STRESSED PLANTS AND HERBIVORES: EXPLORING THE MECHANISMS OF DROUGHT'S IMPACT ON COTTON PHYSIOLOGY AND PLANT-HERBIVORE INTERACTIONS

A Dissertation

by

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ABSTRACT

Drought is expected to become more prevalent in our future and influence plant-insect interactions in natural and agricultural systems. There is an established interest in predicting the effects of drought on plant-insect interactions, with over 500 published studies. Despite this intensive effort, researchers cannot accurately predict the effects of water deficit stress on insect performance. To address this, I tested hypotheses aimed to predict insect performance and abundance and developed a hypothesis that may better predict herbivore performance on stressed plants.

I tested the Pulsed Stress Hypothesis which predicts that insect herbivores feeding on drought stressed plants will increase in abundance on plants that are pulsed stressed rather than continuously stressed. I conducted two, 10-week field studies to test the effects of drought on arthropods using 0.6 hectares of cotton. Stress was implemented by withholding water from continuously stressed plants and using pulsed watering for pulsed stressed plants. Piercing-sucking herbivores (i.e., thrips, stinkbugs, fleahoppers) were more abundant on pulsed stressed plants than continuously stressed plants. In contrast, chewing herbivores (e.g., grasshoppers, caterpillars) were similar in abundance on stressed plants. This suggests that the variation we see in herbivore response to stressed plants is dependent upon the severity and frequency of drought in addition to herbivore feeding guild.

For my third field study, I tested the interactions of the timing of cotton aphid infestation, cotton development, and only pulsed stress. I had herbivore exclusion cages

with only aphids inside and either on seedling or fruiting cotton. I largely found that cotton may compensate for early season damage from aphids and pulsed stress, but the combination of the two greatly impact cotton development.

I conducted a meta-analysis on herbivore performance, macronutrients, and allelochemicals to determine the relationship between stress-induced changes in plants and herbivore performance. I used Metawin 2.0 to analyze the data from 42 published studies and found that macronutrients were the most important factor in determining herbivore performance on stressed plants. With this evidence, I devised the Nutrient Availability Hypothesis which predicted that the concentration of stress-induced changes in macronutrients in stressed plants will determine herbivore performance.

DEDICATION

I dedicate my dissertation to my siblings Wallace Jr. and Maya Sconiers, and to my parents Wallace and Cheryl Sconiers. Without their support and freedom to pursue whatever I wanted I would not have traveled so far. I especially dedicate my dissertation to my father Wallace, who was my first role model in achieving higher education and who gave me many of my life philosophies. Thank you so much.

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NOMENCLATURE

N	Nitrogen
PSH	Plant Stress Hypothesis
PLSH	Pulsed Stress Hypothesis
GDBH	Growth-Differentiation Balance Hypothesis
NAH	Nutrient Availability Hypothesis
PS	Piercing-sucking
ROS	Reactive Oxygen Species
POD	Peroxidase
SOD	Superoxide Dismutase
CAT	Catalase

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CHAPTER I

INTRODUCTION

1.1 Herbivory without water-deficit stress

Plants and insect herbivores are in an evolutionary arms race in which plants protect themselves from insect herbivores and herbivores overcome their defenses. The "World is Green Hypothesis" states that plants and insects have evolved in a manner that restricts one population from limiting another (Hairston et al. 1960). But given the abundance of plant material, why are insect communities limited? The literature suggests that herbivores may need much more nitrogen (N) than can be found in their host plants (based on insect C:N ratios) (Awmack and Leather 2002, Fagan et al. 2002, Denno and Fagan 2003, Matsumura et al. 2004, Wilder and Eubanks 2010). This leads to herbivores needing to consume vast amounts of plant material to acquire the amount of N and other minerals they require to grow and develop. For example, Huberty and Denno (2006) demonstrated that N enriched *Spartina* plants dramatically increased the survival, mass, fecundity, and abundance of *P. dolus* and *P. marginata* planthoppers (Hemiptera: Cicadellidae). Plants, on the other hand, are well defended against herbivores. Mechanical defenses such as surface waxes, tissue toughness, and trichomes reduce herbivore feeding efficiency, while chemical defenses such as alkaloids, cyanogenic glycosides, and tannins reduce palatability and digestibility (Gilbert 1971, Cates and Rhoades 1977, Raupp 1985, Rutledge et al. 2003, Stamp 2003). Mao et al. (2007) demonstrated that higher concentrations of the cotton allelochemical gossypol led to

growth retardation in cotton bollworms (Lepidoptera: Noctuidae). To combat poor nutritional quality and allelochemicals, insects have evolved counter adaptations in this evolutionary arms race. Herbivores have been observed to alternate host plants to reduce the intake of allelochemicals and reach nutritional targets (Behmer et al. 2002). Detoxification of allelochemicals is the most common mechanism for handling host plant defense. *Helicoverpa zea* uses glucose oxidase in its saliva to inhibit the defensive signaling compound jasmonic acid (Felton and Eichenseer 1999, Musser et al. 2002). Without water stress, both plants and their herbivores possess adaptations to counter the weapons they each possess. How is this balance upset when plants are stressed and unhealthy? How are the concentrations of nutrients and allelochemicals in plants altered when plants are water stressed? We must first discuss two guilds of herbivores and their interactions with host plants to address these questions.

1.2 Feeding guilds

Herbivores come in many different forms and feeding styles, some of which are specifically designed to bypass plant defenses. Piercing-sucking (PS) herbivores remove fluid nutrients by employing styli to puncture the plant surface and remove material, whether it is from leaves, stems, or fruiting structures. Nutrients are removed from the phloem, xylem, or even the cells themselves. These types of herbivores can bypass defenses by targeting certain areas of the plant such as with aphids (Hemiptera: Aphididae) targeting phloem and cicadas with xylem. Aphids, for example, can maneuver there styli around cells that may contain defensive compounds to target

phloem tissue and is dependent upon the turgor pressure of the plant. For phloem feeders (i.e. Aphididae), the positive pressure (outward pressure) of the phloem capillaries allows passive feeding (Press and Whittaker 1993, Douglas 2003, Guerrieri and Digilio 2008); meanwhile for xylem feeders, they must use their cibarian pumps (i.e., Cicadellidae) to remove xylem from negative pressure (inward pressure) (Press and Whittaker 1993, Novotny and Wilson 1997). In addition, PS herbivores may aggregate to form nutrient sinks in host plants and prefer younger foliage (Cates 1980, Karban and Agrawal 2002). Nutritionally, phloem sap is a poor quality food, is highly disproportionate in favor of sugars and has a low N content (Douglas 2003, Guerrieri and Digilio 2008). To cope with this, aphids, for example, are known to have bacterial symbionts (*Buchnera aphidicola*) to produce essential amino acids (Guerrieri and Digilio 2008). The PS guild includes crop pests such as aphids (Aphididae), cotton fleahoppers (Miridae), and thrips (Thysanoptera). Plant responses to PS herbivores vary and may include gall formation, discoloration, and viral infection. Defensively, plants utilize the salicylic acid (SA) pathway that initiates both local and systemic responses to herbivore feeding and pathogen infection (Malamy et al. 1990, Raskin 1992, Zarate et al. 2007). For example, SA has been implicated to initiate defenses such as chitinases, peroxidaes, and glucanases against aphids and whiteflies (Mohase and van der Westhuizen 2002, Li et al. 2006). Thrips are categorized as PS, but feed in a way that initiates an increased jasmonic acid (JA) response in some host plants and increased SA response in others (Abe et al. 2008). Their feeding style utilizes the left and only mandible to puncture the cell creating a wound (possibly inducing JA) whereby

haustellate like lacina form a food canal to suck out cell contents (possibly inducing SA). A JA response is usually reserved for our next feeding guild.

Chewing herbivores remove plant tissue with powerful mandibles and include herbivores such as caterpillars (Lepidoptera), grasshoppers (Orthoptera: Acrididae), and leaf beetles (Coleoptera: Chrysomelidae). Chewing herbivores encounter plant defenses directly and have an array of counter defenses. Bernays and Hamai (1987) observed that grasshoppers have developed larger head capsules to feed on tough grasses. Parsnip webworms, *D. pastinacella* (Lepidoptera: Oecophoridae), are known to detoxify toxins that are toxic to other herbivores (Berenbaum and Zangerl 1994). This guild can also avoid defenses all together; Dussourd and Denno (1991) demonstrated that orthopterans, lepidopterans, and coleopterans engage in leaf trenching and vein cutting to disable the circulation of latex defenses (Clarke and Zalucki 2000). Chewers exhibit a stronger preference for high N sites and often engage in diet mixing to achieve nutrient targets. Bernays and Minkenberg (1997) showed that grasshoppers and caterpillars may switch hosts between instars to achieve nutritional targets (Behmer et al. 2001, Raubenheimer and Simpson 2004). Plant response to chewing herbivore damage usually induces the JA defensive pathway, producing several toxic allelochemicals such as alkaloids, proteinase inhibitors, polyphenol oxidases, and volatile compounds, as well as resulting in reduced herbivore feeding preference by caterpillars and thrips (Farmer and Ryan 1992, Thaler 1999, Abe et al. 2008, Smith et al. 2009). Within the past decade, there has been evidence of cross-talk between JA and SA pathways in relation to herbivory and pathogen defense, usually resulting in the inhibition of one to utilize the other (Kunkel

and Brooks 2002, Thaler et al. 2002, Cipollini et al. 2004, Smith et al. 2009). Plant-insect interactions are very complex as it is, so how do these interactions change when water deficit stress is involved?

1.3 Water-deficit stress

To determine the changes in plant-insect interactions associated with water stress, we first need to understand water deficit stress. Water stress alters the chemistry, structure, and metabolism of plants. Photosynthesis is the process by which plants produce ATP and other metabolites for basic functions. It can be described simply as the following reaction: $CO_2 + 2H_2O \longrightarrow (CH_2O) + O_2 + H_2O$, with the addition of photons to catalyze the enzymes and provide electrons (e^-) for the reaction (Malkin and Niyogi 2000). The splitting of water releases two e^- resulting in the production of O_2 and carbohydrate. Water-deficit stress hinders this reaction by reducing the amount of CO_2 available (aside from water). This process begins with excessively warm or cold temperatures, salt, or a decline in water availability forcing the plant to close its stomata (minute openings in leaves) in an attempt to reduce water loss. This closure reduces the amount of CO_2 that is able to enter the cell for photosynthesis, which is believed to be the main factor in causing the detrimental effects of water deficit stress (Tezara et al. 1999). The e^- that enter the cells to be captured for photosynthesis by pigments such as chlorophyll, continue to enter the system to activate the enzymes for the reaction. However, without the proper amounts of CO_2 to continue the reaction, the e^- that are not being used remain in the system. This excess energy leads to the over excitation of

oxygen, creating several important reactive oxygen species (ROS) that lead to photosystem damage and a decline in photosynthetic rate (Hernandez et al. 1999, Lin and Kao 2000, Hernández and Almansa 2002, Jithesh et al. 2006). Excess e^- first overexcite O_2 to create superoxide ($O_2^{\cdot-}$), which damages photosystems and produces hydroxyl radicals (HO^-) through its reaction with cell components. Hydroxyl radicals are very destructive and damage DNA, proteins, and lipid membranes. Another ROS is peroxide (H_2O_2) which is converted into more hydroxyl radicals if not neutralized quickly (Jithesh et al. 2006). In terms of photosynthesis, ROS damage the protein chains that conduct photosynthesis, specifically photosystems II & I, leading to a negative feedback loop of decreasing photosynthesis (Malkin and Niyogi 2000). These ROS compounds are inherent to a photosynthesis system even under healthy conditions, so plants have various non-enzymatic and enzymatic methods of neutralizing them (Malkin and Niyogi 2000, Chaves et al. 2003, Jithesh et al. 2006, Taiz and Zeiger 2010). Highly important enzymes for combatting ROS are those that specifically neutralize the ROS mentioned above. Superoxide dismutase (SOD) reacts with superoxide to produce peroxide and is the first line of defense against ROS, reacting with superoxide at diffusion-limited rates (Salin 1988, Bowler et al. 1992, Jithesh et al. 2006). Peroxidase (POD) and catalase (CAT) work to neutralize peroxide, converting it into O_2 and H_2O (Jithesh et al. 2006). Peroxidase acts as an herbivore deterrent by catalyzing the conversion of plant diphenols to reactive quinones. These quinones bind with amino acids and proteins, reducing their assimilation and leading to malnutrition in herbivores (Ruuhola and Yang 2005). Non-enzymatic compounds that neutralize ROS include

carotenoids, glutathione, and tocopherol (Jithesh et al. 2006). Carotenoids and other pigments are especially important as non-enzymatic ROS neutralizers in that they also aid in the regulation of photosynthesis. Pigments collect e^- at excitation states that are too high for chlorophyll, as well as excess e^- (Malkin and Niyogi 2000). Energy can be dissipated through these pigments to reduce over-excited chlorophyll and oxygen to prevent ROS formation. During stress, however, ROS levels exceed the plant's capacity to neutralize ROS, resulting in the deterioration of photosynthesis. Outside of molecular level changes, many physiological changes occur as well.

Several major physiological changes occur during water stress. With less water to serve as a reagent in photosynthesis, the reaction naturally slows. The decrease in photosynthesis results in a decline growth rate, water potential, and turgor pressure (Ghannoum 2008, Parida et al. 2008). Turgor pressure is the force of fluid pressure within plant cell walls (its turgidity); with less water the plant is more flaccid and fluid transportation and metabolism is impaired. With a decline in growth, the photoassimilates (products from photosynthesis) that would be used for growth may be diverted to stress repair and defense. These photoassimilates take the form of digestible carbohydrates and free amino acids (from a dysfunction in protein synthesis and hydrolysis) (Yoshiba et al. 1997, Yancey 2001, Huberty and Denno 2004, Parida et al. 2008). When diverted to stress repair, carbohydrates and amino acids serve as osmolytes, compounds that aid in reducing water loss and increase water potential. As a result of lower water potential, osmolytes are gathered into stress sensitive areas and reproductive parts of the plant to sequester water from the soil through osmotic gradients (Mattson

and Haack 1987, Trotel-Aziz et al. 2000, Yancey 2001, Parida et al. 2008). For example, the amino acid proline is a prominent, stress-related amino acid and has been observed to increase dramatically during times of water deficit stress (Yoshiba et al. 1997, Trotel-Aziz et al. 2000, Yancey 2001, Parida et al. 2008). During stress, proline stabilizes cytoplasmic enzymes, membranes, protein synthesis and is also known to scavenge free radicals and acts as a reservoir of N (Kandpal and Rao 1985, Kishor et al. 2005, Parida et al. 2008). Stress also leads to the accumulation of ammonia; the detoxification of such increases the amount of free amino acids (Brodbeck et al. 1987).

Table 1-1. Examples of mixed support for the PSH. Studies show mixed support within the same feeding guild. "Benefits from water stress" criteria included increased survivorship, abundance, fecundity, etc. that may lead to increased herbivore fitness. Guilds: PS=piercing-sucking, C=chewing, G=gall formers, B=borers

Author	Herbivore	Guild	Host	Benefited from water stress
White 1969	Hemiptera:Psyllidae	PS	Eucalyptus tree	Yes
Archer et al. 1995	Hemiptera: Aphididae	PS	Wheat	Yes
Braun & Flukiger 1984	Hemiptera: Aphididae	PS	Hawthorn	Yes
Waring & Price 1990	Diptera: Cecidomyiidae	G	Creosote bush	Yes
Schowalter et al. 1999	Lepidoptera: Geometridae	C	Creosote bush	Yes
Schowalter et al. 1999	Thysanoptera: Thripidae	PS	Creosote bush	Yes
Mattson & Haack 1987	Coleoptera: Buprestidae	B	Quercus	Yes
Mattson & Haack 1987	Lepidoptera: Geometridae	C	Pine	Yes
Mattson & Haack 1987	Orthoptera: Acrididae	C	Grasses	Yes
Mattson & Haack 1987	Hymenoptera: Diprionidae	C	Pine	Yes
Bjorkman 2000	Hemiptera: Aphididae	G	Norway spruce	No
Larsson & Bjorkman 1993	Hemiptera: Aphididae	PS	Norway spruce	No
Hoffman & Hogg. 1990	Hemiptera: Cicadellidae	PS	Potato	No
Inbar et al. 2001	Hemiptera: Aleyrodidae	PS	Tomato	No
Hanks & Denno 1993	Hemiptera: Diaspididae	PS	Mulberry tree	No
Schowalter et al. 1999	Diptera: Cecidomyiidae	G	Creosote bush	No
Inbar et al. 2001	Lepidoptera: Noctuidae	C	Tomato	No
Larsson & Bjorkman 1993	Hymenoptera: Diprionidae	C	Norway spruce	No
Wagner & Frantz 1990	Hymenoptera: Diprionidae	C	Pine	No

Aside from repair, these spare photoassimilates also form the ROS scavenging compounds mentioned above, providing aid through both osmosis and active ROS removal. Consequently, the rise in carbohydrates and amino acids has been shown to alter herbivore abundances due to changes in host nutritional quality (White 1969, 1984, Huberty and Denno 2004, Scheirs and Bruyn 2005, Mody et al. 2009). This increase in nutrients forms the basis for the "plant stress hypothesis" and has had mixed support from numerous studies (Table 1-1) since it was originally proposed by White in 1969.

1.4 Water-deficit stress and herbivory

In 1969, T.C.R. White correlated water stress with outbreaks of psyllids (Hemiptera: Psyllidae) on eucalyptus trees in Australia using a "stress index" based upon seasonal rainfall. Trees were determined to be under stress when the amount of summer rainfall was lower than that of the preceding winter's rainfall. His study found that positive stress indices, in which trees were experiencing water deficit stress, were correlated with psyllid outbreaks across several decades throughout Australia. The correlation was so strong that populations of psyllids were practically non-existent during non-stress periods. He later postulated that the cause of this was increasing N content in the trees due to stress induced osmolytes. As discussed earlier these compounds contain N and aid in plant rehydration. White surmised that the basis of psyllid outbreaks was based on increased N and therefore greater host nutritional quality of eucalyptus, allowing the psyllids to thrive on hosts that are usually poor in nutritional quality. With this observation, White formulated what is the "plant stress hypothesis"

(PSH), which states that herbivores may outbreak on water stressed plants due to changes in plant physiology, mainly increases in foliar N (White 1969, 1984, Mattson and Haack 1987, Waring and Price 1990, Huberty and Denno 2004). Since its formulation, numerous studies have tested the PSH, finding mixed support (Table 1-1). For example, Waring and Price (1990) observed that gall midges (Diptera: Cecidomyiidae) had higher abundances on water stressed creosote bush (*Larrea tridenrutu*) compared to non-stressed bushes. Schowalter et al. (1999) found several defoliating lepidopteran species (*Semiothesia*) and thysanopteran (*Frankliniella*) species that preferred creosote bushes under reduced water treatments. Archer et al. (1995) demonstrated that aphids preferred stressed wheat versus fully irrigated wheat. Despite this support, there is also just as much opposition. Schowalter et al. (1999) found gall midges that did not prefer stressed creosote; in fact they highly preferred irrigated bushes, the same bush species these gall midges preferred under stress (Waring and Price 1990). In addition, leaf miners and chewers as well as whiteflies did not perform well on stressed tomato plants and exhibited a preference for more vigorous plants (Inbar et al. 2001). Empirical studies show mixed support for even the same feeding guild and herbivore families (Table 1-1.) With such high variation in herbivore response to stressed plants, attention needed to be directed to the studies themselves.

1.5 Huberty and Denno meta-analysis

There has been great difficulty in supporting the plant stress hypothesis, yet White's observations were sound in 1969. Support from empirical studies and

observations since the formulation of the PSH have been conflicted, with empirical studies unable to support PSH. This begged the question as to what were the differences between empirical studies and observations made in nature. This question and the discrepancies in the literature were addressed more thoroughly by Huberty & Denno in 2004 (HD). They compiled 82 published studies relating to water deficit stress and herbivore response, and discussed relative aspects of plant physiology and insect ecology. HD compared leaf water and N content between stressed and non-stressed plants, assessed the effects of stressed plants on the major feeding guilds (including PS and chewing), and compared herbivore performance on stressed plants.

Overall, HD found that experimental studies did not support the plant stress hypothesis for both PS and chewing herbivores. Density, fecundity, survivorship, oviposition, and growth rate were either negatively affected or responded neutrally to the effects of water stress. Despite increases in foliar N content, both PS and chewing herbivores responded negatively to water stress. For example, chewing herbivores exhibited a statistically neutral response caused by a declining response from free-living chewers and gall formers and an increase in response by stem borers. PS herbivores actually exhibited a greater negative response to water stress compared to chewing herbivores and when comparing sub-guilds (mesophyll and phloem vs. stem borers, gallers, miners, and free-living).

Our knowledge of the negative effects of water stress may help us formulate an understanding of these results. For instance, the accumulation of allelochemicals and decreases in plant turgor may undermine the benefits of increased N content by deterring

herbivores and reducing feeding efficiency. Inbar et al. (2001), observed an increase in peroxidase and chitinases in water stressed plants (supported by Lorio Jr (1986)). Paré and Tumlinson (1999) also stated that water stressed plants produced more volatiles than non-stressed plants. We also know that a reduction in plant turgor pressure may result in reduced feeding efficiency. Kennedy et al. (1958) supported that low turgor pressure may lead to a decrease in aphid populations and even suggested that the return of turgor after a drought may lead to outbreaks of aphids. The reduction in leaf area due to stress may also result in significantly higher densities of trichomes, reducing herbivore feeding preference (Gershenzon 1984). Leaf toughness and increases in defensive peroxidase activity (insect malnutrition) are believed to be responsible (Inbar et al. 2001, Ruuhola and Yang 2005). Tougher leaf foliage lowers the availability of foliar N through mechanical defense and may result in decreased performance (McMillin and Wagner 1996). Stress induces a reduction in growth, allowing plants to produce more structural defenses such as lignin instead of more cells, increasing plant toughness. Despite these negative effects, osmolytes provide greater nutrition for herbivores and there are studies such as White's observations and the handful of experimental studies that do support the PSH. So then what are the differences between the observational studies that support the PSH and the experimental studies that do not? With plant physiology and literature supporting the possibility that herbivores may both benefit or be impaired by stressed plants, HD carefully examined the experimental designs of the 82 published studies to piece the puzzle together. Eventually they identified several empirical issues that may have led to high variation in herbivore response: 1) researchers did not independently

establish that the experimental plants were indeed water stressed (i.e. measurements of photosynthetic rate, turgor, etc.). Therefore, whether the plant was indeed stressed and the possible severity of that stress was not independently determined, allowing for the possibility of discounted variation within "water stress" treatments. Researchers based consequential physiological responses (i.e. fewer seeds, stunted growth) as a measurement of water stress whereas these results may have been due to growing conditions, variation within plant species, or other stresses, leaving room for confounding effects on herbivores. 2) Water stress was not isolated from other forms of stress such as water logging, pollution, excess light, etc., leading to a possible compounding of all sorts of stress effects that affected herbivore response. The third and most important note was that experimental drought situations were usually in the form of continuous stress without any form of recovery, while intermittent or pulsed stress occurs in nature. HD discussed that turgor pressure is key to understanding negative responses from PS herbivores. Turgor is required for efficient PS feeding and they rationalized that if water content dropped below a certain level, feeding efficiency is dramatically reduced. They proposed a threshold level of leaf water content above which feeding is efficient and if below leads to a decline in herbivore performance. For example, *Myzus persicae* and *Brevicoryne brassicae* aphids exhibited lower feeding efficiency when feeding on low turgor plants (Wearing 1972). White's initial documentation of the effects of water stress was in nature, where pulsed stress occurs. Pulsed stress allows for the recovery of foliar water content and turgor pressure, which has been shown to benefit both chewing and PS herbivores (Scriber 1977). HD deduced

that natural water stress scenarios have a periodic return of plant turgor and it is this in combination with increased soluble N levels that lead to herbivore outbreaks, in comparison to most experimental scenarios that do not raise turgor. Thus, the predictive power of PSH depended heavily upon the type of stress, its duration, and its timing. They therefore proposed the "Pulsed Stress Hypothesis" (PLSH) which hypothesized that herbivores will respond positively to pulsed stress plants since these plants have the turgor pressure necessary for herbivores to access stress-induced increases in nutrients.

1.6 After Huberty and Denno

Since HD's meta-analysis, there have been various studies addressing the effects of water stress on herbivore response. However, studies still report conflicting results for PSH and even the PLSH, despite HDs noted experimental concerns. An Nguyen et al. (2007) conducted a 14 day continuous stress study that showed that aphids did not perform well on continuously stressed plants. Plants were only watered at the beginning of the study and not once for 14 days, allowing "severe stress" to occur. An Nguyen's only measurement of stress was the observed stress at the end of the 14 day aphid assay. Additionally, an observational sampling study was conducted by Trotter et al. (2008), using forest systems with and without a historical record of varying degrees of drought stress. They found that larger arthropod communities of herbivores, predators, and parasitoids subsisted on low stress plants. This study conducted sampling in a 6-10 day period during phenological maturation of pinyon-juniper woodlands in Arizona. Once again issues can be brought to light, specifically that this study only provided a snapshot

of the community composition and current drought conditions. Prior water stress conditions, including the type of stress, needed to be addressed in order to accurately determine the significance of the conditions for the arthropod community. Furthermore, a continuous stress experiment was conducted on Brassicaceae plants by Khan et al. (2010), resulting in a positive response from a generalist aphid species (*M. persicae*) and a neutral response from its specialist counterpart (*B. brassicae*). Once again this study did not incorporate an independent measurement of water stress, allowing for similar methodological concerns as noted above.

Mody et al. (2009) conducted a pulsed stress study involving apple plants, *Spodoptera littoralis* caterpillars (Lepidoptera: Noctuidae), and *Aphis pomi* aphids. This study utilized several physiological measurements that measured stress, including stomatal conductance as a direct measurement. They also measured shoot and root growth and N content in leaves. Unfortunately, the pulsed treatment was implemented based on visible symptoms of stress, once again allowing for concerns as to the certainty of stress severity. The low stress plants were watered when "wilting" and high stress plants before visible necrosis. These methods raise concerns in that plants are stressed and begin to accumulate osmolytes before they are wilting and further so before necrosis (Lombardini 2006). The other issue in this study was the brevity of the "pulsed stress". Water was provided to the plants several hours before the addition of caterpillars, in which the researchers stated provided adequate time for turgor recovery, however turgor was not measured. The presence of pulsed stress is thereby questionable. This study, however, was close to addressing the effects of pulsed stress on plant-insect interactions

since the HD review in 2004. Interestingly enough, this study supported the preference of chewing herbivores on highly stressed plants and a negative response from aphids, a contradiction to the pulsed stress hypothesis. Paine and Hanlon (2010) demonstrated that psyllids respond neutrally to water stress even though they utilized a "pulsed" water stress treatment. This study exhibited several flaws that HD specifically addressed as in the study did not have a plant-specific measurement of stress and relied solely on soil moisture fluctuations to indicate pulse stress. The lack of an independent measurement of stress suggests that the researchers did not know how stressed the plants were, if the supposed stress was significantly different between trees, and if the given soil moisture was a direct correlate to water stress and plant water usage. These issues may have led to a neutral response from psyllids.

The literature past and present indicates the need to address the effects of water stress in a concise manner, employing a clear method of measuring water stress and distinctly separating the differences between the effects of pulsed and continuous stress. As I have mentioned, water stress dramatically changes the physiology of host plants with each type of stress altering physiology in a different manner. As supported through observational studies, herbivores may outbreak on water stressed plants; however, currently we are unable to conclusively predict the effects of water stress on insect herbivores. The HD analysis of herbivore response literature has revealed that pulsed water stress may have been responsible for observed herbivore outbreaks, yet empirical studies have yet to support this hypothesis fully.

1.7 Dissertation questions

For my dissertation, I will provide the evidence for PLSH's ability or inability to predict herbivore outbreaks with my main question: Can the pulsed stress hypothesis be used to predict herbivore response to water stressed host plants? This question will be critical to understanding the establishment and population dynamics of herbivores by using the literature's current water stress hypotheses. This question is important because not only will it narrow the knowledge gap in herbivore response literature, but it will also lead to understanding the occurrence of herbivore pest outbreaks in agriculture and natural systems.

CHAPTER II

CONTINUOUS AND PULSED DROUGHT: THE EFFECTS OF VARYING WATER STRESS ON COTTON PHYSIOLOGY

2.1 Introduction

Drought stress is predicted to become more prevalent with climate change and have a greater impact on plant-insect interactions (Mishra and Singh 2010, Dai 2011, Kiem and Austin 2013, Van Lanen et al. 2013). There has been an established interest in predicting the effects of water stress on plant-insect interactions, with over 500 published studies (search results from Web of Knowledge, Google Scholar 2013) and half a dozen formal hypotheses addressing the topic (White 1969, Price 1991, Huberty and Denno 2004). Despite this intensive effort researchers still have difficulty accurately predicting the effects of water stress on insect abundance. Variation in herbivore response to water-stressed plants, for instance, has been attributed to differences in herbivore feeding physiology, taxonomy, and natural history (Waring and Price 1990, Herms and Mattson 1992, Hanks and Denno 1993, Larsson and Bjorkman 1993, Huberty and Denno 2004, Mody et al. 2009, Gutbrodt et al. 2011). Aside from herbivores, however, little attention has been given to determining the variation in stress-induced changes in host plants and how this variation may contribute to differences in herbivore response to water-stressed plants.

During water deficit stress plants accumulate primary metabolites (macronutrients), digestible carbohydrates, antioxidant enzymes, and micronutrients to

alleviate stress (Hsiao 1973, Chaves et al. 2002, Jithesh et al. 2006, Ghannoum 2008, Taiz and Zeiger 2010). For instance, the amino acid proline alleviates water stress by stabilizing cell membranes, cytoplasmic enzymes, and scavenging free radicals (Jithesh et al. 2006, Parida et al. 2008, Taiz and Zeiger 2010). The accumulation of these stress-related compounds can also be beneficial to herbivores because they contain nitrogen and essential nutrients for growth and development. On the other hand, water stress can lead to tougher leaves, more trichomes, thicker surface waxes, and higher concentrations of allelochemicals that negatively affect herbivores (Raupp 1985, Herms and Mattson 1992, Stamp 2003).

The extent to which water-stressed plants accumulate stress-related compounds and negatively affect herbivores may be dependent upon the evolutionary history of the plant and stress severity (Lorio Jr 1986, Ryser and Lambers 1995, Fine et al. 2004). For example, the "Growth-Differentiation Balance Hypothesis" predicts that plants growing in resource-poor conditions (e.g., water deficit limiting carbon uptake) will allocate resources (e.g., chlorophyll, nitrogen, allelochemicals) to maintain and protect leaf tissue to minimize investment in tissue regrowth due to herbivory (Coley et al. 1985, Lorio Jr 1986, Herms and Mattson 1992). The degree to which plants protect existing leaf tissue and invest in potential regrowth, results in differences in photosynthesis and plant biomass (Nash and Graves 1993, Durhman et al. 2006, Taiz and Zeiger 2010). Additionally, the induction and magnitude of these stress-related changes in plants fluctuate with continued and increased severity of water stress (Hsiao 1973, Coley et al. 1985, Chaves et al. 2002, Chaves et al. 2003). For instance, long periods of drought, or

continuous water stress, result in a decline in photosynthesis, stomatal conductance, water potential, and increases in stress-related compounds compared to mildly stressed plants (Baskin and Baskin 1974, Tezara et al. 1999, Huberty and Denno 2004, Parida et al. 2008, Lawlor and Tezara 2009, Taiz and Zeiger 2010). In contrast, the "Pulsed Stress Hypothesis" (intermittent water stress) suggests that when plants recover from stress, plants may reduce the deleterious effects of water stress and improve plant quality for herbivores. Previous studies, however, tend to generalize the effects of water stress on host plants, often overlooking the effects of stress severity and duration on host plants. Incorporating the contrasting effects of pulsed and continuous stress into a single basis to predict herbivore performance may have resulted in the variation in studies testing herbivore response to water-stressed plants. To our knowledge, studies have not directly compared the effects of pulsed and continuous stress simultaneously under the same experimental conditions. Understanding the differences in how stress severity and duration affect stressed host plants may help us more accurately predict herbivore response to water-stressed plants.

In our study, we measured stress-related changes in cotton plants in response to pulsed and continuous water stress in an agro-ecosystem. Our goal was to determine how different types of water stress influence cotton physiology and may lead to differences in host plant quality for herbivores. We measured photosynthesis, stomatal conductance, transpiration efficiency, relative chlorophyll content, nutrients, antioxidant enzymes, stem water potential, soil moisture, and plant development. We hypothesized that pulsed stressed plants will have increased nutrients, water potential, and

photosynthesis to predictably increase host plant quality compared to continuously stressed plants. Continuously stressed plants, however, will have greater chlorophyll content and be less developed in conjunction with the growth-differentiation balance hypothesis. Differences in photosynthesis, nutrients, and plant development between pulsed and continuously stressed plants should produce differences in plant quality for insect herbivores and affect herbivores differently.

2.2 Methods

2.2.1 Study system

We conducted two, 10-week field studies in 2010 and 2011 at the Texas A&M Field Laboratory in Burleson County, Texas (coordinates: 30.548754,-96.436082). The south-central region of Texas experiences subtropical and temperate climates with mild winters lasting no longer than two months. High temperatures range from 25°C in May to 35°C in July, and precipitation ranges from 11.94cm in May to 5.08cm in July. Our field site primarily consisted of Belk series clay soil, known as a very deep, well drained, and slowly permeable soil that is common in Texas flood plains (U.S.A. 2007). Approximately 0.6 hectares of cotton were planted in both 2010 and 2011. We planted commercial cotton (*Gossypium hirsutum* L.), Delta Pine 174RF (no drought/pest resistance), on 3 May 2010. Cotton was furrow irrigated on 14 May and treatments began on 14 June when cotton reached the late seedling, early squaring (flower bud) stage. In 2011, the same cotton variety was planted on 18 April and irrigated on 21 May before treatments began on 13 June during the late seedling-early squaring stage. The

study concluded on 29 and 28 August in 2010 and 2011, respectively. The field was treated with Round-Up herbicide to eliminate weeds and fertilized with 14.69 kg of nitrogen/hectare with a time-release formula for both years.

2.2.2 Experimental design

Cotton was divided into 54, 6 m x 4.5 m plots, separated into 9 blocks and each block randomly received continuous stress, pulsed stress, or control irrigation treatments. Each treatment had 6 plots per block for a total of 18 plots per treatment in a randomized complete block design. Blocks had 9.1 m of untreated cotton on all sides and each plot within a block had 2.7 m of untreated cotton between plots. Continuously water stressed plants were not irrigated for the entire growing season and only received ambient rainfall, while the control plants received irrigation weekly. For the pulsed stressed plants, we used a pressure chamber (model 615, PMS Instrument Co., Albany, OR) to measure water status and determine the appropriate stress level to trigger irrigation. Cotton plants are water-stressed at approximately -1.2 MPa (-12 bars) and begin to accumulate stress-induced increases in foliar nitrogen and other nutrients (Hsiao 1973, Lombardini 2006). Pulsed stressed plants were watered when their stem water potential was below -1.2 MPa. For the pressure chamber measurements, 17 cm x 9 cm aluminum bags were placed over the uppermost, fully-expanded leaf for 20 minutes, and then clipped at the proximal end of the petiole using scissors. The aluminum bag, with the leaf still inside, was then folded gently and inserted into the chamber for a pressure reading. To accommodate for destructive sampling for pressure chamber measurements,

each plot was divided into 32 subplots containing 10-15 plants and one plot was randomly selected for pressure chamber measurements each week, for a total of 18 measurements each week per treatment. This subplot method was used for all measurements to avoid sampling the same plants.

2.2.3 Soil moisture, photosynthesis, stomatal conductance, and transpiration efficiency

Soil moisture data was collected using a soil corer to remove a 212 cm^3 soil sample in every 1st, 4th, and 6th plot of each block, between plants outside of furrows. The soil sample was weighed for wet weight, dried at 60°C for two days in a Thermo Precision drying oven (model 6524, Thermo Fisher Scientific, Inc., Waltham, MA), and the dry weight recorded.

Photosynthetic rate, stomatal conductance of water, and transpiration efficiency were measured using a LI-COR portable photosynthesis system model LI-64000XT (LI-COR, Inc., Lincoln, Nebraska). Transpiration efficiency (TE) was calculated from the LI-COR photosynthetic rate and transpiration data with: TE= photosynthetic rate (µmol CO_2/m^2s)/transpiration rate (mmol H_2O/ m^2s) similarly to Hubick et al. (1988) and Masle et al. (2005). LI-COR measurements were taken during optimal daylight hours from 9am to 2pm on the uppermost, fully expanded leaf with three measurements per leaf for each plot measured. To complete the measurements for all 9 blocks between 9am and 2pm, the 1st, 4th, and 6th plot of each block was measured per treatment for a total of 9 measurements per treatment per week.

2.2.4 Chlorophyll fluorescence, relative chlorophyll content, and peroxidase assay

Chlorophyll fluorescence was measured using a chlorophyll fluorometer (model OS30p, Opti-Sciences Inc., Hudson, NH) on the uppermost, fully expanded leaf. Prior to each measurement, a single 1.5 cm diameter, light-excluding clip was placed over the upper leaf surface for 20 minutes to render the surface dark-adapted. The fluorometer was then inserted into the clip and the measurement was taken. Fluorescence data was recorded as the maximum quantum efficiency: $Fv/Fm = ((F_{\text{Maximum fluorescence}} - F_{\text{O(minimum fluorescence)}})/F_{\text{maximum fluorescence}})$.

Relative chlorophyll content was measured using a chlorophyll meter (SPAD model 502, Konica Minolta Sensing, Inc., Tokyo, Japan) on the uppermost, fully expanded leaf of 5 plants per plot per block each week of the season. The SPAD-502 provides non-destructive measurements of relative chlorophyll content and has been used to monitor the nitrogen nutritional status of maize, rice, potato, and cotton (Vos and Bom 1993, Feibo et al. 1998, Chang and Robison 2003).

Peroxidase is an antioxidant enzyme in plants that neutralizes reactive oxygen species that destroy photosystems in chloroplasts during water stress and has been shown to become more concentrated in plants during water stress (Chaves et al. 2002, Jithesh et al. 2006, Taiz and Zeiger 2010). Each week, the uppermost fully-expanded leaf of 5 plants within a randomly selected subplot was removed. Leaves were quickly and gently placed into 9 cm x 5.75 cm coin envelopes, stored in ice coolers, and quickly transported to the laboratory. The envelopes were placed in -80°C freezers until assayed. Proteins for the peroxidase assay were extracted using 275 mg of plant tissue ground in

15 µl of 0.01M sodium phosphate buffer at pH 6.8. Once ground, samples were centrifuged at 12,000 rpm for 12 minutes, and the supernatant was kept and stored at -20°C until assayed. For the assay, 2 µl of extracted proteins were added to a 96-well plate in duplicate and 150 µl of 0.01M guaiacol solution at pH 6.0 was added to each well. Samples and plates were kept on ice. The plates containing extracted proteins and guaiacol solution were read by a microplate reader (model 680, Bio-Rad Laboratories Inc., Hercules, CA) at 470 nm. Peroxidase activity was quantified by the following equation: POD activity = (absorbance reading from software/1000)/(sample mass*0.015) and expressed as ΔAbs_{470}/min/gFW.

2.2.5 Amino acid and digestible carbohydrate assays

The leaves used for the peroxidase assay were also used for the amino acid and digestible carbohydrate assays. Plant chemistry assays for amino acids were conducted using a modified ninhydrin assay as according to Starcher (2001) and McArthur et al. (2010). Ten samples were randomly chosen per treatment from each week of the study to measure changes in amino acid concentration over time. For each sample, approximately 5 mg of tissue were removed and ground in 10 µl of 80% ethanol in an Eppendorf tube using a manual tissue grinder and placed on ice. Once the tissue was ground, 500 µl of 6N HCl was added to the sample tube and placed in a heating block at 100°C for 24 hours. A large block was placed on top of the tubes to ensure that the caps stayed closed. During the last 2.5 hours of the 24 hour period, the large blocks were removed and each tube was opened to allow the HCl to boil off. The remaining pellet was suspended in 1

ml of water and centrifuged at 12,000 rpm for 1 minute to facilitate sample homogeneity. The ninhydrin stock solution was prepared using 200 mg of ninhydrin, 7.5 ml of ethylene glycol, 2.5 ml of 4N sodium acetate buffer and 200 µl of stannous chloride solution. In a new Eppendorf tube, 20 µl of sample and 100 µl of the ninhydrin solution were added and returned to the 100°C heating block for 10 minutes. Samples were cooled at room temperature and the sample and ninhydrin mixtures were transferred to a 96-well plate. Plates were read at 570 nm using an Epoch microplate reader (BioTek Instruments Inc., Winooski, VT). If samples were too dark to be read at 570 nm due to high amino acid content, 620 nm was used and a simple linear regression equation was generated to convert amino acid concentrations from readings at 620 nm to predicted concentrations at 570 nm. Amino acid standards were prepared using powdered BSA (Sigma-Aldrich, St. Louis, MO) with dilutions prepared at 2 µg, 4 µg, 6 µg, 8 µg, and 10 µg/1 ml of water from 10 mg/1 ml of water. Standards were used to generate a standard curve to approximate µg of amino acids/5 mg of plant tissue sample.

Plant chemistry assays for digestible carbohydrate content were conducted using a phenol-sulfuric acid assay as according to Smith et al. (1964) and Clissold et al. (2006). Ten frozen samples were randomly selected per treatment from each week of the study to measure changes in digestible carbohydrates over time. Approximately 200 mg of leaf tissue from each sample was freeze-dried (model UX-03336-73, Labconco, Kansas City, MO) at -50°C for two days. Once dried, samples were ground to powdered flakes using a MF 10 basic wiley cutting mill (IKA Works, Inc., Wilmington, NC) using a size 20 mesh and 20 mg were removed and added to screw-cap test tubes. Each tube

received 1 ml of 0.1M sulfuric acid and was placed in a boiling water bath for 1 hour. Tubes were cooled in a container of room temperature water, emptied into 1.5 ml Eppendorf tubes, and mixed in a centrifuge at 13,000 rpm for 10 minutes. Each tube had 15 µl of supernatant removed which was added to glass test tubes with 385 µl of distilled water and 400 µl of 5% phenol solution. Tubes then received 2 ml of concentrated sulfuric acid and allowed to sit for 10 minutes. Samples were mixed for several seconds using a vortex mixer then allowed to sit for an additional 30 minutes. Carbohydrate standards were prepared using 0.2 mg/µl glucose to make six 400 µl dilutions containing 0, 15, 30, 45, 60, or 75 µg of glucose. The dilutions were treated in the same manner as the samples with the same concentrations of phenol and sulfuric acid added in the same manner. After sitting, 750µl of the sample, phenol, and sulfuric acid mixture was added to cuvettes for spectrophotometric measurements at 490 nm with the samples being measured in duplicates and the standards in triplicate. The standards were used to generate a standard curve to approximate µg of digestible carbohydrates/20 mg of dried plant tissue.

2.2.6 Cotton development

Plant height and nodes were counted in all plots during weeks 3, 6, and 9 during the 2010 season and during weeks 1, 4, 6, and 9 during the 2011 season. In addition, the quantity of squares and bolls were recorded for the first fruiting position (most economically important bolls) in 2010 and for the entire plant in 2011.

2.2.7 Analysis

All measurements conducted in the study were analyzed using univariate repeated measures ANOVA with JMP 10.0.0 (SAS Institute, Cary, NC) to make comparisons between treatments over time. Sphericity tests were conducted for all repeated measures to ensure that the variance assumptions of repeated measures were not violated and analyses were accurate. If sphericity was violated and a corrective Greenhouse-Geisser test yielded an ε of ≥ 0.75, then the corrected test was used. If the Greenhouse-Geisser test yielded an ε of < 0.75, then a MANOVA was used to generate a Wilk's lambda test statistic (Λ) to compare treatments over time. These adjustments ensured that the most appropriate and powerful test was used to analyze the data. Weekly and season average data were analyzed for relative SPAD values, amino acids, digestible carbohydrates, photosynthetic rate, stomatal conductance, transpiration efficiency, and peroxidase activity. Weekly data was analyzed for stem water potential, soil moisture, chlorophyll fluorescence, and cotton development. In addition, for those measurements with season average data, data was also reported for the weeks in which pulse stressed plants were watered, during which the pulse stressed hypothesis can make predictions of herbivore abundance. There were several weeks in the data during the season in which the season average and pulses were not reported due to unforeseen circumstances encountered in the field. LI-COR data (photosynthetic rate, stomatal conductance, transpiration efficiency) and chlorophyll fluorescence were reported for the 2010 season. Data on season average and pulse concentrations of amino acids and digestible carbohydrates were analyzed relative to the control (control= 0).

2.3 Results

2.3.1 Stem water potential and soil moisture

Water stress had strong effects on stem water potential. In 2010, stem water potential varied throughout the season (stem water potential: $F_{23, 408}$= 78.39, p<0.0001), with treatment (treatment: $F_{2, 429}$= 246.33, p<0.0001), and with treatment over time (treatment*week: $F_{14, 417}$= 24.24, p<0.0001) (Fig. 2-1A). In addition, control plants maintained stem water potential above -1.2 MPa throughout the season, while continuously stressed plants decreased stem water potential -1.2 MPa during week 5 and decreased to -2.5 as the season concluded (Fig. 2-1A). Stem water potential in pulsed stressed plants decreased below -1.2 MPa by week 5 and received irrigation at the end of weeks 5 and 6 (black circles around weeks on x-axis; Fig. 2-1A). Pulsed stressed plants were watered during week 5 and 9 to produce one pulse during week 6 (Fig. 2-1A).

In 2011, stem water potential varied throughout the season (stem water potential: $F_{10.5, 267.8}$= 28.87, p<0.0001), with treatment (treatment: $F_{2, 465}$= 258.18, p<0.0001), and with treatment over time (treatment*week: $F_{10.5, 267.8}$= 28.87, p<0.0001) (Fig. 2-1B). Control plants stem water potential below -1.2 MPa during weeks 8 and 9. Continuously stressed plants decreased stem water potential below -1.2 MPa by week 4 and to -3 MPa as the season concluded (Fig. 2-1B). Pulsed stressed plants decreased pressure below -1.2 MPa by week 4 in 2011 and received irrigation at the end of weeks 4, 7, and 9 (black circles around weeks on the x-axis; Fig. 2-1B). Furthermore, pulsed stressed plants were water-stressed during weeks 4, 7, 8, and 9 and were watered thereafter to give them pulses during weeks 5, 8, and 10 (Fig. 2-1B).

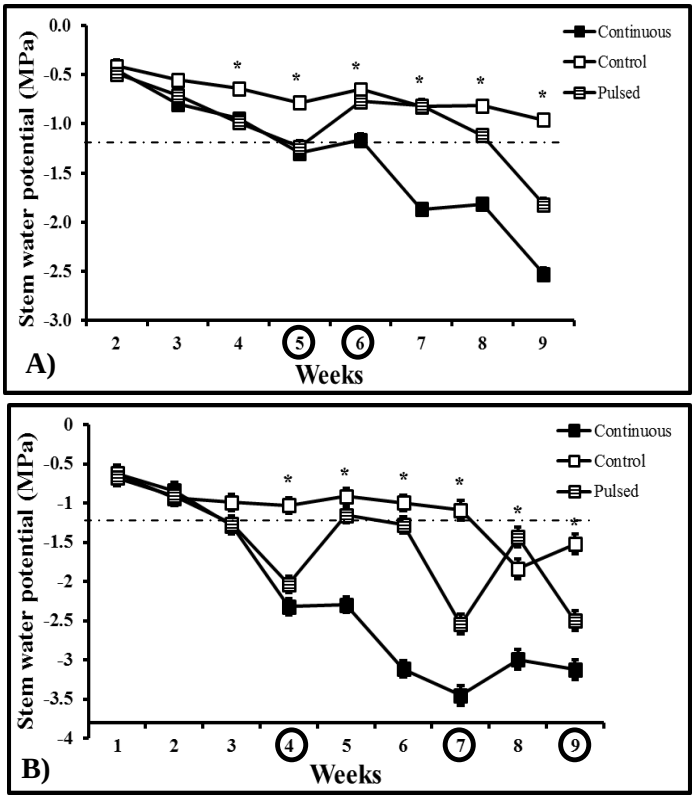

Figure 2-1. Stem water potential for stressed plants in 2010 and 2011. 2010 (A) and 2011 (B). The markers are mean stem water potential ±SE. The dotted line marks -1.2 MPa, when plants are believed to be water-stressed. Circles over certain weeks indicate when the pulsed stress treatment received irrigation to end water stress. The "pulses" were during week 6 in 2010, and during weeks 5, 8, and 10 in 2011. Asterisks indicate significant differences between at least two of the three treatments for a given week.

Soil moisture was reflective of watering treatments during both years. In 2010, soil moisture varied throughout the season (soil moisture: $\Lambda = 0.17$, $F_{8, 42} = 7.65$, $p<0.0001$), with treatment (treatment: $F_{2, 132} = 106.81$, $p<0.0001$) and with treatment over time (treatment*week: $\Lambda = 0.17$, $F_{8, 42} = 7.65$, $p<0.0001$) (Fig. 2-2A). Furthermore, during the pulse, pulsed stressed plants increased in soil moisture and remained greater than that of continuously stressed plants until week nine, while continuously stressed plants remained at approximately 4-6.5% throughout the season (Fig. 2-2A). In 2011, soil

moisture varied throughout the season (soil moisture: $\Lambda = 0.02$, $F_{16, 34} = 12.68$, $p<0.0001$), with treatment (treatment: $F_{2, 240} = 104.48$, $p<0.0001$), and with treatment over time (treatment*week: $\Lambda = 0.02$, $F_{16, 34} = 12.68$, $p<0.0001$). During the pulses in week five and eight, pulsed stressed plants had higher soil moisture than continuously stressed plants and matched the soil moisture of control treated plants. Continuously stressed plants declined in soil moisture from 20% during week 1 to 10% by week 9 (Fig. 2-2B).

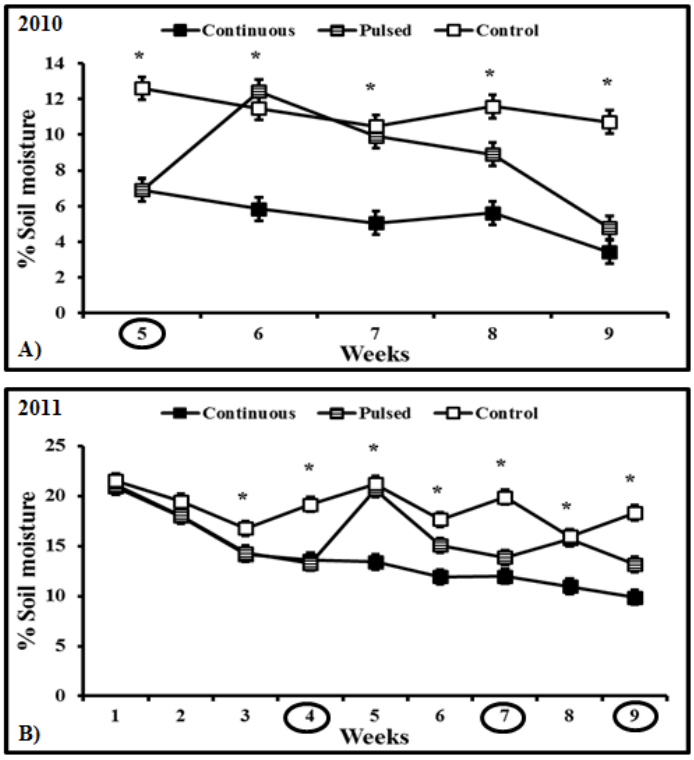

Figure 2-2. Soil moisture % during field studies in 2010 and 2011. 2010 (A) and 2011 (B). The markers are mean soil moisture % ±SE. Circles over certain weeks indicate when the pulsed stress treatment received irrigation to end water stress. The "pulses" were during week 6 in 2010, and during weeks 5, 8, and 10 in 2011. Asterisks indicate significant differences between at least two of the three treatments for a given week.

2.3.2 Photosynthesis, stomatal conductance, and transpiration efficiency

The pulse in 2010 had minimal effect on weekly photosynthetic rate in pulsed stressed plants, but there were strong contrasts between treatments for the weekly measurements and the season average. Photosynthetic rate varied during the season (photosynthetic rate: $F_{20, 168}= 5.94$, $p<0.0001$), with treatment (treatment: $F_{2, 186}= 11.07$, $p<0.0001$) and with treatment over time (treatment*week: $F_{12, 176}= 5.24$, $p<0.0001$) (Fig. 2-3A). In addition, the season average of photosynthetic rate varied among treatments

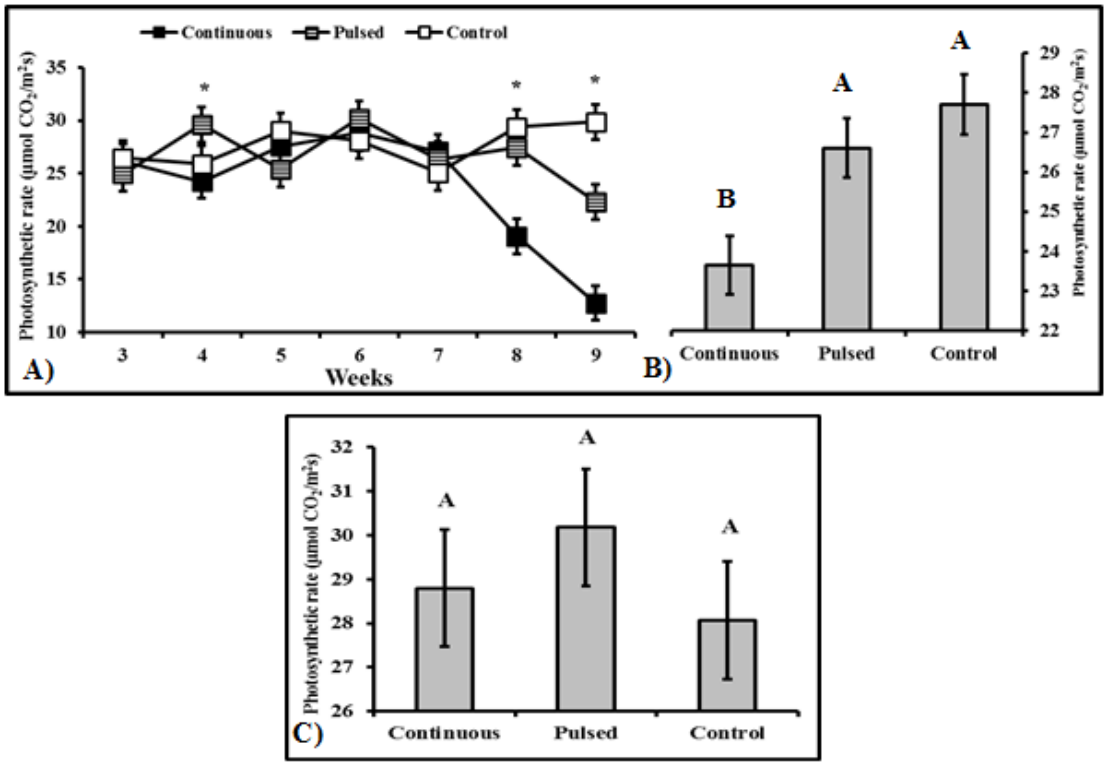

Figure 2-3. Photosynthetic rate for stressed plants in 2010. Photosynthetic rate for the season (A), the average for the season (B), and during the pulse during week 6 in 2010 (C). The markers are mean photosynthetic rate ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars are mean photosynthetic rate ±SE. Bars with different letters above them are significantly different.

(season average: $F_{2, 186}$= 7.84, p= 0.0005; Fig. 2-3B), but treatment did not affect photosynthetic rate during the pulse in week six (treatment: $F_{2, 24}$= 0.65, p= 0.5332; Fig. 2-3C). Moreover, weekly photosynthetic rates were similar between stressed plants for the majority of the season, then diverged during week 7 with continuously stressed plants decreasing to a photosynthetic rate of 12μmol CO_2/m^2s compared to 22 μmol CO_2/m^2s in pulsed stressed plants during week 9 (Fig. 2-3A). Additionally, pulsed stressed plants had a 13% greater photosynthetic rate on average compared to continuously stressed plants throughout the season and were not significantly different than control plants (Fig. 2-3B).

Stomatal conductance of water did not vary during the pulse in 2010, but varied between stress treatments. Stomatal conductance varied throughout the season (stomatal conductance: $F_{20, 167}$= 10.84, p<0.0001), was significantly affected by treatment (treatment: $F_{2, 185}$= 43.30, p<0.0001), and was affected by treatment over time (treatment*week: $F_{12, 175}$= 7.74, p<0.0001). Stomatal conductance was similar between stressed treatments until week 7 in which continuously stressed plants decreased 62% compared to the previous week, while pulse stressed plants decreased 11% (Fig. 2-4A). Season average of stomatal conductance varied between treatments (season average: $F_{2, 185}$= 027.19, p<0.0001) with pulsed stressed plants 30% higher on average compared to continuously stressed plants (Fig. 2-4B).

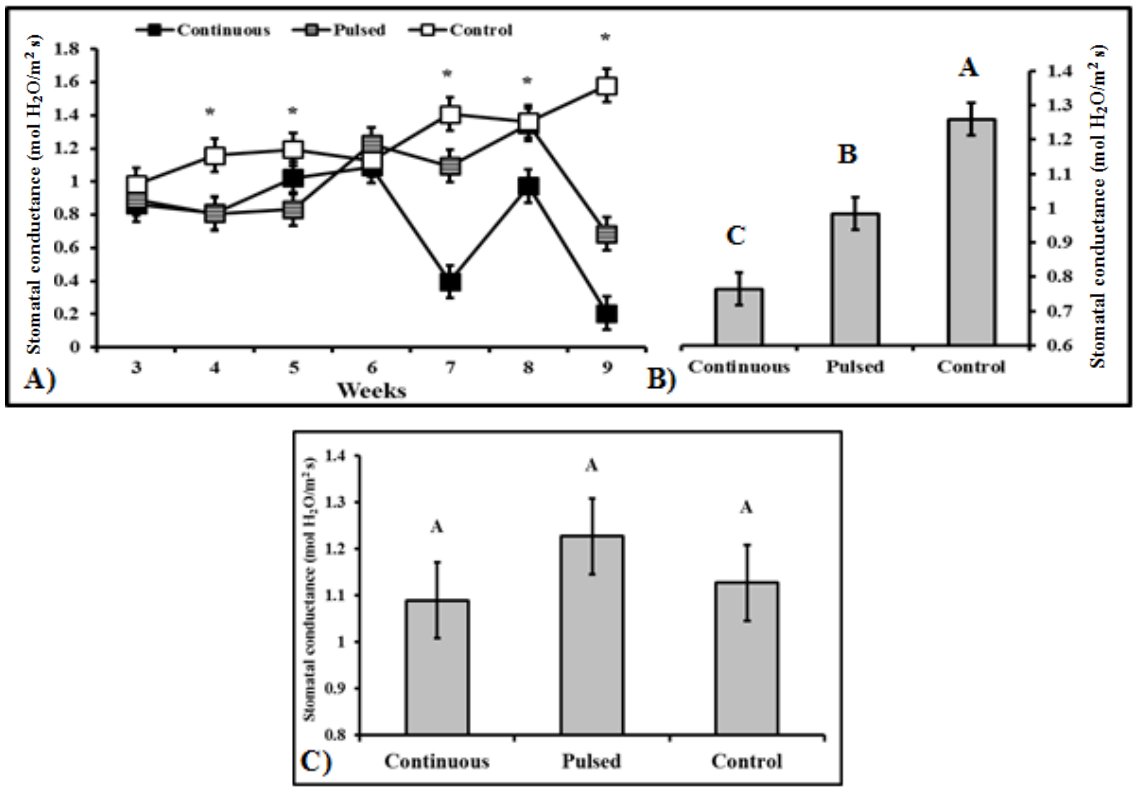

Figure 2-4. Stomatal conductance in stressed plants in 2010. Stomatal conductance of water for the season (A), the average for the season (B), and during the pulse during week 6 in 2010 (C). The markers are mean stomatal conductance ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars are mean stomatal conductance ±SE. Bars with different letters above them are significantly different.

Transpiration efficiency varied among treatments during the second half the season. Overall, transpiration efficiency varied throughout the season (transpiration efficiency: $\Lambda = 0.3$, $F_{12, 38} = 2.66$, $p = 0.0108$), with treatment (treatment: $F_{2, 187} = 12.91$, $p<0.0001$), with treatment over time (treatment*week: $\Lambda = 0.3$, $F_{12, 38} = 2.66$, $p = 0.0108$) (Fig. 2-5A). During week 7, continuously stressed plants had a 13% higher transpiration efficiency than pulsed stressed plants and remained similar in efficiency at week 9 (Fig. 2-5A). In addition, the season average for transpiration efficiency was significantly

affected by treatment (season average: $F_{2, 187}$= 7.36, p= 0.0008; Fig. 2-5B), but was not affected by treatments during the pulse (treatment: $F_{2, 24}$= 1.43, p= 0.2592; Fig. 2-5C). Furthermore, transpiration efficiency was 9% higher in continuously stressed plants compared to pulsed stressed plants on average throughout the season (Fig. 2-5B).

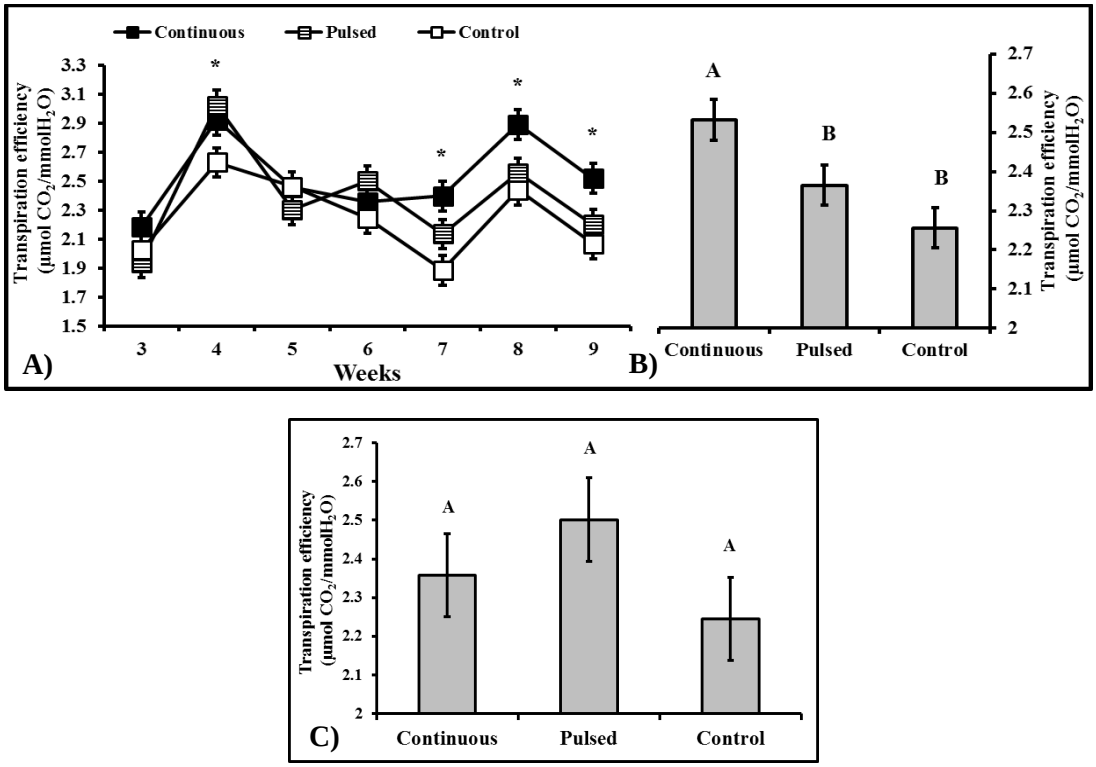

Figure 2-5. Transpiration efficiency in stressed plants in 2010. Transpiration efficiency for the season (A), the average for the season (B), and during the pulse during week 6 in 2010 (C). Transpiration efficiency= photosynthetic rate (µmol CO_2/m^2s)/transpiration rate (mmol H_2O/ m^2s). The markers are transpiration efficiency±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars are mean transpiration efficiency ±SE. Bars with different letters above them are significantly different.

2.3.3 Chlorophyll fluorescence, relative chlorophyll content, and peroxidase assay

Chlorophyll fluorescence (maximum quantum efficiency) did not vary between stress treatments. Fluorescence varied over the season (fluorescence: $F_{20, 351}$= 5.65, p<0.0001) and with treatment over time (treatment*week: $F_{14, 383}$= 3.44, p<0.0001), but treatment alone did not have an effect (treatment: $F_{2, 395}$= 0.21, p= 0.8109) (Fig. 2-6). Fluorescence was significantly different between stressed plants during week 9, in which pulsed stressed plants had a 10% greater quantum efficiency compared to continuously stressed plants.

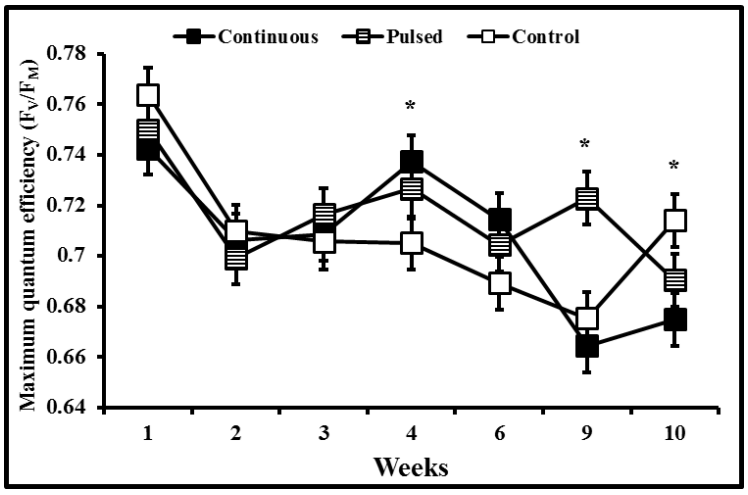

Figure 2-6. Chlorophyll fluorescence in stressed plants in 2011. The markers are mean maximum quantum efficiency ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week.

There were strong differences in relative chlorophyll content between stress treatments in 2010. SPAD values varied throughout the season (chlorophyll content: $F_{26, 435}$= 48.11, p<0.0001), with treatment (treatment: $F_{2, 459}$= 327.47, p<0.0001), and with treatment over time (treatment*week: $F_{16, 445}$= 17.00, p<0.0001) (Fig. 2-7A).

Furthermore, continuously stressed plants contained significantly more chlorophyll during most of the season with SPAD values ranging from 36 to 40 from week 6 to 9, while pulsed stressed plants ranged from 30 to 34 (Fig. 2-7A). SPAD values varied on average (season average: $F_{2, 459}$= 160.05, p<0.0001; Fig. 2-7B) and during the pulse in 2010 (chlorophyll content: $F_{2, 51}$= 51.82, p<0.0001; Fig. 2-7C). Continuously stressed plants were 12% higher in SPAD values compared to pulsed stressed plants on average throughout the season (Fig. 2-7B), and 20% higher during the pulse in 2010 (Fig. 2-7C).

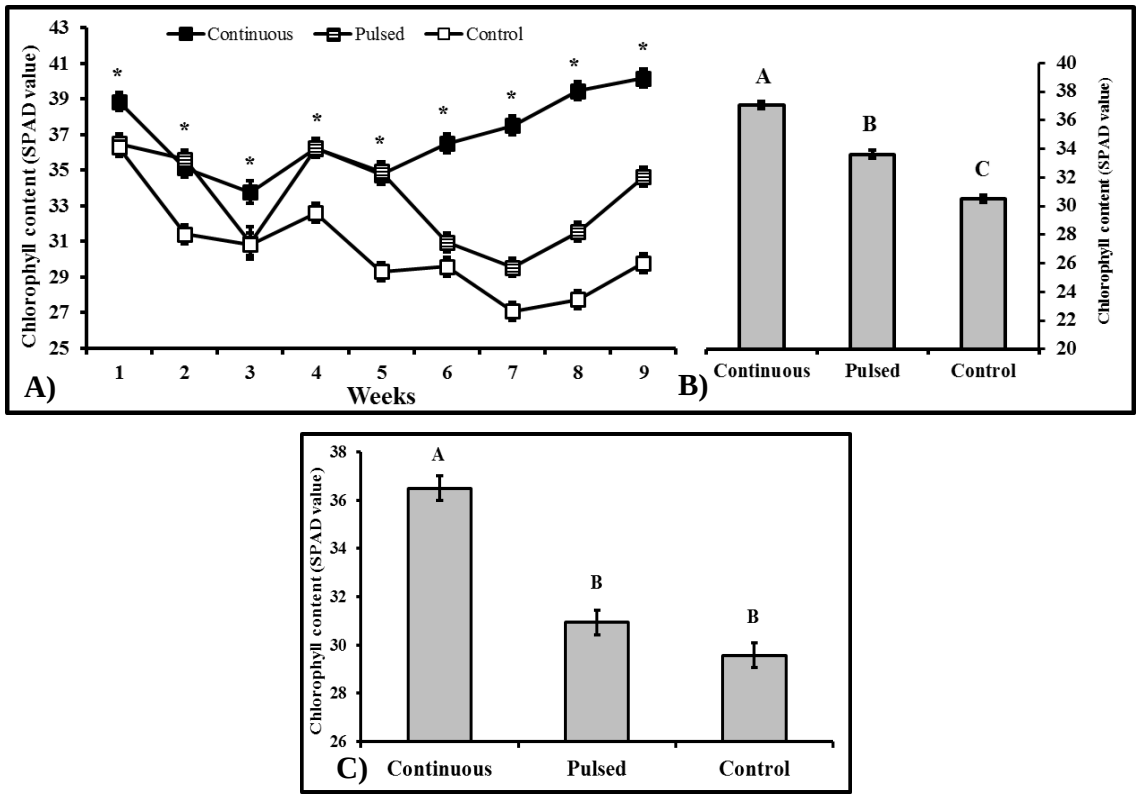

Figure 2-7. Relative chlorophyll content (SPAD values) in 2010. The effects of water stress on relative chlorophyll content using a SPAD meter for the season (A), the average for the season (B), and during the pulse during week 6 in 2010 (C). The markers are mean SPAD values ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars are mean SPAD values ±SE. Bars with different letters above them are significantly different.

In 2011, differences in relative chlorophyll content between stress treatments were not as pronounced. Overall, SPAD values varied throughout the season (chlorophyll content: $F_{20, 357}$= 20.77, p<0.0001), with treatment (treatment: $F_{2, 375}$= 130.03, p<0.0001), and with treatment over time (treatment*week: $F_{12, 365}$= 5.22, p<0.0001) (Fig. 2-8A). Stressed plants were significantly greater in chlorophyll content compared to control plants for the majority of the season (Fig. 2-8A). In addition,

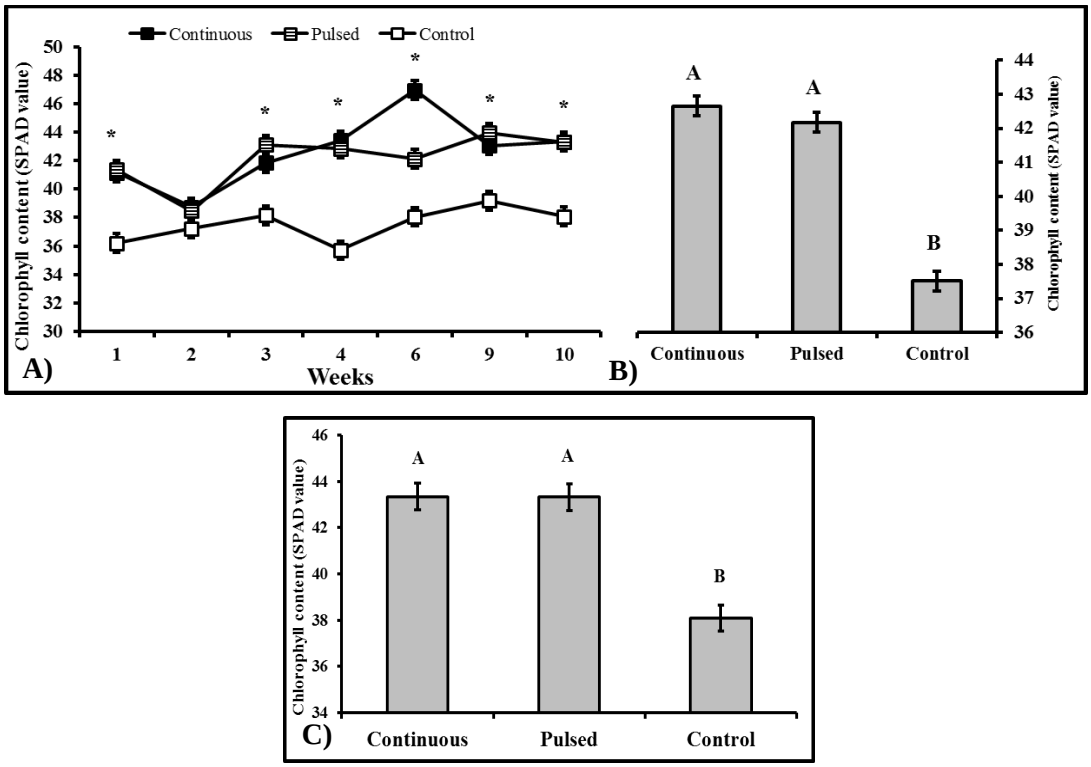

Figure 2-8. Relative chlorophyll content (SPAD values) in 2011. The season (A), the average for the season (B), and during the pulse during the third pulse in week 10 in 2011 (C). The markers are mean SPAD values ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars are mean SPAD values ±SE. Bars with different letters above them are significantly different.

stressed plants were similar in SPAD values on average for the season (season average: $F_{2, 375}$= 95.16, p<0.0001; Fig. 2-8B) and during the pulse in week 10 (treatment: $F_{2, 51}$= 28.07, p<0.0001; Fig. 2-8C), but were significantly greater in chlorophyll content compared to control plants in both cases (Figs. 2-8B and 2-8C).

Peroxidase activity did not vary throughout the season (POD activity: Λ= 0.94, $F_{6, 96}$= 0.52, p= 0.7915), did not differ with treatment (treatment: $F_{2, 274}$= 1.77, p= 0.1716), and did not vary with treatment over time (treatment*week: Λ= 0.94, $F_{6, 96}$= 0.52, p= 0.7915) (Fig. 2-9A). Average peroxidase activity was not affected by treatment

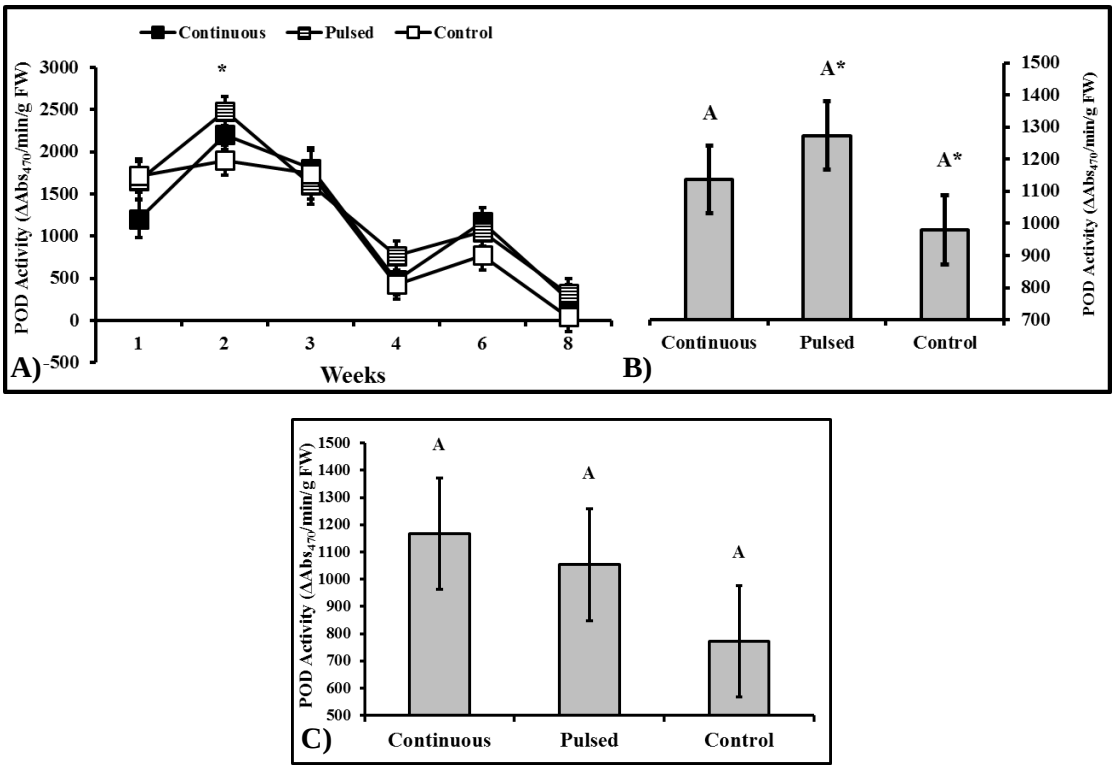

Figure 2-9. Peroxidase activity in stressed plants in 2010. Activity for the season (A), season average (B), and the week 6 pulse in 2010 (C). The markers and bars are mean peroxidase activity ±SE. Asterisks indicate significant differences. Bars with different letters above them are significantly different and letters with asterisks indicate a marginal significant difference.

(season average: $F_{2, 274}$= 1.90, p= 0.1515; Fig. 2-9B), but was marginally different between pulsed stressed and control plants (Fig. 2-9B). Additionally, peroxidase activity was similar for the treatments during the pulse in 2010 (treatments: $F_{2, 51}$= 0.99, p= 0.3777; Fig. 2-9C).

Peroxidase activity in 2011 did not vary throughout the season (POD activity: Λ= 0.6, $F_{16, 62}$= 1.11, p= 0.3622), varied with treatment (treatment: $F_{2, 441}$= 6.11, p= 0.0024), and did not vary with treatment over time (POD activity: Λ= 0.6, $F_{16, 62}$= 1.11, p= 0.3622) (Fig. 2-10). Activity was similar between treatments until a large peak during week 8, but the stress treatments were similar (Fig. 2-10A). Furthermore, peroxidase activity varied in the season average (season average: $F_{2, 441}$= 4.13, p= 0.0167; Fig. 2-10B), but pulsed and continuously stressed plants were similar (Fig. 2-10B). During the pulse in week 5, peroxidase activity significantly varied with treatment (treatment: $F_{2, 46}$= 4.30, p= 0.0194; Fig. 2-10C) while pulsed stressed plants were 500% lower in activity compared to continuously stressed plants (Fig. 2-10C). During the second pulse in week 8, peroxidase activity also varied by treatment (treatment: $F_{2, 50}$= 5.42, p= 0.0074; Fig. 2-10D), but stressed plants were similar (Fig. 2-10D).

2.3.4 Amino acid and digestible carbohydrate assays

Concentrations of amino acids were similar in stressed plants during the season, but there were a few notable contrasts. In 2010, concentrations of amino acids varied throughout the season (amino acids: $F_{20, 188}$= 17.66, p<0.0001), with treatment

(treatment: $F_{2, 206}$= 16.77, p<0.0001), and with treatment over time (treatment*week: $F_{2, 24}$= 2.33, p= 0.0084) (Fig. 2-11A). In addition, there was a significant difference

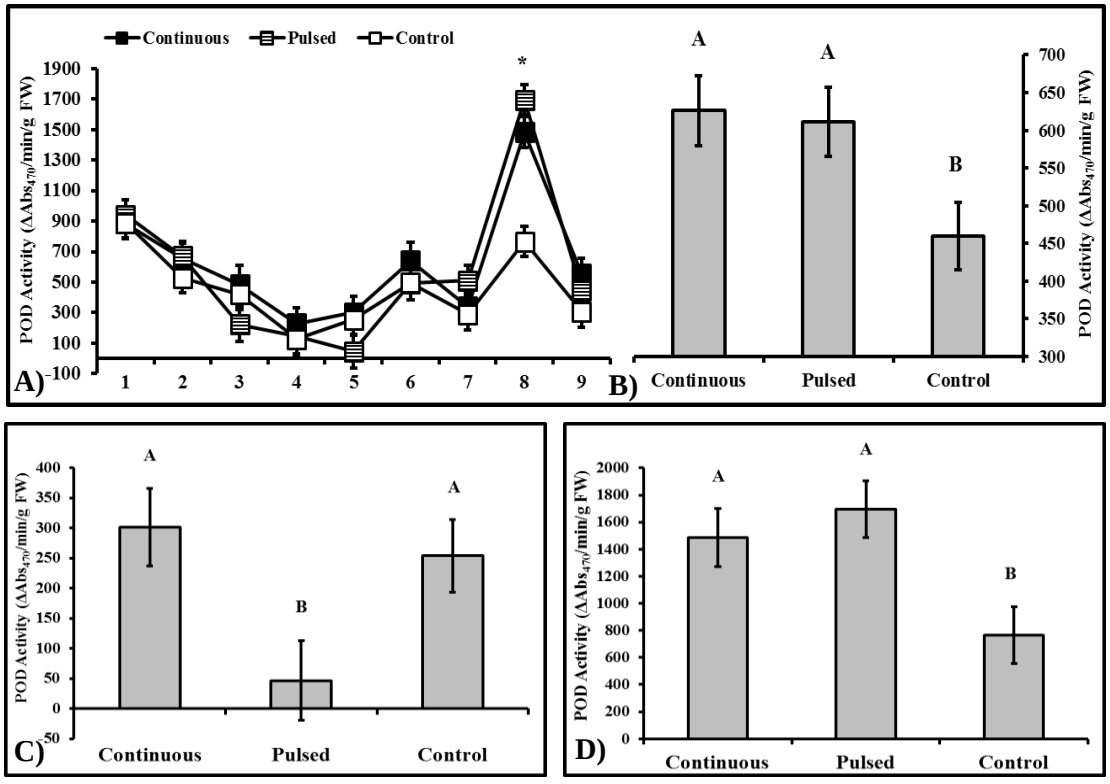

Figure 2-10. Peroxidase activity in stressed plants in 2011. Peroxidase activity for the season (A), the average for the season (B), during the pulse during week 5 in 2011(C), and the pulse during week 8 in 2011. The markers are mean peroxidase activity ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars are mean peroxidase activity ±SE. Bars with different letters above them are significantly different.

between stressed plants for the season average ($F_{1, 138}$= 10.87, p= 0.0012; Fig. 2-11B) and pulsed stressed plants had a 250% lower concentration of amino acids (Fig. 2-11B). During the pulse however, treatments had no effect on amino acids (treatment: $F_{1, 18}$= 1.24, p= 0.2809; Fig. 2-11C).

Water stress did not affect concentrations of amino acids during most of the season in 2011. Overall, amino acids varied throughout the season (amino acids: Λ= 0.28, $F_{12, 42}$= 3.08, p= 0.0034), with treatments (treatment: $F_{2, 265}$= 5.04, p= 0.0072), and with treatment over time (treatment*week: Λ= 0.28, $F_{12, 42}$= 3.08, p= 0.0034) (Fig. 2-12A). In addition, the season average did not differ between stress treatments (treatment: $F_{1, 179}$= 1.03, p= 0.3113; Fig. 2-12B). During the pulse in week 5 in 2011, stressed plants had similar concentrations amino acids ($F_{1, 18}$=1.10, p= 0.3074; Fig. 2-12C), but were marginally different during the second pulse ($F_{1, 18}$= 4.28, p= 0.0532; Fig. 2-12D).

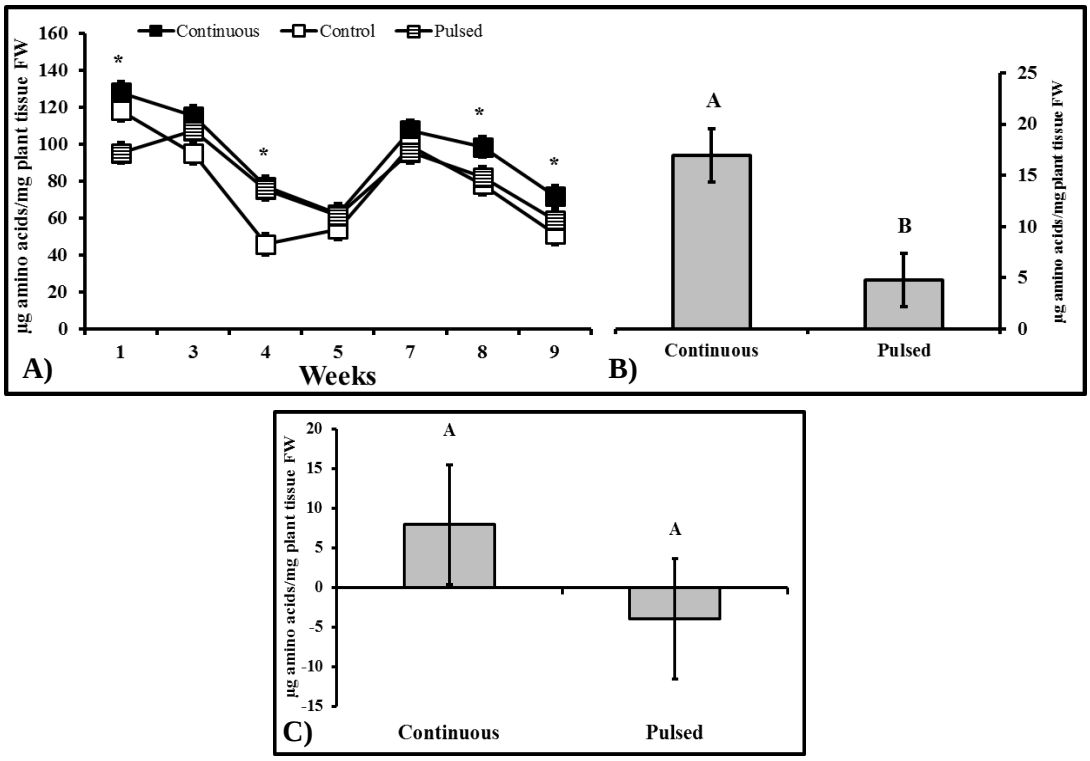

Figure 2-11. Amino acids in stressed plants in 2010. Concentrations of amino acids for the season (A), the average for the season relative to the control (B), and during the pulse during week 6 in 2010 relative to the control (C). The markers are mean amino acid concentrations ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars are mean amino acid concentrations ±SE. Bars with different letters above them are significantly different.

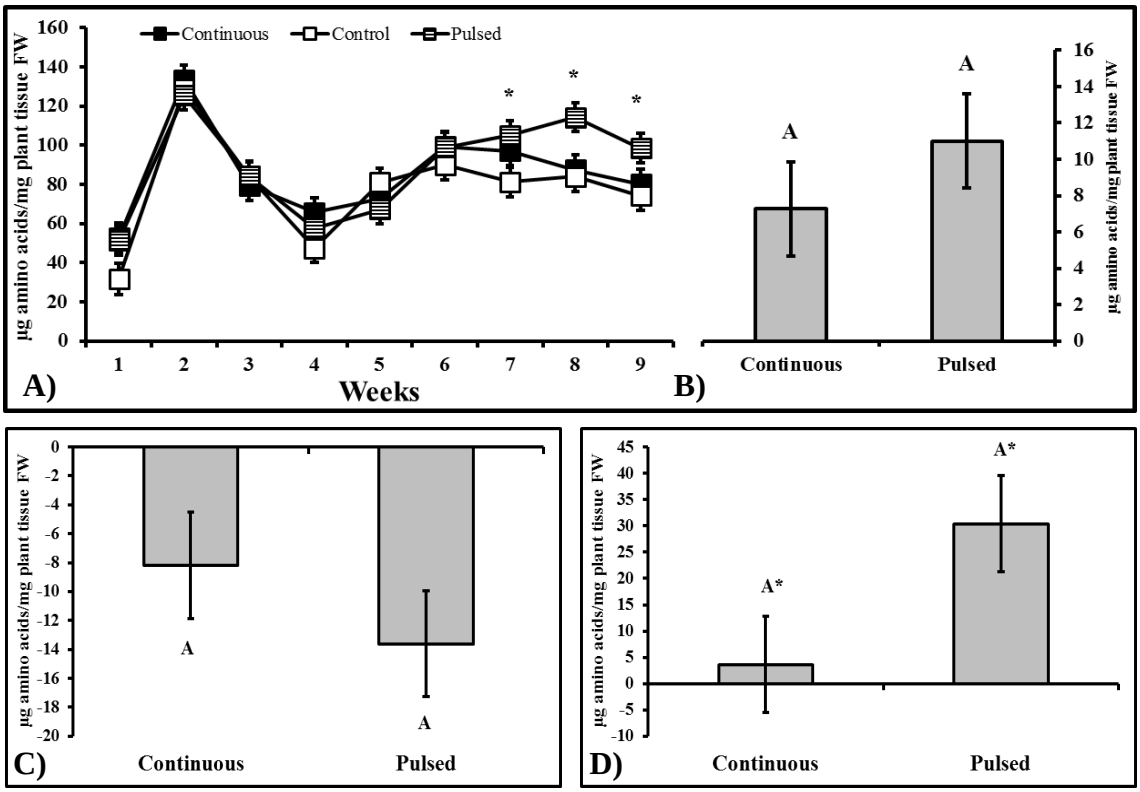

Figure 2-12. Amino acids in stressed plants in 2011. Concentrations of amino acids for the season (A), the average for the season relative to control plants (B), the pulse during week 5 in 2011 relative to control plants (C), and the pulse during week 8 in 2011 relative to control plants. The markers are mean concentrations of amino acids ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars are mean concentrations of amino acids ±SE. Bars with different letters above them are significantly different and letters with asterisks indicate a marginal significant difference.

Water stressed plants had similar concentrations of digestible carbohydrates in 2010. Carbohydrates did not vary throughout the season (carbohydrates: $F_{6.1,\ 79.6}$= 1.28, p= 0.2742), varied with treatment (treatment: $F_{2,\ 146}$= 7.47, p= 0.0008), but did not vary with treatment over time (treatment*week: $F_{6.1,\ 79.6}$= 1.28, p= 0.2742) (Fig. 2-13A). For the season average, carbohydrates were similar among stressed plants (season average: $F_{1,\ 98}$= 1.01, p= 0.3173; Fig. 2-13B), and treatments had a similar effect on

carbohydrates after the pulse during week 7 (treatment: $F_{1, 18}$= 0.20, p= 0.6587; Fig. 2-13C).

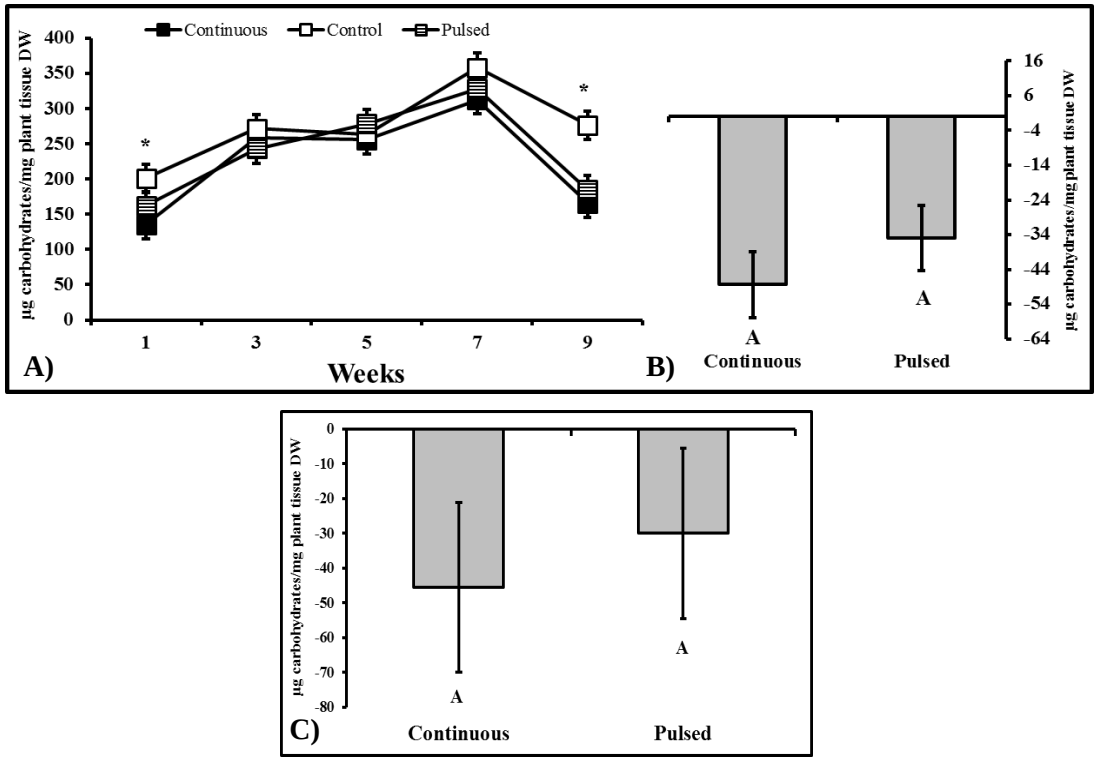

Figure 2-13. Digestible carbohydrates in stressed plants in 2010. Concentrations of digestible carbohydrates for the season (A), the average for the season relative to the control (B), and during the pulse during week 6 in 2010 relative to the control (C). The markers are mean digestible carbohydrates concentrations ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars are mean digestible carbohydrates concentrations ±SE. Bars with different letters above them are significantly different.

In 2011, carbohydrates in stressed plants were similar for the majority of the season. Overall, carbohydrates varied throughout the season (carbohydrates: $F_{14, 133}$= 2.35, p= 0.0060), with treatment (treatment: $F_{2, 145}$= 1.38, p= 0.2545), and with treatment over time (treatment*week: $F_{8, 139}$= 1.68, p= 0.1087) (Fig. 2-14A). Additionally,

carbohydrates were similar in concentration on average throughout the season (season average: $F_{1, 97}$= 2.40, p= 0.1242; Fig. 2-14B) and stressed plants were similar during the first pulse in week five (treatment: $F_{1, 18}$= 0.07, p= 0.8002; Fig. 2-14C). Furthermore, stress type had significantly different effects on carbohydrates during the second pulse in week 8 (treatment: $F_{1, 18}$= 27.56, p<0.0001; Fig. 2-14D) and continuously stressed plants had seven times lower concentrations of carbohydrates compared to pulsed stressed plants (Fig. 2-14D).

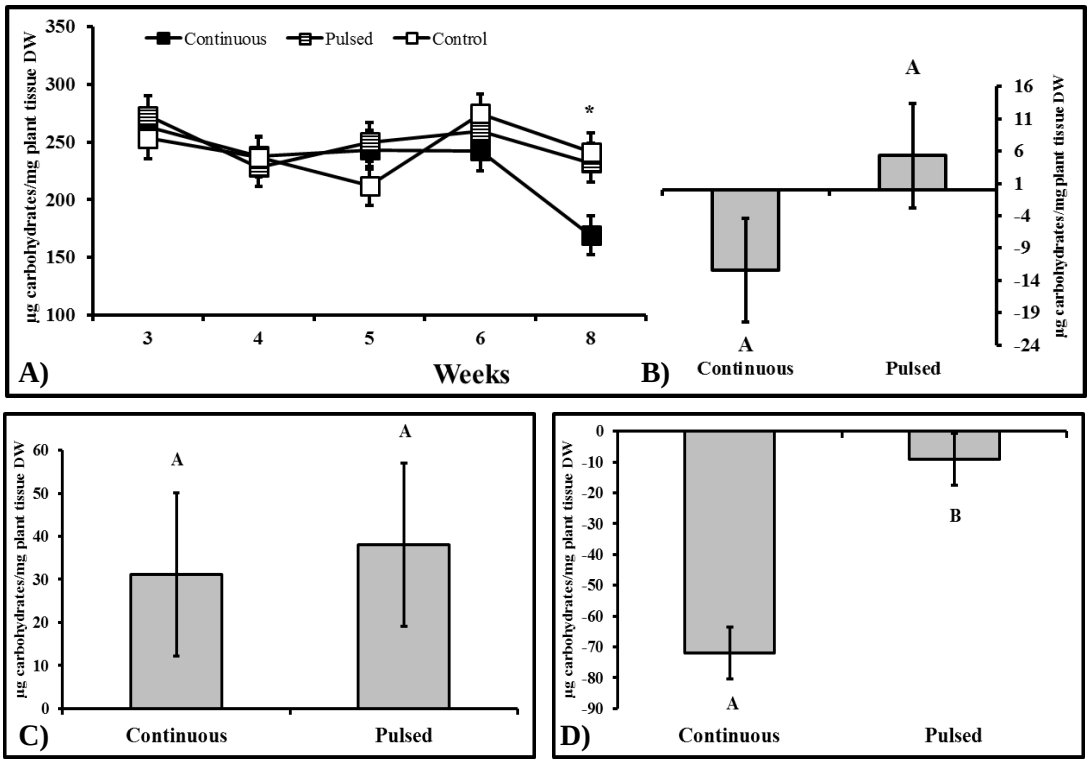

Figure 2-14. Digestible carbohydrates in stressed plants in 2011. Concentrations of digestible carbohydrates for the season (A), the average for the season relative to control plants (B), the pulse during week 5 in 2011 relative to control plants (C), and the pulse during week 8 in 2011 relative to control plants. The markers are mean digestible carbohydrates ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars are mean concentrations of digestible carbohydrates ±SE. Bars with different letters above them are significantly different.

2.3.5 Cotton development

Water stress significantly affected cotton height, nodes, and the quantity of squares and bolls in 2010. Plant height significantly varied throughout the season (height: $F_{8, 152}$= 164.26, p<0.0001), by treatment (treatment: $F_{2, 158}$= 102.19, p<0.0001), and with treatment over time (treatment*week: $F_{4, 156}$= 17.89, p<0.0001) (Fig. 2-15A). For instance, pulsed stressed plants were significantly taller than continuously stressed plants, with pulsed stressed plants 30% taller than continuously stressed plants at week 9. Furthermore, the number of nodes varied throughout the season (nodes: $F_{8, 152}$= 60.39,

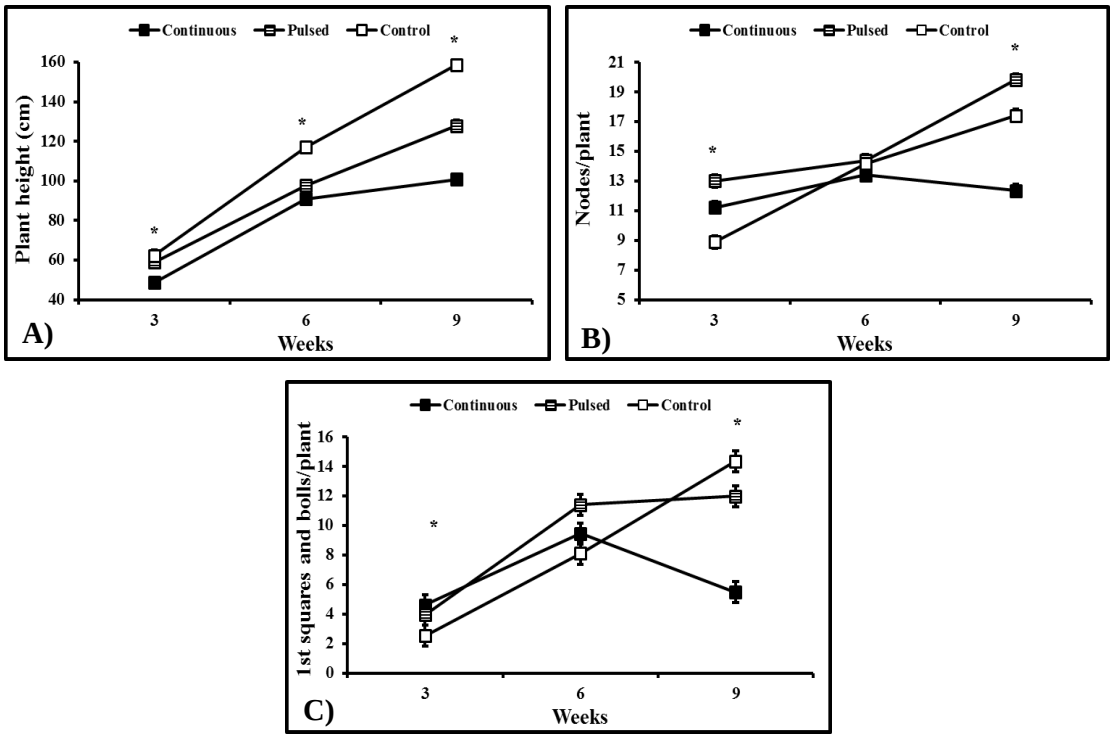

Figure 2-15. Plant height, total nodes, and total 1st position squares and bolls in 2010. Plant height (A), nodes (B), and first position squares and bolls in 2010 (C). The markers are means ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week.

$p<0.0001$; Fig. 2-15B), with treatment (treatment: $F_{2, 158}= 52.03$, $p<0.0001$), and with treatment over time (treatment*week: $F_{4, 156}= 29.19$, $p<0.0001$) (Fig. 2-15B). In addition, there were significant differences in nodes between treatments during week 3, similarities during week 6, and pulsed stressed plants had 60% more nodes than continuously stressed plants by week 9 (Fig. 2-15B). For first position squares and bolls, the total varied throughout the season (squares and bolls: $F_{3.1, 77.3}= 7.52$, $p= 0.0002$), with treatment (treatment: $F_{2, 158}= 10.55$, $p<0.0001$), and with treatment over time (treatment*week: $F_{3.1, 77.3}= 7.52$, $p= 0.0002$). For example, pulsed stressed plants had 220% more first position squares and bolls by week 9 (Fig. 2-15C).

In 2011, water stressed significantly reduced plant height, quantity of nodes, and total squares and bolls. Plant height varied throughout the season (height: $F_{6, 153}= 25.96$, $p<0.0001$), with treatment (treatment: $F_{2, 213}= 116.96$, $p<0.0001$), and with treatment over time (treatment*week: $F_{6, 153}= 25.96$, $p<0.0001$) (Fig. 2-16A). For instance, pulsed stressed plants were 20% taller than continuously stressed plants by week 9. For quantity of nodes, quantity varied throughout the season (nodes: $F_{11, 204}= 67.10$, $p<0.0001$), with treatment (treatment: $F_{2, 213}= 36.47$, $p<0.0001$), and with treatment over time (treatment*week: $F_{6, 209}= 16.22$, $p<0.0001$). In addition, stressed plants were similar in total nodes during weeks 1 and 4 and pulsed stressed plants had 23% more nodes than continuously stressed plants by week 9 (Fig. 2-16B). For total squares and bolls, the total varied throughout the season (squares and bolls: $F_{4.6, 116.6}= 5.56$, $p= 0.0002$), with treatment (treatment: $F_{2, 213}= 16.87$, $p<0.0001$), and with treatment over time

(treatment*week: $F_{4.6,\ 116.6}$= 5.56, p= 0.0002). For instance, pulsed stressed plants had 200% more fruits than continuously stressed plants at week 9 (Figs. 2-16C).

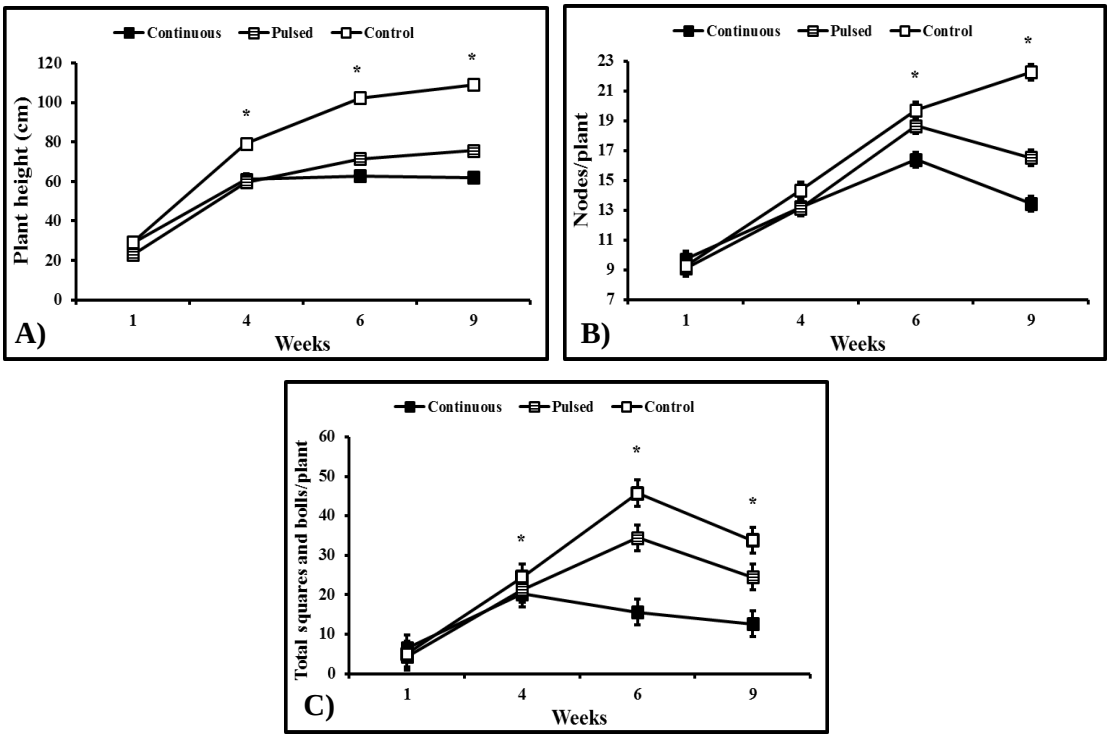

Figure 2-16. Plant height, total nodes, and total squares and bolls in 2011. Plant height (A), nodes (B), and total squares and bolls in 2011 (C). The markers are means ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week.

2.4 Discussion

We found that pulsed and continuously stressed plants had physiological similarities, but there were differences that suggest that different stress types need to be considered when making predictions of herbivore performance on water-stressed plants. Our study suggests that water-stressed plants will affect herbivores through decreased carbon assimilation, alterations in water use (stomatal conductance, transpiration),

increased amino acid content, and resource allocation to preserving leaf material (chlorophyll content).

Continuously stressed plants had decreased photosynthetic rate and stomatal conductance compared to pulsed stressed plants, which may impact herbivore feeding and preference. When plants are under gradual, continuous water stress as in our study, prolonged stomatal closure in response to stress results in a decreased physiological requirement for CO_2 and acclimation to reduced photosynthesis under conditions with decreased water content (Ort et al. 1994, Chaves et al. 2002, Taiz and Zeiger 2010). In conjunction with decreased plant height, nodes, and squares and bolls we observed in stressed plants, continuously stressed plants will further reduce the production of leaves, fruits, and other structures for herbivores to consume and impede their development compared to pulsed stressed plants. Additionally, reduced stem water potential and water content would reduce the feeding efficiency of piercing-sucking herbivores such as aphids (Hemiptera: Aphididae) and protein assimilation in chewing herbivores such as beet armyworms (Lepidoptera: Noctuidae) (Wearing 1972, Scriber 1977, Huberty and Denno 2004, Douglas 2006).

Under the Growth-Differentiation Balance Hypothesis, stressed plants may contain higher allelochemical concentrations and structural carbohydrates than unstressed plants (Coley et al. 1985, Fajer et al. 1992, Herms and Mattson 1992). In our study, continuously stressed plants may have increased structural carbohydrates and chlorophyll in leaves more than pulsed stressed plants as evidenced by greater SPAD values. As predicted by the GDBH, continuously stressed plants would be tougher to

consume, produce greater densities of trichomes, and contain higher allelochemical concentrations compared to pulsed stressed plants (Gershenzon 1984, Raupp 1985, Inbar et al. 2001). For instance, continuously stressed green spruce (Pinaceae: *Picea sitchensis*) had higher amounts of monoterpene allelochemicals compared to pulsed stressed spruce, and the green spruce aphid (Hemiptera: *Elatobium abietinum*) decreased in abundance on continuously stressed spruce compared to pulsed stressed spruce (Major 1990). Continuously and pulsed stressed plants would affect herbivore response differently due to the dissimilarities in resource allocation and water content.

Water stress treatments had minimal effect on concentrations of amino acids and digestible carbohydrates throughout the season and differences between treatments were largely insignificant. Stress-induced changes in nutrients were not as closely associated with water stress or the pulses as expected, and thus our study does not support the nutritional predictions of the plant and pulsed stress hypotheses. Plant nutrients should increase in stressed plants when their stem water potential decreases below -0.4 MPa through -1.2 MPa and as plants become water stressed (White 1969, Hsiao 1973, Chaves et al. 2002, Lombardini 2006), but our stressed plants were also exposed to insect herbivory which may have influenced nutrient concentrations. For instance, *Brassica oleracea* (Brassicaceae) decreased in nitrogen with high densities of *Delia radicum* larvae (Diptera: Anthomyiidae) compared to lower densities and no herbivory (Tariq et al. 2013). In addition, herbivores such as aphids and sawflies (Hymenoptera) may decrease nutrient concentrations by hindering photosynthesis and stomatal conductance (Godfrey and Wood 1998, Shannag et al. 1998, Delaney et al. 2010). For example,

cotton aphids at a density of 25 aphids/leaf decreased the photosynthetic rate of cotton plants by 40% after 27 days of feeding compared to uninfested plants (Shannag et al. 1998), and decreased stomatal conductance by 18.5% on cotton plants with more than 20 aphids/leaf (Godfrey and Wood 1998). This suggests that herbivory could have influenced the nutrient concentrations in our stressed plants, especially if one treatment experienced greater herbivory than the other. Furthermore, protein synthesis at the cellular level in plants is dependent upon desiccation-sensitive ribosomes and as the severity of water stress continues, there is an increase in free amino acids as fewer amino acids are synthesized into proteins (Bewley 1981). The magnitude of stress-induced increases in free amino acids when plants are recovering from water stress is dependent upon the plant's ability to resume protein synthesis following rehydration (Bewley 1981). In our study, concentrations of amino acids were similar in pulsed and continuously stressed plants during pulses, suggesting that the stress treatments did not influence protein synthesis differently despite differences in stress severity. Our study suggests that herbivores will encounter similar concentrations of nutrients in pulsed and continuously stressed plants.

To our knowledge, this is the first study to test the effects of pulsed and continuous water stress on plants to predict herbivore performance. Previous studies have compared these types of stress, but focused on herbivore performance (Major 1990, Huberty and Denno 2004, An Nguyen et al. 2007, Mody et al. 2009). We found that both stress treatments can affect cotton plants in similar ways, but differences in photosynthesis, stomatal conductance, transpiration efficiency, plant development,

chlorophyll content, water content and minor differences in concentrations of nutrients may increase herbivore performance on pulsed stressed plants compared to continuously stressed plants. We believe that differences between stress treatments would have been more distinct if pulsed stressed plants were more stressed and experimental conditions were more controlled. For instance, during the 2010 season, plants were mildly stressed during week five at -1.24 MPa, which may not have been severe enough to truly demonstrate the differences in treatments, especially for nutrients. Whereas in 2011, pulsed stressed plants were not under -1.2 MPa after the first pulse in week five and did not recover from stress after the subsequent pulses (Fig. 2-1). Furthermore, incomplete datasets (missing weeks) due to inherent obstacles of fieldwork excluded potentially critical data that may have distinguished the effects between stress treatments. We do believe, however, that field studies should continue to address the complexity of this topic, but more planning and foresight is needed to reduce the complications inherent to field studies. Despite this, we believe that our study has illustrated that different types of water stress affect plants differently, and that these differences must be considered to accurately predict herbivore performance.

CHAPTER III

NOT ALL DROUGHTS ARE CREATED EQUAL? THE EFFECTS OF PULSED AND CONTINUOUS STRESS ON INSECT HERBIVORE ABUNDANCE

3.1 Introduction

The impact of drought stress on plant-insect interactions has remained a topic of debate for many decades. As drought is predicted to increase in the future, understanding how climate change impacts plant-insect interactions is critical (Dai 2011). The "Plant Stress Hypothesis" states that herbivores will increase in abundance on water-stressed plants due to increases in foliar nitrogen (White 1969). This hypothesis was the first formal attempt to explain the interactions between drought stress and plant-insect interactions. Since its introduction, however, empirical studies have not consistently supported the plant stress hypothesis. For example, results supporting the plant stress hypothesis include studies that found populations of aphids (Hemiptera: Aphididae), geometrid caterpillars (Lepidoptera: Geometridae), and beetles (Coleoptera: *Agrilus, Tetropium, Scolytus*) more abundant on drought stressed plants (Mattson and Haack 1987, Archer et al. 1995, Schowalter et al. 1999). Other studies, however, found that these same insects or their close relatives did not increase in abundance on drought stressed plants (Hanks and Denno 1993, Larsson and Bjorkman 1993, Inbar et al. 2001). This suggests that the potential benefits to insects of increased foliar nitrogen and nutrients that typically increase during stress are not always realized (Larsson 1989, Saikkonen et al. 1995, English-Loeb et al. 1997, Showler and Moran 2003, Huberty and

Denno 2004, Mody et al. 2009). During water stress, declines in water potential and water content may reduce feeding from piercing-sucking and chewing herbivores (Kennedy et al. 1958, Scriber 1978, Archer et al. 1995). Aphids, in particular, require water potential to feed from plant phloem cells (Douglas 2003, Guerrieri and Digilio 2008). Water-stressed plants, therefore, may become more nutritious during water stress, but other physiological properties may limit the impact of these benefits.

The duration and severity of water stress may also determine the availability of nutrients. In addition to increases in foliar nitrogen, water stressed plants accumulate stress-related compounds such as amino acids, sugars, and antioxidant enzymes that alleviate the negative effects of stress (English-Loeb et al. 1997, Sholwer 2002, Jithesh et al. 2006). These stress-related compounds stabilize cytoplasmic enzymes, cell membranes, and scavenge free radicals (Chaves et al. 2002, Lawlor and Tezara 2009, Taiz and Zeiger 2010). The induction and insects benefits of these stress-related compounds, however, decline with the continued and increased severity of water stress (Hsiao 1973, Chaves et al. 2002, Ghannoum 2008). Long periods of drought, or continuous water stress, result in a decline in water potential and water content, and these changes have been associated with decreases in nutrient availability (Hsiao 1973, Huberty and Denno 2004, Taiz and Zeiger 2010). Herbivore exposure to the deleterious effects of continuous stress may explain some variation in herbivore response to water stressed plants. The "Pulsed Stress Hypothesis" suggests that when plants recover from stress, plants may provide adequate water potential and water content for herbivores to take advantage of stress-induced increases in plant nutrients (Huberty and Denno 2004).

While recovering from stress, plants will regain water potential and may still exhibit elevated levels of nutrients for several days after rehydration (Baskin and Baskin 1974, Parida et al. 2008). Few studies have directly tested the pulsed stress hypothesis and even fewer have compared the response of insect herbivores to pulsed and continuous stress simultaneously under the same experimental conditions. In our study, we examined insect herbivore and arthropod abundance in response to pulsed and continuously cotton plants in an agro-ecosystem. Our goal was to determine how different types of water stress influence herbivore abundance on stressed plants and determine the influence of stress-induced increases in nutrients on herbivore abundance. We measured herbivore and arthropod abundance, colony growth of aphids in clip cages, herbivore damage, and amino acid and digestible carbohydrate content in water-stressed plants. We hypothesized that herbivores feeding on pulsed stressed plants should increase in abundance in response to stress-induced increases in nutrients, but insect herbivores feeding on continuously stressed plants should decrease in abundance. The pulsed stress hypothesis predicts that piercing-sucking herbivores (e.g., aphids, stinkbugs, fleahoppers) in particular should increase in abundance on plants with higher water potential, whereas chewing herbivores (e.g., caterpillars, grasshoppers) should prefer well-watered plants.

3.2 Methods

3.2.1 Study system

We conducted two, ten-week field studies in 2010 and 2011 at the Texas A&M Field Laboratory in Burleson County, Texas (coordinates: 30.548754,-96.436082). The south-central region of Texas experiences subtropical and temperate climates with mild winters lasting no longer than two months. High temperatures range from 25°C in May to 35°C in July, and precipitation ranges from 11.94 cm in May to 5.08 cm in July. Our field site primarily consisted of Belk series clay soil, known as a very deep, well drained, and slowly permeable soil that is common in Texas flood plains (U.S.A. 2007). Approximately 0.6 hectares of cotton were planted in both 2010 and 2011. We planted commercial cotton (*Gossypium hirsutum* L.), Delta Pine 174RF (no drought/pest resistance), on 3 May 2010. Cotton was furrow irrigated on 14 May and treatments began on 14 June when cotton reached the late seedling, early squaring (flower bud) stage. In 2011, the same cotton variety was planted on 18 April and irrigated on 21 May before treatments began on 13 June during the late seedling-early squaring stage. The study concluded on 29 and 28 August in 2010 and 2011, respectively. The field was treated with Round-Up herbicide to eliminate weeds and fertilized with 14.69 kg of nitrogen/hectare with a time-release formula for both years.

3.2.2 Experimental design

Cotton was divided into 54, 6 m x 4.5 m plots, separated into 9 blocks and each block randomly received continuous stress, pulsed stress, or control irrigation treatments.

Each treatment had 6 plots per block for a total of 18 plots per treatment in a randomized complete block design. Blocks had 9.1 m of untreated cotton on all sides and each plot within a block had 2.7 m of untreated cotton between plots. Continuously water stressed plants were not irrigated for the entire growing season and only received ambient rainfall, while the control plants received irrigation weekly. For the pulsed stressed plants, we used a pressure chamber (model 615, PMS Instrument Co., Albany, OR) to measure water status and determine the appropriate stress level to trigger irrigation. Cotton plants are water-stressed at approximately -1.2 MPa (-12 bars) and begin to accumulate stress-induced increases in foliar nitrogen and other nutrients (Hsiao 1973, Lombardini 2006). Pulsed stressed plants were watered when their stem water potential was below -1.2 MPa. For the pressure chamber measurements, 17 cm x 9 cm aluminum bags were placed over the uppermost, fully-expanded leaf for 20 minutes, and then cut at the proximal end of the petiole using scissors. The aluminum bag, with the leaf still inside, was then folded gently and inserted into the chamber for a pressure reading. To accommodate for destructive sampling for pressure chamber measurements, each plot was divided into 32 subplots containing 10-15 plants and one plot was randomly selected for pressure chamber measurements each week, for a total of 18 measurements each week per treatment. This subplot method was used for all measurements to avoid sampling the same plants.

3.2.3 Arthropod and herbivore surveys

Using the subplot sampling methods described above, the top 0.61 m of 5 plants were gently angled and shaken over a large bowl for arthropod identification. Afterwards, the top 5 leaves of each plant were carefully examined for arthropods that were not dislodged (e.g., aphids, spider mites). Arthropods that could not be identified in the field were placed in vials of 70% ethanol and identified in the lab to species using Texas A&M Extension field guides for cotton pests and natural enemies (Knutson and Ruberson 1996, Bohmfalk et al. 2011). Arthropods not in these guides were identified to family using Triplehorn et al. (2005). Once arthropods were field identified or placed in vials for laboratory identification, the bowl was emptied over the sampled plants to return as many arthropods as possible back to the field plots once identification was completed.

3.2.4 Aphid clip cages

Aphid clip cages were used to determine the effect of pulsed and continuous water stress on aphid colony growth in enemy-free space during the field season in 2010 (Fig. 3-1). We manufactured clip cages using two 7 cm x 4 cm rectangular pieces of cardboard with an area of 5 cm x 2.5 cm removed. One cardboard piece had black insect-proof mesh (Bioquip Products Inc., Rancho Dominquez, CA) installed into the 5 cm x 2.5 cm that was removed and one short end of each cardboard piece was glued together. The glued cardboard pieces were gently secured onto leaves with the screened cutout on the underside of the leaf to enclose the feeding aphids and the unscreened

cutout over the top of the leaf. The cutouts were secured in place with two hair pins along the long edges of the cage and one hair pin along the unglued, short edge of the cutouts. Clip cages were added to plants on 5 July, 2010 during the second week of the experiment. Twenty-five (25) aphids were collected from adjacent plants and added to the underside of the leaf within the clip cage and the cage was secured in place. One clip cage was placed in each plot in each block for a total of 18 clip cages per treatment per week. Each week, the cages were removed and the aphids were counted. New plants were then randomly selected to receive the clip cages, and 25 aphids were added again and the process repeated each week for the duration of the study.

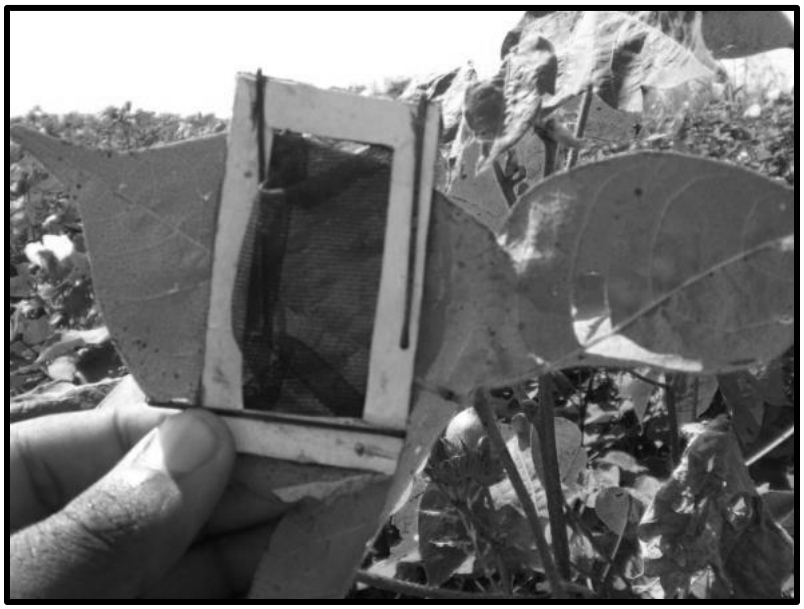

Figure 3-1. Picture of aphid clip cages used in the 2010 herbivore study. The cardboard cages measured 7 cm x 4 cm with 5 cm x 2.5 cm fine black mesh installed. Cages were secured to leaves using 3 hair pins placed as shown with the mesh on the underside of the leaf.

3.2.5 Herbivore damage

Damage from chewing herbivores was measured using visual estimations of the percent of total leaf area removed. Prior to the study, our estimations were calibrated by comparing quantitative measurements of missing leaf area from photographs of damaged leaves using ImageJ software (Bethesda, MD) with our visual estimations. Each week, the percentage of total leaf area removed was estimated for the uppermost fully-expanded leaves on 5 plants within a randomly selected subplot per plot in each block.

3.2.6 Amino acid and digestible carbohydrate assays

Amino acids and digestible carbohydrates were measured in cotton plants to determine the influence of pulsed and continuous stress on nutrient concentrations in water stressed plants and herbivore abundance. Each week, the uppermost fully-expanded leaf of 5 plants within a randomly selected subplot was removed. Leaves were quickly and gently placed into 9 cm x 5.75 cm coin envelopes and stored in ice coolers and quickly transported to the laboratory. Once in the laboratory, the envelopes were placed in -80°C freezers until assayed.

Plant chemistry assays for amino acids were conducted using a modified ninhydrin assay as according to Starcher (2001) and McArthur et al. (2010). Ten samples were randomly chosen per treatment from each week of the study to measure changes in amino acid concentration over time. For each sample, approximately 5 mg of tissue was removed and ground in 10 µl of 80% ethanol in an Eppendorf tube using a manual tissue grinder and placed on ice. Once the tissue was ground, 500 µl of 6N HCl was added to

the sample tube and placed in a heating block at 100°C for 24 hours. A large block was placed on top of the tubes to ensure that the caps stayed closed. During the last 2.5 hours of the 24 hour period, the large blocks were removed and each tube was opened to allow the HCl to boil off. The remaining pellet was suspended in 1 ml of water and centrifuged at 12,000 rpm for 1 minute to facilitate sample homogeneity. The ninhydrin stock solution was prepared using 200 mg of ninhydrin, 7.5 ml of ethylene glycol, 2.5 ml of 4N sodium acetate buffer and 200 µl of stannous chloride solution. In a new Eppendorf tube, 20 µl of sample and 100 µl of the ninhydrin solution were added and returned to the 100°C heating block for 10 minutes. Samples were allowed to cool and the sample and ninhydrin mixtures were transferred to a 96-well microplate. Plates were read for spectrophotometric absorbance at 570 nm using an Epoch microplate reader (BioTek Instruments Inc., Winooski, VT). If samples were too dark to be read at 570 nm due to high amino acid content, 620 nm was used and a simple linear regression equation was generated to convert amino acid concentrations from readings at 620 nm to predicted concentrations at 570 nm. Amino acid standards were prepared using powdered BSA (Sigma-Aldrich, St. Louis, MO) with dilutions prepared at 2 µg, 4 µg, 6 µg, 8 µg, and 10 µg/1 ml of water from 10 mg/1 ml of water. Standards were used to generate a standard curve to approximate µg of amino acids/5 mg of plant tissue sample.

Plant chemistry assays for digestible carbohydrate content were conducted using a phenol-sulfuric acid assay as according to Smith et al. (1964) and Clissold et al. (2006). Ten frozen samples were randomly selected per treatment from each week of the study to measure changes in digestible carbohydrates over time. Approximately 200 mg

of leaf tissue from each sample was dried using a freeze-dryer (Labconco, Kansas City, MO) at -50°C for two days. Once dried, samples were ground to powdered flakes using a MF 10 basic wiley cutting mill (IKA Works, Inc., Wilmington, NC) using a size 20 mesh and 20 mg were removed and added to screw-cap test tubes. Each tube received 1ml of 0.1M sulfuric acid and was placed in a boiling water bath for 1 hour. Tubes were cooled in a container of room temperature water, emptied into 1.5 ml Eppendorf tubes, and mixed in a centrifuge at 13,000 rpm for 10 minutes. Each tube had 15 µl of supernatant removed which was added to glass test tubes with 385µl of distilled water and 400 µl of 5% phenol solution. Tubes then received 2 ml of concentrated sulfuric acid and allowed to sit for 10 minutes. Samples were mixed using a vortex mixer then allowed to sit for an additional 30 minutes. Standards were prepared using 0.2 mg/µl glucose to make six 400 µl dilutions containing 0, 15, 30, 45, 60, or 75 µg of glucose. The dilutions were treated in the same manner as the samples. After sitting, 750 µl of the sample, phenol, and sulfuric acid mixture was added to cuvettes for spectrophotometric readings at 490 nm with the samples being measured in duplicates and standards in triplicate. The standards were used to generate a standard curve to approximate µg of carbohydrates/20 mg of dried plant sample.

3.2.7 Analysis

Data for herbivores other than aphids and chewing herbivores and data for natural enemies, amino acids, digestible carbohydrates, and missing leaf area were compared between treatments. The concentrations of amino acids and digestible

carbohydrates were not able to be determined during the third pulse (week 10) in 2011. All data were analyzed with repeated measures ANOVA or ANOVA using JMP Pro 10.0.0 (SAS Institute, Cary, NC) to make comparisons between treatments over time. Sphericity tests were conducted for all repeated measures to ensure that the variance assumptions of repeated measures were not violated and analyses were accurate. If sphericity was violated and a corrective Greenhouse-Geisser test yielded an ε of > 0.75, then the corrected test was used. If the Greenhouse-Geisser test yielded an ε of < 0.75, then a MANOVA was used to generate a Wilk's lambda test statistic (Λ) to compare treatments over time. These adjustments ensured that the most appropriate and powerful test was used. Regression analyses, also with JMP, were conducted to determine associations between the stress-induced changes in nutrients in plants, herbivore abundance, and natural enemies, and were reported for seasonal aphid abundance.

3.3 Results

3.3.1 Water-deficit stress, stem water potential, and plant nutrients

Water stress significantly affected stem water potential (MPa) during both years. In 2010, stem water potential significantly varied throughout the season (stem water potential: $F_{23, 408}= 78.39$, $p<0.0001$) and the effects of stress were significant over time (week*treatment: $F_{14, 408}= 24.24$, $p<0.0001$). Control plants in 2010 maintained stem water potential above -1.2 MPa throughout the season, while continuously stressed plants decreased below -1.2 MPa during week 5 and below -2.5 MPa as the seasons concluded (Fig. 3-2A). Stem water potential in pulsed stressed plants decreased below -

1.2 MPa by week 5 and was irrigated at the end of weeks 5 and 6. Pulsed stressed plants in 2010 were water-stressed during weeks 5 and 9 and had one pulse during week 6.

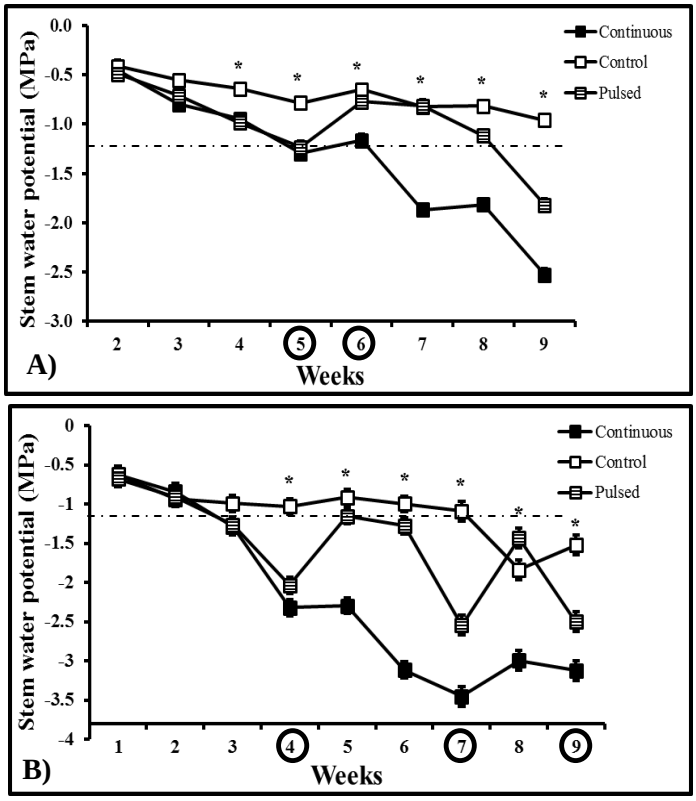

Figure 3-2. Stem water potential for stressed plants in 2010 and 2011. 2010 (A) and 2011 (B). The markers are mean stem water potential ±SE. The dotted line marks -1.2 MPa, when plants are believed to be water-stressed. Circles over certain weeks indicate when the pulsed stress treatment received irrigation to end water stress. The "pulses" were during week 6 in 2010, and during weeks 5, 8, and 10 in 2011. Asterisks indicate significant differences between at least two of the three treatments for a given week.

In 2011, stem water potential significantly varied throughout the season (stem water potential: $F_{10.5,\ 267.8}= 28.87$, $p<0.0001$) and the effects of stress were significant over time (week*treatment: $F_{10.5,\ 267.8}= 28.87$, $p<0.0001$; Fig. 3-2B). Control plants in 2011 plants decreased below -1.2 MPa during weeks 8 and 9 (Fig. 3-2B). Continuously

stressed plants decreased below -1.2 MPa during week 4 and decreased to -3 MPa as the season concluded. Stem water potential in pulsed stressed plants decreased below -1.2 MPa by week 4 and received irrigation at the end of weeks 4, 7, and 9. Pulsed stressed plants were water-stressed during weeks 4, 7, 8, and 9 (Fig. 3-2B). Pulsed stressed plants in 2011 had three pulses during weeks 5, 8, and 10 (Fig. 3-2B).

The effects of water stress on concentrations of amino acids and digestible carbohydrates did not vary among stress treatments. In 2010, concentrations of amino acids were not significantly different among treatments (treatment: $F_{2, 26}$= 0.87, p= 0.4307; Fig. 3-3A) and stressed plants did not differ during the pulse (Fig. 3-3A). In,

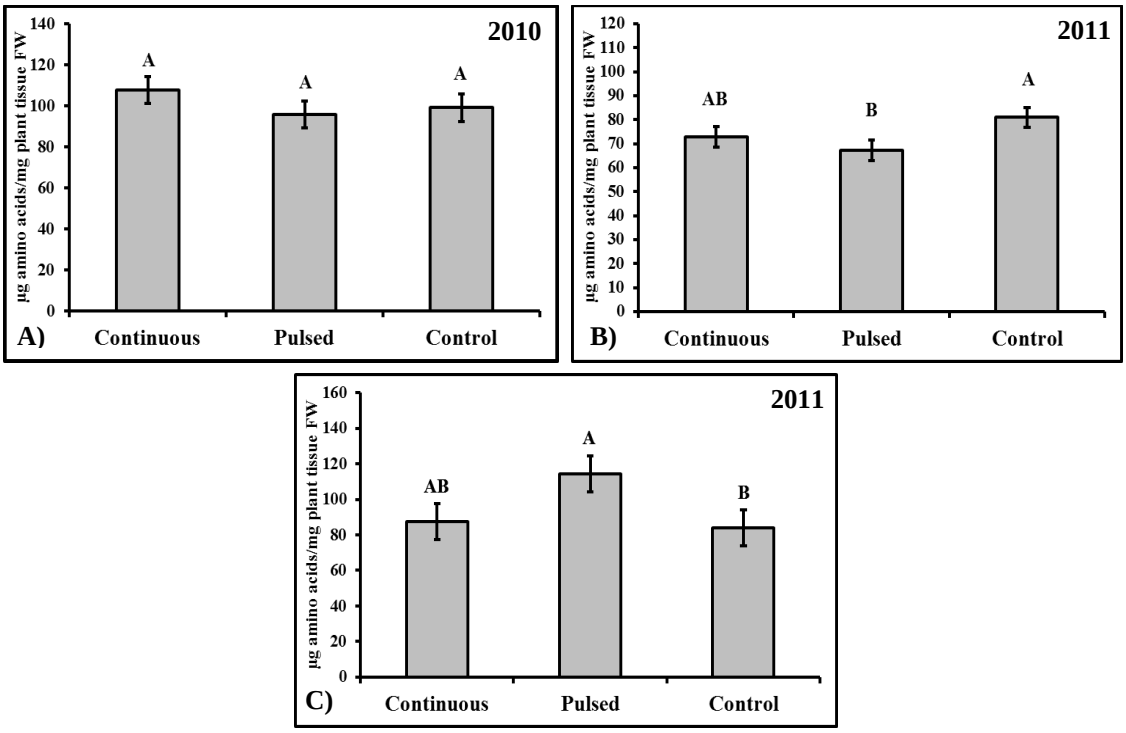

Figure 3-3. Amino acids in stressed plants during pulses in 2010 and 2011. Plants during the pulse in 2010 (A), the pulse during week five (B) and week eight (C) in 2011. Bars are means ±SE and bars with different letters above them are significantly different.

addition, treatments had no effect on digestible carbohydrates during the pulse in 2010 (treatments: $F_{2, 26}$= 0.49, p= 0.6191; Fig. 3-4A). In 2011, concentrations of amino acids were marginally different between treatments during two pulses (pulse 1: treatment: $F_{2, 27}$= 2.64, p= 0.0896; pulse 2: $F_{2, 27}$= 2.66, p= 0.0880; Figs. 3-3B and 3-3C). Amino acids were 7% lower in pulsed stressed plants compared to continuously stressed plants during

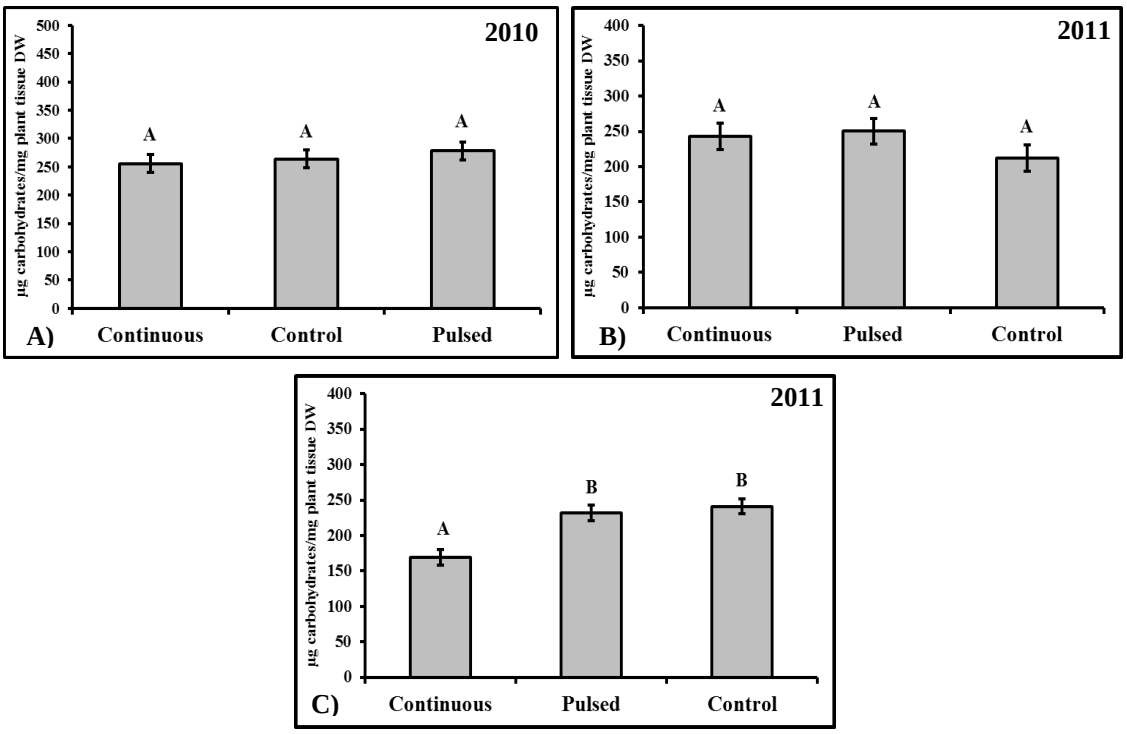

Figure 3-4. Digestible carbohydrates in plants during pulses in 2010 and 2011. Digestible carbohydrates in water-stressed plants during the pulse in 2010 (A), for the pulse during week five (B), and week eight (C) in 2011. Bars are means ±SE and bars with different letters above them are significantly different.

the first pulse in 2011, then 30% higher than continuously stressed plants during the second pulse in 2011 (Figs. 3-3B and 3-3C). Water stress had no effect on digestible carbohydrates during the first pulse in 2011 (pulse 1: treatment: $F_{2, 27}$= 1.20, p= 0.3172),

but the effects of stress were significant during the second pulse (pulse 2: treatment: $F_{2, 26}$= 13.36, p<0.0001) with carbohydrates increasing 36% higher in pulsed stressed plants than in continuously stressed plants (Figs. 3-4B and 3-4C).

3.3.2 Water stress and piercing-sucking herbivores

The communities of piercing-sucking herbivores in our cotton plots were different in the two years of our study. In 2010, cotton aphids (Hemiptera: *Aphis gossypii*), western flower thrips (Thysanoptera: *Frankliniella occidentalis*), stink bugs (Hemiptera: *Nezara viridula* and *Euschistus servus*), the cotton leafhopper (Hemiptera: *Amrasca terraereginae*), and the cotton fleahopper (Hemiptera: *Pseudatomoscelis seriatus*) were the most abundant piercing-sucking herbivores. In 2011, tube-tailed thrips (*Phlaeothrips sp.*), double-banded thrips (*Aeolothrips sp.*), and silverleaf whiteflies (Hemiptera: *Bemesia tabaci*) became abundant and both the southern green and the brown stink bugs were rare.

The effects of water stress on piercing-sucking herbivores were inconsistent. In 2010, aphid abundance significantly varied throughout the season (abundance: Λ= 0.41, $F_{18, 86}$= 2.70, p= 0.011) and with stress over time (treatment*week: Λ= 0.41, $F_{18, 86}$= 2.70, p= 0.011), but the stress treatments alone had no effect on aphids (treatment: $F_{2, 510}$= 1.24, p= 0.2892) (Fig. 3-5). In addition, average aphid abundance was similar among treatments ($F_{2, 537}$= 0.90, p= 0.4070; Fig. 3-5B), but differed during the pulse in week six (treatment: $F_{2, 51}$= 6.17, p= 0.0040; Fig. 3-5E) with three times the abundance on pulsed stressed plants compared to continuously stressed plants (Fig. 3-5E).

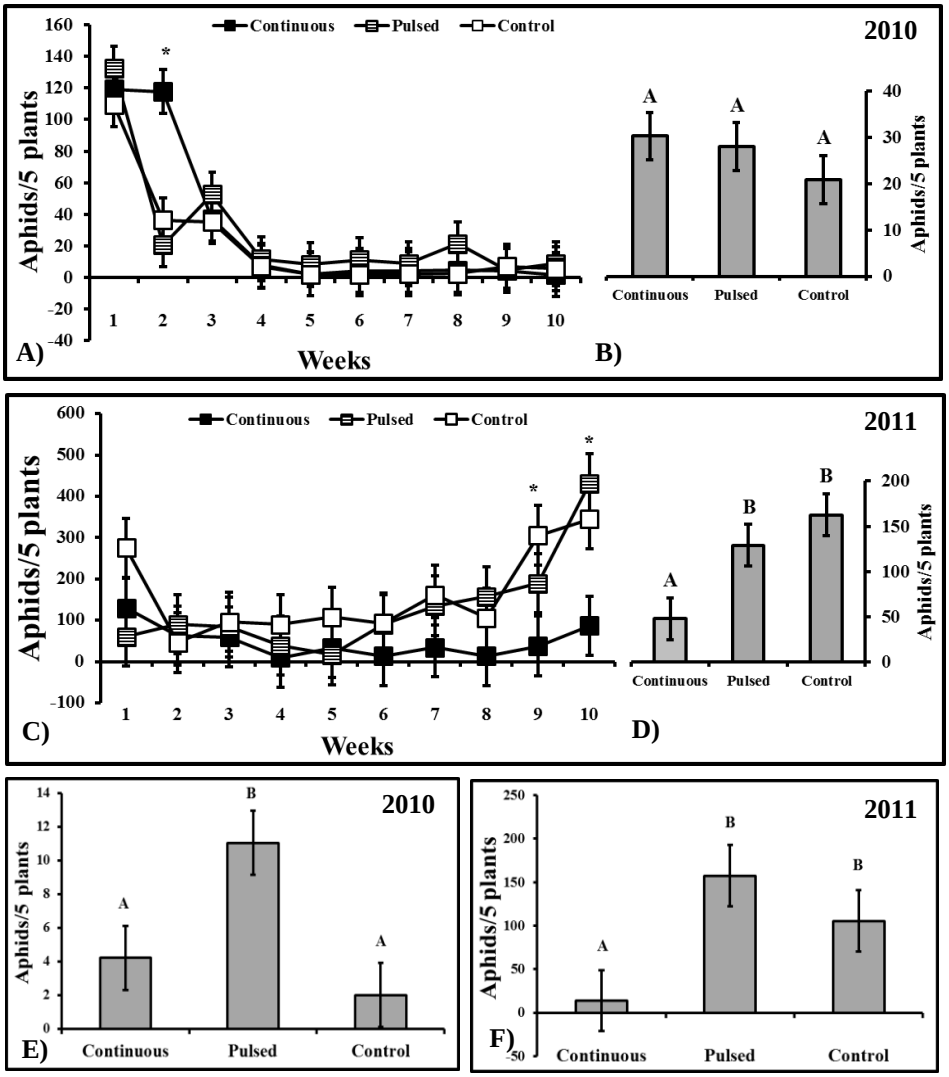

Figure 3-5. Aphid abundance over time on stressed plants in 2010 and 2011. Aphid abundance on water-stressed plants in 2010 (A), 2011 (C), and average aphid abundance on stressed plants in 2010 (B) and 2011 (D). Also, aphid abundance during the pulse in 2010 (E) and during the second pulse in week 8 in 2011 (F). Markers and bars are means $\pm$SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars with different letters above them are significantly different.

In 2011, aphid abundance marginally varied over the season (abundance: Λ= 0.56, $F_{18, 86}$= 1.6, p= 0.0791), treatment had an effect (treatment: $F_{2, 510}$= 6.71, p= 0.0013), and the treatments were marginally significant over time (week*treatment: Λ=

0.56, $F_{18, 86}$= 1.6, p= 0.0791) (Fig. 3-5C). Furthermore, average aphid abundance differed among treatments (treatment: $F_{29, 537}$= 6.48, p= 0.0017; Fig. 3-5D) with 129 aphids/5 plants on pulsed stressed plants compared to 48 aphids/5 plants on continuously stressed plants (Fig. 3-5D). In addition, aphid abundance was significantly different among treatments during the first pulse in week 5 (pulse 1: treatment: $F_{2, 51}$= 4.19, p= 0.0206, data not shown) but not between stress treatments. Moreover, aphid abundance was significantly different among treatments during the second pulse in week 8 (pulse 2: treatment: $F_{2, 51}$= 4.26, p= 0.0194; Fig. 3-5F) with 157 aphids/5 plants on pulsed stressed plants compared to 14 aphids/5 plants on continuously stressed plants (Fig. 3-5F). During the third pulse in 2011, aphid abundance did not differ among treatments ($F_{2, 51}$= 1.30, p= 0.2813), but there were 431 aphids/5 plants on pulsed stressed plants compared to 86 aphids/5 plants on continuously stressed plants and was not significantly different due to high variation (data not shown).

Aphid abundance in clip cages did not vary throughout the season (abundance: Λ= 0.93, $F_{8, 96}$= 0.42, p= 0.905) and the treatments had no effect (treatment: $F_{2, 255}$= 0.54, p= 0.5829, week*treatment: Λ= 0.93, $F_{8, 96}$= 0.42, p= 0.905; Fig. 3-6A). In addition, average aphid abundance for the season in clip cages did not differ among treatments (treatment: $F_{2, 267}$= 0.44, p= 0.6441; Fig 3-6B). Aphids in clip cages declined in abundance to zero aphids after week 7.

Water stress influenced the associations between aphids and nutrients, but not for natural enemies. In 2010 on continuously stressed plants, changes in aphid abundance were positively associated with changes in concentrations of amino acids (R^2 = 0.2554,

$F_{1, 48}$= 16.47, p= 0.0002) and aphid abundance was more strongly associated with amino acids on control plants (R^2 = 0.4313, $F_{2, 46}$= 34.88, p<0.0001) (Figs. 3-7A and 3-7B). In contrast, aphids on pulsed stressed plants in 2010 were negatively associated with digestible carbohydrates (R^2 = -0.2114, $F_{1, 48}$= 12.87, p= 0.0008; Fig. 3-7C). In 2011, aphids were positively associated with the abundance of natural enemies on control and water-stressed plants cotton plants (R^2 =0.4853, $F_{1, 178}$= 167.81, p<0.0001; Fig. 3-7D).

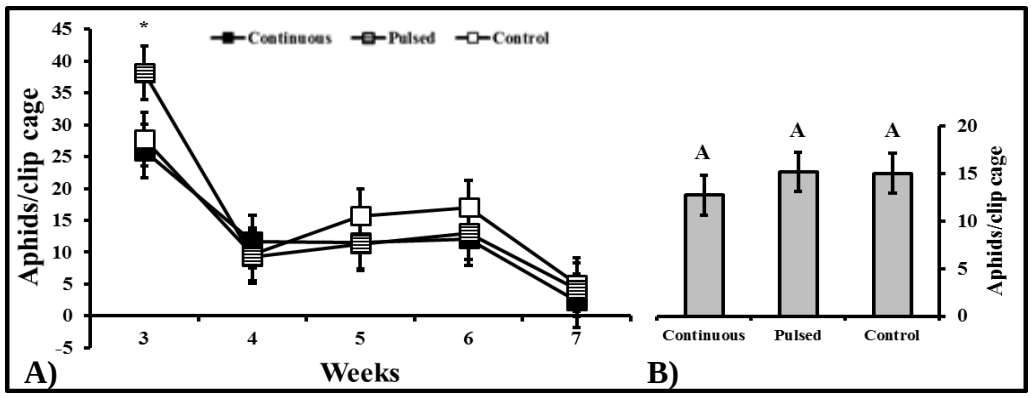

Figure 3-6. Aphid abundance in clip cages on stressed plants in 2010. Aphid abundance in clip cages on water-stressed and control plants in 2010 (A) and average aphid abundance in clip cages (B). Markers and bars are means ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars with different letters above them are significantly different.

Thrips were more abundant on pulsed stressed plants compared to continuously stressed plants during both years. During the first pulse in 2010, water stress had a significant effect on thrips abundance (treatment: $F_{2, 51}$= 34.06, p<0.0001; Fig 3-8A) and pulsed stressed plants had significantly more thrips with an average of 34 thrips/5 plants compared to 7 thrips/5 plants on continuously stressed plants (Fig. 3-8A). For the first pulse in 2011, there was no significant difference between treatments (treatment: $F_{2, 50}$=

1.97, p= 0.1494; Fig. 3-8B). During the next two pulses in 2011, stress had significant effects on thrips abundance (first pulse: $F_{2, 51}$= 4.33, p= 0.0184; second pulse:

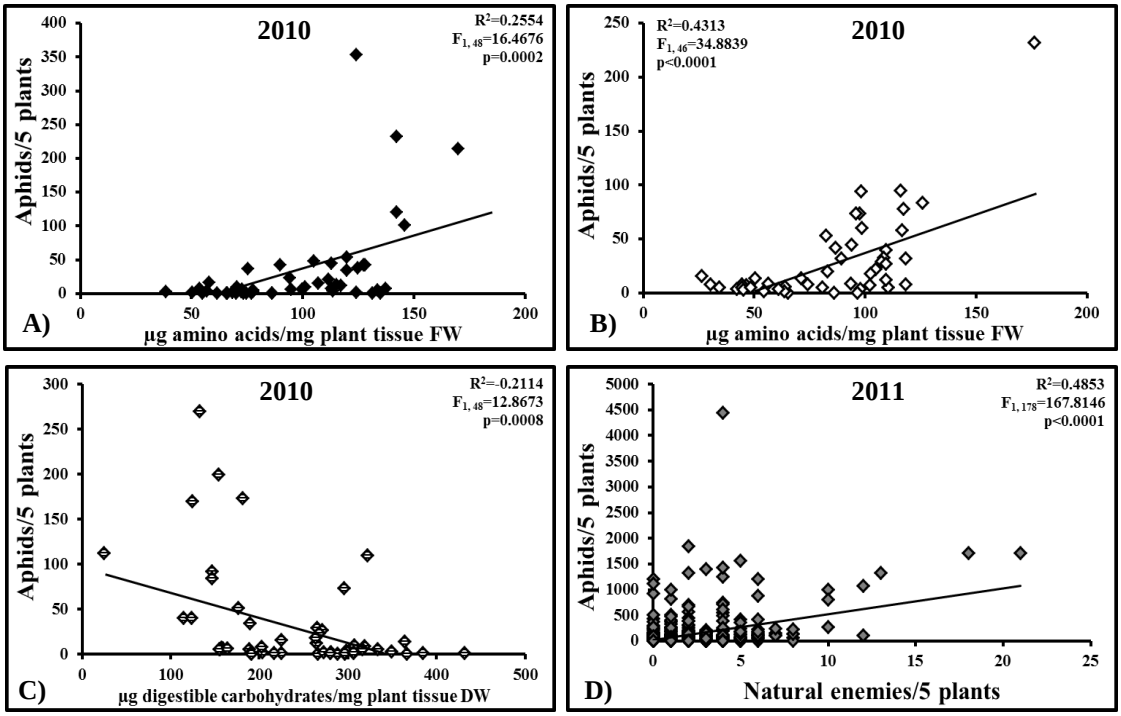

Figure 3-7. Regression analyses between aphids and nutrients in stressed plants. Seasonal aphid abundance with amino acids on continuously stressed plants in 2010 (A), with amino acids in control plants in 2010 (B), with digestible carbohydrates in pulsed stressed plants in 2010 (C), and with the seasonal abundance of natural enemies on all cotton plants in 2011 (D).

$F_{2, 51}$= 14.05, p<0.0001; Figs. 3-8C and 3-8D) and thrips were significantly more abundant on pulse stressed plants with an average of 15 and 7 thrips/5 plants compared to 3 and 2 thrips/5 plants, respectively, on continuously stressed plants (Figs. 3-8C and 3-8D).

Stink bugs and leafhoppers were significantly more abundant on pulsed stressed plants in 2010. Water stress marginally affected stink bug abundance (treatment: $F_{2, 51}$=

2.46, p= 0.0956; Fig. 3-9A) and stink bugs were 10 times more abundant on pulsed stressed plants compared to continuously stressed plants (Fig 3-9A). Leafhoppers were affected by stress (treatment: $F_{2, 51}$= 6.54, p= 0.0030; Fig. 3-9B) and only occurred on pulsed stressed plants with an abundance of 0.27 leafhoppers/5 plants (Fig. 3-9B).

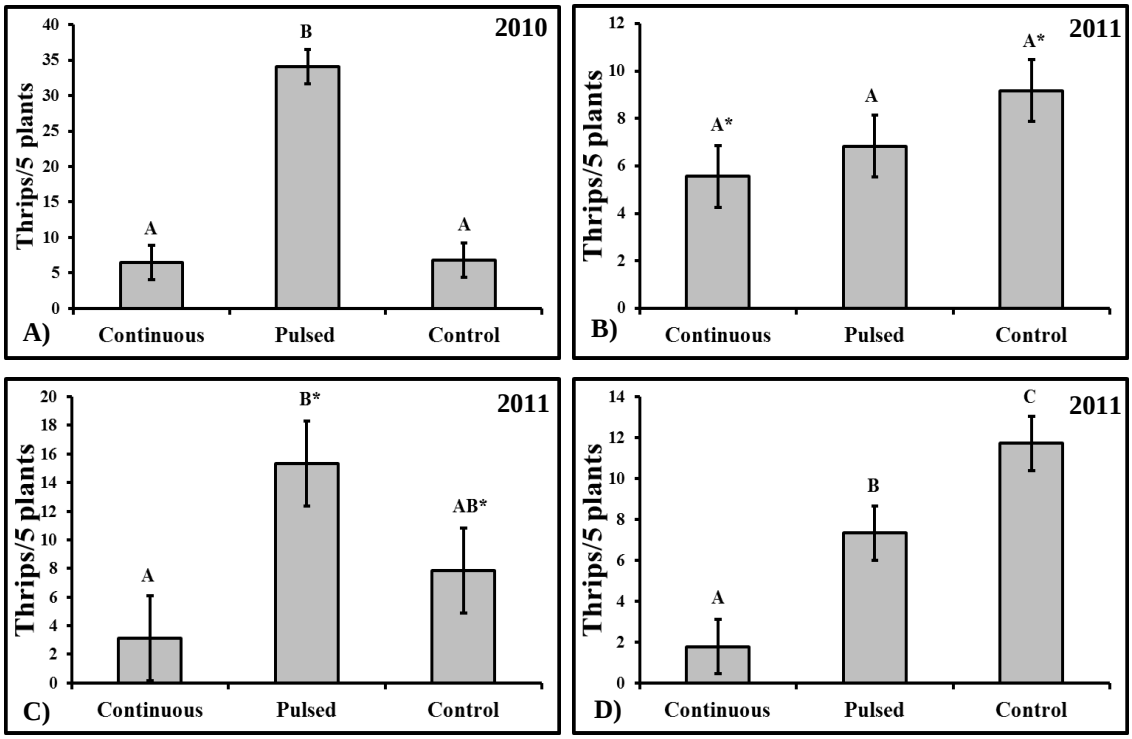

Figure 3-8. Thrips abundance on stressed plants during pulses in 2010 and 2011. During the pulse in 2010 (A) and for the pulses during week five (B), week eight (C), and week ten (D) in 2011. Bars are abundance means ±SE. Bars with different letters above them are significantly different and letters with asterisks indicate a marginal significant difference.

Cotton fleahoppers varied in abundance on water-stressed plants, but were most abundant on pulsed stressed plants. The treatments significantly affected fleahopper abundance during the pulse in 2010 (treatment: $F_{2, 51}$= 6.16, p= 0.0040; Fig. 3-10A) and

there were 3.3 fleahoppers/5 plants on pulsed stressed plants compared to 1.7 on continuously stressed plants (Fig. 3-10A).

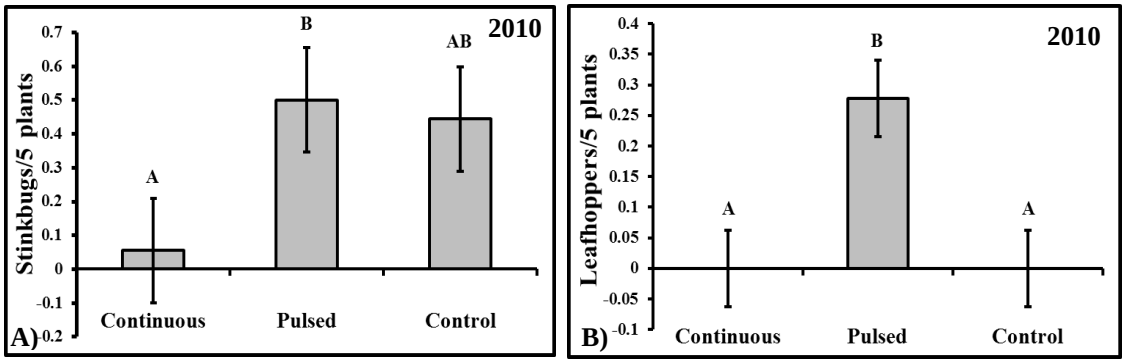

Figure 3-9. Stink bug abundance on stressed plants during pulses in 2010 and 2011. Stink bug (A) and leafhopper (B) abundance. Bars are means ±SE and bars with different letters above them are significantly different.

During the first two pulses in 2011, there were no significant differences between stress treatments (first pulse: $F_{2, 51}= 0.29$, $p= 0.7486$; second pulse: $F_{2, 51}= 15.24$, $p<0.0001$; Figs. 3-10B and 3-10C). During the third pulse in 2011, however, there were differences ($F_{2, 51}= 6.77$, $p= 0.0025$; Fig. 3-10D) with 2.7 fleahoppers/5 plants on pulsed stressed plants compared to 0.1 fleahoppers/5 plants on continuously stressed plants (Fig. 3-10D).

Whiteflies varied in abundance on water-stressed plants, but there were some notable differences. There was no significant difference between treatments for the first pulse in 2011 (treatment: $F_{2, 51}= 1.72$, $p= 0.1885$; Fig. 3-11A), but there was a marginally significant difference between whitefly abundance on pulsed and continuously stressed plants (Fig. 3-11A). Additionally, whitefly abundance differed

between treatments during the second pulse (treatments: $F_{2, 51}$= 2.74, p= 0.0742) and were 10 times more abundant on pulsed stressed plants compared to continuously stressed plants, but there was no difference between treatments during the third pulse (treatment: $F_{2, 51}$= 1.0961, p= 0.3419) (Figs. 3-11B and 3-11C).

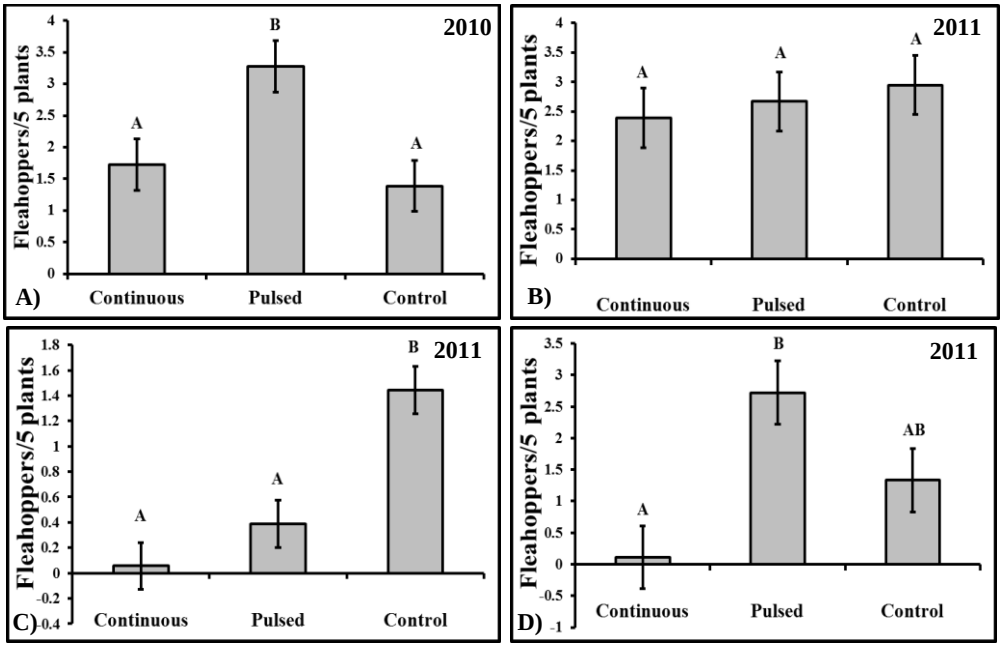

Figure 3-10. Fleahopper abundance on stressed plants during pulses in 2010 and 2011**.** During the pulse in 2010 (A) and for the pulses during week five (B), week eight (C), and week ten (D) in 2011. Bars are means ±SE and bars with different letters above them are significantly different.

3.3.3 Chewing herbivores and natural enemies

Caterpillars such as cabbage loopers (Lepidoptera: *Trichoplusia ni*), cotton bollworms (Lepidoptera: *Heliothis zea*), beet armyworms (Lepidoptera: *Spodoptera exigua*), various inchworm species (Lepidoptera: Geometridae), and American and

lubber grasshoppers (Orthoptera: *Schistocerca sp.* and *Brachystola sp*.) were the dominant defoliating herbivores in our cotton plots.

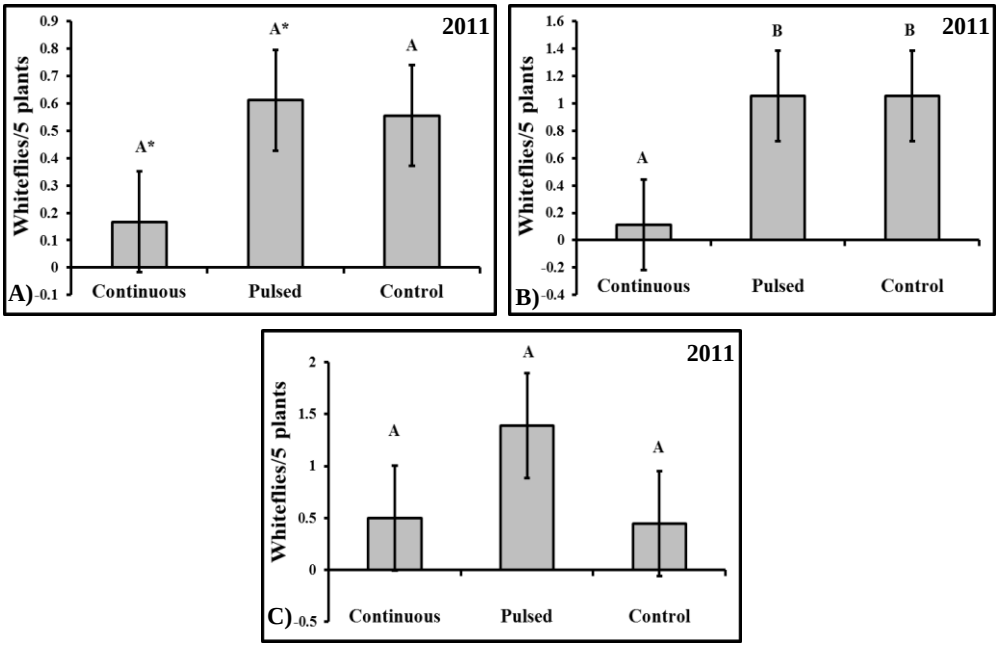

Figure 3-11. Whitefly abundance on stressed plants during pulses in 2010 and 2011. Abundance for the pulses during week five (A), week eight (B), and week ten (C) in 2011. Bars are means ±SE and bars with different letters above them are significantly different and letters with asterisks indicate a marginal significant difference.

In 2010, the abundance of chewing herbivores did not vary throughout the season (abundance: $\Lambda = 0.59$, $F_{18, 86} = 1.43$, $p = 0.1388$), the stress treatments had a significant effect (treatment: $F_{2, 510} = 8.13$, $p = 0.0003$), and the treatments had no effect over time (week*treatment: $\Lambda = 0.59$, $F_{18, 86} = 1.43$, $p = 0.1388$) (Fig. 3-12A). In addition, the average abundance of chewing herbivores varied between treatments (treatment: $F_{2, 51} = 7.46$, $p = 0.0006$; Fig. 3-12B) and was 2.5 times greater on pulsed stressed plants compared to continuously stressed plants (Fig. 3-12B), but there were no differences in

abundance between treatments during the pulse (treatment: $F_{2, 51}$= 0.58, p= 0.5612, data not shown) (Fig. 3-12B). In 2011, the abundance of chewing herbivores was not affected (abundance: Λ= 0.66, $F_{18, 86}$= 1.09, p= 0.3731), nor by water stress (treatment: $F_{2, 510}$= 0.83, p= 0.4365), and not affected by stress over time (week*treatment: Λ= 0.66, $F_{18, 86}$= 1.09, p= 0.3731) (Fig. 3-12C). Chewing herbivores on average were not affected by stress treatment (treatment: $F_{2, 537}$= 0.82, p= 0.4415; Fig. 3-12D) and were not affected during the pulses (pulse 1: treatment: $F_{2, 51}$= 1.3, p= 0.2818; pulse 2: treatment: $F_{2, 51}$= 0.5, p= 0.61; pulse 3: treatment: $F_{2, 51}$= 1.19, p= 0.3137; data not shown).

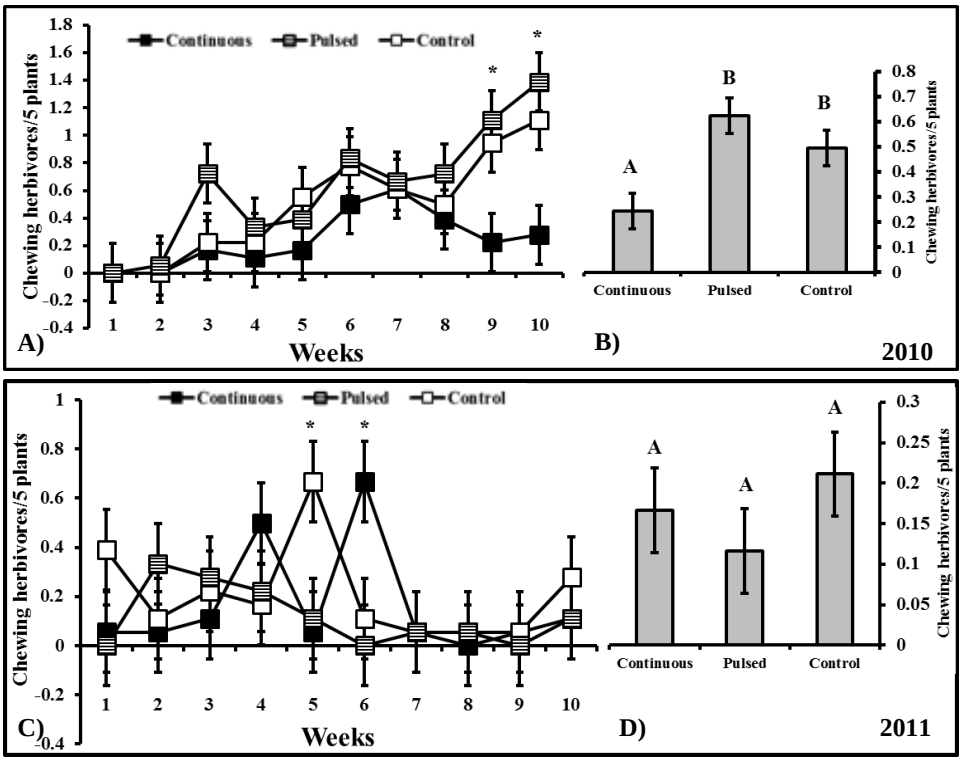

Figure 3-12. Abundance of chewing herbivores on stressed plants in 2010 and 2011. Weekly abundance in 2010 (A), 2011 (C), and average abundance of chewing herbivores on stressed plants in 2010 (B) and 2011 (D). Markers and bars are means ±SE. Asterisks indicate significant differences between at least two of the three treatments for a given week. Bars with different letters above them are significantly different.

Water stress influenced the amount of leaf tissue removed from our cotton plots. During the pulse in 2010, the amount of leaf area missing differed between treatments (treatment: $F_{2, 51}$= 16.98, p<0.0001; Fig. 3-13A), pulsed stressed plants had 9% leaf area removed compared to 6% removed from continuously stressed plants. For the first two pulses in 2011, there were no significant differences between the treatments (pulse 1: treatment: $F_{2, 51}$= 0.31, p= 0.7315; pulse 2: treatment: $F_{2, 51}$= 4.40, p= 0.0173; Figs. 3-13B and 3-13C). During the last pulse in 2011, however, treatments had an effect (treatment: $F_{2, 51}$= 5.47, p= 0.0070; Fig. 3-13D) and pulsed stressed plants had 11% leaf area removed compared to 6% from continuously stressed plants.

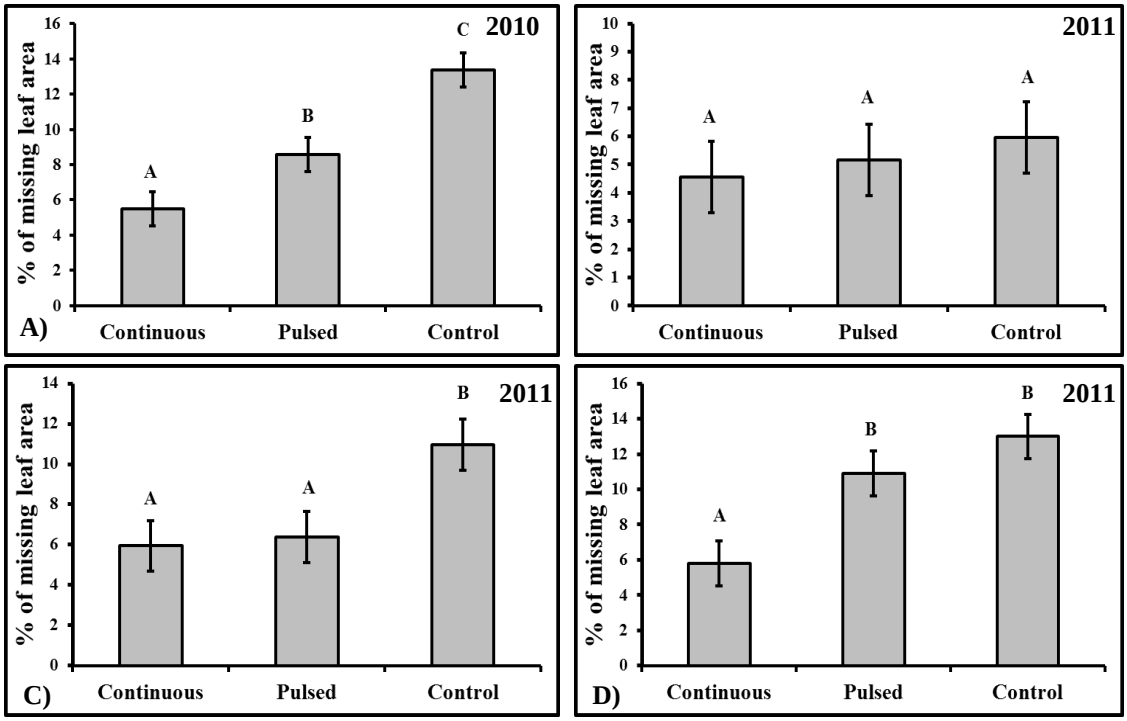

Figure 3-13. Missing leaf area from stressed plants in 2010 and 2011. During the pulse in 2010 (A) and for the pulses during week five (B), week eight (C), and week ten (D) in 2011. Bars are means ±SE and bars with different letters above them are significantly different.

Predominant natural enemies included: convergent ladybird beetles (Coleoptera: *Hippodamia convergens*), spotted lady beetles (Coleoptera: *Coleomegilla maculata*), lacewings (Neuroptera: *Chrysopa spp.*), big-eyed bugs (Hemiptera: *Geocoris sp.*), minute pirate bugs (Hemiptera: *Orius spp.*), red imported fire ants (Hymenoptera: *Solenopsis invicta*), orb weaver spiders (Araneae: *Acanthepeira stellata*, *Tetragnatha laboriosa*), crab spiders (Araneae: *Misumenoides formosipes*, *Misumenops celer*), striped lynx spiders (Araneae: *Oxyopes salticus*), winter spiders (Araneae: *Cheiracanthium inclusum*), and grey dotted spiders (Araneae: *Aysha gracilis*).

The abundance of natural enemies significantly differed between treatments during the pulse in 2010 (treatment: $F_{2, 51}= 13.24$, $p<0.0001$; Fig. 3-14A), with 7 times more natural enemies on pulsed stressed plants than continuously stressed plants (Fig. 3-14A). During the first two pulses in 2011, there were no significant differences between stress treatments (pulse 1: treatment: $F_{2, 51}= 0.19$, $p= 0.8252$), but there were differences during the second pulse (pulse 2: treatment: $F_{2, 51}= 4.03$, $p= 0.0237$). In addition, there were no differences between pulsed and continuously stressed plants during the first two pulses (Figs. 3-14B and 3-14C). During the third pulse, however, there were significant differences among treatments ($F_{2, 51}= 10.81$, $p= 0.0001$; Fig. 3-14D) with five times more natural enemies on pulsed stressed plants compared to continuously stressed plants (Fig. 3-14D).

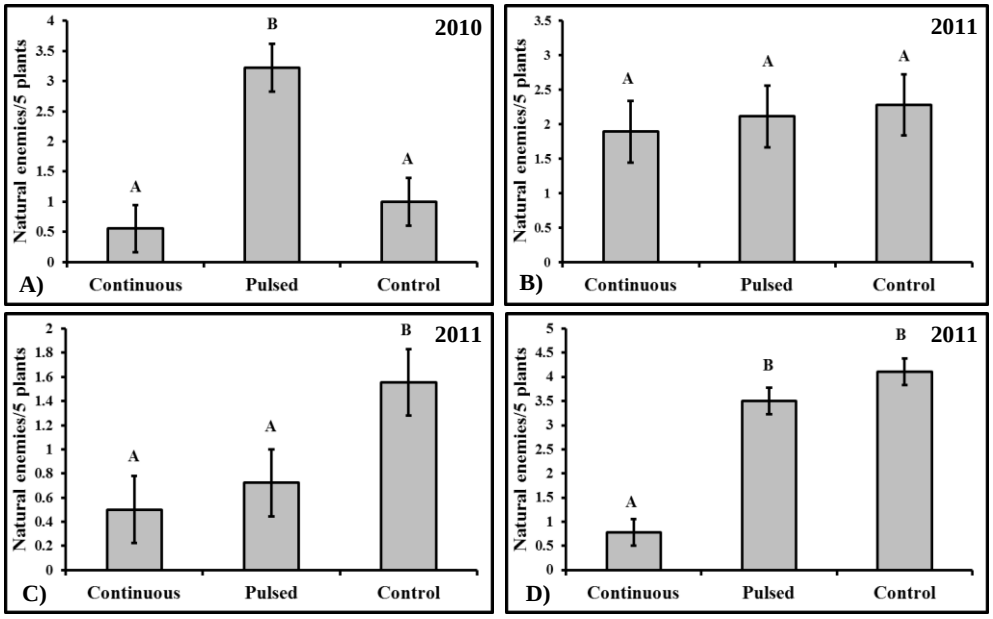

Figure 3-14. Natural enemy abundance on stressed plants in 2010 and 2011. During the pulse in 2010 (A) and for the pulses during week five (B), week eight (C), and week ten (D) in 2011. Bars are means ±SE and bars with different letters above them are significantly different.

3.4 Discussion

We found that pulsed and continuous water stress had contrasting effects on insect herbivores, suggesting that the type of water stress influences herbivore abundance. Piercing-sucking herbivores were significantly more abundant on pulsed stressed plants than continuously stressed plants, especially thrips and stink bugs (Figs. 3-8, 3-9). Chewing herbivores, on the other hand, were not affected by water stress in general and their abundance was inconsistent even on control plants (Fig. 3-12).

Cotton aphids feeding on water stressed plants were not affected by stress type or duration for the majority of the study. In another study, green spruce aphids (*Elatobium abietinum*), however, increased in abundance when feeding on pulsed stressed spruce trees, with up to 300% differences in aphid abundance on pulse stressed plants compared

to continuously stressed plants (Major 1990). In addition, apple aphids (*Aphis pomi*), preferred well-watered plants compared to pulsed stressed apple trees (Mody et al. 2009). Furthermore, a meta-analysis on water stress and arthropod herbivores suggests that aphids decrease in abundance on water-stressed plants (Chapter V). Previous studies suggested that aphid response would vary in response to water stress, but in our study cotton aphids significantly increased in abundance in the beginning of the season in 2010 and at the end in 2011 regardless of water stress (Fig. 3-5). While in enemy-free space (clip cages), aphids still decreased in abundance on control and stressed plants, suggesting that there may be factors besides predation and stress-induced changes in plants that influence aphid abundance (Fig. 3-6).

Aphids may have been more sensitive to developmental and ontological changes in the defensive chemistry of cotton plants throughout the season. For instance, seedlings are predicted to have the lowest defensive capabilities against herbivores as they allocate resources to root growth and establishment (Boege and Marquis 2005). After the seedling stage, plant defenses increase significantly and are at their peak during the reproductive stage, and decline after the reproductive stage as plants mature (Ritchie et al. 2004, Weiner 2004, Boege and Marquis 2005). Aphid abundance in our study followed this pattern in response to plant defense with aphid abundance highest when plants were most vulnerable during the late seedling stage in 2010 and when plants were maturing in 2011. During the pulse in 2010 and the second pulse in week 8 in 2011, however, aphids were significantly more abundant on pulsed stressed plants, but were only more abundant during these particular pulses (Fig. 3-5E and 3-5F). In addition,

aphids were strongly associated with nutrients in stressed plants, but these associations were positive for amino acids and negative for carbohydrates (Fig. 3-7A and 3-7B). This suggests that the ontogeny and development of cotton plants may be important in influencing aphid abundance and stress has inconsistent effects on aphid abundance. Chapter IV addresses aphid response to stressed plants by directly testing the interactive effects of water stress and cotton development on aphid abundance.

Very few studies have determined the effects of continuous and pulsed water stress on insect herbivores, but our study illustrates that different types of water stress should be considered when predicting herbivore response to water-stressed plants. Other studies show that piercing-sucking herbivores are more abundant on irrigated plants or slightly stressed plants. For example, piercing-sucking herbivores feeding on creosote bush increased in abundance on fully irrigated to slightly water-stressed bushes and declined in abundance on severely stressed bushes (Lightfoot and Whitford 1987). Scale insects (Hemiptera: Coccoidea), whiteflies, psyllids (Hemiptera: Psyllidae), sharpshooters (Hemiptera: Cicadellidae), thrips, and other piercing-sucking herbivores responded variably to water-stressed creosote bush, suggesting that herbivores within the same feeding guild will respond differently to water stress (Schowalter et al. 1999). In our study, thrips had the greatest response to pulsed stressed plants, and were significantly more abundant during pulses. In addition, leafhoppers, fleahoppers, stink bugs, and whiteflies were more abundant on pulsed stressed plants; however, these herbivore responses were not consistent and varied in magnitude. The variation we see

in herbivore response, therefore, may be partly due to herbivores within the same feeding guild responding differently to the same stressed plants.

Throughout our study, continuously stressed plants never had a significantly greater abundance of herbivores compared to pulsed stressed plants, while pulsed stressed plants had greater herbivore abundance more often, suggesting that there may be distinct physiological differences in plants that cause increased herbivore vulnerability in pulsed stressed plants. Stress severity is known to have a differential impact on plants; for instance, alterations in CO_2 assimilation rate, protein synthesis, and hormone signaling can occur in some plants at approximately -0.4 MPa of stem water potential, whereas wilting becomes visible at -1.2 MPa or lower (Hsiao 1973, Chaves et al. 2002, Lombardini 2006, Taiz and Zeiger 2010). Continuously stressed plants were typically below -1.2 MPa during our study and herbivores feeding on those plants may have experienced leaf tissue with lower water content, tougher leaves, and other physiological changes associated with leaf wilting and necrosis (Chaves et al. 2002, Jithesh et al. 2006, Anjum et al. 2011). Prolonged drought can cause concentrations of proline to decrease from peak accumulations after 10 days of stress (Anjum et al. 2011). Mild and moderately stressed plants may still photosynthesize and produce non-structural carbohydrates whereas photosynthesis declines dramatically in severely stressed plants (Hsiao 1973, Lombardini 2006, Taiz and Zeiger 2010). Our study illustrates that different intensities of water stress, as seen between the pulsed and continuously stressed treatments, impact herbivores differently and stress intensity plays a role in determining herbivore abundance.

The plant stress and pulsed stress hypotheses were not consistently accurate in predicting herbivore abundance on water-stressed plants. Water stress did not significantly influence concentrations of nutrients during pulses, but herbivores were occasionally more abundant during pulses on pulsed stressed plants. Our study is not consistent with other studies that found significant changes in concentrations of nutrients in water-stressed cotton plants (Sadras et al. 1998, Sholwer 2002, Showler and Moran 2003), or in other plants (Barnett and Naylor 1966, Franzke and Reinhold 2011, Gutbrodt et al. 2011, Tariq et al. 2013), but the variation in herbivore response we observed is consistent with the literature (Larsson and Bjorkman 1993, Archer et al. 1995, Schowalter et al. 1999, Inbar et al. 2001, Huberty and Denno 2004). This suggests that increased water content and other factors associated with the alleviation of stress outside of increased nutrients may play a role in determining the abundance of herbivores on stressed plants.

Our study demonstrated the complex interactions between water-stressed plants, herbivore abundance, and stress-related nutrients. Stress frequency and severity influenced herbivore abundance and may have influenced nutrient preferences for herbivores. Herbivore abundance on stressed plants was inconsistent and we could not support the plant and pulsed stress hypotheses. To accurately predict herbivore abundance on water-stressed plants, we need to consider the roles that stress frequency, stress severity, nutrient concentration, and plant development serve and the impact these interactions have on herbivore abundance.

CHAPTER IV

THE IMPACTS OF THE TIMING OF APHID INFESTATION AND WATER STRESS ON COTTON DEVELOPMENT, PHYSIOLOGY, AND YIELD

4.1 Introduction

Water availability is one of the most limiting factors of crop productivity (Hsiao et al. 1976, Alishaha and Ahmadikhah 2009, Sinclair and Rufty 2012). Drought is expected to become more frequent with climate change and have a greater impact on crop yield and pest resistance (Mishra and Singh 2010, Dai 2011). In June 2012, drought alone was responsible for the loss of 45 million tons of maize and 4.2 million tons of soybeans in the USA (Gilbert 2012). In addition to increased drought, climate change is expected to lengthen growing seasons, potentially increasing crop exposure to pests that may develop faster and become more abundant (Smith et al. 2012, Wolkovich et al. 2012, Malcolm et al. 2013). Drought-stressed plants also become more attractive to insect pests as amino acids and other nutrients become concentrated and can lead to pest outbreaks (White 1969, Huberty and Denno 2004, Jithesh et al. 2006). For example, populations of inchworms (Lepidoptera: Geometridae), gall midges (Diptera: Cecidomyiidae), and aphids (Hemiptera: Aphididae) have been known to increase in abundance on stressed plants, particularly during seasonal events such as El Niño (Waring and Price 1990, Archer et al. 1995, Schowalter et al. 1999, Garrett et al. 2013). Determining the interactions between the effects of water stress and increased pest

abundance and their effects on crop productivity is essential to developing crop management strategies for the future.

The cotton aphid (*Aphis gossypii*) is an economically important pest of cotton plants (*Gossypium hirsutum* L.). Cotton aphids are quite abundant during the early season, but are not typically a pest later in the season due to strong compensatory abilities of cotton to early season damage and pressure from natural enemies such as ladybird beetles (Coleoptera: Coccinellidae) and parasitoid wasps (Hymenoptera) (Rosenheim et al. 1995, Rosenheim et al. 1997, Cisneros and Godfrey 2001). Drought stress, on the other hand, greatly influences cotton lint yield throughout the growing season, particularly during boll development (Guinn and Service 1982, Rosenheim et al. 1995, Freeland et al. 2006, Bengough et al. 2011). Drought stress can decrease lint yield by impeding nutrient absorption, reducing the production and expansion of sympodial leaves, impairing photosynthesis, and altering sink-source relationships between leaves and squares (Daniel et al. 1999, Pettigrew 2004, Pregitzer and King 2005, Freeland et al. 2006, Taiz and Zeiger 2010). Cotton aphids, however, may increase in abundance when feeding on water-stressed plants and increase their impact on cotton yield throughout the season. The Plant Stress Hypothesis predicts that herbivores should increase in abundance on water-stressed plants due to foliar increases in nitrogen and the Pulsed Stress hypothesis predicts that intermittent or pulsed stress is best for piercing-sucking herbivores such as aphids (White 1969, Huberty and Denno 2004, Mody et al. 2009). During drought stress, plants accumulate stress-related compounds such as nitrogen containing compounds, antioxidant enzymes (e.g., peroxidase) that alleviate the negative

effects of water stress. These stress-related compounds, however, may be beneficial for herbivores since they contain essential nutrients and nitrogen. In addition, aphids may induce symptoms of drought stress in fully irrigated plants and the occurrence of drought stress and aphid infestation simultaneously, individually, or in sequence may result in significant changes in plant development (Riedell 1989, Willis et al. 1993, Carbrera et al. 1994). The benefits of water stress may increase the abundance of aphids later in the season, making the timing of aphid infestation an important factor to consider. To our knowledge, the effects of simultaneous drought and the timing of aphid infestation have not been tested on cotton development and lint production. Previous studies have tested drought and aphid interactions on other plants, but these studies focused on plant growth and not yield or aphid abundance (Riedell 1989, Willis et al. 1993, Carbrera et al. 1994). As drought is predicted to become more prevalent in the future, understanding the interactions between the effects of drought and herbivory is fundamental to maintaining crop productivity with our expanding global food and material demands. Our goal was to determine the combined effects of drought and the timing of aphid infestation on cotton development and lint yield. Pulsed water stress was imposed in field conditions in an agro-ecosystem and aphids were added to cotton plants in enemy-free cages during the seedling or squaring stage. We predicted that water deficit stress will be more problematic for plants that also have aphids added at the seedling stage, than for those with aphids at the squaring stage. In addition, well-watered control plants should produce the most lint and outperform water-stressed plants. We also predict that cotton plants will compensate for aphid damage early in the season, but should still develop

slower and produce less lint that well-watered control plants. This was the first study to directly assess the interaction between drought stress, timing of aphid infestation, cotton development, and their impacts on cotton lint yield.

4.2 Methods

4.2.1 Study system

We conducted a 10-week field study in 2012 at the Texas A&M Field Laboratory in Burleson County, Texas (coordinates: 30.548754,-96.436082). The south-central region of Texas experiences temperature and subtropical climates with mild winters lasting less than two months. High temperatures range from 25°C in May to 35°C in July, and precipitation ranges from 11.94 cm in May to 5.08 cm in July. Our field site primarily consisted of Belk series clay soil, known for very deep, well drained, slowly permeable soils common of Texas flood plains (U.S.A. 2007). On 24 April, 2012 we planted approximately 0.3 hectares of commercial cotton (*Gossypium hirsutum L.*), Delta Pine 174RR Flex (no drought/pest resistance). Cotton was irrigated on 8 and 21 May, and treatments began on 4 June (week 0) when plants were in the seedling (4-6 leaves) and squaring stage. Prior to the implementation of treatments, all plants were treated with two applications of spinosad to remove thrips and other herbivores from plants prior to the study by mixing from concentrated "Green Light Lawn & Garden Spray with SPINOSAD" (Green Light Co., San Antonio, TX). Concentrated formula was diluted to 59.2 ml/3.8 L of water and each plant received two applications with three days between

applications. The field was treated with Roundup herbicide to eliminate weeds and fertilized with 14.69 kg of nitrogen/hectare with a time-release formula.

4.2.2 Experimental design

Cotton plants were grown in 60, 182.88 cm^3, UV-resistant Lumite field cages, with 1 mm^2 fine mesh cages (Lumite Inc., Gainesville, GA), with 10 cotton plants per cage. Cages were arranged into eight blocks with each block randomly receiving stress or control irrigation on a weekly basis. Plants were randomly chosen to receive aphids during the seedling or squaring stage. Plants that were going to receive aphids at the squaring stage were watered to emerge early and reach squaring stage by the start of the study. Plants that were going to receive aphids at the seedling stage had their emergence delayed by withholding water to synchronize their seedling emergence with the squaring treatment plants. Eighteen (18) cages received aphids at the seedling stage, 27 at the squaring stage (fruiting), and 15 cages were the non-aphid control. For the stress treatment, plants were pulsed stressed (rather than continuously stressed) to allow the plants to survive the 10-week growing season to produce lint and to simulate dry field conditions. Plants in cages were stressed or watered weekly as a control (field capacity) with furrow irrigation, and aphids were added during the seedling or squaring stage, or no aphids (aphid control) in a randomized complete block design. All cages were separated by three meters of cotton. For the stressed plants, we used a pressure chamber (model 615, PMS Instrument Co., Albany, OR) to measure water status and to trigger irrigation. Cotton plants are water-stressed at approximately -1.2 MPa (-12 bars) and

begin to accumulate stress-induced increases in foliar nitrogen and other nutrients (Hsiao 1973, Lombardini 2006). Stressed plants received water when their water potential was below -1.2 MPa. For the pressure chamber measurements, 17 cm x 9 cm aluminum bags were placed over the uppermost, fully-expanded leaf for 20 minutes, and then cut at the proximal end of the petiole using scissors. The aluminum bag, with the leaf still inside, was then folded gently and inserted into the chamber for a pressure reading. To accommodate for the destructive sampling for the pressure chamber measurements, the 1st and 4th cages within a block had a single leaf removed for this measurement one week, the 2nd and 6th cages the next week, and the 3rd and 8th cages the following week. This cycle was alternated throughout the season and a single plant was only sampled once. Data for the pressure bomb was analyzed based on water treatment and not for individual aphid infestation-water treatment combinations due to limited plants within each cage for a total of 8 repetitions per water treatment per week.

For aphid additions, 25 aphids were added to the plant with a fine tipped paintbrush from adjacent plants outside the cage. For plants with the aphid addition during the squaring stage, seedling plants had aphids removed by hand using cotton balls and 25 aphids were added once the plants started squaring. Aphids were added a week before the study began (week zero) to allow the aphids to establish prior to water stress. For the aphid-free control plants, aphids were removed by hand using cotton balls. Aphids were counted weekly on the top 15 cm of 3 randomly selected plants (including leaves, stems, squares) per cage. During the second week of the season (June 18th-24th), aphids reached populations large enough to induce “black sooty mold” on the honeydew

covering infested plants which can lead to decreased plant vigor and photosynthetic rate, and lead to severe economic injury (Shannag et al. 1998, Rondon et al. 2005). To temporarily lower aphid populations and reduce mold, 20 convergent lady beetles (Coleoptera: *Hippodamia convergens*, purchased from Rincon-Vitova Insectaries, Inc., Ventura, CA) were placed within cages (including aphid-free cages) for 3 days and were then removed along with any lady beetle eggs.

4.2.3 Amino acid and digestible carbohydrate assays

Amino acids and digestible carbohydrates were measured in cotton plants to compare the influence of stress and the timing of aphid herbivory on nutrient concentrations in cotton plants. Each week, the uppermost fully-expanded leaf of 5 plants within a randomly selected subplot was removed. Leaves were quickly and gently placed into 9 cm x 5.75 cm coin envelopes and stored in ice coolers and quickly transported to the laboratory. Once in the laboratory, the envelopes were placed in -80°C freezers until assayed.

Plant chemistry assays for amino acids were conducted using a modified ninhydrin assay as according to Starcher (2001) and McArthur et al. (2010). Ten samples were randomly chosen per treatment from each week of the study to measure changes in amino acid concentration over time. For each sample, approximately 5 mg of tissue was removed and ground in 10 µl of 80% ethanol in an Eppendorf tube using a manual tissue grinder and placed on ice. Once the tissue was ground, 500 µl of 6N HCl was added to the sample tube and placed in a heating block at 100°C for 24 hours. A large block was

placed on top of the tubes to ensure that the caps stayed closed. During the last 2.5 hours of the 24 hour period, the large blocks were removed and each tube was opened to allow the HCl to boil off. The remaining pellet was suspended in 1ml of water and centrifuged at 12,000 rpm for 1 minute to facilitate sample homogeneity. The ninhydrin stock solution was prepared using 200 mg of ninhydrin, 7.5 ml of ethylene glycol, 2.5 ml of 4N sodium acetate buffer and 200 µl of stannous chloride solution. In a new Eppendorf tube, 20 µl of sample and 100 µl of the ninhydrin solution were added and returned to the 100°C heating block for 10 minutes. Samples were allowed to cool and the sample and ninhydrin mixtures were transferred to a 96-well micotitre plate. Plates were read for spectrophotometric absorbance at 570 nm using an Epoch microplate reader (BioTek Instruments Inc., Winooski, VT). If samples were too dark to be read at 570 nm due to high amino acid content, 620 nm was used and a simple linear regression equation was generated to convert amino acid concentrations from readings at 620 nm to predicted concentrations at 570 nm. Amino acid standards were prepared using powdered BSA (Sigma-Aldrich, St. Louis, MO) with dilutions prepared at 2 µg, 4 µg, 6 µg, 8 µg, and 10 µg/1 ml of water from 10 mg/1 ml of water. Standards were used to generate a standard curve to approximate µg of amino acids/5mg of plant tissue sample.

Plant chemistry assays for digestible carbohydrate content were conducted using a phenol-sulfuric acid assay as according to Smith et al. (1964) and Clissold et al. (2006). Ten frozen samples were randomly selected per treatment from each week of the study to measure changes in digestible carbohydrates over time. Approximately 200 mg of leaf tissue from each sample was dried using a freeze-dryer (Labconco, Kansas City,

MO) at -50°C for two days. Once dried, samples were ground to powdered flakes using a MF 10 basic wiley cutting mill (IKA Works, Inc., Wilmington, NC) using a size 20 mesh and 20 mg were removed and added to screw-cap test tubes. Each tube received 1 ml of 0.1M sulfuric acid and was placed in a boiling water bath for 1 hour. Tubes were cooled in a container of room temperature water, emptied into 1.5 ml Eppendorf tubes, and mixed in a centrifuge at 13,000 rpm for 10 minutes. Each tube had 15 µl of supernatant removed which was added to glass test tubes with 385 µl of distilled water and 400 µl of 5% phenol solution. Tubes then received 2 ml of concentrated sulfuric acid and allowed to sit for 10 minutes. Samples were mixed using a vortex mixer then allowed to sit for an additional 30 minutes. Carbohydrate standards were prepared using 0.2 mg/µl glucose to make six 400 µl dilutions containing 0, 15, 30, 45, 60, or 75 µg of glucose. The dilutions were treated in the same manner as the samples with the same concentrations of phenol and sulfuric acid added in the same manner. After sitting, 750 µl of the sample, phenol, and sulfuric acid mixture was added to cuvettes for spectrophotometric measurements at 490 nm with the samples being measured in duplicates and the standards in triplicate. The standards were used to generate a standard curve to approximate µg of digestible carbohydrates/20 mg of dried plant tissue.

4.2.4 Chlorophyll and peroxidase assay

Relative chlorophyll content was measured using a chlorophyll meter (SPAD model 502, Konica Minolta Sensing, Inc., Tokyo, Japan) on the uppermost fully expanded leaf of 5 plants per cage (averaged into one value per cage) each week for the

season. The SPAD-502 provides non-destructive measurements of relative chlorophyll content and has been used to monitor the nitrogen nutritional status of maize, rice, potato, and cotton (Vos and Bom 1993, Feibo et al. 1998, Chang and Robison 2003).

Peroxidase is an antioxidant enzyme in plants that neutralizes reactive oxygen species that destroy photosystems in chloroplasts during water stress and has been shown to become more concentrated in plants during water stress (Chaves et al. 2002, Jithesh et al. 2006, Taiz and Zeiger 2010). The same leaves used for the amino acid and digestible carbohydrate assays were also used for the peroxidase assays. Proteins for the peroxidase assay were extracted using 275 mg of plant tissue ground in 15 µl of 0.01M sodium phosphate buffer at pH 6.8. Once ground, samples were centrifuged at 12,000 rpm for 12 minutes, and the supernatant was kept and stored at -20°C until assayed. For the assay, 2 µl of extracted proteins was added to a 96-well plate in duplicate and 150 µl of 0.01M guaiacol solution at pH 6.0 was added to each well. Samples and plates were kept on ice. The plates containing extracted proteins and guaiacol solution were read by a microplate reader (model 680, Bio-Rad Laboratories Inc., Hercules, CA) at 470 nm. The quantity of peroxidase protein was quantified by the following equation: POD activity = (absorbance reading from software/1000)/(sample mass*0.015) and expressed as ΔAbs_{470}/min/gFW.

4.2.5 Cotton development and lint harvest

Plant height and nodes were counted weekly on 5 plants in each cage for the duration of the season. In addition, the number of fruits in the 1st (produces the most lint)

and 2nd (produces the second most lint) fruiting positions and fruit retention for 1st position bolls was recorded weekly for 5 plants in each cage. Cotton lint was removed by hand on October 13th, 2012 before the end of the Texas statewide growing season at the end of October. All the cotton lint was removed from each plant in each cage and separated by 1st and 2nd fruiting position, and the remainder (lint from vegetative and all other bolls). Cotton was ginned by hand using small scale gins for low weight samples at the Texas A&M Cotton Improvement Laboratory.

4.2.6 Analysis

Weekly and average aphid abundance, concentrations of amino acids and digestible carbohydrates, SPAD values, and peroxidase activity were analyzed to compare the effects of water stress and plants with different aphid infestations were analyzed separately. In addition, seasonal height and the number of nodes on cotton plants, boll production, lint yield, % of unopened bolls, and fruit retention were analyzed to compare the effects of the timing of aphid infestation and control and stressed plants were analyzed individually. All data collected in the study were analyzed using repeated measures ANOVA using JMP 10.0.0 (SAS Institute, Cary, NC) to make comparisons between treatments over time. Full factorial ANOVA was also conducted with JMP on pooled data to determine interactions between water stress, timing of aphid herbivory, and time.

4.3 Results

4.3.1 Stem water potential and aphid abundance

Stem water potential (MPa) was significantly different during the season (stem water potential: $F_{11, 84}$= 12.5229, p<0.0001), stress had an effect on stem water potential (treatment: $F_{2, 93}$=23.98, p<0.0001), and stress had an effect over time (stress*week: $F_{5, 90}$=4.41, p= 0.0013) (Fig. 4-1). Control plants maintained stem water potential above -1.2 MPa throughout the season, while stressed plants became water-stressed during weeks four and eight. Stressed plants reached similar stem water potential levels compared to control treated plants when they were watered (Fig. 4-1). Stressed plants went through one cycle of low to high stem water potential during the season.

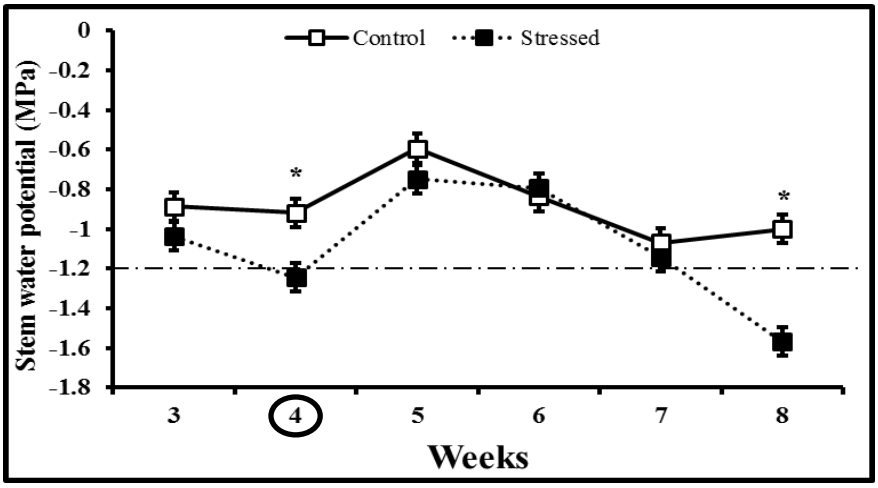

Figure 4-1. Stem water potential in stressed plants for 2012 field study. The markers are mean stem water potential ±SE. The circle over "4" indicates when water stressed plants received irrigation to end water stress. The dotted line marks -1.2 MPa, when plants are water-stressed. Asterisks indicate significant differences between treatments.

Water stress and the timing of aphid infestation had little impact on aphid abundance in general, but there were some notable differences. When added during the seedling stage, aphids significantly varied throughout the season (abundance: $F_{21, 159}$= 6.26, p<0.0001), but water stress did not have an effect (stress: $F_{1, 179}$= 0.0360, p=0.8497), and there was no effect over time (stress*week: $F_{11, 84}$= 1.36, p= 0.2047) (Fig. 4-2A). Aphid abundance, however, was 3 times greater on control plants during week 2 and 56% higher on stressed plants during week 3 (Fig. 4-2A). When aphids were added during the squaring stage, aphid abundance varied throughout the season

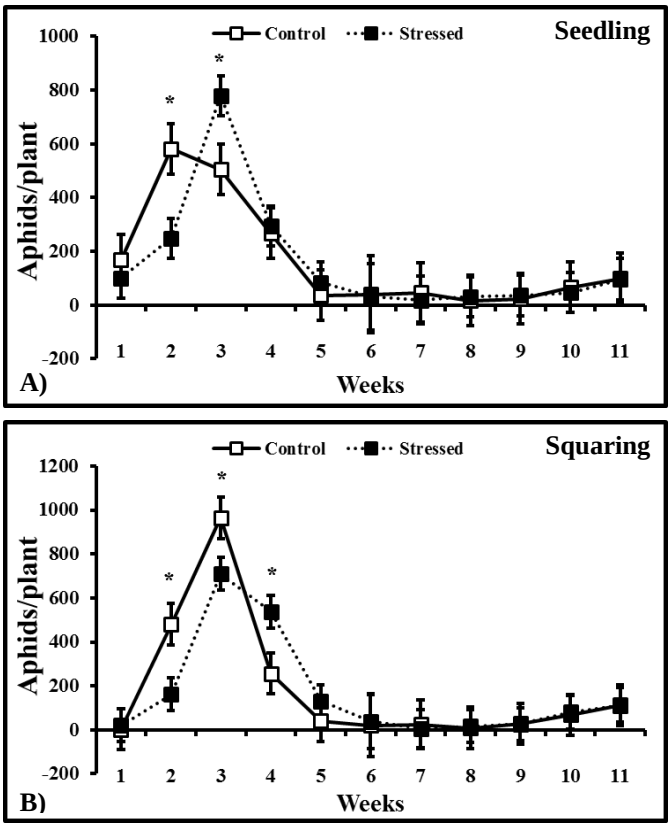

Figure 4-2. Aphids on cotton plants for 2012 field study. Abundance for plants with aphids added during the seedling stage (A) and during the squaring stage (B). Markers are the mean ±SE. Asterisks represent significant differences between treatments.

(abundance: $F_{21, 229}$= 10.65, p<0.0001) and varied with stress over time (stress*week: $F_{10, 240}$= 2.07, p= 0.0274), but stress alone did not have an effect (stress: $F_{1, 249}$= 0.16, p= 0.6871) (Fig. 4-2B). In addition, aphid abundance was significantly lower on stressed plants compared to control plants during weeks two and three, but was two times greater on stressed plants during week 4 (Fig. 4-2B). Furthermore, with pooled data with all treatments, aphid abundance was strongly affected by time (week: $F_{10, 421}$= 27.99, p<0.0001) and by the interaction of time and stress (stress*week: $F_{10, 421}$= 1.94, p= 0.0389) (Table 4-1).

Table 4-1. Factorial effects of treatments on aphids**.** Effects of water stress, week, and the timing of aphid infestation on aphid abundance. "Week" indicates the effect of time and "stage" is the timing of aphid infestation (at seedling, at squaring, or no aphids). ** indicates significance at 0.05 and * indicates marginal significance at 0.1

Response variable	Factors and interactions	df 1	df 2	F ratio	p-value
Aphid abundance	Model	43	388	8.3518	<0.0001**
	Stress	1	430	0.1666	0.6834
	Week	10	421	27.986	<0.0001**
	Stress*Week	10	421	1.9383	0.0389**
	Stage	1	430	0.1394	0.7091
	Stage*Week	10	421	1.0506	0.4
	Stage*Stress	1	430	0.0199	0.8879
	Stage*Week*Stress	10	421	1.2723	0.2441

4.3.2 Amino acids and digestible carbohydrates

Amino acids in plants were not significantly affected by the interactions between water stress and the timing of aphid infestation, but other factors had a significant impact. In plants without aphids, amino acids varied throughout the season (amino acids: $F_{11, 46}$= 11.98, p<0.0001), but did not vary with stress (stress: $F_{1, 56}$= 1.43, p= 0.2384) or with stress over time (stress*week: $F_{5, 52}$= 0.17, p= 0.9719) (Fig. 4-3A). When aphids

were added to plants during the seedling stage, amino acids varied throughout the season (abundance: $F_{11, 46}$= 9.74, p<0.0001) and with stress over time (stress*week: $F_{5, 52}$= 3.33, p= 0.0120), but stress alone did not have an effect (stress: $F_{1, 56}$= 1.82, p= 0.1842) (Fig. 4-3B). In addition, for plants with aphids added during the seedling stage, the concentration of amino acids was twice as high in stressed plants compared to control plants during week three (Fig. 4-3B). In plants with aphids added during the squaring stage, however, amino acids varied throughout the season (amino acids: $F_{11, 48}$= 12.5, p<0.0001) and with stress (stress: $F_{1, 58}$= 15.8, p= 0.0002), but not with stress over time (stress*week: $F_{5, 54}$= 0.95, p= 0.4578) (Fig. 4-3C). Amino acids in these plants, however,

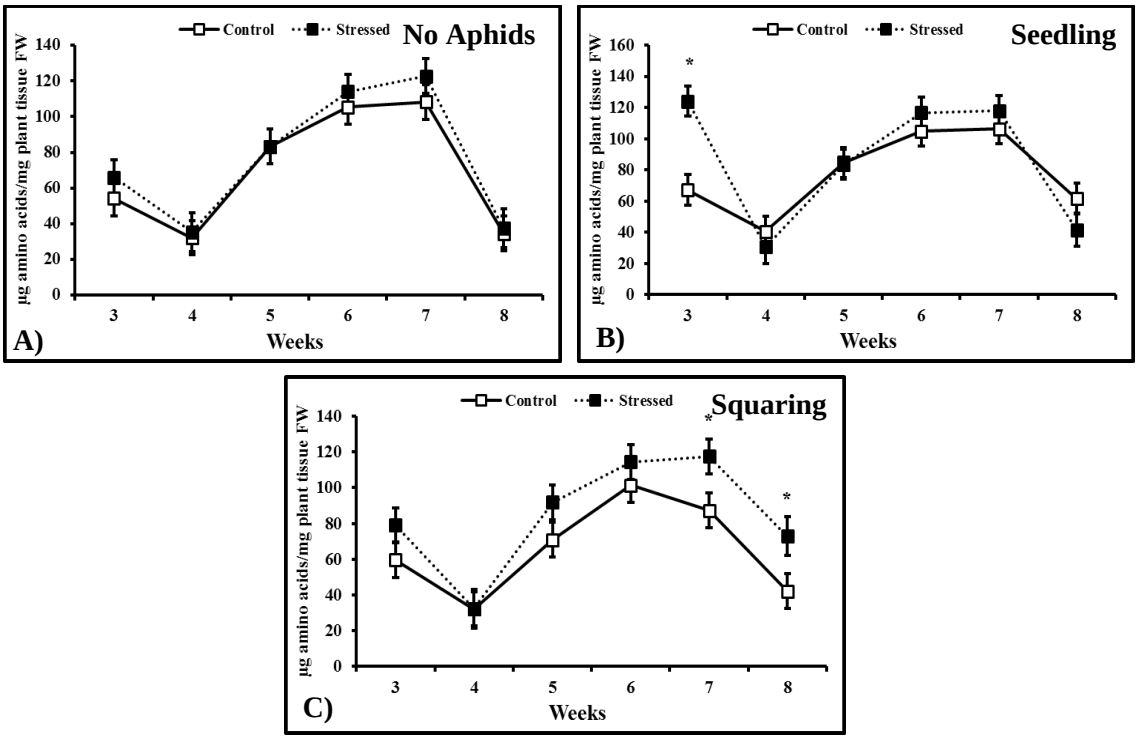

Figure 4-3. Amino acids in treated plants for 2012 field study. Amino acids in plants without aphids (A), with aphids added during the seedling stage (B), and during the squaring stage (C). Markers are the mean ±SE. Asterisks represent significant differences between treatments.

were 40% and 70% greater with water stress during weeks 7 and 8, respectively (Fig. 4-3C). With pooled data including all aphid treatments, amino acids were significantly affected by stress, week (time), the timing of aphid infestation, the interaction of stress and time, and interaction between the timing of aphid infestation and week (Table 4-2).

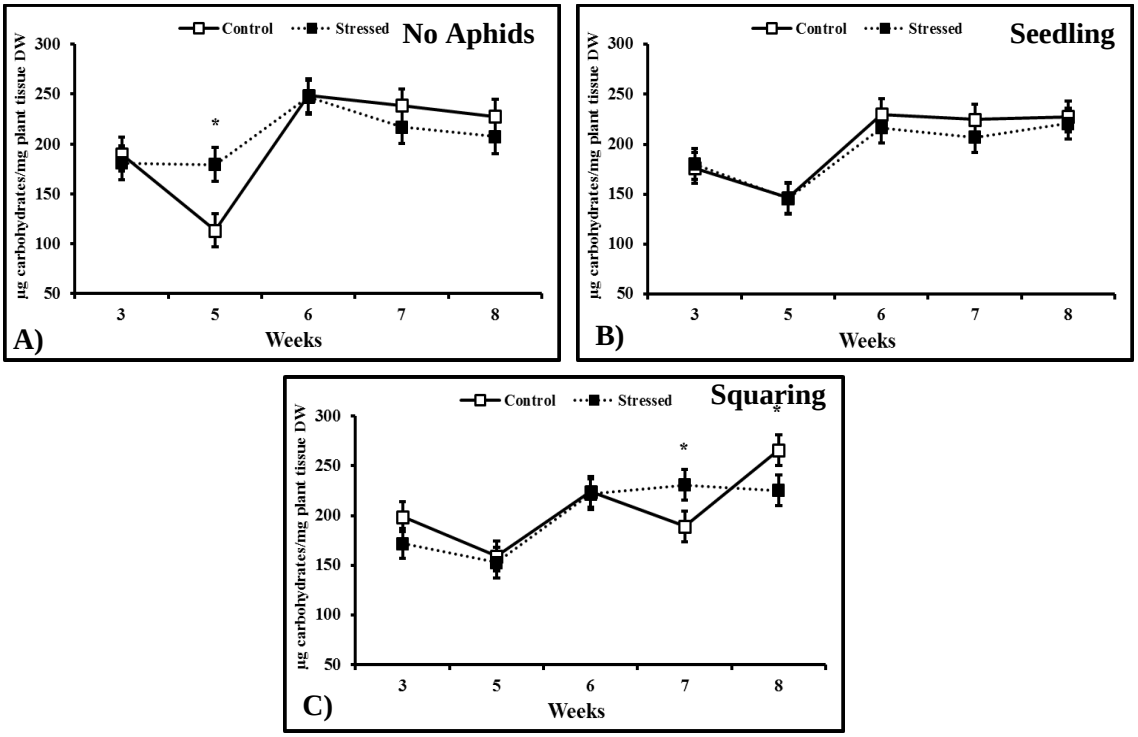

Figure 4-4. Digestible carbohydrates in treated plants for 2012 field study. Digestible carbohydrates in plants without aphids (A), with aphids added during the seedling stage (B), and during the squaring stage (C). Markers are the mean ±SE. Asterisks represent significant differences between treatments.

Digestible carbohydrates in plants were not affected by stress or the timing of aphid infestation. In plants with no aphids, carbohydrates varied throughout the season (carbohydrates: $F_{9, 90}= 5.98$, $p<0.0001$), did not vary with stress (stress: $F_{1, 48}= 0.07$, $p= 0.7994$), but stress over time had a marginal effect (stress*week: $F_{5, 45}= 2.33$, $p= 0.0724$)

(Fig. 4-4A). In addition, under this aphid treatment, carbohydrates were 58% higher in stressed plants compared to control plants (Fig. 4-4A). In plants with aphids added during the seedling stage, carbohydrates significantly varied over the season (carbohydrates: $F_{9, 40}$= 4.61, p= 0.0003) and did not vary with stress (stress: $F_{1, 48}$= 0.52, p= 0.7994) or with stress over time (stress*week: $F_{4, 45}$= 0.17, p= 0.9544) (Fig. 4-4B). In

Table 4-2. Factorial effects of treatments on nutrients**.** The effects of water stress, time, and the timing of aphid infestation on concentrations of amino acids and digestible carbohydrates. "Week" indicates the effect of time and "stage" is the timing of aphid infestation (at seedling, at squaring, or no aphids). ** indicates significance at 0.05 and * indicates marginal significance at 0.1

Response variable	Factors and interactions	df 1	df 2	F ratio	p-value
Amino acids	Model	35	140	10.7972	<0.0001**
	Stress	1	174	12.646	0.0005**
	Week	5	170	61.6833	<0.0001**
	Stress*Week	5	170	2.0348	0.0774*
	Stage	2	173	2.5575	0.0811*
	Stage*Week	10	160	1.7406	0.0774*
	Stage*Stress	2	173	1.4972	0.2273
	Stage*Week*Stress	10	160	1.4047	0.1841
Digestible carbohydrates	Model	29	120	5.5007	<0.0001**
	Stress	1	148	0.4504	0.5034
	Week	4	145	32.1025	<0.0001**
	Stress*Week	4	145	1.5745	0.1855
	Stage	2	147	0.6924	0.5024
	Stage*Week	8	141	1.0117	0.4309
	Stage*Stress	2	145	0.3374	0.7143
	Stage*Week*Stress	8	141	1.7761	0.0883*

plants with aphids added during the squaring stage, carbohydrates varied throughout the season (carbohydrates: $F_{9, 40}$= 7.32, p<0.0001), with stress over time (stress*week: $F_{5, 44}$= 2.76, p= 0.0404), but stress alone did not have an effect (stress: $F_{1, 48}$= 0.68, p= 0.4142) (Fig. 4-4C). Furthermore, carbohydrates were 20% higher in stressed plants compared to control plants during week 7, but were 17% lower in stressed plants during

week 8. With data pooled across all treatments, carbohydrates were significantly affected by week and marginally affected by the interaction between stage, week, and stress (Table 4-2).

4.3.3 Relative chlorophyll content and peroxidase activity

Water stress had strong effects on relative SPAD values regardless of the time of aphid infestation. In plants with no aphids, SPAD values varied throughout the season (SPAD values: $F_{19, 131}$= 75.62, p<0.0001) and stress (stress: $F_{1, 149}$= 114.10, p<0.0001) and stress over time had significant effects (stress*week: $F_{9, 141}$= 8.72, p<0.0001). In addition, stressed plants had higher SPAD values than control plants during 7 of the 10 weeks (Fig. 4-5A). In plants with aphids added during the seedling stage, SPAD values varied throughout the season (SPAD: $F_{19, 161}$= 13.53, p<0.0001), varied with stress (stress: $F_{1, 179}$= 53.90, p<0.0001), and with stress over time (stress*week: $F_{9, 171}$= 8.53, p<0.0001) (Fig. 4-5B). Furthermore, stressed plants had higher SPAD values compared to control stressed plants during 5 of the 10 weeks (Fig. 4-5B). In plants that had aphids added during the squaring stage, SPAD values significantly varied throughout the season (SPAD: $F_{19, 238}$= 37.56, p<0.0001), with stress (stress: $F_{1, 256}$= 254.44, p<0.0001), and with stress over time (stress*week: $F_{9, 248}$= 13.35, p<0.0001) (Fig. 4-5C). Chlorophyll was greater in stressed plants compared to control plants during 8 of the 10 weeks (Fig. 4-5C). With data pooled across all treatments, SPAD values were significantly affected by all factors and combinations except stage and week and the interaction between stage, week, and stress (Table 4-3).

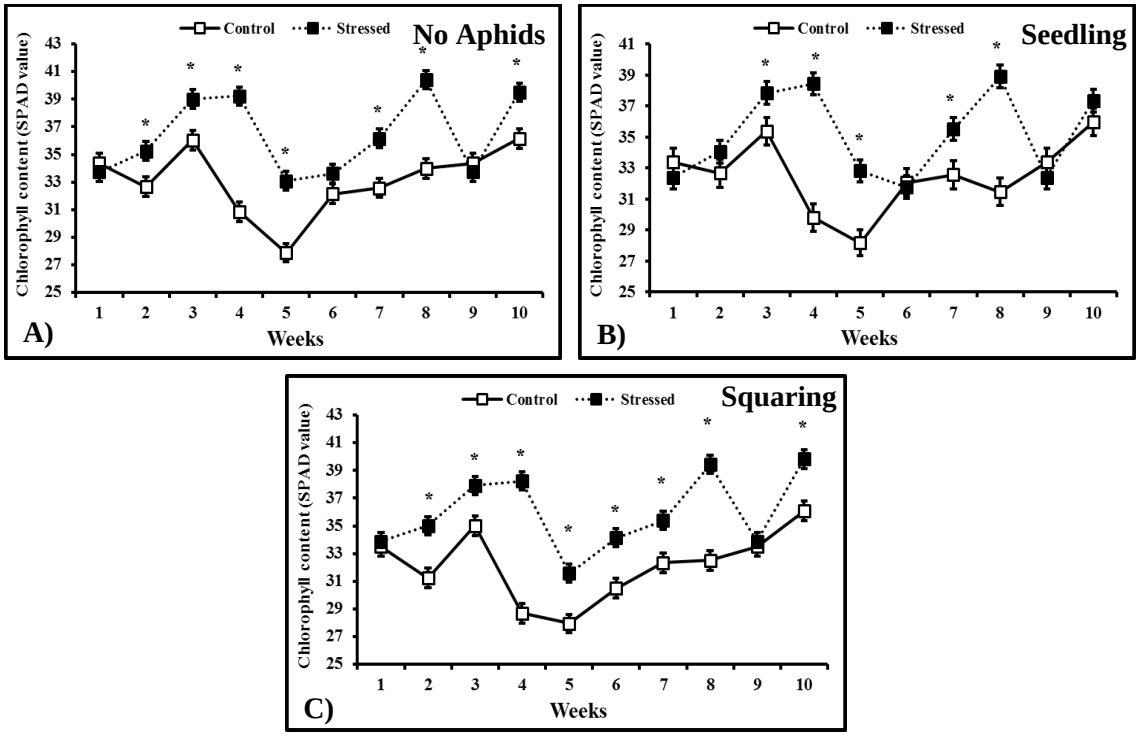

Figure 4-5. Relative chlorophyll content (SPAD values) for 2012 field study. SPAD values for plants without aphids (A), with aphids added during the seedling stage (B), and during the squaring stage (C). Markers are the mean ±SE. Asterisks represent significant differences between treatments.

Peroxidase activity was inconsistently affected by water stress, but varied with time and the timing of aphid infestation. In plants without aphids, peroxidase activity varied throughout the season (peroxidase activity: $F_{11, 113}= 5.28$, $p<0.0001$), marginally varied with stress (stress: $F_{1, 123}= 4.03$, $p= 0.0567$) and with stress over time (stress*week: $F_{5, 119}= 3.60$, $p= 0.0047$) (Fig. 4-6A). In addition, in plants without aphids, peroxidase activity was 600% greater in control plants compared to stressed plants during week 5 and then decreased activity lower than stressed plants the next week (Fig. 4-6A). In plants with aphids added during the seedling stage, peroxidase activity varied throughout the season (peroxidase activity: $F_{11, 83}= 3.57$, $p=0.0004$), but stress and stress

over time had no effect on activity (stress: $F_{1, 93}$= 0.32, p= 0.5750; stress*week: $F_{5, 89}$= 0.73, p= 0.6030) (Fig. 4-6B). In plants with aphids added during the squaring stage, peroxidase activity varied throughout the season (peroxidase activity: $F_{11, 111}$= 7.05, p<0.0001) and varied with stress over time (stress*week: $F_{5, 117}$= 4.57, p=0.0008), but stress alone did not have an effect (stress: $F_{1, 121}$= 0.0067, p=0.9348) (Fig 4-6C).

Table 4-3. Factorial effects of treatments chlorophyll content and POD activity. The effects of water stress, time, and the timing of aphid infestation on relative Chlorophyll content (SPAD values) and peroxidase activity. "Week" indicates the effect of time and "stage" is the timing of aphid infestation (at seedling, at squaring, or no aphids). ** indicates significance at 0.05 and * indicates marginal significance at 0.1

Response variable	Factors and interactions	df 1	df 2	F ratio	p-value
Chlorophyll content	Model	59	530	22.2712	<0.0001**
	Stress	1	588	342.5486	<0.0001**
	Week	9	580	61.6217	<0.0001**
	Stress*Week	9	580	27.7224	<0.0001**
	Stage	2	587	8.92	0.0002**
	Stage*Week	18	571	0.7931	0.7095
	Stage*Stress	2	587	3.959	0.0196**
	Stage*Week*Stress	18	571	0.8263	0.6695
Peroxidase activity	Model	35	307	5.4496	<0.0001**
	Stress	1	341	0.1885	0.6645
	Week	5	337	21.2172	<0.0001**
	Stress*Week	5	337	4.0426	0.0014**
	Stage	2	340	9.3519	0.0001**
	Stage*Week	10	332	2.2659	0.0144**
	Stage*Stress	2	340	1.5736	0.209
	Stage*Week*Stress	10	332	0.8794	0.5529

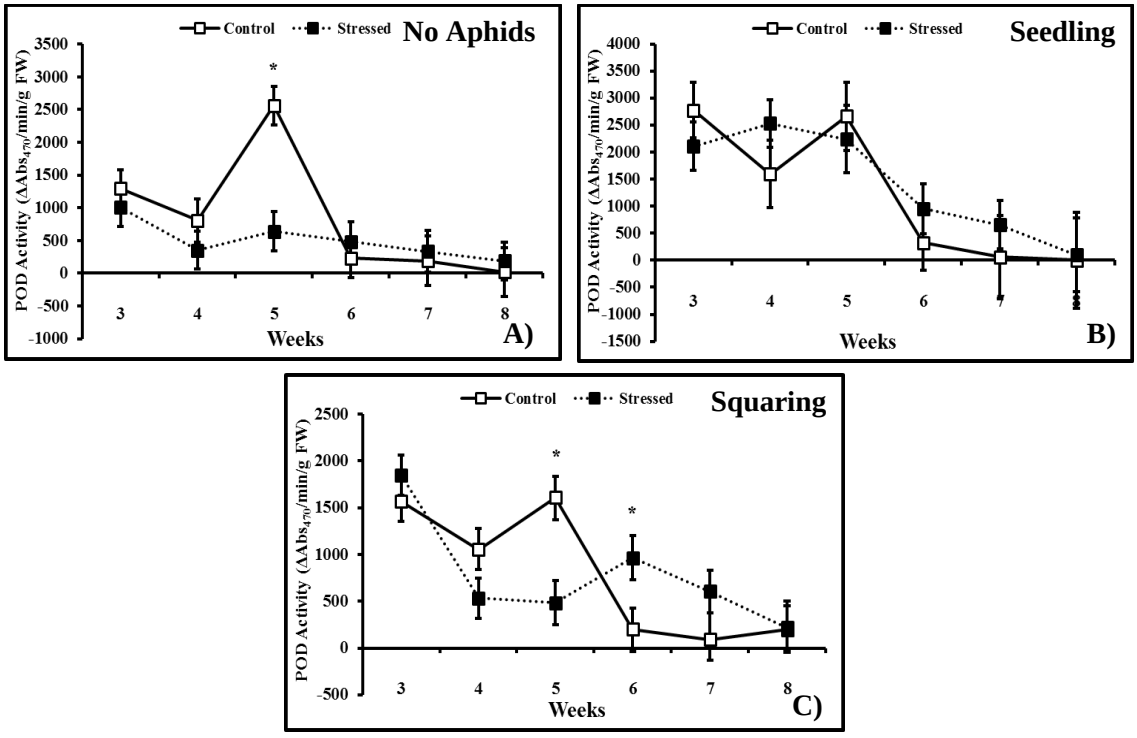

Figure 4-6. Peroxidase activity for treated plants for 2012 field study. The effects of water stress on peroxidase activity for plants without aphids (A), with aphids added during the seedling stage (B), and during the squaring stage (C). Markers are the mean ±SE. Asterisks represent significant differences between treatments.

Furthermore, with these same plants, peroxidase activity was 330% lower in stressed plants compared to control plants during week 5, but 480% greater in stressed plants during week 6 (Fig. 4-6C). With all data pooled across treatments, peroxidase activity was significantly affected by time, timing of aphid infestation, the interaction of time and timing of aphid infestation, and the interaction of time and stress (Table 4-3).

4.3.4 Cotton development and lint yield

Water stress and the timing of aphid infestation (stage) had a strong effect on plant height. For water stress-free plants, plant height varied throughout the season (height: $F_{31, 201}$= 89.87, p<0.0001) and was significantly affected by the timing of aphid infestation (stage: $F_{1, 231}$= 16.06, p<0.0001) and the interaction of time and the timing of aphid infestation (stage*week: $F_{19, 223}$= 8.72, p=0.0028) (Fig. 4-7A). In addition, plants that had aphids added during the seedling stage and were stress-free were significantly shorter than plants with no aphids and plants with aphids added during the squaring stage during weeks two through six (Fig. 4-7A). For stressed plants, plant height varied throughout the season (height: $F_{31, 237}$= 57.25, p<0.0001) and with the timing of aphid infestation (stage: $F_{1, 267}$= 41.48, p<0.0001), but did not vary with the timing of aphid infestation over time (stage*week: $F_{19, 223}$= 0.77, p=0.7451) (Fig. 4-7B). Moreover, with stressed plants, plants with aphids added during the seedling stage were significantly shorter than plants with no aphids and plants with aphids added during the squaring stage during week 2 and weeks 4 through 10 (Fig. 4-7B). With the data pooled across all treatments, plant height was significantly affected by all factors and combinations except the interaction between time, stress, and the timing of aphid infestation (Table 4-4).

Water stress and the timing of aphid infestation had significant effects on the number of plant nodes on cotton plants. For control plants, the number of nodes significantly varied during the season (nodes: $F_{31, 201}$= 66.33, p<0.0001) with the timing of aphid infestation (stage: $F_{1, 231}$= 17.32, p<0.0001), and was marginally affected by the timing of aphid infestation over time (stage*week: $F_{19, 213}$= 1.54, p= 0.0743) (Fig. 4-8A).

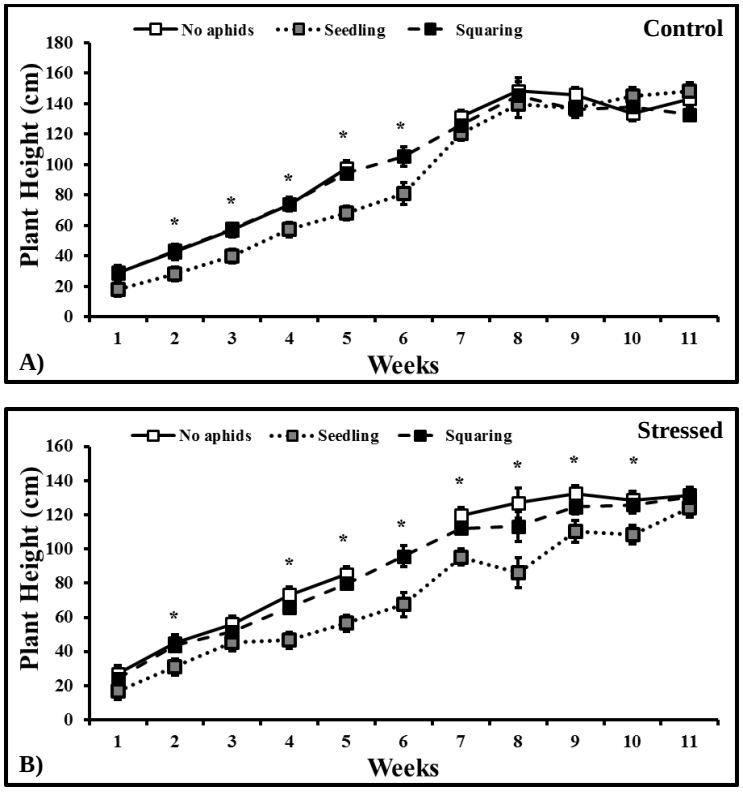

Figure 4-7. Plant height for treated plants in 2012 field study. Plant height for plants with water stress (A) and for plants without water stress (B). Markers are the mean ±SE. Asterisks represent significant differences between at least two of the three treatments.

In addition, for control plants, nodes on plants that had aphids added during the seedling stage were significantly fewer in number than aphid-free plants and plants with aphids added during the squaring stage during weeks one through six (Fig. 4-8A). On stressed plants, the number of nodes significantly varied during the season (nodes: $F_{31, 237}$= 52.93, $p<0.0001$), with the timing of aphid infestation (stage: $F_{1, 267}$= 37.33, $p<0.0001$), but did not significantly vary with time and the timing of aphid infestation (stage*week: $F_{19, 249}$= 1.54, p= 0.5601) (Fig. 4-8B). Furthermore, plants with aphids added during the seedling stage had significantly fewer nodes compared to aphid-free plants and plants with aphids added during the squaring stage during week one and two, and during

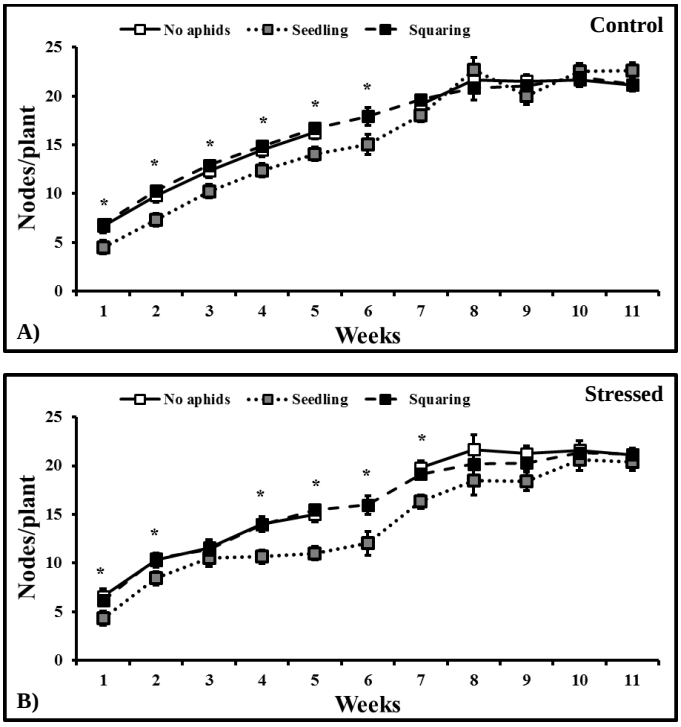

Figure 4-8. Total nodes on treated plants in 2012 field study. Plant nodes for plants with water stress (A) and for plants without water stress (B). Markers are the mean ±SE. Asterisks represent significant differences between at least two of the three treatments.

Table 4-4. Factorial effects of treatments on plant height and total nodes. Effects of water stress, time, and the timing of aphid infestation on plant height and the number of nodes. "Week" indicates the effect of time and "stage" is the timing of aphid infestation (at seedling, at squaring, or no aphids).

Response variable	Factors and interactions	df 1	df 2	F ratio	p-value
Plant height	Model	59	427	75.8075	<0.0001**
	Stress	1	485	71.7365	<0.0001**
	Week	9	477	439.1176	<0.0001**
	Stress*Week	9	477	4.5566	<0.0001**
	Stage	2	484	37.0658	<0.0001**
	Stage*Week	18	468	1.787	0.0246**
	Stage*Stress	2	484	4.3064	0.0141**
	Stage*Week*Stress	18	468	0.984	0.4771
Nodes	Model	59	427	62.1243	<0.0001**
	Stress	1	485	15.0677	<0.0001**
	Week	9	477	363.1891	<0.0001**
	Stress*Week	9	477	1.3753	0.1969
	Stage	2	484	27.2088	<0.0001**
	Stage*Week	18	468	1.7657	0.0271**
	Stage*Stress	2	484	3.3339	0.0366**
	Stage*Week*Stress	18	468	0.6281	0.8781

weeks four through seven (Fig. 4-8B). With the data pooled across all treatments, the number of nodes was significantly affected by all factors and combinations except the interaction between stress and week and the interaction between week, stress, and the timing of aphid infestation (Table 4-4).

The timing of aphid infestation had no effect on the number of first position bolls produced and had no effect on first position bolls on stressed plants. For control plants, the timing of aphid infestation did not have an effect (stage: $F_{2, 76}$= 0.66, p= 0.5158) and the number of bolls were similar (Fig. 4-9A).

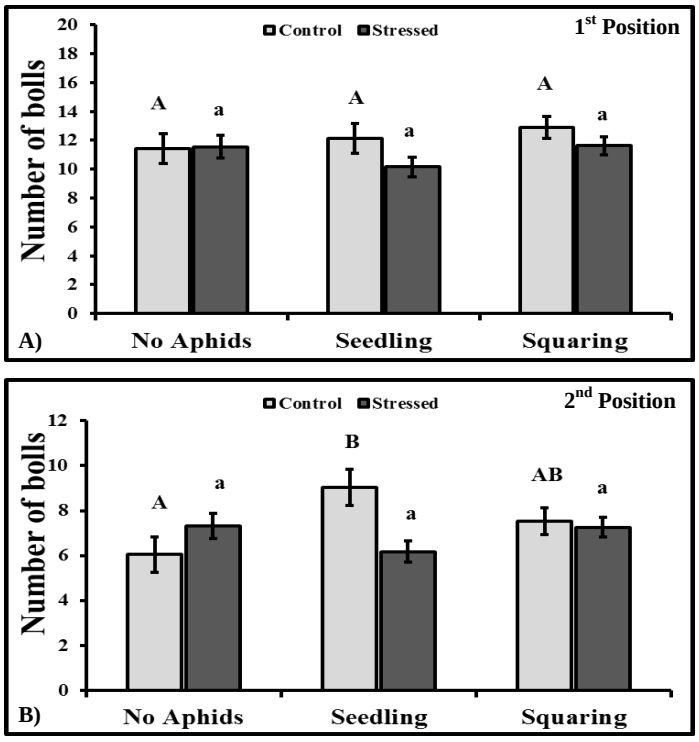

Figure 4-9. Number of 1st and 2nd position bolls on treated plants in 2012. 1st position bolls for plants with and without water stress (A) and for 2nd position bolls for plants with and without water stress (B). Bars are the mean ±SE. Bars with letters of the same size were statistically compared and bars with different letters above them are significantly different.

Stressed plants were similar to control plants with the timing of aphid infestation having no effect on the number of first position bolls produced (stage: $F_{2, 76}$= 1.48, p= 0.2330) and the number of bolls were similar across the different timings of aphid infestation (Fig. 4-9B). With pooled data, first position bolls (as well as all boll and lint data) were analyzed for effects of stress, timing of aphid infestation, and the interaction of stress and the timing of aphid infestation (no time comparison). First position bolls were not significantly affected by any of the factors or interactions (Table 4-5).

Second position bolls on control plants were significantly affected by the timing of aphid infestation, but stressed plants were unaffected. For control plants, the timing of aphid infestation had an effect on the number of second position bolls (stage: $F_{2, 76}$= 3.53, p= 0.0342) with plants infested with aphids during the seedling stage have 30% more bolls than plants without aphids (Fig. 4-9B). For stressed plants, there was no effect of timing of aphid infestation (stage: $F_{2, 93}$= 1.77, p= 0.1761) and the number of bolls were similar for all timings of aphid infestation (Fig. 4-9B). With pooled data, second position bolls were significantly affected by the interaction of water stress and the timing of aphid infestation (Table 4-5).

The timing of aphid infestation had different effects on the percent of bolls unopened by the harvest date on October 12th, 2012 for control plants and stressed plants. For control plants, the timing of aphid infestation did not have an impact on the percent of bolls unopened (stage: $F_{2, 76}$= 0.95, p= 0.3924) (Fig. 4-10A). For stressed plants, the timing of aphid infestation had an effect (stage: $F_{2, 93}$= 4.71, p= 0.0112) and plants with aphids added during the seedling stage had 12% of their bolls unopened

compared to 4.5% for plants with no aphids and plants with aphids added during the squaring stage (Fig. 4-10A). With pooled data, bolls unopened by harvest were

Table 4-5. Factorial effects of treatments on 1st and 2nd position bolls and lint**.** Effects of water stress, time, and the timing of aphid infestation on the number of bolls and lint yield. "Week" indicates the effect of time and "stage" is the timing of aphid infestation (at seedling, at squaring, or no aphids). ** indicates significance at 0.05 and * indicates marginal significance at 0.1

Response variable	Factors and interactions	df 1	df 2	F ratio	p-value
1st position bolls	Model	5	169	1.5029	0.1914
	Stress	1	173	2.4718	0.1178
	Stage	2	172	1.1179	0.3294
	Stage*Stress	2	172	0.7437	0.4769
2nd position bolls	Model	5	169	2.7356	0.021**
	Stress	1	173	1.5596	0.2135
	Stage	2	172	1.1045	0.3338
	Stage*Stress	2	172	5.3043	0.0058**
1st position lint	Model	5	54	0.932	0.4677
	Stress	1	58	0.1098	0.7417
	Stage	2	57	0.3375	0.715
	Stage*Stress	2	57	1.6699	0.1978
2nd position lint	Model	5	60	1.6202	0.1685
	Stress	1	64	4.4332	0.0394**
	Stage	2	63	0.1294	0.8789
	Stage*Stress	2	63	2.0868	0.133
Total lint	Model	5	61	0.7652	0.5784
	Stress	1	65	0.2181	0.6422
	Stage	2	64	0.3531	0.7039
	Stage*Stress	2	64	1.272	0.2876

only affected by the interaction of stress and the timing of aphid infestation (Table 4-6).

Fruit retention was not affected by the timing of aphid infestation. For control plants, the timing of aphid infestation did not have an effect (stage: $F_{2, 200}$= 0.70, p= 0.4992) and fruit retention was similar among timings of aphid infestation (Fig. 4-10B). For stressed plants, aphid infestation did not have an effect (stage: $F_{2, 229}$= 1.28, p= 0.2805) and fruit retention was the same among aphid treatments (Fig. 4-10B). With pooled data, fruit retention was marginally affected by water stress (Table 4-6).

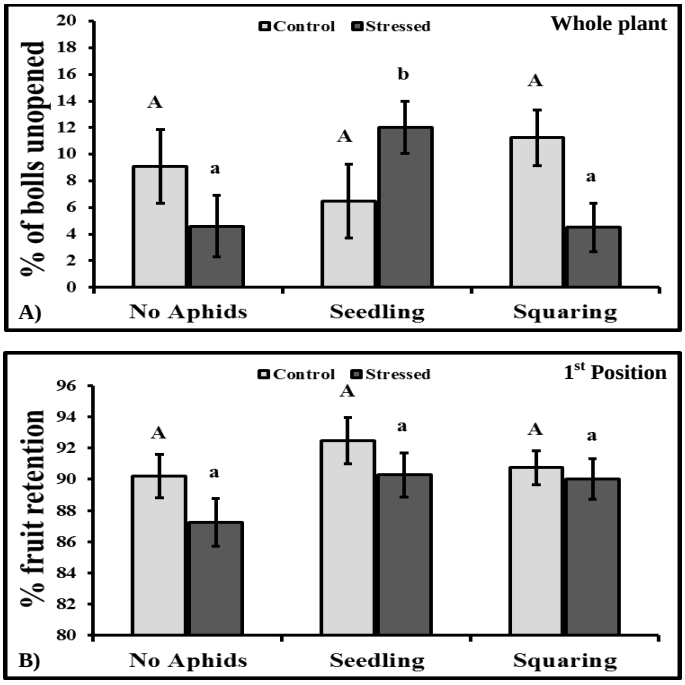

Figure 4-10. The % of unopened bolls and fruit retention for treated plants in 2012. Bolls unopened by the harvest date of October 12th, 2012 for plants with and without stress (A) and fruit retention of 1st position bolls for plants with and without water stress (B). Bars are the mean ±SE. Bars with letters of the same size were statistically compared and bars with different letters above them are significantly different.

The timing of aphid infestation had different effects on lint yield from first position bolls. For control plants, there was no effect of aphid infestation (stage: $F_{2, 25}$= 0.23, p= 0.7929) on lint yield from first position bolls and the quantity of lint did not differ among timings of aphid infestation (Fig. 4-11A). For stressed plants, however, there was a marginal effect of aphid infestation on lint yield (stage: $F_{2, 29}$= 3.15, p= 0.0578) and plants with aphids added during the seedling stage produced 21 g of lint per plant compared to 26 g from plants with no aphids and plants with aphids added during the squaring stage (Fig. 4-11A). With pooled data, lint yield from first position bolls was not significantly affected by any factors or interactions (Table 4-5).

Lint yield from second fruiting positions was not affected by the timing of aphid infestation, but there were differences between control and stressed plants. In control plants, lint yield was not affected by the timing of aphid infestation (stage: $F_{2, 28}$= 0.98, p= 0.3862) and was similar among all timings of aphid infestation (Fig. 4-11B). For stressed plants, the timing of aphid infestation also had no effect (stage: $F_{2, 32}$= 1.19, p= 0.3178) and lint yield was similar among timings of aphid infestation (Fig. 4-11B). With pooled data, however, lint yield from second fruiting positions was significantly affected by water stress (Table 4-5).

Table 4-6. Factorial effects of treatments on % bolls unopened and retention**.** Effects of water stress and the timing of aphid infestation on % of bolls unopened by October 12, 2012 and % of bolls retained by the end of the season. "Week" indicates the effect of time and "stage" is the timing of aphid infestation (at seedling, at squaring, or no aphids). ** indicates significance at 0.05 and * indicates marginal significance at 0.1.

Response variable	Interactions	df 1	df 2	F ratio	p-value
% bolls unopened	Model	5	169	2.4379	0.0366**
	Stress	1	173	1.0449	0.3081
	Stage	2	172	0.4936	0.6113
	Stage*Stress	2	172	4.2317	0.0161**
% retention	Model	5	429	1.2806	0.2712
	Stress	1	433	2.9759	0.0852*
	Stage	2	432	1.6488	0.1935
	Stage*Stress	2	432	0.3764	0.6866

Total lint yield from all bolls on the cotton plant was not affected by the timing of aphid infestation or water stress. For control plants, total lint yield was not affected by the timing of aphid infestation (stage: $F_{2, 27}$= 0.66, p= 0.5248) and there were no differences in lint yield among the timings of aphid infestation (Fig. 4-12). For stressed plants, lint yield was not affected by the timing of aphid infestation (stage: $F_{2, 34}$= 1.05, p= 0.3616) and there were no differences among timings of aphid infestation (Fig. 4-12).

With pooled data, total lint yield was not significantly affected by any factors or the interaction of factors.

Characteristics of cotton quality (micronaire, strength, etc.) were similar between plants regardless of the timing of aphid infestation and water stress. Micronaire (measure of fineness and maturity), for instance, ranged between 4.5 to 4.9 with lower values indicating higher cotton fineness and higher values indicating coarser fibers (Raskopf 1966, Montalvo Jr 2005) (Table 4-7). Plants without water stress had longer fibers compared to stressed plants, with stress-free plants in the "long" fiber category and stressed plants in the "medium-long" fiber category (Bradow and Davidonis 2000) (Table 4-7). Cotton fiber uniformity varied between 82.5 and 84.2 with similar uniformity indices between water stressed plants and plants with different timings of aphid infestation. In addition, the strength of cotton fibers ranged from 27.9 (average) to 31.7 (very strong) and elongation of cotton fibers were similar among all treated plants (Table 4-7) (Raskopf 1966, Montalvo Jr 2005, USDA 2005).

Table 4-7. Effects of treatments on cotton lint quality.

Timing of aphid infestation	Water stress	Micronaire	Length (mm)	Uniformity Index ($kNmkg^{-1}$)	Strength ($kNmkg^{-1}$)	Elongation (%)
No aphids	**Stress**	4.9	28.70	82.7	28.4	7.9
Seedling	**Stress**	4.9	27.9	84.2	31.7	7.6
Squaring	**Stress**	5.1	28.96	83.5	29.2	7.7
No aphids	**No stress**	4.9	29.21	82.5	27.9	7.2
Seedling	**No stress**	4.5	29.97	82.9	29.1	7.2
Squaring	**No stress**	4.7	29.72	83.4	29.7	7.4

4.4 Discussion

Water stress had the strongest effects on cotton development and lint yield. In general, the timing of aphid infestation significantly affected cotton development when it was combined with water stress. The low impact of the timing of aphid infestation was most likely due to a sharp and persistent decline in aphids during the first half of the season, resulting in relatively low aphid herbivory as plants reached maturity. The sharp decline in aphids and consistently low abundances on both stressed and control plants while in enemy-free cages suggest that overcrowding or ontogenetic changes in plants may have led to declines in aphid abundance (Boege and Marquis 2005). Previous studies suggested that aphid abundance would vary on water-stressed plants and aphids would decline over time. For instance, green spruce aphids (*Elatobium abietinum*) feeding on pulse stressed and watered green spruce trees reached maximum abundances of 90 and 68 aphids per tree, respectively, after 60 days of feeding on green spruce trees, after which both treatments decreased to 32 and 10 aphids per tree over the next 30 days (Major 1990). Furthermore, cotton aphids on six different, unstressed cotton cultivars reached peak aphid densities of 300-350 aphids per leaf between 195-202 Julian days (14-24 July), after which densities decreased to under 50 aphids for all six cultivars in the next week (Weathersbee III and Hardee 1994). Even when plants were not stressed (the latter example) aphids decreased in abundance on host plants, suggesting that other factors aside from stress and plant cultivar dictate aphid abundance on host plants. In addition, temperature may have influenced aphid abundance and led to declining aphid populations. Aphid reproductive capacity decreases from 22 to 32°C (Slosser et al.

1989), while temperatures during week three and four of our study reached 31 to 39°C. Various aspects of cotton ontogeny, temperature, and overcrowding may have contributed to the decline and persistently low abundances of aphids; further examination will be required to address the full extent of this phenomenon.

Water stress inconsistently affected concentrations of amino acids and digestible carbohydrates and fluctuations in nutrients generally followed similar patterns regardless of aphid infestation. When data was pooled, however, amino acids were significantly affected by many factors, suggesting that the timing of aphid infestation and aphids over time also have a strong influence on nutrient concentrations. Consequently, our results do not follow the Plant Stress Hypothesis that suggested that water stress should increase nutrient concentrations compared to unstressed plants (White 1969, Price 1991). Unfortunately, aphid abundance was minimal during many of the instances in which concentrations of amino acids differed between plants with different aphid infestation times. During week three, however, when aphids were reached their greatest abundance on plants with aphids added during the seedling stage, amino acids were at their highest concentration, suggesting an interaction between aphid abundance and amino acids (Figs. 4-2A and 4-3B). Despite this and other differences in amino acids in stressed plants compared to control plants, aphids continued to decline in abundance. This is further evidence suggesting that other factors besides nutrients in host plants influence aphid abundance. Additionally, we did add lady bugs to cages to reduce populations due to increasing cases of black sooty mold on honeydew. This, however, should not have

resulted in the collapse of colonies as the predators were only in the cages for three days and removed afterwards, after which populations should have recovered.

Cotton plants with varying aphid infestation times and under stressed or stress-free conditions did not vary in boll and lint production, suggesting that cotton compensated or avoided the negative effects of these abiotic and biotic stresses. After herbivory, plants in natural and agricultural systems have been known to compensate (match the fitness of unconsumed plants) and even overcompensate (exceed the fitness of unconsumed plants) for herbivore damage (Trumble et al. 1993, Strauss and Agrawal 1999, Wilson et al. 2003). Compensating plants regrow lost tissue, increase photosynthetic rate, and/or reallocate photoassimilates to damaged tissue (Trumble et al. 1993, Sadras 1995). Additionally, cotton may completely compensate for aphid herbivory during the pre-reproductive stages of plant growth and the timing of cotton maturation, quantity of yield, and the quality of the fiber are unaffected (Rosenheim and Wilhoit 1993, Rosenheim et al. 1997, Godfrey et al. 2000). For example, Rosenheim et al. (1997) found that cotton plants with aphid herbivory were similar to aphid-free plants six weeks after aphid populations declined. In addition, total above-ground biomass and allocation of biomass to leaf, stem, and fruit production was similar between treatments. In our study, cotton plants may have compensated for aphid herbivory within weeks after the decline of aphid abundances. On the other hand, stressed plants may not have been stressed long enough to induce boll shed and may have avoided production losses due to stress rather than compensated for them. Cotton squares and bolls are primarily affected by impaired photosynthesis, while photosynthesis may be maintained or

actually increase with initial water stress and functionally supported by stress-related nutrients and compounds (Guinn and Service 1982, Chaves et al. 2003, Jithesh et al. 2006, Taiz and Zeiger 2010). Squares and bolls, however, are sensitive to the loss of photoassimilates from nearby source leaves, in which the abscission of younger squares and bolls may occur (Guinn and Service 1982, Freeland et al. 2006). It may have been possible, therefore, that the water stress our plants experienced was not severe enough to induce boll shed in our plants (-1.24 MPa, Fig. 4-1). Furthermore, antioxidant enzymes such as peroxidase migrate from leaves to squares and bolls when plants become water stressed (Sandhu et al. 2007), which may support why peroxidase activity declined in the leaves of stressed plants compared to stress-free control plants (Fig. 4-6A). Cotton plants, therefore, demonstrated several physiological mechanisms that allowed for the compensation of aphid herbivory and avoidance of stress-induced fruit shed, resulting in similar lint production and quality between treatments.

Our study illustrated that aphid infested and water-stressed cotton plants may produce comparable lint yields to uninfested plants. Aphid abundance may be more influenced by ontogenetic and abiotic factors than stress-induced changes in host plants, resulting in early season population declines in enemy-free space. Future work would need to address the extent to which aphid population dynamics depend upon ontogenetic changes in host plants, and the degree to which allelochemistry and resource allocation in host plants play a role. In addition, increasing the severity and frequency of water stress may more thoroughly address how water stress and ontogenetic changes in plants influence aphid abundance and subsequent lint yield. Our study is one of the first to

address the combined effects of the timing of aphid infestation and water stress, however, more research needs to be done further explore the complexity of these interactions.

CHAPTER V

THE NUTRIENT AVAILABILITY HYPOTHESIS: DEVELOPMENT OF A UNIFYING PLANT STRESS-HERBIVORE HYPOTHESIS

5.1 Introduction

There has been a long-standing interest in accurately predicting the effects of water deficit stress on plant-insect interactions (Loomis 1932, White 1969, Coley et al. 1985, Herms and Mattson 1992, Koricheva et al. 1998, Huberty and Denno 2004, Jactel et al. 2012). Over 500 published studies have addressed this topic (search results from the Web of Knowledge and Google Scholar 2013) and nearly half a dozen formal hypotheses have been developed to explain the effects of water deficit stress on insect herbivores (Loomis 1932, White 1969, Coley et al. 1985, Price 1991, Huberty and Denno 2004). Despite this intensive effort, it is still difficult to accurately predict the effects of water deficit stress on insect abundance and performance. Some studies, for example, have found that insect herbivores including gall midges (Diptera: Cecidomyiidae), inchworms (Lepidoptera: Geometridae), and aphids (Hemiptera: Aphididae) perform better and are more abundant on water deficit-stressed plants (Waring and Price 1990, Archer et al. 1995, Schowalter et al. 1999). Other studies, however, have shown that these same insects or their close relatives perform better and are more abundant on non-stressed plants (Hanks and Denno 1993, Larsson and Bjorkman 1993, Inbar et al. 2001). Variable results such as these are extremely common in the literature, so understanding the sources that contribute to variation in the effects of

water deficit stress on plant-insect interactions is of vital importance to the management of agricultural and natural ecosystems. Climate change predicts more intense and frequent droughts, which makes it even more critical that we understand the factors that determine the impact of water deficit stress on plant-insect interactions (Dai 2011, Kiem and Austin 2013, Van Lanen et al. 2013).

Water deficit stress can be broadly defined as alterations in normal plant functions and physical-chemical equilibrium due to water deficit (Bray 1997, Shao et al. 2008), leading to alterations in how plants manage water, hormones, enzymes, and macro- and micronutrients (Hsiao 1973, Herms and Mattson 1992, Chaves et al. 2002, Chaves et al. 2003, Hu et al. 2006, Jithesh et al. 2006, Shao et al. 2008, Taiz and Zeiger 2010). Predicting the impacts of water deficit stress on herbivore performance is difficult because stress changes plant physiology in ways that can increase primary metabolites (macronutrients) and secondary metabolites (toxic allelochemicals) in host plants. During water stress, plants accumulate macronutrients and antioxidant enzymes to alleviate the deleterious effects of stress (White 1969, English-Loeb et al. 1997, Sholwer 2002, Huberty and Denno 2004, Jithesh et al. 2006, Ghannoum 2008, Mody et al. 2009). These compounds stabilize cell membranes, cytoplasmic enzymes, and scavenge free radicals (Jithesh et al. 2006, Parida et al. 2008, Taiz and Zeiger 2010). The accumulation of macronutrients, however, can be beneficial to herbivores because they contain amino acids and essential nutrients for insect growth and development. For example, the concentration of the amino acid phenylalanine increases in stressed plants and is required for insects to synthesize tyrosine to stabilize and pigment cuticle and form

proteinaceous structures (Kramer and Hopkins 1987, Daubner et al. 2011, Vavricka et al. 2014) . Water stress, however, can also lead to tougher leaves, more trichomes, thicker surface waxes, and possibly higher concentrations of toxic secondary metabolites that all negatively affect herbivores (Raupp 1985, Herms and Mattson 1992, Stamp 2003). Focusing on stress-induced changes in plants may provide an objective, mechanistic basis for predicting herbivore performance on water deficit-stressed plants by concentrating on the changes in plant physiology that directly influence herbivore performance.

In addition, most studies tend to generalize the effects of water deficit stress on macronutrients and allelochemicals across plant taxa, ignoring potential inherent differences among plants (Coley et al. 1985, Price 1991, Huberty and Denno 2004, Jactel et al. 2012). Variation in herbivore response to stressed plants may arise due to intrinsic differences in how different plants allocate macronutrients and allelochemicals in response to water stress. Any assessment of the effects of water deficit stress on plant-insect interactions should explicitly address potential variation among plant groups.

The goal of this meta-analysis was to determine the relationship between stress-induced changes in water deficit-stressed plants and herbivore performance. Specifically, we determined: 1) how concentrations of key macronutrients and allelochemicals in plants change during water stress, 2) the performance of herbivores on water-stressed plants, 3) the relationship between stress-induced changes in plant macronutrients, allelochemicals, and herbivore performance, and 4) variation among plant taxa and the effects of water stress on concentrations of macronutrients and allelochemicals. The

analysis included studies that reported changes in plant macronutrients and allelochemicals in water-stressed plants in addition to herbivore performance (e.g., growth, survival, etc.). Our approach was novel in that it specifically focused on potential mechanisms that influence herbivore performance on water-stressed plants by directly examining stress-induced changes in plants and the correlated changes in herbivore response.

5.2 Methods

5.2.1 Selection criteria for meta-analysis

We selected experimental and observational studies from 1967 (the earliest study we found) to 2013 that examined the influence of water deficit stress on macronutrients, allelochemicals, and herbivore performance. Macronutrients from selected studies were compounds that can be beneficial to herbivore growth and development, including plant primary metabolites, amino acids, proteins (excluding defensive proteins), and digestible carbohydrates. Allelochemicals from selected studies were any secondary metabolites produced by the plant that could negatively affect herbivore growth and development such as phenolics, alkaloids, terpenes, etc. Herbivore performance from selected studies referred to individual herbivore growth, development, survival, and as well as population growth. Our literature search used the Web of Knowledge and Google Scholar databases, and phrases such as “water stress and insects”, “plant stress and nutrients”, and “plant stress and insects”. We also searched the literature cited from all published studies that we selected. Studies were excluded if they: 1) did not include a well-watered

or stress-free control, 2) implemented water stress without water deficit (e.g., root excision, polyethylene glycol), or 3) lacked the statistical information required for a meta-analysis (i.e., sample size, standard error). Studies included in our meta-analysis did not have plants that were primed for water deficit stress (exposed to water deficit stress prior to study).

5.2.2 Meta-analysis

Changes in macronutrients, allelochemicals, and herbivore performance between control and water deficit-stressed plants were collected from published studies using Grab It! Software (Datatrend Software, 1998-2001) and analyzed using MetaWin 2.0 (Rosenberg et al. 1997). Observations of changes included differences between concentrations of individual macronutrients and allelochemicals between control and water deficit-stressed plants. For example, if a study reported changes in the concentration of the amino acids glutamine, methionine, and phenylalanine, those three differences were counted as individual observations for changes in macronutrients, not averaged and counted as a single observation. Amino acids and other macronutrients are used differently by plants and insects (Chapman 1998, Taiz and Zeiger 2010), thus averaging the changes in concentrations of individual amino acids into a single number may underestimate the degree of change that occurs within plants and the implications those changes have for plant and insect growth and development (e.g., essential versus non-essential amino acids). Treating differences between individual amino acids as

individual observations is an inclusive way to highlight the biological significance of changes in macronutrients and nutrient profiles in water-stressed plants.

We estimated effect size by calculating Hedge's *d*, which measured the magnitude of effect that a treatment had on an experimental unit versus the effect of the control. An effect size of "0" (95% CI includes zero) meant that the treatment had the same effect as the control and differences in effect sizes indicate differences in the magnitude of change. The meta-analytical program MetaWin 2.0 was used to calculate Hedge's *d* which accounts for differences in sample sizes and weights categorical responses according to sample size (Rosenberg et al. 1997). Weighted values are added to each analysis based on the differences between sample sizes, the total variance of categorical data, and estimated with a 95% CI. Hedge's *d* values are compared similarly to ANOVA and between-group heterogeneity (Q_B) was tested against a chi-square distribution to determine if significant differences exist between groups of categorical variables (Rosenberg et al. 1997, Kaplan and Denno 2007). A minimal effect size ranges from $\pm$0.1-0.3, a moderate effect from $\pm$0.3-0.7, and a large effect size is a value larger than $\pm$0.7. In our analyses, we searched for relationships between changes in plant chemistry (e.g., macronutrients, allelochemicals) and herbivore response in various taxonomic groups for plants and insects (including feeding guild and diet breadth for insects). To determine the influence of water deficit stress on different macronutrients in the analyses, we divided "macronutrients" into "total macronutrients" (amino acids, proteins, digestible carbohydrates, including nitrogen), "nitrogenous macronutrients" (amino acids, proteins, including nitrogen), and "digestible carbohydrates" (sugars).

Herbivore performance data was pooled and analyzed as "performance" due to insufficient data to analyze each measurement individually (survivorship compared to fecundity, etc.). In addition, we compared concentrations of macronutrients and allelochemicals in stressed plants that either increased or decreased herbivore performance. For these analyses, we analyzed data from studies with data on macronutrients, allelochemicals, and herbivore performance. Effect sizes were reported using the between-group heterogeneity test statistic (Q_B), the p-value from the test against the chi-square distribution, and the mean effect size $\pm$ a 95% confidence interval.

5.3 Results

5.3.1 Plant responses to water deficit stress

We found 42 published studies that met our selection criteria for the meta-analysis. For the analysis of stress-induced changes in total macronutrients, 11 plant taxa were sufficiently represented in the data to be analyzed. Water deficit stress affected concentrations of total macronutrients (Q_B= 412.06, p<0.0001, df= 10, N= 634; Fig. 5-1) and led to increases in 8 of the 11 plant taxa. Plants in the family Malvaceae greatly increased total macronutrients with an effect size of 0.97$\pm$0.06, followed by plants in the family Brassicaceae (0.69$\pm$0.30). Furthermore, plants in the families Betulaceae, Poaceae, and Salicaceae moderately increased concentrations of total macronutrients (Betulaceae: d= 0.45$\pm$0.31, n= 10; Poaceae: d= 0.34$\pm$0.08, n= 127; Salicaceae: d= 0.32$\pm$0.16, n= 24; Fig. 1) and plants in the families Solanaceae and Rutaceae increased

concentrations minimally (Solanaceae: d= 0.24±0.22, n= 29; Rutaceae: d= 0.12±0.11, n= 147; Fig. 5-1).

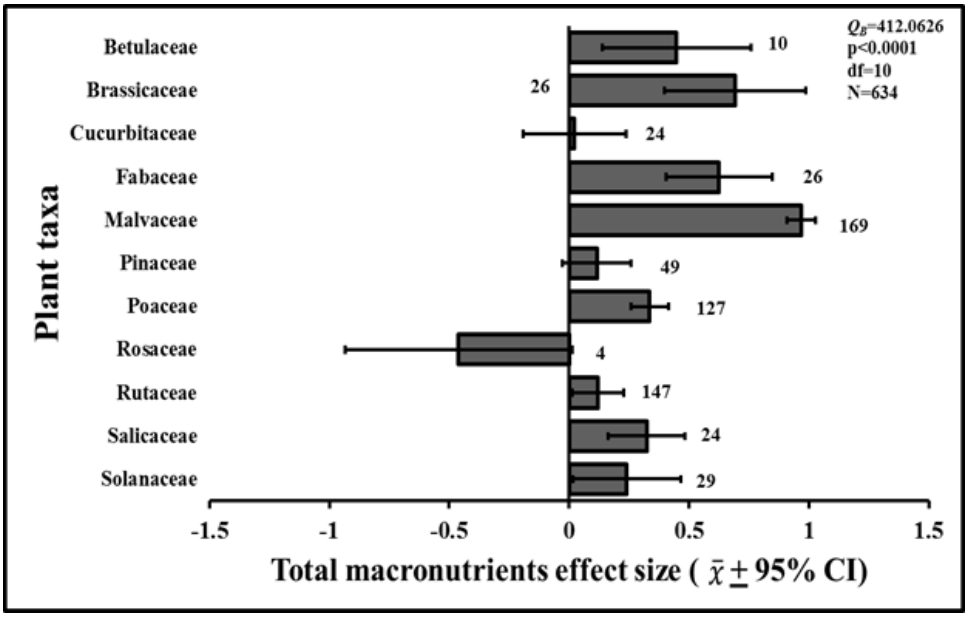

Figure 5-1. Total macronutrients in stressed plants by plant taxa. Mean effect size ± 95% CI. Numbers above bars indicate the number of observations per plant taxa.

Ten plant taxa were sufficiently represented in the data to be analyzed for stress-induced changes in nitrogenous macronutrients and there was significant variation in the effects of water deficit stress among plant taxa (Q_B= 379.33, p<0.0001, df= 9, N= 546; Fig. 5-2A). In 7 of the 10 plant taxa, concentrations of nitrogenous macronutrients increased. There were very large increases in plants from Malvaceae and Brassicaceae (Malvaceae: d= 0.96±0.06, n= 167; Brassicaceae: d= 0.96±0.30, n= 23; Fig. 5-2A). In addition, plants in the families Fabaceae and Betulaceae increased concentrations of nitrogenous macronutrients moderately (Fabaceae: d= 0.63±0.22, n= 26; Betulaceae: d=

0.45±0.31, n= 10; Fig. 5-2A) and plants in the families Pinaceae and Rutaceae increased concentrations minimally (Pinaceae: *d*= 0.12±0.14, n= 49; Rutaceae: *d*= 0.12±0.11, n= 147; Fig. 5-2A).

Concentrations of digestible carbohydrates were analyzed for 7 plant taxa and stress-induced changes varied among plants (Q_B= 27.37, p= 0.0012, df= 6, N= 91; Fig. 5-2B). Water deficit stress significantly increased digestible carbohydrates in 3 of the 7 plant taxa. Salicaceae and Cucurbitaceae plants increased carbohydrates the greatest in response to water deficit stress (Salicaceae: *d*= 0.91±0.58, n= 6; Cucurbitaceae: *d*= 0.87±0.65, n= 5), while four plant taxa exhibited no change (Fig. 5-2B). For instance,

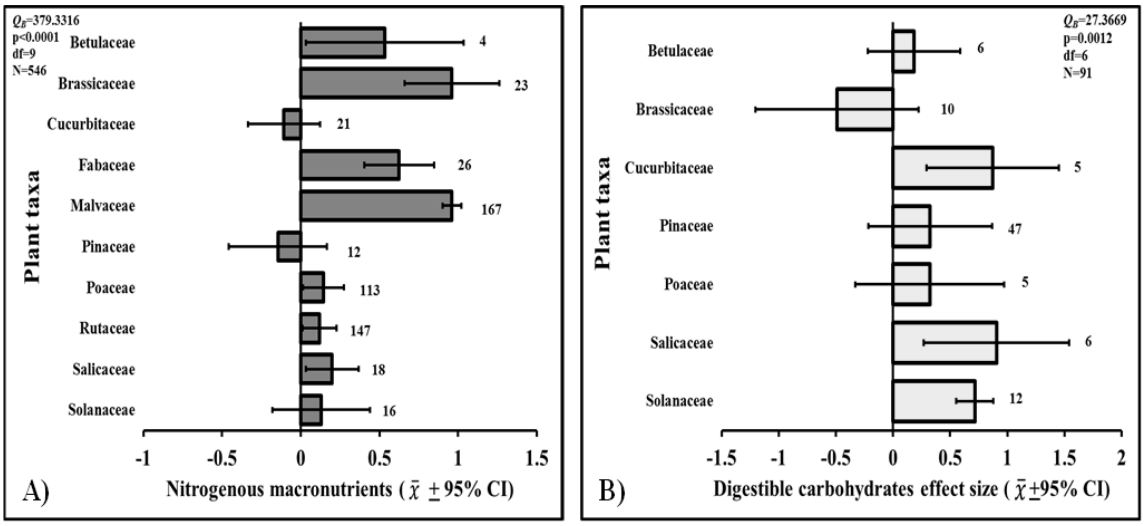

Figure 5-2. Nitrogenous macronutrients and digestible carbohydrates. The effects of water deficit stress on nitrogenous macronutrients (A) and digestible carbohydrates (B) in different plant families. Mean effect size ± 95% CI. Numbers above bars indicate the number of observations per plant taxa.

in one of the studies in our analysis, water deficit-stressed *Populus spp.* (Salicaceae) increased sucrose concentrations by 450% compared to unstressed *Populus spp*

(Tschaplinski and Blake 1989).

Stress-induced changes in allelochemicals were analyzed for five plant taxa that were sufficiently represented in the data to be analyzed. Water deficit stress significantly varied concentrations of allelochemicals among plants (Q_B= 35.14, p<0.0001, df= 4, N= 288; Fig. 5-3) and led to increases in 3 of the 5 plant taxa. Plants in the Solanaceae increased allelochemicals the most in response to water deficit stress (d= 0.39±0.17, n= 48) followed by plants in Ericaceae, while plants in Pinaceae and Salicaceae exhibited no change (Fig. 5-3). For example, in one of the studies in our analysis, water deficit-stressed *Calluna vulgaris* (Ericaceae) increased anthocyanins by 30% compared to unstressed *C. vulgaris* (Bucchetti et al. 2011).

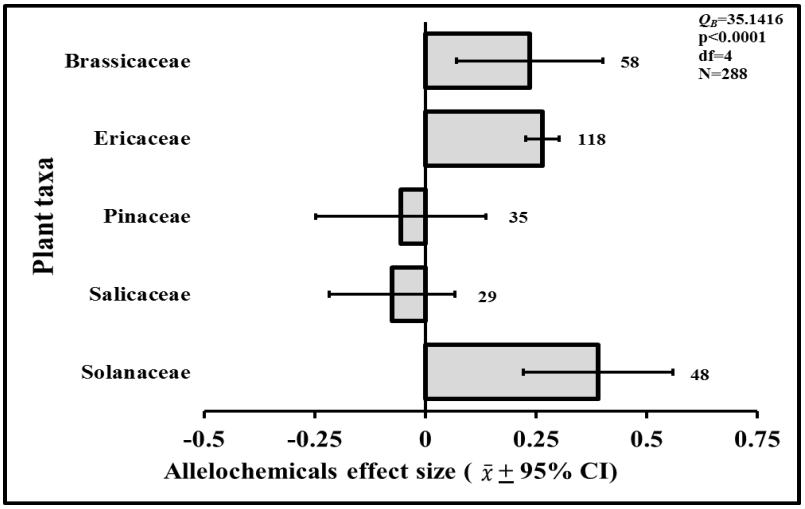

Figure 5-3. Allelochemicals in stressed plants by plant taxa. Mean effect size ± 95% CI. Numbers above bars indicate the number of observations per plant taxa.

5.3.2 Herbivore response to water-stressed plants

Herbivore response to water deficit-stressed plants was analyzed for six herbivore taxa that were sufficiently represented in the data to be analyzed (Fig. 5-4). Herbivore performance was significantly affected on stressed host plants and performance varied among herbivore taxa (Q_B= 162.47, p<0.0001, df= 5, N= 171; Fig. 5-4). Grasshoppers (Orthoptera: Acrididae) increased their performance the most, with a large, positive response to water deficit-stressed plants (d= 0.73±0.17, n= 7) followed by cabbage butterflies (Lepidoptera: Pieridae, d= 0.51±0.39, n= 6) and decreased performance from aphids (Hemiptera: Aphididae, d= -0.37±0.16, n= 40) (Fig. 5-4). For example, in one of the studies in our analysis, green apple aphids (Hemiptera: *Aphis pomi*) decreased in abundance by 82% on water-stressed apple trees (Rosaceae: *Malus domestica*) compared to green apple aphids on unstressed apple trees (Mody et al. 2009).

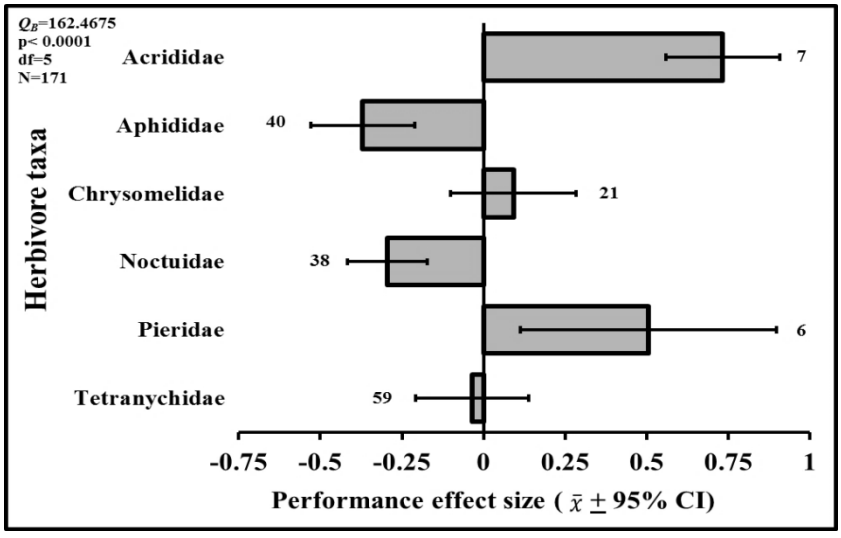

Figure 5-4. Herbivore performance on stressed plants by herbivore taxa. Mean effect size ± 95% CI. Numbers above bars indicate the number of observations per herbivore taxa.

Herbivore diet breadth influenced how herbivores responded to water deficit-stressed plants (Fig. 5-5A). Monophagous herbivores benefited more when consuming stressed plants compared to oligophagous and polyphagous herbivores (Q_B= 17.48, p=0.0016, df= 2, N= 178; Fig. 5-5A). Oligophagous herbivores exhibited no change in performance when feeding on water deficit-stressed plants (d= 0.06±0.12, n= 81; Fig. 5-5A), and polyphagous herbivores minimally increased performance (d= 0.15±0.08, n= 87; Fig. 5-5A).

Chewing and piercing-sucking herbivores in feeding guilds did not vary in performance when feeding on water deficit-stressed plants (Q_B= 1.03, p=0.31, df= 1, N= 173; Fig. 5-5B). Water stress significantly affected the performance of chewing herbivores on stressed plants (d= 0.18±0.08, n= 80, Fig. 5-5B), but did not affect piercing-sucking herbivores (d= 0.10±0.13, n= 93; Fig. 5-5B).

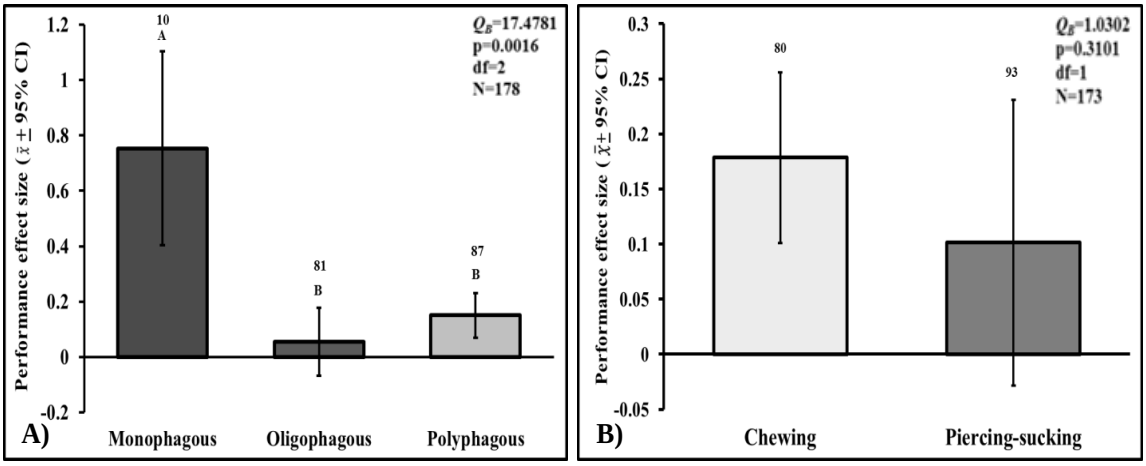

Figure 5-5. Herbivore performance on stressed plants by diet breadth and guild. Herbivore performance by diet breadth (A) and feeding guild (B). Mean effect size ± 95% CI. The "piercing-sucking" guild consisted of phloem, xylem, cell content, and mesophyll feeders, and the "chewing" guild composed of defoliators. Numbers above bars indicate the number of observations per mean. Bars with different letters are significantly different.

5.3.3 Macronutrients, allelochemicals, and herbivore performance

Across all 42 studies, water deficit stress significantly increased concentrations of total macronutrients and allelochemicals in plants, with greater increases in total macronutrients than allelochemicals (Q_B= 137.07, p<0.0001, df= 1, N= 935; Fig. 5-6). Concentrations of total macronutrients increased twice as much as allelochemicals in water deficit-stressed plants (total macronutrients: d= 0.54±0.04, n= 641 and allelochemicals: d= 0.24±0.03, n= 294; Fig. 5-6).

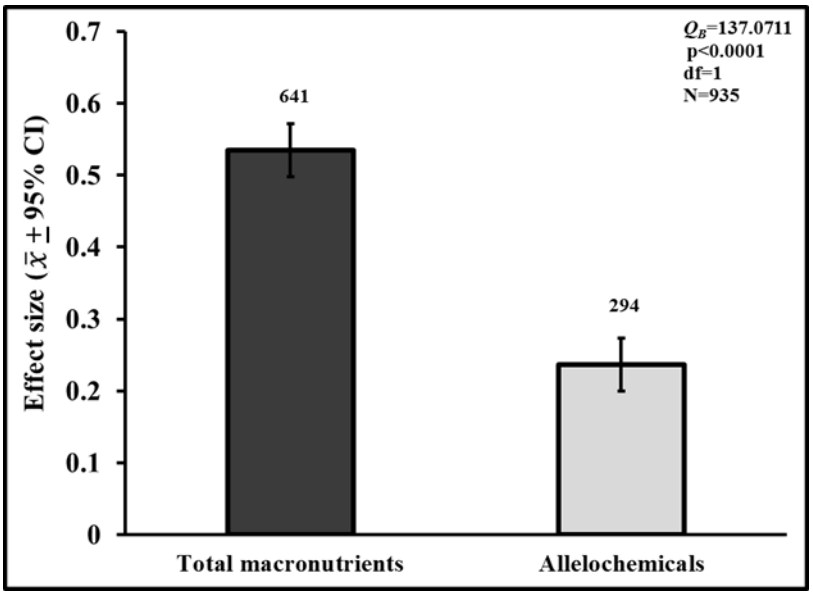

Figure 5-6. Total macronutrients and allelochemicals in stressed plants. Mean effect size ± 95% CI. Numbers above bars indicate the number of observations.

We found strong evidence that stress-related changes in total macronutrients were the most important factors in determining herbivore performance on water deficit-stressed plants. Concentrations of total macronutrients significantly differed between

stressed plants that increased or decreased herbivore performance (Q_B= 41.43, p<0.0001, df= 1, N= 343; Fig. 5-7). Water deficit-stressed plants with moderate to large increases in concentrations of total macronutrients increased herbivore performance (d= 0.46±0.08, n= 76; Fig. 5-7). In contrast, herbivores decreased performance on stressed plants with a minimal increase in total macronutrients (d= 0.19±0.04, n= 267; 5-7). On the other hand, concentrations of allelochemicals in stressed plants were not associated with changes in herbivore performance (Q_B= 0.56, p=0.46, df= 1, N= 76; increased performance: d= 0.11±0.16, n= 62; decreased performance: d= 0.21±0.21, n= 14; Fig. 5-7).

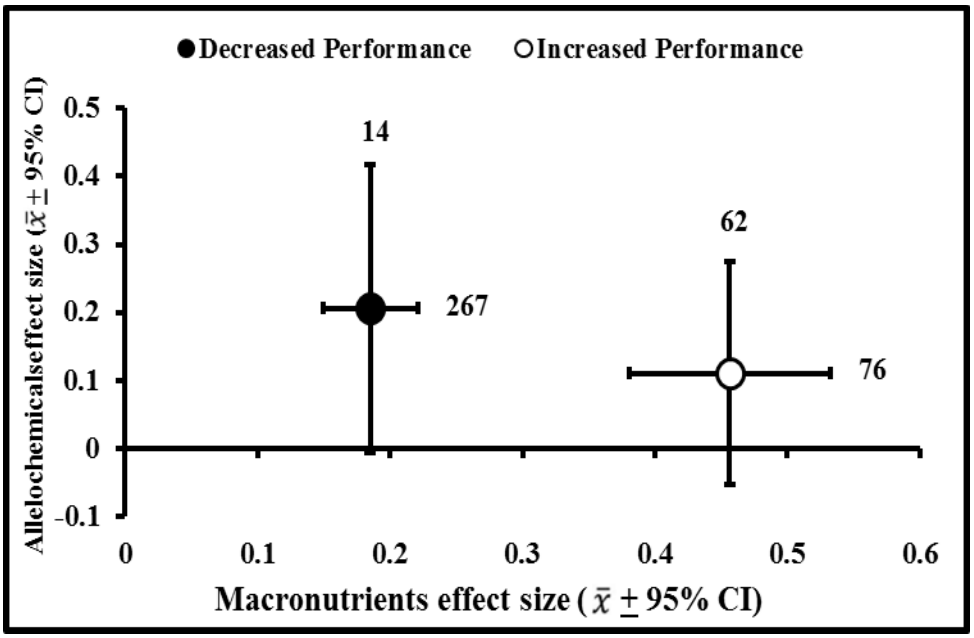

Figure 5-7. Bicoordinate plot with herbivore performance by macronutrients and allelochemicals. A comparison of the concentrations of macronutrients and allelochemicals in water deficit-stressed plants that either decreased herbivore performance (black circle) or increased herbivore performance (white circle). The x-axis is the effect size of macronutrients and the y-axis is the effect size of allelochemicals. The circles are mean effect size ± 95% CI. Numbers above CI intervals indicate the number of observations. Macronutrients: Q_B= 41.43, p<0.0001, df= 1, N= 343; Fig. 9A), allelochemicals: Q_B= 0.56, p=0.46, df= 1, N= 76.

5.4 Discussion

Our analysis provided strong evidence that stress-induced increases in host plant macronutrients determine herbivore performance on water deficit-stressed plants. Based on this result, we propose the Nutrient Availability Hypothesis (NAH), which predicts that changes in the concentration of macronutrients are the most important factor in determining herbivore abundance on water deficit-stressed plants and, conversely, that changes in allelochemicals are not important. This hypothesis is novel because it focuses on stress-induced changes in macronutrients as the mechanism driving insect herbivore performance and abundance on water deficit-stressed plants. In addition, our hypothesis incorporates the predictions of other plant-insect herbivore hypotheses. The Plant Stress Hypothesis predicts that herbivores will outbreak on water stressed plants due to increased foliar nitrogen (White 1969). Research since White developed this hypothesis, however, has shown that not all insect herbivores outbreak on water deficit-stressed plants. The Nutrient Availability Hypothesis expands this idea not only to take into account variation among different plants in their response to water deficit stress, but our associated meta-analysis also indicates that stress-induced changes in carbohydrates and not just changes in nitrogen are responsible for changes in herbivore performance.

The Growth-Differentiation Balance Hypothesis (GDBH) predicts that plants growing in resource-poor conditions (e.g., water deficit limiting carbon uptake) will have increased allelochemicals and overall plant defense as a strategy to reduce resource demand for regrowth due to herbivory (Coley et al. 1985, Lorio Jr 1986, Fajer et al. 1992, Herms and Mattson 1992, Fine et al. 2004). Furthermore, the GDBH predicts that

the defensive strategies resource-limited plants employ may be linked to their evolutionary and life history, leading to differences in macronutrient allocation and plant defense between plant taxa under similar environmental conditions (Coley et al. 1985, Ryser and Lambers 1995, Fine et al. 2004). Our meta-analysis strongly suggests that water stress-induced changes in allelochemicals are relatively unimportant, thus NAH focuses on stress-induced changes in nutrients. The simple predictive nature of NAH may be applied across host plants regardless of the magnitude of stress in their evolutionary history.

Our study was the first comprehensive assessment of how water stress-induced changes in macronutrients and allelochemicals affect herbivore performance. Previous reviews of herbivore performance on water deficit-stressed plants focused on differences between herbivore feeding guilds and often focused on a specific plant taxa or group of plants (e.g., forest species, woody plants) (Mattson and Haack 1987, Price 1991, Koricheva et al. 1998, Huberty and Denno 2004, Cornelissen et al. 2008, Jactel et al. 2012). Inherent differences among plants may be an underlying cause for the inconsistencies observed in studies of interactions between water deficit-stressed plants and herbivores. We found significant variation in the magnitude of change in macronutrients and allelochemicals among 11 plant taxa with different evolutionary histories (Figs. 5-1 and 5-2). This suggests that water deficit stress may induce changes in the concentration of macronutrients and allelochemicals in inherently different ways in different plant taxa. Few studies have directly examined or reported the differences between multiple plant taxa (i.e., plant family) in response to water stress. These few

studies outside our meta-analysis illustrate that various plant taxa respond differently to water stress (Nash and Graves 1993, Koricheva et al. 1998, Durhman et al. 2006). For example, water deficit-stressed *Acer rubrum* (Aceraceae) had a 50% greater net assimilation rate of carbon compared to that of water deficit-stressed *Asimina triloba* (Annonacea) and 30% greater compared to that of water-deficit stressed *Nyssa sylvatica* (Cornaceae) (Nash and Graves 1993). In addition, *Sedum acre, S. kamtschaticum,* and *S. reflexum* (Crassulaceae) produced significantly more biomass during water deficit stress compared to stressed grass and aster species (Asteraceae) (Durhman et al. 2006). These studies did not report differences in macronutrients or allelochemicals, but differences in net assimilation rate and total biomass between different plant taxa during water deficit stress suggest that there are substantial physiological differences among plants while stressed. These physiological differences may contribute to differences in macronutrient and allelochemical concentrations in stressed host plants, effectively increasing variation in herbivore response.

In conclusion, we found that stress-induced changes in concentrations of macronutrients were key in determining herbivore performance on water deficit-stressed plants. We propose the Nutrient Availability Hypothesis which predicts that changes in concentrations of macronutrients will determine herbivore performance on water deficit-stressed plants. We believe focusing on stress-induced changes in macronutrients will greatly improve our ability to accurately predict plant stress-herbivore interactions.

Table 5-1. Published studies analyzed in meta-analysis. The table includes authors, water stress treatments, macronutrients, allelochemicals, host plants, herbivores, diet breadth, guild, performance, and the effect of water deficit-stress on herbivore performance (positive, negative, or both).

Author(s)	Treatment(s)	Macronutrients	Allelochemicals
Amundson et al. 1993	Water stress	General sugar content	---
Barnett and Naylor 1966	Severe and moderate water stress	Amino acids, protein	---
Baskin and Baskin 1974	Water and pulsed water stress	Amino acids	---
Bjorkman 2000	Water stress	---	Phenolic RT40, phenolics
Bucchetti et al. 2011	Water stress	---	Anthocyanins, tannins
Croise and Lieutier 1993	Water stress	Carbohydrates	---
English-Loeb et al. 1997	Water stress for 2-14 days	Total N, carbohydrates	Proteinase inhibitor, chlorogenic acid, rutin
Franzke and Reinhold 2011	Water stress	Amino acids, protein	---
Griffin et al. 1991	Water stress	Amino acids	---
Guehl et al. 1992	Water stress	Carbohydrates	---
Gutbrodt et al. 2011	High and low water stress	Nitrogen	Glucosinolates, alliarinoside, isovitexin
Hale et al. 2003	Water stress	Amino acids	---
Hare et al. 1989	Water stress	Amino acids, protein, total nitrogen	---
Inbar et al. 2001	Water stress	Nitrogen, carbon	Chitinase, glucanase, peroxidase, phenolics
Isaacs et al. 1998	Water stress	Amino acids, carbohydrates	---
Joern and Mole 2005	Water stress	Nitrogen, carbohydrates	---
Kainulainen et al. 1992	Water stress	---	Terpenes
Khan et al. 2010	Water stress	Carbohydrates	---
Koppenaal et al. 1991	Water stressed roots and shoots	Carbohydrates, nitrogen	---
Lehto 1992	Water stress	Nitrogen	---
McQuate and Connor 1990	Water stress	Amino aicds	---
Meyer et al. 2006	Water stress	Carbohydrates, proline	---
Miles et al. 1982	Water stress	Amino acids, carbohydrates, nitrogen	Phenolics
Miles, Aspinall, Rosenberg 1982	Water stress	Amino acids, carbohydrates	Phenolics
Mody et al. 2009	High and low water stress	Nitrogen	---
Pritchard et al. 1997	Water stress	---	Phenolics, tannins
Roth et al. 1997	Water stress	Nitrogen, protein, carbohydrates	Tannins
Sadras et al. 1998	Water stress	Nitrogen	---
Scheirs and Bruyn 2005	High, moderate, and low water stress	Amino acids, protein	---
Showler 2002	Severe to low water stress	Amino acids	---
Showler and Moran 2003	High to low water stress	Amino acids	---
Stewart and Lieffers 1993	Water stress	Nitrogen, minerals, carbohydrates, amino acids	---
Talhouk et al. 1990	Water stress	Sucrose	---
Tariq et al. 2011	High, moderate, low water stress	Nitrogen, carbon	Glucosinolates
Tariq et al. 2013	High and moderate water stress	Nitrogen	Glucosinolates
Thomas and Hodkinson	Low water stress	Nitrogen	---
Tschaplinski and Blake 1989	Water stress	Carbohydrates	---
Turtola et al. 2005	Water stress	---	Salicylates, flavinoids, chlorogenic acids
Waring 1988	High and moderate water stress	Protein	Phenols
Waring and Price 1990	Water stress	Protein, nitrogen	Phenolic resin
Wellburn et al. 1996	Water stress	Protein, nitrogen	Phenols
Young and Hall 1986	Water stress	Protein	---

Table 5-1. Continued

Host Plant Family	Herbivore species	Herbivore (Family: Order)
Pinaceae	---	---
Poaceae	---	---
Leguminosae	---	---
Pinaceae	---	---
Ericaceae	---	---
Pinaceae	---	---
Solanaceae	Spodoptera exigua	Noctuidae: Lepidoptera
Poaceae, Fabaceae	Chorthippus biguttulus	Acrididae: Orthoptera
Salicaceae	---	---
Pinaceae	---	---
Brassicaceae	Pieris brassicae, Spodoptera littoralis	Pieridae, Noctuidae: Lepidoptera
Poaceae	Rhopalosiphum padi	Aphididae: Hemiptera
Rutaceae	Panonychus citri	Tetranychidae: Trombidiformes
Solanaceae	Bemisia argentifolii; Liriomyza trifolii; Heliothis zea	Aleyrodidae: Hemiptera; Agromyzidae: Diptera; Noctuidae: Lepidoptera
Cucurbitaceae	Bemisia tabaci	Aleyrodidae: Hemiptera
Poaceae	---	---
Pinaceae	---	---
Brassicaceae	Myzus persicae	Aphididae: Hemiptera
Pinaceae	---	---
Pinaceae	---	---
Fabaceae	---	---
Piperaceae	Atta colombica	Formicidae: Hymenoptera
Brassicaceae, Myrtaceae	Pieris rapae, Paropsis atomaria	Pieridae: Lepidoptera; Chrysomelidae: Coleoptera
Brassicaceae	Brevicoryne brassicae	Aphididae: Hemiptera
Rosaceae	Spodoptera exigua, Aphis pomi	Noctuidae: Lepidoptera; Aphididae: Hemiptera
Pinaceae	---	---
Salicaceae	Malacosoma disstria	Lasiocampidae: Lepidoptera
Malvaceae	Tetranychus urticae	Tetranychidae: Trombidiformes
Poaceae	Chromatomyia milii	Agromyzidae: Diptera
Malvaceae	---	---
Malvaceae	Spodoptera exigua	Noctuidae: Lepidoptera
Pinaceae	---	---
Betulaceae	Lymantria dispar	Lymantriidae: Lepidoptera
Brassicaceae	Brevicoryne brassicae, Myzus persicae	Aphididae: Hemiptera
Brassicaceae	Brevicoryne brassicae, Myzus persicae	Aphididae: Hemiptera
Salicaceae, Betulaceae	---	---
Salicaceae	---	---
Salicaceae	---	---
Salicaceae	Euura lasiolepis	Tenthredinidae: Hymenoptera
Zygophyllaceae	Asphondylia spp	Cecidomyiidae: Diptera
Pinaceae	---	---
Ulmaceae	Xanthogaleruca luteola	Chrysomelidae: Coleoptera

Table 5-1. Continued

Diet Breadth	Guild	Performance	Effect on Herbivore Performance
---	---	---	---
---	---	---	---
---	---	---	---
---	---	---	(-)
---	---	---	---
---	---	---	---
Polyphagous	Chewing	Growth, survivorship	(-)
Polyphagous	Chewing	Growth, fecundity	(+)
---	---	---	---
---	---	---	---
Monophagous, polyphagous	Chewing	Development, growth	(+) (-)
Polyphagous	Piercing-sucking	Population growth	(-)
Oligophagous	Piercing-sucking	Egg production, density	(+) (-)
Polyphagous	Piercing-sucking, chewing, chewing	Egg production, growth	(-)
Polyphagous	Piercing-sucking	Weight	(-)
---	---	---	(-)
---	---	---	---
Polyphagous	Piercing-sucking	---	(+)
---	---	---	---
---	---	---	---
---	---	---	---
Polyphagous	Chewing	---	(+)
Polyphagous, oligophagous	Chewing	Pupae weight	(+)
Oligophagous	Piercing-sucking	---	(+)
Polyphagous	Chewing, piercing-sucking	Growth, abundance	(-)
---	---	---	---
Polyphagous	Chewing	Growth, development	(-)
Polyphagous	Piercing-sucking	Fecundity, abundance	(-)
Oligophagous	Miner	---	(-)
---	---	---	---
Polyphagous	Chewing	Fecundity, growth, development	(-)
---	---	---	---
Polyphagous	Chewing	Growth	(+) (-)
Oligophagous, polyphagous	---	Fecundity, population growth	(+)
---	---	Fecundity, population growth	(+)
---	---	---	(+)
---	---	---	---
---	---	---	---
Monophagous	Galler	Survivorship	(-)
---	Galler	---	(+)
---	---	---	---
Oligophagous	Chewing	Development, fecundity	(+)

CHAPTER VI

CONCLUSION

Variation in herbivore response to water deficit-stressed plants can be contributed to differences in the effects of pulsed and continuously stress, to differences in herbivore response to these plants, due to interactions between the timing of herbivory and plant development, and variation in the stress-induced changes in stressed plants in not only cotton, but across plant taxa. I found that there were significant physiological differences in the effects these different stress types had on cotton plants (Chapter II). Furthermore, these physiological differences were not always consistent, but ultimately increased the abundance of different herbivores who fed on these plants (Chapter III). In addition, the interactions between the timing of herbivory and plant development differentially affected aphid abundance, the duration of aphid feeding on stressed plants, and cotton development (Chapter IV). Finally, stress-induced changes in water deficit-stressed plants, primarily changes in macronutrients, were the most important factor in determining herbivore performance on stressed plants (Chapter V).

Pulsed and continuous stress had different impacts on photosynthesis, stomatal conductance, transpiration efficiency, plant development, and slight differences in nutrients. This suggests that we need to consider the effects of stress severity and frequency when predicting herbivore response to stressed plants. These developmental differences in stressed plants will determine the amount of plant material herbivores have to consume through significant changes in plant physiology and development such

as through changes in metabolic requirements of CO_2 (Ort et al. 1994, Chaves et al. 2002, Taiz and Zeiger 2010), overall plant height, leaves, and lower water content which would impede the feeding of PS and chewing herbivores (Wearing 1972, Scriber 1977, Huberty and Denno 2004, Douglas 2006). In my dissertation, however, we did not find consistent differences in concentrations of amino acids and digestible carbohydrates and could not support the nutritional predictions of the PSH and PLSH hypotheses. I do believe that the -1.2 MPa may not have been severe enough of a water stress to induce enough differentiation of stress types. Based on my results from Chapter II, I would conclude that herbivores may be responding to other factors aside from nutrients that may influence their abundance, however, this conflicts with the results from my meta-analysis (Chapter IV). Indeed, there were not clear differences in nutrients during my three field studies from 2010-2012, but perhaps the studies in my meta-analysis induced enough stress to make significant differences between stressed and unstressed plants to provide the evidence I needed to develop my NAH hypothesis. In the future, I will increase the amount of stress to ensure that there are strong differences between stressed and unstressed plants. Despite this, I still demonstrated differences in herbivore response to stressed plants.

In Chapter III I demonstrated clear differences in how herbivores respond to pulsed and continuously stressed plants. Herbivores such as thrips, stink bugs, fleahoppers, and whiteflies were more abundant on pulsed stressed plants compared to continuously stressed plants. Aphids, however, were more sensitive to possibly ontological and developmental changes in plants aside from water stress, but were

significantly associated with stress-induced changes in nutrients, however, this could not been supported in Chapter IV. Furthermore, very few studies have compared the effects of pulsed and continuous water stress on herbivores (Lightfoot and Whitford 1987, Schowalter et al. 1999), but these few studies find various effects of stress on herbivore abundance from the same feeding guild and even the same species having contrasting responses to different stressed plants or even plants from the same taxa (Larsson and Bjorkman 1993, Archer et al. 1995, Schowalter et al. 1999, Inbar et al. 2001, Huberty and Denno 2004). With this variation and my field studies illustrating the differences between pulsed and continuously stressed plants, there is further evidence that variation in these studies may be due to differences in stress severity and duration. To compliment my dissertation work, future research should address differences between particular nutrients (i.e., essential versus non-essential amino acids) and the effects these specific nutrients have in herbivore performance. In addition, increasing the controlled level of stress using pressure chambers, but allowing stress to continue further than -1.2 MPa may produce the significant differences between pulsed and continuously stressed plants and clarify herbivore response.

In Chapter IV, the timing of aphid infestation, water deficit-stress, and cotton development yield interesting results and implications, but several aspects made accurate predictions difficult. First, aphid populations declining during the first few weeks of the study has been observed in other studies (Slosser et al. 1989, Major 1990, Weathersbee III and Hardee 1994) and gave cotton time to compensate for early season aphid damage, decreasing the impacts these interactions had on aphids, cotton development, and yield

(Rosenheim and Wilhoit 1993, Rosenheim et al. 1997, Godfrey et al. 2000). Cotton's compensation for aphid herbivory renders constructive predictions of the effects of aphid herbivory and cotton development on water deficit-stressed cotton difficult to make. I did see, however, that aphids added during the seedling stage and water stress had the greatest effect on cotton development and yield, and these plants were consistently underdeveloped compared to counterparts with different treatments. This further highlights, the variation underlying herbivore response to water deficit-stressed plants because there are factors beyond the ones I tested that must have an impact on aphid abundance and performance on stressed plants.

Interestingly, regardless of stress and timing of aphid infestation, cotton plants produced similar cotton lint in terms of quality and quantity. There were several differences between a few treatments, but overall quality was similar. This further supports cotton's compensatory ability to overcome herbivory and stress.

The Nutrient Availability Hypothesis in Chapter V predicts that herbivore performance on water deficit-stressed plants can be predicted by the concentration of stress-related macronutrients in stressed plants. This simple, testable hypothesis may allow us to accurately predict herbivore performance by determining concentrations of nutrients. On stressed plants, herbivores have been shown to increase or decrease in performance and abundance and on many plant taxa, yet nutrients were found to be associated with differences in herbivore performance while considering many changes that occur in stressed plants. Our hypothesis, however, will need to be tested to determine its broad applicability as it may overlook potentially important nuances in

insect ecology with its current broad predictions. Macronutrients will need to be determined in various parts of leaf tissue, not just total foliar or general plant macronutrients as concentrations differ in different parts of the plant and throughout its development. Furthermore, herbivores from different feeding guilds and with different diet breadths feed in different locations on that plant, suggesting that even if a plant increases concentrations of macronutrients, where those changes occur in the plant or even in specific foliar tissues (i.e., leaf surface versus phloem tissue) will be key in determining which herbivores actually benefit from stress-induced increases in macronutrients and to what degree (Hanway and Weber 1971, Boege and Marquis 2005). Additionally, the concentration of allelochemicals may differ in different plant parts (i.e., roots versus leaves) which may affect their unimportance and the predictive value of macronutrients in the hypothesis. These are very important subtleties to consider when testing the broad applicability of my hypothesis and I acknowledge these potentially discrepancies. I do believe, however, that the Nutrient Availability Hypothesis is a significant step forward in predicting herbivore performance on water deficit-stressed plants, but future tests will be needed to determine just how broadly it can be applied.

My dissertation has clarified much of the variation we observe in herbivore response to water deficit-stressed plants and has brought attention to various aspects of plant physiology and insect ecology that we must consider when predicting these interactions. I hope that my research has helped clarify why we observe so much variation in herbivore response to stressed plants and provided some insights into how to

make more accurate predictions and why those insights must be considered. In the future, I will be continuing my passion in studying how water stress affects insect ecology and plan to incorporate other aspects into the predictive equation such as microbes and soil processes, plant-fungal interactions such as with endophytes, and asking my research questions in different systems such as with urban forests. Hopefully incorporating this wide array of perspectives will help clarify the variation we see in these interactions and allow us to accurately predict herbivore response to water deficit-stressed plants.

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