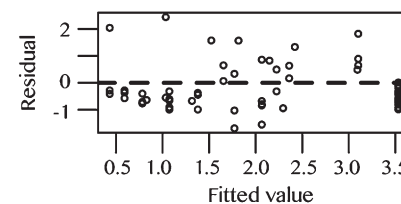
Parameters	SE	Significance
$a = 98.87$	3.22	<0.001
$b = 14.708$	2.45	<0.001



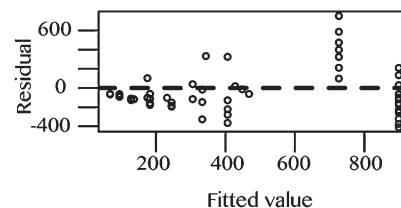
Parameters	SE	Significance
$a = 0.29$	0.01	<0.001
$b = 31.89$	6.23	<0.001



Parameters	SE	Significance
$a = 4.12$	0.26	<0.001
$b = 59.54$	13.05	<0.001



Parameters	SE	Significance
$a = 1186.97$	145.33	<0.001
$b = 115.32$	37.77	<0.01



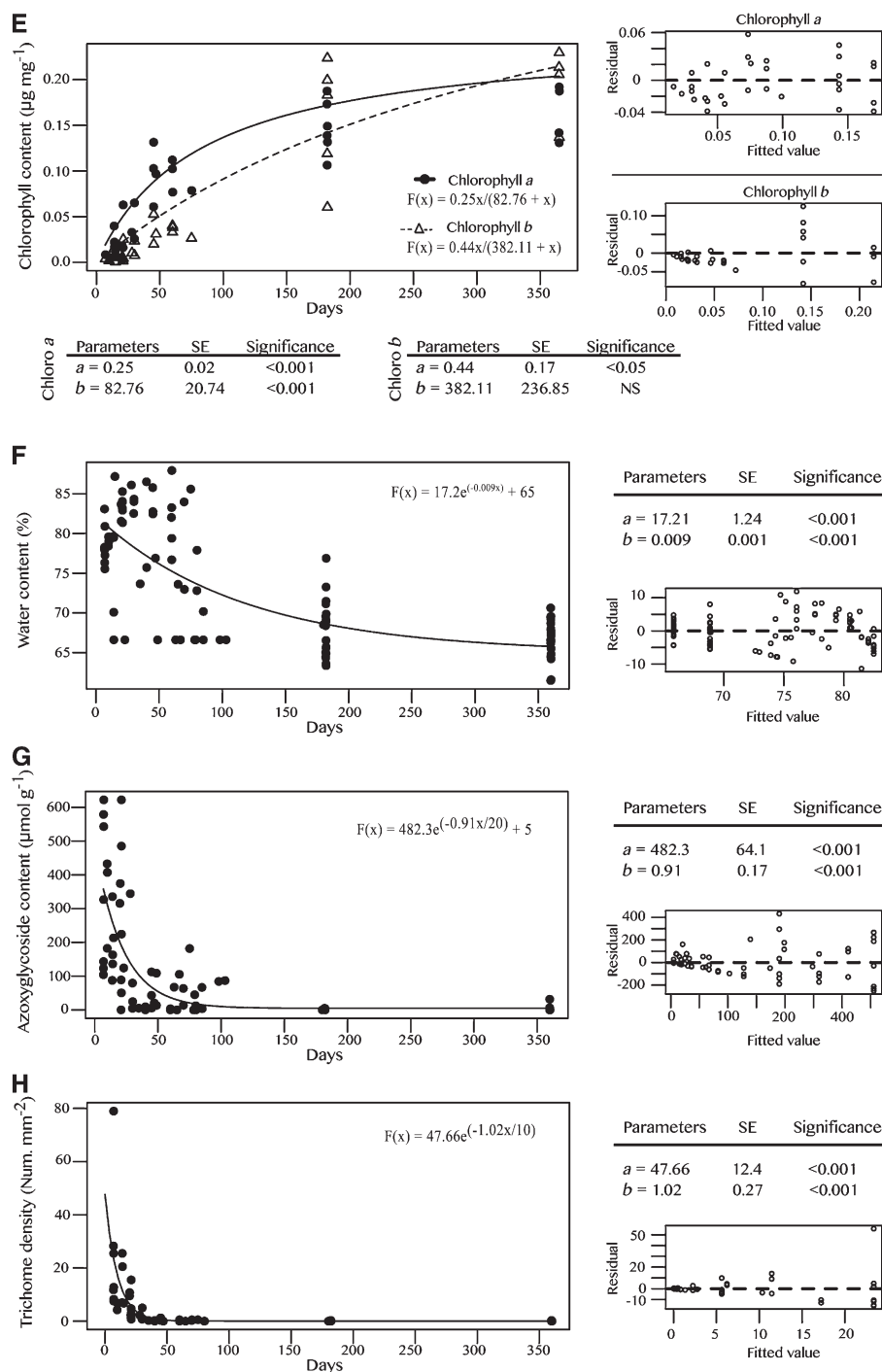


Fig. 2. Continued.

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. By contrast, a negative exponential model,  $F(x) = ae^{-bx}$ , was fitted to the water content, AZG content, and trichome

← Fig. 2. Changes in the leaf traits of *Zamia stevensonii* over a 1-yr period. The phenology of leaf traits was measured during 2011. Day 0 equals the day when leaf primordia protruded from 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 the data in A–E. Negative exponential models  $F(x) = ae^{-(bx)+65}$ ,  $F(x) = ae^{-(bx/20)+5}$ , and  $F(x) = ae^{-(bx/10)}$  were fitted to data in F, G, and H, respectively. Residual plots and a summary of the parameters for each model are at the right of the main graph.

density of leaves of age  $x$ , because these traits decreased quickly but reached an asymptote slowly. 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.

**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 whether 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 Akaike's information criterion corrected for small sample size (AIC<sub>c</sub>) and  $r^2$  values.

**Protection of the young leaves**—Preliminary analysis of the 3-yr survey showed a constraint on herbivory during the first 10 d of leaf development. This restriction on herbivory was supported by PCA results. Therefore, we tested whether 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 were 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) deterred 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; Zuur et al., 2009; Crawley, 2012). 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. Because 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); in  $F$  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.

## RESULTS

**Phenology**—The total number of leaves on individual *Z. stevensonii* ranged from 1 to 23 ( $8.8 \pm 4.7$  [average  $\pm$  SD]), with 4–16 leaflet pairs leaf<sup>-1</sup> ( $9.5 \pm 3.0$ ). As expected, older, larger plants tended to have longer leaves with more leaflets (data not shown). More than half (62% and 55%, respectively) of all *Z. stevensonii* produced a synchronous flush of new leaves during

the first 2 mo of the wet season in 2012 and 2013 (Fig. 1A, 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 individual *Z. stevensonii* studied produced similar numbers of leaves during 2012 and 2013 (217 and 223, respectively). However, the peak flush during 2013 was more synchronous than the one in 2012 (Fig. 1 and Table 2). The difference could reflect the smaller number of plants that contributed to the 2013 peak flush (Table 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 ( $>500 \mu\text{mol g}^{-1}$  DW) and water content ( $\approx 85\%$ ). Within 1 wk, the faintly pigmented leaves began to unfold. Soon thereafter, greening began, with chlorophyll content increasing over the subsequent year (Fig. 2E). Chlorophyll *a* concentrations reached half-maximal values at 80 d, whereas chlorophyll *b* increased at a slightly lower rate, taking up to 1 yr to reach half-maximal values. Leaf water content declined relatively slowly over the course of an entire year (Fig. 2F; minimum value = 60%), whereas both trichome density and AZG content decreased very rapidly and closely followed changes in leaf size (Fig. 2G, H). Unbranched, nonglandular trichomes densely covered the youngest leaves ( $>20 \text{ mm}^{-2}$ ), but their density decreased rapidly as the leaf expanded. All trichomes had fallen away within 3 wk and were not replaced (Fig. 2H). The AZG levels decreased at a slower pace, going from  $>500 \mu\text{mol g}^{-1}$  DW to  $\sim 100 \mu\text{mol g}^{-1}$  DW in 2 mo (Fig. 2G). The AZG levels declined as leaf size increased ( $\rho = -0.539$ ,  $P < 0.001$ ; Table 3), suggesting that plants protect young leaves with high AZG levels but that these become diluted as leaflets expand. In leaves older than 6 mo, AZG levels were below the HPLC detection limit ( $\approx 1 \mu\text{mol g}^{-1}$  DW).

Two weeks after leaf flush, leaves of *Z. stevensonii* reached half their full size, which was attained at  $\sim 100$  d. Leaf size and lamina thickness increased quickly and reached an abrupt asymptote at  $\sim 80$  d and  $\sim 110$  d, respectively (Fig. 2A, B). By contrast, resistance-to-fracture increased at a slower rate (Fig. 2C). Therefore, leaves first invested in growth, with half-maximal size reached at 14 d and half-maximal lamina thickness reached at 31 d. Toughness, indicated by the half-maximal resistance-to-fracture, was achieved only 59 d after leaf flush.

TABLE 3. Correlation coefficients (Spearman's  $\rho$ ) between *Zamia stevensonii* leaf traits (defined in Table 1; NA = not applicable). All coefficients are significant to at least  $P < 0.001$ . Values above the diagonal are numbers of pairwise comparisons.

	Age	Size	Thick	Res fract	Work F	Chl <sub>A</sub>	Chl <sub>B</sub>	H <sub>2</sub> O	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 <sub>A</sub>	0.915	0.696	0.663	0.830	0.845		50	50	50	49
Chl <sub>B</sub>	0.927	0.744	0.669	0.842	0.854	0.983		50	50	49
H <sub>2</sub> O	-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	



**Herbivore incidence**—Most leaf flushes occurred within a 2-mo 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. Insect herbivory occurred only on leaves between 10 and 103 d of age, and herbivory was highest on leaves between 20 and 30 d of age (Fig. 1C). In the 3 yr of the study, only four exceptions were noted: three incidences at 9 d by *A. appendiculata* beetles, and one incidence at 305 d by *E. godartii* caterpillars (Fig. 1C).

Loss of leaflets of the newly produced leaves due to insect herbivores was 41.01% in 2012 and 33% in 2013 (Table 2). These sizable losses in photosynthetic potential resulted from damage by three species: *E. godartii*, and adult *A. appendiculata* and *Nomotus* sp.

Groups of  $\geq 20$  *A. appendiculata* beetles on a single leaf were not uncommon during the last 2 wk of April and the first 2 wk of May in each of the 3 yr of the study. Frequently, feeding by *A. appendiculata* 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* and *A. appendiculata*, 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 3 yr of the study, *A. appendiculata* accounted for 51–65% and *E. godartii* for 12–27% of damage by herbivores (Table 2). An additional 8–20% of damage was caused by both these species feeding together on the same leaf. Adult *Nomotus* sp. accounted for the rest of the herbivory (5–12%).

**Herbivory and leaf traits**—Herbivory on the youngest and most tender leaves (<9 d) was not observed. Herbivory rarely

occurred on fully toughened leaves ( $>750$  J m<sup>-2</sup>) but was restricted to leaves with toughness values of 10–697 J m<sup>-2</sup>. The highest levels of herbivory were recorded on leaves containing 10–200  $\mu\text{mol g}^{-1}$  DW AZGs, few trichomes (<35 trichomes mm<sup>-2</sup>), and high water content (66–86%).

Principal component 1 (PC1) explained >75% of the variation in *Z. stevensonii* leaf traits (Fig. 3A and Table 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. 3B).

The proportion of leaflets eaten is a linear function of leaf age and AZG concentration ( $P < 0.001$ ), whereas time of exposure and, surprisingly, trichomes had no significant effect ( $P = 0.747$  and  $P = 0.1058$ , respectively; Table 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 chance of herbivory on leaves  $\geq 180$  d old is near zero and that high levels of toxins can suppress herbivory despite the age of the leaves (Fig. 4). These results suggest that older leaves are well protected because of their toughness, whereas younger, tender leaves are protected by toxins, even against AZG-sequestering herbivores. However, as leaves expand and AZG levels drop, and before leaves reach maximum toughness, cycads are vulnerable to herbivory by specialist insects.

## 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). Our study examined leaf traits of the slow-growing Neotropical cycad *Z. stevensonii* under the pressure of several highly specialized

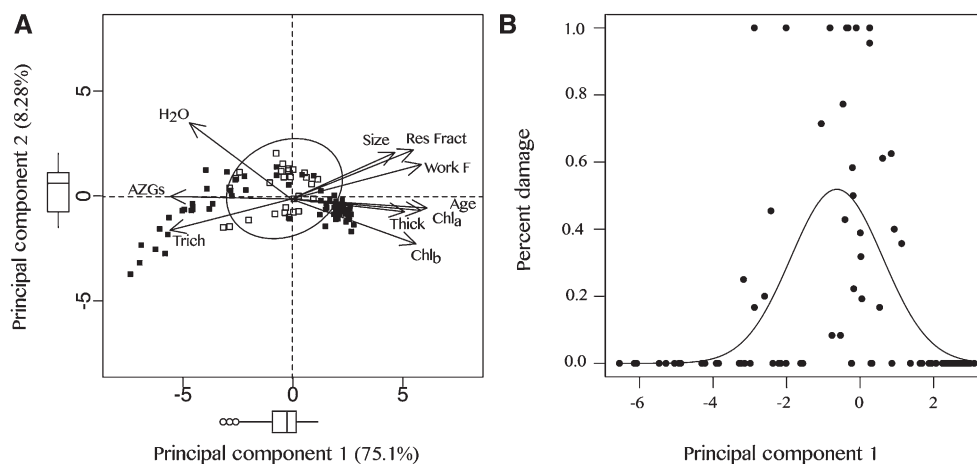


Fig. 3. Correlations between leaf traits (defined in Table 1) and leaf damage. (A) Principal component analysis biplots of *Zamia stevensonii* leaf traits. Open squares represent damaged leaves, and filled squares represent undamaged. A 95% confidence interval ellipse has been drawn around the damaged 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 fitted to the data ( $F(x) = a * e^{-0.5 * ((x - b) / c)^2}$ ).

TABLE 4. First (PC1) and second (PC2) principal component loadings for leaf traits (defined in Table 1).

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 <sub>A</sub>	0.91325	-0.1597
Chl <sub>B</sub>	0.85378	-0.475
H <sub>2</sub> O	-0.75097	0.55471
AZGs	-0.85165	-0.0993
Trich	-0.868	-0.30442

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, b; Kursar and Coley, 2003; Poorter and Bongers, 2006). Our results are consistent with the “resource availability hypothesis,” which predicts that under high risk of predation, slow-growing species will invest heavily in constitutive defenses (Coley et al., 1985), thereby highlighting 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. 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, b; 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 <20% as low and those >60% as high. In the 2 yr of our study, 33–41% of leaflets were damaged by herbivores; >40% of the leaves were at least partially damaged during leaf expansion. Herbivory by specialist insects began ~10 days after leaf flush and then dropped drastically at ~100 d. Two important components of leaf toughness, lamina thickness and resistance-to-fracture, were close to their maximum by 100 d. Lamina thickness (mean  $\pm$  SD = 0.25  $\pm$  0.09 mm) and resistance-to-fracture (3.28  $\pm$  0.53 MN m<sup>-2</sup>) exhibited by mature leaves of *Z. stevensonii* were higher than in shade leaves of 177 woody angiosperm trees meta-analyzed by Onoda et al. (2011; mean lamina thickness = 0.187; mean force to punch = 2.35 MN m<sup>-2</sup>). Leaves of *Z. stevensonii* 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* Hüb. can feed

on *Z. skinneri* only if the leaves are <65 d old, whereas the later instars are able to eat mature leaves. Similarly, the first instar of *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 d of age may substantially reduce populations of *E. godartii* and pose a formidable barrier to herbivory by *A. appendiculata* (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 that it applies to mobile adult aulacoscelid beetles. Several chrysomeloid beetle species are known to aggregate in response to a blend of plant-derived volatiles and pheromones released by the males (Dickens et al., 2002; Beran et al., 2011). 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 that 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. stevensonii* in 1 d. These specialists sequester and use cycad AZGs for their own defense (Rothschild, 1992; Schneider et al., 2002; Prado et al., 2011, 2012). *Aulacoscelis 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). So how do leaves <10 d of age escape herbivory by these AZG-sequestering insects? Our data show that specialist herbivores feed on cycad leaves that contain few trichomes and have AZG levels in the range of 10–480  $\mu\text{mol g}^{-1}$  DW, but do not feed on the youngest leaves, which are covered with trichomes (>20 mm<sup>-2</sup>) and contain AZG levels >500  $\mu\text{mol g}^{-1}$  DW. This suggests that specialist herbivores are unable to feed on young, pubescent leaves with high AZG levels. However, an alternative 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 herbivory by *E. godartii* as the adult female butterfly anticipates new leaf production and lays its eggs on *Z. stevensonii* trunks, old leaf bases, and, occasionally, 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 (Clark and Clark, 1991; A. Prado et al., personal observation). Furthermore, a small forest plot has cycad leaf flushes of many ages, so *E. godartii* caterpillars and adult *A. appendiculata* have a range of leaf ages from which to choose. Finally, our generalized linear model predicted that the highest AZG levels are negatively correlated with herbivore damage, whereas exposure time and trichomes did not significantly affect herbivory (Table 5 and Fig. 4). In fact, most herbivore damage occurred on leaves 20–30 d old, when AZG levels were between 50 and 200  $\mu\text{mol g}^{-1}$  DW.

TABLE 5. Analysis of deviance of binomial general-linear-model 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

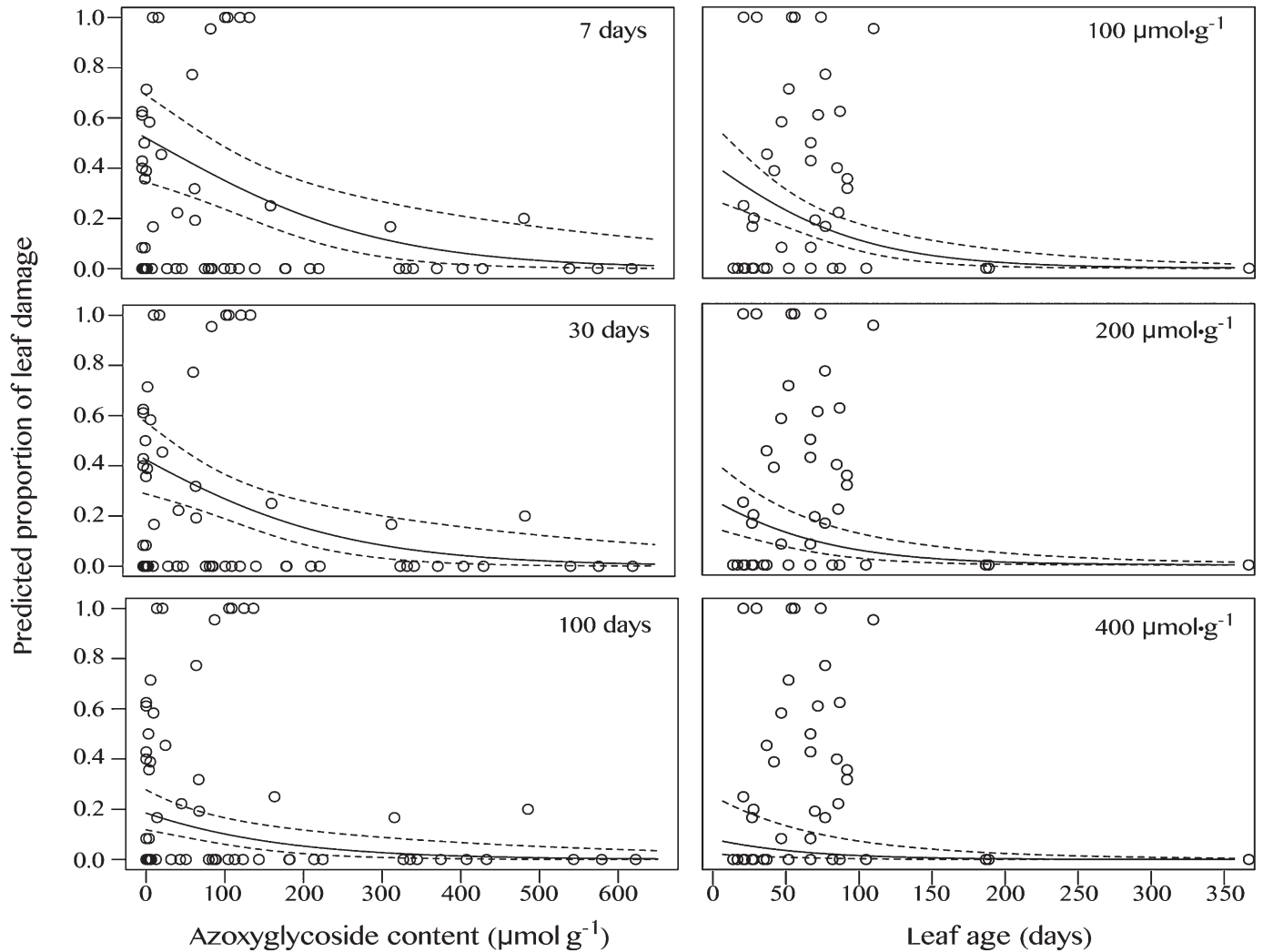


Fig. 4. Generalized linear model predictions for the proportion of leaf damage based on leaf age and azoxyglycoside (AZG) concentration, with fitted values (solid lines) and 95% confidence bands (dashed lines) for the optimal binomial GLM. In the left panels, predictions were performed at the specific leaf ages 7, 30, and 100 d. In the right panels, predictions were performed at the specific AZG toxin concentrations 100, 200, and 400  $\mu\text{mol}\cdot\text{g}^{-1}$ . Open circles are the actual observations; the same data are used in all six panels.

Therefore, taken 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 made in other plant-specialist insect systems in which specialist herbivores are not completely immune to the plants' toxins, but rather are capable of tolerating moderately high doses (Baldwin, 1988; Berenbaum et al., 1989; Adler et al., 1995; Ali and Agrawal, 2012). However, we cannot dismiss the possibility that specialist herbivores may be avoiding young leaves for reasons not accounted for 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. They are not stored in trichomes, because the trichomes are nonglandular, and leaves still contain significant AZG levels even after trichomes are lost ( $\approx 3$  wk; Fig. 2H). Norstog and Fawcett (1989) proposed that AZGs are compartmentalized in idioblasts in the male and female cones; it is unknown whether 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.

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