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## Herbivores and mutualistic ants interact to modify tree photosynthesis

### Introduction

In the arms race between plants and herbivores, plants have evolved a variety of strategies to reduce the fitness costs of herbivore damage by investing resources in defense compounds and structures. Because both herbivore damage and defense investments are costly in terms of plant's resources, tradeoffs have emerged as a central theme in the evolutionary ecology of plant responses to herbivory, shaping the extent and the forms of defense that plants evolve (Coley *et al.*, 1985; van der Meijden *et al.*, 1988). While understanding allocation tradeoffs has fuelled prolific research and debate (Nuñez-Farfan *et al.*, 2007), empirical studies have not generated consistent support for tradeoff-based arguments. Plant physiology research, which focuses on resource acquisition and metabolic processes upstream of phenotypic plant defense traits, offers an under-explored perspective that may yield deeper insights into costs and allocation patterns of plant defense. Several studies have demonstrated that herbivory can trigger increases in leaf-level photosynthetic rates (Welter, 1989; Trumble *et al.*, 1993; Thomson *et al.*, 2003). For plants that utilize carbon-based defense strategies, this photosynthetic up-regulation has the important consequence of increasing the available pool of carbohydrates that can potentially be allocated to defense. Such varying rates of carbon assimilation fundamentally change the playing field for evaluating the costs of defense investments.

Plants employ three main strategies to mitigate the costs of herbivory: resistance, indirect defense and tolerance (Karban & Baldwin, 1997). Resistance strategies, such as thorns and noxious chemicals, and indirect defenses,

whereby plants attract enemies of herbivores, both reduce damage to plants by decreasing the rates of herbivore attack. Tolerance strategies, by contrast, reduce the fitness cost of herbivory by allocating more resources to growth in order to replace lost tissue. *Acacia drepanolobium*, a dominant tree through much of East Africa's savannas, exhibits inducible carbon-based investments in all three classes of defense strategies. In response to herbivory, trees allocate photosynthate to produce longer and more numerous spines, which deter mammalian browsers (resistance strategy) (Young, 1987; Young *et al.*, 2003). Herbivory can also stimulate increased rates of leaf and shoot growth (tolerance strategy) (Huntzinger *et al.*, 2004). Finally, *A. drepanolobium* trees provide inducible extrafloral nectar and bulbous hollow thorns as food and housing for resident ants, which in turn help to protect trees from herbivory (indirect defense strategy) (Madden & Young, 1992; Stapley, 1998).

In the savannas of Laikipia District in northern Kenya, nearly every *A. drepanolobium* tree (*c.* 100% of trees > 1 m tall) is occupied by one of four mutually exclusive ant species, with ant species turnover rates of *c.* 15% yr<sup>-1</sup> (Palmer *et al.*, 2000). Each ant species exhibits its own suite of distinctive behaviours towards its tree host (Young *et al.*, 1997; Palmer *et al.*, 2000). *Crematogaster mimosae* behaves as a faithful mutualist, forming dense populations, recruiting aggressively to attack browsers and patrolling to remove insect herbivores (Young *et al.*, 1997). *Crematogaster nigriceps* also defends strongly against herbivory, with dense colonies and an aggressive response to browsers and insects (Palmer & Brody, 2007), but these ants eat and destroy most of the host trees' axillary and terminal shoots (Stanton *et al.*, 1999). The two other ant species, *Crematogaster sjostedti* and *Tetraponera penzigi*, offer little, if any, mutualistic benefit to their host trees; they defend weakly against mammalian browsers and ignore or even promote insect damage to host trees (Palmer & Brody, 2007; Palmer *et al.*, 2008).

While numerous previous studies in this system have shown that resident ant species differentially affect the extent to which various defensive traits are induced or relaxed in response to changing browsing pressure (Gadd *et al.*, 2001; Young *et al.*, 2003; Huntzinger *et al.*, 2004; Palmer *et al.*, 2008), here we investigate for the first time whether interactions with browsers and resident ants also modulate leaf-level photosynthetic rates, thereby affecting available carbon pools and metabolic pathways to induced defenses.

### Materials and Methods

We conducted the study at Mpala Research Center, Kenya (36°52'E, 0°17'N) within the Kenya Long-term Exclosure Experiment (KLEE), a replicated array of 4-ha plots maintained with access to all wildlife (hereafter 'browsed') or electric-fenced to allow no wildlife access (hereafter 'unbrowsed') since 1995, where *A. drepanolobium* (Sjostedt)

trees constitute 95% of the tree canopy (Young *et al.*, 1998). In three browsed and three unbrowsed plots, we marked 12 similarly sized trees, three occupied by each ant species. In January 2009 (growing season) and April 2009 (dry season), we measured gas-exchange rates on one unshaded, fully expanded leaf per tree, using an LI-6400XT photosynthesis system (LI-COR Biosciences, Lincoln, NE, USA) at fixed photon flux density, CO<sub>2</sub> concentration, humidity and leaf temperature, from which net photosynthesis ( $P_n$ ), transpiration (Tr) and water use efficiency (WUE) were calculated. In January, we collected 10 unshaded, fully expanded leaves from one tree per ant species per plot for total nitrogen and  $\delta^{15}\text{N}$  analysis (University of California, Davis Stable Isotope Facility). Using JMP Statistical Software (SAS, Inc., Cary, NC, USA), we analyzed the effects of browsing and occupying ant species on  $P_n$ , Tr and WUE for each season using two-way ANOVA, and planned Student's pairwise contrasts between browsing levels within each ant species. Relationships between metabolic parameters and tissue chemistry in January were analyzed using ANOVA techniques. Distributions met assumptions of normality and homoscedasticity.

## Results and Discussion

During the active growing season, photosynthetic rates per unit area ( $P_n$ ) in the browsed plots were *c.* 50% greater on trees occupied by *C. mimosae* and *C. nigriceps* than on trees occupied by *C. sjostedti* and *T. penzigi*. In unbrowsed plots, all trees exhibited similar  $P_n$  regardless of ant occupant (Fig. 1a). From unbrowsed to browsed treatments,  $P_n$  increased significantly for *C. mimosae* and *C. nigriceps* trees (contrasts:  $P = 0.040$  and  $P = 0.006$ , respectively), while the slight decreases in  $P_n$  for trees occupied by *C. sjostedti* and *T. penzigi* were not significant (contrasts:  $P = 0.081$  and  $P = 0.149$ , respectively). Transpiration rates showed nearly identical patterns of variation in response to ants and browsing as  $P_n$  (Fig. 1b). As a consequence, there were no significant differences in WUE (the ratio of  $P_n$  : Tr) between treatments or ant occupants (Fig. 1c), meaning that trees with elevated photosynthesis were therefore using larger absolute quantities of water to support those rates.

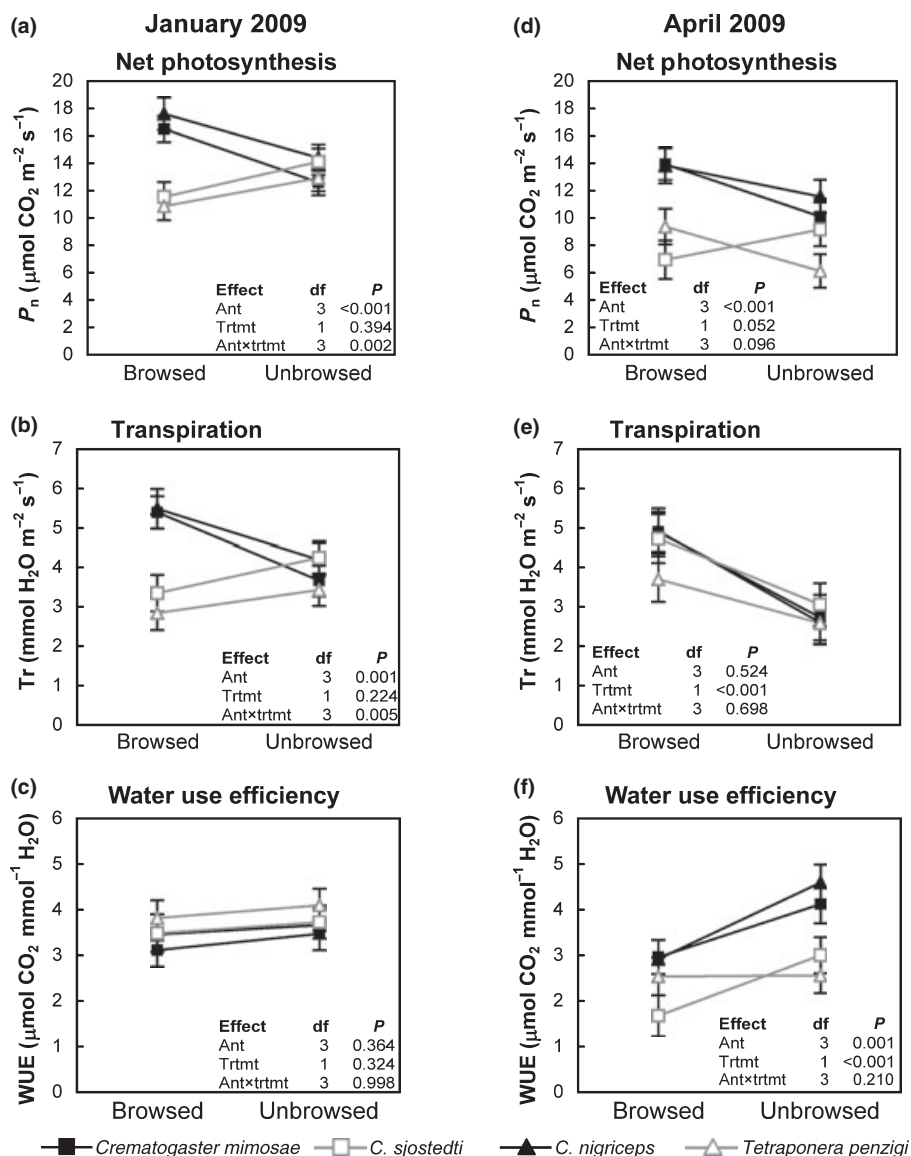
In the dry season,  $P_n$  was generally lower and all metabolic rates showed greater variation, which made ant-specific effects harder to detect. *A. drepanolobium* is deciduous, and trees were in variable stages of leaf senescence by the April census. Despite that variability, we again found that browsed trees occupied by *C. mimosae* and *C. nigriceps* had significantly higher  $P_n$  than trees occupied by *C. sjostedti* or *T. penzigi*, while unbrowsed trees showed no ant-specific differences (Fig. 1d, contrasts *C. mimosae*, *C. nigriceps* vs *C. sjostedti*, *T. penzigi*: browsed  $P < 0.0001$ , unbrowsed  $P = 0.061$ ). Unlike the growing season, dry season Tr was significantly higher in browsed trees, with no significant ant

or interaction effects (Fig. 1e). This translated into an ant-specific effect on WUE, namely that unbrowsed trees with *C. mimosae* and *C. nigriceps* had greater WUE than other ant-browsing combinations (Fig. 1f). While all browsed trees showed higher rates of Tr, only the trees occupied by *C. mimosae* and *C. nigriceps* achieved higher  $P_n$  by doing so.

The key finding of this study is that *A. drepanolobium* trees exhibit elevated photosynthetic rates in response to browsing only when occupied by strongly mutualistic ants. We discuss key implications of this novel result in the context of previous studies of defense expression in this system, as well as studies of induced variation in photosynthetic rates in other systems.

We compared our findings with previously documented effects of browsing and resident ant species on defense traits in this system. The strongest parallel was between  $P_n$  and tolerance, assessed as new leaf growth (Gadd *et al.*, 2001) (Table 1). Photosynthetic up-regulation has been postulated to enable tolerance-related growth (Trumble *et al.*, 1993; Karban & Baldwin, 1997; Strauss & Agrawal, 1999). A few recent studies have tested for correlated changes in  $P_n$  and tolerance by crossing herbivory treatments with another environmental variable known to affect  $P_n$  and/or tolerance (Gonzales *et al.*, 2008; Stevens *et al.*, 2008; Suwa & Maherali, 2008). Yet, to our knowledge, this is the first study that has in fact found congruent increases in  $P_n$  and tolerance when tested across another environmental variable, which in our case was the species of occupying ant. The ant-specific photosynthetic responses we observed were not congruent, however, with patterns of resistance or indirect defense, assessed via thorn length (Young *et al.*, 2003), extrafloral nectary and swollen thorn production (Palmer *et al.*, 2008), (Table 1). Instead, each ant species is associated with a distinct pattern of host tree photosynthetic rates and subsequent allocation to defense in response to herbivory. The environmentally variable correlations between  $P_n$ , tolerance and other defense traits highlight the need to understand, in more detail, external drivers of metabolic resource assimilation rates and subsequent allocation before assessing the costs of defense.

Current research has focused on herbivore-induced tissue loss and damage as the cueing mechanism for photosynthetic up-regulation, usually through the creation of carbon sinks and gradients (Schwachtje & Baldwin, 2008). In our study system, the behaviours of the two strong mutualist ant species cause very different degrees of tissue damage on their host trees. *C. nigriceps* ants cause continual damage by chewing and destroying nearly all axillary buds, while *C. mimosae* ants do not display this behaviour (Young *et al.*, 1997; Stanton *et al.*, 1999). The steep carbon gradient resulting from *C. nigriceps*' damage-causing behaviour is thought to explain why their host trees continue to produce more nectar and longer thorns even when herbivores are absent, whereas *C. mimosae* trees only elevate nectar



**Fig. 1** Means ( $\pm 1$  SE) of leaf-level net photosynthesis ( $P_n$ ), transpiration ( $Tr$ ) and water use efficiency (WUE) of *Acacia drepanolobium* trees located in browsed plots and plots from which browsers had been excluded for 15 yr, grouped by resident ant species. Measurements were performed during the growing season in January 2009 (a–c) and during the dry season in April 2009 (d–f). Results of two-way ANOVAs are given for each variable. In January, 66 trees were measured,  $n = 8$  or 9 trees in each ant  $\times$  browsing treatment combination. In April, 60 trees were measured,  $n = 7, 8$ , or 9 trees in each treatment combination.

production and thorn elongation in the presence of browsers (Young *et al.*, 2003; Palmer *et al.*, 2008). In contrast to their differential expression of defense traits, *C. nigriceps* and *C. mimosae* trees both show the same reduction in photosynthesis when browsers are excluded. Thus, damage-based mechanisms can adequately explain documented patterns of allocation of photosynthate to defense traits, but it appears that some additional mechanism, driven by the interaction between herbivores and occupying ants, is responsible for regulating the rate of photosynthesis itself.

In addition to intrinsic cueing mechanisms,  $P_n$  up-regulation can depend on resource availability, such as

nitrogen (Ripley *et al.*, 2006) or water (Gonzales *et al.*, 2008). We investigated whether the quantity of nitrogen per leaf area, as tissue nitrogen concentration and as leaf thickness, differed between treatments that exhibited elevated vs 'baseline'  $P_n$  (i.e. browsed *C. mimosae* and *C. nigriceps* trees, vs all other ant-browsing treatments) in the growing season. The percentage of nitrogen in leaves did not vary based on treatment  $P_n$  elevation (ANOVA, degrees of freedom (df) = 1,  $F = 0.04$ ,  $P = 0.52$ ), nor did specific leaf area ( $\text{cm}^2 \text{g}^{-1}$ ) (ANOVA, df = 1,  $F = 2.51$ ,  $P = 0.13$ ). These results imply that the photosynthetic advantage of browsed *C. mimosae* and *C. nigriceps* trees was not the result

**Table 1** Comparison of effects of browsing on *Acacia drepanolobium* tree traits, which vary depending on the species of ant occupying trees

Tree trait	Occupying ant species			
	<i>Crematogaster mimosae</i>	<i>Crematogaster nigriceps</i>	<i>Crematogaster sjostedti</i>	<i>Tetraponera penzigi</i>
Primary metabolism: net photosynthesis	↑	↑	○	○
Tolerance: new leaves per shoot <sup>1</sup>	↑	↑	○	○
Resistance: thorn length <sup>2</sup>	↑	○	○	○
Indirect defense: nectaries <sup>3</sup>	↑	○	↑	○
Indirect defense: swollen thorns <sup>3</sup>	○	○	○	○

Tissue-level photosynthesis responses were measured in this study; responses of tolerance, resistance and indirect defense traits were drawn from cited studies.

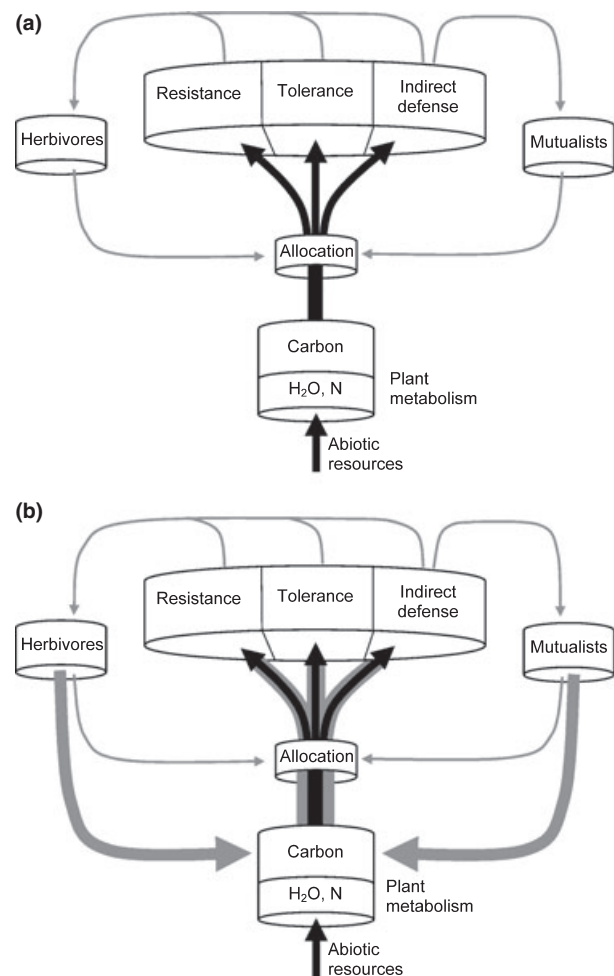
↑, Significant increase in browsed trees; ○, no significant change.

<sup>1</sup>, From Gadd *et al.* (2001); <sup>2</sup>, from Huntzinger *et al.* (2004); <sup>3</sup>, from Palmer *et al.* (2008).

of greater nitrogen allocation per leaf area. Given the observed trends in WUE, water availability may be a factor impacting the costs of herbivory and photosynthetic up-regulation. However, we are unable to propose a plausible way that *C. mimosae* and *C. nigriceps* and browsing could interact to increase a tree's water availability as a *mechanism* of  $P_n$  up-regulation. Nevertheless, most studies in this system point to the complex, multivariate nature of interactions, in terms of both upstream cues and downstream responses (Young *et al.*, 1997; Palmer, 2003; Palmer *et al.*, 2008). Further studies that manipulate resources may help to determine whether, and how, resource availability plays a mechanistic role in  $P_n$  modulation in response to ants and browsers.

Our findings suggest that the strong mutualist ant species *C. mimosae* and *C. nigriceps* may reduce the costs of herbivory to their host trees in two distinct ways. First, given that *C. mimosae* and *C. nigriceps* ants are known to swarm much more aggressively to disturbed branches than *C. sjostedti* or *T. penzigi* (Palmer & Brody, 2007), and browser deterrence is proportional to the abundance of swarming ants (Madden & Young, 1992; Stapley, 1998), the aggressive behaviour of these species should directly reduce the rates of herbivore damage. Second, by enabling photosynthetic up-regulation, the presence of the strong mutualist ants can further mitigate costs of herbivory by increasing pools of photosynthate available for additional defense or for re-growth of lost tissue. To our knowledge, this is the first study to identify a metabolic benefit of indirect defenders via increased photosynthesis.

The tritrophic structure of this plant–herbivore system, with multiple indirect defenders and all three inducible defense strategies, presents a particularly powerful lens for seeing how biotic interactions modulate plant responses to herbivory in terms of cueing mechanisms, metabolic response and allocation to induced defense (Heil & McKey, 2003; Heil, 2008). The potential for metabolic variation, however, hypothetically exists in every plant–herbivore system. Despite growing attention to herbivory–



**Fig. 2** (a) Schematic diagram of the prevailing paradigm for evaluating biotic interactions and plant defense. Effects of herbivores and indirect defenders are recognized to affect plant allocation of resources to defense, but not primary metabolic acquisition of resources. (b) Revision of the paradigm to include critical effects of herbivores and indirect defenders on rates of carbon assimilation, which subsequently alters pools of resources available for allocation to defense traits.

induced metabolic variation in ecophysiological research (Schwachtje & Baldwin, 2008), most evolutionary ecology studies of defense do not consider photosynthetic up-regulation as an integral factor affecting resource availability for defense, or that it can respond to biotic and abiotic environmental conditions independently of defense trait expression (Fig. 2). Tritrophic regulation of photosynthesis provocatively illustrates the need to consider primary metabolism in order to unravel the costs of plant defense.

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