

# Nitrogen fertilizer and gender effects on the secondary metabolism of yaupon, a caffeine-containing North American holly

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**Abstract** Yaupon (*Ilex vomitoria*) is a caffeine-containing dioecious shrub native to the southeastern United States that was historically brewed into a stimulating beverage. We tested predictions of the carbon/nutrient balance (CNB) hypothesis by determining whether nitrogen availability and gender influence production of caffeine and related alkaloids as well as phenolic compounds in leaves of pot-grown yaupon plants fertilized with ammonium nitrate. The CNB hypothesis predicts that additional nitrogen should result in increased alkaloid concentrations and decreased phenolic concentrations. An extension of the CNB hypothesis to dioecious plants predicts that females have higher C/N ratios and therefore higher phenolic concentrations and lower alkaloid concentrations than male conspecifics. In our study, caffeine and total alkaloid concentrations were 5–10 times higher in fertilized than control plants but did not vary by gender. Nevertheless, an observed interaction between gender and fertilization suggests that females respond more to fertilization than males in caffeine production. In addition, fertilized plants not only contained higher concentrations of alkaloids and total nitrogen but also allocated a larger proportion of their nitrogen to alkaloid production than control plants. Total phenolic concentrations

were higher in control females than control males as predicted by the CNB hypothesis, but did not vary by treatment nor were there differences by gender among fertilized plants. We also found high correlations between antioxidant capacity and both classes of phenolic compounds detected in our study (cinnamic acid derivatives and flavonoids) indicating that in addition to their putative defensive function against herbivores, phenolics protect yaupon from oxidative stress. Explanation of the inconsistencies between our data and predictions of the CNB hypothesis may benefit from a re-appraisal of the physiological mechanisms by which resource availability affects secondary metabolism as well as consideration of the selective pressures to which secondary metabolism responds.

**Keywords** Antioxidants · Carbon/nutrient balance hypothesis · Dioecy · *Ilex vomitoria* · Methylxanthine alkaloids

## Introduction

Leaves of yaupon (*Ilex vomitoria*, Aquifoliaceae), a dioecious shrub native to the coastal plain of the southeastern United States, were traditionally brewed into a caffeinated beverage widely consumed by indigenous peoples and European colonists (Hudson 1979). Although yaupon was the principal ingredient of a beverage imbibed by Timucuan and Creek Indians during a ceremony that included ritual vomiting (Merrill 1979), qualitative chemical analyses detected no emetic properties (Fuller et al. 2002). Partially due to its repellent and misleading specific epithet, yaupon's virtue as a caffeine source is now mostly ignored, while markets for its close South American relative, *I. par-*

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*aguariensis* (yerba maté) grow rapidly (Graham 1998). To revive interest in yaupon, we investigated whether nitrogen fertilization increases foliar concentrations of caffeine and other methylxanthine alkaloids. In addition, we determined the effects of nitrogen fertilization on concentrations of phenolic compounds, which confer antioxidant properties on yerba maté (Carini et al. 1998). Finally, we used this dioecious and easily cultivated alkaloid and phenolic-producing species to test a well-known theory that addresses nutrient allocation to plant secondary metabolites.

The carbon/nutrient balance (CNB) hypothesis (Bryant et al. 1983, 1988; Tuomi et al. 1988; Reichardt et al. 1991; Herms and Mattson 1982) is used to explain the effects of nutrient availability on the concentration of plant secondary metabolites (e.g., alkaloids and phenolics). The CNB hypothesis asserts that plants allocate carbon and nitrogen to secondary metabolism only after growth requirements are met and that growth is constrained more by nutrient limitations than by photosynthesis. According to this theory, the excess carbohydrates that accumulate in nutrient-limited plants when photosynthesis outpaces growth are diverted to the production of carbon-based secondary compounds (e.g., phenolics). Removal of nutrient limitation by fertilization increases tissue nutrient concentrations and allows growth to outpace photosynthesis. Therefore, according to the CNB hypothesis, production of nitrogen-based secondary metabolites (e.g., alkaloids) should increase as nitrogen is acquired in excess of growth requirements. Conversely, concentrations of carbon-based phenolic compounds are predicted to decrease with fertilization due to decreased rates of photosynthesis relative to growth.

Although some studies on the effects of nitrogen fertilization on secondary metabolites support the CNB hypothesis, others contradict it (Herms and Mattson 1982; Koricheva et al. 1998). Critics of the CNB hypothesis explain that its failures can be attributed to its assumption that plants respond to varying levels of resource availability in a physiologically passive manner driven by simple mass-action (Hamilton et al. 2001; Nitao et al. 2002). These critics contend that although phenotypic plasticity in secondary metabolite production is influenced by resource availability, it is also subject to selective pressures and is therefore genetically regulated and possibly adaptive. For example, plasticity in nicotine production maximizes seed production in *Nicotiana attenuata* by allowing individuals to regulate resource allocation in response to herbivore pressure (Baldwin 1998, 1999).

Although variation in production of caffeine and phenolics has apparently not yet been shown to be

adaptive, there is evidence that their production is genetically regulated. For example, high narrow-sense heritability for caffeine concentration ( $h^2 = 0.80$ ) in seeds of robusta coffee (*Coffea canephora*) indicates that most phenotypic variation in its caffeine output is attributed to genetic and not environmental factors (Montagnon et al. 1998). In other species, phenylalanine ammonia-lyase activity, which catalyzes the first step of phenolic biosynthesis, is finely regulated both by a multi-gene family and by negative feedback inhibition (Dong et al. 1991; Kervinen et al. 1998). This fine genetic regulation contrasts with the CNB hypothesis that presumes mass flow, as directed by environmental factors, exerts the strongest influence on the production of secondary metabolites (Reichardt et al. 1991).

The CNB hypothesis was extended by Herms and Mattson (1982) to predict gender differences in secondary metabolism. The basis for these predictions is the assumption that due to the nutrient requirements of fruit and seed maturation, female plants allocate higher proportions of nutrients to reproductive structures than males. If this assumption is correct, female plants should have higher C/N ratios in vegetative tissue and higher concentrations of carbon-based secondary compounds than males. The logic of this argument notwithstanding, studies examining differences in nitrogen concentration and carbon-based secondary compounds by gender have yielded mixed results (Ågren et al. 1999; Doormann and Skarpe 2002; Bañuelos et al. 2004) indicating that the mechanisms underlying gender differences in secondary metabolism are not yet fully understood.

Given the inconsistent evidence for the CNB hypothesis, we sought to contribute to the debate by determining how nitrogen fertilization and gender influence the production of methylxanthine alkaloids and phenolics in yaupon holly. In addition, we measured total foliar nitrogen concentrations and antioxidant capacities to further explore the effects of gender on responses to increased nitrogen availability. We hope that while exploring the mechanisms that govern resource allocation to secondary metabolism in yaupon, we can stimulate interest in this long-used but recently forgotten native plant.

## Materials and methods

### Plant material and nitrogen fertilization

Yaupon holly (*Ilex vomitoria*) grows from North Carolina south to central Florida and west to east Texas in

coastal scrub and in the understories of coastal and inland hardwood forests and pinelands. For this pot study, a rooted leaf-bearing stem was collected randomly from each of 26 male and 26 female *I. vomitoria* genets growing in the understory of an oak savanna near Gainesville, Florida (29°39'N, 82°19'W). Genets were defined as clusters of stems separated by at least 5 m from the nearest conspecific of the same gender (as determined during the flowering season). Each rooted ramet was planted in a 1-l pot filled with surface soil (0–20 cm) from the collection site. Potted plants were grown outdoors in partial shade on the University of Florida campus. Percentage full sun for both the source area ( $\bar{x} = 0.23$ ,  $s = 0.16$ ,  $n = 10$ ) and pot study site ( $\bar{x} = 0.29$ ,  $s = 0.16$ ,  $n = 4$ ) were similar as estimated by Gap Light Analyzer software version 2.0 (copyright 1999; Simon Fraser University, British Columbia and Institute of Ecosystem Studies, New York) from photographic images taken with a 180° hemispheric lens ( $t = 0.62$ ,  $P = 0.55$ ). Although light availability can influence secondary metabolite production (Herms and Mattson 1982; Koricheva et al. 1998), this experiment only allowed assessment of nitrogen fertilization and gender effects.

After 8 weeks and continuing for the next 12 weeks, half of the plants of each gender were fertilized with ammonium nitrate at a rate of 250 mg nitrogen per week. Four plants died during cultivation resulting in a final sample size of 13 control males, ten fertilized males, 12 control females, and 13 fertilized females. All leaves produced after the commencement of the fertilization treatment were harvested for chemical analysis.

#### Chemical analyses

Collected leaves were dried at 105°C to a constant weight and total nitrogen content was determined using the Kjeldahl method (Bradstreet 1965). Dried leaf tissue for determination of methylxanthine alkaloid and phenolic concentrations was extracted in a 100% methanol for 1 min and filtered through Whatman no. 4 filter paper. Isolates were then diluted with an equal volume of water, centrifuged for 5 min at ca. 2,000 r.p.m., and filtered through a 0.45-mm filter for HPLC analysis. Separation was conducted with a Waters 2695 Alliance HPLC system using a Supelcosil LC-18 column (250 × 4.6 mm) and detected at 280 nm with a Waters 996 photodiode array (PDA) detector scanned from 200 to 400 nm.

Following the protocol of Talcott et al. (2000), a gradient mobile phase was run consisting of a 2% aqueous solution of acetic acid in phase A and a 30%

acetonitrile plus 2% acetic acid solution in phase B. The gradient ran phase B from 0 to 30% for 20 min, 30–50% for 10 min, 50–70% for 20 min, and 70–100% for 5 min at 0.8 ml/min. Phase B ran for an additional 15 min to elute remaining non-polar compounds followed by equilibration of the column with 100% phase A prior to injection of the next sample. Compounds were identified by UV/VIS spectral interpretation and compared to authentic standards and retention times when available. Alkaloid and phenolic concentrations were calculated on percent dry weight basis.

Isolates extracted in methanol were also used to quantify total reducing compounds by the Folin–Ciocalteu method (Swain and Hillis 1958), which provides an estimate of total soluble phenolics. Data were expressed in chlorogenic acid equivalents. The same isolates were used to determine antioxidant capacity by the oxygen radical absorbance capacity (ORAC) method as modified by Ou et al. (2001). Fractions were diluted 400-fold in pH 7.2 phosphate buffer prior to pipetting into a 96-well microplate. Peroxyl radicals were generated by 2, 2'-azo-bis (2-amidinopropane) dihydrochloride with fluorescein as the fluorescent probe. Fluorescence loss was measured on a Molecular Devices  $f_{max}$  Microplate Reader (485 nm excitation, 538 nm emission, 37°C) every 2 min for 70 min. Areas under decay curves generated by these measurements were compared to areas for blank preparations and a standard curve of Trolox, a water-soluble analogue of tocopherol (vitamin E) and expressed as  $\mu\text{M}$  Trolox equivalents/g.

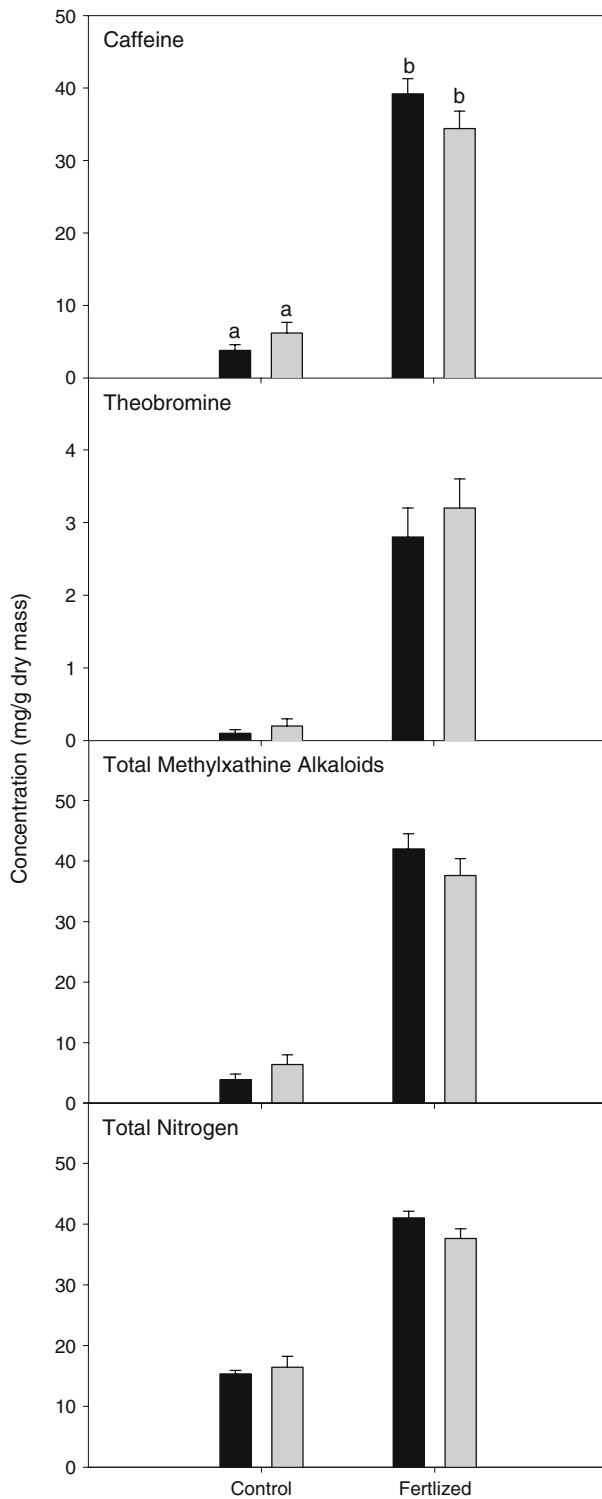
#### Statistical analyses

Differences in chemical concentrations between treatments were analyzed with a two-way full factorial ANOVA followed by Tukey's HSD tests for post hoc pair-wise contrasts (SAS software version 8.2; SAS Institute, Cary, N.C.). Correlations were determined by Pearson product-moment correlation.

## Results

### Methylxanthine alkaloids

Concentrations of caffeine, theobromine, and total methylxanthine alkaloids (Fig. 1) were substantially higher in leaves of fertilized than control plants (Table 1). Although concentrations of these compounds did not vary by gender, there was a significant interaction between fertilization and gender in the caffeine response (Table 1). This interaction may be explained



**Fig. 1** Mean (+1 SE) foliar concentrations of alkaloids and total nitrogen by treatment and gender in *Ilex vomitoria* (note difference in scales). Black bars represent female plants, grey bars represent male plants. Lowercase letters specify groupings as indicated by Tukey's test for multiple comparisons when interactions were indicated ( $P < 0.05$ )

**Table 1** ANOVA results for foliar concentrations of methylxanthine (MX) alkaloids in *Ilex vomitoria*

Source	df	Caffeine	Theobromine	Total MX alkaloids
Treatment	1	329.04****	88.29****	348.89****
Gender	1	0.47	0.79	0.25
Treatment	1	4.26*	0.18	3.55 <sup>a</sup>
× Gender				
Error	44			

\* $P < 0.05$ , \*\*\*\* $P < 0.0001$

<sup>a</sup>  $P < 0.1$

by a greater response to fertilization in females (10.4-fold increase) than males (5.6-fold increase). In contrast to the caffeine effect, there were no significant interactions in either the theobromine or total methylxanthine alkaloid responses.

### Phenolics

Concentrations of total soluble phenolics in yaupon foliage did not differ by treatment or by gender but were influenced by an interaction between the two effects (Table 2). Post hoc analysis (Fig. 2) indicates that the interaction is at least partially attributed to slightly higher total phenolic concentrations in control females than control males ( $P < 0.05$ ).

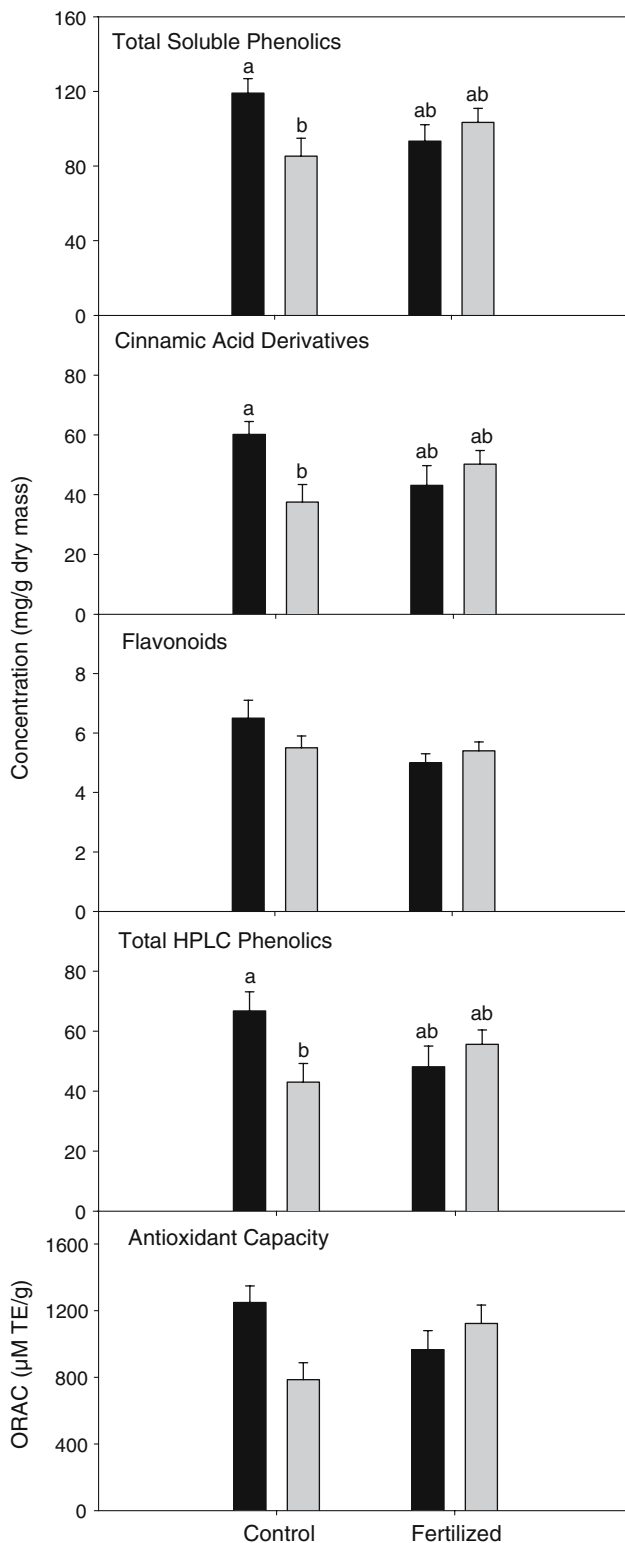
HPLC analysis of yaupon leaf issue detected two classes of phenolic compounds, cinnamic acid derivatives and flavonoids. Based on retention times, maximum wavelength of absorbance, and UV spectroscopic analysis by PDA, the cinnamic acid derivatives were composed of chlorogenic acid, chlorogenic acid isomers, and chlorogenic acid di-isomers (dicafeoylquinic acids). Based on the above indicators as well as a PDA library of flavonoid samples, the flavonoids are most likely rutin and luteolin.

**Table 2** ANOVA results for foliar concentrations of phenolic compounds in *I. vomitoria*

Source	df	Total phenolics	Total cinnamic derivatives	Total flavonoids	Total phenolics by HPLC
Treatment	1	1.83	1.67	0.58	1.65
Gender	1	0.19	0.13	3.48 <sup>a</sup>	0.22
Treatment	1	6.21*	6.10*	2.84 <sup>a</sup>	6.12*
× Gender					
Error	44				

\* $P < 0.05$

<sup>a</sup>  $P < 0.1$



**Fig. 2** Mean (+1 SE) foliar concentrations of phenolics and antioxidant capacity by treatment and gender in *I. vomitoria* (note difference in scales). Black bars represent female plants, grey bars represent male plants. Lowercase letters specify groupings as indicated by Tukey's test for multiple comparisons when interactions were indicated ( $P < 0.05$ ). ORAC Oxygen radical absorbance capacity, TE Trolox equivalents

Concentrations of cinnamic acid derivatives, total flavonoids, and total phenolics as detected by HPLC did not vary by gender or in response to fertilization (Table 2) but these treatments interacted to affect the production of cinnamic acids and total phenolics detected by HPLC (Table 2). As found with total soluble phenolics, post hoc pair-wise tests suggest that these interactions are attributable to greater concentrations of both cinnamic acid derivatives and total HPLC phenolics in control females than control males ( $P < 0.05$ ). Fertilized samples, in contrast, differed neither by gender nor from control plants in concentrations of cinnamic acid derivatives or total HPLC phenolics and there was no gender-by-fertilization interaction in the flavonoid response.

### Nitrogen Content

Although foliar nitrogen concentrations (Fig. 1) were much higher in fertilized than control plants ( $F = 233.2$ ,  $P < 0.0001$ ), nitrogen concentration did not vary by gender nor were there significant interactions. In all plants, nitrogen concentrations were closely correlated with concentrations of caffeine ( $r = 0.98$ ,  $P < 0.0001$ ), theobromine ( $r = 0.67$ ,  $P < 0.0001$ ), and total methylxanthine alkaloids ( $r = 0.98$ ,  $P < 0.0001$ ). Furthermore, a far greater proportion of nitrogen was invested in alkaloid production by fertilized plants. For example, female and male control plants invested 7.4 and 12.8% of their total nitrogen in the production of methylxanthine alkaloids, respectively, whereas in fertilized females and males, the respective proportions were 29.8 and 28.3%.

### Antioxidant capacity

Measures of ORAC were extremely high in all plants and did not vary by treatment or gender (Fig. 2). ORAC values were highly correlated with total phenolics ( $r = 0.94$ ,  $P < 0.0001$ ), total cinnamic acid derivatives ( $r = 0.93$ ,  $P < 0.0001$ ), total flavonoids ( $r = 0.58$ ,  $P < 0.0001$ ), and total phenolics as measured by HPLC ( $r = 0.93$ ,  $P < 0.0001$ ).

### Discussion

The results of this study both support and contradict predictions of the CNB hypothesis. Yaupon responded to nitrogen fertilization with large increases in concentrations of caffeine and total methylxanthine alkaloids but not with decreases in concentrations of

cinnamic acid derivatives, flavonoids, or total phenolics. Furthermore, neither alkaloid nor total nitrogen concentrations differed by gender, although an interaction between gender and fertilization influenced caffeine production. As predicted by the CNB hypothesis, cinnamic acid derivative and total phenolic concentrations were higher in females than males among the control group but did not differ by gender among fertilized plants nor did flavonoid concentrations differ by gender in either control or fertilized groups.

The lack of phenolic responses by yaupon to nitrogen addition indicates the need for alternatives to the mechanism set forth by the CNB hypothesis for interpretation of these results. Although the CNB hypothesis predicts that carbon allocation to carbon-based secondary metabolism decreases with nitrogen fertilization due to faster rates of growth relative to photosynthesis (Bryant et al. 1983), nitrogen fertilization does not necessarily favor growth over photosynthesis (Kannan and Paliwal 1997). Furthermore, even when nitrogen additions stimulate biomass accumulation but not photosynthetic rates, phenolic concentrations do not always decrease (Reichardt et al. 1991; Bezemer et al. 2000). Similarly, Donaldson et al. (2006) found no correlation between relative growth rates and the relative foliar mass of phenolic glycosides among nitrogen-fertilized *Populus tremuloides*.

The CNB hypothesis places an importance on relative increases in growth over photosynthesis in response to nitrogen fertilization because it posits that secondary metabolite production is driven by carbon and nitrogen overflow beyond allocation to growth (Bryant et al. 1983; Reichardt et al. 1991). By this mechanism, relatively lower photosynthetic rates with additional nitrogen constrain carbohydrate availability for carbon-based secondary metabolism while nitrogen supplied in excess of growth requirements is allocated to nitrogen-based secondary metabolism. Studies have revealed, however, that nitrogen fertilization does not necessarily decrease starch or total sugar concentrations even when it decreases phenolic concentrations (Balsberg Pålsson 1992) and that nitrogen additions may even stimulate increases in concentrations of total nonstructural carbohydrates (Kaakeh et al. 1992).

Closer examination of the influence that carbohydrate metabolism exerts on all secondary metabolic pathways may resolve patterns of resource allocation to secondary metabolite production. In particular, biosynthesis of nitrogen-based secondary compounds is dependent on carbon skeletons generated by photosynthesis (Dennis and Blakeley 2000) and therefore, like the biosynthesis of carbon-based secondary compounds, should also be limited by carbon availability.

For example, biosynthesis of phenylalanine, the amino acid precursor of phenolic compounds, is dependent on organic acids derived from carbohydrate pools generated by glycolysis and the pentose phosphate pathway (Weaver and Herrmann 1997). Similarly, biosynthesis of purine, the nucleotide precursor of caffeine and related methylxanthine alkaloids (Suzuki et al. 1992), also begins with an organic acid derived from the pentose phosphate pathway and derives nitrogen from amino acids with carbon skeletons also supplied by organic acids (van der Graaff et al. 2004). Carbon partitioning obviously warrants further investigation in yaupon and other plant species that employ numerous secondary metabolic pathways.

A refined understanding of the mechanisms by which nitrogen availability influences carbohydrate, nitrogen, and secondary metabolism can clarify its influence on carbon and nitrogen allocation for the production of both alkaloids and phenolics. For example, recent studies have shown that nitrate, and not merely downstream nitrogen metabolites, signals the induction of organic acid, amino acid, and nucleotide metabolism but represses starch biosynthesis (Scheible et al. 1997, 2004). By a similar mechanism, nitrate also induces nicotine but inhibits phenylpropanoid and flavonoid biosynthesis in *Nicotiana tabacum* (Fritz et al. 2006). These studies indicate that carbohydrate, nitrogen, and secondary metabolism are, in fact, coordinated according to nitrogen availability. Carbon/nitrogen ratios alone, however, may be insufficient for predicting resource allocation to secondary metabolites because their production is influenced by nitrate-induced signals and not merely by the mass flow of carbon and nitrogen through various metabolic pathways (Stitt and Krapp 1999).

Root/shoot ratios are also known to reflect carbon/nitrogen ratios (e.g., Ågren and Ingstead 1987) and therefore may shape the effect of nitrogen additions on carbohydrate, nitrogen, and secondary metabolic responses. For example, studies that showed a decrease in root/shoot ratios with additional nitrogen also showed concomitant increases in soluble sugar and amino acid concentrations but decreases in starch concentrations (Green et al. 1994) as well as decreased concentrations of condensed tannins but no changes in benzoic acid derivatives (Donaldson et al. 2006). Because our study on yaupon involved the use of rooted vegetative offshoots with presumably low root/shoot ratios (e.g., Ritchie et al. 1992), the potential influence of root/shoot ratios on secondary metabolic output should be considered.

Determining the influence of selective agents may also inform our understanding of the physiological

mechanisms that drive variation in secondary metabolic output (Berenbaum 1995; Hamilton et al. 2001; Nitao et al. 2002). For example, given that many plant secondary metabolites are putative defenses against pathogens and herbivores, predictions about allocation patterns for their production should reflect the selective pressures to which they are a response (Berenbaum 1995). Induction of caffeine and/or theobromine synthesis upon attack by fungal pathogens in tea (*Camellia sinensis*; Kumar et al. 1995; Punyasiri et al. 2005) and cacao (*Theobroma cacao*; Aneja and Gianfagna 2001), for example, provides evidence that selective agents may shape the production patterns of these alkaloids. Although plant nutrient concentrations have long been correlated with herbivore preferences (e.g., Mattson 1980), the effect of nitrogen availability on chemical responses to herbivores and pathogens has not been extensively explored. Lou and Baldwin (2004) found, however, that plants with low nitrogen availability maintain higher constitutive levels of nicotine than plants with high nitrogen availability but that only plants of the latter group increased nicotine production in response to application with larval oral secretions in *Nicotiana attenuata*.

Secondary metabolites may serve adaptive functions in addition to defense, which may also help explain their responses to additional nitrogen (Herms and Mattson 1982; Nitao et al. 2002). For example, our results indicating that fertilized yaupon plants allocated a higher proportion of nitrogen to methylxanthine alkaloid production than control plants suggest that alkaloid biosynthesis is not only actively regulated in yaupon holly but that caffeine may also serve multiple functions. Caffeine concentrations in fertilized female and male plants (3.9 and 3.4%, respectively), far exceed those necessary to deter or even kill herbivores (Nathanson 1984; Slansky and Wheeler 1992; Hollingsworth et al. 2003). Their high allocation of nitrogen to caffeine after nitrogen fertilization suggests that caffeine also serves as a storage metabolite for nitrogen accumulated in excess of immediate plant needs (Chapin et al. 1990). The suitability of caffeine for nitrogen storage, however, requires verification by studies addressing both the metabolic and ecological costs of storing caffeine.

The multiple functions of phenolic compounds may render it difficult to predict their production in response to nitrogen additions as well. For example, chlorogenic acid and flavonoids are known to inhibit herbivores (e.g., Ikonen et al. 2001, 2002; Onyilagha et al. 2004) but both also protect plants from light stress by scavenging free radicals and absorbing potentially harmful UV radiation (Grace and Logan

2000; Harborne and Williams 2000). Correlations between antioxidant capacity and all measures of phenolic concentrations indicate that both cinnamic acid derivatives and flavonoids provide yaupon with protection from oxidative stress. That a particular secondary metabolite serves numerous functions supports the notion that its production is under genetic control and most likely regulated by numerous stress factors (Nitao et al. 2002), making it difficult to ascertain the mechanisms driving its phenotypic variation without first isolating the effects of each factor.

Our results also largely contradict the CNB hypothesis prediction that female plants have higher foliar C/N ratios, lower levels of nitrogen-based defensive compounds, and higher levels of carbon-based defenses than male conspecifics (Herms and Mattson 1982; Ågren et al. 1999). In particular, concentrations of neither alkaloids nor total nitrogen differed by gender in yaupon. The observed interactive effect between gender and fertilization on caffeine production, however, suggests that female plants respond more to nitrogen fertilization than males, and therefore that resource allocation to secondary metabolism may differ by gender. Furthermore, higher concentrations of total phenolics and total cinnamic acid derivatives in control females than control males also suggest gender differences in resource allocation to secondary metabolism. Measurements of other traits by gender such as growth rates, herbivory, and investment in structural defenses (Jing and Coley 1990; Ågren et al. 1999) may resolve the mechanism and selective forces that shape variation in secondary metabolic output. If gender-based phenotypic differences in life history traits are observed, however, they should be appraised in terms of their effects on reproductive success or some other measure of their adaptive significance (Berenbaum 1995).

The dramatic increases in caffeine and theobromine concentrations in response to increased nitrogen availability observed in yaupon may relate to its success across a broad environmental range (Sultan 2000). For example, yaupon occurs both in coastal scrub communities, where it is adapted to high light, salt spray, and drought (Johnson and Barbour 1990), as well as in the understories of mesic hardwood forests, which are typically moister and contain higher levels of soil organic matter (Platt and Schwartz 1990). Because foliar nitrogen concentrations affect plant susceptibility to herbivory (Mattson 1980) and because the native habitats of yaupon vary in soil nutrient availability, an ability to mediate between herbivore pressure and defensive metabolite production in response to nitrogen availability may indicate a plastic response that is possibly

adaptive. Such an adaptive strategy may be suggested by our finding that 2.4- and 2.3-fold increases in foliar nitrogen concentration resulted in 10.4- and 5.6-fold increases in the caffeine concentrations of female and male plants, respectively. Future studies that include the identification of yaupon's herbivores and pathogens may reveal the significance of these differences in allocation to caffeine in relative proportion to total nitrogen concentration. Common garden and reciprocal transplant experiments could also help to clarify the role that phenotypic plasticity in secondary metabolic output plays in determining the success of yaupon across its large geographical and ecological range.

## Conclusions

Because yaupon holly (*Ilex vomitoria*) is dioecious, easily cultivated and produces large amounts of both nitrogen-based and carbon-based secondary compounds, it is an excellent species on which to test theories about resource allocation to secondary metabolism. For example, although an increase in methylxanthine alkaloid concentrations in response to nitrogen fertilization is predicted by the CNB hypothesis, the lack of phenolic responses indicates that a re-evaluation of the mechanism by which nitrogen availability influences secondary metabolism is necessary. In addition, the lack of differences in concentrations of both total foliar nitrogen and methylxanthine alkaloids between genders contradicts an extension of the CNB hypothesis to dioecious species. An interactive effect between gender and fertilization on caffeine production as well as higher phenolic concentrations in female conspecifics, however, indicates that resource allocation to secondary metabolite production may differ by gender in yaupon. The precise mechanisms underlying these gender differences, however, warrant further investigation. Finally, ample production of both caffeine and antioxidant-rich phenolics indicate the potential for renewed commercial use of yaupon holly.

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