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Developmental Changes in Direct and Indirect Defenses in the Young Leaves of the Neotropical Tree Genus *Inga* (Fabaceae)

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ABSTRACT

Plant fitness is affected by herbivory, and in moist tropical forests, 70 percent of herbivore damage occurs on young leaves. Thus, to understand the effects of herbivory on tropical plant fitness, it is necessary to understand how tropical young leaves survive the brief, but critical, period of susceptibility. In this study, we surveyed three species of *Inga* during young leaf expansion. Three classes of toxic secondary metabolites (phenolics, saponins, and tyrosine), extrafloral nectar production, leaf area, and extrafloral nectary area were measured at randomly assigned young leaf sizes. In addition, all defenses were compared for potential trade-offs during leaf expansion. No trade-offs among defenses were found, and the concentration of all defenses, except tyrosine, decreased during leaf expansion. We suggest that plants continued to increase phenolic and saponin content, but at a rate that resulted in decreasing concentrations. In contrast, tyrosine content per leaf steadily increased such that a constant concentration was maintained regardless of young leaf size. Nectar production remained constant during leaf expansion, but, because young leaf area increased by tenfold, the investment in extrafloral nectar per leaf area significantly decreased. In addition, nectary area did not change during leaf expansion and therefore the relative size of the nectary significantly decreased during young leaf expansion. These results support the predictions of the optimal defense hypothesis and demonstrate that the youngest leaves have the highest investment in multiple defenses, most likely because they have the highest nitrogen content and are most susceptible to a diversity of herbivores.

Abstract in Spanish is available in the online version of this article.

Key words: ant–plant mutualism; defense chemistry; extrafloral nectary; optimal defense; Panama; plant–herbivore interactions.

HERBIVORY HAS MAJOR IMPACTS ON PLANT FITNESS AND INDIRECT EFFECTS ON THE OUTCOMES OF other biotic interactions (Marquis 1984, Eichhorn *et al.* 2010). In the understory of tropical moist forests, over 70 percent of a leaf's lifetime herbivore damage occurs while it is young and expanding (Coley & Barone 1996). As young leaves are inherently less tough and more nutritious than mature leaves, they are particularly attractive to herbivores (Brunt *et al.* 2006, Yadav *et al.* 2010). Consequently, tropical plants invest an impressive amount of resources into young leaf defenses. Typically, chemical defenses alone comprise 10–50 percent of the dry weight of young leaves (Coley & Aide 1991, Coley & Barone 1996, Boege 2004, Brenes-Arguedas *et al.* 2006, Lokvam *et al.* 2006). In addition, they invest in other indirect biotic defenses or physical defenses such as spines and hairs. Once leaves have reached their adult size, they become tough and low in nutrients, such that they are difficult for herbivores to consume. As a consequence, herbivory rates decrease to nearly zero (Kursar & Coley 2003). Therefore, because herbivory affects tropical plant performance, it is important to understand how young leaves survive the brief, but critical, period of susceptibility.

While developmental changes in defenses of tropical leaves have been examined in other studies (Heil *et al.* 2000a, b, Brenes-Arguedas *et al.* 2006, Lokvam *et al.* 2006), few have compared the investment trade-off between direct and indirect defenses. Direct defenses are produced by a plant, and directly impact the performance of an herbivore and reduce future herbivory. Common examples are toxic chemicals or trichomes. In contrast, indirect defenses rely on an external agent to reduce herbivore performance and consequently, future herbivore damage (Kost & Heil 2008). Common examples of indirect defenses are volatile organic compounds that attract predators or parasitoids of herbivores and extrafloral nectaries that attract ants that act as bodyguards. The optimal defense hypothesis predicts that plants only invest in a subset of defenses because they are costly and resources are limited (McKey 1974, 1979, Rhoades 1979). Therefore, a trade-off among defenses would exist, which could take place spatially or temporally at different developmental stages. For example, ant bodyguards may be more effective than toxic compounds when leaves are small and easily patrolled, but, less effective than toxic compounds when leaves are large. It is also possible, however, that defenses are additive and that the complete suite of different defenses is required to effectively protect a plant against a diversity of potential herbivores.

In this study, we examine the relative investment in direct and indirect defenses during young leaf expansion in three Neotropical tree species in the genus *Inga* (Fabaceae) on Barro Colorado Island, Panama. At the genus level, *Inga* has a diverse array of toxic chemical defenses (Lokvam *et al.* 2004, 2006, Coley *et al.*

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2005, Lokvam & Kursar 2005, Brenes-Arguedas *et al.* 2006). Within the genus, phenolic compounds are the most abundant class of defense compounds that have been shown to reduce herbivore performance (Coley *et al.* 2005, Lokvam & Kursar 2005, Brenes-Arguedas *et al.* 2008). Saponins and even tyrosine at the levels found in *Inga* leaves, however, have also been shown to reduce herbivore growth (Lokvam *et al.* 2004, 2006, Coley *et al.* 2005, Brenes-Arguedas *et al.* 2008). Most species of *Inga* also have extrafloral nectaries that produce nectar and use ant protection to varying degrees (Koptur 1984, 1985, Brenes-Arguedas *et al.* 2006, Bixenmann *et al.* 2011). Extrafloral nectaries in *Inga* are located on the rachis between leaflets pairs and only secrete nectar when the leaves are young and expanding. In this study, we examined the investment in extrafloral nectar production and extrafloral nectary structures relative to young leaf area during leaf expansion. In addition, we examined the investment in toxic secondary metabolites and compared the response variables for trade-offs. Recognizing the extensive divergence in the defense traits among species of *Inga* (Kursar *et al.* 2009), we compared the ontogenetic changes in defenses for several species.

METHODS

Study site.—Field research was conducted on Barro Colorado Island (BCI), Panama from May to July 2007 and 2008. BCI is located in the Panama Canal (9°N 80°W) and is administered by the Smithsonian Tropical Research Institute. The island is a tropical moist lowland forest that experiences a 4-mo dry season (January–April). For *Inga*, most young leaves are produced during the rainy season (P.D. Coley & T.A. Kursar, pers. obs.). This is also the season that herbivores and their predators are most abundant in both gaps and understories (Richards & Windsor 2007).

Field survey.—The young leaves of three focal *Inga* species (*I. marginata*, *I. multijuga*, and *I. umbellifera*) were selected and monitored along the trails of BCI. All three species are shade-tolerant trees from 20 to 30 m tall. *Inga multijuga* is distributed throughout lowland tropical forests in Central America near water and swamps (Pennington 1997). *Inga marginata* and *I. umbellifera* are widely distributed throughout Central and South America in a diversity of lowland forest microhabitats (Pennington 1997). The three species were of similar size and life stage at the time of this experiment: saplings 1–4 m tall. The three species were selected based on their abundance along the trails on BCI and the ease of access to the nectaries. In addition, we chose these particular three species of *Inga* because they represent the range of young leaf expansion rates (*I. marginata* 72% per day, *I. umbellifera* 45% per day, and *I. multijuga* 23% per day). Due to the differences in the expansion rates, the young leaves of the three species are present for different lengths of time (*I. marginata ca* 1 wk, *I. umbellifera ca* 2 wk, and *I. multijuga ca* 4 wk).

One branch per individual sapling between one and four meters tall was sampled. No manipulative treatments were applied to any of the plants, but each focal plant was sampled at a randomly, preassigned young leaf size, and care was taken to sample evenly from plants in light gaps and the understory (microhabitat). If more than 10 percent of the canopy cover was open, then plants were designated as in a gap. If less than 10 percent of the canopy was open, plants were designated as in the understory. Mature and young leaf areas from each focal plant were used to calculate young leaf age expressed as percent of mature leaf size (area of young leaf/area of mature leaf). Extrafloral nectar and leaf samples were collected in tandem and were vacuum-dried and stored at −20°C until they were prepared for analysis (see below). Individual leaves were not sampled more than once and only one leaf per plant was sampled to avoid potential effects of induced defenses on the remaining tissue. In these *Inga* species, however, herbivory does not induce extrafloral nectar production (Bixenmann *et al.* 2011) and herbivore damage only induces chemical defenses by a marginal amount (R. J. Bixenmann, unpubl. data). Nonetheless, efforts were made to collect tissue and nectar from leaves with minimal herbivore damage (average 8%).

Leaf and extrafloral nectary area.—Each time a focal leaf was visited, the leaf was photographed with a scale (Fig. 1). The leaf was oriented perpendicular to the lens of the camera with the entire leaf blade in the same plane. We used NIH ImageJ (http://rsbweb.nih.gov/ij/) to calculate the absolute young leaf area (cm^2), area lost to herbivores (cm^2), and the absolute nectary area (cm^2). In addition, we calculated percent damage (area of herbivore damage/absolute young leaf area), relative extrafloral nectary area (absolute nectary area/absolute young leaf area), and percent adult size (absolute young leaf area/absolute mature leaf area) using mature leaves from each focal plant.

Extrafloral nectar production.—To collect extrafloral nectar from the young leaves, nectaries were first washed with distilled water to remove accumulated nectar. Then, the entire leaf was placed in a plastic bag to prevent rain or insects from removing nectar. We did not use mesh bags because they would not have prevented disturbance by rain. Although plastic bags may influence temperature and humidity, the bags were not airtight and samples were collected mostly in the shaded understory and during the rainy season when air temperatures were cooler. In addition, condensation on the inside of the bags was rarely observed in either habitat (gap or understory), indicating no significant heat difference between the inside and outside of the bag. After 24 h, extrafloral nectar was collected and its volume was measured using microcapillary tubes (initial collection). To collect any residual nectar, one microliter drops of distilled water were placed on nectaries, collected, and added to the initial collection. The nectar was collected into glass GC vials, dried under vacuum, and frozen at −50°C until analysis. Bixenmann *et al.* (2011) demonstrated that an increase in extrafloral nectar production on the leaves of these *Inga* species resulted in an increase in the number of ant bodyguards. Therefore, ant numbers are not reported here. Instead, we treat extrafloral nectar production as a proxy for the investment in ant defense.

An HP 6890 gas chromatograph with a DB-1 capillary column and FID was used to identify and to quantitate the sugars

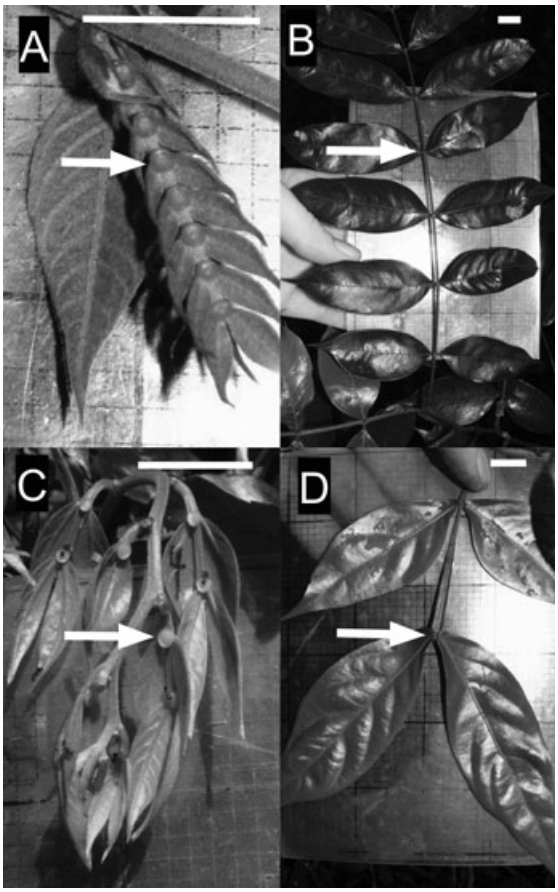

FIGURE 1. The ratio of extrafloral nectary to leaf area significantly decreases as young leaves expand. Nectary area does not change during leaf expansion, but leaf area does. White arrows point to extrafloral nectaries on leaves less than 25 percent (left) and greater than 80 percent (right) of adult size on *Inga multijuga* (A and B) and *Inga marginata* (C and D). Scale bars in the top right corner of each picture represent 1.27 cm.

in the nectar. The majority of the components in the nectar were fructose, glucose, and sucrose. The dried nectar samples were dissolved in 50 μL of pyridine. Due to the polarity of sugars, samples were derivatized by adding 50 μL of BSTFA with 1 percent TCMS to the pyridine solution and allowed to remain for 10 h. External standards and calibration curves were used to identify and to quantitate the three sugars in the nectar. The total mass of the three sugars was summed for each sample (*I. marginata* = 32, *I. umbellifera* = 32, and *I. multijuga* = 16) and corrected by the number of nectaries per leaf over a 24-h period (μg sugar/nectary/24 h) or by the leaf area over a 24-h period (μg sugar/cm^2/24 h).

SECONDARY METABOLITE ANALYSIS.—We quantitated two classes of secondary metabolites (phenolics and saponins) that are known to be toxic to herbivores based on previous feeding trials using extracts of *Inga* species and other reports on these compounds (Potter & Kimmerer 1989, Agrell *et al.* 2003, Coley *et al.* 2005). Secondary metabolites were extracted, separated, and quantitated gravimetrically following modified protocols from previous work on *Inga* secondary metabolite research (Lokvam *et al.* 2004, 2006, Coley *et al.* 2005, Lokvam & Kursar 2005). In addition, preliminary sample fractions were checked for extraction efficiency, yield, and fraction purity using HPLC-DAD and HPLC-MS. For *I. marginata* and *I. multijuga*, 70–80 mg of sample were homogenized using grinding beads in a 1 mL Nunc Cryo Tubes™ (Thermo Fisher Scientific, Rochester, New York, USA) and a Wig-l-bug® (Sigma-Aldrich, St. Louis, Missouri, USA) mixer at 46 Hz for a totally of 3 min. The grinding beads were removed and 1 mL of 80 percent ethanol was added and mixed. Samples were then centrifuged for 10 min at 9055 RCF and at 5°C. The supernatant was retained and the extraction was repeated a total of five times with 80 percent ethanol. The same process was repeated twice with 70 percent acetone and all collected supernatants were combined. Pellet and extract were dried under nitrogen, then under a vacuum (0.8 torr) at ambient temperature and weighed.

Three milliliters of 60 percent methanol and 3 mL of hexane were added to each sample extract vial to remove lipids. Vials were shaken and allowed to settle. Once two distinct layers formed, the lipid-containing hexane layer was removed and placed in a preweighed vial, 3 mL of hexane was added and the separation was repeated for a total of five times. Both the polar organic fraction and lipids were dried under nitrogen and then under a vacuum (0.8 torr) at ambient temperature and weighed.

The polar organic fraction was separated on a preparatory liquid chromatography column packed with octadecylsilane (ODS). Columns were prepared using 10cc syringes plugged with glass wool and filled with 1.9 g of ODS. Water–methanol solutions were used in the following concentrations: (1) 5 percent MeOH (organic acids); (2) 60 percent MeOH (phenolics); and (3) 100 percent MeOH (saponins). Each fraction was collected separately into a labeled preweighed vial, dried under nitrogen, and then under a vacuum (0.8 torr). Fractions were quantitated gravimetrically and HPLC was used to verify the class of compounds in each fraction (*I. marginata* = 15, *I. umbellifera* = 25, and *I. multijuga* = 14).

TYROSINE AND PHENOLICS IN INGA UMBELLIFERA.—Tyrosine is toxic at the high concentrations found in *I. umbellifera* (Lokvam *et al.* 2006). In addition to its high concentration, the low solubility of tyrosine required a special extraction protocol. First, 25 mg of dried leaf sample was homogenized using grinding beads in a 1 mL Nunc Cryo Tubes™ and a Wig-l-bug® mixer at 46 Hz for a totally of 3 min. The homogenized, dried leaf sample was extracted in 2 mL of 10 percent MeOH (acidified with acetic acid to pH = 3) for 20 min at 80°C. Samples were centrifuged at 13250 RCF and ambient temperature for 5 min. The resulting supernatant was retained and the extraction was repeated once more. The combined supernatants were separated on preweighed Agilent (Santa Clara, California, USA) SampliQ C18 solid phase extraction columns (500 mg ODS). The supernatant was added to the prepared column and washed with an additional 2 mL of

10 percent MeOH (pH3). The 10 percent MeOH wash containing tyrosine was dried under vacuum (0.8 torr) and redissolved in 20 mL of 10 percent MeOH. Samples were then separated on a Hitachi (Pleasanton, California, USA) LaChrom Elite with an Omnisphere C18 250 × 2.0 mm column with an isocratic gradient of 10 percent MeOH/90 percent HOH with 0.1 percent formic acid. Tyrosine was detected and quantitated using a diode array detector and external calibration curves. The SampliQ columns were dried and reweighed and the difference was considered to be the mass of the phenolic fraction trapped on the column (n = 25). *Inga umbellifera* did not contain saponins.

STATISTICS.—We used analysis of covariance (ANCOVA) to test for significance because we had a mix of continuous and categorical data. We conducted separate ANCOVAs for nectar volume, absolute nectary area, relative nectary area, nectar production per nectary, nectar production per leaf area, percent phenolics, percent saponins, and percent tyrosine. The percent phenolics, percent saponins, and percent tyrosine were arcsine-transformed to meet the assumptions of ANCOVA. In addition, absolute nectary area, average nectary area, nectary area per leaf area, and nectar production per leaf area were log-transformed to meet the assumptions of ANCOVA. The same explanatory variables (young leaf size, percent damaged, plant species, and microhabitat) were used for each ANCOVA, and nonsignificant parameters were removed from each model using the 'step' function in R (R Development Core Team 2009). All main effects and interaction terms were included in the original model. Step then creates all possible models with one term removed from the model and compares the Akaike Information Criteria (AIC) values. Step then passes the new model with the lowest AIC (*i.e.*, best fit) to another iteration of model selection until no better fit can be found. For tyrosine (in *I. umbellifera*) and saponins (in *I. marginata*), plant species was not used because they were each found in only one species. In addition, Pearson's product-moment correlations were computed for all possible combinations of the response variables.

RESULTS

EXTRAFLORAL NECTAR AND NECTARIES.—As young leaves expanded, the nectary area became an increasingly smaller proportion of the total leaf area (Figs. 1 and 2A, $F_{1,76}$ = 150.41, r^2 = 0.65, P < 0.001). In addition, there was a difference in the ratio of extrafloral nectary area to young leaf area among species ($F_{2,76}$ = 8.48, P < 0.001), but average nectary area was not different among species, nor did it change during leaf expansion (Table 1). Similarly, the volume of nectar produced per leaf (μL/young leaf/24 h, Fig. 3B) and nectar production per nectary (μg/nectary/24 h) did not decrease as leaves expanded (Fig. 2B), but nectar production per leaf area (μg/cm^2/24 h) and volume of nectar per leaf area (μL/cm^2/24 h) did significantly decrease

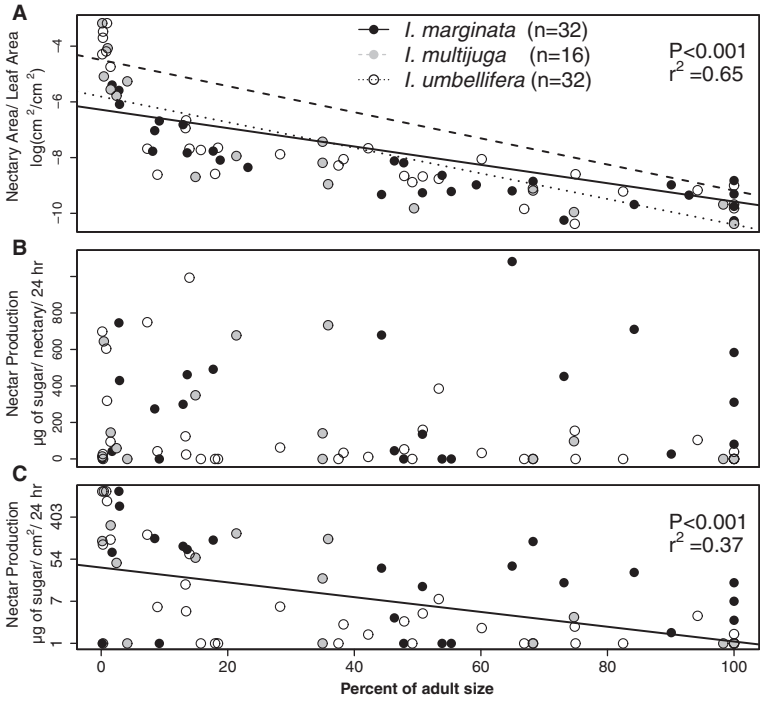

FIGURE 2. Nectary area and nectar production per leaf area decrease as leaves expand. The ratio of nectary area to leaf area for *I. marginata* (black circles and solid line), *I. multijuga* (gray circles and dashed line), and *I. umbellifera* (open circles and dotted line) significantly decreased as leaves expanded (A; $F_{1,76}$ = 150.41, r^2 = 0.65, P < 0.001) and the mean nectary area/leaf area was significantly different among species ($F_{2,76}$ = 8.48, P < 0.001). There was no change in nectar production per nectary for any species (B, ns), but nectar production per leaf area significantly decreased for all species (C; $F_{1,70}$ = 34.43, r^2 = 0.37, P < 0.001).

TABLE 1. *ANCOVA Table for the response variables. Models started with all factors and were reduced using 'step' in* R.

Source	df	Sum of Squares	Mean of Squares	*F*-value	*P*-value
Volume					
Plant species	2	261.24	130.619	5.979	$P < 0.010$
Residuals	67	1463.69	21.846		
log(average nectary area)					
Plant species × Microhabitat	5	6.7115	1.3423	6.262	$P < 0.001$
Residuals	74	15.8633	0.21437		
log(nectary area/leaf area)					
Leaf age	1	176.785	176.785	150.414	$P < 0.001$
Plant species	2	19.928	9.964	8.478	$P < 0.001$
Residuals	76	89.324	1.175		
Nectar production (μg sugar/nectary/24 h)					
Plant species	2	639,559	319,780	2.271	0.111
Residuals	69	9,714,596	140,791		
log(nectar production [μg sugar/cm^2/24 h])					
Leaf age	1	111.67	111.671	34.432	$P < 0.001$
Residuals	70	227.03	3.243		
Phenolics (mg/mg)					
Leaf age	1	0.14351	0.143513	17.379	$P < 0.001$
Plant species	2	0.07976	0.039880	4.829	$P < 0.050$
Residuals	50	0.4129	0.008258		
Saponins (mg/mg)					
Leaf age	1	0.017516	0.0175160	43.861	$P < 0.001$
Percent damage	1	0.0044131	0.0044131	11.051	$P < 0.010$
Microhabitat	1	0.0065342	0.0065342	16.362	$P < 0.010$
Residuals	11	0.0043929	0.0003994		
Tyrosine (mg/mg)					
Leaf age	2	0.000102	0.000102	0.011	$P < 0.918$
Residuals	23	0.214935	0.009345		

as leaves expanded (Fig. 2C, $F_{1,70} = 34.43$, $r^2 = 0.37$, $P < 0.001$; Fig. 3C, $F_{1,61} = 24.08$, $r^2 = 0.13$, $P < 0.05$). In addition, the volume of nectar produced per leaf (μL/young leaf/24 h) was significantly different among species ($F_{2,67} = 5.98$, $P < 0.01$). There was no significant relationship between herbivore damage and nectary area or nectar production nor was there a significant relationship between microhabitat and nectary area or nectar production.

CHEMICAL DEFENSES.—There was a significant difference in phenolic concentration among species (Fig. 4A, $F_{2,50} = 4.82$, $P < 0.05$) and phenolic concentrations decreased similarly for all species as leaves expanded (*I. marginata* = 67%, *I. umbellifera* = 37%, and *I. multijuga* = 46%; Fig. 4A, $F_{1,50} = 17.38$, $r^2 = 0.23$, $P < 0.001$). Only *I. marginata* had saponins and the saponin concentration also decreased (51%) as young leaves expanded (Fig. 4B, $F_{1,11} = 43.86$, $r^2 = 0.85$, $P < 0.001$). In addition, there was an increase in saponins in gap microhabitats (Table 1, $F_{1,11} = 16.36$, $P < 0.010$). *Inga umbellifera* was the only species that contained tyrosine and there was no significant relationship between tyrosine and leaf age or any of the other explanatory variables or other defenses (Fig. 3C). The only significant relationship between response variables was a positive correlation between saponins and phenolics in *I. marginata* (Fig. 5D; $R^2 = 0.88$, $P < 0.001$). There was no significant relationship between herbivore damage and any of the three chemical defenses.

DISCUSSION

Nectaries were a substantial proportion of the youngest leaves (8.1% of total leaf area in the youngest size class measured, Fig 1A) and did not change size throughout leaf development. Investment in extrafloral nectaries early rather than late in leaf development could either be a developmental constraint or an adaptive trait. Given that other tissues such as the rachis and leaflets expand considerably, physiological constraints on expansion of nectaries are unlikely. Most likely, early investment in nectaries is advantageous. The nectaries on *Inga* are large and elevated and require an investment in tissue, suggesting a one-time construction cost that may be balanced by anti-herbivore defense benefits (Elias 1983, Pennington 1997). Extrafloral nectaries on many other species are much less developed and demonstrate that an investment in a structure is not required to attract ant bodyguards (Elias 1983). There are advantages, how-

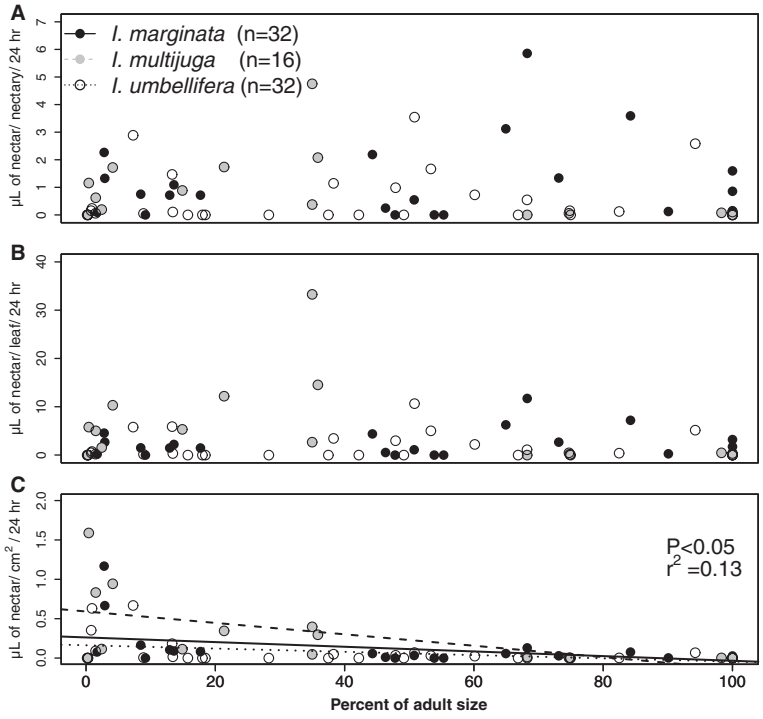

FIGURE 3. Extrafloral nectar volume per nectary and per young leaf do not change as young leaves expand (A and B) for the three *Inga* species: *I. marginata* (black circles and solid line), *I. multijuga* (gray circles and dashed line), and *I. umbellifera* (open circles and dotted line). However, the nectar volume per young leaf area significantly decreases as the young leaves of all three species expand (C; $F_{1,61} = 24.08$, $r^2 = 0.13$, $P < 0.05$).

ever, to investing in nectary tissue: (1) nectar is less affected by environmental factors; (2) nectary parenchyma tissue can photosynthesize and provide photosynthate to the nectary; (3) nectaries can be conspicuous and therefore act as a visual target to attract ants (Mondor & Addicott 2003, Pacini & Nepi 2007). Although we have not demonstrated the adaptive significance of nectary tissue in *Inga,* the presence of this nonessential structure suggests an advantage to producing the tissue. Furthermore, an early one-time investment in the nectary structure may be adaptive when leaf tissue is most susceptible, has the highest potential value, and requires the most defense.

As nectar concentration (μg sugar/μL nectar), nectar volume (μL/leaf/24 h), and nectar production per nectary (μg/nectary/24 h) did not significantly change during young leaf expansion, nectar production per leaf area ($\mu g/cm^2/24$ h) dropped considerably. In other words, the decrease in nectar production per leaf area was caused by leaf expansion rather than by a change in total investment in nectar. In addition, Bixenmann *et al.* (2011) showed that there is variation in the relative concentration of the three sugars present (sucrose, fructose, and glucose), but that there was no significant change in the relative sugar concentrations among different stages of young leaves. In addition, they showed that mature leaves do not produce extrafloral nectar. As ants positively respond to higher nectar concentrations and nectar production per nectary (Bixenmann *et al.* 2011), the constant nectar production rate per nectary in this study suggests that the same number of ants will visit nectaries and patrol a leaf regardless of the leaf size. Thus, the area an ant would have to patrol to effectively reduce herbivory would become increasingly larger and potentially dilute the effectiveness of the ant bodyguard. These results highlight the important difference between total defense investment per leaf and investment per leaf tissue (Koricheva 1999) and their different ecological outcomes.

Phenolics and saponins were most concentrated in the youngest leaves (*ca* 29% DW), suggesting that defense at this stage is important. Furthermore, both phenolic and saponin concentrations decreased as leaves expanded. This may be because (1) investment in phenolics and saponins happened as the young leaf was formed and were then diluted; or (2) phenolics and saponins were continuously synthesized during young leaf expansion, but, at a rate that still resulted in a decrease in concentration. Brenes-Arguedas *et al.* (2006) found that flavanoid content (a class of phenolics) increased throughout leaf expansion in *I. goldmanii* and during the first half of leaf expansion in *I. umbellifera*. However, the flavanoid concentration during leaf expansion decreased for both species, by 44 and 65 percent, respectively, despite the continued investment in phenolic content. In this study, the total phenolic concentration decreased by half during leaf expansion despite a tenfold increase in leaf area. This suggests that total phenolic content per leaf increased during leaf expansion, but at a rate that resulted in decreasing phenolic concentration. Similarly, saponins in *I. marginata* decreased by almost half (49%), while the leaf area increased nearly ninefold. Despite the continued synthesis of phenolics and saponins during leaf expansion, the

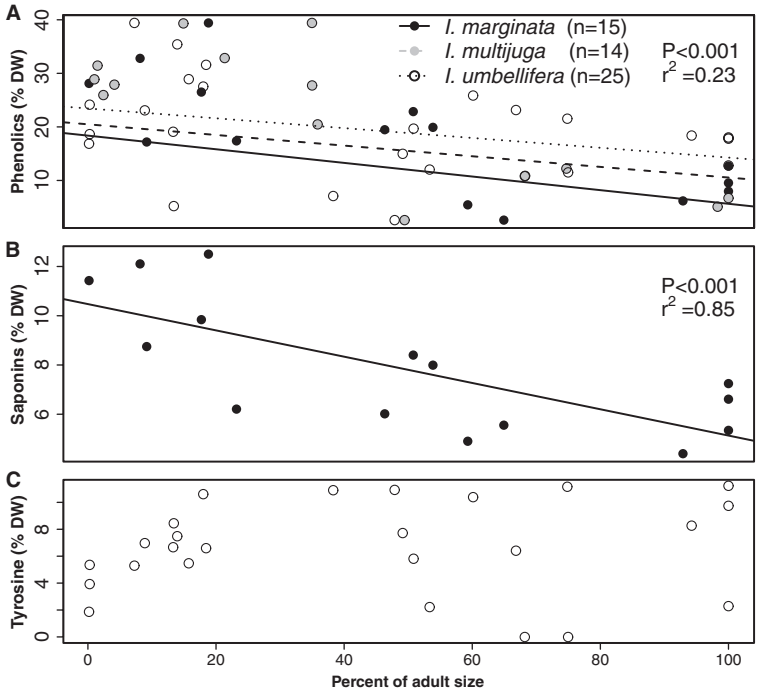

FIGURE 4. Phenolics and saponins decrease as young leaves expand. Phenolic concentration for *I. marginata* (black circles and solid line), *I. multijuga* (gray circles and dashed line), and *I. umbellifera* (open circles and dotted line) significantly decreased as leaves expanded (A; $F_{1,50} = 17.38$, $r^2 = 0.23$, $P < 0.001$) and phenolic concentration was significantly different among species ($F_{2,50} = 4.83$, $P < 0.001$). Saponins were only present in *I. marginata* and significantly decreased as leaves expanded (B; $F_{1,11} = 43.86$, $P < 0.001$, $r^2 = 0.85$). There was no relationship between tyrosine and leaf expansion in *I. umbellifera* (C; ns).

concentration in the youngest leaves was still 50 percent higher than the concentrations of older leaves near the adult size. Thus, it appears that young leaves are physiologically able to synthesize phenolics and saponins during leaf expansion, but do not maintain the same concentration throughout development. This suggests that there is a physiological constraint that prohibits leaves from maintaining higher concentrations of phenolics and saponins, or, that there is an adaptive significance to investing relatively more defense to younger, smaller leaves.

Tyrosine concentration had no relationship with young leaf size and remained at 7 percent of leaf dry weight throughout development. This indicates that the total tyrosine content per leaf had to increase as young leaves expanded to maintain the same concentration. Indeed, Lokvam *et al.* (2006) also showed that total tyrosine content in *I. umbellifera* increased to maintain a constant concentration until the leaf matured, at which point tyrosine was almost completely removed. The continual investment in tyrosine during leaf expansion and its subsequent removal from mature leaves suggest that tyrosine is metabolically labile and can therefore be reallocated to other tissues. Furthermore, other studies have demonstrated that tyrosine can be catabolized and that free tyrosine or its catabolized components can be transported via the phloem (Ellis 1973, Simpson & Dalling 1981, Arias-Barrau *et al.* 2004, Dixon & Edwards 2006). In addition, tyrosine in the young leaves of *Lupinus albus* (Fabaceae) is synthesized in the developing leaves and exported via the phloem after the leaves mature (Atkins *et al.* 1983). Therefore, an increasing investment in tyrosine during leaf expansion would not be a lost investment, as it is removed from mature leaves when they become effectively defended by toughness.

Taken together, our results demonstrate that tropical young leaves invest more in defense, both direct and indirect, than mature leaves. Although few studies have demonstrated detailed developmental changes in defenses of tropical leaves, greater protection of young plant tissue has been demonstrated in other systems (Wooley *et al.* 2007, Radhika *et al.* 2008, Rostás & Eggert 2008). For example, volatile organic compounds and extrafloral nectar are both indirect defenses used to attract enemies of herbivores. Radhika *et al.* (2008) demonstrated that the youngest leaves of *Phaseolus lunatus* and *Ricinus communis* produced more of both volatile and extrafloral nectar. However, the mature leaves of a temperate tree, *Paulownia tomentosa,* produced more nectar than young leaves, which were defended by direct defenses (Kobayashi *et al.* 2008). Other studies examining direct defenses have also demonstrated higher investment in young leaves which corroborate our findings (Coley & Aide 1991, Coley & Barone 1996, Haukioja *et al.* 2002, Kursar & Coley 2003, Kobayashi *et al.* 2008). Tropical young leaves are of greater value than mature leaves because they have higher nitrogen content (Kursar & Coley 2003). In addition, the youngest leaves are sinks for photosynthate from mature leaves (Kursar & Coley 1992) and have not yet contributed enough photosynthate to the plant to compensate

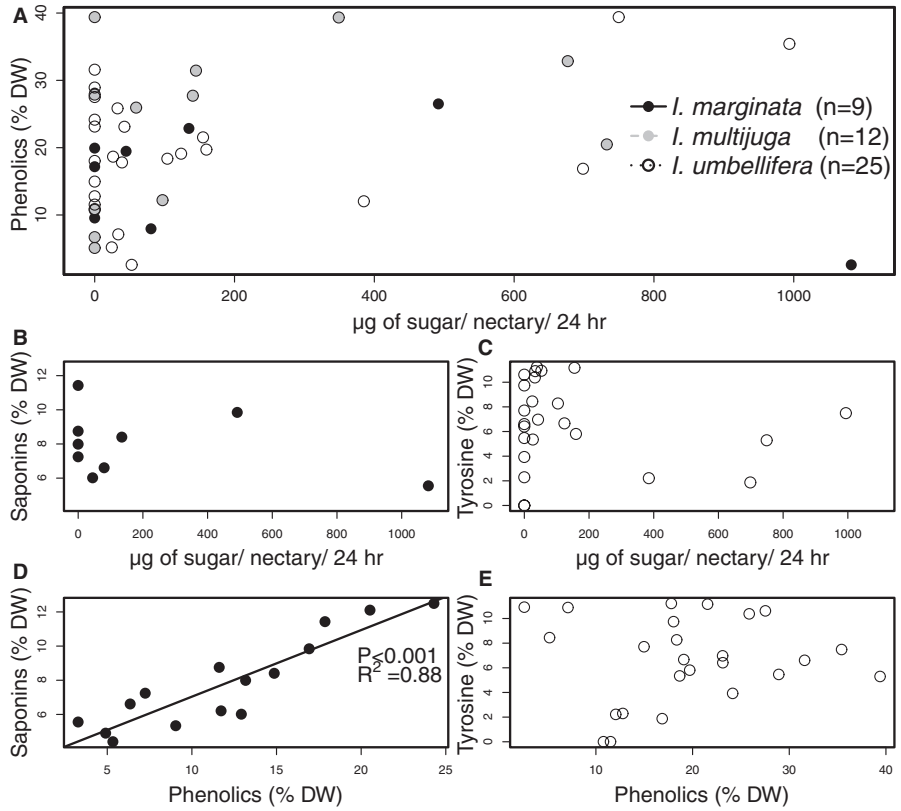

FIGURE 5. Correlations among response variables. There was no significant relationship between nectar production and phenolics (A), saponins (B), or tyrosine (C). However, there was a significant correlation between saponins and phenolics (D; $P < 0.001$, $R^2 = 0.88$). However, there was no significant relationship between phenolics and tyrosine (E). Responses are for *I. marginata* (black circles and solid line), *I. multijuga* (gray circles), *and I. umbellifera* (open circles).

for the high construction costs they already have accrued (Radhika *et al.* 2008). Therefore, our results support one prediction of the optimal defense hypothesis: The more valuable, younger leaf tissue found earlier in development is better defended than leaves that are near the end of development.

Despite the predictions of the optimal defense hypothesis, we found no trade-offs among the different observed defenses. Indeed, we saw a lack of correlation among all the defenses except for a positive relationship between saponins and phenolics in *I. marginata*. This positive correlation is due to higher concentrations of both defensive compounds in younger leaves. Although this result highlights the importance of young leaves, it also suggests a redundancy of defenses that is not predicted under the optimal defense hypothesis. Rasmann and Agrawal (2009) and Rasmann *et al.* (2011), however, posited that if the predictions of the optimal defense hypothesis are valid, then seemingly redundant defenses may be necessary to effectively defend against multiple herbivore species. Indeed, Kursar *et al.* (2006) found seven herbivore species on *I. marginata* and *I. umbellifera* and four herbivore species on *I. multijuga,* which highlight the potential need for multiple defenses of one plant species against multiple herbivore species. Alternatively, seemingly redundant defenses may work concurrently to increase the effectiveness of the separate defenses against a single herbivore species (Rasmann *et al.* 2011). For example, direct defenses may slow the development of herbivores exposing them to predators for a longer period of time, whereas indirect defenses increase the probability of attack from predators through attractants such as extrafloral nectar or volatile compounds. Although trade-offs among direct and indirect defenses have been demonstrated in obligate ant–plant mutualisms (Heil *et al.* 1999, Heil *et al.* 2000a, b, Dyer *et al.* 2001, Eck *et al.* 2001, Rudgers *et al.* 2004, but see Heil *et al.* 2002), the trade-offs do not appear to extend to facultative ant–plant mutualisms such as *Inga*. Therefore, our results support previous findings that defenses in facultative ant–plant mutualisms may, in fact, be complementary rather than redundant (Steward & Keeler 1988, Rudgers *et al.* 2004, but see Kobayashi *et al.* 2008).

CONCLUSION

The high nitrogen content in young leaves makes them more nutritious for herbivores and more valuable to the plant (Kursar & Coley 1991, 2003; Coley *et al.* 2005, Kursar *et al.* 2006). These valuable expanding leaves cannot be defended by toughness, an extremely effective defense for mature leaves (Coley 1983). Thus, the vulnerability and value of the youngest leaves would select

for high investment in defenses. In this report, we demonstrate that indeed young leaves do invest relatively more in nectary tissue, extrafloral nectar production, and defensive chemistry (except for tyrosine). The high investments in young leaf defenses are consistent with predictions of the optimal defense hypothesis (McKey 1974, 1979, McCall & Fordyce 2010). The theory also predicts trade-offs between classes of defenses, although trade-offs between direct and indirect defenses have rarely been demonstrated. In *Inga*, the early investment in all defenses and the lack of any negative correlations among defenses suggest that there is no trade-off among defenses, but rather an additive effect resulting in better protection for the youngest leaves.

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