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Water availability, carbon-based plant defenses, and the outcome of tritrophic
interactions in an ant-plant mutualism

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ABSTRACT

There have been few studies that examine the influence of soil water content on the outcome of interactions between ants, insect herbivores, and extrafloral nectary (EFN)-producing plants despite the potential of water to alter such interactions. Here I asked two questions: 1) Does water availability mediate the phenotypic expression of EFN, chemical and physical defenses? 2) Does water availability influence the outcome of tritrophic interactions between ants, herbivores, and EFN producing plants? Plants of *Chamaecrista fasciculata* (Fabaceae) growing naturally in a remnant prairie were randomly assigned to water addition or control and ant removal or control in a two factorial crossed design. Rainfall was below the 95% confidence interval for a 16-year average throughout the season resulting in low water availability for control plants. Plants subjected to ambient water conditions produced a higher concentration of hydrolysable tannins in leaves than plants in the water addition treatment. There were no other differences in EFN, chemical, or physical defenses among treatments, either water addition or ant exclusion, or their interaction. Plants subject to ambient water had fewer herbivores, greater leaf area per plant, and produced more fruit and seeds than plants in the water addition group. Herbivores in the control for water addition ingested more leaf area per plant and per individual insect than those plants in the water addition treatment yet this did not reduce fruit or seed production. Ant presence increased plant survivorship early in the season but had no significant impact on leaf damage, leaf production or seed and fruit production of the survivors. An interaction between chemical and ant defense may have lowered leaf damage, increasing reproductive output. Overall these results demonstrate the potential for a direct influence of water availability

on chemical defense and an indirect effect of water availability on tritrophic interactions

between ants, herbivores, and plants.

CHAPTER 1

Abiotic factors and the phenotypic expression of carbon-based anti-herbivore defenses in extrafloral nectary plant systems

INTRODUCTION

Extrafloral nectaries (EFNs) are plant glands located on non-floral parts of plants that

secrete nutritious liquid termed extrafloral nectar. Like floral nectar, EFN nectar contains

constituents such as water, multiple sugar types and amino acids, and in some cases

lipids. The concentration and identity of these constituents are termed EFN nectar traits.

EFNs are considered to be an indirect defense, as EFN nectar attracts ants to the plant,

which in turn often protect the plant from vertebrate and invertebrate herbivore damage

to leaf and reproductive tissues. Yet EFN producing plants also employ other direct

defenses for protection against herbivores such as trichomes and secondary metabolites

found inside or on the surface of leaf and reproductive tissue. This direct and indirect set

of defenses may be co-expressed with variable outcomes for the EFN producing plant as

it interacts with its herbivores, and the predators of those herbivores (Rico Gray and

Oliveira 2007).

The fitness outcome of tritrophic interactions, derived from ant-guard protection, is

often but not always beneficial to the EFN producing plant (Tempel 1983, Kelly 1986,

Rashbrook et al. 1992). The range in fitness outcomes for EFN producing plants may be

influenced by the expression of non-ant defenses (i.e. direct defenses such as chemical

and physical defenses)(Rico Gray and Oliveira 2007), non-ant herbivore predators that

are members of the third trophic level (Rico Gray and Oliveira 2007), ant guard densities

(Kelly 1986, Barton 1986), ant guard species aggression towards herbivores, and the

severity of herbivore pressures (Rico-Gray and Oliveira 2007). One commonly

overlooked set of factors that may also impact the phenotypic expression of defense as

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well as the density, diversity, and behavior of all trophic levels, is abiotic factors, such as soil moisture, soil nutrients, temperature, and light.

Abiotic factors can have direct effects on the phenotypic expression of anti-herbivore carbon-based defenses, such as phenolics, trichomes, and EFN nectar (Gershenzon 1984, Rico Gray and Oliveira 2007, Dalin et al. 2008). There are several advantages to studying the direct influence of the abiotic environment on carbon-based defenses. First, defenses of the same class may have similar physiological constraints and may be synthesized in similar organelles and regions of the cell/plant (Gershenzon 1994). Second, within a particular class, defense expression may be constrained because different compounds are using the same molecular resource for production, resulting in an apparent tradeoff in phenotypic expression in resource-limited environments.

Here I will discuss findings regarding the influence of the most commonly investigated abiotic factors (light, nutrients, and water) on the phenotypic expression of direct and indirect carbon-based defenses. Direct defenses refer to those defenses that are synthesized by the plant and directly impact herbivore feeding and/or behavior. Indirect defenses refer to those defenses that are synthesized by the plant yet require an intermediary, such as ants or wasps, to impact herbivore feeding and/or behavior. In EFN producing plant systems, the commonly investigated carbon-based indirect defense is EFN nectar, while the most commonly investigated carbon-based direct defenses include the chemical defense (phenolics and terpenoids, the former discussed here), and the physical defense (trichomes). I also will discuss how abiotic factors may affect the simultaneous phenotypic expression of multiple defenses by an EFN producing plant.

THE ABIOTIC ENVIRONMENT AND CARBON-BASED DIRECT DEFENSES

Phenolics and tannins

Phenolics are a carbon-based class of secondary metabolites that are structurally defined by a hydroxyl group bonded to an aromatic hydrocarbon ring in a variety of structures. One of the most frequently examined types of phenolic compounds is tannins, including condensed tannins, hydrolysable tannins, and phlorotannins (not discussed here). Tannins (and phenolics) are often concentrated in leaf tissue, and when eaten by herbivores can prolong herbivore larval development, decrease protein absorption in the herbivore gut, and increase an herbivore's time spent feeding on leaf tissue (Hagerman 1991). Yet the negative effects of phenolics (condensed tannins) in ingested leaf tissue may not exist for some herbivore species (Ayres 1997) and some herbivore species may counteract the negative effects of ingesting tannins (hydrolysable) by compensatory feeding (Barbehenn et al. 2008). Such impacts on digestion rates and feeding time however may cause herbivores to be more susceptible to predation from members of the third trophic level, such as ant-guards or wasps (Rico Gray and Oliveira 2007).

Abiotic factors may influence the phenotypic expression of phenolics in plant tissue via the quantity of carbon and nitrogen found in a plant's local environment. For example, studies have shown that nitrogen fertilization decreases the percent dry weight of phenolics and tannins found in the leaves of various plant species (Bryant et al 1987, McKey 1979, Richardson et al. 1999, Coley et al. 2002). This result is not always consistent, as Dudt and Shure (1994) found nitrogen enrichment had little effect on phenolic and tannin concentrations in the leaves of *Liriodendron tulipifera* and *Cornus florida*. Bryant et al. (1983) proposed that in the presence of high nitrogen levels

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(fertilization) plants favor the use of carbon pools for growth that would otherwise be converted to phenolics under low nitrogen (non-fertilized) conditions.

Subjecting plants to artificially elevated levels of atmospheric CO_2 concentrations can increase phenolic and tannin concentrations in leaf tissue. For example, Bazin et al. (2002) found that phenolic concentrations were higher in the leaf tissue of *Lotus corniculatus* plants that were subjected to elevated levels of atmospheric CO_2 than plants exposed to ambient CO_2. Coley et al. (2002) found similar results with saplings of multiple tropical tree species exhibiting increases in phenolic leaf concentrations due to elevated CO_2. Additional studies corroborate that tannin concentrations in the leaves of various plant species may increase due to exposure to elevated levels of atmospheric CO_2 (Penueles and Esttarte 1998, Lindroth et al. 2001, Stiling and Cortnelissem 2007). These results suggest that an increase in the quantity of carbon available to a plant (here via air) may potentially alter the phenotypic expression of phenolics in leaf tissue.

Variation in light exposure levels to leaf tissue may also alter phenolic concentrations in leaf tissue. For example, increased exposure to sunlight increased phenolic concentrations in the leaves of *Salix dasyclados* (Larrson et al. 1986). Similarly, Dudt and Sure (1994) found that higher sunlight exposure increased phenolic and tannin concentrations in the leaves of *Liriodendron tulipifera* but less markedly so in the leaves of *Cornus florida.* Additionally, high elevation (synchronous with elevated light exposure) has been shown to influence the expression of phenolics: Koptur (1985) found that high elevation *Inga* trees had higher phenolic concentrations in leaves than low elevation *Inga* plants. What remains unclear is whether increased phenolic concentration in leaves is due to increased light exposure and subsequent increased photosynthetic

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output (i.e. increase in available carbon) or rather a response to the stress of ultraviolet light exposure (Heil et al. 2002). Many plant species may gain UV protection from phenolics (Lattanzio et al 2008), thus these above results suggest that phenolic concentrations in the leaf tissue of plants may increase in those plants exposed to high UV/light exposure and/or high elevations (relative to low light/elevation plants).

Water availability also impacts the concentrations of phenolics in leaf tissue. For example, drought has been shown to increase phenolic concentrations in the leaf tissue of many plant species (Gershenzon 1984, Mattson and Hack 1987, Estiarte et al. 1994), yet Shure et al. (1998) found that condensed tannin concentrations in the leaves of oak, chestnut and maple species were the greatest in high precipitation years. Furthermore, Blodgett and Stanosz (1998) found that water stress did not affect phenolic concentration in the needles of red pine. Thus the influence of water availability/stress on the expression of various classes on phenolic compounds in leaves may vary by plant system and species. More studies are needed to elucidate the direct effect of this abiotic factor on differing classes of phenolic compounds among environmental gradients of soil moisture.

Abiotic factors also may alter the degree to which plants can induce the production of phenolic compounds in leaves. For example, Held and Baldwin (2005) found that soil nutrient levels influenced both the jasmonic acid (JA) induction pathway for phenolics in *Artemisia vulgaris* as well as subsequent expression of phenolics in leaf tissue. Plants growing on low nutrient soil had inhibited jasmonic acid induction and low phenolic concentrations in leaves, while the leaves of plants grown on high nutrient soils exhibited non-inhibited JA induction and high phenolic concentrations in leaves. These results suggest a cost associated with the induction of phenolic compounds. Ontogenetic

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variation in the ability of plants to induce various defensive compounds, such as

phenolics (Baldwin 1994), further suggests that a cost exists for the induction of

phenolics. Resource restraints imposed upon induction pathways and phenotypic

expression due to the cost of expression may be exacerbated by abiotic factors (Boege

and Marquis 2005). Thus, the degree of induction (and concentration) of phenolics in leaf

tissue may be a function of resource availability and the abiotic environment, yet this area

is relatively under-researched.

Trichomes

Trichomes, found on the surface of leaf tissue, are carbon-based defenses that can act as

both physical and chemical defenses from herbivore damage to leaf tissue. Glandular

trichomes (not covered here) produce anti-herbivore chemical toxins, while non-

glandular or structural trichomes contain no form of chemical defense. Structural

trichomes act as a physical barrier between nutrients in leaf tissue and herbivores and

reduce the ability of herbivores to move across leaf tissue (potential for increased

detection by the third trophic level). These characteristics limit herbivore damage to leaf

tissue covered in structural (Levin 1973, Mauricio 1998, Dalin et al. 2008).

The abiotic environment, specifically in the form of CO_2 and light, may affect

trichome density. For example, Wilkens et al. (1996) found that elevated CO_2 increased

trichome density on the leaves of *Lycopersicon esculentum* suggesting alterations in

available carbon may impact trichome densities on leaves. Additionally, high UV-

radiation has been shown to increases trichome densities on leaves (Skaltsa et al. 1994,

Wilkens et al. 1996, Liakoura et al. 1997). Whether such increases in trichome density

under high light intensity/UV-radiation are due to increased carbon fixation or rather an

adaptation to dampen negative UV effects is unclear. More research is needed to examine the exact cause for such increased trichome density under high light intensity conditions.

Water availability also may influence the density of structural trichomes found on the surface of leaf tissue. For example, Gonzales et al. (2008) subjected *Madia sativa* plants to either drought or non-drought conditions in the greenhouse. It was found that plants subjected to drought had a greater density of structural trichomes on leaves than non-drought plants. In contrast, Forkner and Hare (2000) found that the density of non-glandular (structural) trichomes in *Datura wrightii* was not affected by water availability. These studies give a partial picture of plant responses to drought in terms of structural trichome production; however subjecting plants to a greater range of water treatments may further explain the relationship between structural trichome production and water availability. More research is needed to clarify the role of water availability upon the phenotypic expression of structural trichomes in multiple water regimes and plant systems.

Finally, the density of structural trichomes on leaves is also inducible. Artificial and actual herbivore damage (Agrawal 1999, 2000, Dalin et al. 2008, Gonzales et al. 2008), along with jasmonic acid application (Traw and Bergelson 2003) has been shown to increase trichome density. Because trichomes may be costly for plants to produce (Björkman et al. 2008, Dalin et al. 2008), changes in resource availability may influence the trichome induction. For example, in a greenhouse setting Gonzales et al. (2008) found that drought increased induction of structural trichomes of *Madia sativa* plants following artificial herbivory relative to non-drought plants. Aside from Gonzales et al.

(2008), the relationship between trichome induction and varying abiotic conditions has yet to be examined, especially in a field setting.

THE ABIOTIC ENVIRONMENT AND CARBON-BASED INDIRECT DEFENSE

Extrafloral nectar and ant defense

The effectiveness of EFN nectar as an anti-herbivore defense is influenced by the direct effects of the environment on both the phenotypic expression of EFN nectar traits and ant activity, as well as the indirect effects of EFN nectar itself upon insect herbivore damage. EFN nectar traits include water, sugar, and amino acid concentration, and volume. The mechanism by which EFNs (and EFN nectar) function as a indirect defense in the context of the abiotic environment involves the direct effects of abiotic factors on the quantity of resources available for the phenotypic expression of various EFN traits, the direct effects of abiotic factors on EFN traits (i.e. sugar/amino acid concentration and/or nectar volume via evaporation and deterioration rates or resource availability), the response of ant-guards to EFN nectar (i.e. attraction to EFNs and aggressiveness towards herbivores) due to potential changes from the abiotic environment in EFN nectar traits, and the direct effects of the abiotic environment on ant activity independent of EFN nectar's influence on ant behavior (i.e. abiotic stress affecting ant behavior or metabolism). Here I cover past investigations on the effects of the abiotic environment on EFN defense.

Abiotic stresses associated with elevation such as solar radiation and water/nutrient availability may influence the phenotypic expression of EFN nectar traits. For example, Inouye and Taylor (1979) compared investment in EFN nectar traits between high (at 2896 m and 3091 m) and low elevation (2734 m) plants of the aspen sunflower

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(*Helianthella quinquernervis* (Asteraceae)). Amino acid type and concentrations found in nectar did not vary by elevation, but EFN nectar volume was lower in high elevation plants (EFN sugar concentration was not examined). Despite lower nectar volume at higher elevations the benefit of ant protection was greater at the high elevation site. The authors speculated that this benefit of ant protection might be due to lower herbivore pressures at higher elevations or alternatively physiological mechanisms (not specified by the authors) affecting EFN nectar. Potential physiological effects on EFN nectar may be due to abiotic factors associated with elevation, such as solar radiation and/or water stress, that can cause changes in EFN volume or sugar concentration. For example, a decrease in EFN volume may impact sugar concentration if EFN nectar water content decreases yet sugar content remains constant. In this way sugar concentration would increase perhaps increasing the attractiveness of EFN nectar to ants. More research is needed to evaluate the impact of varying elevations and associated abiotic factors on EFN traits.

In an experiment similar to that of Inouye and Taylor (1979), Koptur (1985) investigated the influence of elevation on investment in ant defense in two species of *Inga* trees. Koptur found that elevation did not influence the number of active nectaries per plant, but that ant attendance/attraction to EFNs was lower at higher elevation. Nectar traits such as volume and constituents were not measured in this experiment, but it is possible that changes in nectar traits, influenced by the abiotic environment and elevation stresses, may have altered the attractiveness of EFN nectar to ant-guards at higher elevations. Alternatively, there may have been fewer ants at higher elevations due to direct effects of the abiotic environment on ant behavior. This study suggests that

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elevation and associated abiotic factors/stresses have the potential to influence ant activity and/or ant attraction to EFN.

Variation in the quantity of water available to an EFN producing plant may alter the phenotypic expression of EFN traits. In the only manipulative experiment to investigate the influence of abiotic factors on EFN nectar traits, Chen (2003) subjected two groups of field-grown *Chamaecrista fasciculata* (Fabaceae) plants to either water addition or ambient water levels and found that water supplemented plants produced a greater volume of EFN, produced more leaves (i.e. more EFNs per plant), and accrued less leaf damage. However, counter-intuitively, water supplementation did not result in increased ant attendance, suggesting that water availability may have influenced either the quality of EFN nectar and/or the behavior of the ants. Alternatively, other non-ant defenses, such as chemical or physical defenses (see discussion above) may have been favored in expression due to excess water availability, resulting in greater non-ant defense and perhaps lowered herbivore damage in the water addition group. Such results suggest that the abiotic environment may alter EFN nectar traits and the attractiveness of EFN nectar to ants.

Further evidence from the *C. fasciculata* system demonstrates that soil nutrients may influence the attractiveness of EFN nectar to ants and perhaps the phenotypic expression of EFN nectar traits. Abdala and Marquis (2008) conducted a reciprocal transplant experiment between three populations of *C. fasciculata* that varied in soil nutrient concentration and measured the degree of ant protection between sites. They found that, regardless of source site, all plants transplanted to the site with high soil nutrients received greater ant activity and reduced leaf area loss to herbivores. However,

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EFN volume did not vary as a function of site, suggesting increased ant activity (and decreased leaf area lost) on plants found in high nutrient soils sites may have been due to changes in nectar quality, such as amino acid or sugar concentration, driven by the abiotic environment. Alternatively, the phenotypic expression of other non-ant defenses, such as secondary metabolites or physical defenses, due to the abiotic environment may have lowered herbivore damage. Such findings may also be a result of direct effects of the abiotic environment on ant behavior. More experimentation is needed to determine the degree to which soil nutrients alter EFN nectar traits, non-ant defenses, and ant behavior in EFN plant systems.

Multiple abiotic factors may also simultaneously affect elements of the ant-EFN-plant mutualism. Kersch and Fonseca (2005) studied the effects of light and soil nutrients on ant protection of the EFN producing plant *Inga vera*. Despite equal ant abundance between the sun and shade plants, it was found that shaded plants did not benefit from ant attendance in terms of herbivore damage, growth, survival, and biomass, while non-shaded plants did so. Furthermore, nutrient addition lowered herbivore damage in the shade yet had no effect in the sun. Kersch and Fonseca (2005) hypothesized that differences in herbivore densities drove the observed patterns, as thrips (the primary herbivore) densities were greatest in shaded regions. No measures were made of EFN nectar traits or non-ant defenses so it is also possible that light and nutrient availability may have directly influenced the phenotypic expression of defense and ant behavior. For example, sun exposure may increase the attractiveness of EFN nectar to ants, in comparison to shaded plants, via a evaporation driven decrease in nectar water content (volume) and a subsequent increase in EFN nectar constituent (sugars/amino acids)

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concentration. In some systems ants appear to prefer concentrated EFN nectar (high ratio of constituents to water) to a high volume of EFN nectar (low ratio of constituents to water) (Heil and McKey 2003). Thus, for Kersch and Fonseca (2005) the increase in concentration of EFN nectar constituents relative to shaded plants of *Inga vera* also may have increased ant aggression towards herbivores and therefore overall ant protection. More research is needed to disentangle the influence of light exposure and nutrient addition on the phenotypic expression of EFN traits and the outcome of tri-trophic interactions, especially in the context of varying herbivore pressures between treatments.

EFN nectar traits can be induced by damage to leaf tissue, which may be influenced by the abiotic environment. Following both natural or artificial herbivore damage, induction has been noted for a number of EFN traits including increases in nectar constituents such as sugar concentration (Ness et al. 2003), amino acid concentration (Heil 2008), EFN nectar volume (Heil 2008, Holland et al. 2009, J. Flunker unpublished data), and the number of EFNs per plant (Mondor and Addicott 2003, Wolley et al 2007). Evidence also suggests that the abiotic environment may influence the induction of the number of EFNs produced per plant. For example, Mondor et al. (2006) found that, following artificial herbivory, nutrient rich (high levels of N, P and K in soil) *Vicia fava* plants induced a greater number of EFNs per plant than nutrient-poor plants. No studies to date have examined the potential influence of the abiotic environment on the induction of EFN nectar traits such as sugar and amino acid concentrations and EFN nectar volume. It seems likely that the amount of resources available for the production and induction of EFN defenses, in response to herbivory, may be dictated by the abiotic environment.

The abiotic environment may also directly influence ant activity and/or aggression towards herbivores on EFN nectar producing plants. The efficacy of EFN nectar as an indirect defense is entirely dependant on the ant response to EFN nectar traits, thus the direct impact of the abiotic environment on ant activity may affect the protective benefit of ants to EFN producing plants. Temperature seems to be one influential factor dictating ant activity on EFN producing plants with periods of peak ant activity and ant density varying as a function of temperature: very low and high temperatures appear to correlate with low ant densities at EFNs while mid-range temperatures correlate with high ant densities at EFNs (Rico-Gray and Oliveira 2007). Differences in elevation perhaps associated with abiotic stress also may impact ant activity as Koptur (1985) found that high elevation *Inga* (Fabaceae) plants had low ant densities compared to *Inga* plants found at lower elevation. These findings suggest that the abiotic environment may directly influence ant activity and therefore overall ant protection provided to plants.

Rainfall also may be an abiotic factor that can directly affect ant activity and therefore ant protection in EFN producing plants. For example, Wirth and Leal (2001) demonstrated that ant activity declined at the EFNs of *Passiflora coccinea* after simulated rainfall events. This result suggests that ants may patrol EFN producing plants less during rainy periods or in wet environments thereby lowering the degree of ant protection available to a plant during periods of rain or in wet habitats. Conversely, Cogni et al. (2003) found that ant activity at baits adjacent to EFNs on leaves of *Hibiscus* sp. was higher in a wet mangrove habitat than in a sandy, dry mangrove habitat. Thus, the abiotic environment may directly affect ant activity on EFN producing plants, yet this relationship may vary by environment and may also be due to changes in EFN nectar

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traits such as nectar volume or quality. The direct impact of the abiotic environment on ant activity is important to evaluate, especially when determining the overall effectiveness of EFN nectar as an indirect defense against herbivores.

TRADE-OFFS IN CARBON-BASED DEFENSES AND THE INFLUENCE OF THE ABIOTIC ENVIRONMENT

EFN producing plants most likely employ multiple types of anti-herbivore defense to protect against an array of potential herbivore threats that vary in time and space. Because anti-herbivore defenses are costly to produce (Stamp 2003) resource availability may dictate both the type and quantity of particular defenses expressed by a plant. As plants do not always have access to the required nutrients for all physiological processes, resource constraints may originate from the costs of growth and reproduction (Herms and Mattson 1992) and from limitations on resources available to the plant imposed by the abiotic environment (variability in available light, water and/or nutrients). Subsequently, the resource costs for the production of anti-herbivore defense combined with limited resource availability may result in trade-offs between the phenotypic expressions of differing anti-herbivore defenses (Rhoades 1979, Coley 1985). Although genetic influences on the expression of defense are likely important, as well as the interaction of genotype with the abiotic environment (Reznick 1985), phenotype trade-offs are the focus of this discussion.

The molecular basis of defenses may determine whether a phenotypic trade-off is likely to occur between anti-herbivore defenses under differing abiotic conditions. The probability that a trade-off may occur depends on the synthesis pathways of the defenses,

the degree to which molecular precursors to synthesis are shared between defenses, the relative costs of production per defense, and resource availability (Gershenzon 1994). Given these details, I hypothesize that defenses with the same molecular basis, for example, carbon-based ant defense (i.e. EFN nectar) and carbon based non-ant defenses (i.e. chemical and physical defenses), may share synthesis pathways and molecular precursors and could thus be prone to trade-offs in phenotypic expression, especially in a resource-limited environment. Alternatively, defenses with a different molecular basis (i.e. nitrogen versus carbon-based defenses) often respond differently to abiotic stresses (Waterman and Mole 1989) such as resource limitation. This differential response to environmental stress also may make trade-offs in phenotypic expression between nitrogen and carbon-based defenses probable under resource limiting conditions.

The presence of trade-offs in the phenotypic expression of anti-herbivore defenses varies by plant species and defense type (Heil et al. 2002). For example, in a meta-analysis of 31 studies examining trade-offs in the phenotypic expression of defenses, Koricheva et al. (2004) concluded that, in general, across many plant systems, trade–offs in phenotypic expression of defenses were not frequent. Nonetheless, Koricheva et al. (2004) found that trade-offs did occur under certain conditions. While examples of trade-offs were limited among differing chemical classes of defense, similar classes of chemical compounds, such as carbon based chemical defense, did exhibit trade-offs. Examples of such trade-offs include condensed tannins versus phenolic glycosides in *Populus tremuloides* (Lindroth and Whang 1996) and condensed tannins versus cyanogenic glycosides in *Lotus corniculatus* (Ross and Jones 1983). The synthesis pathways of these carbon-based compounds may overlap (Gershenzon 1994) supporting

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the above hypothesis that trade-offs in phenotypic expression between carbon-based

defenses may be prone to occur.

Predicted and observed trade-offs (negative correlations) between the phenotypic

expression of ant and non-ant defenses have been investigated in a select few EFN-

producing plant systems (Rico-Gray and Oliveira 2007) yet more studies are needed.

Here I discuss trade-offs in carbon-based ant and non-ant defenses. As described above

these defenses may have the greatest probability of competing for resources needed in

production, due to perhaps similar synthesis pathways and a common molecular

backbone.

Trade-offs between ant and non-ant defense

Phenotypic trade-offs between investment in ant (EFN traits) and non-ant defenses

(chemical and physical) have been relatively under-explored. One of the few studies to

investigate trade-offs between investment in EFN traits and non-ant defense was

conducted by Rudgers et al. (2004), in which three populations of *Gossypium thurberi*

were measured for negative correlations (i.e. trade-offs) between the non-ant defenses

defenses (trichomes and leaf glands) and the ant defenses (the number of EFNs per plant

and average extrafloral nectary area per plant). Rudgers found no evidence for trade-offs

between ant and non-ant defense. However, specific measurements of EFN traits such as

volume and constituents were not made. Thus, it is possible that trade-offs may have

existed between nectar traits, such as EFN nectar volume, amino acid and sugar

concentration, versus non-ant defenses (trichomes and leaf glands).

Evidence suggests that trade-offs between the phenotypic expression of ant defense

and non-ant defense may exist. For example, trade-offs between ant activity/presence and

tannin concentrations in leaves appear to be prevalent in many plant species (Heil et al 2002), while trade-offs between food body production and chemical defense (Dyer et al. 2001) and ant activity/presence and leaf silica body concentration per leaf (physical defense) (Korndorfer and Del-Claro 2006) have also been noted. Yet Wooley et al. (2007) found no evidence for trade-offs between EFN density per leaf and phenolic concentration. Thus the detection of trade-offs between investment in ant defense and non-ant defense may vary per system, in addition to the particular defenses of interest.

The abiotic environment may alter the potential for trade-offs between plant investment in ant and non-ant defenses. For example, Koptur (1985) found high elevation EFN producing *Inga* (Fabaceae) plants had low ant densities and high leaf tannin concentrations, while low elevation *Inga* plants had high ant densities and low leaf tannin concentrations. These results suggest a trade-off between investment in ant defense and chemical defense related to changing environmental conditions across elevation. Koptur hypothesized that low ant density at the high elevation sites may have led to increased tannin production. Whether low ant density or variation in abiotic factors between elevations influenced this phenotypic trade-off was not examined. EFN nectar may be costly to produce (Rutter and Rausher 2004), therefore, for Koptur (1985), if elevation promoted such potential resource restraints as light, water and nutrient stresses, plants may favor the phenotypic expression of the least costly defense. In this way plants thereby increase synthesis of phenolics (Gershenzon 1984) and lower investment in ant defense/EFN traits depending on abiotic conditions. The degree to which abiotic factors affect the relationship between the phenotypic expression of EFN traits and non-ant defenses has yet to be explicitly explored via manipulative experimentation.

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CONCLUSIONS/FUTURE DIRECTIONS

Abiotic factors have the potential to mediate the phenotypic expression of carbon-based defenses in EFN producing plants. Yet the abiotic conditions that dictate the phenotypic expression of anti-herbivore defense strategies remain largely under-investigated. Despite a myriad of quality work examining the role of biotic factors in mediating the expression of defense in EFN plant systems (Bronstein 1998, Koptur 1992, Rico Gray and Oliveira 2007), few studies have examined the direct impact of abiotic factors on the expression of ant and non-ant defenses in EFN producing plants. Moreover, no studies to date have simultaneously combined both the investigation of abiotic factors and their influence on potential trade-offs in the phenotypic expression of defenses in systems involving EFN producing plant-herbivore interactions.

Given considerable spatiotemporal variability in ant densities (Inouye and Taylor 1979, Horvitz and Schemske 1990) it may be selectively advantageous for an EFN producing plant engaged in a protective mutualism with ants, particularly a facultative mutualism, to be phenotypically plastic for alternative non-ant anti-herbivore defenses. In the event of low ant densities, high herbivore pressure, and perhaps resource limitation, a plant may need to employ chemical and/or physical defensive traits (direct defenses) that may limit herbivore damage. The ability to express defenses however may be mediated by the abiotic environment via resource constraints on defense production. Thus, it is crucial to be aware of the abiotic environment in which ant-EFN producing plant interactions are occurring in order to explain, at least in part, variable patterns of phenotypic defense expression as well as the outcomes of tritrophic interactions between EFN producing plants, ant guards, and herbivores.

I advocate future experiments that examine tritrophic interactions between plants, herbivores, and the third trophic level (i.e. ants) to measure the role of abiotic factors on such interactions. These abiotic factors can be measured simultaneously, using them as covariates in statistical analysis. More preferably, they may be manipulated experimentally to determine the direct influence of a chosen abiotic factor on all three trophic levels. In this way the direct influence of the abiotic factor on trophic levels may be controlled for, making the relative strengths of direct and indirect interactions between trophic levels less obscured by unexplained variability. Future experiments geared to examine phenotypic trade-offs between ant and non-ant defense in EFN plants systems, especially in the context of the abiotic environment, must determine which level of measurement is appropriate for quantifying investment in ant defense. The ability to detect a trade-off may be due in part to how defenses are measured. For example, a plant's investment in the number of EFNs per plant versus the volume/constituents of EFN nectar may suggest very different conclusions regarding trade-offs as both measures may respond differently to selective pressure.

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CHAPTER 2

Water availability, carbon-based plant defenses, and the outcome of tritrophic

interactions in an ant-plant mutualism

INTRODUCTION

Abiotic factors are hypothesized to mediate fitness outcomes for plants that produce extrafloral nectar to attract ants for protection against potential herbivores (Huxley 1991, Hunter and Price 1992, Bronstein 1998). Such mutualisms can be beneficial for the plant because ant-guards often reduce damage to plant tissue caused by insect herbivores (Bentley 1977, Koptur 1992, Rico Gray and Oliveira 2007). Despite attention to the impact of light and nutrient levels (de la Fuente and Marquis 1999, Kersh and Fonseca 2005) and elevation (Inouye and Taylor 1979, Koptur 1985) on interactions between ant-guards and extrafloral nectar producing plants, the likely role of soil moisture on the conditional outcome of mutualisms between ant-guards and extrafloral nectar producing plants remains relatively unexplored (but see Chen 2003).

Abiotic factors may impact one or more of the three trophic levels in ant-plant mutualisms. For example, the abiotic environment may alter the types, quantity and quality of ant and non-ant antiherbivore defenses expressed by plants (Gershenzon 1984, Rico Gray and Oliveira 2007, Dalin et al. 2008). Relevant defenses influenced by the abiotic environment include the quantity and quality of ant rewards, secondary compound concentrations in plant tissue, and structural aspects of leaves including leaf toughness and pubescence. In addition, herbivore activity, density and diversity (Hunter and Price 1992, Brown et al. 2001) and ant guard activity, density, and diversity (Inouye and Taylor 1979, Koptur 1985, Chen 2003, Kersh and Fonseca 2005, Rico-Gray and Oliveira 2007, Abdala and Marquis 2008, Rios et al. 2008) can be affected by abiotic factors such as light level, humidity, and temperature.

Studies of ant-plant interactions often focus solely on the ant contribution to defense, overlooking the role of secondary compounds (Rico Gray and Oliveira 2007). For example, Folgarait and Davidson (1995) and Kersch and Fonseca (2005) both found that the abiotic environment may affect ant-plant mutualisms, but neither examined the influence of the abiotic environment on non-ant defenses. The amount of herbivore damage to a plant defended by ants, however, is likely to be a consequence of both defenses. Ant protection is never perfect (Rico Gray and Oliveira 2007), leaving plant tissue quality as the backup defense. Combined ant and chemical defense may be more effective than a singular defense alone (Fincher et al. 2008). Subsequently, the abiotic environment may modify this synergy between ant and non-ant defenses.

Soil water availability is one under-investigated abiotic factor that lends itself to experimental manipulation in the context of ant protective mutualisms. Under water stress, a plant may close its stomata to minimize evaporative water loss, reducing intake of CO_2. Reduced photosynthesis may result, lowering carbon availability for growth and reproduction. In addition, allocation to carbon-based secondary compounds, structural defenses (e.g., trichomes) and extrafloral nectar, composed mainly of water and sugars, might all be affected. For example, Shure et al. (1998) found that condensed tannin concentrations in oak, chestnut and maple trees were low in below average precipitation years and high in wet years. Alternatively, water stress may increase foliar concentrations of phenolics and tannins (Rosenthal and Janzen 1979, Waterman and Mole 1989). Extrafloral nectar traits also have been found to respond to water availability. Chen (2003) found that *Chamaecrista fasciculata* increased nectar production by extrafloral nectaries (EFN) and produced more leaves (i.e. more EFNs per plant) in response to

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water supplementation. In turn, this increase in nectar volume attracted more ant-guards, resulting in lowered herbivore damage to leaf tissue and an overall positive benefit to plant reproductive output.

The impact of water availability, and resources in general, on the outcome of tritrophic interactions will depend on how plant phenotype is affected, and in turn, how herbivores and ant-guards respond to this change in phenotype. Limited resources may result in trade-offs in the phenotypic expression among defense types, these trade-offs becoming apparent as resource availability changes (Rhoades 1979, Coley 1985). A plant may increase extrafloral nectar production as a function of increased water availability (Chen 2003), but may simultaneously lower chemical defense. Alternatively, the phenotypic expression of all defenses could be upregulated, resulting in a plant that is superior in many defense types. Changes in the phenotypic expression of ant and non-ant defense may alter the outcome of an ant-plant mutualism as both are likely to influence the level of tissue loss to herbivores. For example, extrafloral nectar producing plants subject to high water availability may upregulate the production of carbon-based defenses such as trichomes and secondary compounds, resulting in decreased herbivore movement, increased herbivore feeding time, and an increased probability of ant-guards detecting and pursuing herbivores. Such a case would be an example of a synergy between ant and non-ant defense. Synergy between ant and chemical defense has been noted in the *Piper* system (Fincher et al. 2008). In contrast to synergy between ant and non-ant defenses, non-ant defenses also may limit ant movement on the plant, increasing herbivore detection time (Belcher and Thurston 1982, Davidson 1989, Lovinger et al. 2000).

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Here I examine how soil moisture influences plant defense and ant protection in the extrafloral nectar producing plant *Chamaecrista fasciculata*. I addressed the following two questions: 1) Does soil water availability mediate the phenotypic expression of EFN traits, chemical, and physical defenses? 2) Does soil water availability influence the outcome of tritrophic interactions between ants, herbivores, and EFN producing plants? I supplemented natural rainfall with additional water for a set of experimental plants, and compared their plant defense traits and resulting tritrophic interactions with that of control plants. Because I manipulated only the local environment of the plant, any differences between control and treatment plants would be due to changes in plant phenotype, potentially affecting ants and herbivores indirectly, and not due to direct effects of the environment on the animal components of the system. I predicted that EFN defense, carbon-based chemical defense, and carbon-based physical defense expression would be higher in watered plants due to greater access to carbon via water addition. As a result, more ants would be attracted to the plant, offering greater protection against vulnerable herbivores. Watered plants would suffer less damage than control plants because both ant defense and defenses in leaves would be greater in watered plants. Because of lower leaf area loss to herbivores, watered plants would have greater reproductive output than control plants.

MATERIALS AND METHODS

Study System

Chamaecrista fasciculata (Fabaceae: Caesalpinioideae) is an annual legume distributed throughout the central and eastern United States in natural prairies and disturbed areas

(Foote and Jacobs 1965). Because this species is an annual, it is possible to estimate the effects of manipulations on lifetime fitness of individual plants within a single growing season. Yellow, non-nectar secreting flowers are produced from July until late September with fruits maturing in September and October. Compound pinnate leaves are produced throughout the growing season with one EFN on the petiole of each leaf. These EFNs secrete extrafloral nectar containing sucrose, glucose and fructose (Rios et al. 2008), which in turn attracts ant-guards that can function as an anti-herbivore defense and promote plant fitness (Barton 1986; Kelly 1986; Rios et al. 2008). The volume of EFN nectar that *C. fasciculata* produces as well as the number of leaves (i.e. number of EFNs) can increase due to high soil moisture (Chen 2003). *C. fasciculata* also contains the carbon-based defenses tannins in leaves (see Results), trichomes on the midrib and peripheral edge of each leaflet (J. Flunker, personal observation), and leaf rachis hairs on stems (Rios et al. 2008). Comparison of multiple populations shows that negative correlations exist at the population level between EFN volume and trichome density (Rios et al. 2008).

This study was conducted at the Sherwood Prairie (39°04′ N 90°92′ W) of Cuivre River State Park, near Troy, MO. The prairie is a remnant tall-grass prairie, approximately 8 ha in size, and is comprised of a matrix of grasses and forbs dominated by *Andropogon gerardii* and *Schizachyrium scoparium* surrounded by an oak/hickory hardwood forest. The primary ant species known to visit the EFNs on *C. fasciculata* in this region include *Monomorium minimum* (Buckley) and *Crematogaster lineolata* (Say) (Rios et al. 2008). The primary herbivores found in the region include the free-feeding *Colias eurytheme* and *Eurema lisa* (both Pieridae), the leaftier, *Polyhymno luteostrigella*

(Gelechiideae), that forms small leaf ties with silk and feeds on leaf tissue inside of the

tie, various grasshopper species, an unidentified "bud feeder" of the Gelechiidae that

sews sepals together and feeds on internal reproductive structures, and curculionid beetles

(Coleoptera) that feed on both leaves and fruit (Rios et al. 2008, J. Flunker personal

observation).

Experimental design

On June 12, 2007, 160 *C. fasciculata* seedlings of approximately equal height and leaf

number were randomly assigned to four treatments (40 plants per treatment) in a two-way

cross factorial design with either water added or not and ants excluded or not.

Examination of *C. fasciculata* root area showed that plant roots extended outwards from

the plant stem in a circle with a radius of 13 cm. Plants in the water addition treatment

received natural rainfall in addition to approximately 200 mL of supplemental water

every 3-4 days (from June 14 to October 2 2007) poured within a 13 cm radius of each

plant. Plants in the ambient water group received only natural rainfall. Throughout the

duration of the field season each plant in the water addition treatment received a total

25.0 L water (17.0 L natural rainfall + 8.0 L addition; 5,500 mL per month) compared to

the ambient water group that received 17.0 L of natural rainfall (3,800 mL per month).

This additional 1,600 mL of water per month in the water addition treatment was above

the 95% confidence interval for average rainfall per month at the field site (mean monthly

rainfall between the years of 1990 and 2006 = 4878 mL +/- 1312 mL (95% C.I.)) (Figure

1).

Ants were excluded from all plants in the ant exclusion treatment using Tanglefoot

(Grand Rapids, MI), a sticky resinous substance that was applied to a foam barrier

(Tangleguard; Grand Rapids, MI) on the plant's stem. Tanglefoot plus a foam barrier has no direct effects on plant growth or flower production compared to non-manipulated control plants (Flunker and Marquis, in prep.). A circle of approximately 15 cm in radius was cleared of all plant matter around each experimental and control plant to eliminate all ant access points to the plant. Tanglefoot was reapplied as needed throughout the season to plants in the ant exclusion treatments. Plant matter was removed as needed throughout the season within the 15 cm radius circle of each plant in the ant access and ant exclusion treatments. Ant exclusion plants were surveyed for ant exclusion efficacy at the same time as all other plants in each arthropod census (see below).

EFFECT OF WATER AVAILABILITY ON EFN, CHEMICAL AND PHYSICAL DEFENSES

EFN defense

To estimate the effects of experimental treatments on extrafloral nectar production, thirty plants were randomly selected per treatment in the evening of August 10 and standing nectar was removed from nectaries on the five most apical leaves. All ants were removed from the plant, and then plants were covered with plastic bags and a twist tie was used to cinch the bag close on the base of the plant stem. This prevented EFN visitors from accessing nectar and minimized evaporation. After 12 hours, plants were re-visited, plastic bags were carefully removed, and nectar was collected with a 5-microliter-microcapillary from the nectaries of the three apical leaves. The proportion of the total length (cm) of the capillary filled with nectar was recorded. This proportion was multiplied by the total capillary volume (5 μL) and the product then multiplied by 2 to

determine total nectar volume over a 24-hour period. Nectar samples were transported to the lab on ice and stored at -80°C until sugar analysis.

The quantity and types of sugars (glucose, fructose, and sucrose) in the EFN nectar were measured via high pressure liquid chromatography (HPLC) (Perkin Elmer ISS- 100 sampling system and Perkin Elmer LC pump series 250: Perkin Elmer, Norwalk, CT). Samples were thawed to approximately 3°C and then diluted with 30 mL of distilled de-ionized water. Five µL of each sample were injected into a 250 cm $\times$ 4.6 mm Amino Column (Reztek Amino Column) with a solvent and mobile phase of 75/25% HPLC grade acetonitrile/distilled dionized water and a flow rate of 1 mL per minute. Refractive index was measured with a refraction detector (RefractoMonitor: Milton Roy, Riviera Beach, FL.) and the area of each sugar's refractive index peak was determined with the software Turbochrome Navigator (Perkin Elmer). Each sugar refractive index peak area was then converted to mg sugar via a standard curve generated from sucrose, fructose, and glucose standards. After accounting for the 30 mL dilution necessary for HPLC analysis, total sugar produced per plant over 24 hr was calculated as the proportion of mg sugar per mL of EFN nectar sample multiplied by the total volume of EFN nectar produced per 24 hours (mg sugar/sample volume $\times$ total volume per 24 hrs = 24 hr total sugar production). Nectary area was measured with a calibrated microscope as the area (cm^2) of an ellipse (area = $\pi \times$ length $\times$ width) on three leaves collected per experimental plant on 21 August used for defense measurements (see below for leaf collection method).

Chemical and physical anti-herbivore defense

On 21 August, three fully-expanded leaves, one from the top, middle, and lowest portion of the plant (to account for variation in leaf quality due to leaf age), were collected from each experimental plant. To minimize impacts on overall leaf area, these three leaves were the only leaves collected throughout the duration of the experiment. Leaves were transported to the lab on ice, weighed to determine wet mass, and stored at -80°C until laboratory measurements were completed. I measured investment in EFN defense (nectary area), non-EFN defense (chemical and physical), leaf area, the proportion of leaf area lost to herbivores, and the proportion of leaf weight due to water, nitrogen and carbon.

Two measures of trichome number were made on the three collected leaves per experimental plant: the number of rachis hairs occurring on the mid-vein of the leaf between the third and fourth pair of leaflets, and the number of peripheral trichomes occurring on the peripheral circumference of the third leaflet. No trichomes were found on the actual leaf surface.

Following measures of EFN area, trichome number and leaf area, leaves were freeze-dried (Labonco 6). The mass of dry leaves was then determined to estimate the proportion of leaf weight due to water. Leaves were then ground for quantification of nitrogen, carbon, and phenolic concentration. Nitrogen and carbon proportion of leaf weight were determined via microcumbustion (Perkin Elmer Series II CHNS/O Analyzer 2400). Phenolics were extracted using alternating washes of ether and acetone. The concentration of hydrolyzable and condensed tannins was measured respectively with the KIO_3 assay and the N-butanol assay following Hagerman (2000). Total phenolics were

measured with the Folin-Denis assay (Hagerman 2000). Light absorbance was measured on a microplate reader (Molecular Devices, VERSmax microplate reader) and concentrations of condensed tannins, hydrolysable tannins, and total phenolics were determined from standard curves based on light absorbance versus concentration plots of phenolic standards. Standard curves were generated from bulk samples of leaves collected from 30 non-experimental plants (3-5 leaves per plant) at the Sherwood prairie in close proximity to experimental plants. Negative ion electrospray mass spectrometry was conducted on bulk extracts from experimental plants to determine the potential molecular structures for phenolics found in leaf tissue as a function of water and ant availability.

Effect of water availability on plant-insect interactions

The proportion of leaf area eaten by herbivores was measured as the area of leaf matter present versus the potential leaf area (i.e. estimated leaf size in the absence of any herbivore damage). Leaves obtained from each plant were scanned and each leaf's area was estimated with the pixel counting program, Sigma Scan Pro 5.0 (SYSTAT, San Jose, CA). Potential leaf area per leaf was generated from a regression equation that utilized the average length $\times$ width measurements of three leaflets (1st, 3rd, and 5th from the petiole) per leaf (X) to estimate total potential area (potential leaf area = 0.7011X + 0.0038, r^2 = 0.9701). This regression line was based on 90 leaves from 30 non-experimental *C. fasciculata* plants taken from Sherwood prairie in the vicinity of the experimental plants, yet not from the same plants used for bulk tannin samples. Leaf area present per leaf was divided by the potential area per leaf to give the estimated proportion of leaf area lost to herbivores per leaf. Total leaf area eaten per plant was calculated by

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multiplying the average leaf area remaining post-herbivory per leaf by the total number of leaves per plant present at the time that leaves were collected. Total leaf area eaten per herbivore per plant was calculated by dividing total leaf area eaten by the seasonal total herbivore density per plant. Total potential leaf area per plant was calculated by multiplying the potential leaf area per leaf by the total number of leaves per plant at the date of leaf collection.

All experimental and control plants were censused for arthropods (herbivores and ants) at three periods throughout the experiment with each census period requiring eight days. Censuses were conducted on June 25 – July 1 (census 1), July 20 – July 27 (census 2), and August 14 – August 21 (census 3). Visits to plants occurred on alternating days throughout the census period for a total of four visits per plant over an eight-day period for each census. Arthropod density per census was calculated by averaging the four-day density measurements per each census period. This multi-day census period potentially controlled for temporal fluctuations in arthropod densities and diversity. All censuses were conducted between 09:00 and 12:00 hours to coincide with the daily peak of ant activity (J. Flunker, personal observation). Herbivores were classified as free-feeding caterpillars, leaf-tying caterpillars, flower bud feeding caterpillars, grasshoppers, or coleopterans. These categories were summed to estimate total herbivore density per census. The total number of ants was also recorded per plant at the same time as herbivores. Ant density was calculated as the number of ants per nectar resource (number of ants/ total number of leaves) to account for the fact that *C. fasciculata* produces one nectary per leaf (Rios et al. 2008). Herbivore densities (total and per group) were calculated as the total number of individuals divided by the total potential leaf area per

plant (= mean potential area per leaf (cm^2) $\times$ total number of leaves). Total number of leaves per plant was recorded once per census period for all experimental plants. Plant height was measured at the termination of the experiment. Plant survival was recorded throughout the experiment.

Flower, fruit and seed production/florivory, fruit damage, and seed predation

Flower number was counted on all experimental plants twice weekly throughout the duration of the experiment as a measure of reproductive investment. Fruits were collected as they matured and seeds were counted at the end of the experiment (2 October) as a measure of reproductive output. Floral damage was measured by visually inspecting all flowers on a plant (at same time as flower number counts) and determining the proportion of flower damage based on a six category scale: 1) no damage, 2) sepals damaged, 3) one petal damaged, 4) more than one petal damaged, 5) androecium damaged, or 6) gynoecium damaged (modified from Chen 2003). These categories were used to estimate the proportion of floral damage per flower, with each category (aside from category 1) accounting for 20% damage. For example, a flower with all floral parts eaten would be given a score of 100% damage while a flower with one category of the flower eaten would be given a score of 20% damage. The proportion of the flower damage per flower was then averaged for all flowers on a plant to obtain the average percent floral damage per flower per plant. The number of fruit locules damaged per fruit was recorded as a measure of fruit damage. The number of seeds damaged was also recorded but this figure was extremely low (three seeds damaged out of 1300 seeds examined); therefore seed predation was not included in the analysis.

Statistical analyses

All statistical analyses were conducted with SPSS 17 (SPSS 2009). Transformations to account for non-normal distributions or heterogeneity of variances are listed per response variable in the statistical methods below. All ANOVAs were of 2×2 design with ant presence/absence crossed with water addition/control as the independent variables. All conclusions are based on type III sums of squares. In some of the following analyses (noted below in "Results") statistical power was low (<60%; recommended power is >80%) due to low sample sizes. In such cases Bonferroni corrections of the alpha value for multiple comparisons tests were not conducted as they are discouraged when power is low (Nakagawa 2004). Also Bonferroni corrections may make differences among groups difficult to detect. As the number of comparisons increases, the alpha value becomes smaller and makes tests more conservative (Moran 2003). In cases where statistical power (calculated with alpha $= 0.05$) was low (i.e. power < 0.80) for multivariate analyses, cases of $P < 0.20$ were investigated graphically for *post hoc* differences among treatments and then analyzed accordingly. Due to high rates of plant mortality throughout the experiment a total of 87 plants were included in statistical analysis, with 37 plants in the group lacking water addition (water-), 50 in the water addition group (water+), 61 plants in ant present (ant+) group, and 26 plants in the ant exclusion (ant-) group.

    To determine differences in survivorship per treatment, a Chi-square test was conducted on the proportion of individuals alive and dead for the factors water addition/absent and ant present/absent, as well as well for each of the four combinations

of the treatments. Following a significant Chi-square test, pairwise Chi-square analyses were conducted to examine which treatments differed.

To assess differences in various response variables due to treatments, a series of 2×2 multivariate and univariate analyses were conducted with the factors water and ant as independent factors in the models. All significant models were followed with protected ANOVAs and t-tests to determine which groups differed and what variables contributed to such differences. Repeated measures analysis of variance (rmANOVA) was used to determine the effectiveness of the ant exclusion treatment and water on total ant density. The effectiveness of the water addition treatment on percent water content of leaves was analyzed via ANOVA. To determine differences in ant, chemical, and physical defenses, a multivariate analysis of variance (MANOVA) was conducted that included the dependant variables EFN nectar volume production per 24 hr, EFN nectar sugar production per 24 hours, EFN area (cm^2), condensed, hydrolysable, and total phenolics proportion of leaf weight (mg), number of peripheral trichomes on the third leaflet, and the number of rachis hairs between the 3rd and 4th leaf (rachis hairs log transformed). A MANOVA was conducted to examine potential differences in leaf quality as measured by carbon and nitrogen weight per leaf. Differences in total herbivore density were assessed via a repeated measures analysis of variance (rmANOVA). To determine differences in herbivore feeding groups a MANOVA was conducted with seasonal totals (low herbivore densities per census prevented repeated measures analysis) for free-feeding caterpillars, leaf-tying caterpillars, flower bud feeding caterpillars, grasshoppers, or coleopterans. Differences in plant damage were assessed with one MANOVA, with average proportion of leaf area eaten by folivores per plant (arcsin), average proportion of floral damage per

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plant, and proportion of fruit cells eaten per plant (arcsin) as dependent variables.

Treatment effects on total leaf area eaten per plant and total leaf area eaten per herbivore

were assessed with univariate ANOVAs. A MANOVA was conducted for reproductive

effort by treatment that included total flower production (log transformed), total fruit

production, and total seed production. Treatment differences in leaf production (leaf

number per plant) were assessed via rmANOVA. Treatment effects on final plant height

and total leaf area per leaf (post-herbivory) were assessed via MANOVA (Table 1).

RESULTS

Effectiveness of treatments

Percent water content in leaves was not affected by water availability (ANOVA, $F_{3,83}=$

0.752, $P = 0.38$), ant access (ANOVA, $F_{3,83}= 2.203$, $P = 0.15$), or the water $\times$ ant

interaction (ANOVA, $F_{3,83}= 0.470$, $P = 0.53$), affected). The ant exclusion treatment

significantly lowered the seasonal average ant density: ant+ = 0.015 ants/leaf $\pm$ 0.002 SE;

ant- = 0.003 $\pm$ 0.003 (rmANOVA $F_{1,75} = 6.43$, $P = 0.01$). The effect of the ant exclusion

treatment on ant density did not vary with time (time $\times$ ant interaction: rmANOVA $F_{1,75} =$

0.86, $P = 0.36$).

EFFECT OF WATER AVAILABILITY ON ANT, CHEMICAL, AND PHYSICAL

DEFENSES

Water availability had no detectable influence on ant density (rmANOVA $F_{1,75} = 0.02$, P

$= 0.88$). Ant density did not vary with time ($F_{1,75} = 1.194$, $P = 0.28$) and water had no

impact on the time $\times$ ant treatment interaction ($F_{1,75} = 0.860$, $P = 0.36$). The MANOVA

conducted to determine differences in EFN, chemical, and physical defenses showed no

significant effects on defense for water (Wilks' lambda = 0.86, $F_{9,75}$= 1.35, P = 0.20), ant

(Wilks' lambda = 0.87, $F_{9,75}$= 1.20, P = 0.30), or the water × ant interaction (Wilks'

Lambda = 0.69, $F_{9,75}$= 0.32, P = 0.96). Due to low power for the MANOVA (observed

power = 0.60) and a P value equal to 0.20 for water, *post hoc* graphical investigation and

post hoc ANOVAs for water were calculated revealing that control plants had a 10%

higher concentration of hydrolyzable tannins in leaves compared to plants with water

addition (ANOVA, $F_{1,83}$ = 3.92, P = 0.05) (Figure 2). No other treatment differences

existed in anti-herbivore defenses. Additionally, across all treatments a negative

correlation existed between percent leaf water content and the concentration of

hydrolyzable tannins (Pearson correlation = -0.255, P = 0.01), suggesting low water

availability was in part responsible for increased tannin concentrations in the leaves of

plants lacking water addition. No treatment differences existed in the expression of other

measured anti-herbivore defenses. Overall means for defenses are listed in Table 2.

There were no significant effects of the water treatment (Wilks' Lambda = 0.95,

$F_{2,21}$ = 0.36, P = 0.79), ant treatment (Wilks' Lambda = 0.97, $F_{2,21}$ = 0.21, P = 0.88), or

the water × ant treatment interaction (Wilks' Lambda = 0.70, $F_{2,21}$ = 4.34, P = 0.30) on

mg carbon and nitrogen per leaf. Overall means for carbon and nitrogen are listed in

Table 2.

Mass Spectrometry

Based on calculations for hypothetical phenolic structures, there were multiple condensed

tannin oligomers and multiple oligomers of an unidentified phenolic similar in structure

to a hydrolyzable tannin found in *C. fasciculata* leaf tissue (Fig. 3a). This unidentified

phenolic was an oligomer of multiple flavanoid units (~272 Daltons, $C_{15}H_{12}O_5$). Several

oligomers of varying molecular mass were identified (Figure 3a,b). I was unable to

unequivocally verify the presence of other types of phenolics. No differences existed in

detectable phenolic types as a function of water availability or ant access.

INFLUENCE OF WATER ON PLANT-INSECT INTERACTIONS

Plant survivorship

Plants with ants present were more likely to survive than plants with ants excluded (Chi-

square, 1 df = 112.29, $P < 0.001$), while water availability had no influence on

survivorship (Chi-square, 1 df = 0.49, $P = 0.48$). *Post-hoc* pairwise comparisons of 2×2

factorial treatment combinations revealed that treatment 1 (-water/-ant) and treatment 3

(+water/-ant) did not differ in survivorship and that treatment 2 (-water/+ant) and

treatment 4 (+water/+ant) were not different in survivorship, while all other pair-wise

comparisons were significant ($P \leq 0.05$). Survivorship was 29% for treatment 1 (-water/-

ant), 81% for treatment 2 (-water/+ant), 32% for treatment 3 (+water/-ant), and 85% for

treatment 4 (+water/+ant). Overall these results suggest that water availability had little

influence on survivorship and that ant presence increased survivorship in the study season

(Fig. 4).

Arthropod density

The rmMANOVA for differences in herbivore density (herbivores/cm^2) showed plants

with and without ant access differed marginally in total herbivore density ($F_{1,74} = 2.95$, P

$= 0.09$). Plants with and without water addition also differed marginally in total herbivore

density ($F_{1,74} = 3.19$, $P = 0.07$). Plants with ants excluded had 145% higher herbivore

density than plants with ants present (ant+ = 0.01 herbivores/cm^2 ± 0.004; ant- = 0.023 ±

0.006). Plants with water addition had 275% higher herbivore density than plants with

only natural rain (water+ = 0.022 ± 0.005 herbivores/cm^2; water- = 0.008 ± 0.006). Water availability in conjunction with ant access (water × ant interaction) had no impact on herbivore density ($F_{1,74}$ = 1.40, P = 0.24). Time had no influence on herbivore and ant densities ($F_{1,74}$ = 1.37, P = 0.25), and all interactions between the treatments and time were non-significant ($P > 0.20$). These results suggest that during the study year ants provided some protection to plants, in the form of reducing herbivore density, and that plants under ambient water conditions may have been less desirable to folivores than watered plants.

The MANOVA analysis for seasonal totals per herbivore group revealed that plants with water addition differed from control plants lacking water addition in their assemblage of herbivore groups (Wilks' Lambda = 0.85, $F_{5,74}$ = 2.42, P = 0.04), while plants with ants did not differ in assemblage of herbivore groups compared to plants from which ants were excluded (Wilks' Lambda = 0.90, $F_{5,74}$ = 1.67, P = 0.15). The water × ant interaction had no impact on herbivore assemblage (Wilks' Lambda = 0.92, $F_{5,74}$ = 1.26, P = 0.30). A *post-hoc* ANOVA revealed plants with water addition had a 510% greater density of free-feeders per leaf than plants with water addition ($F_{1,79}$ = 3.85, P = 0.05; Fig. 5). Bud feeder density was very low in both treatments (water - = 0.003 ± 0.001; water + = 0.0012×10^{-17} ± 0.001) and resisted transformations for normality. A *post-hoc* Mann-Whitney U test revealed that plants lacking water addition had a greater density of bud feeders than plants with water addition ($Z_{1,79}$ = -2.462 P = 0.014). As power was low for the ant treatment in the overall MANOVA model (power = 0.55) and the P value for the factor ant was < 0.20, the impact of ant presence on herbivore groups was investigated via *post-hoc* ANOVAs. It was found that plants with ants excluded had

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a 688% higher density of free-feeders than plants with ants present ($F_{1,79} = 4.66$, $P = 0.034$; Fig. 6). No other differences in densities of leaf-tiers, bud feeders, beetles, or grasshoppers were found due to water availability, ant access, or the water × ant interaction. These results suggest that plants with water addition and plants without ants present may have been attractive to free-feeding caterpillars and that this group contributed the most to treatment differences in herbivore density.

Herbivore damage

There was a significant difference in total leaf area ingested by herbivores per plant due to the water treatment (ANOVA: $F_{1,79} = 3.58$, $P = 0.05$). The ant treatment had no impact on total leaf area ingested ($F_{1,79} = 1.11$, $P = 0.30$), nor did the water × ant interaction ($F_{1,79} = 0.170$, $P = 0.68$). Plants without water addition had 77% more leaf area ingested by herbivores per plant than plants with water addition, 7.6 cm^2 (± 1.3 SE) versus 4.3 cm^2 (± 1.2).

There were no differences in the proportion of herbivore damage to the leaves, fruits and flowers of plants with and without water addition (Wilks' Lambda = 0.95, $F_{3,81} = 1.20$, $P = 0.30$), or for plants with and without ant access (Wilks' Lambda = 0.98, $F_{3,81} = 0.51$, $P = 0.67$). Plants without water addition had 8.8% damage per leaf, 19% damage per fruit, and 25% damage per fruit, while plants with water addition had 9.1% leaf damage, 11% fruit damage, and 27% flower damage. Plants with ants present had 10% leaf damage, 17% fruit damage, and 26% flower damage while plants with ants excluded had 10% leaf damage, 13% fruit damage, and 25% flower damage. The water by ant interaction was significant for plant damage (Wilks' Lambda = 0.88, $F_{3,81} = 4.99$, $P = 0.003$). *Post-hoc* analyses revealed that the interaction for water by ant treatment was

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significant for flower damage ($F_{1,83}$ = 11.77, P < 0.001) and non-significant for other measures of folivory. Pairwise comparisons (P = 0.05) demonstrated that plants with water addition and without ant access (+water/-ant; mean = 0.32 ± 0.02 standard error) had greater proportion flower damage than both plants without water addition and without ants present (-water/-ant; mean = 0.21 ± 0.03 SE) and plants with water addition and ant access (+water/+ant; mean = 0.22 ± 0.02). Plants lacking water addition and with ants present (–water/+ants; mean = 0.29 ± 0.02) were not different from any of the other treatments. These results suggest that water addition to plants may make flowers more attractive to florivores but that ant presence may provide some protection from florivory in this case. Chemical defense may also play a role in flower palatability for florivores.

Across all treatments average seasonal herbivore density was negatively correlated with total leaf area post-herbivory per plant (Pearson r = -0.212, P = 0.05). This significant negative correlation allowed me to combine leaf area eaten per plant with average seasonal herbivore density to estimate total leaf area eaten per herbivore. Herbivores on plants lacking water addition ate 63% more leaf area per herbivore than on plants with water addition, yet this was marginally signifigant ($F_{1,79}$ = 2.93, P = 0.09; Fig. 7). The ant treatment ($F_{1,79}$ = 2.08, P = 0.15) and the ant treatment by water interaction did not affect leaf area ingested per herbivore ($F_{1,79}$ = 1.09, P = 0.30).

Plant Size and Reproduction

The overall model MANOVA for measures of plant size (leaf area per leaf post herbivory and final plant height) was significant for water (Wilks' Lambda = 0.90, $F_{2,74}$ = 3.73, P = 0.03), and non-significant for both the ant treatment (Wilks' Lambda = 0.98, $F_{2,74}$ = 0.49, P = 0.61) and the ant treatment by water interaction (Wilks' Lambda = 0.96, $F_{2,74}$ = 1.24,

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$P = 0.29$). *Post-hoc* analyses revealed that plants lacking water addition had 28% greater post-herbivory leaf area per leaf than plants with water addition ($F_{1,75} = 5.05$, $P = 0.03$; Fig. 8). There were no significant effects of water ($F_{1,75} = 0.006$, $P = 0.938$), ant ($F_{1,75} = 0.07$, $P = 0.75$), or the water by ant interaction ($F_{1,75} = 0.165$, $P = 0.69$) on final plant height.

Separate ANOVAs revealed that plants lacking water addition had 70% greater total potential leaf area per plant (water- = 91.05 cm^2 ± 11.07, water+ = 54.25 cm^2 ± 9.57 SE; ANOVA: $F_{1,75} = 6.32$, $P = 0.01$) as well as 67% greater total post-herbivory leaf area per plant (water- = 83.40 ± 10.37, water+ = 49.94 cm^2 ± 8.97 SE; ANOVA: $F_{1,75} = 5.96$, $P = 0.02$) than plants with water addition. No detectable differences existed in total potential leaf area per plant due to ant (ANOVA: $F_{1,75} = 0.12$, $P = 0.73$) or the water by ant interaction (ANOVA: $F_{1,75} = 0.35$, $P = 0.56$). Total leaf area per plant post-herbivory also was not affected by ants (ANOVA: $F_{1,75} = 0.26$, $P = 0.61$) or the water by ant interaction ($F_{1,75} = 0.46$, $P = 0.50$).

The number of leaves per plant did not differ for water ($F_{1,75} = 2.29$, $P = 0.13$), ant ($F_{1,75} = 0.79$, $P = 0.37$), or the water by ant interaction treatments ($F_{1,75} = 0.20$, $P = 0.65$). However, there was an increase in number of leaves found on plants as the season progressed (Wilks' Lambda = 0.50, $F_{2,74} = 35.99$, $P < 0.001$), and the relationships among treatments did vary over time as evidenced by significant interactions of time by water (Wilks' Lambda = 0.89, $F_{2,74} = 4.62$, $P = 0.01$), time by ant (Wilks' Lambda = 0.77, $F_{2,74} = 10.48$, $P < 0.001$), and time by water by ant (Wilks' Lambda = 0.89, $F_{2,74} = 4.17$, $P = 0.02$) interactions. *Post hoc* univariate analyses revealed that in the third census, plants

with ants had a greater number of leaves than plants without ants. No other differences existed among time periods due to water or the interaction of water and ant.

Plants with and without water addition (Wilks' Lambda = 0.86, $F_{3,81}$ = 4.11, P = 0.01) differed in reproductive output, while ant access had no effect (Wilks' Lambda = 0.96, $F_{3,81}$ = 1.17, P = 0.36). The water by ant interaction was also not significant (Wilks' Lambda = 0.99, $F_{3,81}$ = 0.28, P = 0.83). *Post-hoc* analyses revealed that plants without water addition produced 41% more flowers than plants with water addition but this was marginally significant ($F_{1,83}$ = 2.75, P = 0.10). Plants without water addition produced 76% more fruit ($F_{1,83}$ = 10.13, $P < 0.001$) and 60% more seeds ($F_{1,83}$ = 4.44, P = 0.038) than plants with water addition (Fig. 9, 10).

Pearson correlation analysis indicated that across all treatments fruit production was positively correlated with total potential leaf area per plant (Pearson r = 0.73, P = 0.001) and with total leaf area post-herbivory per plant (Pearson r = 0.73, P = 0.001). Seed production across all treatment also was positively correlated with total potential leaf area per plant (Pearson r = 0.73, P = 0.001) and total leaf area post-herbivory per plant (Pearson correlation = 0.74, P = 0.001). These results indicate that the more leaf area a plant possessed the greater its reproductive output. Across all treatments I did not detect any negative correlations between defense and fruit/seed production or between herbivore damage and fruit/seed production. This suggests defense potentially had little negative consequence for reproductive output and that herbivore damage had little consequence for plant reproduction in this study season.

I found, via multiple regression, that among those plants with ants present, both ant density and hydrolysable tannin concentration per leaf significantly predicted post-

herbivory leaf area per leaf (R^2 = 0.155, P = 0.05) and marginally predicted seed

production (R^2 = 0.083, P = 0.08). This suggests the potential for defensive synergy

between ant and non-ant defenses in limiting herbivore damage to leaf tissue in *C.*

fasciculata. Yet such synergy was not directly observed via manipulation here.

DISCUSSION

My treatment applications were effective in lowering ant density. Counter to my

prediction, water addition did not increase leaf water content, defense, or reproductive

output. In fact water addition appeared to negatively impact defense and plant

performance measures compared to plants exposed to ambient levels of water.

Effects of water availability on the phenotypic expression of EFN traits, chemical and

physical defenses

Plants lacking water supplementation produced more hydrolysable tannins than watered

plants. An increase in tannin leaf concentration due to water stress has been found in

other temperate herbaceous and woody plant species (Gershenzon 1984), yet this is the

first example in a system involving an ant-plant mutualism. Contrary to my predictions,

water availability had no measurable direct influence on the protective mutualism

between ant-guards and *C. fasciculata* via changes in extrafloral nectar traits or ant-guard

activity. Water availability also had no influence on the phenotypic expression of

condensed tannins, total phenolics, and physical defenses.

   The production of extrafloral nectar and non-ant defenses may be costly (Stamp

2003, Rutter and Rausher 2004). In a resource-limited environment, plants may be forced

to choose whether to express ant or non-ant defenses depending on the density and

identity of herbivores (Rico Gray and Oliveira 2007). In this study the abiotic

environment directly impacted the phenotypic expression of non-ant chemical defense

(i.e. below average precipitation was associated with high concentrations of hydrolysable

tannins in leaves). Here if leaf chemistry is favored in phenotypic expression due to

abiotic stress associated with low water availability then plants may lower investment in

ant defense. This would be considered a phenotypic trade-off between ant and non-ant

defense driven by resource limitations (Koptur 1985, 1989, Dyer et al. 2001, Heil et al.

2002, Rudgers et al. 2004). In contrast to Rios et al. (2008), who detected a trade-off in

the phenotypic expression of EFN nectar volume and rachis hairs per leaf across

locations, I did not detect trade-offs (i.e. negative correlations) in the phenotypic

expression of ant and non-ant defense due to environmental variation in water

availability. Furthermore, across all experimental treatments no negative correlations

were noted between defense and fruit/seed production or between herbivore damage and

fruit/seed production. This lack of correlations suggests that minimal costs may exist for

the phenotypic expression of the measured anti-herbivore defenses for *C. fasciculata.*

Measuring the cost of a defense in the presence of herbivores may be confounded by

other non-measured costs associated with the loss of leaf area to herbivores. A lack of

trade-offs may also be due to minimal resource limitation at our field site or synergy

between ant and non-ant defenses (Koricheva et. al 2004).

Does water availability influence the outcome of plant-insect interactions?

This is the first demonstrated example of the abiotic environment, in the form of water

availability, altering interactions between plant and herbivore in a temperate ant-plant

mutualism system. Plants at ambient rainfall level had fewer free-feeding herbivores per

plant and had a greater amount of leaf tissue eaten per plant than plants with water addition. Plants without water addition also had larger leaves post herbivory and greater overall leaf area per plant (potential and post-herbivory) than plants with water addition yet had more leaf area lost to herbivores. Greater leaf area per plant may have potentially allowed for plants without water addition to tolerate herbivore damage and produce more fruit and seeds than plants with water addition. The positive correlation of both potential and post-herbivory leaf area per plant with fruit and seed production among all plants suggests that one mechanism by which plants lacking water addition produced a greater number of fruits and seeds than plants with water addition may have been a greater total leaf area per plant.

My results demonstrate that the effects of the abiotic environment on leaf chemistry in plants lacking water addition may have resulted in compensatory feeding by herbivores. I found that plants lacking water addition had higher levels of hydrolysable tannins than plants with water addition and herbivores on these plants ate more leaf tissue per insect than on plants with water addition. This difference in herbivore feeding between treatments appears to be due to hydrolysable tannins and leaf quality, as all other anti-herbivore defenses were equal between treatments. My results may be the first field observed compensatory feeding due to leaf chemistry variability driven by the abiotic environment. Such compensatory feeding due to hydrolysable tannin levels in leaf tissue have been demonstrated in a laboratory setting: Barbehenn et al. (2009) found herbivores fed tannin-free *Populus* leaves coated with high concentrations of hydrolysable tannins ate a greater quantity of leaf tissue than herbivores fed control leaves lacking the hydrolysable tannin coating. This behavior by herbivores is believed to be due to reduced

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conversion of leaf material eaten into body mass which was counteracted by increased ingestion of leaf material. In the *C. fasciculata* system many herbivores do not frequently travel between plants (J. Flunker pers. obs.), thus caterpillars that hatch on those *C. fasciculata* plants that are high in hydrolysable tannins may be forced to compensate for less nutritious leaf tissue by eating more of that leaf tissue in comparison to a caterpillar eating a more nutritious, lower hydrolysable tannin concentration leaf found on plants with water addition.

Changes in the phenotypic expression of anti-herbivore defense, mediated by the abiotic environment, may also have profound effects on the distribution of herbivores on plant tissue within and among plants (Speight et al. 1999). I found that plants lacking water addition had lower densities of herbivores than plants with water addition. This difference in density may be due in part to changes in leaf chemistry driven by the abiotic environment that may have altered both the feeding preferences of herbivores with the ability to move from plant to plant (Hunter et. al 1994) and oviposition preferences (Abrahamson et al 2003). Further investigation is necessary to elucidate the role of these mechanisms in affecting herbivore density in the *C. fascicluata* system.

Synergy between ant and non-ant defenses, driven by the abiotic environment, may help to defend plants from herbivore damage more effectively than a singular defense. I found that among plants with ants present, ant density combined with chemical defense significantly predicted average leaf size (post-herbivory) and marginally predicted seed production. These results suggest that ant-guards may be a more effective defense against herbivores when aided by chemical defense and that this synergy may help to elevate plant reproductive output. One potential mechanism to explain this synergy between ant

and chemical defense is that herbivores may spend a greater amount of time feeding on leaves high in chemical defense and may therefore have an increased probability of being detected by ant guards compared to herbivores on plants with lower concentrations of secondary metabolites in leaves. Synergy between and ant chemical defense has been previously noted by Fincher et al. (2008) who found that *Piper cenocladum* utilized both ant and chemical defense to decrease herbivore damage to leaves more than plants with only one of either ant or chemical defense. Furthermore, it is believed that ants defend *Piper* against specialist herbivores while chemical defense deters feeding by generalist herbivores (Dyer et al. 2004). However, neither of these examples shows both a positive leaf area and reproductive benefit to defensive synergy. This is the first example in an ant-plant mutualism in which both a positive leaf area and reproductive benefit is associated with synergy between ant and non-ant defenses. I did not explicitly find that those plants high in both chemical defense and ant–guard density produced a greater number of fruits and seeds than those plants low in both chemical and ant defense. Perhaps in environments with greater herbivore pressures, such as tropical environments, synergy between ant and non-ant defense may be more pronounced.

My results suggest that the abiotic environment may alter a plant's tolerance to herbivore damage via impacts on leaf chemistry and leaf size/area. The primary mechanism by which plants lacking water addition produced more fruit than plants with water addition appears to be larger leaves (post herbivory) with greater leaf area per plant, as plants in the treatment lacking water addition were not taller. Plants with larger leaves and greater leaf area may be able to capture more sunlight and thus transfer more energy fruit and seed production. Larger leaves and greater leaf area may also buffer

herbivore damage in that larger leaves may sustain losing a greater amount of leaf area while still possessing the minimum leaf area necessary for fruit and seed production. In this experiment plants without water addition had larger leaves and total leaf area post herbivory yet despite greater leaf area eaten per plant than those plants with water addition, still produced more fruits and seeds. Surprisingly, plants without water addition only produced a marginally greater number of flowers than plants with water addition. However, plants without water addition had a greater number of bud feeders and this may have lowered the number of flowers able to produce fruit.

Increased water availability for *C. fasciculata* may actually be detrimental to leaf growth and reproduction, depending on environment. I found that plants with water addition had smaller leaves and overall leaf area post-herbivory compared to plants lacking water addition. This may have made plants with water addition less able to tolerate herbivore damage resulting in diminished fruit and seed production. Plants high in hydrolysable tannins are believed to be more resistant to ultraviolet light damage (Lattanzio et al 2008) therefore plants lacking water addition may have also been subject to greater UV damage than plants lacking water addition due to lower concentrations of hydrolyzable tannins in leaf tissue. It is possible that *C. fasciculata* may actually perform better in soils with lower moisture levels. Plants in tallgrass prairies may be less susceptible to water limitations, due perhaps to the architecture of neighbor plants and intermingled roots that may buffer plants from stresses associated with low levels of precipitation. Such architecture may also leave those plants with water addition over-watered and forced to divert resources away from leaf growth and reproduction towards eliminating excess water.

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In comparing total water received in the experimental season versus the 16-year average for the site I found that plants with water addition received a volume of water close to that of the 16-year average. What is unclear however is why plants supplemented with water did not grow and produce more seeds than control plants if these plants received a level of water that was close to the 16-year average. Here the assumption is that the 16-year average level of water availability is close to the ideal water availability for *C. fasciculata.* Counter to this assumption my results suggest that an average level of water availability found in a tallgrass prairie may not be ideal for *C. fasciculata* and that *C. fasciculata* plants may actually perform better in conditions of lower precipitation than in tallgrass prairie soils. One past study from the *C. fasciculata* system demonstrates that *C. fasciculata* exhibits variation in plant performance in different environments/field sites. Abdala and Marquis (2008) found that variation in *C. fasciculata* flower production among experimental sites was due to soil related abiotic factors and that herbivores and ants had little impact on reproductive output. Perhaps *C. fasciculata* may perform better in soils with lower moisture availability such as disturbed and poor nutrient soils in which it is frequently observed (Foote and Jacobs 1966). Further greenhouse experiments are needed to determine to what degree soil moisture influences plant traits in the absence of herbivore pressures.

Upon cursory inspection my results regarding the effects of soil moisture on tritrophic interactions in the *C. fasciculata* system differ markedly from those of Chen (2003): Chen found that plants with water addition produced greater EFN nectar volume, produced a greater number of leaves, had lower herbivory, and produced more fruit/seeds than plants without water addition; while conversely, my results show plants lacking

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water addition had greater leaf area eaten by herbivores and greater fruit/seed production

than plants with water addition. Natural precipitation levels at the time of Chen's

experiment in comparison those during my experiment may influence such apparent

differences in results. During Chen's experiment in 2002 plants lacking water addition

received a seasonal total of 7413.54 mL of water and plants with water addition received

approximately 9812 mL of water. Plants in my experiment lacking water addition

received 10057.23 mL and plants in the water addition treatment received 18057.23 mL

(calculated at 10 cm radius scale used by Chen for area around the plant in which water

was poured). Thus precipitation levels varied greatly per experiment such that Chen's

water addition treatment received a total amount of water close to the volume of plants

lacking water addition in my experiment (9812 mL versus 10057 mL, respectively)

potentially explaining the similar results between Chen's water addition plants and my

plant's lacking water addition. Additional levels of water manipulation both above and

below natural precipitation levels may have helped with further comparisons between the

two experiments. Soil type may also impact elements of tritrophic interactions (Abdala

and Marquis 2008) and water dynamics in the *C. fasciculata* system. Chen's study site

contained soil essentially of pure sand while Sherwood prairie is a prairie soil much

lower in sand content. Future experiments in the *C. fasciculata* system examining the role

of soil moisture on the outcome of tritrophic interactions may benefit from incorporating

multiple levels of water availability (above and below natural precipitation volumes) and

multiple study sites to account for site-to-site variation in soil composition and tritrophic

interactions.

The mechanism by which ant-guards promote plant survival in this experiment is not clear. Marginal differences in herbivore density between ant present and ant exclusion treatments (i.e. higher herbivore density on ant exclusion plants) may explain a drop in survival early on for plants lacking ant-guards. It is also possible (I have no direct evidence for this) that plants in the ant exclusion group had reduced survivorship in comparison to plants with ants present not because of ant-guards alone per say, but due to potential adverse effects of the ant exclusion method itself on plant survival in extremely hot temperatures. The black foam barrier used (1 cm diameter) to prevent the contact of tanglefoot with plant tissue may potentially cause heat build up on the stem in very hot and high light intensity conditions thereby compromising plant survival. I did not find such adverse effects in a greenhouse setting (Flunker; unpublished data).

Conclusions

Plants lacking water addition were subject to potentially stressful environmental conditions, in the form of low water availability, which increased chemical defense in leaves. This subsequently increased leaf area and, despite compensatory feeding by herbivores, lead to increased fruit and seed output and thus a positive impact on the outcome of tri-trophic interactions between ant-guards, plants, and herbivores. During the study year at my study site I found few strong treatment impacts, thus the exact mechanism by which plants lacking water addition out-performed those plants with water addition remains somewhat unclear. All evidence points to a potential benefit of larger leaves and higher chemical defense towards reproductive output in plants lacking water addition. Further investigation of the impact of water availability on plant defenses and growth/reproduction traits in a greenhouse setting combined with manipulative field

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studies across a range of herbivore and ant-guard densities, as well as soil types and water availability levels, may help to elucidate the mechanism by which low water availability and associated abiotic factors promotes reproductive output in *C. fasciculata*. Future experiments aimed at examining the influence of water availability on plants defense and tri-trophic interactions may also benefit from examining the impact of water availability at multiple levels of soil moisture both above and below average rainfall amounts (i.e. average rainfall ± 95% confidence interval). These experimental design elements may help to elucidate a more complete picture of the role that water availability plays in mediating tri-trophic interactions between ant-guards, herbivores and EFN producing plants.

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TABLES

Table 1: Multivariate statistical analyses, sample size, census periods.

	MANOVA		rmMANOVA		
Hypothesis	Variables	Sample size	Variables	Sample size	Number of census periods
1	Ant, chemical, physical defense	87	Herb, ant density	79	3
2	Leaf, flower, fruit damage	87	Leaf number	87	3
2	Flower, fruit, seed production	87			
2	Height, leaf area	87			

Table 2: Overall means across all treatments for non-significant variables.

Dependent Variable	Mean	Std. Error (+/- mean)
EFN nectar volume (µL per 24 hours)	.043	.012
Total sugars (mg per 24 hours)	.042	.021
Sucrose (mg per 24 hours)	.020	.010
Glucose (mg per 24 hours)	.010	.005
Fructose (mg per 24 hours)	.013	.007
EFN nectary area (cm^2)	2.566	.106
Rachis hairs per leaf	39.544	1.361
Peripheral trichomes per leaf	73.101	1.815
% Total phenolics per leaf	10.196	.433
% Condensed tannins per leaf	10.264	.518
% Leaf damage	9.0	0.90
Flowers per plant	8.386	.908
% Flower damage per flower	26.045	1.304
% Fruit damage per fruit	15.1	2.0
Nitrogen per leaf (mg)	.543	.046
Carbon per leaf (mg)	10.826	.888
Number of Leaves per plant	20	2.00
Height	41.69	1.42

FIGURES

Figure 1: Water volumes received per month among the water addition treatment (natural precipitation plus water addition), the ambient water treatment, and the average monthly rainfall over a 16-year period (1990-2006) at the Sherwood prairie field site. Rainfall in the supplemental rainfall group (small dashed line) received a supplemental amount of water that was above the 16-year 95% CI for natural precipitation.
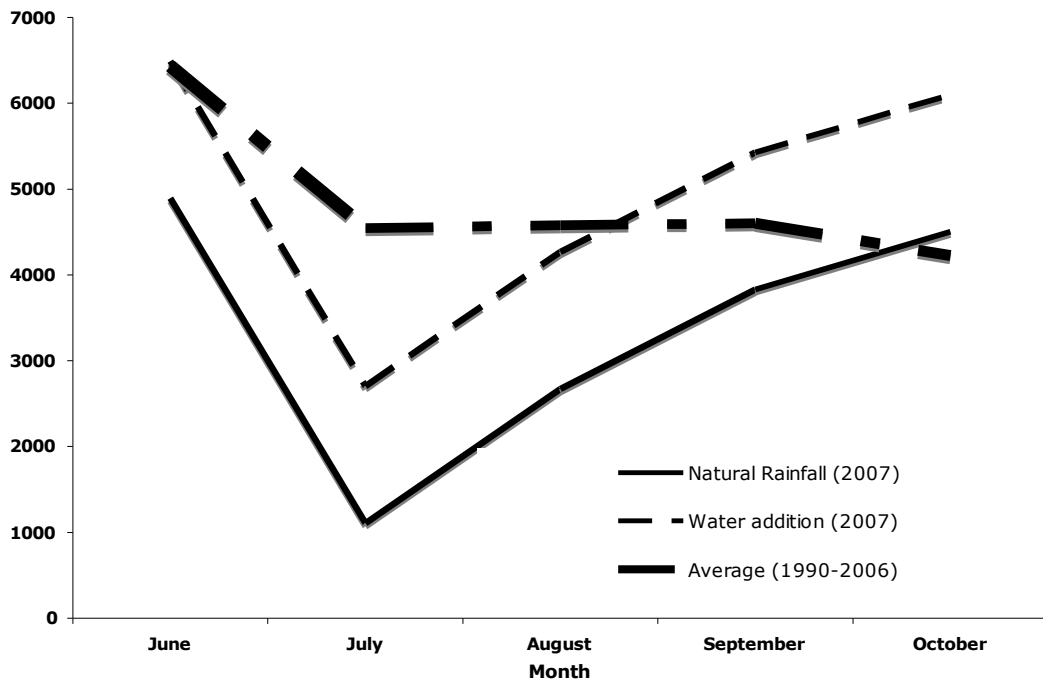
Figure 2: Hydrolyzable tannin proportion of leaf weight due to water addition (water+)

and ambient water (water-). Error bars represent standard error. Asterisk represents a

significant difference between groups at or below p=0.05.
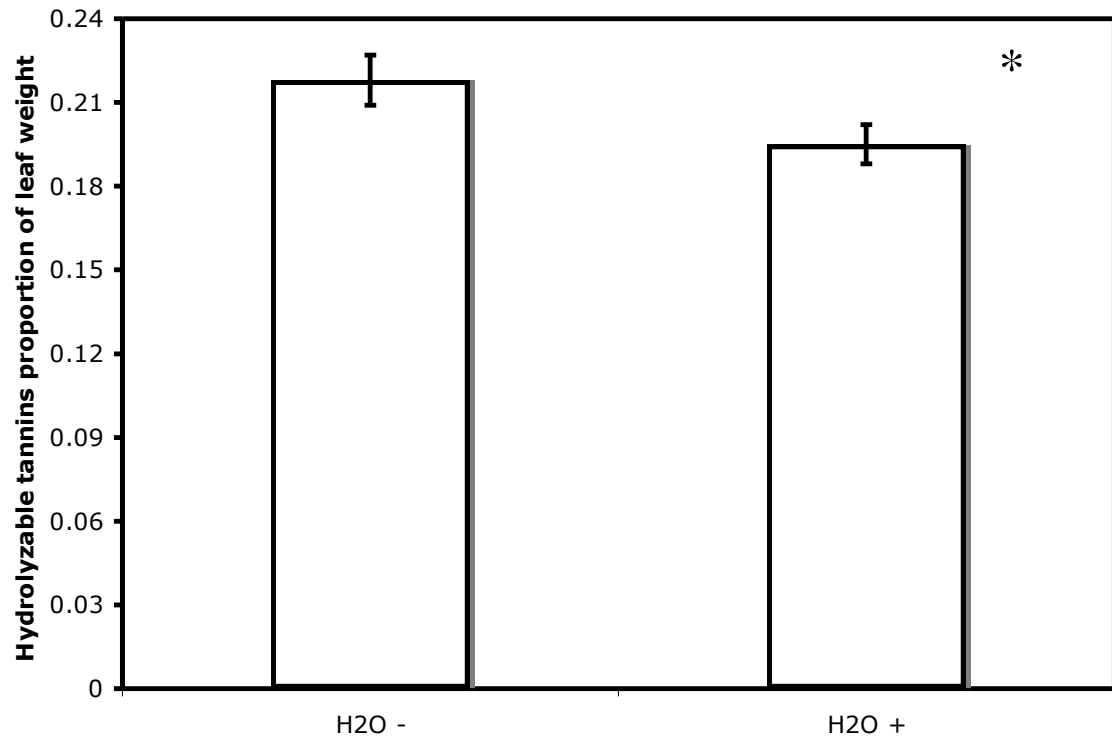
Figure 3a. Mass spectrometry absorbance peak results. Each peak within a cluster

represents sets of condensed tannin oligomers with similar molecular weights differing

by approximately one or two carbons and/or hydrogen atoms. Each peak cluster is

separated by a flavanoid unit ($C_{15}H_{12}O_5$).
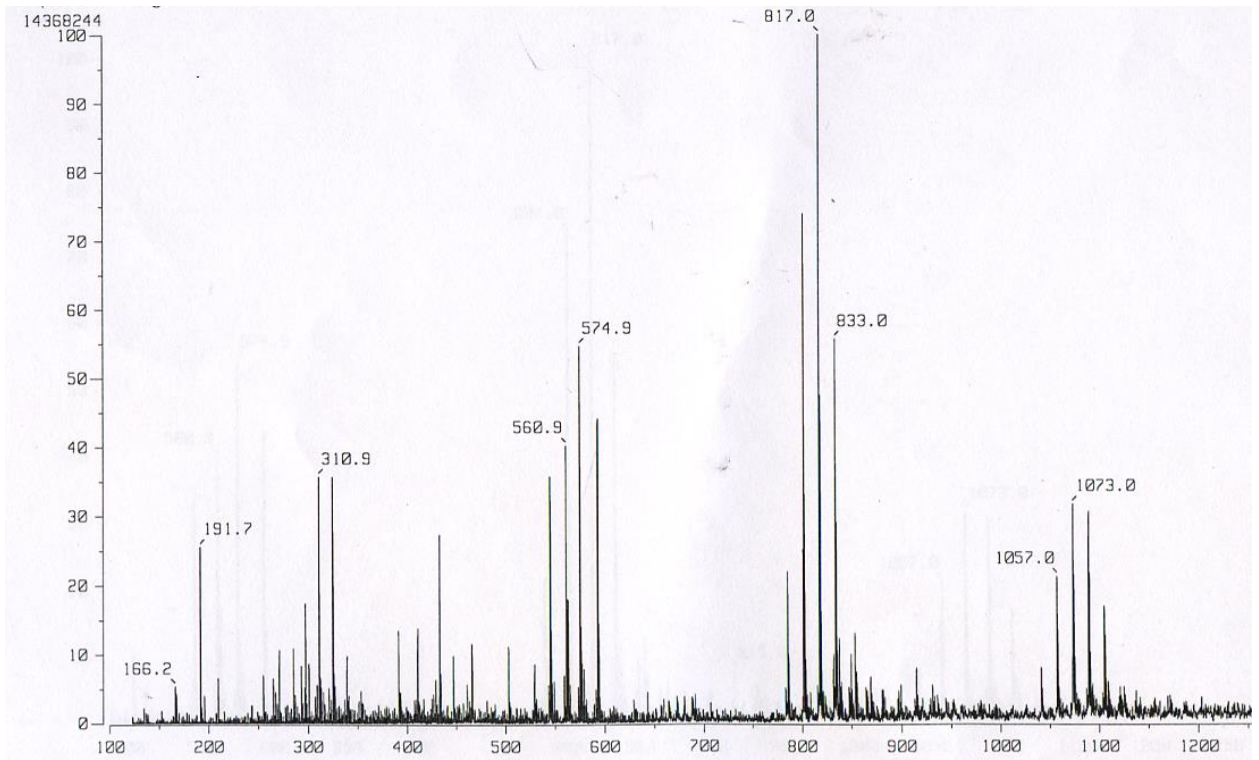
Figure 3b. Mass spectrometry hypothetical structures of two condensed tannin oligomers

found in *C. fasciculata* leaf tissue. Oligomer a) has a molecular mass of 816.22 Daltons

while oligomer b) has a molecular mass of 1090.29 Daltons. The difference between a)

and b) is the addition of approximately one flavanoid unit (~272 Daltons, $C_{15}H_{12}O_5$).

a)

Molecular Formula = $C_{45}H_{38}O_{15}$
Monoisotopic Mass = 818.221071 Da
M+ = 818.220522 Da
M- = 818.221619 Da
[M+H]+ = 819.228347 Da
[M+H]- = 819.229444 Da
[M-H]+ = 817.212697 Da
[M-H]- = 817.213794 Da

b)

Molecular Formula = $C_{60}H_{50}O_{20}$
Monoisotopic Mass = 1090.289544 Da
M+ = 1090.288995 Da
M- = 1090.290093 Da
[M+H]+ = 1091.29682 Da
[M+H]- = 1091.297918 Da
[M-H]+ = 1089.28117 Da
[M-H]- = 1089.282268 Da
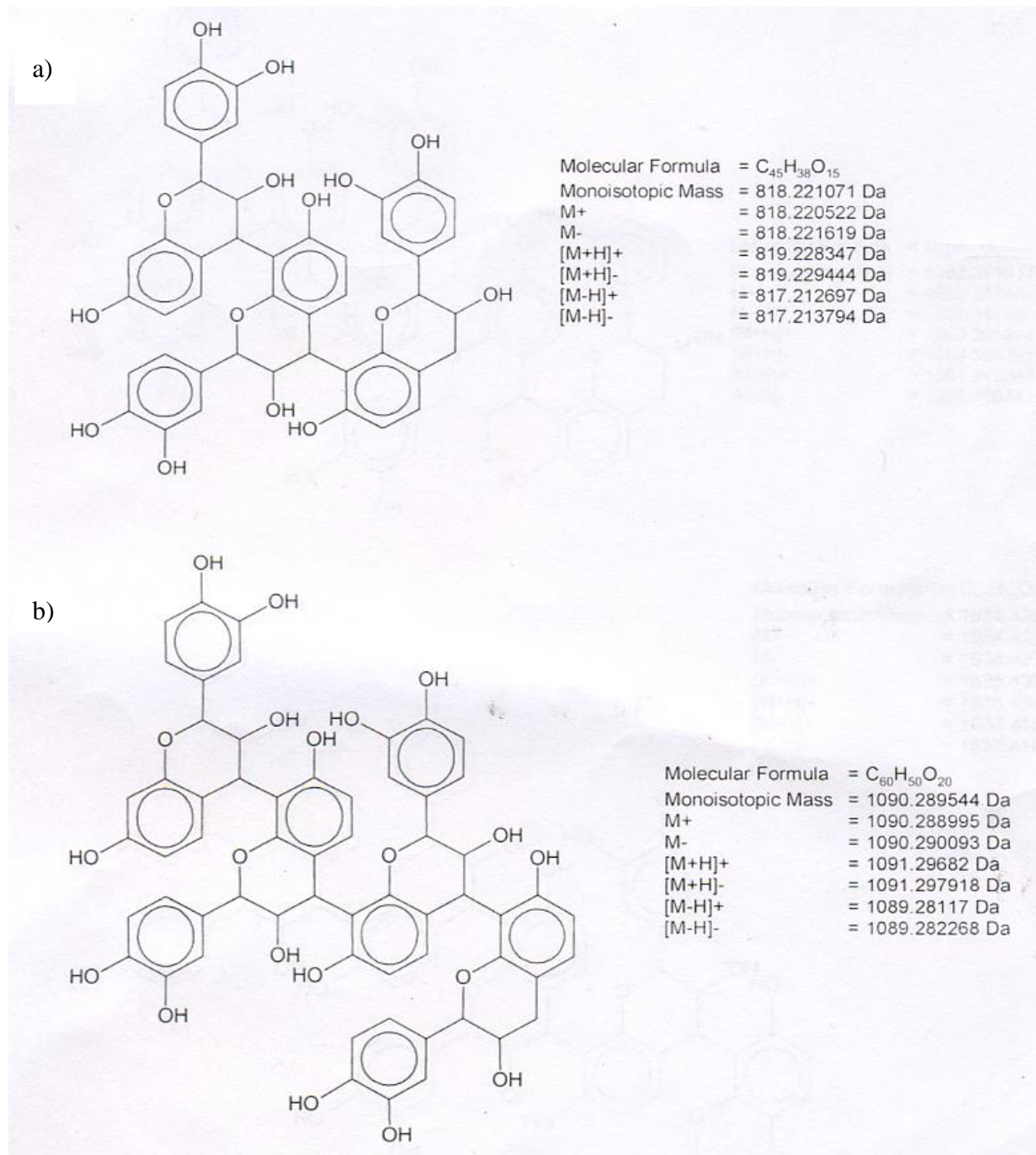
Figure 4: Survivorship curves among treatments. Letters represents groups based on post-hoc chi-square pair-wise comparisons at significance levels less than or equal to p=.05.
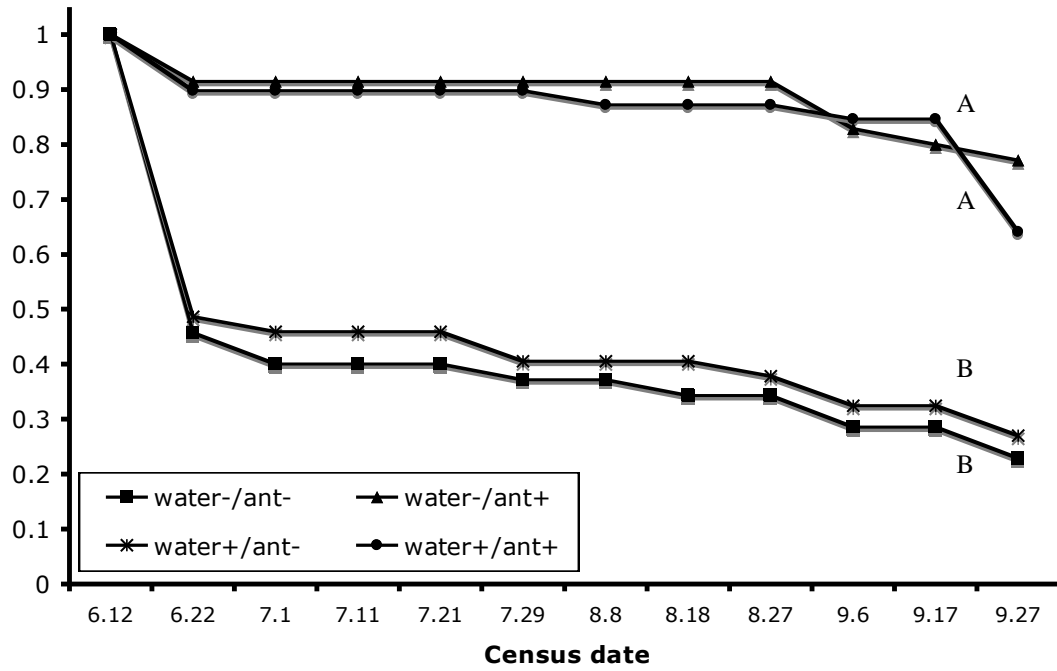
Figure 5. Free-feeder density per total plant leaf area due to water treatments. Error bars represent standard error. Asterisk represents a significant difference between groups at or below p=.05.
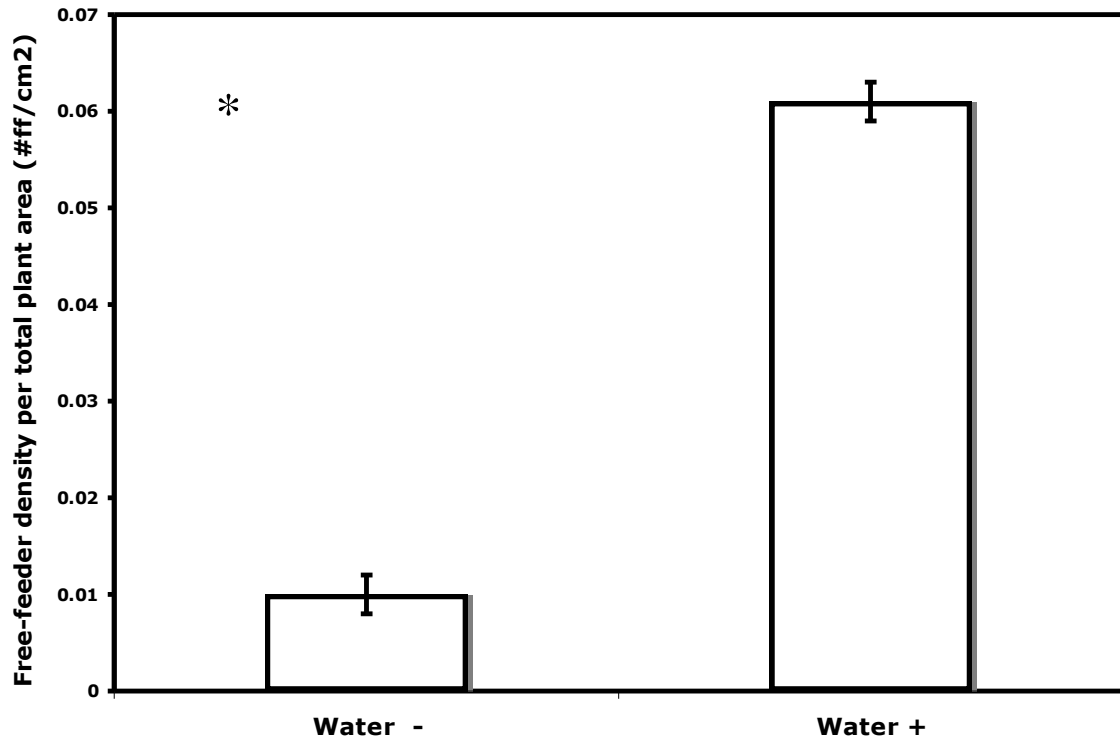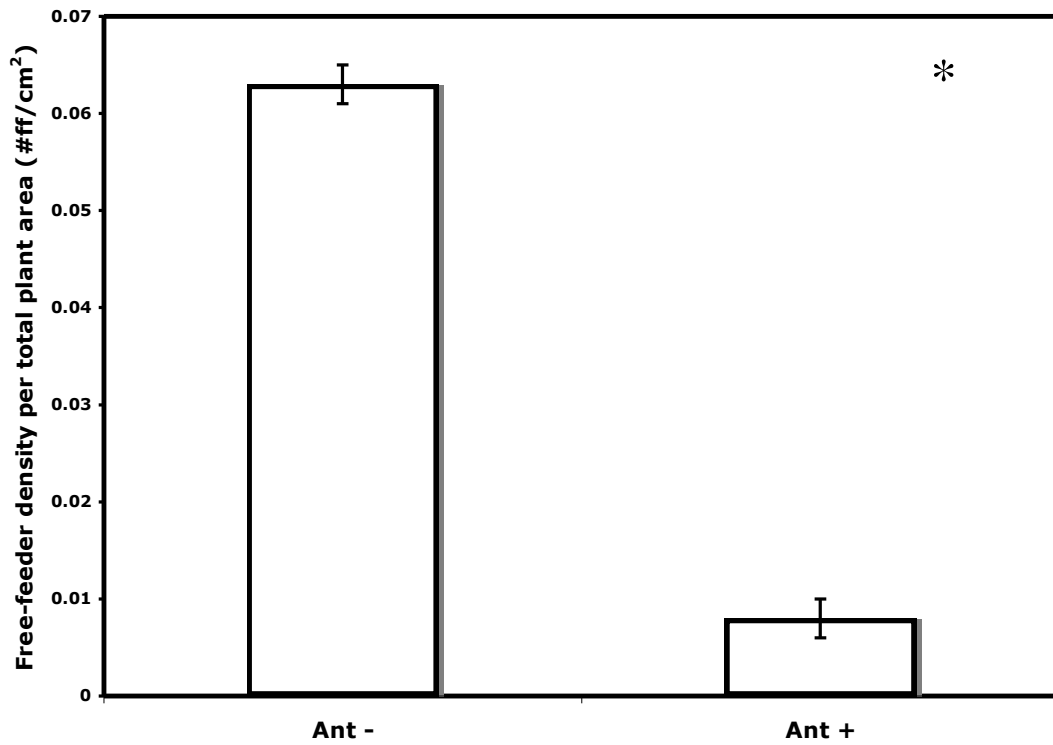
Figure 6. Free-feeder density per total plant leaf area due to ant treatments. Error bars represent standard error. Asterisk represents a significant difference between groups at or below p=.05.

Figure 7: Total leaf area ingested per herbivore due to water addition (water+) and

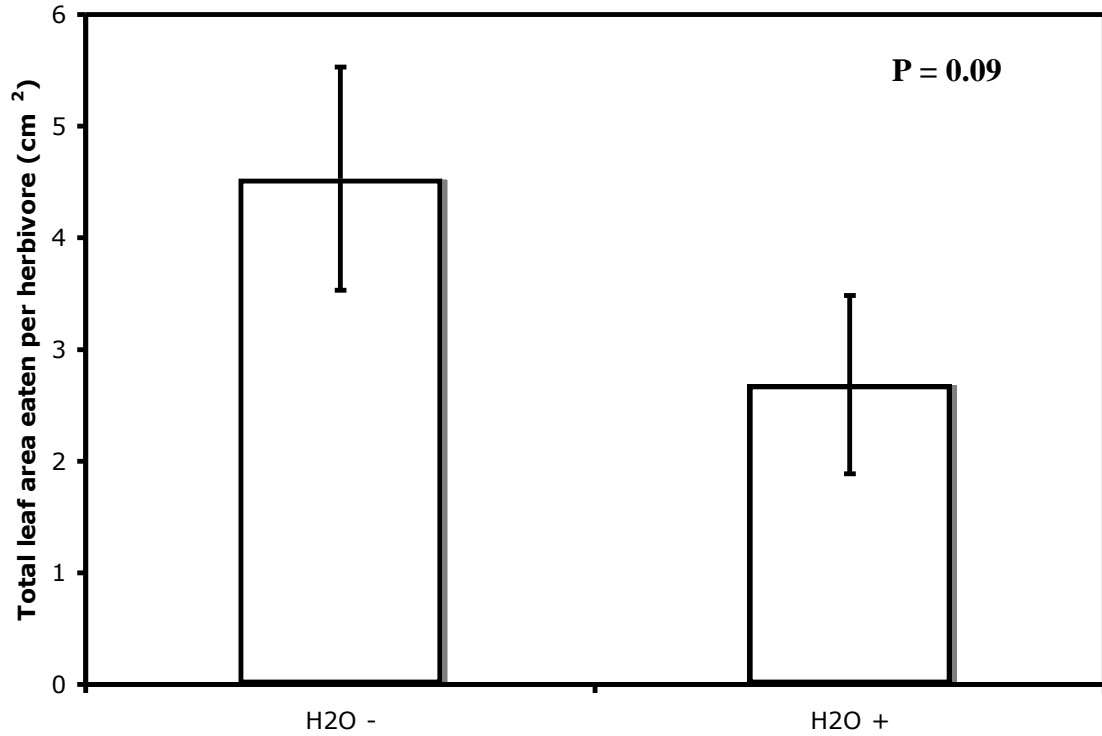ambient water (water-). Error bars represent standard error.

Figure 8: Leaf area per leaf post-herbivory due to water addition (water+) and ambient water (water-). Error bars represent standard error. Asterisk represents a significant difference between groups at or below p=.05.
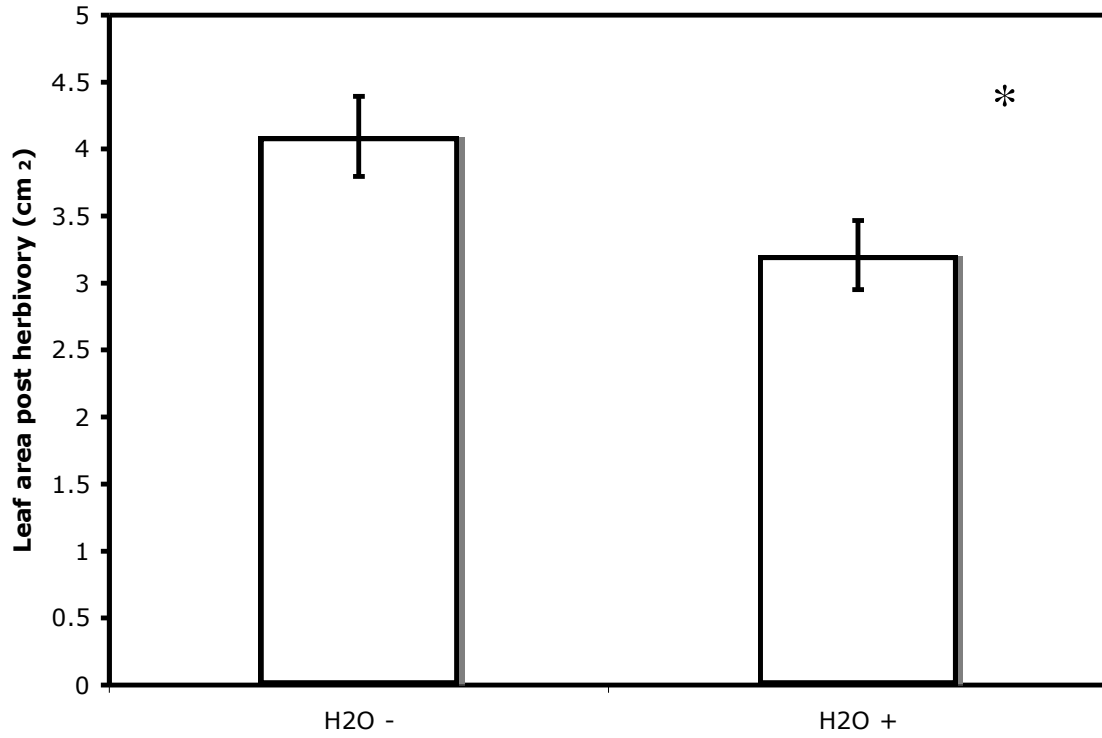
Figure 9: Fruit production due to water addition (water+) and ambient water (water-).

Error bars represent standard error. Asterisk represents a significant difference between
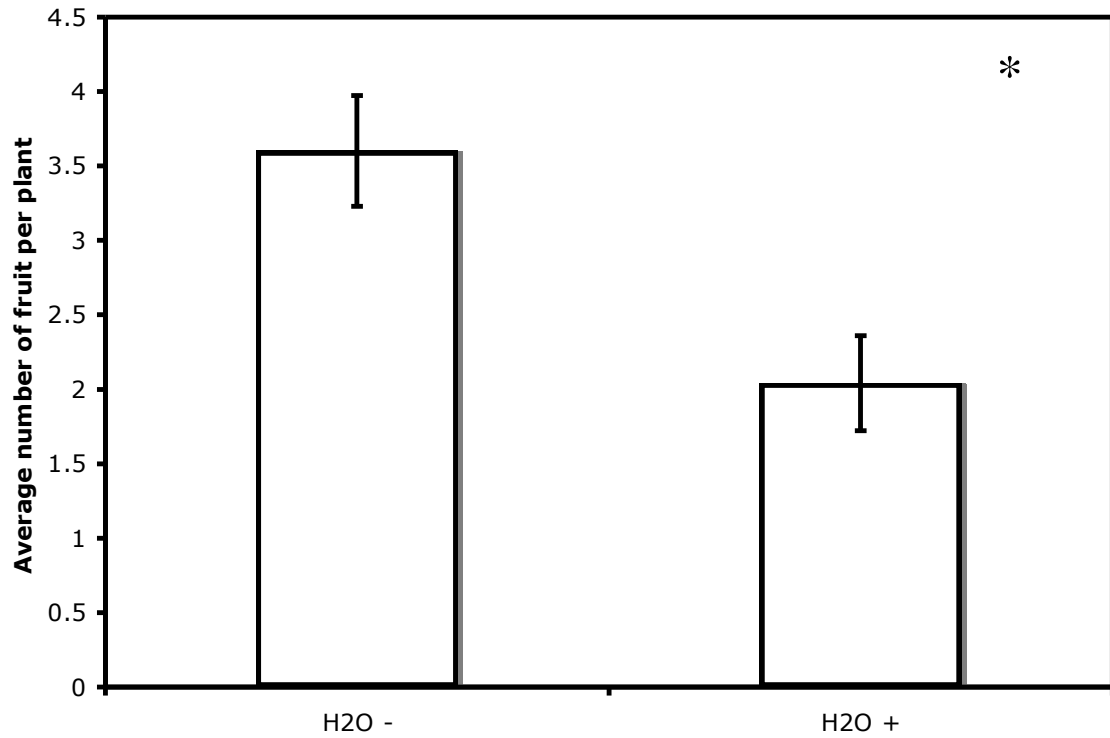
groups at or below p=.05.

Figure 10: Seed production due to water addition (water+) and ambient water (water-). Error bars represent standard error. Asterisk represents a significant difference between groups at or below p=.05.