TRITROPHIC EFFECTS OF NITROGEN ON COTTON ECOSYSTEM

by

YIGEN CHEN

(Under the direction of John R. Ruberson)

ABSTRACT

Nitrogen (N) fertilization is one of the most common agronomic practices in crop production, and can have profound impacts on tritrophic interactions, affecting both bottom-up and top-down forces in pest management, and consequently on community structures of ecosystems. In the study the effects of N fertilization (42, 112, 196, and 280 ppm N) on tritrophic inteactions among cotton plants, *Gossypium hirsutum* L., beet armyworm, *Spodoptera exigua* (Hübner), and the parasitoid, *Cotesia marginiventris* (Cresson), were investigated in the laboratory, greenhouse and field.

N addition increased plant biomass and plant nutritional quality as indicated by total N content of leaf blade and petiole nitrate-N. N enhancement decreased the major non-volatile plant defensive compounds (hemigossypolone and heliocides 1-4) of cotton leaf tissue. *Spodoptera exigua* larvae developed faster on plants receiving high N fertilization and significantly fewer *S. exigua* larvae underwent a sixth larval instar than those reared on low N plants. In dual-choice tests, both *S. exigua* larvae and adult females preferentially chose cotton plants with high N fertilization for feeding and oviposition, respectively. Increased nutritional quality and weaker defense of cotton plants receiving high N fertilization might both contribute to faster development and feeding preference of *S. exigua* larvae, and oviposition preference of *S.*

exigua adult females on high N plants compared to low N plants. N fertilization of host plants subsequently fastened the development of *C. marginiventris*, possibly due to more balanced protein:carbohydrate (P:C) ratios, or less defensive compounds in the hemolymph of the hosts.

N enhancement ameliorated the production of plant hormones (jasmonic acids and salicylic acids) and most of the herbivore-induced volatile plant secondary metabolites, except for the major two green leaf volatiles (*Z*-2-hexenal and *Z*-3-hexenal), the releasing of which was increased. The parasitisms of early instar *S. exiuga* larvae by *C. marginiventris* were not significantly affected by N treatments.

The slowed growth of *S. exiuga* larvae feeding on low N plants did not translated into higher mortality inflicted by *C. marginiventris* mainly due to a shift of timing of susceptibility.

N fertilization in the field increased plant growth, and some populations of pests and predators. N fertilization had variable effects on cotton lint yield.

INDEX WORDS: Nitrogen, Plant-herbivore interactions, Tritrophic interactions, *Gossypium hirsutum*, *Spodoptera exigua*, *Cotesia marginiventris*, Plant defense, Plant direct defense, Plant indirect defense, Slow-growth-high-mortality

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YIGEN CHEN

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Major Professor: John R. Ruberson

Committee: Michael Strand
Glen Rains

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

To my lovely and selfless Mom.

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CHAPTER 1

INTRODUCTION

Nitrogen (N) is one of the most important nutrients for both plants and animals. The "building block" of proteins and enzymes that play fundamental roles in all metabolic processes, and genetic materials (DNA and RNA) all need N to function properly. The photosynthetic pigment chlorophyll which sustains life on our planet by absorbing sunlight and making carbohydrates from carbon dioxide (CO_2) and H_2O can not operate normally with the absence of N.

There are differences in N concentrations across trophic levels in terrestrial as well as aquatic ecosystems suggesting that organisms differ in their need for N. The percentage of dry biomass of N of herbivores generally are much higher than that of plants (McNeill and Southwood, 1978; Elser et al., 2000). Fagan et al. (2002) found that insect predators overall possessed 15% more N than herbivorous insects based on reports of 152 species of insects. The disparity of N content was also observed within herbivores of different phylogenetic positions. Mostly newly derived lepidopteran and dipteran herbivorous insects have lower N content than the more primitive coleopteran and hemipteran insects, in particular when terrestrial insects are considered (Fagan et al., 2002). One explanation for the tendency of decreasing percentage of N in recently originated insects would be to lower the dependency on dietary N.

The widespread occurrence of omnivory, defined as insects that feed on more than one trophic level (Menge and Sutherland, 1987; Polis and Strong, 1996), might also reflect that N is a limiting factor for herbivores and those with higher trophic levels. Omnivory is found in diverse habitats and across a large number of taxa (Coll and Guershon, 2002; Denno and Fagan, 2003),

and it is considered to be a strategy to cope with low-N food. Through feeding on non-plant sources of nutrients such as cannibalism (e.g. herbivores) and on other natural enemies (e.g. predators and parasitoids) these omnivorous insects may gain supplemental nutrients.

Although the atmosphere is largely made up of N (ca. 78%), both plants and animals are limited in usable N, because the atmospheric form of N (N_2) is not directly available for living organisms due to the triple bond between the two N atoms. Some soil microorganisms (mostly bacteria from genera *Rhizobium* and *Bradyrhizobium*) both free-living and symbiotic with plants can directly convert some amount of atmospheric N to ammonia (NH_3) which is usable to plants. Microorganism N-fixation is unique to a few types of plants such as legumes (Fabaceae). Examples of leguminous plants are soybeans, peas, clover, and alfalfa. The natural and environment-friendly N fixation pathway, in contrast to industrial fixation, makes the trait desirable. With the advent of agricultural biotechnology, incorporation of these traits into non N-fixing plants may be more promising than ever, since genetic engineers now can transfer the responsible genes from those possessing the ability to those not having the ability. However, the enthusiasm of early genetic engineers has faded because of the limited numbers of genes that can be transferred and the complex interactions of involved genes (Rissler and Mellon, 1996). The soil bacterium *Rhizobia* works closely with legumes and the fixation process involves a large number of bacterial and plant genes (Hirsch, 1992). The complexity of these interactions may limit our ability to design plants with N-fixing properties in the foreseeable future. For non N-fixing plants, the main source of N is artificial fertilizer manufactured through industrial fixation. The problem with industrial fertilizer application is that they often disappear rapidly in nature. Nitrate-N in most inorganic fertilizers does not stay in the soil very well and can be leached readily. In addition, ammonia-N can be lost by volatilization.

Tritrophic interactions (plant—herbivore—natural enemy) are basic components of almost any ecosystem. The potential N effects on tritrophic interactions are complex and are described in Fig. 1.1. Firstly, in plant—herbivore interactions, N availability alters plant quality (from the herbivore's nutritional perspective) as food. This can occur through the plant's direct defenses which are referred to as negative effects of plants directly on herbivores, in contrast to the plant's indirect defenses against herbivores through employing natural enemies of herbivorous insects in plant—natural enemy interactions. Plant direct defenses entail antixenosis, antibiosis and tolerance (Painter, 1951). These direct plant defenses can also affect natural enemies. N effects are relayed to natural enemies of herbivores by changes in herbivore quality (from the natural enemy's nutritional perspective) as host/prey, and may provide herbivore's with a defense against natural enemies. N can also affect the plant's indirect defense via quantitatively and/or qualitatively changing herbivore-induced plant volatiles that are crucial for natural enemies' foraging, plant architecture that might affect natural enemy foraging efficiency, and the quality of food and shelter for natural enemies (collectively referred as to plant indirect defenses).

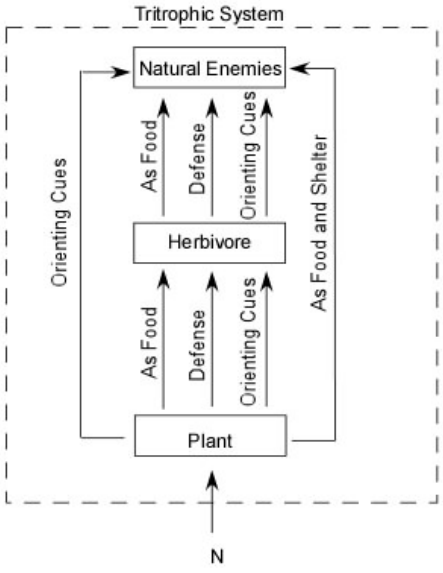

Fig. 1.1 Scheme of potential N effects on tritrophic system.

The impacts of N on tritrophic interactions can also be indirectly executed through changes in atmospheric CO_2 concentration. That the atmospheric CO_2 globally is constantly rising is evident. The concentration has increased to a present level of ca. 370 ppm from the level of 270-280 ppm in the beginning of industrial revolution (Houghton et al., 1996). Although the accurate prediction of CO_2 in the atmosphere in decades to come is difficult and the predicted levels vary greatly, most analyses foresee that the levels will rise to over 700 ppm (Sundquist, 1993). Plants grown under enriched CO_2 typically have a lower percentage of total nitrogen of dry mass, and higher carbon (C) to N ratios (Rogers et al., 1996; Lawler et al., 1997).

N alters suitability of plants as host plant

Nutritional quality as a food plant

The nutritional quality of plants is dependent on plant structures (within plant variation), developmental stages (ontogenetic variation), and species (between plant variation). Within an individual plant the N levels can vary between 0.03 to 7.0% of dry weight with higher N contents in young and expanding plant parts or reproductive structures (e.g., seeds, Mattson, 1980). In the early developmental stages, cotton leaf tissue contains ca. 4% N, but it decreases to less than 3% shortly after flowering (Bassett et al., 1970). Plants can be categorized into competitive or C-selected, ruderal or R-selected, and stress or S-selected on the basis of their primary growing strategies (Grime, 1977). Those plants with C- and R-selected strategies typically have rapid growth, higher N content and occur in habitats with higher resource availability in comparison to S-selected plants.

Plants of the same species subject to different N regimes also have different N contents expressed as percentage N of dried mass or total protein. The higher N available to plants, the

greater N contents they have. As discussed above, N is a limiting resource for phytophagous insects. Thus, changes in N contents can have profound effects on insect physiology and biochemistry, and finally on insect behavior and their survival.

Phytophagous insects that had fed on diets or host plants of lower nutritional qualities overall had lower growth rates, lower efficiency of conversion of ingested food, and lower fecundity (Dixon, 1970; Mattson, 1980; Weibull, 1987; Karowe and Martin, 1989; Lindroth et al., 1995; Awmack and Leather, 2002; Chen et al., 2004), although the degree of response to changes in N can be dependent on herbivore feeding guilds. For example, addition of N to white sagebrush *Artemisia ludoyiciana* Nutt. (Asteraceae) increases performance of seed- and phloem-feeding insects but not chewing insects (Strauss, 1987). The abundance of leaf feeding cereal aphid *Metopolophium dirhodum* (Walker) (Hemiptera: Aphididae) is greater on fertilized wheat *Triticum aestivum* L. (Gramineae) and barley *Hordeum vulgare* L. (Gramineae), and the performance of the ear-feeding grain aphid *Sitobion avenae* F. is unaffected (Honek, 1991).

Given choices, many insect herbivores can distinguish host plants of high quality from those of low quality. Females of two *Pieris* butterflies, *Pieris rapae crucivora* and *P. canidia canidia* (Lepidoptera: Pieridae) (Chen et al., 2004) and buckeye butterfly, *Junonia coenia* Hübner (Lepidoptera: Nymphalidae) (Prudic et al., 2005) prefer fertilized over unfertilized host plants for oviposition. The two *Pieris* species caterpillars also performed better on fertilized compared to unfertilized cabbages (*Brassica oleracea* var capitata L.) (Brassicaceae) in no-choice bioassays (Chen et al., 2004). In a small-scale field study, the diamond-back moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), was more abundant in high N plots than in low N plots (Fox et al., 1990)

To compensate for low N availability in challenged plants, insects generally increase their total food consumption by either increasing consumption rates or prolonging feeding periods, or a combination of the two (Mattson, 1980). Paper birch *Betula papyrifera* (Betulaceae) grown under elevated CO_2 environments had decreased N content (Lindroth et al., 1995). Fourth instar Saturniid caterpillars, *Hyalophora cecropia* L., *Actias luna* L., and *Antheraea polyphemus* Cramer (Lepidoptera: Saturniidae), grown on these birch plants consumed more plant material than on those grown under ambient atmospheric CO_2 (Lindroth et al., 1995). Insect herbivores typically need to reach a certain size before molting to the next stage of development, and N availability will influence this process. Furthermore, the nutritional indexes such as approximate digestibility index (AD), efficiency of conversion of ingested food (ECI) and/or efficiency of conversion of assimilated or digested food (ECD) of insects feeding on low N food are decreased (Mattson, 1980; Chen et al., 2004). This means more food is needed by insects to complete their development.

Both increased consumption rates and feeding periods may increase exposure of herbivores to potential predators, parasitoids, and pathogens. This can be dangerous to herbivores as proposed by the slow-growth-high-mortality (SG-HM) hypothesis. The SG-HM hypothesis states that slower developing herbivores would suffer higher mortality (Feeny, 1976; Augner, 1995; Häggström and Larsson, 1995; Benrey and Denno, 1997; Fordyce and Shapiro, 2003), although the validity of the hypothesis might depend on the system of study and the underlying assumptions (Clancy and Price, 1987; Williams, 1999). Outcomes may vary with the life history of herbivorous insects and their natural enemies, and the extent to which plant characteristics that impair herbivore growth interfere with the foraging efficiency of the natural enemies (Benrey and Denno, 1997). Protracted growth of herbivores can be due to low host plant nutritional

quality, increased allelochemicals, and any morphological traits of host plants that slow down herbivore development. The interactive effects of tri-trophic systems, however, can not be neglected because plant traits that confer resistance to herbivores are not always compatible with the foraging efficiency natural enemies of the herbivores (Cortesero et al., 2000; see Hare, 2002 for a review). For example, adverse effects of plant morphological traits on parasitic insect foraging have been noted in tobacco (*Nicotiana tabacum* L.) (Rabb and Bradley, 1968; Kantanyukul and Thurston, 1973; Elsey and Chaplin, 1978), cotton (*Gossypium hirsutum*) (Treacy et al., 1986), wild potato (*Solanum berthaultii* Hawkes) (Obrycki and Tauber, 1984; Obrycki et al., 1985), Alfalfa (Medicago sativa L.) (Lovinger et al., 2000), and soybean (*Glycine max* L.) (McAuslane et al., 1995).

Direct resistance traits

Instead of being helpless, plants have innate lines of defense to ward off herbivores before their colonization and resisting their effects after colonization. These defensive traits entail escaping infestation by phytophagous insects through desynchronizing growth in space and time with that of herbivores'. Warding off insect colonization can occur via morphological and chemical deterrents, employing mutualistic arthropods, and diverse morphological and chemical defensive traits. For instance, black spruce *Picea mariana* (P. Mill) (Pinaceae) and shrubs such as *Sheperdia Canadensis* (Elaeagnaceae) possess thorny leaves or twigs as part of their defense (Janzen and Marten, 1981).

Plant resistance can be broadly grouped into direct and indirect resistance. Indirect resistance includes any plant traits that increase fitness through interactions with organisms other than herbivore, for example, recruiting entomophagous insects of herbivores. Contrarily, direct

resistance refers to traits such as gossypol in cotton that directly confer negative effects on herbivores. Direct resistance can be further divided into constitutive and induced. Besides maintaining diverse plant secondary metabolites that are presented independent of herbivory, plants can be induced to manufacture large categories of defensive compounds. These nitrogen-containing (e.g., alkaloids, non-protein amino acids, and gossypols) and non-nitrogen-containing (e.g., flavonoids, phenolics, tannins, and terpenes) plant secondary metabolites had previously been considered as "waste products" because they were thought to have no clear functions in plant survival. More and more evidence is emerging showing diverse ecological, physiological and biochemical roles of these chemicals (Seigler and Price, 1976; Bennett and Wallsgrove, 1994; Constabel and Ryan, 1996; Zangerl and Rutledge, 1996; Simmonds, 2003; Wink, 2003; Zagrobelny et al., 2004), albeit there is no unifying theory to explain how and why plants produce, transport, and store such a diverse armory of chemicals (see Firn and Jones, 2000; Dudareva et al., 2004; Peñuelas and Llusià, 2004; Owen and Peñuelas, 2005, 2006(a), 2006(b); Firn and Jones, 2006(a), 2006(b); Pichersky et al., 2006 for discussion). Moreover, resistance can not only be induced locally (induced local resistance, ILR) but systemically as well (induced systemic resistance, ISR) (Loughrin et al., 1994; Röse et al., 1996; Chen et al., 2006).

The mechanisms of plant direct defense to herbivorous insects can be antixenosis, antibiosis, and tolerance (Painter, 1951; Beck, 1965; Glynn et al., 2003; Horber, 1980). A large number of insect and mite pests grow slower or have greater mortality feeding on host plants with antibiotic nature (Karban and Carey, 1984; Jouanin et al., 1998; Carlini and Grossi-de-Sa, 2002; Coviella et al., 2002; Lawrence and Koundal, 2002; Thaler et al., 2002; Ranjekar et al., 2003). A good example for the antibiotic resistance of plants is the performance of beet armyworm larvae on wild-type and genetically-modified tomato plants deficient in jasmonate (Thaler et al., 2002).

Jasmonate is one of the putative plant hormones that transfer wounding signals from wounded sites to other parts of the plant (Dubt and Shure, 1994; Karban and Baldwin, 1997; Zhang and Baldwin, 1997; Schmelz et al., 2003). Significantly more beet armyworm larvae survived on jasmonate-deficient tomato plants compared to wild tomato, and the biomass of larvae reared on jasmonate-free plants more than doubled those reared on wild plants. Given choices between host plants with and without defensive compounds, many insect herbivores avoid those with defensive compounds and consume less on these plants (Alborn et al., 1996; McAuslane and Alborn, 1998; Glynn et al., 2003).

Soil nutrient availability affects the expression of constitutive and induced allelochemicals in a wide range of plant species (Koricheva et al., 1998; Stout et al., 1998; Darrow and Bowers, 1999; Cipollini and Bergelson, 2001; Coviella et al., 2002; Hol et al., 2003; Orians et al., 2003), albeit the magnitude may increase, remain neutral or decline depending on the study systems. For example, total concentration of the carbon-based iridoid glycoside from *Plantago lanceolata* (Plantaginaceae) was decreased by fertilization (Darrow and Bowers, 1999; Prudic et al., 2005). Nitrogen addition also lowered constitutive phenolics in tomato plants, *Lycopersicon esculentum* (Solanaceae) (Stout et al., 1998) and condensed tannins of quaking aspen *Populus tremuloides* (Salicaceae) (Hemming and Lindroth, 1999). However, fertilization had no effect on the phenolics of tulip poplar *Liriodendron tulipifera* and dogwood *Cornus florida* (Dudt and Shure, 1994). Proteinaceous trypsin inbibitor concentrations in *Brassica napus* L. (Brassicaceae) seedlings (Cipollini and Bergelson, 2001) and in tobacco *Nicotiana attenuate* (Solanaceae) (Lou and Baldwin, 2004), and nicotine content in tobacco (Lou and Baldwin, 2004) were enhanced by nutrient fertilization. Proteinase inhibitor levels of tomato plants *L. esculentum* grown under low, medium, and high N conditions remained at the same levels, although leaflet total protein

concentrations increases as N availability went from low to high (Stout et al., 1998). In stinking willie *Senecio jacobaea* L. (Asteraceae), N-based defensive compound pyrrolizidine alkaloid level was lessened by addition of nutrients (Hol et al., 2003).

Besides the effects on C- and N-based constitutive chemicals discussed above, N may also affect plant induced defense at the time of herbivory. For example, N fertilization increased the degree of induced resistance in poplar (*Populus nigra*) after continuous feeding of gypsy moth (Lymantria dispar) for 72 hours (Glynn et al., 2003). Likely, the magnitude of induced trypsin inhibitor in high nutrient treatment was greater than in low nutrient treatment in *Brassica napus* following mechanical damage (Cipollini and Bergelson, 2001).

Since 1970, a number of models have been proposed to explain plant direct defense. Among them are the Growth-Differentiation Balance Hypothesis (GDB; Loomis, 1932, 1953; Herms and Mattson, 1992), Optimal Defense Hypothesis (OD; McKey 1974, 1979; Rhoades, 1979), Plant Apparency Hypothesis (PA; Feeny, 1976), Carbon-Nutrient Balance Hypothesis (CNB; Bryant et al., 1983), and Growth Rate Hypothesis (GR; Coley et al., 1985). However, so far none of these hypotheses can explain the effects of N on the production of plant defensive compounds, or to account for the complex of ecological factors that may influence these processes (for a detailed review of the strengths and drawbacks of the hypotheses, see Stamp 2003).

N alters suitability of herbivore as prey/host of natural enemies

Nutritional quality

The development time of natural enemies is typically positively related to host size, although the relationship can be neutral and negative in some cases (King, 1987; Sequeira and Mackauer, 1992). The dependency of development time upon host size differs regarding idiobiont and

koinobiont parasitoids (Salt, 1941; Vinson and Iwantsch, 1980; Kouamé and Mackauer, 1991; Godfrey, 1994). Host size, in turn, is closely related to nutritional quality of host plants.

The fitness component of predators can be affected by their diet (Jervis and Kidd, 1986; Li and Jackson, 1997; Thompson, 1999; Mayntz and Toft, 2001). For example, when jumping spiders *Portia fimbriata* (Araneae: Salticidae) were provided with prey composed of intra-guild spiders, they had greater survival, in comparison to those supplied with N-poor phytophagous insects (Li and Jackson, 1997). Compared to predacious stink bugs (*Podisus maculiventris*) reared on caterpillars fed on diets made of mature-leaf power, their conspecifics reared on caterpillars fed on diets made of new-leaf powder grew faster (Strohmeyer et al., 1998). The young leaf tissue not only contained higher protein, but also iridoid glycosides. The higher growth when feeding on a young leaf diet was attributed to higher nutrients, even in the presence of higher amounts of iridoid glycosides (Strohmeyer et al., 1998).

Host-feeding parasitoids are restricted to the insect order Hymenoptera and 140 species from 17 families were noted to have this behavior (Jervis and Kidd, 1986). The fecundity of host-feeding parasitoids is also affected by the host they feed on (Jervis and Kidd, 1986; Thompson, 1999). For example, fecundity of hymenopteran parasitoids such as *Bracon hebetor* Say (Braconidae), *Aphytis lingnanensis* Compere. (Aphelinidae), and *Pimpla turionellae* (L.) (Ichneumonidae) was greatest when supplied with haemolymph of their hosts, compared to when starved or only water was available (Edwards, 1954; Debach and White, 1960; Benson, 1973; Lum, 1977), because they obtain amino-nitrogen for egg development (Jervis and Kidd, 1986).

Herbivore defense to natural enemies

Lower nutritional quality of host plants may lower an herbivore's encapsulation ability. The herbivore's chances of encapsulating invading parasites is generally correlated with the herbivore's developmental stage (instar) and physical strength (Salt, 1968; Smith and Smilowitz, 1976; Blumberg and Debach, 1981; van Driesche and Bellows, 1988), which is, in turn, influenced by N availability.

Many plant allelochemicals that function as defensive compounds are sequestered by various herbivorous insects in the hemolymph. The predators and host-feeding parasitoids that feed on those insects, and larval offspring of parasitic wasps that live part of their life time inside such insects will in many cases suffer in terms of developmental time and survivorship (Campbell and Duffey, 1979; Duffey and Bloem, 1986; van Emden, 1995; Kester and Barbosa, 1991; for a review, see Turlings and Benrey, 1998). The adverse effect of the antibiotic tobacco compound nicotine absorbed in tobacco hornworm, *M. sexta*, hemolymph on parasitism and survival of the gregarious parasitoid *Cotesia congregata* (Say) is a good example (Morgan, 1910; Gilmore, 1938; Thurston and Fox, 1972). *M. sexta* is a specialist herbivore in tobacco and can process nicotine effectively mostly through excretion. However, some amount of the nicotine is sequestered in the *M. sexta* hemolymph without any ill-effect to the herbivore (Self et al., 1964). The parasitic wasp *C. congregata*, is more sensitive to nicotine, which reduces their survival (Parr and Thurston, 1972; Thorpe and Barbosa, 1986; Barbosa et al., 1991). No detrimental effects of herbivore-resistance compounds on the fitness of some other natural enemies have been found. For example, *Cotesia vestalis*, a solitary endoparasitoid of diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), grown on *Bt*-resistant caterpillars develop normally (Schuler et al., 1999).

As far as we are aware, there is no study directly linking N fertilization, plant allelochemicals, herbivore defense and natural enemies. Lou and Baldwin (2004) noted that N addition increased tobacco nicotine production. In separate studies, Thorpe and Barbosa (1986), and Parr and Thurston (1972) found lower survival of *C. congregata* on *M. sexta* larvae that had fed on tobacco plants with higher nicotine levels and artificial diets containing nicotine compared to cotton plants with lower nicotine and artificial diets without nicotine, respectively. Therefore, addition of N to tobacco plants will adversely affect the performance of *C. congregata*. In contrast, as shown previously, the quantities of many constitutive defensive plant secondary metabolites are negatively related to N levels. Consequently, in such cases predators and parasitoids that feed or live inside herbivores that are grown on host plants of higher N levels may perform better.

N affects plant indirect resistance/defense incurred through natural enemies

The recruitment of entomophagous natural enemies by plants is referred to as plant indirect defense. Because the relationship can appear mutualistic, these natural antagonists of herbivores are sometimes called 'plant bodyguards' (Dicke and Sabelis, 1988; Whitman, 1994; Cortesero et al., 2000). Herbivore-induced volatile organic compounds (VOCs) that natural enemies rely on when foraging, as well as food and shelter of natural enemies, can be altered by plant N status.

N changes volatile release pattern (orienting cues)

Plants release a blend of volatile chemicals following wounding by herbivore. Some of them are released around the actual feeding site, while others can be induced from plant tissue distal to and above the wounded site. Green leaf volatiles (GLVs) (e.g., (Z)-3-hexenal, (Z)-3-

hexenol, and (Z)-3-hexenyl acetate), some acyclic monoterpenes, sesquiterpenes, homoterpenes, and indole are among the typical locally induced volatile organic compounds (VOCs) (Loughrin et al., 1994; McCall et al., 1994; Turlings et al., 1995; Paré and Tumlinson, 1997, 1998). (Z)-3-hexenyl acetate, some acyclic monoterpenoids, sesquiterpenes and homeoterpenes can be systemically induced (Loughrin et al., 1994; Röse et al., 1996; Paré and Tumlinson, 1997, 1998). Many of these herbivore-induced plant-originated VOCs provide foraging natural enemies essential cues to locate potential host/prey. Both parasitoids and predators have been observed to respond actively to VOCs. For example, the parasitoids *Cotesia marginiventris* (Cresson) (Röse et al., 1998), *Microplitis croceipes* (Cresson) (Röse et al., 1998) and *Cardiochiles nigriceps* Viereck (De Moraes et al., 1998) fly more frequently to host-damaged plants. The predatory mite *Phytoseiulus persimilis* (Acari: Phytoseiidae) and two insect predators, *Scolothrips takahashii* (Thysanoptera: Thripidae) and *Oligota kashmirica benefica* (Coleoptera: Staphylinidae), were attracted to spider mite (*Tetranychus urticae*)-infested lima bean plants (Dicke et al., 1990; Shimoda et al., 2002; Choh et al., 2004)

Nitrogen levels can alter the production and release of these volatiles. Depending upon the plant, positive, negative and no effects have been observed. In corn (*Zea mays* var Delprim), the peak of volatile release was detected when N concentration in the solution was the lowest, both after mechanical wounding and addition of volicitin (an elicitor isolated from oral secretion of beet army worm, *Spodoptera exigua* (Hübner) (Schmelz et al., 2003). Low N availability also increased production of the main sesquiterpenes ((*E*)-*α*-berganotene, *β*-caryophyllene and (*E*)-*β*-farnesene) to a greater extent after volicitin application, compared with mechanical damage. In addition, reduced N levels made the concentration of jasmonic acid (a chemical messenger thought to be crucial to the induction of volatiles) decline at a slow rate. Likewise, in a second

system studied, celery with additional N had a lower quantity of volatile compounds (Van Wassenhove et al., 1990). Nevertheless, Gouinguené and Turlings (2002) found that unfertilized corn plants (*Zea mays* var Delprim) emanated less volatiles when compared with those that had received a complete nutrient solution. The role of N was not implied in this study as all the nutrients were varied (Schmelz et al., 2003). In tobacco (*Nicotiana attenuata*), oral secretion from tobacco hornworm *Manduca sexta* (L.) and methyl jasmonate (MeJA) induced volatile release was not affected by N, though low N availability attenuated the jasmonate and salicylate levels and reduced two N-containing anti-herbivore defense compounds, nicotine and trypsin proteinase inhibitors (Lou and Baldwin, 2004). No other studies on VOC release patterns are available to date. However, the studies to date suggest that the effects of N on the release pattern of VOCs might be system- or species-specific.

Plant as food and shelter of natural enemies

Many insect predators and parasitoids feed on pollen, floral and extrafloral nectar as supplemental food (Keeler, 1977, 1978; Hespenheide, 1985; Koptur, 1985, 1992; Kelly, 1986; Bugg et al., 1989; Pemberton and Vandenberg, 1993; Idris and Grafius, 1995; Jervis and Kidd, 1999; Stapel et al., 1997; Rahat et a., 2005; Lee et al., 2004, 2006). Various fitness correlates of many natural enemies such as longevity, movement and fecundity are increased by feeding on these plant foods (Hagley and Barber, 1992; Wäckers and Swaans, 1993; Olson and Nechols, 1995; Morales-Ramos et al., 1996; Baggen and Gurr, 1998; Eijs et al., 1998; Jervis and Kidd, 1999; Irvin and Hoddle, 2007). Male and female parasitoids *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae), of the tephritid fruit fly for example, lived up to 15 and 28 days, respectively, when cotton extrafloral nectaries are available (Sivinski et al., 2006).

Conversely, with provision of only water male and female parasitoids lived a maximum of 7 days. *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae), an important egg parasitoid of southern green stink bug (*Nezara viridula* (L.)) (Hemiptera: Pentatomidae), lives longer when floral nectars are available (Rahat et al., 2005). Provision of food sources can attract more natural enemies and increase the mortality of herbivorous insects. For instance, parasitism of the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae), was higher on plants with extrafloral nectaries, although the parasitoid species richness between nectaried and nectariless plants was not different (Pemberton and Lee, 1996). More bollworm *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) eggs were parasitized by *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) in cotton plants with extrafloral nectaries than those without nectar (Treacy et al., 1987).

Plants not only provide natural enemies with food, but also shelter. Many plant structures such as leaf domatia and leaf veins can provide shelter and over wintering sites to various natural enemies (Karban et al., 1995; Walter, 1996; Hance and Boivin, 1993; Whitman, 1994; Corbett and Rosenheim, 1996; Elkassabany et al., 1996; Maschwitz et al., 1996; Agrawal and Karban, 1997). However, the effects of N on plant nectar production and shelter provision were not well known.

Predation/parasitism rate changed by N

Natural enemies (predators, parasitoids, and pathogens) of herbivores employ chemical, visual, and vibrational cues (both from hosts/preys and food plants of hosts/preys) to search for potential preys/hosts. The selection of the different categories of cues might be shaped by different selection pressure and may reflect the species'various ecological niches. The

hymenopteran parasitoids *M. croceipes* (Cresson) (Hymenoptera: Braconidae) (Wäcker and Lewis, 1994, 1999), *Cotesia rubecula* (Marshall) (Hymenoptera: Braconidae) (Wäcker, 1994), *Diachasmimorpha juglandis* (Muesebeck) (Hymenoptera: Braconidae) (Henneman, et al., 2002) and dipteran parasitoid *Apocephalus paraponerae* (Borgmeier) (Diptera: Phoridae) (Morehead and Feener, 2000) can use collected visual information to orient to hosts. Vibrational sounding is also commonly used by foraging parasitoids, in particular, by those whose hosts are hidden (such as in wood or plant stem) and immobile (such as pupae). The hymenopteran pupal parasitoid *Pimpla turionellae* L. (Hymenoptera: Ichnemonidae) utilizes vibration to detect their hosts (Fischer et al., 2001). Broad and Quicke (2000) summarized the phylogenetic distribution of parasitoids that possess vibrational detection ability within the order Hymenoptera and discussed the adaptive significance. Among the 56 families studied, only 2 families (Orussidae and Ichneumonidae) were found to have this ability.

The chemical cues (also called semiochemicals) are, in most cases, the most important cues used by natural enemies to locate prey/hosts. Among the numerous crop systems in which herbivore natural antagonists exploit semiochemicals to find their hosts/prey are cotton (*Gossypium hirsutum* L.), corn ((*Zea mays* L.), lima bean (*Phaseolus lunatus*), and Brussels sprout (*Brassica oleracea*). The generalist larval parasitiods *C. marginiventris* and *M. croceipes* were attracted to cotton plant volatiles induced by their corresponding hosts, beet armyworm *S. exigua*, and corn earworm, *Helicoverpa zea* (Boddie) (Wäcker and Lewis, 1994; Röse et al., 1998). The specialist parasitoid *C. nigriceps* utilizes tobacco budworm, *Heliothis virescens* (Fabricius), induced plant volatiles to orient (Cortesero et al., 1997; De Moraes et al., 1998). The predatory mite *P. persimilis* and two insect predators, *S. takahashii* and *O. kashmirica benefica*, were oriented to spider mite, *T. urticae*, infested lima bean plants (Dicke et al., 1990; Shimoda

et al., 2002; Choh et al., 2004). In Brussels sprouts, the *Peiris brassicae*-damaged leaves release a blend of volatiles that are attractive to the parasitoid *Cotesia glomerata* (L.) (Hymenoptera: Braconidae) (Mattiacci et al., 2001). All these examples demonstrated that semiochemicals have their own advantages. Chemical cues, in contrast to other cues, can be used in a distance. They can also be more specifically and reliably (such as those associated with host/prey, feces, and prey/host-induced plant volatiles).

Some parasitoids are more attracted to high N plants and exert a greater control on herbivores. The parasitoid of whitefly, *Bemisia argentifolii* Bellows and Perring (Hemiptera: Aleyrodidae), *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae), was more frequently observed on fertilized and whitefly-infested poinsettia plants, *Euphorbia pulcherrima* than on whitefly-infested but not fertilized plants under choice-tests (Bentz et al., 1996). Significantly more whiteflies per leaf in the high N treatment were parasitized than the number in the low N treatment. The mean counts of whitefly per leaf (sum of parasitized, fed upon and unparasitized) were about the same across the treatments (29.3 or 29.4 individuals) (see Table 1 of Bentz et al., 1996), so the possibility that the parasitoids were responding to greater sucking damage could be excluded. In a study of the impact of collard plant (*Brassica oleracea*) quality on parasitism rate and sex ratio of diamondback moth *P. xylostella* parasitoid *Diadegma insulare* (Hymenoptera: Ichneumonidae), Fox et al. (1990) found more parasitoids in the well-fertilized treatment and parasitism rates were lowest under regimes without application of fertilizer, although foliar N level and protein concentration was marginally correlated with parasitism rate. Additionally, parasitoids that had emerged from high N treatments were more female-biased. Loader and Damman (1991) also found that parasitism rates were higher on cabbage butterfly *Pieris rapae* (Lepidoptera: Pieridae) developing on high N collards.

Predators, in contrast, kill more herbivores in low N conditions (Loader and Damman, 1991). Herbivores feeding on plants with low N availability are generally low in nutrition and predators need to ingest higher number of prey to compensate.

The cues that natural enemies respond to in the studies discussed remains unknown. The crop systems utilized to investigate VOC release patterns differ from those selected to examine natural enemy effects. Based on limited information available at this point, it is hard to draw conclusions on whether or not the observed parasitism/predation patterns are consistent with that of VOC release. However, Olson et al. (2000) showed under field-type conditions, that *M. crociepes* spent 80% of their time searching host-infested and induced cotton plants, parasitized most of the larvae present, and increased their performance over time through learning. Other orienting cues such as visual cues may also play a role in some of the cases because plants with low and high N availability not only differ in height, but also in color and architecture. Plant morphological traits also interact with foraging efficiency of natural enemies, and mutualistic, antagonistic, and neutral relationships between plant trichomes and predators and natural enemies have been documented (Elsey and Chaplin, 1978; Price et al., 1980; Treacy et al., 1986; Kauffman and Kennedy, 1989; McAuslane et al., 1995; Sutterlin and van Lenteren, 1997; Bottrell et al., 1998; Cortesero et al., 2000; Lovinger et al., 2000; Gassmann and Hare, 2005; Simmons and Gurr, 2005; Olson and Andow, 2006; Styrsky et al., 2006).

Summary

N fertilization has profound bottom-up influences on ecosystems— interactively extending across trophic levels. The negative biomass loss of low N plants due to compensatory consumption of herbivores might be compensated for by increased plant direct and indirect

defenses. On the contrary, high N availability to plants promotes plant biomass production, but the increase might be offset by increased removal of biomass by attraction of more phytophagous herbivores. Efficient use of N fertilization is to minimize the negative but maximize the positive components of the interactions, and eventually maximize the net benefit of production. N efficiency can also reduce N contamination of the environment.

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CHAPTER 2

NITROGEN FERTILIZATION RATE AFFECTS LARVAL PERFORMANCE AND FEEDING, AND OVIPOISTION PREFERENCE OF THE BEET ARMYWORM, SPODOPTERA EXIGUA, ON COTTON[1]

[1]Chen, Y., J.R. Ruberson and D.M. Olson. Submitted to *Entomologia Experimentalis et Applicata*. 09/17/2007

ABSTRACT: Nitrogen (N) is one of the most critical chemical elements for plant and animal growth, exerting a variety of bottom-up effects. Development and oviposition of the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), were studied in relation to varying nitrogen fertilization levels in cotton (42, 112, 196, and 280 ppm). Low N fertilization of cotton plants (*Gossypium hirsutum* L.) led to reduced plant biomass, and a lower percentage of N in leaf blades and in leaf petioles. Development of *S. exigua* larvae fed on plants with reduced N applications (42 and 112 ppm) was prolonged relative to treatments receiving higher N fertilization. Larvae reared on artificial diets almost all underwent only 5 larval instars before pupation. However, most larvae reared on cotton plants, irrespective of N levels, experienced a supernumerary sixth larval instar. Further, significantly more larvae reared on lower N cotton plants underwent supernumerary development compared to larvae reared on higher N cotton plants. Lifetime feeding damage per larva ranged from 55 to 65 cm^2, depending upon the nutritional quality of the food plant, although the differences were not statistically significant. Larvae distinguished between cotton plants with various nutritional qualities and fed preferentially on higher N plants. Female moth oviposition choice was also affected by host plant nutritional quality in the study -- cotton plants with higher N levels were preferentially chosen by *S. exigua* females for oviposition. The mechanisms of these effects are unclear, but they can have important implications for population dynamics and pest status of beet armyworms in the field.

KEY WORDS: Nutrients, Sublethal effect, Supernumerary instar, Developmental polymorphism, Plant-herbivore interactions, Lepidoptera, Noctuidae, *Gossypium hirsutum* L., Malvaceae

INTRODUCTION

Nitrogen (N) is a critical element for plants and herbivores, profoundly influencing development and reproduction. Plants with added N typically grow more vigorously, to an upper limit. They also have greater N content, expressed as percentage N of dried mass or total protein, and biomass (Dudt & Shure, 1994; Wilkens et al., 1996; Darrow & Bowers, 1999; Glynn et al., 2003; Stiling & Moon, 2005). Correspondingly, insects that feed on diets or host plants high in N generally have greater growth rates, higher efficiency of conversion of ingested food (ECI) and shorter developmental times (Mattson, 1980; Lindroth et al., 1995; Chen et al., 2004).

Many herbivorous insects can qualitatively distinguish among host plants, and feed and oviposit preferentially on high quality plants (White, 1984; Fox et al., 1990; Jauset et al., 1998; Chen et al., 2004; Prudic et al., 2005). For instance, females of the buckeye butterfly, *Junonia coenia* Hübner (Lepidoptera: Nymphalidae), selected fertilized over unfertilized host plants for oviposition (Prudic et al., 2005). Whitefly, *Trialeurodes vaporariorum* (Westwood), females preferred to oviposit on tomato plants (*Lycopersicon esculentum* Mill.) with high N levels over those with low N (Jauset et al., 1998). Therefore, high N fertilization might contribute to higher pest populations in crops and may lead to yield loss due to preferential feeding. In cropping systems, high N inputs also increase production costs and environmental risks such as contamination of water resources due to fertilizer runoff (Ojeda et al., 2006; Kyllmar et al., 2006; Udawatta et al., 2006).

In contrast to the situation on plants with high N, phytophagous insects feeding on low N plants generally have prolonged developmental times, lower growth rates, and potentially increased windows of vulnerability to their natural antagonists. These changes may result in higher herbivore mortality (termed the “slow-growth high–mortality” hypothesis; Feeny, 1976;

Moran & Hamilton, 1980; Benrey & Denno, 1997; but see Clancy & Price, 1987; Williams, 1999). Therefore, reduced plant biomass due to low N might be compensated for by higher mortality of pests due to natural enemies.

The beet armyworm, *Spodoptera exigua* (Hübner), is a generalist herbivore with over 90 known host plant species, including a number of economically important crops such as tomatoes, cotton, corn, soybeans, peanuts, and peppers (Pearson, 1982). Beet armyworm populations are often suppressed by a complex of parasitoids, predators, and pathogens (Ruberson et al., 1994a; Mohaghegh et al., 2001; Bianchi et al., 2002; Ehler 2007). However, beet armyworm outbreaks can be triggered by applications of insecticides that deplete or remove the natural enemy complex (Eveleens et al., 1973; Ruberson et al., 1994a). Severe beet armyworm outbreaks in cotton have occurred in the US intermittently from the late 1980's, but the factors involved in those outbreaks are poorly understood.

In this study we investigate the impact of N fertilization on cotton plant growth and beet armyworm feeding, growth and oviposition behavior. Specifically, we tested four hypotheses: (1) beet armyworm development will be prolonged due to lower host plant nitrogen fertilization, (2) the lifetime feeding damage of beet armyworm will be inversely related to host plant N fertilization rates, (3) beet armyworm larvae will prefer to feed on host plant tissues receiving higher N fertilization, and (4) beet armyworm females will oviposit preferentially on host plants with higher nitrogen fertilization.

MATERIALS AND METHODS

Cotton plants, *Gossypium hirsutum* L. (Malvaceae) (cv FiberMax 989), were individually grown in 1-liter pots using sphagnum peat moss (Premier Horticulture Inc., Quakertown, PA,

USA) and landscape top soil (Hood Timber Co., Adel, GA, USA) with a ratio of 3:1 as a growing medium in the greenhouse. The photoperiod was L14:D10. The temperature was set at approximately 32 °C during the day and 28 °C during the night.

Four growing regimes (treatments) were utilized, all involving manipulations of nitrogen in the water solution comprising 42, 112, 196, and 280 ppm N (see Table 2.1 for macronutrient formulas). The micronutrients contained in all N treatments were 1 ml each of $MnCl_2$ (0.004 M), $CuSO_4$ (0.0003 M), H_3BO_3 (0.05 M), MoO_3 (0.0001 M), $ZnSO_4$ (0.0008 M), and Fe sequestrene (10% Fe^{3+}) sodium ferric diethylenetriamine penta-acetate in 1 l of water. The four N treatments in ppm are roughly equivalent to 19, 50, 87, and 125 kg/ha N in the field, respectively. The nutrient solutions with varying N levels were generated by altering the volumes of the first two macronutrient chemicals in the solutions to maintain the ratios of NH_4^+ and NO_3^- forms of N within the desirable range (Jones, 1997).

Following seedling emergence, cotton plants were watered daily with 100 ml of 112 ppm N nutrient solution for ca. 2 weeks, when the plants attained the 2-true-leaf stage (the third leaf was still small), at which time plants were assigned to the different treatments and were fertilized with appropriate nutrient solutions. The experimental design was a randomized complete block. Plants were first arranged into blocks by matching leaf size and plant height. Within a block, plants were randomly assigned to each treatment. Plants were fertilized 5-6 times weekly. Leaching (watering without nutrients) followed every fourth nutrient solution application in order to reduce salt (salinity) buildup. About 2 weeks later and at the time of the experiments cotton plants had 3-5 mature true leaves. All plants used in the study were prepared this way and were at this stage unless otherwise noted.

Beet armyworm larvae and adults used in the experiments were laboratory-reared on modified Pinto bean diet (Burton, 1969) at 25 ± 1 °C and photoperiod of L14:D10, except otherwise noted.

N effect on cotton plant growth and nutritional quality

We first characterized the effects of the N treatments on the cotton plants. Nitrogen effects on plant height, and above- and below-ground plant biomass were examined on 20 cotton plants (in 4 blocks). At the time of the experiment, plant height from the first lateral root to the top of the terminal was determined. Plant height at time of first treatment fertilization was determined as a covariate. Above- and below-ground fresh plant material was then cleaned and dried in an oven at 65 °C for 2 days before weight was determined.

Plant tissue N analysis was conducted on 30 plants for each treatment. Leaf blades of the same leaf position for a group of 6 plants per treatment were pooled and placed in paper bags to assess total N. Likewise, leaf petioles of the same treatment within a group were pooled for total nitrate-N analysis. Leaf blade samples were separately prepared by leaf positions (true leaves 1 and 2 combined (L1/2), true leaf 4 (L4), and true leaf 5 (L5)), to understand the within-plant pattern of N, and analyzed for percentage of total N as a percentage of dry mass. Samples were immediately oven-dried at 65 °C for 2 days before submission to the Soil, Plant, and Water Laboratory at the University of Georgia for N analysis. Total nitrogen plant tissue analysis uses a sulfuric (H_2SO_4)-salicylic ($C_7H_6O_3$) acid mixture as a digestion reagent (Buresh et al., 1982). Petiole tissue nitrate-N analysis utilizes a H_2O_2-H_2SO_4 mixture for digestion of plant material in the absence of heavy metals that were previously used in the plant and soil analysis (McGill & Figureiredo, 1993).

Hypothesis 1: Beet armyworm development will be prolonged due to lower host plant nitrogen fertilization

Two trials were set up to assay short-term larval development on cotton plants of different N levels. In the first trial, neonate larvae were bioassayed in Petri dishes kept in an environmental chamber at 25 ± 1°C and photoperiod of L14:D10. Development of individual larvae can vary significantly even on the same diet with all other environmental variables held identical (Y. Chen, personal observation). In order to stabilize variance, groups of 10 larvae were kept in Petri dishes supplied with an excised cotton leaf that was changed daily. Leaves from different treatments used on the same day were of the same leaf node (true leaf 1, 2, 3, and 4 from the beginning to the end). Larvae were weighed after 1, 3, and 4 days in groups of 10, when larvae were 1, 3, and 4 days old, respectively.

To simulate *S. exigua* normal feeding behavior in the nature, a second trial was conducted in which larvae were allowed to move freely throughout the caged plant. In this trial, 10 3-day-old (reared on artificial diets for the first 3 days following eclosion) larvae were put on the second true leaf of cotton plants that were individually caged in a mesh bag in the greenhouse. We used 3-day-old larvae instead of neonate larvae to reduce loss from dislodgement and to facilitate manipulation. The greenhouse conditions were the same as described above. Larvae from each plant were removed after 4 days of feeding and weighed as a group. They were put back on the second true leaf after weighing. Larvae were collected and weighed again 3 days later. Because most of the leaves of caged plants were consumed after 7 days of feeding, recovered larvae were subsequently kept on modified Pinto bean diet (Burton, 1969) to equalize rearing conditions, in groups of 4-5 larvae per diet cup until pupation. Adult emergence was recorded.

In both trials a randomized complete block design was used. Cotton plants were arranged into 8 blocks before randomly assigning them to 4 treatments within each block.

To examine the impact of host plant N levels on lifetime development of larvae, neonate larvae (less than 16-h-old) were reared individually in Petri dishes (d = 50 mm, Becton Dickinson and Company, Franklin Lakes, NJ, USA) with excised leaf tissue from cotton plants of 42 and 196 ppm N treatments in an environmental chamber set at 25 ± 1°C and with a photoperiod of L14:D10. The leaf tissue used was obtained from node 1 but leaf tissue from higher nodes was progressively used as leaves were depleted. Leaf tissue used for all treatments was from the same node on the same day to reduce variability. A small cotton ball soaked with water was placed inside the dish to maintain humidity and the turgidity of leaf tissue. Larvae were not observed feeding or resting on the cotton ball. Fresh leaf tissue was replaced once or twice daily. Larvae were checked daily for molting until pupation. Cast head capsules were measured with an ocular micrometer in a stereomicroscope (model Wild M3C, Leica Microsystems Ltd, Heerbrugg, Switzerland) under 40x magnification, to determine instar. The number of instars each larva went through before pupation was recorded. Each treatment was replicated 5 times with 10 individually-reared larvae per replicate for a total of 50 larvae.

Hypothesis 2: The lifetime feeding damage of beet armyworm will be inversely related to host plant N fertilization rates

The experiment was conducted in the greenhouse described above. Three 3-day-old larvae were caged on the first true leaf of each plant for 2-3 days. Three-day-old larvae were used because they were easy to handle, could not easily escape the cages, and feed sufficiently to permit measurement. Cages were of a clamshell design made with plastic soft drink lids in the

center of which a disk was cut and glued with fine mesh gauze (see Chen et al., 2006 for detail). Larvae were progressively moved to the next upper leaf as they consumed the leaves on which they were caged. Larvae of all treatments were moved to new leaves of the same node on the same day to manage variability. The leaf area eaten was measured 2, 4, and 7 days after initial caging (see Chen et al., 2006 for measurement), and cumulative damage to pupation also was measured, which correspond to leaf tissue consumed within 5, 7, 10 days, and preimaginal lifetime (feeding in the first three days following eclosion is negligible). A randomized complete block design was used with 10 blocks (replicates, 3 larvae/block) and the 4 nitrogen treatments described above.

Hypothesis 3: Beet armyworm larvae will prefer to feed on host plant tissues receiving higher N fertilization

Larval feeding preference was evaluated in cages made of 2 wooden rings (d = 30 cm). The rim of each ring was 2 cm wide and 1.8 cm high with two small notches cut into opposite sides of the ring to accommodate leaf petioles. One side of each ring was covered with fine mesh, and the two rings were clamped together to enclose the experimental leaves and larvae. The petioles of the third true leaf from plants of 2 different N treatments were clamped into the cages wrapped with cotton to prevent escape of the larvae. Eight 5-day-old larvae were placed in the middle of each arena and were recovered 24 h later. The number of larvae on each leaf was recorded at recovery.

Six combinations of 2-choice tests among N treatments were conducted. These were 42 vs. 112 ppm, 42 vs. 196 ppm, 42 vs. 280 ppm, 112 vs. 196 ppm, 42 vs. 280 ppm, and 196 vs. 280 ppm N. Each combination was replicated 4 times.

Hypothesis 4: Beet armyworm females will oviposit preferentially on host plants with higher nitrogen fertilization

Pupae were obtained from the laboratory colony reared on artificial diet. Pupae were sexed and pupae of the same sex were kept in the same cup. After adult emergence, 1 male and 1 female were paired and kept in a 5-ml diet cup. Maximum egg production by female *S. exigua* occurs in the first 1-2 nights following initial oviposition (Fye & McAda, 1972), so females were used immediately following their initial oviposition in the cups. All females used were 3-5 days old.

The same test arena used to assess larval feeding preference was used in the tests. The petioles of the third true leaves from 2 different treatments were placed in the ring cages. Petioles were wrapped with cotton to avoid escape of the adult female. One gravid female *S. exigua* was released in the middle of each test arena and allowed to choose between cotton leaves from 2 N treatments. The test leaves were checked for egg masses 24 h later. The number of egg masses and the number of eggs on each leaf were recorded. The experiment was repeated 8 times for each pairing. The N treatments tested in the experiment were 42, 112, and 196 ppm N. The 2-choice tests were 42 vs. 196 ppm N and 112 vs. 196 ppm N. All tests were done in the greenhouse described previously.

Experimental design and statistical analysis

Plant height, dry shoot and root weights, total plant mass, and nitrate-N were analyzed with one-way ANOVA (SAS Institute, 1999). Percentage total N of dried leaf blades was analyzed with a non-parametric Kruskal-Wallis test (SAS Institute, 1999). Larval weight, time to pupation, and time to adult emergence in relation to N levels were analyzed by one-way ANOVA.

Percentage of *S. exigua* larvae pupated in the free-moving developmental trial was analyzed with Kruskal-Wallis test. Progressive larval weights were analyzed with repeated measures ANOVA, with weighing dates as the repeated measure. Data in supernumerary development experiment were analyzed with one-way ANOVA. Percentages of larvae undergoing 5 or 6 larval instars before pupation were transformed ($\arcsin\sqrt{x}$) before analysis. In the larval preference experiment, percentages of larvae found on a leaf were compared to the percentage of leaf area of the respective leaf with χ^2 tests (PROC FREQ) (SAS Institute, 1999) because differences in leaf size could affect the probability of random larval encounter with the leaves. If the proportions of larvae on the leaves were proportionate to respective leaf areas, then movement was considered random. Otherwise, larval movement was considered non-random, or directional. One-way ANOVA was utilized to analyze beet armyworm feeding damage on days 2, 4, and 7, and lifetime damage. Feeding damage was also analyzed with repeated measures ANOVA. Beet armyworm adult female oviposition results were analyzed with one-way ANOVA. Data were not transformed unless otherwise noted. In all ANOVA analyses, if the null hypothesis was rejected at $\alpha = 0.05$, means were further separated by a two-tailed t-test with $\alpha = 0.05$.

RESULTS

N effect on cotton plant growth and nutritional quality

Plant growth was significantly affected by N supplied in the water (Table 2.2). Plants with higher N applied were taller, had greater dry shoot weight, dry root weight and total biomass compared to those with low N input.

Treatment significantly affected percentage of total N in leaf blade dry mass, regardless of leaf position (L1/2: $\chi^2 = 14.95$, $P = 0.0019$; L4: $\chi^2 = 16.71$, $P<0.001$; L5: $\chi^2 = 16.28$, $P = 0.001$;

Figure 2.1). Total N did not differ significantly between 196 and 280 ppm regardless of leaf position (L1/2: $\chi^2 = 0.01$, P = 0.9168; L4: $\chi^2 = 2.45$, P = 0.1172; L5: $\chi^2 = 3.15$, P = 0.0758; Figure 2.1). Plants receiving 196 ppm N had consistently higher percentages of total N compared to those receiving 112 ppm N, regardless of leaf position (L1/2: $\chi^2 = 6.82$, P = 0.009; L4: $\chi^2 = 6.82$,P = 0.009; L5: $\chi^2 = 4.81$, P = 0.028; Figure 2.1). Likewise, the percentage of total N of leaf blade dry mass in 112 ppm plants was higher than those receiving 42 ppm N across the leaf positions (L1/2: $\chi^2 = 5.77$, P = 0.0163; L4: $\chi^2 = 6.82$, P = 0.009; L5: $\chi^2 = 6.82$, P = 0.009; Figure 2.1).

Leaf petiole nitrate-N status was strongly affected by treatment ($F_{3,19} = 219.20$, P<0.0001; Figure 2.2). The average nitrate-N amount in the 280 ppm treatment was 14416.60 ± 766.49 ppm. The corresponding value for the 196 ppm treatment was 8120.40 ± 700.89 ppm, which was significantly lower than that in 280 ppm (Figure 2.2). Similarly, nitrate-N level was higher in plants of the 196 ppm treatment compared to the 112 ppm treatment (1622.00 ± 33.20 ppm). The nitrate-N level of 112 ppm was in turn higher than that of 42 ppm (163.40 ± 12.42 ppm) (Figure 2.2).

Hypothesis 1: Beet armyworm development will be prolonged due to lower host plant nitrogen fertilization

N had a significant effect on beet armyworm development regardless of the age of the larvae used at the onset of trials. In the neonate bioassay, larval weight differed across treatments only 3 days after beginning the trial (weight was not assessed on days 1 or 2) (Figure 2.3). The difference was greater on day 4. The effects of date, N treatment, and their interaction on larval weight were all significant (P<0.0001 in all cases; Figure 2.3). The interaction of date and N

treatment was due to weight interactions of larvae in 196 ppm N and 280 ppm N between days 3 and 4. On day 3, larvae in the 196 ppm treatment weighed significantly more than those in 280 ppm, while on day 4 those in the 280 treatment weighed more.

In the cage trials that permitted intra-plant movement of 3-day-old larvae, larvae reared on 196 and 280 ppm N plants weighed significantly more than those reared on 112 and 42 ppm N plants after 4 days of feeding (larvae were 7-day-old) (Table 2.3). The same pattern was observed 3 days later (10-day-old larvae) (Table 2.3). The time from onset of bioassay to pupation and to adult emergence was significantly affected by N treatment ($P < 0.0001$ in both cases; Table 2.3). Pupal weight and percentage of larvae pupating were not significantly influenced by N (Table 2.3).

Nitrogen fertilization of host plants affected the number of larval instars beet armyworm larvae underwent before pupation (Table 2.4). Of larvae reared on cotton plants in the 42 ppm N treatment, an average of 96.0 ± 2.45% underwent 6 instars before pupation, which is significantly higher than the percentage (76.0 ± 0.51%) of supernumerary larvae reared on 196 ppm N treatment plants ($F_{1,8} = 14.05$, $P < 0.0056$). Regardless of N treatment, however, more beet armyworm larvae reared on cotton plants underwent 6 instars (42 ppm N treatment: fifth:sixth instar = 4%:96%, $\chi^2 = 7.26$, $P < 0.0071$; 196 ppm N treatment: fifth:sixth = 24%:76%, $\chi^2 = 6.90$, $P<0.0086$; Table 2.4) than is the case on artificial diets (Y Chen, unpublished data).

Developmental times of larvae were affected by the N levels of host plants on which they were reared (Table 2.4). The developmental time from egg hatch to the second instar, fifth instar, sixth instar, and pupation was significantly longer for larvae reared on 42 ppm N cotton plants than corresponding times of larvae reared on 196 ppm N host plants, although differences from egg hatch to the third and fourth instars were not significant among N treatments (to the second

instar: $F_{1,8} = 8.71$, $P < 0.02$; to the third instar: $F_{1,8} = 2.82$, $P < 0.13$; to the fourth instar: $F_{1,8} = 2.71$, $P < 0.14$; to the fifth instar: $F_{1,8} = 7.82$, $P < 0.02$; to the sixth instar: $F_{1,8} = 21.52$, $P < 0.0017$; to pupation: $F_{1,8} = 41.88$, $P < 0.0002$).

Hypothesis 2: The lifetime feeding damage of beet armyworm will be inversely related with host plant N fertilization rates

Originally there were 10 replicates for each treatment. Some replicates were excluded due to escape of larvae. So, only those replicates in which all 3 larvae were recovered (8 replicates for 42 and 280 ppm N; 7 for 112 and 196 ppm N) at the end of trial were included.

Beet armyworm larvae consumed little leaf tissue before 7 days of age (Figure 2.4), when in the later portion of the third instar. The total area eaten by that time was less than 5 cm^2 per larva. The damage increased rapidly thereafter until pupation (Figure 2.4). The time from bioassay to pupation varied between 1 and 3 days among larvae. The lifetime leaf areas fed upon by larvae reared on 42, 112, 196, and 280 ppm N plants were 64.7 ± 6.18, 60.1 ± 3.63, 54.6 ± 3.18, and 55.5 ± 2.61, respectively (Figure 2.4). The difference across treatments was not statistically significant ($P = 0.11$). However, the difference between highest damage (42 ppm N) and lowest damage (196 and 280 ppm N) reached ca. 10 cm^2 per larva, which was a ca. 15% difference.

Hypothesis 3: Beet armyworm larvae will prefer to feed on host plant tissues receiving higher N fertilization

N affected beet armyworm larval feeding choice (Figure 2.5). In all 6 combinations of 2-choice tests between 42, 112, 196, and 280 ppm N treatments, more larvae were found on the leaves with higher N content. Based on the percentage of larvae found on a leaf compared to the

percentage of leaf area, the movement of larvae was not random ($P < 0.01$ in all cases) (Figure 2.5).

Hypothesis 4: Beet armyworm female will oviposit preferentially on host plants with higher nitrogen levels

N significantly affected beet armyworm female oviposition preference. There were significantly more egg masses laid on higher N cotton leaves (42 vs. 196 ppm N: $F_{1,14} = 21.00$, $P < 0.001$; 112 vs. 196 ppm N: $F_{1,14} = 14.97$, $P < 0.01$; Table 2.5). There were significantly more eggs laid on higher N cotton leaves (42 vs. 196 ppm N: $F_{1,14} = 23.84$, $P < 0.001$; 42 and 196 ppm N: $F_{1,14} = 16.40$, $P < 0.01$; Table 2.5). In a few cases there were some egg masses laid on the test cages. But compared to the surface areas of the cage and the leaves, this was negligible.

DISCUSSION

As expected, greater N fertilization increased plant total N content in the study. Within a treatment the percentages of total N in younger leaves were higher than those of older leaves. This may reflect the generally greater fitness value of younger leaves, and that nutrients of older leaves translocate nutrients to young leaves as leaves age (Ohnmeiss & Baldwin, 2000). Leaf petiole nitrate-N is a reliable indicator of cotton N status (Keisling, 1995; Weir et al., 1996), and N fertilization increased leaf petiole nitrate-N.

Increased N application enhanced plant growth and increased plant nutritional quality for *S. exigua* larvae. Larvae that had fed on higher N diets or host plants had greater growth rates, and shorter developmental times, which is similar to what has been found for other herbivores on other host plants (Mattson, 1980; Lindroth et al., 1995; Chen et al., 2004). *S. exigua* larvae also

were capable of detecting the nutritional difference in various food plants and preferentially fed on host plants with higher nutritional quality. This is also similar to what has been found for other herbivores on other plants (White, 1984; Fox et al., 1990; Chen et al., 2004; Prudic et al., 2005).

The developmental times of *S. exigua* larvae feeding on lower N cotton plants were prolonged even though some of the larvae were fed upon lower N plants for less than half of their larval developmental lives. The lifetime feeding damage of *S. exigua* larvae on host plants with varying N levels were not statistically significant from each other, although larvae feeding on lower N plants tended to consume more plant tissue. The lack of compensatory feeding of *S. exigua* larvae on low nutritional quality foods was unexpected because many lepidopteran herbivores have been shown to compensate for low nutritional quality by increasing consumption amounts (Woods, 1999; Lavoie & Oberhauser, 2004; Lee et al., 2004a). Therefore, the reduced suitability of low N plants can increase feeding damage on these plants. Nevertheless, beet armyworm larvae are capable of choosing higher N plant tissues for feeding as shown in this study. Therefore, if beet armyworm larvae are able to choose and can move to higher N plants, overall feeding damage may be reduced on the less-preferred plants than on high N plants.

Furthermore, larvae on lower N plants would likely be exposed to higher mortality due to increased exposure to natural enemies through longer developmental times and increased movement. However, as found by DM Olson and AM Cortessero (unpublished) cotton plants with too low or too high N and those that are water-stressed and fed upon by the caterpillar *Heliocoverpa zea* (Boddie) are less attractive to the caterpillar's parasitoid, *Microplitis croceipes* Cresson (Hymenoptera: Braconidae). Although beet armyworm is not a host of *M. croceipes*, beet armyworm parasitoids may be similarly affected. Thus, N effects on the third trophic level

may need to be considered before we more fully understand the relationship between N fertilization and cotton plant damage by beet armyworm.

Female moths in this study showed a significant oviposition preference for cotton plants with higher N applications. Female oviposition choice in large part determines the fate of the offspring, particularly in those insects with low-mobility immature stages. One would expect oviposition preference to coincide with larval suitability as shown in this study. However, this is not always the case (Courtney, 1981; Singer et al., 1994; Berdegué et al., 1998; Showler & Moran, 2003). The beet armyworm is a generalist herbivore with over 90 known host plant species (Pearson, 1982). Both positive and negative correlations between beet armyworm oviposition preference and offspring performance have been reported in the literature. In tests with different host plant species, Showler (2001) showed that beet armyworm adult females preferred pigweed, *Amaranthus hybridus* L., over cotton for oviposition, and that third-instar beet armyworm larvae also chose *A. hybridus* over cotton in choice tests. Nevertheless, in choices between the host plant species *Chenopodium murale* L. (Chenopodiaceae) and *Apium graveolens* L. (Umbelliferae), beet armyworm adult females preferred *C. murale* over *A. graveolens* for oviposition, although the development of larvae reared on *C. murale* was prolonged and pupal weights were lower in comparison to those reared on *A. graveolens* (Berdegué et al., 1998). Given choices between drought-stressed and control cotton, beet armyworm females oviposited more on drought stressed plants (Showler & Moran, 2003). This relationship was suggested earlier by from field observations (Ruberson et al., 1994b; Ruberson, 1996). Again, larval performance expressed as larval survivorship was greater on non-stressed cotton plants which were less preferred for oviposition (Showler & Moran, 2003). There is so far no satisfactory explanation for the lack of a positive correlation between larval feeding

performance and female oviposition preference in these studies. One possible explanation is intra-specific competition or interference in resource-limited areas, because in the preliminary experiment with beet armyworm females, they not only showed no oviposition preference between cotton plants with various N levels when 6 females were released per cage, but they also oviposited the majority of eggs on walls of cage and flower pots (Y Chen, personal observation). But this explanation cannot account for the lack of a positive correlation between larval feeding performance and female oviposition preference shown by Showler & Moran (2003). In that study, only individual females were used in choice tests. Other mechanisms such as selection for enemy-free space (Lawton & McNeill 1979) or several possibilities such as the parasite/grazer hypothesis proposed by Thompson (1988) may also account for the differential relationship between beet armyworm larval feeding performance and adult female oviposition choice on water-stressed and variable N levels in cotton. More work needs to be done to fully understand these phenomena.

The mechanisms by which N fertilization increases *S. exigua* larval growth, and modifies larval feeding and female oviposition preferences are unknown. It is possible that the variable N applications shifted the balance of the plants' protein to carbohydrate ratio (P:C), or reduced levels of plant defensive compounds, or a combination of the two. An appropriate P:C ratio is important for growth and development of many phytophagous insects (Simpson & Raubenheimer, 1993; Bede et al., 2007). *Spodoptera exigua* is a generalist herbivore and prefers higher protein diets than specialist herbivores such as *Spodoptera exempta* (Walker) (Lepidoptera: Noctuidae) in choice tests (Lee et al., 2004b). The self-selected dietary P:C ratio for *S. exigua* larvae was 22:20 (Bede et al., 2007). *Spodoptera exigua* larvae have also been shown capable of distinguishing between glanded (containing constitutive defensive terpenoid

aldehydes) and glandless isogenic lines of cotton plants and preferentially chose to feed on glandless cotton plants (McAuslane & Alborn, 1998). Many plant defensive allelochemicals have been reported to be decreased by N addition (Stout et al., 1998; Darrow & Bowers, 1999; Hemming and Lindroth, 1999; Schmelz et al., 2003; Prudic et al., 2005), so it is possible that some elements of preference are attributable to changes in plant defensive capacity. These qualitative and quantitative attributes may be malleable by varying nitrogen fertilization, and may further modify the herbivore-plant interactions.

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Table 2.1 Macronutrient solution formulas (ml/l water) for generating four N levels (modified Hoagland solution, Hoagland & Arnon 1950).

	N treatments (ppm N)			
Macronutrients	**42**	**112**	**196**	**280**
NH_4NO_3 (1M)	0.5	1	2	5
$Ca(NO_3)_2$ (1M)	1	3	5	5
KH_2PO_4 (1M)	4	4	4	4
$MgSO_4$ (1M)	2	2	2	2

Table 2.2 Cotton plant growth parameters in response to N availability in the greenhouse (cotton plants were at 3-5 mature leaf stages at time of experiment)

N treatment (ppm)	Plant height Mean ± SE (cm)	Dry shoot weight Mean ± SE (g)	Dry root weight Mean ± SE (g)	Total mass Mean ± SE (g)
42	29.67 ± 2.40a	2.01 ± 0.16a	0.51 ± 0.23a	2.53 ± 0.17a
112	34.95 ± 2.26b	3.56 ± 0.42b	0.72 ± 0.08b	4.28 ± 0.49b
196	40.13 ± 1.37c	5.15 ± 0.27c	0.89 ± 0.09bc	6.04 ± 0.33c
280	43.08 ± 1.29c	6.46 ± 0.33d	0.87 ± 0.08c	7.34 ± 0.39d

Source	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
Block	4	5.56	0.01	4	4.17	0.02	4	5.40	<0.01	4	5.62	<0.01
Treatment	3	18.2	<0.01	3	69.5	<0.01	3	12.1	<0.01	3	71.6	<0.01
Covariate[1]	1	13.2	<0.01									

[1]Plant height at the time of being first fertilized with corresponding nutrients. Means of plant height, dry shoot weight, dry root weight, and total mass were separated by pair-wise t–test after the null hypothesis of equality was rejected at $\alpha = 0.05$. Means followed by different lower-case letters imply they were significantly different at $\alpha = 0.05$.

Table 2.3 Growth and developmental times of *Spodoptera exigua* larvae on cotton plants grown with various N levels

N treatment (ppm N)	Larval weight (mean ± SE)(mg)		Time to pupation[1] (mean ± SE) (days)	Time to adult emergence[2] (mean ± SE) (days)	Pupal weight (mean ± SE) (mg)	% larvae pupated (mean ± SE)
	7-day-old	10-day-old				
42	9.00 ± 0.52a	42.34 ± 2.48a	15.82 ± 0.13a	23.04 ± 0.25a	107.59 ± 3.16	47.62 ± 7.43
112	11.20 ± 0.29b	63.34 ± 1.60b	15.09 ± 0.18b	22.28 ± 0.20b	100.27 ± 3.74	53.57 ± 5.10
196	13.10 ± 0.62c	82.19 ± 5.54c	14.72 ± 0.16bc	21.99 ± 0.30bc	101.28 ± 3.22	54.76 ± 7.01
280	12.91 ± 0.44c	93.26 ± 8.65c	14.49 ± 0.10c	21.59 ± 0.19c	102.89 ± 1.93	63.10 ± 4.40

ANOVA

Source	d.f.	F	P	F	P	F	P	F	P	F	P	χ^2	P
Block	6	0.64	0.70	1.06	0.42	0.91	0.51	2.12	0.07	2.80	0.0466	--	--
Treatment	3	14.25	<0.0001	17.68	<0.0001	16.21	<0.0001	10.06	<0.0001	0.88	0.4721	3.1899	0.3633

[1]Time from onset of bioassay to pupation.

[2]Time form onset of bioassay to adult emergence.

Means followed by lower case letters within a column imply the difference was significant at $\alpha = 0.05$ level. Percentage of larvae pupated was analyzed with Kruskal-Wallis test. Other data were analyzed with ANOVA and means were separated with Duncan's test after the overall null hypothesis was rejected at $\alpha = 0.05$.

Table 2.4 Supernumerary larval development of *Spodoptera exigua* due to low nutritional quality of cotton

N treatment	No.	Developmental time[1]						Maximum larval instars[2]	
	larvae	Second instar	Third instar	Fourth instar	Fifth instar	Sixth instar	Pupation	Fifth instar (%)	Sixth instar (%)
42 ppm N	50	4.16 ± 0.18b	7.12 ± 0.19	9.44 ± 0.28	12.64 ± 0.43b	16.04 ± 0.41b	22.36 ± 0.29b	4.00 ± 2.45a	96.00 ± 2.45*
196 ppm N	50	3.52 ± 0.12a	6.72 ± 0.15	8.90 ± 0.16	11.22 ± 0.27a	13.60 ± 0.32a	19.00 ± 0.43a	24.00 ± 5.10b	76.00 ± 5.10*

[1]Time from egg hatch to various developmental stages.

[2]*Spodoptera exigua* have 5-6 instars.

*Within N treatment, significantly more *S. exigua* underwent a sixth instar before pupation. Data were analyzed with one-way ANOVA. Percentages of individuals undergoing 5-6 instars before pupation were transformed (arcsin$\sqrt{x}$) before analysis.

Table 2.5 The number of egg masses and eggs laid on test leaves in *Spodoptera exigua* female oviposition choice tests[1].

Choice test	42 vs. 196 ppm N		112 vs. 196 ppm N	
No. egg and mass	42 ppm N	196 ppm N	112 ppm N	196 ppm N
No. egg mass (Mean ± SE)	0.00 ± 0.00	1.50 ± 0.33***	0.38 ± 0.26	2.00 ± 0.33**
No. eggs (Mean ± SE)	0.00 ± 0.00	38.00 ± 7.78***	9.88 ± 6.50	57.75 ± 9.87**

[1] The third true leaves from cotton plants grown with 2 N treatments were used in the bioassay; ** P<0.01; *** P<0.001.

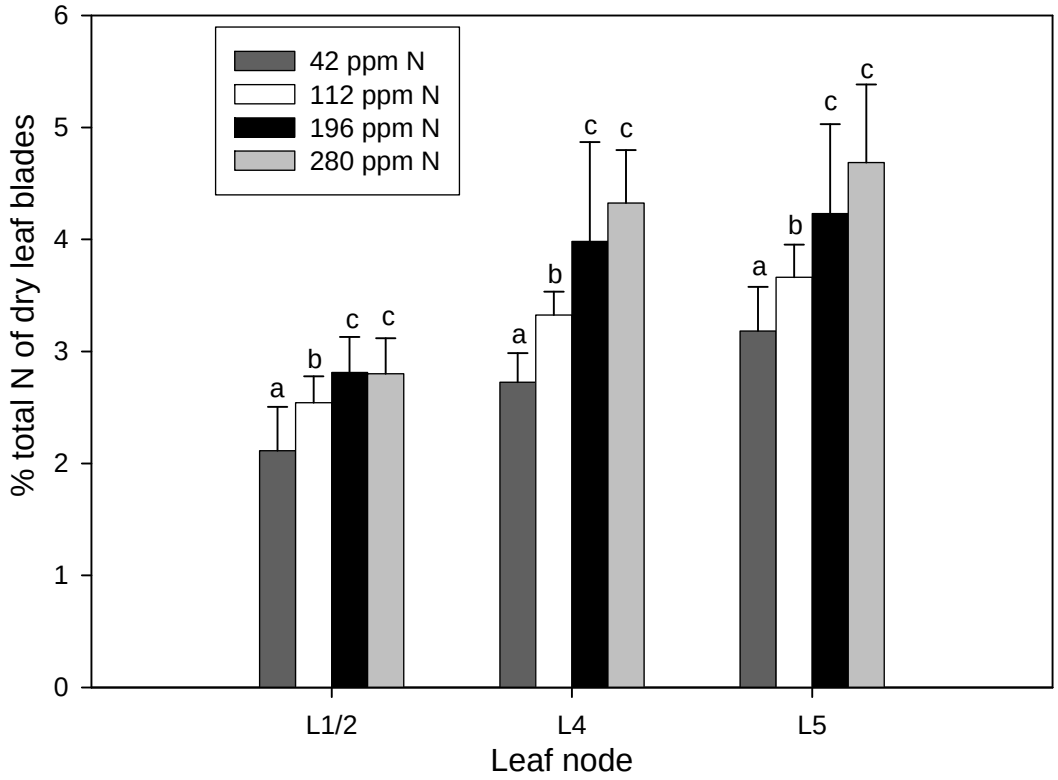

Figure 2.1 Percentage of total N of dried leaf mass in relation to different N levels. L1/2: true leaves of node 1 and 2; L4: true leaf of node 4; and L5: true leaf of node 5. The bars represent averages of 5 replicates. Different lower-case letters above N treatments of the same leaf position mean significant difference at $\alpha = 0.05$. Data were analyzed with Kruskal-Wallis test.

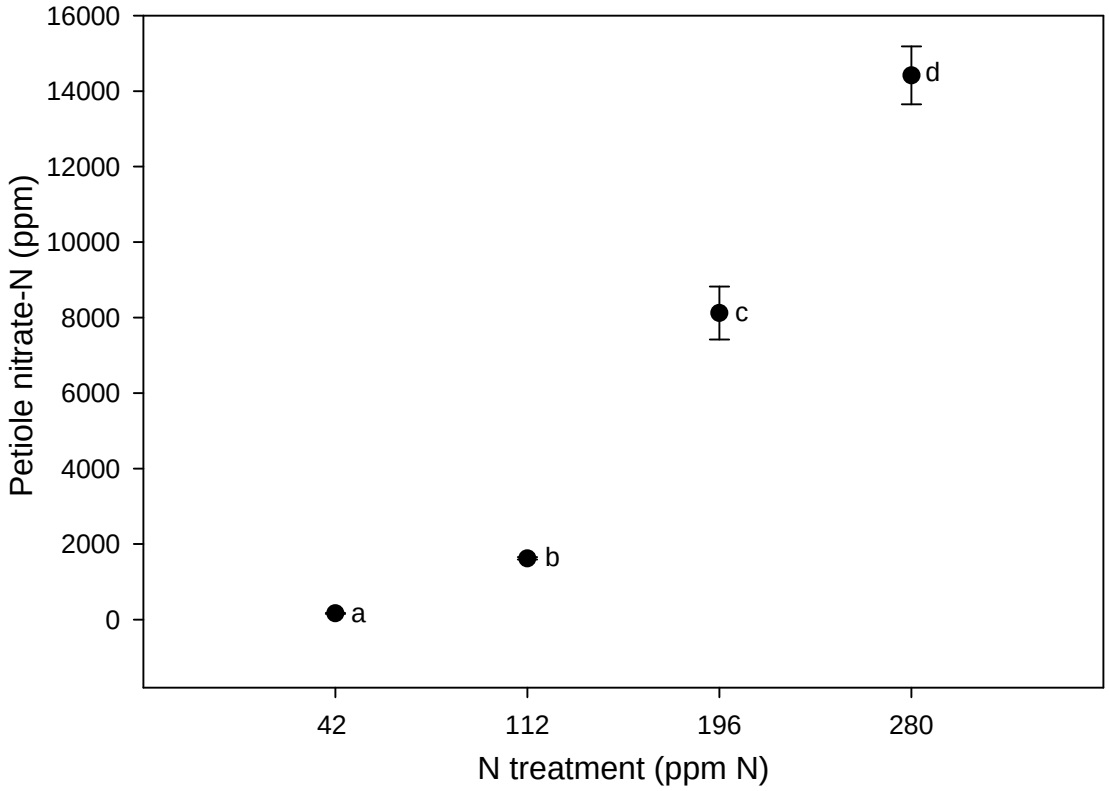

Figure 2.2 Nitrate-N (ppm) of petioles of different treatments. Different lower-case letters to the right of data points denote the difference was significant between each other at $\alpha = 0.05$ level. Standard error bars of 42 and 112 ppm N treatments do not appear because they are smaller than symbols representing these two data points. Data were analyzed with ANOVA and means were separated with t-test after the overall null hypothesis was rejected at $\alpha = 0.05$.

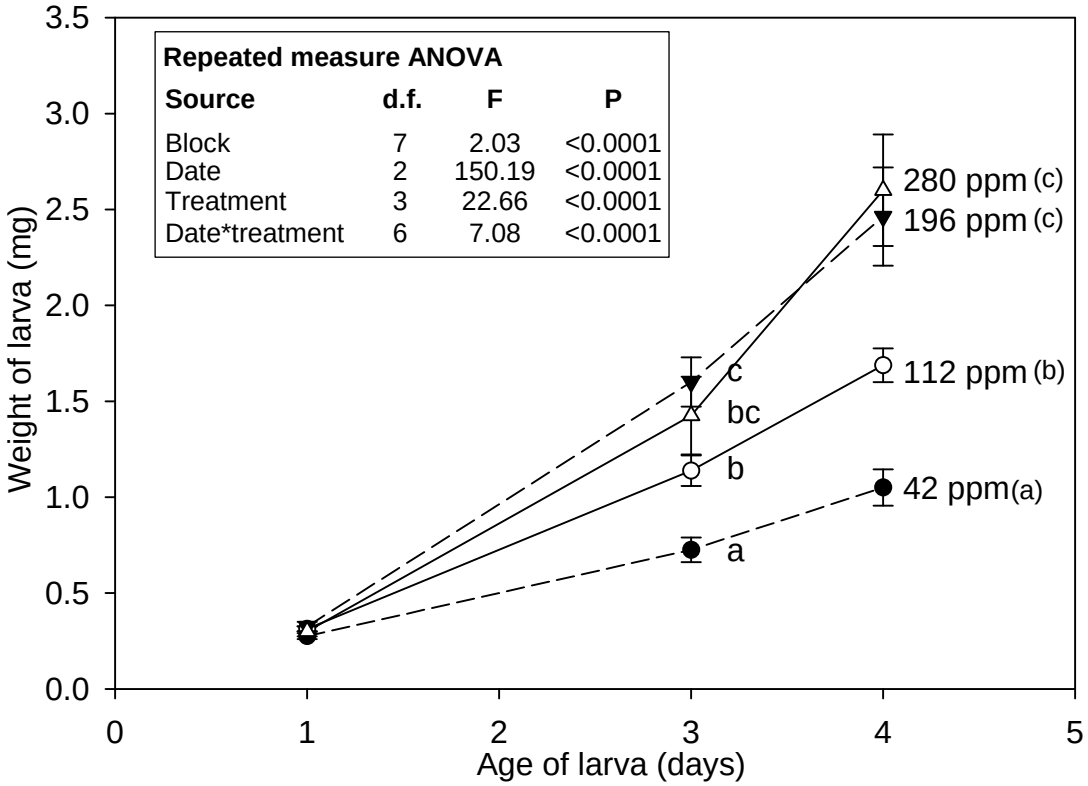

Figure 2.3 Effects of cotton plant N application on short-term development of young *Spodoptera exigua* in no-choice condition. Different lower-case letters to the right of data points of same day imply the difference was significant between each other at $\alpha = 0.05$ level. Data were analyzed with ANOVA and means were separated with Duncan's test after the overall null hypothesis was rejected at $\alpha = 0.05$.

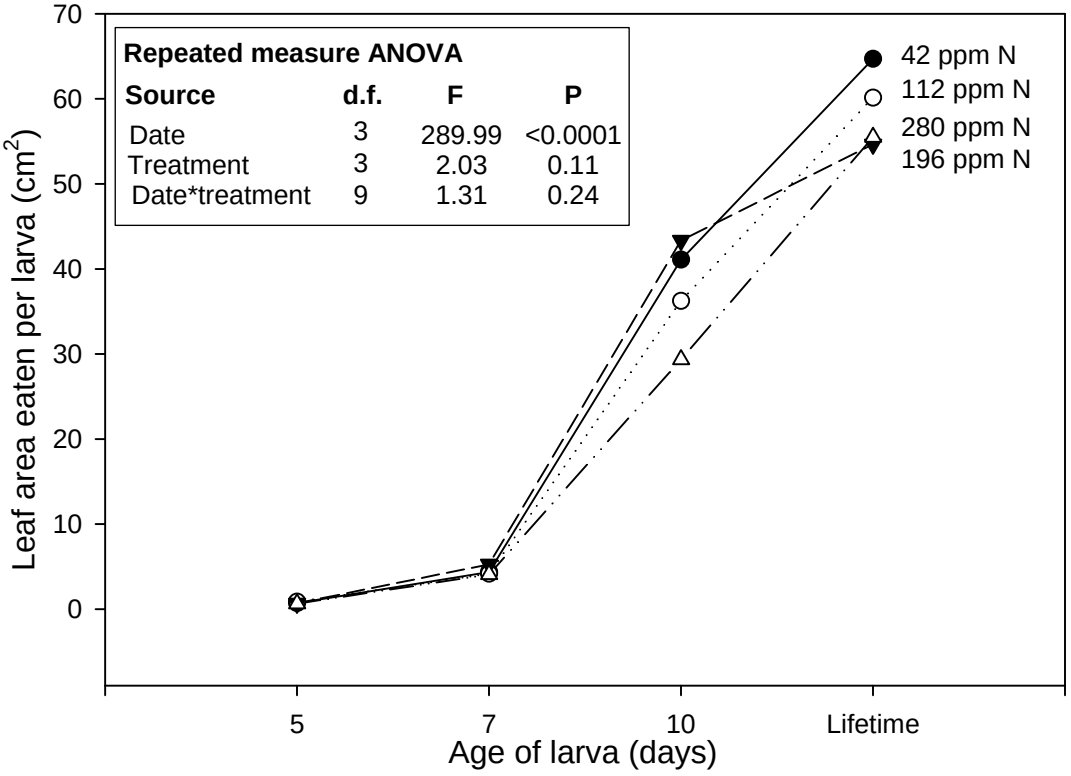

Figure 2.4 Cumulative leaf damage by *Spodoptera exigua* larvae in relation to plant nitrogen. Data were analyzed with ANOVA.

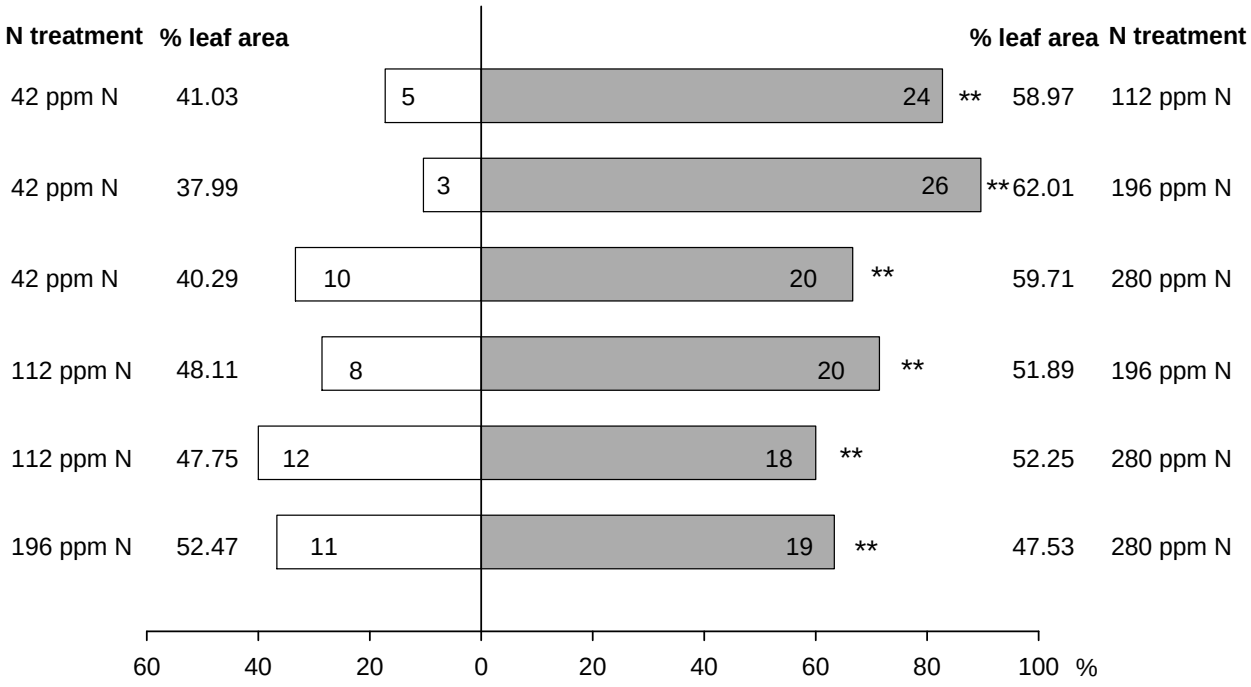

Figure 2.5 Percentage of *Spodoptera exigua* larvae on leaves with different N levels during 2-choice tests. Numbers on the bar represents total count of larvae found on corresponding leaves of 4 replicates. ** means P<0.01 during the 2-choice test. Data were analyzed with χ^2 test.

CHAPTER 3

COTTON PLANT, *GOSSYPIUM HIRSUTUM* L., DEFENSE IN RESPONSE TO NITROGEN FERTILIZATION AND BEET ARMYWORM, *SPODOPTERA EXIGUA* (HÜBNER), DENSITY[1]

[1]Chen, Y., E.A. Schmelz, F. Wäckers and J.R. Ruberson. To be submitted to *Journal of Chemical Ecology*.

ABSTRACT: Many plants respond to insect herbivory by producing non-volatile and volatile plant secondary metabolites. Plants capable of distinguishing herbivory gradations should be favored by selection because induced defenses are costly to produce and release, and a scaled response to herbivory gradations would be valuable where nutrients are limiting. N fertilization significantly reduced the production of non-volatile terpenoid aldehydes (hemigossypolone, heliocides H_1, H_2, H_3, and H_4) of cotton plant (*Gossypium hirsutum* L.) mature leaves. Herbivore density did not significantly affect the production of the terpenoids of mature leaves. However, bioassay beet armyworm larvae preferred young leaves that one of the lower leaves had been damaged by lower number of induction (5 larvae) beet armyworm larvae over those the lower leaves had been damaged by more induction larvae (20 larvae). Besides, the weights of bioassay beet armyworm larvae decreased as the feeding damage on the lower leaves increased until a certain level. This might suggest that either beet armyworm larvae were capable of detecting the density effects on induction of terpenoid aldehydes, or other factors might be involved. N fertilization also decreased the production of herbivore-induced plant hormones (jasmonic acid and salicylic acid) and volatile compounds, except *Z*-3-hexenal and *Z*-2-hexenal. However, parasitism of beet armyworm larvae by the parasitoid *Cotesia marginiventris* in field cages did not differ between N treatments.

Keywords Plant-herbivore interactions · Tritrophic interactions · Plant resistance · Direct defense · Indirect defense · *Spodoptera exigua* · Malvaceae · Hymenoptera · Lepidoptera Noctuidae · Braconidae · *Cotesia marginiventris*

INTRODUCTION

Plant secondary metabolites were originally considered as chemical wastes because they have no direct metabolic importance in plant survival (Janzen, 1969; Whittaker and Feeny, 1971). However, evidence is emerging showing diverse ecological, physiological and biochemical roles of these compounds (Ehrlich and Raven, 1967; Constabel and Ryan, 1996; Zangerl and Rutledge, 1996; Simmonds, 2003; Zagrobelny et al., 2004), although there is no unifying theory explaining how and why plants produce, transport, and store such a diverse array of chemicals (see Firn and Jones, 2000; Peñuelas and Llusià, 2004; Owen and Peñuelas, 2006a, 2006b; Firn and Jones, 2006a, 2006b; Pichersky et al., 2006 for discussion). Many of these compounds are shown to deter or resist herbivore colonization (direct plant defense) (Harborne, 1988; Berenbaum, 1995; Duffey and Stout, 1996; Glynn et al., 2003), and/or attract natural enemies of herbivores (indirect plant defenses) (Dicke et al., 1990; Röse et al., 1998; Park et al., 2001; Shimoda et al., 2002; Choh et al., 2004).

Nitrogen application is one of the most important agronomic practices in crop production and can exert a variety of bottom-up effects and potentially significantly alter tritrophic interactions through qualitative and quantitative alteration of plant direct and indirect defensive compounds (McNeil and Southwood, 1978; Stiling and Moon, 2005). Soil nutrient availability affects the expression of plant constitutive and induced plant allelochemicals in a wide range of plant species (Stout et al., 1998; Darrow and Bowers, 1999; Cipollini and Bergelson, 2001; Coviella et al., 2002; Orians et al., 2003). The magnitude of these effects may increase (Cipollini and Bergelson, 2001; Lou and Baldwin, 2004), remain neutral (Dudt and Shure, 1994) or decline (Stout et al., 1998; Hemming and Lindroth, 1999) with the N fertilization depending on the study systems.

Plant indirect defensive compounds can also be changed by plant N fertilization. Plants release a blend of volatile chemicals following wounding by herbivores. Many of these herbivore-induced plant-originated VOCs provide foraging natural enemies essential cues to locate potential host/prey (Dicke et al., 1990; De Moraes et al., 1998; Röse et al., 1998; Shimoda et al., 2002; Choh et al., 2004). N fertilization can alter the production and release of these volatiles. For example, in corn (*Zea mays* var Delprim) the peak of volatile release was detected when N concentration in the solution was the lowest, both after mechanical wounding and addition of volicitin (an elicitor isolated from oral secretion of beet armyworm, *Spodoptera exigua* (Hübner) (Schmelz et al., 2003b). In a second system, celery with additional N had a lower quantity of volatile compounds (Van Wassenhove et al., 1990). Nevertheless, Gouinguené and Turlings (2002) found that unfertilized corn plants (*Zea mays* var Delprim) emanated less volatiles when compared with those that had received a complete nutrient solution. The role of N itself was not implied in this study as all the nutrients were varied (Schmelz et al., 2003b). In tobacco (*Nicotiana attenuata*), induction of volatiles released by oral secretion from tobacco hornworm, *Manduca sexta* (L.), and methyl jasmonate (MeJA) was not affected by N, though low N availability attenuated the jasmonate and salicylate levels and reduced two N-containing anti-herbivore defensive compounds, nicotine and trypsin proteinase inhibitors (Lou and Baldwin, 2004). No other studies on VOC release patterns are available to date. However, the studies to date suggest that the effects of N on the release pattern of VOCs might be system- or species-specific.

Herbivory pressures that plants face spatially and temporally are not uniform. In the evolutionary arms race with host plants, and intra- and interspecific competition, herbivores also have evolved various feeding adaptations, such as gregarious and solitary feeding, to mitigate

plant defenses and natural enemies. These various feeding behaviors inflict damage on plants at different rates. Plants capable of distinguishing herbivory gradations should be favored by selection because induced defenses are assumed to be metabolically costly to produce, maintain, and release in most models of evolution of resistance (Gulmon and Mooney, 1986; Simms and Rausher, 1987) and the costs have been shown in some systems (Han and Lincoln, 1994; Baldwin, 1998; Mauricio, 1998). Further, a scaled response to herbivory gradations would be valuable where nutrients are limiting (Bazzaz et al., 1987).

We, for the first time, investigated cotton plant, *Gossypium hirsutum* L., defense in relation to nitrogen fertilization and beet armyworm, *Spodoptera exigua* (Hübner), density. Specifically, we tested 3 hypotheses: 1) cotton plant direct defense will be affected by N fertilization and beet armyworm can distinguish the difference and choose plants with lower defense; 2) cotton plant direct defense will be positively related to beet armyworm feeding damage and bioassay beet armyworm preferentially choose cotton plants with lower induced defense; 3) plant indirect defense will be affected by N fertilization and the parasitoid *Cotesia marginiventris* will inflict differential mortality of sentinel beet armyworm feeding on these plants.

MATERIAL AND METHODS

Cotton plants. *Gossypium hirsutum* L. (cv. FiberMax 989) plants were grown using the methods described elsewhere (Chapter 2), except otherwise noted. Cotton plants were fertilized with 100ml of 112 ppm N nutrient solution for ca. 2 weeks, at which time cotton plants of the same height and similar size of leaves at the same leaf position were randomly assigned to different N treatments. Cotton plants were fertilized with corresponding N nutrient solutions for ca. 2 weeks until experimentation. Leaching (watering without nutrients) followed every fourth N nutrient

solution application in order to reduce salt (salinity) buildup. All experimental plants were at the 3 to 5 mature true leaves stage. Because the petiole nitrate-N and percentage total N of dry leaf blade of cotton plants grown in this method were significantly affected by N regimes (Chapter 2), these variables were not determined in this study, unless otherwise noted.

Spodoptera exigua *and* Cotesia marginiventris. Beet armyworm larvae and the parasitoids were from laboratory colonies maintained in Biological Control Laboratory at the University of Georgia-Tifton in Tifton, GA.

Cotton plant non-volatile terpenoid aldehyde production in relation to N fertilization and beet armyworm density. The experiment was a 3 (N fertilization: 42, 112, 196 ppm N) × 3 (beet armyworm density) factorial design. At the time of experiment 5 and 20 3-d-old *S. exigua* larvae were caged on the third true leaf to induce production of the terpenoid aldehydes (see Chen et al., 2006 for cage description). In control (no insect feeding) plants, a cage with no larvae was placed on the third true leaf to account for possible physiological changes caused by cages. Each treatment was repeated 4 times. The cages were checked twice daily for escape of larvae and availability of leaf tissue. Cages and *S. exigua* larvae were removed after 48 h continuous feeding. The third leaves (mature; local resistance) of all treatments were briefly cleaned with fine brush and immediately excised at the distal of the petiole and stored at -80ºC until lyophilization. The sixth true leaves (young; systemic resistance) of all treatments were collected as well to understand the effects of N fertilization on systemic production of terpenoid aldehydes. Samples were then lyophilized with a VIRTIS freeze dryer (model Freezemobile 25 ES, Gardiner, NY) for 48 h.

The high performance liquid chromatography (HPLC) procedure outlined by Stipanovic et al (1988) was used to analyze terpenoid concentrations. Samples, each 100 mg of freeze-dried ground plant material, were shaken for 30 min in a capped 125-ml Erlenmeyer flask with 15 ml of glass beads, 10 ml of 3:1 hexane:ethyl acetate (HEA) and 100 ml of 10% HCL. The solution was filtered over a glass-fritted filter funnel into a 50-ml pear-shaped flask, and the beads and residue were rinsed three times with 3-ml HEA. The solvent was left to evaporate in a hot water bath, and the residue in the flask was redissolved with four 150 µl HEA washes. Each wash was transferred to a Maxi-clean silica cartridge (Alltech, Breda, The Netherlands). The silica cartridge was dried with compressed air, and terpenoid compounds were eluted with 5-ml isopropyl alcohol, acetonitrile, water and ethyl acetate (35:21:39:5). The eluent was filtered through a 45-µm nylon filter and transferred to a crimp top vial for HPLC analysis. Twenty microliters of each sample were injected onto a DIONEX HPLC-system (DIONEX Corp., Sunnyvale, CA, USA), equipped with a single wavelength absorbance detector and a 250-mm long, 4.6-mm (*d*) Alltima C-18 column (Alltech, Breda, The Netherlands). The column was eluted with EtOH:MeOH:IPA:CAN:H^2O:EtOAc:DMF:PPAcD (16.7:4.6:12.1:20.2:37.4:3.8:5.1:0.1) at a flow rate of 1.25 ml per minute and kept at 55ºC during analysis (Stipanovic et al., 1988). Detection was conducted at 272 nm. Standards of hemigossypolone (HGQ), gossypol (G), and heliocides 1 and 4 (H_1 + H_4), heliocides 2 (H_2) and 3 (H_3) were used to assess retention times of the individual terpenoids. Terpenoids were calculated as microgram per gram dried plant material.

S. exigua *larval growth bioassay*. In order to examine the effects of herbivore density on cotton plant response, *S. exigua* larval growth was bioassayed under no-choice conditions. The cotton

plants in this experiment were grown using methods described elsewhere (Chen et al., 2006). *S. exiuga* larvae were reared on modified Pinto bean diet in a group of 20 to 30 until early second instars. Five, 10, 20, and 30 early second instar larvae (induction larvae) were then caged and allowed to feed on the third fully expanded true leaf for 24 h to inflict differential levels of feeding damage. Cotton plant resistance can be induced in as short as 6 h by *S. exigua* feeding (Alborn et al., 1996), so 24 h induction time was selected in the study. Total feeding damage was measured using the method of Chen et al. (2006). Another 5 *S. exigua* larvae (bioassay larvae) of the same age were caged on the sixth leaf (a young expanding leaf) right after the removal of induction larvae. The number of bioassay *S. exigua* larvae recovered was recorded and total weight determined daily for 5 d with a Mettler Analytical Balance (AE 100, Mettler Instrument Corp., Switzerland). Average bioassay larval weights were calculated as total weights of bioassay *S. exigua* larvae divided by the number of larvae recovered. Because the sixth leaf was too small for larvae to feed for 5 d, larvae were moved to the fifth leaf on Days 4 and 5. The weights of bioassay *S. exigua* larvae were regressed against feeding damage amounts caused by inducing *S. exigua* larvae. The experiment was arranged as a randomized complete block design with four treatments and control each blocked 5 times (replicates).

S. exigua *larval choice tests*. To examine cotton plant defense in relation to herbivore density, A choice test using 8 5-d-old *S. exigua* larvae were conducted. To induce cotton plant resistance, 5 and 20 3-d-old (early second instars) larvae (induction larvae) were caged on the third fully expanded true leaf (mature) of cotton plants grown with one of the 4 N levels (42, 112, 196, and 280 ppm N) for 48 h. The induction larvae were then removed from the plants and the petioles of the sixth true leaf (young and expanding) were placed in the notches of the choice test arena

described elsewhere (Chapter 2). Eight bioassay larvae were placed in the center of the test arena and allowed to feed freely. After 24 h, the number of bioassay larvae on each of the two test leaves was recorded. Percentages of bioassay larvae on test leaves were calculated as the number of larvae on either of the test leaves divided by total larvae on 2 test leaves. Larvae on the sides of the cages were considered as making no choices and excluded from the data sets. If the movement of bioassay larvae was random, then we would predict the percentages of bioassay larvae on test leaves be 50/50 % since leaf areas of the 2 test leaves were approximately the same, otherwise the movement was considered non-random. Each N treatment was replicated 7 times. Because N levels can significantly affect the choices of *S. exigua* larvae (Chapter 2) and the leaf chlorophyll content was a good indicator of N status (Wood et al., 1992; Chen et al., unpublished data), the leaf chlorophyll levels were determined between 1000 to 1200 h with a chlorophyll meter (SPAD-502, Konica Minolta Sensing, Inc., Japan) on the leaf blades of test leaves immediately before the bioassay. Two measurements were made (one on each side of the mid-vein at the base of the leaf blade) on each leaf blade.

Cotton plant volatile production in response to N fertilization. The experimental design was a 3 (N fertilization: 42, 112, and 196 ppm N) × 2 (induced and control) factorial design. Twenty 3-d-old *S. exigua* larvae were caged on the third true leaf on the main stem to induce plant volatile production in induced treatments (see Chen et al., 2006 for cage description). In control treatments, a cage with no larvae was placed on the third true leaf to account for possible physiological changes caused by cages. The cages were checked frequently for escape of larvae and availability of leaf tissue. About 150-200 mg fresh leaf tissue was collected and weighed with a Mettler balance to the nearest 0.0000 g (model AB104-S, Mettler Toledo, Switzerland)

immediately following continuous feeding for 48 h, and immediately stored in FastPrep® tubes containing ca. 1 g Zirmil beads (1.1 mm; SEPR Ceramic Beads and Powders, Mountainside, NJ, USA) and liquid N_2 frozen as described in Schmelz et al. (2004). Samples were collected from both true leaf 3 (local; mature leaf) and true leaf 6 (systemic; expanding at the time of experiment). Samples were stored at -80ºC until analysis. Vapor phase extraction was used to extract plant metabolites because this method allowed us to extract phytohormones and herbivore-induced volatile organic compounds (VOCs) simultaneously (Schmelz et al., 2004). The details of sample preparation and extraction were described in Schmelz et al. (2004). Briefly, the plant metabolites were extracted with 300 µl of H_2O: 1-propanol:HCl (1:2:0.005) and dichloromethane ($MeCl_2$). The $MeCl_2$:1-propanol layer into which plant metabolites were extracted was then transferred to a glass vial and 2 µl of 2.0 M trimethylsilyldiazomethane in hexane were added to form methyl esters of plant metabolites. Excess trimethylsilyldiazomethane was neutralized with 2 µl of 2.0 M acetic acids in hexane. These methyl esters were trapped with approximately 30 mg Super Q (Alltech Associates, Inc., Deerfield, IL, USA) at 200 ºC and eluted with $MeCl_2$. Elution was later analyzed with chemical ionization-gas chromatography/mass spectrometry (CI-GC/MS) profiling method. The settings of CI-GC/MS were described elsewhere (Engelberth et al., 2003; Schmelz et al., 2004). Plant hormones analyzed were jasmonic acid and its volatile methyl ester (MeJA) (hereafter they are collectively presented as jasmonic acid) and salicylic acid. Plant volatiles analyzed were (*Z*)-3-hexenal, (*Z*)-2-hexenal, (*Z*)-3-hexenyl acetate, indole, *α*-pinene, *β*-pinene, myrcene, (*E*)-β-ocimene, caryophyllene, (*E*)-β-farnesene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-trideca-tetraene, beragmotene, *α*-humulene, *γ*-bisabolene, and limonene. All

reagents and solvents used in the experiments were purchased from Sigma-Aldrich (St Louis, MO, USA). All treatments were replicated 4 times.

C. marginiventris *foraging tests*. To examine the effects of N in cotton plants on parasitoid foraging, 3 cotton plants of one N treatment were placed into one side of 2 x 2 x 2 m cages covered with fine mesh (L = 0.1 cm). Three plants of the other N treatment were placed into the opposite side of the cage. Plants were so arranged that plants of same N treatments were touching each other but plants with different N treatments were ca. 50 cm apart. N treatments tested were 42 vs. 196 ppm N and 112 vs. 280 ppm N. Forty (42 vs. 196 ppm N trial) or 30 (112 vs. 280 ppm N trial) neonate *S. exigua* larvae were placed on the top leaves of each cotton plant (a total of 120 and 90 larvae/treatment for 42 vs. 196 ppm N and 112 vs. 280 ppm N trials, respectively) and allowed to feed for 24 h before 5 *C. marginiventris* females were released into the center of the cage. *C. marginiventris* females were prepared as follows: (1) 1-d-old male and female wasps were provided with 2-d-old *S. exigua* larvae for 24 h; the 2-d-old male and female wasps were transferred to a new cage without hosts but supplied with a cotton ball soaked with honeydew water for an additional 24 h before experimentation. Remaining *S. exigua* larvae were recovered and placed in groups of 5 into 5-ml plastic cups filled with 3 ml modified Pinto bean diets (Burton, 1969). Parasitoid offspring emergence was checked daily. Recovery rate was calculated as the total number of *S. exigua* larvae recovered divided by 120 (42 vs. 196 ppm N trial) or 90 (112 vs. 280 ppm N trial). Parasitism rate was calculated as the number of *C. marginiventris* offspring emerged from hosts irrespective of making cocoon or not divided by the total number of *S. exigua* larvae recovered. Total mortality was calculated as the total number of dead *S. exigua* larvae divided by the total number of larvae recovered. The causes of *S. exigua*

larval death fell into 3 categories: (1) death due to successful development of parasitoid offspring (those made cocoons); (2) death due to physical damage incurred by parasitoid female ovipoistion (those larvae died shortly after parasitoid oviposition); and (3) death due to physiological fights with parasitoid offspring (those host larvae died later but from which no parasitoid emerged).

Statistical analyses

The feeding damage on induction leaves and non-volatile terpenoid aldehydes tested were analyzed with two-way ANOVA (SAS Institute, 1999). The experimental designs of volatiles and plant hormones production were a 3 (nitrogen levels) × 2 (undamaged and damaged plants) factorial, so the amounts of chemical compounds were analyzed by two-way ANOVA. The data were $\sqrt{(x + 3/8)}$ transformed for normality before analysis. Damage on the induction leaf, weights of bioassay *S. exigua* larvae, leaf chlorophyll levels were analyzed with one-way ANOVA. The weights of bioassay *S. exigua* larvae were regressed against feeding damage caused by inducing *S. exigua* larvae using PROC REG. Recovery rate, parasitism and total mortality of *S. exigua* larvae were arcsine (square-root) transformed before being subjected to one-way ANOVA. Percentages of test larvae on test leaves of different N levels under 2-choice conditions were analyzed with X^2 tests (PROC FREQ), under the assumption of 50:50 probabilities. In all ANOVAs, means were separated by 2-tailed *t*-tests if the null hypothesis was rejected.

RESULTS

Cotton plant non-volatile terpenoid aldehyde production in relation to N fertilization and beet armyworm density. The effects of N fertilization and herbivore density on production of non-volatile terpenoid aldehydes of local mature leaf (true leaf 3) and systemic young leaf (true leaf 6) are summarized in Table 3.1. N fertilization did not significantly affect feeding damage caused by inducing beet armyworm larvae ($P = 0.50$) (Table 3.1). Herbivore density significantly increased feeding damage caused by induction beet armyworm larvae ($P < 0.0001$). The main terpenoids in the leaves were HGQ, heliocides (H_1, H_2, H_3, and H_4). N fertilization significantly decreased production of HGQ, heliocides and total tepenoids in local mature leaf (HGQ: $P < 0.0001$; H_1+H_4: $P < 0.05$; H_2: $P < 0.001$; H_3: $P < 0.001$; total: $P < 0.0001$). Gossypol was marginally affected by N fertilization ($P = 0.06$). Herbivore density had no significant effect on any terpenoid aldehydes tested in local leaf, except gossypol (G: $P = 0.03$).

N fertilization significantly decreased the production of HGQ, H_2, H_3, and total terpenoids in systemic leaf (HGQ: $P < 0.0001$; H_2: $P < 0.01$; H_3: $P < 0.0001$; total: $P < 0.0001$). Herbivore density had no significant effect on individual terpenoid aldehydes tested in systemic leaf.

Young expanding leaves had significantly higher HGQ, H_2, H_3, and total tepenoids leaves than mature leaves (HGQ: $P < 0.0001$; H_1+H_4: $P < 0.05$; H_2: $P < 0.01$; H_3: $P < 0.0001$; total: $P < 0.0001$).

S. exigua *larval growth bioassay*. Feeding damage to the inducing leaf was significantly affected by the densities of early second instar *S. exigua* larvae ($P < 0.0001$) (Table 3.2). The weights of bioassay larvae reared on previously damaged cotton plants were consistently lower that those reared on control plants (no previous damage) over a period of 5 d (1 d bioassay: $P < 0.05$; 2 d

bioassay: $P < 0.01$; 3 d bioassay: $P < 0.01$; 4 d bioassay: $P = 0.09$; 5 d bioassay: $P < 0.01$) (Table 3.2). One and 4 d after bioassay, the weights of bioassay larvae reared on cotton plants previously fed on by 10, 20, and 30 larvae were significantly lower than those reared on cotton plants previously damaged by 5 larvae (Table 3.2). The relationship between weights of bioassay *S. exigua* larvae and feeding damage were well expressed as quadratic as the weights of bioassay larvae decreased with increasing feeding damage to a certain level at which weight level off (Fig. 3.1). The pattern was consistent over the bioassay period.

S. exigua *larval choice tests*. Leaf chlorophyll levels of test leaves of the treatments (5 and 20 *S. exigua* larvae) were not significantly different from each other (42 ppm N: $P = 0.97$; 112 ppm N: $P = 0.84$; 196 ppm N: $P = 0.58$; 280 ppm N: $P = 0.92$) (Table 3.3). The feeding damage on the induction leaf caused by 20 *S. exigua* larvae was significantly greater than that caused by 5 larvae, irrespective of N treatments (Table 3.3). Significantly more *S. exigua* larvae chose test leaves that one lower leaf had been fed by 5 larvae than that had been fed by 20 larvae, regardless of N treatments (Table 3.3).

Cotton plant volatile production in response to N fertilization. N fertilization did not significantly affect feeding damage inflicted by inducing *S. exigua* larvae ($P = 0.38$). The average damage on induction leaves receiving 42, 112, and 196 ppm N were 13.93 ± 1.30, 15.62 ± 0.87, and 15.64 ± 0.50 cm^2, respectively.

Because the patterns of volatile and plant hormone production of the local leaf (true leaf 3) and systemic leaf (true leaf 6) were of the same and to simplify data presentation, only data on local leaf were presented (Table 3.4). Plant N levels significantly and negatively affected

constitutive (control) salicylic acid ($P < 0.01$) (Fig. 3.2). Induced jasmonic acid and salicylic acid were significantly and negatively affected by N levels ($P < 0.05$) for both jasmonic acid and salicylic acid) (Fig. 3.2). Damaged plants had significantly higher jasmonic acid content than undamaged plants ($P < 0.01$). Damaged plants had significantly less salicylic acid than undamaged plants ($P < 0.05$). The interactive effect of N levels and damage levels on jasmonic acid was significant ($P < 0.05$), and not significant on salicylic acid ($P = 0.34$).

Plant N addition significantly increased constitutive (*Z*)-3-hexenal and (*Z*)-2-hexenal levels ($P < 0.01$ and $P < 0.001$, respectively). Damaged plants had significantly higher levels of induced (*Z*)-3-hexenal under 42 and 112 ppm N. Damaged plants had significantly higher levels of induced (*Z*)-2-hexenal under 42 and 112 ppm N. Plants receiving 196 ppm N had significantly lower constitutive (*Z*)-3-hexenyl acetate than those receiving 42 ppm N ($P < 0.05$). Plant N levels significantly and negatively affected induced (*Z*)-3-hexenyl acetate, (*E*)-*β*-ocimene, caryophyllene, (*E*)-*β*-farnesene, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-trideca-tetraene, beragmotene, and *α*-humulene. Undamaged plants had significantly higher levels of caryophyllene than damaged plants under 196 ppm N. Under 42 ppm N conditions, damaged plants had significantly higher levels of (*E*)-*β*-farnesene than undamaged plants, while undamaged plants had higher amount of (*E*)-*β*-farnesene than damaged under 196 ppm N. Damaged plants had greater levels of (*E*)-4,8-dimethyl-1,3,7-nonatriene than control plants under 42 ppm N. Under 42 ppm N conditions, damaged plants had significantly higher levels of beragmotene than undamaged plants, while undamaged plants had higher amount of beragmotene than damaged under 196 ppm N. Control plants had higher *α*-humulene concentrations than damaged plants under 196 ppm N.

C. marginiventris *foraging tests*. N treatment did not significantly affect the recovery rate, parasitism rate, and total mortality of sentinel *S. exigua* larvae (Table 3.5).

DISCUSSION

Cotton plant non-volatile production in relation to N fertilization and beet armyworm density.

The main terpenoids of leaves in the study were HGQ and heliocides (H_1, H_2, H_3, H_4), and HGQ was the richest (Stipanovic et al., 1988). N fertilization significantly reduced the production of HGQ and heliocides in the study. As shown by Chen et al. (unpublished data, Chapter 2) in beet armyworm larval feeding and adult oviposition preference choice tests, both larvae and adult females preferred plant leaves receiving high N fertilization for feeding and oviposition. Both higher N content (Chapter 2) and weaker defense of plants grown with high N fertilization may contribute to beet armyworm larval and adult female preference of plants receiving high N fertilization over those receiving low N fertilization. Therefore, from applied perspective, the increased plant biomass of cotton plants due to high N application might be offset by rising damages inflicted by increased pest populations. On the contrary, lower biomass production of cotton plants due to low N fertilization might be compensated for by reduced herbivory in these plants.

Density of beet armyworm had no significant effects on the induced terpenoid aldehydes of mature and young expanding leaves. However, beet armyworm no-choice and choice test results might indicate that cotton plants responded differentially to densities of feeding beet armyworm larvae. In choice tests, bioassay beet armyworm larvae preferentially fed on young cotton leaves with lower defense (5 induction larvae) compared to leaves with higher defense (20 induction larvae), regardless of N levels of test cotton plants. In no-choice tests, the weights of bioassay

beet armyworm larvae decreased with increasing feeding damage until ca. 6 cm^2 damage in the study. The weights leveled off or even increased with additional feeding damage. This might suggest that either beet armyworm larvae were able to distinguish the density effects on induction of terpenoid aldehydes, or that other factors might be involved. This may also suggest that either cotton plants actively avoid over-investment of resources in defense or they experience metabolic constraints for stronger defense. Herbivore-induced headspace volatile release was exhibited or implied to be positively correlated with spider mite (*Tetranychus urticae*) density in kidney bean plants (Meada and Takabayashi, 2001; Horiuchi et al., 2003), *S. exigua* density (Schmelz et al., 2003a) and *Helicoverpa zea* feeding damage (Dean and De Moraes, 2006) in maize (*Zea mays*), and diamondback moth *Plutella xylostella* damage in oilseed rape, *Brassica napus* (cv. Oscar, line O52) (Schuler et al., 1999). No limitation of volatile production was reported in these studies, however, probably because the experiments were not designed to detect the limitation. From the beet armyworm's perspective, this may suggest aggregation is an adaptive feeding strategy to mitigate plant defense. A good example of host plant defense exhaustion by overwhelming defensive capacity is bark beetle, *Dendroctonus ponderosae* Hopkins, attack on lodgepole pine, *Pinus contorta* Dougl. ex Loud. var. *latifolia* (Raffa and Berryman, 1983). Beet armyworm eggs are laid in masses averaging 100 eggs each (J.R.R. unpublished). Hatched larvae remain congregated until the third instar when they start to disperse. To what extent this feeding behavior benefits beet armyworm development and reproduction needs further investigation.

Cotton plant volatile production in relation to N fertilization. Jasmonic acid is a 12-carbon fatty acid derivative synthesized via the lipoxygenase pathway with the precursor 18-carbon linolenic

acid (Vick, 1993). It's a major plant hormone induced by herbivory and widely accepted to be responsible for the induction of various plant chemical and physiological responses (Reinbothe et al., 1994; Karban and Baldwin, 1997; Schmelz et al., 2003b). In this study, beet armyworm herbivory significantly increased jasmonic acid production. N fertilization had no significant effects on the constitutive jasmonic acid levels, but it was inversely related to the amount of jasmonic acid in damaged leaves and induced jasmonic acid content. This is overall consistent with the few studies investigating N effects on jasmonic acid production (Schmelz et al., 2003b). Salicylic acid is a plant hormone widely considered to be induced in response to pathogen attack or pathogen-like damage caused by phloem-feeding insects, such as whiteflies and aphids (Pieterse and van Loon, 1999; Walling, 2000). N addition to cotton plants significantly decreased salicylic acid contents of both control and damage leaves. However, in contrast to jasmonic acid, insect herbivory generally reduced salicylic acid levels. This indicates possible antagonistic interactions of jasmonic acid and salicylic acid, which has been exhibited in tomato plants (Pena-Cortés et al., 1993) and tobacco (Niki et al., 1998).

Cotton plants receiving higher N (196 ppm N) had lower amounts of most individual VOCs and total amounts of VOCs compared to plants receiving lower N (42 ppm N) in the study. Nevertheless, N treatment did not significantly affect beet armyworm parasitism rate and total mortality due to the parasitoid *C. marginiventris*. *Cotesia marginiventris* females are known to exploit insect-induced volatiles to locate the hosts (Turlings et al., 1991b; Hoballah et al., 2002; Gouinguené et al., 2005), and both qualitative and quantitative differences in VOC blend are suggested to affect the attraction of the parasitoid (Novartis Foundation, 1999; Hoballah et al., 2002). At first glance, the lack of dose-dependent responses of the parasitoid to VOCs is surprising, since attractiveness was shown to increase with rising quantity of VOCs in some

systems (Turlings et al., 1991a; Vaughn et al., 1996; Weissbecker et al., 1999; Hoballah et al., 2002). However, little is known about how individual VOCs and qualitative and quantitative differences in the blend affect *C. marginiventris* behaviors, in particular, their foraging behaviors in nature. Minor, if any, qualitative differences of VOC blend were assumed because the only difference in the treatments in the study was N fertilization level, and big qualitative and quantitative differences generally stems from different plant species and/or varieties (Takabayashi et al., 1994; Loughrin et al., 1995; Hoballah et al., 2002). It is likely that the increased release of most VOCs in lower N treatments was obscured by decreased emanation of (Z)-2-hexenal and (Z)-3-hexenal. Another explanation of the lack of N effects on parasitoid foraging is simply due to the unnatural test arena. The parasitoid foraging process is hierarchically divided into host habitat location, host location, host acceptance, and host suitability (Nordlund et al. 1981). In cage studies, like this study, the cues involved in host habitat location might be obscured, thereby possibly rendering the results of limited ecological significance. Therefore, parasitoid foraging tests in the field would be very valuable because they would incorporate multiple elements of the herbivore's and enemy's behaviors.

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Table 3.1 Cotton plant non-volatile terpenoid aldehyde (mean ± SE mg/g dry material) production in relation to N fertilization and beet armyworm density.

N	42 ppm N			112 ppm N			196 ppm N		
Density[1]	0	5	20	0	5	20	0	5	20
Damage	0	3.5 ± 0.5	14.5 ± 2.1	0	4.9 ± 0.5	15.9 ± 0.5	0	3.6 ± 0.2	14.9 ± 2.1
HGQ	1334.3 ± 229.2	1400.3 ± 203.3	1206.0 ± 293.2	1110.8 ± 84.8	907.3 ± 164.1	736.0 ± 110.3	574.3 ± 13.8	591.8 ± 38.1	623.8 ± 31.0
G	27.8 ± 27.8	73.3 ± 24.9	0	0	48.0 ± 27.9	19.0 ± 19.0	0	0	0
H1 + H4	467.5 ± 29.2	827.5 ± 117.6	428.75 ± 101.5	578.0 ± 141.4	689.5 ± 97.5	780.5 ± 368.2	291.0 ± 36.9	461.0 ± 118.9	297.0 ± 17.3
H3	322.3 ± 30.5	294.3 ± 34.8	249.3 ± 29.3	298.5 ± 13.6	318.3 ± 25.9	314.0 ± 44.9	205.5 ± 14.2	236.0 ± 17.2	208.8 ± 21.5
H2	1025.5 ± 80.4	996.8 ± 112.6	740.3± 95.8	808.0 ± 50.4	725.5 ± 94.1	828.5 ±138.8	551.5 ± 20.8	635.0 ± 49.2	569.8 ± 71.3
Total	3177.3 ± 260.5	3592.0 ± 182.7	2624.3 ± 495.0	2795.3 ± 226.5	2688.5 ± 290.4	2678.0 ± 660.5	1622.3 ± 49.3	1923.8 ± 170.8	1699.3 ± 62.5

1 Number of 3-d-old beet armyworm larvae used to induced plant resistance in mature leaf (true leaf 3). HGQ, hemigossypolone; G, gossypol; H_1-H_4, heliocides 1-4.

Table 3.2 Damage and *S. exigua* growth (mean ± SE) in response to herbivore density over 5 d period in no-choice tests.

Induction *S. exigua* density (larvae)	Damage on induction leaf (cm^2)	Weight of bioassay *S. exigua* larvae (mg)[1]				
		1 d	2 d	3 d	4 d	5 d
Control	0.00 ± 0.26 a	2.25 ± 0.08 b	4.21 ± 0.31 b	11.32 ± 0.68 b	16.50 ± 1.45 b	36.45 ± 3.23 b
5	1.29 ± 0.26 b	2.11 ± 0.08 b	3.13 ± 0.31 a	8.73 ± 0.68 a	12.82 ± 1.45 b	26.23 ± 3.23 a
10	2.65 ± 0.26 c	1.96 ± 0.08 a	2.75 ± 0.31 a	7.31 ± 0.68 a	11.51 ± 1.45 a	20.10 ± 3.23 a
20	5.08 ± 0.26 d	1.84 ± 0.08 a	2.36 ± 0.31 a	6.57 ± 0.68 a	10.65 ± 1.45 a	17.07 ± 3.23 a
30	8.23 ± 0.26 e	1.94 ± 0.08 a	2.56 ± 0.31 a	7.17 ± 0.68 a	11.36 ± 1.45 a	18.93 ± 3.23 a

1 Weight made 1 to 5 d after onset of experiment; means within columns followed by different lower-case letters denote significant difference at $P < 0.05$.

Table 3.3 Cotton plant resistance in response to *S. exigua* larvae density—group larvae bioassay.

Nitrogen treatment (ppm)	Damage on induction leaf Mean±SE (cm^2)		N levels of test leaf Mean±SE (SPAD units)		Larvae on test leaf Mean±SE (%)	
	5 larvae[1]	20 larvae[2]	5 larvae[1]	20 larvae[2]	5 larvae[1]	20 larvae[2]
42	2.688 ± 0.57*	10.072 ± 2.86	27.007 ± 1.61	27.079 ± 1.12	63.213 ± 0.49	36.787 ± 10.49****
112	1.180 ±0.26***	8.063 ± 1.27	30.029 ± 1.09	30.379 ± 1.32	65.391 ± 7.58	34.609 ± 7.58****
196	2.306 ± 0.60***	11.852 ± 1.74	27.357 ± 1.75	28.721 ± 1.61	73.214 ± 7.49	26.786 ± 7.49****
280	2.096 ± 0.69*	7.753 ± 1.94	30.921 ± 1.58	30.707 ± 1.36	67.091 ± 9.25	32.909 ± 9.25****

[1] and [2] denotes 5 and 20 *S. exigua* larvae used to induce plant resistance, respectively; *, ***, and **** denote $P < 0.05$, 0.001, and 0.0001, respectively.

Table 3.4 Plant volatile production (Mean ± SE ng/g fresh weight) in response to various N levels – Local resistance.

Volatile compounds	42 ppm N		112 ppm N		196 ppm N	
	Control[1]	20 larvae[2]	Control[1]	20 larvae[2]	Control[1]	20 larvae[2]
(*Z*)-3-Hexenal	2781.3 ± 305.6 b	1.7×10^4 ± 2546.5 ***	7395.4 ± 2538.6 b	1.9×10^4 ± 2683.5 *	1.7×10^4 ± 3291.3 a	1.9×10^4 ± 4276.4
(*Z*)-2-Hexenal	2588.0 ± 213.9 c	1.2×10^4 ± 2592.3 *	1.1×10^5 ± 2337.6 b	2.0×10^4 ± 2554.7 *	$2.2\times10^4$5 ± 3579.4 a	1.7×10^4 ± 2756.7
(*Z*)-3-Hexenyl acetate	277.4 ± 58.6 ab	355.2 ± 76.5 A	279.3 ± 99.1 a	119.9 ± 16.4 B	101.6 ± 6.2 b	125.9 ± 26.3 B
Indole	1.2 ± 0.3	13.3 ± 9.9	1.1 ± 0.8	1.6 ± 0.7	0.4 ± 0.1	1.4 ± 1.2
α-Pinene	3.0×10^4 ± 1.7×10^4	7.5×10^4 ± 7327.5	8813.2 ± 8741.2	12×10^4 ± 6723.5	1.7×10^4± 1.0×10^4	1.7×10^4 ± 8299.7
β-Pinene	1.1×10^4 ± 5285.2	6843.6 ± 2443.4	3977.7 ± 2192.0	4525. 7 ± 1915.5	6160.1 ± 2680.2	6164.7 ± 2418.3
Myrcene	1.6×10^4 ± 8408.5	1.9×10^4 ± 6846.0	8357.2 ± 2702.0	7701.0 ± 2672.5	1.4×10^4 ± 5776.2	1.3×10^4 ± 3075.2
(*E*)-*β*-Ocimene	3302.4 ± 1724.0	5038.1 ± 1372.7 A	2323.1 ± 445.4	1687.1 ± 179.4 B	3537.0 ± 663.8	2193.6 ± 549.0 B
Caryophyllene	1.8×10^5 ± 2.4×10^4	2.2×10^5 ± 3.9×10^4 A	1.3×10^5 ± 1.5×10^4	1.1×10^5 ± 1.1×10^4 B	1.2×10^5 ± 9870.0 *	9.1×10^4 ± 5399.7 B
(*E*)-*β*-Farnesene	1.1×10^4 ± 1017.8	1.5×10^5 ± 1.8×10^4 A*	1.0×10^4 ± 1529.8	7.2×10^4 ± 8947.3 B	1.2×10^4 ± 791.9 *	8753.9 ± 247.1 B
(*E*)-4,8-Dimethyl-1,3,7-Nonatriene	47.1 ± 9.9	153.6 ± 22.7 **	49.1 ± 13.0	95.6 ± 18.6	41.4 ± 8.4	54.8 ± 14.2
(*E*,*E*)-4,8,12-Trimethyl-1,3,7,11-trideca-tetraene	20.4 ± 7.9	24.3 ± 5.2 A	14.9 ± 3.0	9.6 ± 2.0 B	10.7 ± 1.3	9.6 ± 0.9 B
Beragmotene	8353.9 ± 1562.8	1.4×10^4 ± 1897.8 A *	7713.5 ± 1204.1	6991.6 ± 822.6 B	8771.6 ± 5555.3 *	6683.3 ± 185.3 B
α-Humulene	5. $\times10^4$ ± 8013.7	7.8×10^4 ± 1.2×10^4A	4.4×10^4 ± 4963.46	3.8×10^4 ± 4055. 8 B	4.3×10^4 ± 3178.6 *	3.1×10^4 ± 1669.4 B

γ-Bisabolene	$8.1\times10^4 \pm 6476.2$	$1.5\times10^5 \pm 1.8\times10^4$	$7.7\times10^4 \pm 1.0\times10^4$	$7.2\times10^4 \pm 8.9\times10^3$	$8.8\times10^4 \pm 6772.9$	$12\times10^5 \pm 3651.6$
Limonene	13002.4 ± 6356.6	7378.9 ± 2628. 9	5043.85 ± 2462.62	5831.4 ± 2267.9	7733.7 ± 3177.0	7320. 8 ±2611.2

[1] means followed by different lower-case letters denote significant difference among N treatments of control plants at $\alpha = 0.05$; [2] means followed by different upper-case letters denote significant difference among N treatments of damaged plants at $\alpha = 0.05$; *, **, and *** denote significant difference between control and damaged plants within the same N treatment at $\alpha = 0.05$, 0.01, and 0.001, respectively.

Table 3.5 *Cotesia marginiventris* foraging in response to host plant N fertilizations in choice tests.

Trials	42 vs. 196 ppm N		112 vs. 280 ppm N	
Items	42 ppm	196 ppm	112 ppm	280 ppm
Recovery rate (Mean ± SE) (%)	62.00 ± 5.20	70.50 ± 6.56	72.50 ± 3.94	77.83 ± 1.98
Parasitism rate (Mean ± SE) (%)	17.39 ± 6.27	13.21 ± 4.68	26.71 ± 1.69	23.35 ± 2.95
Total mortality (Mean ± SE) (%)	23.30 ± 7.62	21.46 ± 7.47	38.75 ± 1.77	40.16 ± 5.63

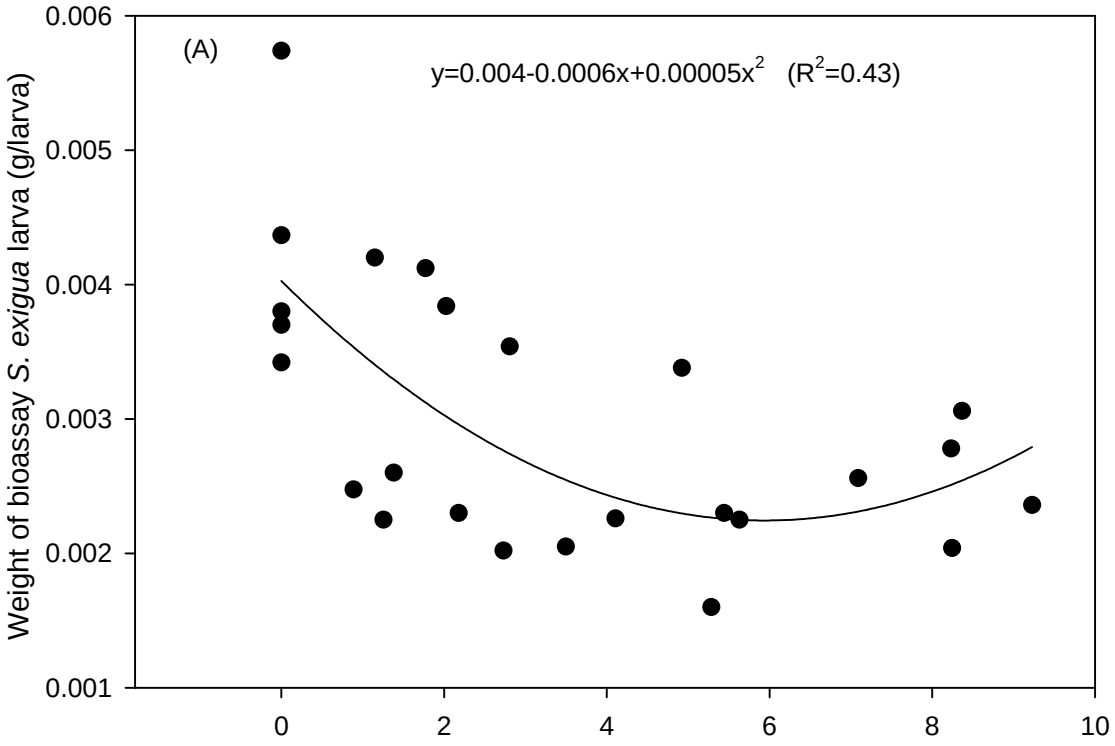
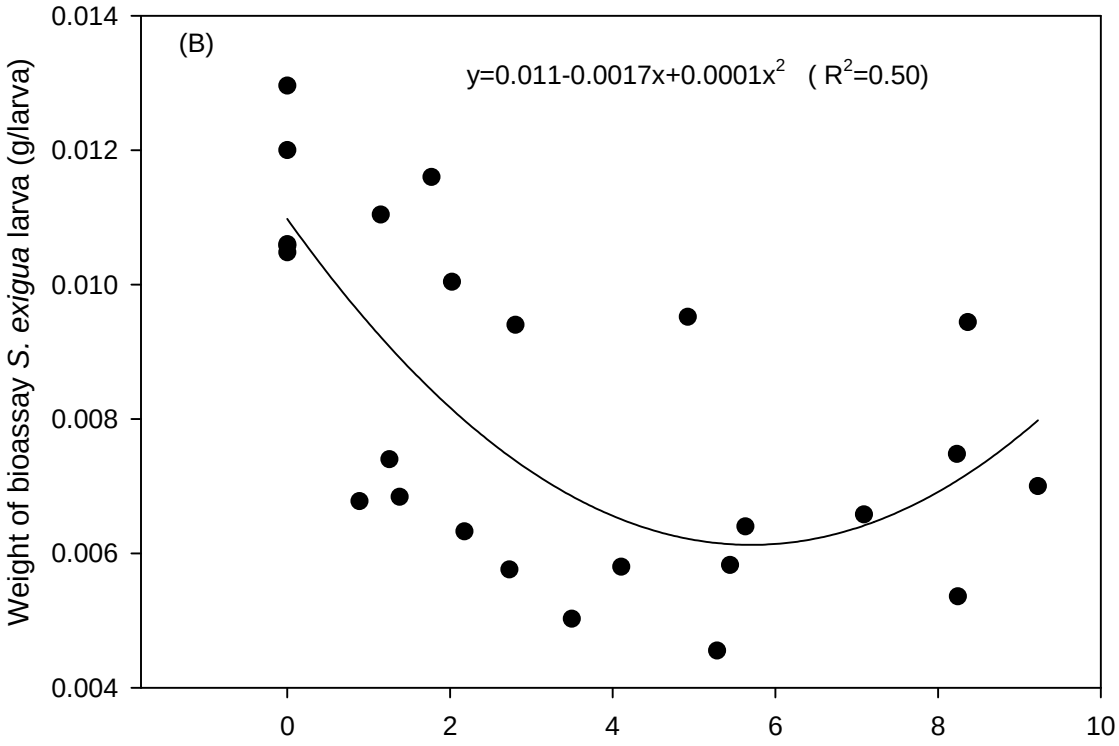


Fig. 3.1 Quadratic regression ($y = y_0 + ax + bx^2$) of weight of bioassay *S. exigua* larvae against induction feeding damage over a period of 4 d. (A) 2 d after bioassay, $P(y_0) < 0.0001$, $P(a) = 0.0036$, and $P(b) = 0.0244$; (B) 3 d after bioassay, $P(y_0) < 0.0001$, $P(a) = 0.0008$, and $P(b) = 0.0064$; (C) 4 d after bioassay, $P(y_0) < 0.0001$, $P(a) = 0.0066$, and $P(b) = 0.0281$; (D) 5 d after bioassay, $P(y_0) < 0.0001$, $P(a) = 0.0009$, and $P(b) = 0.0077$;



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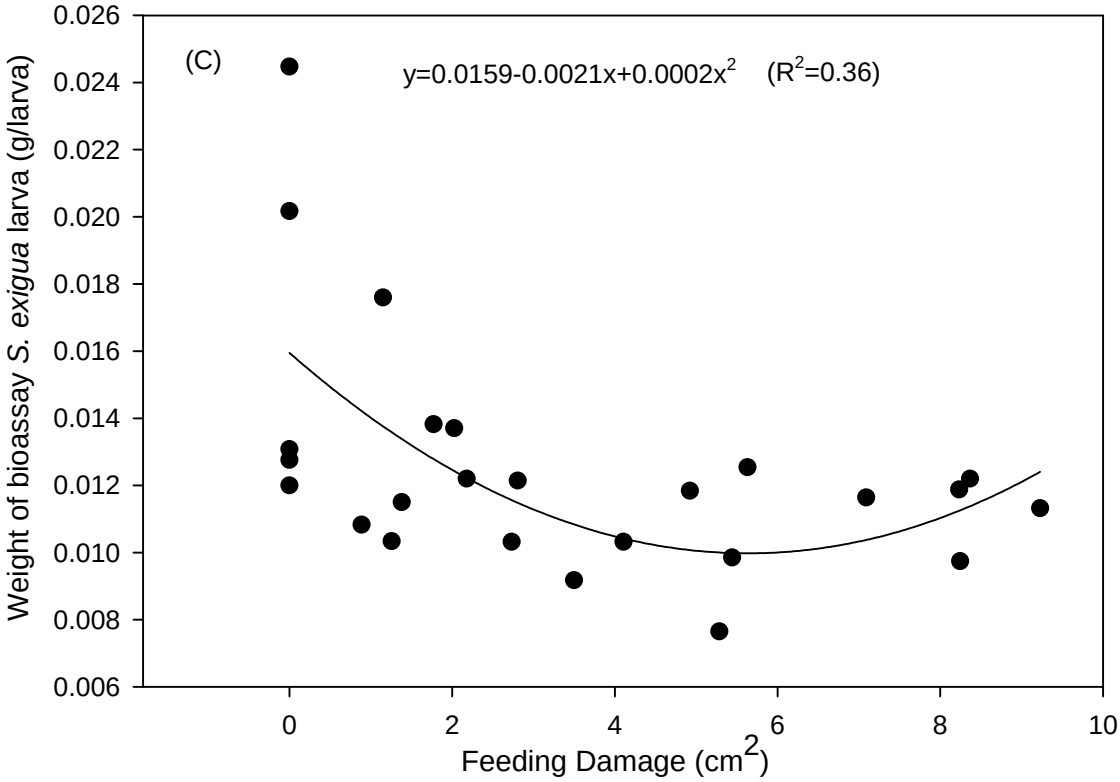

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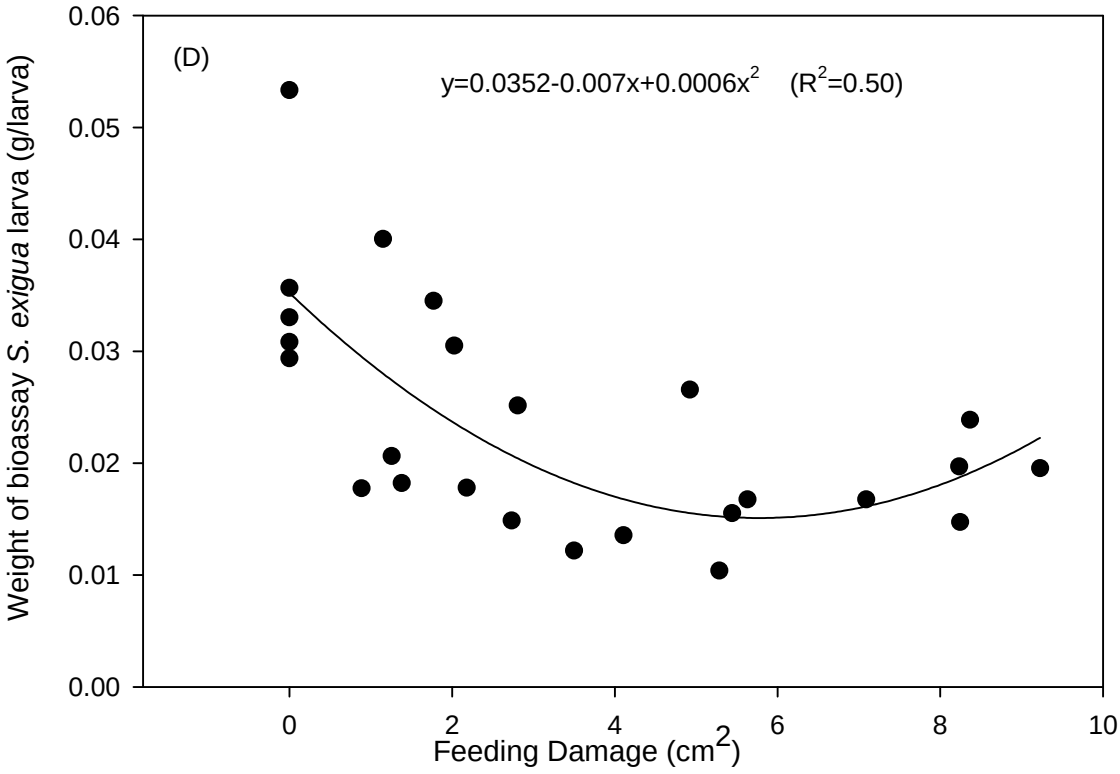

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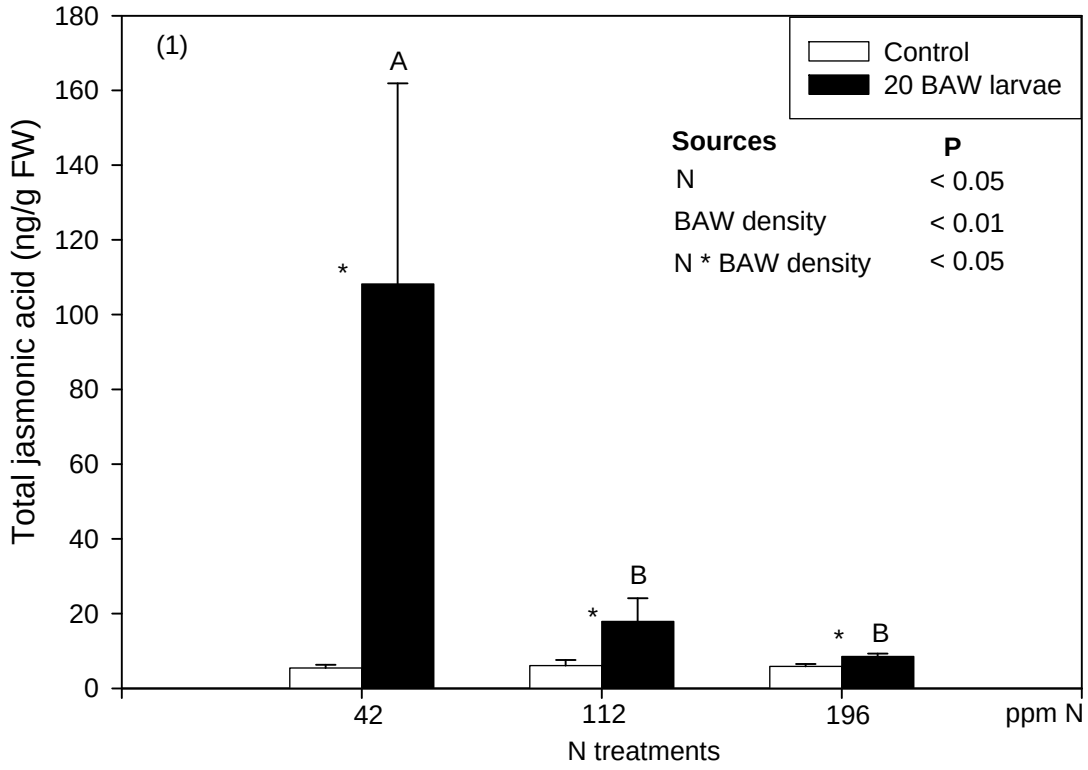
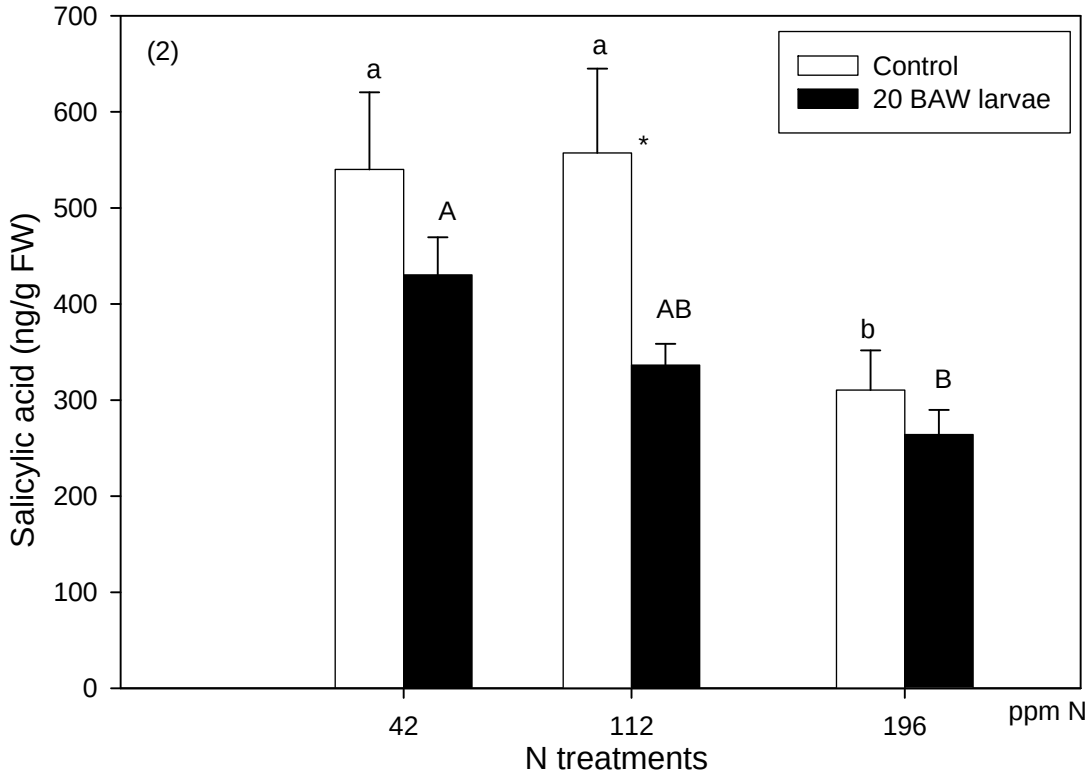


Fig. 3.2 Plant hormone production in response to nitrogen fertilization. (1) total jasmonic acid; (2) salicylic acid. Different lower-case letters above mean bars denote significant difference between N treatments of control plants at $\alpha = 0.05$; Different upper-case letters above mean bars denote significant difference between N treatments of damaged plants at $\alpha = 0.05$; * denotes significant difference between control and damaged plants of the same N treatment at $\alpha = 0.05$.



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CHAPTER 4

INFLUENCE OF HOST PLANT NITROGEN FERTILIZATION ON HAEMOLYMPH PROTEIN PROFILES OF HERBIVORE *SPODOPTERA EXIGUA* AND ON THE ENDOPARASITOID *COTESIA MARGINIVENTRIS* DEVELOPMENT[1]

[1]Chen, Y., J.R. Ruberson and X Ni. To be submitted to *Biological Control*.

ABSTRACT: Nitrogen (N) has complex effects on plant-herbivore-parasitoid tritrophic interactions. The negative effects of host plant low nitrogen fertilization on insect herbivores in many cases can be amplified to the higher trophic levels. In the present study, we examined the impact of nitrogen fertilization levels in cotton (42, 112, 196, and 280 ppm) on the interactions between the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), and the hymenopteran endoparasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae). We predicted that the development and fitness of *C. marginiventris* would be adversely affected by the host plant N fertilization through the herbivore beet armyworm. The percentage of *C. marginiventris* offspring developed to cocoon, and total mortality of parasitized *S. exigua* larvae were all unaffected by the nitrogen treatments. However, the developmental time of *C. marginiventris* larvae in *S. exigua* larvae feeding on low (42 ppm N) nitrogen cotton plants was approximately 30% and significantly longer than those feeding on high (112, 196, and 280 ppm N) nitrogen plants. Parasitoid fitness correlate of *C. marginiventris* males (hind tibia of right leg) was also affected by N treatments. Total amounts of *S. exigu*a haemolymph proteins were not affected by nitrogen treatments. Two proteins with molecular weights of ca. 84 and 170 kDa dominated the *S. exigua* larval haemolymph proteins. The concentrations of the 170 kDa haemolymph proteins of *S. exigua* larvae reared on four N treatments were not significantly different from each other. The concentrations of the 84 kDa haemolymph proteins of *S. exigua* larvae reared on 42 ppm N plants were significantly lower than those reared on other three N treatment plants. However, the prolonged development of *C. marginiventris* larvae in *S. exigua* larvae feeding on cotton plants treated with 42 ppm N was most likely not due to the relative deficiency of the protein, because the amount of the same protein in parasitized *S. exigua* larvae was significantly higher than those unparasitized. Unparasitized *S. exigua* larvae had

significantly higher amounts of 84 kDa protein than parasitized larvae reared on the same N level plants, except 42 ppm N plants which the reverse was true. Possible mechanisms for the extended development of *C. marginiventris* on *S. exigua* hosts grown on 42 ppm N plants were discussed.

Keywords: *Gossypium hirsutum*; *Spodoptera exigua*; *Cotesia marginiventris*; Haemolymph; Proteins; Development; Total protein assay; Gel electrophoresis; Parasitoid; Tritrophic interactions; Biological control

INTRODUCTION

Nitrogen (N) fertilization is one of the most widely-used agronomic practices in crop production. N has profound effects across trophic levels. In plant-herbivore interactions, low N availability in many cases decreases plant quality (from the herbivore's nutritional perspective) as food and increases plant defensive compounds (Mattson, 1980; Scriber, 1984; Stout, et al., 1998; Hemming and Lindroth, 1999; Hol et al., 2003; Prudic et al. 2005). Herbivores fed on these host plants consequently suffer detrimental effects (Loader and Damman, 1991; Kaneshiro and Johnson, 1996; Glynn et al., 2003). The negative effects can be amplified to natural enemies of these herbivores (Campbell & Duffey, 1979; Duffey and Bloem, 1986; van Emden, 1995; Kester & Barbosa, 1991; for a review, see Turlings & Benrey, 1998). For example, predacious stink bugs (*Podisus maculiventris* Say) reared on caterpillars fed on diets of powdered young leaves of *Plantago lanceolata* grew faster compared with conspecifics reared on caterpillars of same species fed on powdered mature leaves (Strohmeyer et al., 1998). The higher growth rate when feeding on a young leaf diet was attributed to higher nutrients, even in the presence of higher amounts of iridoid glycosides. The antibiotic effect of nicotine absorbed in tobacco hornworm, *Manduca sexta*, haemolymph on parasitism and survival of the gregarious parasitoid *Cotesia congregata* (Say) is another good example (Morgan, 1910; Gilmore, 1938; Thurston & Fox; 1972). *Manduca sexta* is a specialist herbivore in tobacco and can process nicotine effectively mostly through excretion. However, some amount of the nicotine is sequestered in the *M. sexta* haemolymph without any negative effect to the herbivores (Self et al., 1964). The parasitic wasp *C. congregata*, is more sensitive to nicotine, which in turn reduces their survival (Parr & Thurston, 1972; Thorpe & Barbosa, 1986; Barbosa et al., 1991). N fertilization in tobacco *Nicotiana attenuate* was shown to affect nicotine contents (Lou and Baldwin, 2004).

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is an important crop pest and a generalist herbivore with over 90 known plant species (Pearson, 1982). Its populations are often suppressed by the parasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) and other natural enemies (Ruberson et al. 1994, Mohaghegh et al. 2001, Bianchi et al. 2002). *C. marginiventris* is mainly a koinobiont larval endoparasitoid, although it is also a facultative egg-larval parasitoid (Ruberson and Whitfield 1996). The developmental time of *C. marginiventris* on different hosts at 30 ºC ranged from 6 to 10 d, with most emergence of parasitoid larvae from the hosts occurring 7 d after oviposition (Boling and Pitre, 1970). *Cotesia marginiventris* undergoes three immature instars before emerging from posterior abdominal segments and starting to spin cocoon (Boling and Pitre, 1970). *Cotesia marginiventris* is an effective biological control agent of beet armyworm and the mortality incurred by feral *C. marginiventris* in the field can reach up to 45 % in a 2-d window (Chen et al. *unpublished data*). In the current study we examined the impact of the N fertilization of cotton, *Gossypium hirsutum* (L.), on the beet armyworm and its endoparasitoid *C. marginiventris*. The objectives of this experiment were: 1) To examine the impact of N nutrient treatments of cotton plants on *C. marginiventris* development, as modulated by its herbivore host *S. exigua*; and 2) If development of *C. marginiventris* development was affected by host plant N fertilization, then to investigate changes in haemolymph protein profiles of *S. exigua* larvae fed on cotton plants treated with different levels of N nutrient for possible mechanism.

MATERIALS AND METHODS

Plants

Cotton plants (cv. FiberMax 989) were grown with the method described elsewhere (Chapter 2) Briefly, cotton plant seedlings were fertilized with 100 ml of 112 ppm N nutrient solution for ca. 2 wk, at which time 4 cotton plants of the same height and similar size of leaves at the same leaf position were assigned to a block. The 4 plants within the block were randomly assigned to 4 N treatments (42, 112, 196, and 280 ppm N). Cotton plants were fertilized with corresponding N nutrient solutions for ca. 2 wk until the initiation of the experiment. Leaching (watering without nutrients) followed every fourth N nutrient solution application in order to reduce salt (salinity) buildup. All experimental plants were at the 3- to 5-mature-leaf stage.

Insects

Neonates of *S. exigua* and adults of *C. marginiventris* were from laboratory colonies maintained in Biological Control Laboratory at the University of Georgia-Tifton in Tifton, GA.

Development of C. marginiventris *in S. exigua*

Neonates of *S. exigua* (less than 16-h-old) were placed in a 5-ml diet cup filled with 3ml of modified Pinto bean diet (Burton 1969) in groups of 50 per cup and maintained in an environmental chamber at 25 ± 1ºC and L14:D10 for 2 d before exposure to parasitoids. In this manner all hosts were equivalent qualitatively at the time of stinging. A 3- to 4-d-old *C. marginiventris* female prepared as the methods described elsewhere (Chapter 5) was allowed to sting 2-d-old larvae. The selection of 2- or 3-d-old (in later experiments) *S. exigua* larvae as hosts is because *C. marginiventris* females prefer early instar larvae to oviposit and their offspring

develop within the host body and emerge when host larvae develop to the third instar (Beckage et al., 2003). Parasitoid oviposition was visually verified and only one stinging was allowed per host to avoid confounding effects due to superparasitism and/or excess physical injury that might cause the death of hosts. Ten parasitized larvae were then placed in Petri dishes (d = 50 mm, h = 9 mm; Becton Dickinson and Company, Franklin Lakes, NJ, USA) provided with excised leaves of one of the 4 N treatments. We assumed that each observed stinging event would result in egg deposition or that successful egg deposition rates across N treatments were the same. Cotton leaves were changed twice daily. The leaves used on each change were from the same nodes of plants receiving 4 N treatments. *Spodoptera exigua* larvae were examined twice daily (early morning and later afternoon) for emergence of *C. marginiventris* larvae, cocoon formation, and adult emergence from cocoon. The lengths of the right metathoracic tibiae were measured with an ocular micrometer. Because almost all emerged parasitoid adults were males, only male tibia length was presented. Each treatment was repeated 8 times. Because the leaf chlorophyll content is a good, non-destructive indicator of N status (Wood et al., 1992; Chen et al., unpublished data), the leaf chlorophyll levels were determined between 1000 to 1200 h with a chlorophyll meter, SPAD-502 (Konica Minolta Sensing, Inc., Japan) on the leaf blades of true leaves 1 to 4 immediately before the rearing of *S. exigua* larvae. Two measurements were made (one on each side of the mid-vein at the base of the leaf blade) on each leaf blade.

Quantification of total haemolymph proteins of S. exigua *larvae*

Because the development of *C. marginiventris* in *S. exigua* larvae feeding on cotton plants with low N treatment (42 ppm N) was significantly prolonged, and because *C. marginiventris* larvae are exclusively haemolymph feeders (Gauld and Bolton, 1988; Wharton 1993; Strand,

2000; M. Strand, pers. comm.), the total amount of proteins was determined to grossly assess possible changes that might affect parasitoid development.

Neonate *S. exigua* larvae were reared on excised leaves from one of the 4 N treatments for 48 h. Larvae were then stung by *C. marginiventris* as described in the previous experiment. Stung larvae were thereafter reared on corresponding leaf tissues for 6 d when total haemolymph proteins of *S. exigua* larvae were determined with the Pierce® original BCA™ protein assay kit (Rockford, IL) using bovine serum albumin (BSA) as the protein standard. Control (unstung) larvae were provided. Therefore, the experiment utilized a 2 (stung and unstung) x 4 (N levels) factorial design. To collect *S. exigua* larval haemolymph, a larva was pinned down on both head and the last segment of abdomen. A proleg on the second or the third last segment of abdomen was cut off and 1 µl haemolymph was collected from individual larvae with a micropipette and diluted into a pre-prepared microcentrifuge tube containing 49 µl Ringer solution (Farquharson 1974). The tube was briefly vortexed to achieve a homogenous solution. A volume of 25 µl sample was pipetted into one of the 96-well Microplate Reader. A volume of 200 µl working reagents provided by Pierce was then added to the well. The whole sample preparation procedure was conducted at low temperature (on top of ice). Each treatment was replicated 8 times (1 individual larva/replicate). The samples in the microplate were shaken on a plate shaker for 30 sec and incubated at 37 ºC for 30 min before cooling down to room temperature. The sample was read with a Packard FluoroCount™ fluorescent plate reader (Packard Instrument Company, Meriden, CT) at the wavelength of 562 nm.

Quantification of protein profiles of S. exigua *larval haemolymph*

To further delineate possible protein differences among *S. exigua* larvae reared on four N treatments, we determined relative amounts of selected individual haemolymph proteins by staining densities and comparing the densities by their molecular weights.

Stung (presumably parasitized) and unstung (unparasitized) *S. exigua* larvae were prepared as described in the previous experiment. A volume of 6 µl of haemolymph was collected from 6-8 larvae as described in the previous experiment, and was pipetted into a spin cup with a cellulose acetate filter provided in the Pierce® SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) Sample Prep Kit. The sample was then cleaned with the kit following the instructions. Briefly, 20 µl of PAGE-prep protein binding resin and 55 µl of dimethylsulfoxide (DMSO) were pipetted into the spin cup containing the sample. The mixture was briefly vortexed and centrifuged at 2000 g with a Fisher Marathon Micro A centrifuge (Fisher Scientific, St. Louis, MO) for 2 minutes at 4 ºC. The resin was subsequently washed with DMSO twice and eluted with PAGE-prep elution buffer. One microliter of the elution was pipetted into a microcentrifuge tube containing 29 µl of Ringer solution for total protein assay as in the preceding experiment. The remaining sample (40 µl) was mixed with 10 µl of sample buffer containing 0.3 M Tris·HCl, 5% SDS, 50% glycerol with a ratio of 4:1, and lane marker tracking dye. The sample was heated at 95ºC for 5 minutes and cooled to room temperature before being loaded into a Pierce® 12% polyacrylamide gel. Preliminary experiments with 8, 12, and 8-16% gels indicated that 12% gel was optimum for SDS-PAGE electrophoresis of *S. exigua* haemolymph. Two loadings each with 10 µl of sample (a total of 20 µl) were loaded into each sample well of the polyacrylamide gel. Pierce® BlueRanger pre-stained protein molecular weight marker mix (7 µl) was loaded into a separate well. The marker contains 7 proteins (lysozyme,

trypsin inhibitor, carbonic anhydrase, ovalbumin, BSA, phosphorylase B, and myosin with a molecular weight of 18.3, 28, 39.2, 60, 84, 120, and 215 K, respectively). BIO-RAD mini-protein® II dual vertical slab gel electrophoresis cell (Bio-Rad Laboratories, Hercules, CA) was used. The running buffer was Pierce® Tris-HEPES-SDS buffer containing 100 mM Tris, 100 mM HEPES, and 3 mM SDS with a pH of 8 ± 0.25. Pierce® Coomassie brilliant blue G-250 was used to stain the gels to reveal the proteins. The destained gels were then digitally-recorded with an Olympus camera (CAMEDIA C-5060, Olympus, Japan) and the quantities of the main proteins quantified with BIO-RAD universal Hood II (BIO-RAD Laboratories, Segrate, Italy). The quantities of the proteins were calculated as stained density (intensity·mm^2/µl haemolymph).

Statistical analysis

ANOVA (PROC GLM in SAS) (SAS institute 1999) was used for statistical analysis of all data. All data were analyzed as completely randomized block design. Parasitism and total mortality of larvae were arcsine square-root transformed before analysis. The male right metathoracic tibia length was regressed against N treatments to see their correlation (PROG REG in SAS).

RESULTS

Development of C. marginiventris *in* S. exigua *hosts*

N application significantly affected cotton plant leaf chlorophyll levels, irrespective of the leaf position (true leaf 1: $F_{3,21} = 20.03$, $P < 0.0001$; true leaf 2: $F_{3,21} = 58.47$, $P < 0.0001$; true leaf 3: $F_{3,21} = 99.75$, $P < 0.0001$; true leaf 1: $F_{3,21} = 63.31$, $P < 0.0001$; Fig. 4.1). N did not affect percentage of stung hosts in which *C. marginiventris* offspring successfully completed larval

development ($F_{3,21}$ = 2.08, P = 0.13) or total mortality of *S. exigua* larvae ($F_{3,21}$ = 0.82, P = 0.50; Table 4.1). The percentage of *C. marginiventris* cocoons yielding adults was marginally affected by N treatment ($F_{3,28}$ = 2.83, P = 0.06), with no obvious pattern. The percentage of *C. marginiventris* offspring yielded by stung *S. exigua* larvae reared on 112 and 280 ppm N treatments and developing to adulthood was twice as high as in those reared on 42 and 196 ppm N treatments (Table 4.1). High N treatment significantly reduced the developmental time of *C. marginiventris* offspring from oviposition to pupation and adult emergence (from oviposition to cocoon formation: $F_{3,21}$ = 29.06, P < 0.0001; from oviposition to adult: $F_{3,12}$ = 4.76, P = 0.02; Table 4.1). Male parasitoid fitness, as indicated by the proxy of right metathoracic tibia length was not significantly influenced by N treatment ($F_{3,12}$ = 2.61, P = 0.10; Table 4.1). However, there existed a positive correlation between N treatments and male right metathoracic tibia length (Fig. 4.2).

Quantification of total haemolymph proteins of S. exigua *larvae*

The total haemolymph protein of unstung *S. exigua* larvae reared on 42, 112, 196, and 280 ppm N cotton plants varied between 17.46 ± 0.90 and 19.72 ± 3.18 µg/µl, and the protein of stung larvae ranged between 13.53 ± 1.00 and 16.91 ± 1.44 µg/µl (Table 4.2). The N treatment also did not affect the total haemolymph protein of control ($F_{3,28}$ = 0.29, P = 0.83) and stung larvae ($F_{3,28}$ = 2.09, P = 0.12; Table 4.2). However, stung larvae tended to have lower concentrations of proteins compared to controls within the N treatment, but only in the 196 ppm N treatment were the differences between stung and control *S. exigua* larvae statistically significant (42 ppm N: $F_{1,14}$ = 3.45, P = 0.08; 112 ppm N: $F_{1,14}$ = 2.91, P = 0.11; 196 ppm N: $F_{1,14}$ = 10.31, P = 0.0063; 280 ppm N: $F_{1,14}$ = 1.63, P = 0.22).

Quantification of protein profiles of S. exigua *larval haemolymph*

Two proteins dominated the haemolymph of *S. exigua*, with molecular weights of ca. 170 (protein 1) and 84 (protein 2) kDa. No different proteins were detected in haemolymph of stung *S. exigua* when compared with unstung larvae. In control *S. exigua* larvae, N did not significantly affect the amount of protein 1 ($F_{3,8} = 0.01$, $P = 1.00$), while it significantly affected the amount of protein 2 ($F_{3,8} = 14.21$, $P < 0.01$) (Table 4.3) in contrast, the amounts of both proteins 1 and 2 in stung *S. exigua* larvae were unaffected by N (protein 1: $F_{3,8} = 1.29$, $P = 0.34$; protein 2: $F_{3,8} = 1.01$, $P = 0.44$) (Table 4.3). The quantitative differences of 170 kDa protein between unstung and stung *S. exigua* larvae reared on the same levels of N plants were not significantly different from each other (42 ppm N: $F_{1,4} = 1.29$, $P = 0.32$; 112 ppm N: $F_{1,4} = 2.74$, $P = 0.17$; 196 ppm N: $F_{1,4} = 2.34$, $P = 0.20$; 280 ppm N: $F_{1,4} = 0.95$, $P = 0.39$) (Table 4.3). Unstung S. exigua larvae had significantly higher quantities of 84 kDa proteins than parasitized larvae reared on the same N level plants, except for 42 ppm N which the reverse was true (42 ppm N: $F_{1,4} = 31.16$, $P < 0.01$; 112 ppm N: $F_{1,4} = 13.87$, $P < 0.05$; 196 ppm N: $F_{1,4} = 52.62$, $P < 0.01$; 280 ppm N: $F_{1,4} = 48.71$, $P < 0.01$) (Table 4.3).

DISCUSSION

Depending on the plant-insect study systems, the negative effects of host plants on insect herbivores may not always be detected at higher trophic levels. For example, the generalist feeder, *Vanessa cardui* L., feeding on mature-leaf diets that contained less allelochemical iridoid glycoside grew faster than those reared on young-leaf diets which contained higher iridoid glycoside (Strohmeyer et al., 1998). However, the growth rates of a predatory stinkbug (*P. maculiventris*) were higher when predators were raised on *V. cardui* larvae that fed on young-

leaf than on mature-leaf diets. Therefore, the higher iridoid glycoside contents in *V. cardui* larva haemolymph did not negatively affect the performance of the predator. On the contrary, the survival, developmental times and larval weights of *Spodoptera littoralis* larvae feeding on transgenic maize (*Zea mays*) expressing endotoxin gene of *Bacillus thuringiensis* were significantly lower compared to larvae feeding on normal diets, and *C. marginiventris* offspring that developed in *S. littoralis* larvae fed on *Bt* maize showed reduced survival rate, extended developmental times, and reduced cocoon weights, although those negative effects on *C. marginiventris* fitness were considered to be host-mediated – i.e., host quality was reduced due to intoxication, rather than the toxin exerting a direct effect on the parasitoid (Vojtech et al., 2005).

Larval development of *C. marginiventris* in *S. exigua* larvae fed on low N (42 ppm N) cotton plants was greatly extended in our study. The larval developmental time of *C. marginiventris* on different hosts at 30ºC is reported to range from 6 to 10 d, with most emergence 7 d after egg deposition (Boling and Pitre, 1970). The average larval developmental time of *C. marginiventris* offspring on *S. exigua* larvae reared on 42 ppm N cotton plants was over 11 d, approximately 30% longer than the approximately 8 d required by those developing on 112, 196, and 280 ppm N host plants at 25 ± 1ºC. Moulting and metamorphosis of endoparasitoids is either conformed to that of hosts or regulated by juvenile hormone (JH) and ecdysteroids of their own (Beckage, 1985; 1991; Lawrence, 1986; Quicke, 1997). Unlike other insects that the main JHs are JH-I and JH-II, however, the primary JH of parasitic wasps is JH-III (Beckage, 1991). Metamorphosis occurs when the titer of JH-III starts to decline shortly after the final instar parasitoid larvae emerge from the host and the ecdysteroids peak before pupation. The effect of host plant N fertilization on hormone dynamics of both herbivore and parasitoid

remains unknown. The environmental cues that trigger the emergence of *C. marginiventris* larvae from hosts also remain unknown.

Many braconid endoparasitoid species from subfamily Microgastrinae, such as *C. marginiventris*, are almost exclusively haemolymph feeders (Gauld and Bolton, 1988; Wharton 1993; Strand, 2000). For these parasitoids the developmental time in hosts is typically independent of host size at the time of oviposition (Strand et al., 1988; Harvey et al., 1999). Apparently, changes in beet armyworm larvae induced by lower N fertilization of their hosts had constrained the development of *C. marginiventris*. Presence of certain specific nutrients in the host haemolymph can accelerate the growth of larval parasitoids. For example, the growth of *Exeristes roborator* (F.) (Hymenoptera: Ichneumonidae) increased with increasing glucose content when cultured in artificial medium, and the addition of lipid to the medium greatly accelerated the growth rate (Thompson 1979). Amino acids were also shown to be critical and they interact with carbohydrates (Thompson, 1981). In the current study, the total concentrations of haemolymph proteins between N treatments of unparasitized *S. exigua* larvae were not significantly different from each other. The protein profiles of *S. exigua* larvae feeding on various N host plants were the same, and two proteins with molecular weights of ca. 84 and 170 kDa were the most abundant. The amounts of the l70 kDa protein in unparasitized *S. exigua* larvae were not significantly affected by N treatment. In contrast, the density of the 84 kDa protein in unstung *S. exigua* reared on 42 ppm N plants was significantly lower than those reared on other N treatments. However, the differences in developmental time of *C. marginiventris* offspring were most likely not caused by changes in this protein because the quantity of this protein was consistent across all N treatments for stung larvae.

An appropriate ratio of protein to digestible carbohydrates (P:C) in food plants was shown to be important for the development of many phytophagous insects (Simpson and Raubenheimer, 1993; Clissold et al., 2006; Bede et al., 2007). Carbohydrates and amino acids were also shown to interactively affect development of *Exeristes roborator* (F.) (Hymenoptera: Ichneumonidae) (Thompson, 1981). Therefore, the imbalance of P:C in host haemolymph due to feeding on host plants with varying N fertilization and their interactions may be a cause for the protracted development of *C. marginiventris* offspring. The possible changes of allelochemicals such as gossypols and tannins in haemolymph of *S. exigua* larvae feeding on cotton plants with low N fertilization might also delay the development of *C. marginiventris* larvae, because a variety of plant defensive compounds have been reported to be enhanced by N deficiency in host plants (Stout et al., 1998; Darrow and Bowers, 1999; Hemming and Lindroth, 1999; Prudic, et al., 2005), and the development time of male *C. marginiventris* was observed to be significantly affected by host plant species (differ in content of glucosinolates which function as feeding deterrents or exert toxic effects of herbivores) of the hosts *S. exigua* (Sznajder and Harvey, 2003). The development of *Diadegma terebrans* (Grav.) (Hymenoptera: Ichneumonidae), an endoparasitoid of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) was slowed when developing on hosts fed on allelochemical 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a hydroxamic acid which confers resistance of crops such as maize (*Zea mays* L.) to herbivores (Campos et al., 1990). *Cotesia marginiventris* is a generalist endoparasitoid, which were exhibited to be more susceptible to plant chemistry than specialist endoparasitoids in some cases (Barbosa et al., 1991; Sznajder and Harvey, 2003; Harvey et al., 2005). Plant defensive chemicals sequestered in host haemolymph can not only directly and detrimentally affect parasitoid performance, but may also interact with specific nutrients and make them unavailable

to parasitoid larvae. For example, tomatine can directly cause cytolysis and can intervene with many β-sterols in host haemolymph and impede the utilization of these critical nutrients by larvae of *Hyposoter exiguae* (Viereck) (Hymenoptera: Ichneumonidae) (Campbell and Duffey, 1979).

Whether or not the protracted development of *C. marginiventris* was due to longer development in individual or multiple larval instars or may be the result of supernumerary development remains unknown. Boling and Pitre (1970) reported 3 larval instars of *C. marginiventris*. However, lower nutritional qualities of host food or unfavorable conditions induce supernumerary development of many herbivorous insect (Pipa 1976, Boguś and Cymborowski 1984; Pittendrigh et al. 1997; Chen and Ruberson, Chapter 2). The differences in most proteins between unparasitized and parasitized *S. exigua* larvae were not significant. However, unparasitized *S. exigua* larvae had significantly higher levels of 84 and 170 kDa proteins than those parasitized in most cases. This is because unparasitized larvae were at the late fourth or early fifth instars, whereas parasitized larvae were in the third instar at the time of bioassay, and late instars larvae tends to accumulate storage proteins (M. Strand, pers. comm.).

The developmental times of *C. marginiventris* offspring reared on *S. exigua* larvae fed on cotton plants receiving varying N levels from cocoon to adult eclosion were not affected by N treatments. One of the life history trade-offs faced by parasitoids is to grow bigger at the expense of longer developmental time or to grow faster at the expense of smaller adult size (Strand, 2000). This trade-off can be affected by feeding ecology of the host insects -- parasitoids attacking exposed herbivorous insects should favor faster growth to avoid prolonged exposure to predators, while parasitoids attacking concealed herbivores should favor larger size (Harvey and Strand, 2002). *Spodoptera exigua* larvae are typically leaf feeders and are exposed hosts for *C.*

marginiventris. The longer developmental time of *C. marginiventris* offspring developed in *S. exigua* larvae fed on 42 ppm N plants and smaller male size would probably increase exposure time of the parasitoid to predation and parasitism, because immature parasitoids generally suffer the same mortality as their hosts (Price, 1980; Hawdins, 1994) and extended exposure time of hosts may lead to higher mortality due to abiotic and biotic factors as the slow grow high mortality hypothesis predicts (Feeny, 1976). Thus, reductions in nitrogen availability to plants, whether through reduced fertilization or increased atmospheric carbon dioxide decreasing the ability of plants to acquire nitrogen, may have important consequences for survival of parasitoids in exophytic hosts.

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Table 4.1 Development of *C. marginiventris* on beet armyworm larvae reared on cotton plants receiving various levels of N.

N treatment (ppm)	Parasitoid cocoon (%)	Total mortality of *S. exigua* larvae (%)	Percent cocoon developed to adults	Oviposition to cocoon (d)	Oviposition to adult (d)	Tibia length (mm)
42	36.81 ± 4.70	79.86 ± 5.94	30.63 ± 14.03	11.57 ± 0.53 b	17.25 ± 0.14 b	0.72 ± 0.02
112	52.50 ± 7.01	91.25 ± 3.50	74.48 ± 12.66	8.51 ± 0.21 a	14.63 ± 0.26 a	0.75 ± 0.02
196	34.62 ± 4.52	85.17 ± 4.76	33.13 ± 12.68	8.09 ± 0.17 a	13.98 ± 0.22 a	0.77 ± 0.02
280	41.25 ± 7.43	83.75 ± 5.65	61.79 ± 11.91	8.26 ± 0.19 a	15.07 ± 0.56 a	0.80 ± 0.01

Means within a column followed by different low-case letters denote significant difference at $P < 0.05$.

Table 4.2 Total proteins µg/µl of unstung *S. exigua* larvae and larvae stung by the parasitoid *C. marginiventris* and reared on cotton plants receiving various N levels (mean ± SE).

N levels (ppm)	Unparasitized *S. exiuga* larvae	Parasitized *S. exigua* larvae
42	19.72 ± 3.18	13.53 ± 1.00
112	17.46 ± 0.90	15.18 ± 0.99
196	19.47 ± 1.61 **	13.78 ± 0.75
280	19.42 ± 1.34	16.91 ± 1.44

** $P < 0.01$ between unparasitized and parasitized *S. exigua* larvae within 196 ppm N treatment.

Table 4.3 Stained densities (mean ± SE) (intensity·mm^2/µl) of two abundant haemolymph proteins of *S. exigua* larvae reared on cotton plants receiving various N levels. Larvae were either stung by *C. marginiventris* (stung) or were unexposed to parasitoids (unstung).

N levels	Unstung		Stung	
(ppm)	Protein 1[1]	Protein 2[2]	Protein 1	Protein 2
42	883.44 ± 254.68	267.43 ± 40.20 a	571.08 ± 104.53	571.90 ±36.86 **
112	942.79 ± 262.42	1977.40 ±361.54 c	484.70 ± 88.40	624.25 ± 36.43 *
196	886.10 ± 288.01	1318.14 ± 104.40 b	434.72 ± 63.26	541.31 ± 23.81 **
280	922.57 ± 267.24	1738.22 ± 133.00 bc	652.29 ± 75.90	651.24 ± 81.05 **

Means within a column followed by different low-case letters denote significant difference at $P < 0.05$. * and ** represent significant difference of protein quantity between haemolymph of unparasitized and parasitized *S. exigua* larvae reared on the same levels of N at $P < 0.05$ and 0.01, respectively. [1] and [2] denote proteins with molecular weight of ca. 170 and 84 kDa, respectively.

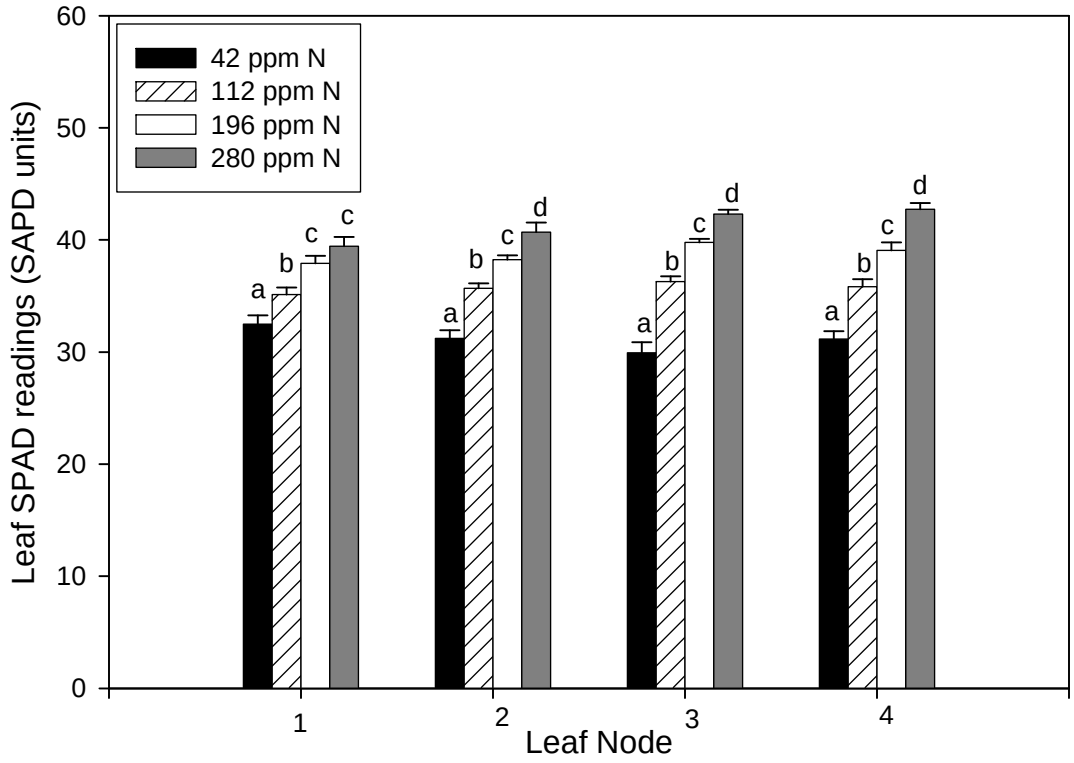

Fig. 4.1 Leaf chlorophyll levels of cotton plants receiving various levels of N. Low-case letters above the bars denote significant difference among treatments within the same leaf position at $P < 0.05$.

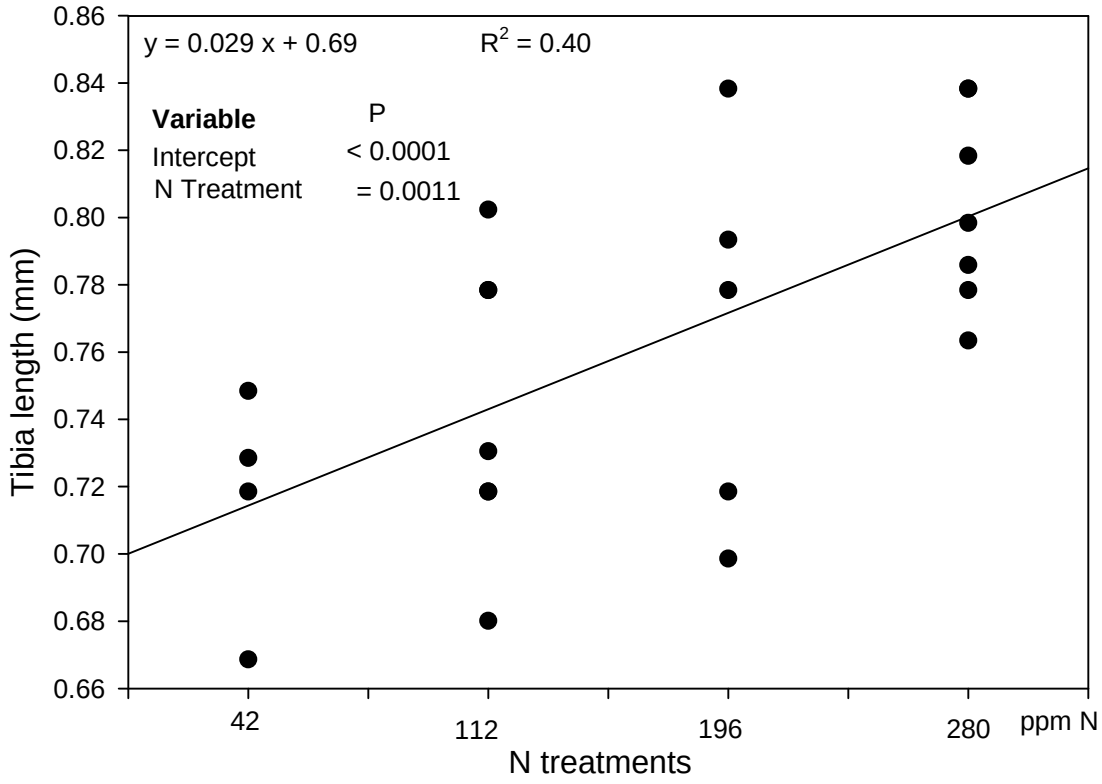

Fig. 4.2 Linear regression of N treatments against male right metathoracic tibia length.

CHAPTER 5

NITROGEN AND BIOLOGICAL CONTROL OF BEET ARMYWORM *SPODOPTERA EXIGUA*, WITH THE PARASITOID, *COTESIA MARGINIVENTRIS*: TESTING THE SLOW-GROWTH-HIGH-MORTALITY HYPOTHESIS[1]

[1]Chen, Y. and J.R. Ruberson. To be submitted to *Ecology*.

ABSTRACT: The slow-growth-high-mortality (SG-HM) hypothesis is a debated hypothesis, and available literature has mixed support for the hypothesis, particularly for parasitoids. We evaluated the SG-HM hypothesis with cotton plants (*Gossypium hirsutum* L.), beet armyworm (*Spodoptera exigua* (Hübner)), and a hymenopteran parasitoid (*Cotesia marginiventris* (Cresson)) over the entire period during which beet armyworm larvae are susceptible to the parasitoid. We expected that slower-growing beet armyworm larvae would experience higher mortality due to the parasitoid than the faster-growing larvae. Differential growth rates of beet armyworm were generated by rearing them on cotton plants fertilized with 42 (low) and 196 (high) ppm nitrogen (N) levels. The results demonstrated that there is a defined range of host sizes (from ca. 0.08 mg/larva to ca. 8 mg/larva) below and above which *S. exigua* larvae, while acceptable for stinging, were not suitable for *C. marginiventris* offspring development. The mean weights of individual *S. exigua* larvae reared on high N in the laboratory were consistently and significantly greater than those reared on low N treatment plants. The percentages of *C. marginiventris* offspring successfully emerging from stung 3- and 5-d-old larvae in no-choice tests were not significantly different, while significantly more 6-d-old larvae from low N treatment succumbed to *C. marginiventris* parasitization, compared to 6-d-old larvae reared on high N plants. *S. exigua* larvae reared on whole cotton plants in cages in a greenhouse also grew faster. Parasitism rates were not significantly different from each other in most ages tested (except for 6-d-old trial). In the early developmental stages (3-d-old and younger) of *S. exigua* larvae, higher percentages of larvae from high N treatment were parasitized, while in the later stages more larvae from low N treatment were parasitized. As such, the timing of vulnerability of *S. exigua* larvae grown on host plants with different N levels to *C. marginiventris* shifted a little, but the window of vulnerability remained mainly the same and the SG-HM hypothesis was rejected in the study.

Key words: Tri-trophic interactions; Nutrients; Cotton plants; Gossypium hirsutum*; Lepidoptera; Noctuidae; beet armyworm;* Spodoptera exigua*; Hymenoptera; Braconidae;* Cotesia marginiventris*; Slow growth; high mortality.*

INTRODUCTION

Herbivorous insects that feed on diets or host plants of lower nutritional quality generally have lower growth rates, lower efficiency of conversion of ingested food, and prolonged developmental time (Mattson 1980, Lindroth et al. 1995, Chen et al. 2004). It is intuitive that prolonged developmental times of herbivores will increase their susceptibility to natural enemies by extending the period when the herbivores are small and presumably more readily subdued (termed the "slow growth high mortality" (SG-HM) hypothesis; see Feeny 1976, Moran and Hamilton 1980, Clancy and Price 1987). Besides developmental delays, increased mortality of herbivores feeding on plants of lower nutritional quality also may result from increased feeding activity and movement within and between plants to locate food of higher nutritional quality. Increased feeding activity and movement may expose herbivores to greater predation and risk of fatal dislodgement (Bernays 1997).

The available literature has provided mixed support for the SG-HM hypothesis (Clancy and Price 1987, Benrey and Denno 1997, Fordyce and Shapiro 2003, Medina et al. 2005; see Williams 1999 for a review). Nevertheless, as pointed out by Williams (1999), the scarcity of experiments specifically designed to test the hypothesis hampers any generalizations. Studies conducted so far almost all measured daily mortality (Häggström and Larsson 1995, Williams 1999), which is not a good indicator of total mortality throughout the vulnerable period. Factors such as plant traits and life histories of herbivorous insects and their enemies may also affect the results of the tests. For example, the survey conducted by Benrey and Denno (1997) showed that the SG-HM was not supported when the same herbivore species was tested on different host plant species. Host plant species differ dramatically from each other in morphology and chemistry, influencing the herbivore and its natural enemies. Thus, comparisons among host

plant species are severely confounded. It has been observed that systems with exophytic herbivores are more likely than those with endophytic herbivores to support the hypothesis (Benrey and Denno 1997), because structures such as galls in which herbivores feed can protect them from natural enemies (Price and Pschorn-Walcher 1988). Equally important in interpretation of the test results are the biologies of the natural enemies involved. When only exophytic herbivores are considered, systems involved with predators are more likely than those with parasitoids to support the hypothesis (Williams 1999).

The biology of natural enemies differs enormously. The koinobiont/idiobiont dichotomy suggested by Askew and Shaw (1986) is a widely used and useful approach for classifying parasitoid biology. Idiobiont parasitoids develop on hosts after the ovipositing female or the deposited parasitoid offspring has terminated host development, essentially fixing the resource pool for the duration of the immature parasitoid's development. Therefore, idiobiont parasitoids would be best served by concentrating on hosts with significant pools of available resources, which would be larger hosts. Thus, idiobionts would not necessarily be expected to conform to the SG-HM hypothesis since there is no growth of hosts after parasitism.

In the case of koinobionts, Williams (1999) noted that 4 of 6 studies involving koinobionts supported the SG-HM hypothesis. Koinobionts might be expected to conform more readily to the SG-HM hypothesis because they have more flexibility in host size choices, and exploitation of smaller hosts would reduce risks of host defensive behaviors and physiological defenses. Even if the koinobiont is restricted to a particular range of host sizes in its preference, then prolonging that window of appropriate sizes could be expected to increase mortality, unless there is a lower size threshold that also is delayed. If there are both lower and upper thresholds of host acceptability/suitability, and the developmental delays are proportional throughout development,

the window of vulnerability may be merely shifted rather than extended. In this case, we would expect to see no effect of slowed development on overall parasitism. In the present study we tested the SG-HM mortality hypothesis for an important koinobiont parasitoid, and evaluated the effect of developmental delays on the window of vulnerability.

Our study system consisted of cotton plants, *Gossypium hirsutum* L., beet armyworm larvae, *Spodoptera exigua* (Hűbner), and a koinobiont parasitoid, *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), and parasitism was examined over the entire vulnerable period of beet armyworm. We manipulated host development using low and high nitrogen (N) fertilization treatments. The questions we addressed were: (1) Are there lower and upper size thresholds beyond which beet armyworm larvae are not suitable for *C. marginiventris* development? (2) Do beet armyworm larvae reared on lower N plants suffer higher mortality over the whole vulnerable period as a result of increased parasitism? and (3) Is the window of vulnerability of beet armyworm larvae to *C. marginiventris* increased when hosts feed on lower N plants compared to higher N plants, or does the vulnerable period simply shift for hosts on low quality plants?

MATERIAL AND METHODS

Study system

Spodoptera exigua is an exophytic generalist herbivore with a known host range of over 90 plant species (Pearson, 1982). Eggs are laid in masses averaging 100 eggs each (J.R.R. unpublished). Larvae feed gregariously until the third instar, when they disperse and become solitary feeders. Beet armyworm larvae are generally exposed foliage feeders, but occasionally bore into fruit. Populations of *S. exigua* are heavily attacked by parasitoids in the southeastern

US, of which the overwhelmingly predominant species is *C. marginiventris* (Ruberson et al. 1994, Mohaghegh et al. 2001, Bianchi et al. 2002). *Cotesia marginiventris* is a solitary larval koinobiont parasitoid, although it can also function as a facultative egg-larval parasitoid (Ruberson and Whitfield 1996). *Cotesia marginiventris* females prefer early-instar larvae for oviposition and their eggs develop within the host's body before generally emerging when host larvae develop to the third instar (Beckage et al., 2003). However, the third or fourth instar larvae are also physiologically suitable for *C. marginiventris* development and a few hosts can even continue to develop to the fifth instar before the ultimate larval instar of *C. marginiventris* emerges from the host to pupate (Chen et al. *unpublished data*), depending on the stage in which the host was parasitized.

Are there lower and upper size thresholds beyond which beet armyworm larvae are not suitable for C. marginiventris development?

Ten *S. exigua* larvae of each age group (2-, 3-, 4-, 5-, 6-, and 7-d-old) were reared on artificial diet and stung by 3- to 4-d-old *C. marginiventris* females prepared as follows: (1) 1-d-old male and female wasps were provided with 2-d-old *S. exigua* larvae for 24h to permit females to obtain oviposition experience; the 2-d-old male and female wasps were subsequently transferred to a new cage without hosts but supplied with a cotton ball soaked with a 9:1 (v/v) water:honey solution for an additional 24h before being exposed to hosts for stinging. The diet used was a modified Pinto bean diet (Burton 1969). Host larvae were exposed to female parasitoids in a 5-ml diet cup. Parasitoid females were temporarily anesthetized before being placed into the cup for ease of manipulation. Stinging of larvae was visually verified and stung larvae were immediately removed from the arena to prevent superparasitism. Stung larvae were weighed as a

cohort of 10 larvae, and the value was transformed to weight per larva. Stung larvae were kept in a 5-ml diet cup filled with 3 ml of modified Pinto bean diet in groups of 5 per cup and maintained in an environmental chamber at 25 ± 1 ºC and L14:D10. We used artificial diet to control for potential effects of host diet on success of parasitoid offspring, because diet quality can affect the vigor of parasitoids' hosts, and thus hosts' encapsulation ability (Salt 1964, van den Bosch 1964, Cheng 1970). Emergence of *C. marginiventris* from hosts was checked daily thereafter and the number of wasps emerged was recorded. Each age group was replicated 8 times.

Because the preceding test (No-choice trial 1) was insufficient to detect a lower size threshold below which *S. exigua* larva were not suitable for *C. marginiventris* oviposition or development, another no-choice test (No-choice trial 2) was conducted. The procedure was the same as for the first No-choice test except the age groups of the *S. exigua* larva were 14, 24, and 48h. There were 8 replicates for the 14 and 24h age groups, and 5 replicates for the 48h age group.

Testing SG-HM in the laboratory

Cotton plants, *Gossypium hirsutum* (L.), were grown with the method described elsewhere (Chapter 2). Cotton plants were fertilized with 100 ml of 112 ppm N nutrient solution for ca. 2 wk, at which time two cotton plants of the same height and with similar-sized leaves at the same leaf position were assigned to a block (a total of 12 blocks). The two plants within the block were each randomly assigned to one of two N treatments (42 and 196 ppm N). Cotton plants were fertilized with corresponding N nutrient solutions for ca. 2 wk until experimentation. Leaching (watering without nutrients) followed every fourth N solution application in order to reduce salt (salinity) buildup. All experimental plants had 3-5 mature true leaves. Neonate *S.*

exigua larvae were reared in groups of 40 to 50, on fresh leaf tissue excised from cotton plants of the two N treatments, in Petri dishes for 3, 5, and 6 d (age group). Leaf tissue was changed twice daily and the Petri dishes were maintained in the environmental chamber described previously. Ten larvae reared on each N treatment were randomly selected and weighed. These larvae were then exposed to stinging by *C. marginiventris* as described in the no-choice trials, and placed in groups of 5 in an artificial diet cup filled with 3 ml of diet. Larvae were checked daily for *C. marginiventris* larval emergence or formation of cocoons. The 3-d-old age group was repeated 4 times, and the 5- and 6-d-old age groups were repeated 8 times.

To determine the effects of N fertilization treatments on the plants, leaf petioles from 4 cotton plants of each N treatment were immediately oven-dried at 65 °C for 2 days and sent to the Soil, Plant, and Water Laboratory at the University of Georgia for petiole nitrate-N analysis. The nitrate-N analysis was repeated 3 times for each treatment.

Testing SG-HM hypothesis in the Greenhouse

Cotton plants with different nutritional qualities were grown as described above with 42 and 196ppm N nutrient solutions and were at the 3-4-mature-leaf stage at the time of the experiments. Two to three plants from the 42 ppm N treatment were randomly placed at one end of the test arena (L × W × H = 100 × 60 × 60 cm cage made of PVC pipes covered with fine mesh) which was placed in a greenhouse held at 32°C during the day and 28°C during the night. The photoperiod of the greenhouse was L14:D10. Two plants from the 196 ppm N treatment were randomly placed at the other end of the cage Plants from the same treatment had some leaves touching each other.

In order to simulate *S. exigua* behaviors and to reduce larval mortality due to movement of neonate larvae, beet armyworm eggs were used in most trials to initiate larval infestation. Eggs less than 16h old were kept in environmental chambers for 2d before being placed on the upper side of the fourth true leaf of each cotton plant inside the cage. *S. exigua* eggs normally hatch in 3 days at 25 ± 1°C. Newly-hatched larvae were allowed to move and feed freely on plants for 1 through 6 days (1-, 2-, 3-, 4-, 5-, and 6-day trials, respectively). At each time interval 5 *C. marginiventris* females were released in the middle of the cage. The number of eggs used for the 1-day trial was ca. 35 per plant (about 70 per treatment per cage). About 5 more eggs per treatment than the previous trial were added in the following trails in an attempt to account for larval mortality due to dislodgement and unknown loss. As such there was a total of 75, 80, 85, 90, and 95 eggs per treatment per cage at the onset of the 2-, 3-, 4-, 5-, and 6-day trials, respectively. For the 7-day trial, 7-d-old larvae laboratory-reared on corresponding cotton plant tissues were placed on plants in the cages to promote beet armyworm recovery rate. Leaves of cotton plants were excised and examined carefully for larvae 24h following the release of *C. marginiventris* females. Larvae that were recovered were weighted and placed in diet cups as in the previous experiment. The larvae had been feeding for 2, 3, 4, 5, 6, and 7 days, respectively, at the time of recovery. Larvae from plants of the same N treatment within a cage were placed together in groups of 5 in the diet cup and maintained in the environmental chamber. Emergence of *C. marginiventris* larvae or formation of cocoons was checked daily. The number of hosts yielding offspring of *C. marginiventris*, irrespective of the success or failure of making cocoons, was considered equivalent to the number of *S. exigua* larvae parasitized. Total mortality of *S. exigua* larvae was calculated by dividing the total number of dead larvae at the end of the trials by total number of larvae recovered.

Leaf petioles of 4 randomly selected cotton plants in each treatment were immediately oven-dried at 65 °C for 2 days and sent to the Soil, Plant, and Water Laboratory at the University of Georgia for petiole nitrate-N analysis. The nitrate-N analysis was repeated 2 times for each treatment.

Statistical analysis

ANOVA (PROC GLM in SAS) (SAS Institute, 1999) was used for statistical analysis of all data. Parasitism and total mortality of *S. exigua* larvae were arcsine (square-root) transformed before analysis.

RESULTS

Are there lower and upper size thresholds beyond which beet armyworm larvae are not suitable for C. marginiventris development?

Weights of 2- to 7-d-old beet armyworm larvae reared on artificial diet differed significantly from one other (Table 5.1; No-choice trial 1: $F_{5,42} = 2228.58$, $P < 0.0001$). *S. exigua* larval weight at time of parasitism significantly affected the success of parasitism by *C. marginiventris* (Table 5.1; No-choice trial 1: $F_{5,42} = 14.02$, $P < 0.0001$). The patterns of total mortality of stung 2- to 7-d-old *S. exigua* larvae paralleled those of parasitism by *C. marginiventris* (Table 5.1; No-choice trial 1: $F_{5,42} = 11.91$, $P < 0.0001$), indicating that most of the mortality was probably parasitoid induced.

The developmental time of *C. marginiventris* in *S. exigua* hosts was similar regardless of host size at parasitism, although the differences between the longer and the shorter developmental times was a little over 1 d and this was statistically significant ($F_{4,35} = 9.82$, $P < 0.0001$) (Table

5.1; No-choice trial 1). In No-choice trial 2 with young host larvae, *S. exigua* weight differed significantly with larval age (Table 5.1, $F_{2,18} = 158.74$, $P < 0.0001$). The parasitism rate and total mortality of very young *S. exigua* larvae were significantly affected by host size (Table 5.1; parasitism rate: $F_{2,18} = 2.79$, $P = 0.09$; total mortality: $F_{2,18} = 4.50$, $P < 0.03$). In all three host size treatments, the stung larvae suffered high mortality.

Testing SG-HM in laboratory

The petiole nitrate-N content of cotton plants of the 196 ppm N treatment was significantly higher than that of the 42 ppm N treatment (Fig. 5.1A), and these differences translated into developmental differences in *S. exigua* larvae on the respective plant treatments. Mean weights of beet armyworm larvae reared on both 42 and 196 ppm N cotton plants after 3 and 5 days were less than 6 mg (Fig. 5.1B). Weights of beet armyworm larvae reared on the 196 ppm N treatment for 3, 5, and 6 d were significantly higher than those of larvae reared on the 42 ppm N treatment (Fig. 5.1B; 3 d: $F_{1,6} = 81.99$, $P = 0.0001$; 5 d: $F_{1,14} = 122.57$, $P < 0.0001$ 6 d: $F_{1,14} = 142.08$, $P < 0.0001$). The percentage of 3- and 5-d-old beet armyworm larvae parasitized by *C. marginiventris* among the 2 N treatments was not significantly different (Fig. 5.2; 3-d-old larvae: $F_{1,6} = 0.63$, $P = 0.46$; 5-d-old larvae: $F_{1,14} = 1.27$, $P = 0.28$). However, successful parasitism of beet armyworm larvae reared on 196 ppm N treatment cotton plants for 6 days was less than half of that for hosts reared in the 42 ppm N treatment (Fig. 5.2; $F_{1,14} = 14.19$, $P = 0.0021$).

Testing SG-HM hypothesis in the Greenhouse

N treatment had no significant effect on *S. exigua* larval recovery rates, although the recovery rates on 196 ppm N treatment tended to be slightly higher than those from the 42 ppm N

treatment, irrespective of the age of the larvae (Fig. 5.3). In any case, recovery rates should not differentially confound parasitism assessment between N treatments. The average recovery rates of larvae on the 42 and 196 ppm N treatments were 30.1 ± 4.45% and 32.4 ± 4.52%, respectively. The age of the larvae had a significant effect on their recovery rate ($F_{6,42}$ = 45.00, P < 0.0001); however, the interaction between age and treatment was not significant ($F_{6.42}$ = 0.11, P = 0.99).

N treatment had a significant effect on cotton petiole nitrate-N contents in all trials except the 7-d-trial (Fig. 5.4A). *S. exigua* larval weights reared on 196 ppm N treatment were all higher than their counterparts reared on 42 ppm N treatment, and the weight differences of *S. exigua* larvae in the 2-, 6-, and 7-d-trials were statistically different (Fig. 5.4B).

Parasitism rates of *S. exigua* larvae in all trials were not affected by N levels, except for the 3-d-trial (marginally affected) and 6-d-trial (Fig. 5.5). When combined over the 7 trials, an average of 46.9 ± 4.39 and 46.8 ± 4.76% larvae from the 42 and 196 ppm N treatments, respectively, were parasitized, and the difference between treatments was not significant ($F_{1,42}$ = 0.03, P = 0.85). The age/size of larvae significantly affected parasitism rates ($F_{6,42}$ = 9.35, P < 0.0001), and over 40% of the larvae 5-d-old and less were parasitized, while the parasitism rates of 6- and 7-d-old larvae declined dramatically (Fig. 5.5). The interaction between age and treatment was not significant ($F_{6,42}$= 0.50, P = 0.80).

Total mortality of *S. exigua* larvae in all trials was not affected by N levels, except for the 6-d-trial (Fig. 5.6). When combined over the 7 trials the difference between treatments was not significant ($F_{1,42}$ = 2.07, P = 0.16). Although the age/size of larvae had a significant effect on total mortality (Fig. 5.6; $F_{6,42}$ = 9.19, P < 0.0001), there was no significant interaction between age and treatment ($F_{6,42}$= 0.73, P = 0.63).

DISCUSSION

The SG-HM hypothesis was not supported in our study for a significant mortality agent of *S. exigua*. The N treatments effectively prolonged the development of the host in the laboratory and greenhouse, but the protracted development did not translate into increased mortality in either location. We postulate that the failure of the SG-HM hypothesis in the laboratory was due to the parasitoid having a discrete range of host sizes over which the host is suitable for parasitoid acceptance and development, and the mere shifting of this suitability period rather than effectively prolonging it. *Spodoptera exigua* larvae over 8 mg (approximately 6- to 7-d-old) and below 0.08 mg (approximately 14-h-old) in this study were much less suitable for development of *C. marginiventris* offspring than were larvae within these endpoints. *Spodoptera exigua* larvae reared on high N (196 ppm N) plants reached the upper size threshold in a shorter time than those reared on low N (42 ppm N) plants. However, because there is a lower size threshold below which hosts were not as suitable for *C. marginiventris* oviposition or development, *S. exigua* larvae reared on high N plants also reached the lower size threshold earlier than their counterparts reared on low N plants. Furthermore, as shown by Chen et al. (unpublished data), the difference in developmental time between *S. exigua* larvae grown on 42 and 196 ppm N plants was mainly at later stages (between the fifth and sixth larval instars) and the difference was small in early stages (until fourth instar) although it's significant. The susceptible stage of *S. exigua* larvae to *C. marginiventris* was before the fourth instars. Therefore, the timing of vulnerability of *S. exigua* larvae reared on low and high N plants to parasitoids shifted, but the window of vulnerability of *S. exigua* larvae (time between lower and upper size thresholds) was overall unchanged or not significantly changed, resulting in no significant changes in overall parasitoid-induced mortality.

The range of host sizes and ages that are acceptable for oviposition and suitable for parasitoid development may be constrained by various factors, including host defensive behaviors, host immune responses, and changes in host cuticle (van Alphen and Janssen, 1982; Godfray, 1994; Quicke, 1997). In our laboratory study, only stung larvae were used, so in each case the host larvae had been accepted for stinging by female parasitoids. Thus, all of the sizes offered were considered acceptable for stinging. We did not verify oviposition, so it is possible that females stung some hosts without actually ovipositing, and may have done so in a differential manner in response to host size. Conversely, females may have oviposited in all or nearly all stung hosts, but the host suitability may have changed with host size/age to exert differential effects on immature parasitoid survival. Our experimental design did not permit more detailed differentiation of these mechanisms. Regardless, there were clear differences in overall mortality of stung hosts in relation to host size, so that mortality inflicted by the parasitoid was confined to a restricted window of host sizes.

The laboratory results were confirmed in cage tests in the greenhouse. In both test environments, the parasitism rates of *S. exigua* larvae in early developmental stages (1- to 3-d-trial) on high N plants were consistently numerically higher than those of larvae reared on low N plants at the same time period. Conversely, the pattern was reversed in the later larval developmental stages (4- to 7-d-trial), indicating a shift in the window of susceptibility to parasitoid-induced mortality.

It is possible that the results observed here may differ from results in the open field because of possible effects of differential nitrogen levels or herbivore feeding on production and availability of foraging cues. Foraging *C. marginiventris* females rely heavily on host-induced volatile chemicals emanating from damaged plants (Turlings et al., 1991; Hoballah et al., 2002). If

feeding of very small larvae is reduced on plants of lower nutritional quality, then foraging cues may not be produced and released in sufficient quantities to permit females to locate hosts. Conversely, hosts developing faster and feeding more may be more readily located by foraging parasitoids. Many entomophagous predators and parasitoids utilize herbivore-induced volatile organic compounds as long-range foraging signals (Dicke and Sabelis 1988, Röse et al. 1998, Choh et al. 2004), and these behaviors may significantly affect the extent to which natural enemies respond to host or prey developmental delays.

Testing of the SG-HM hypothesis in a larger test arena or in the field would be very valuable because it would incorporate multiple elements of the herbivore's and enemy's behaviors. The parasitoid foraging process is hierarchically divided into host habitat location, host location, host acceptance, and host suitability (Nordlund et al. 1981). In small cage studies, like this study, the cues involved in host habitat location might be obscured, thereby possibly rendering the results of limited ecological significance. The causal mechanism of slower growth of *S. exigua* larva on plants with lower N levels might also enhance the emission of these volatiles and foraging efficiency of *C. marginiventris* because N deficiency has been shown to increase volatile production in some plants (van Wassenhove et al. 1990, Schmelz et al. 2003, but see Lou and Baldwin 2004).

One of the problems with many studies testing SG-HM hypothesis is that herbivore mortality was based on daily or short-term mortality (Häggström and Larsson 1995, Williams 1999), which is not predictive of mortality across entire vulnerable stages. In our study, mortality of *S. exigua* larvae was tested throughout the developmental time that is vulnerable to *C. marginiventris* in the lab and greenhouse. *Spodoptera exigua* larval parasitism difference between 2 N treatments was not significant in all stages except for the 6-d-trial, in which a

significantly higher percentage of larvae on low N plants were parasitized compared to those on high N plants. If only daily mortality had been considered in this study, then conflicting conclusions could be made, based on which developmental stages were chosen. However, when all daily parasitism rates were combined together, the difference between low and high N plants was not significant, in contrast with the predictions of the SG-HM hypothesis.

ACKNOWLEDGEMENTS

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Table 5.1 Effects of *S. exigua* age on development of *C. marginiventris* in no-choice tests – Detection of host upper size threshold.

Age groups	No. larvae	Weight/larva Mean ± SE (mg)	Parasitism Mean ± SE (%)	*S. exigua* mortality[1] Mean ± SE (%)	Emergence time[2] Mean ± SE (d)
No-choice trial 1					
2-d-old	80	0.52 ± 0.01 a	47.50 ± 8.81 b	60.00 ± 10.00 b	9.45 ± 0.20 b
3-d-old	80	1.10 ± 0.04 b	67.50 ± 8.61 c	81.25 ± 7.66 bc	8.12 ± 0.12 a
4-d-old	80	1.33 ± 0.06 c	66.25 ± 6.80 c	81.25 ± 6.66 c	8.34 ± 0.15 a
5-d-old	80	2.75 ± 0.05 d	48.75 ± 8.75 bc	65.00 ± 8.86 bc	9.26 ± 0.25 b
6-d-old	80	3.35 ± 0.06 e	58.75 ± 3.98 bc	65.00 ± 2.67 bc	9.22 ± 0.21 b
7-d-old	80	8.80 ± 0.12 f	3.75 ± 1.83 a	8.75 ± 2.27 a	--
No-choice trial 2					
14-h-old	80	0.08 ± 0.00 a	43.75 ± 8.85 a	82.50 ± 4.12 a	--
24-h-old	80	0.13 ± 0.00 b	60.00 ± 7.79 ab	90.00 ± 3.27 ab	--
48-h-old	50	0.38 ± 0.03 c	72.00 ± 5.83 b	98.00 ± 2.00 b	--

[1]parasitized plus dead divided by total number of *S. exigua* larvae in each replicate; [2] Time from egg-laying to emergence from hosts; [3]Head capsule width of *S. exigua* larva when *C. marginiventris* emerged; * *S. exigua* larvae were at the fourth or fifth instar stage according Pearson (1982). Means within each trial followed by different lower-case letters are significantly different at $p < 0.05$.

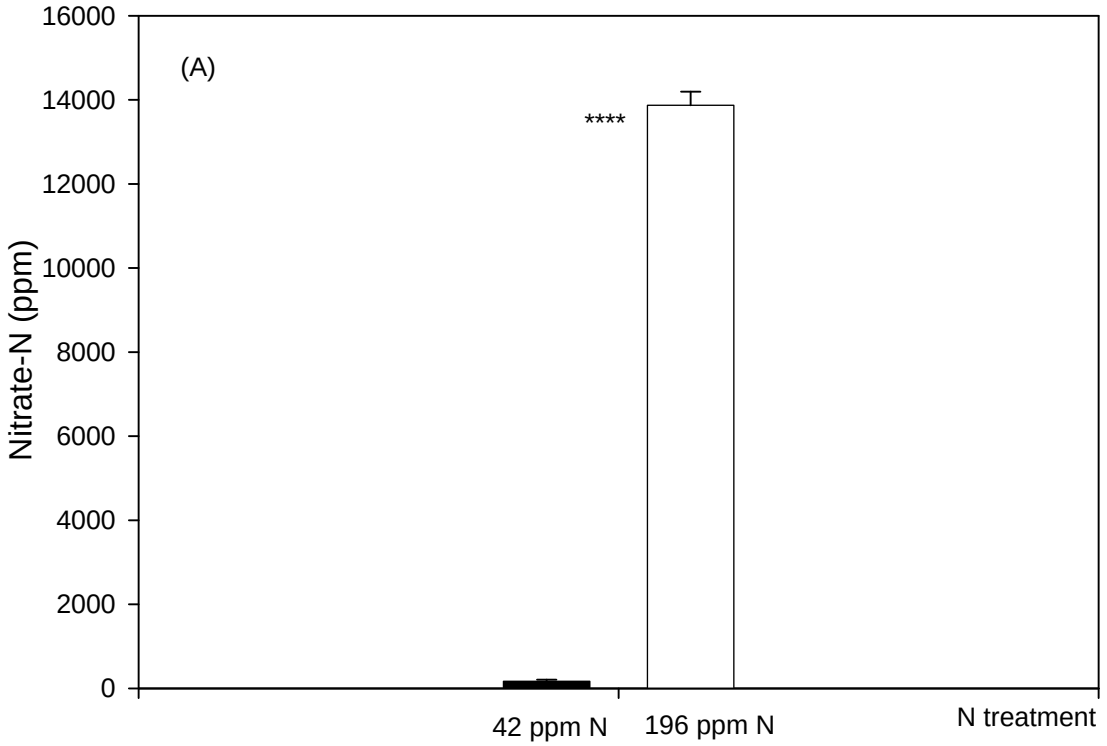

Fig. 5.1 Nitrate-N contents of leaf petioles from 2 nitrogen treatments (A) and weights of *S. exigua* larvae reared from those plants for 3, 5, and 6 days (B). **** $P < 0.0001$.

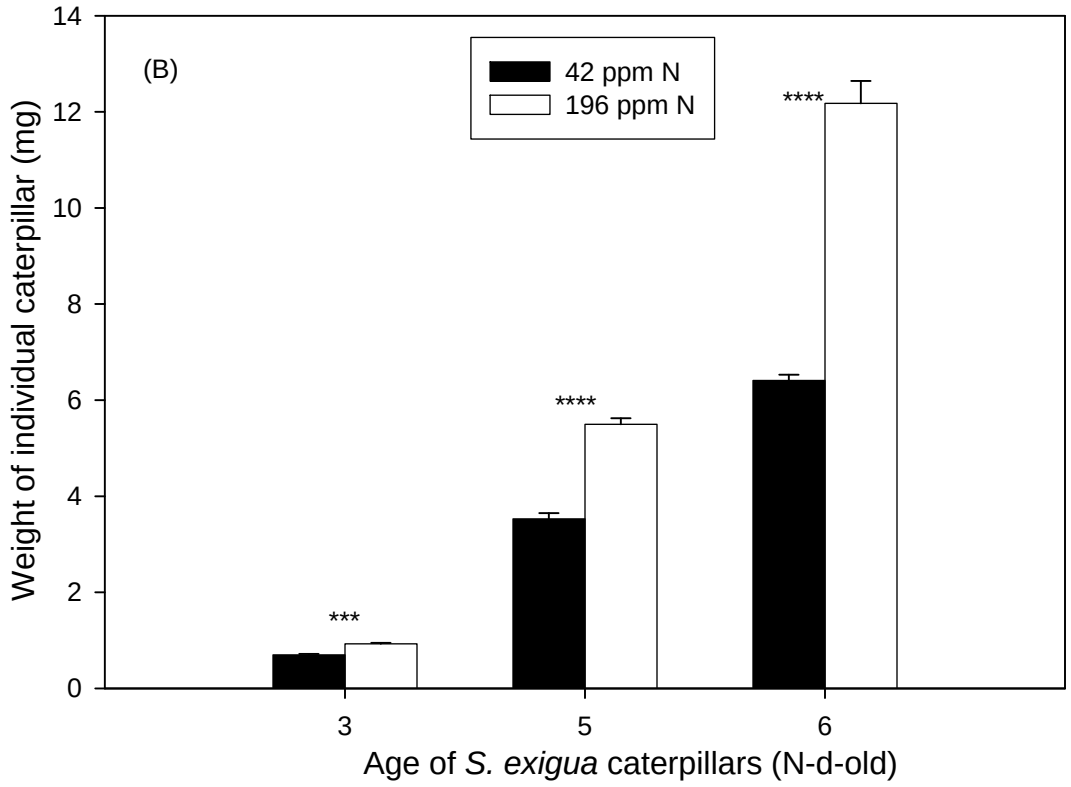

Fig. 5.1 Nitrate-N contents of leaf petioles from 2 nitrogen treatments (A) and weights of *S. exigua* larvae reared from those plants for 3, 5, and 6 days (B). **** $P < 0.0001$.

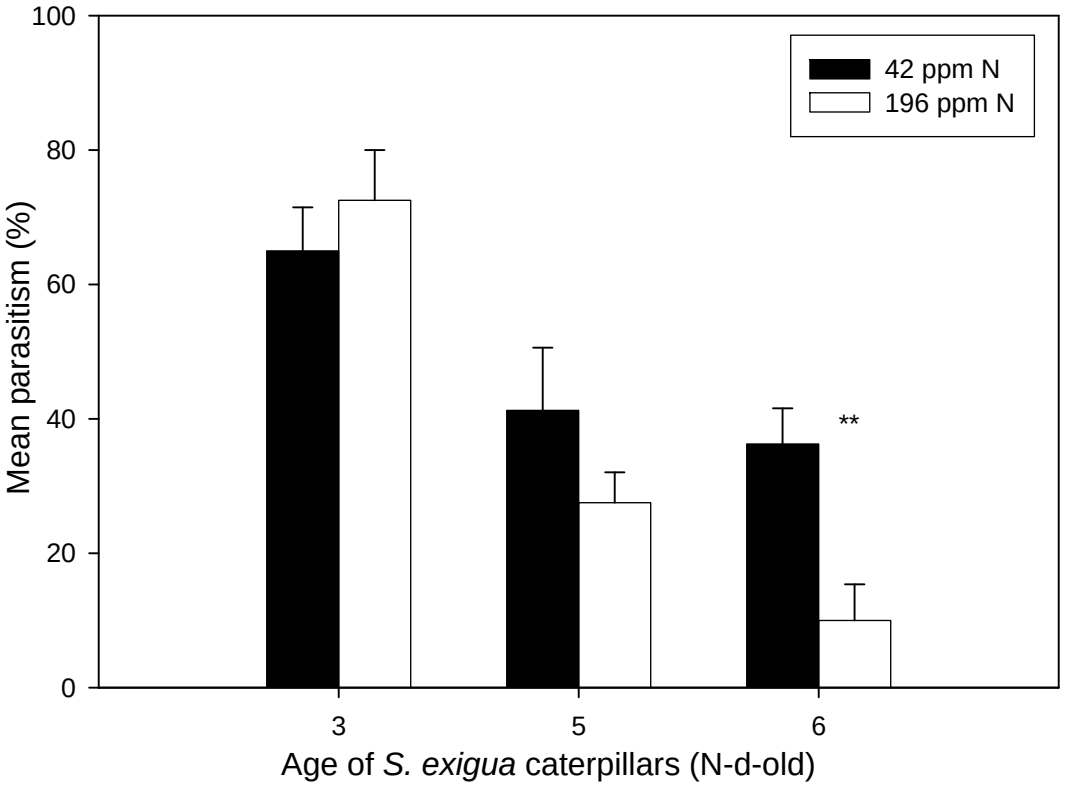

Fig. 5.2 Parasitism of *S. exigua* larvae reared in cotton plants with 42 and 196 ppm N for 3, 5, and 6 days by *C. marginiventris* in the laboratory; ** $P < 0.01$.

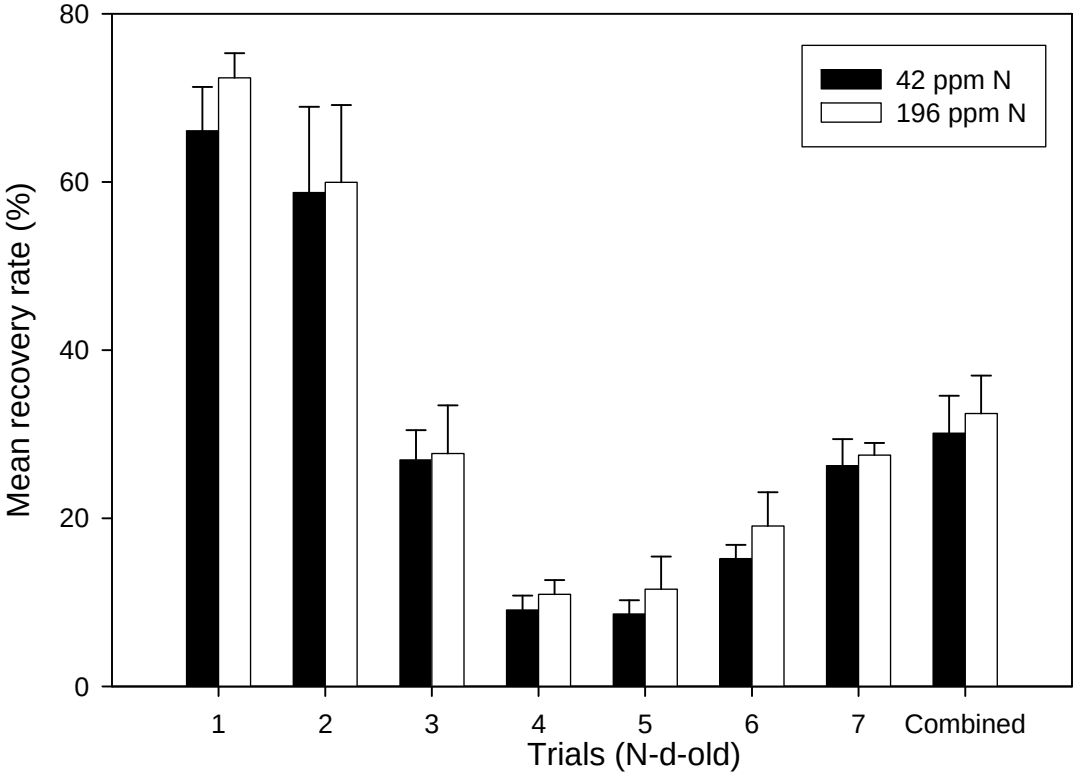

Fig. 5.3 Percentage of sentinel *S. exigua* larvae recovered in 1- to 7-d-trials conducted in greenhouse cages. Larvae in 1-, 2-, 3-, 4-, 5-, 6-, and 7-d-old trials were 2-, 3-, 4-, 5-, 6-, 7-, 8-d-old at the time of recovery, respectively.

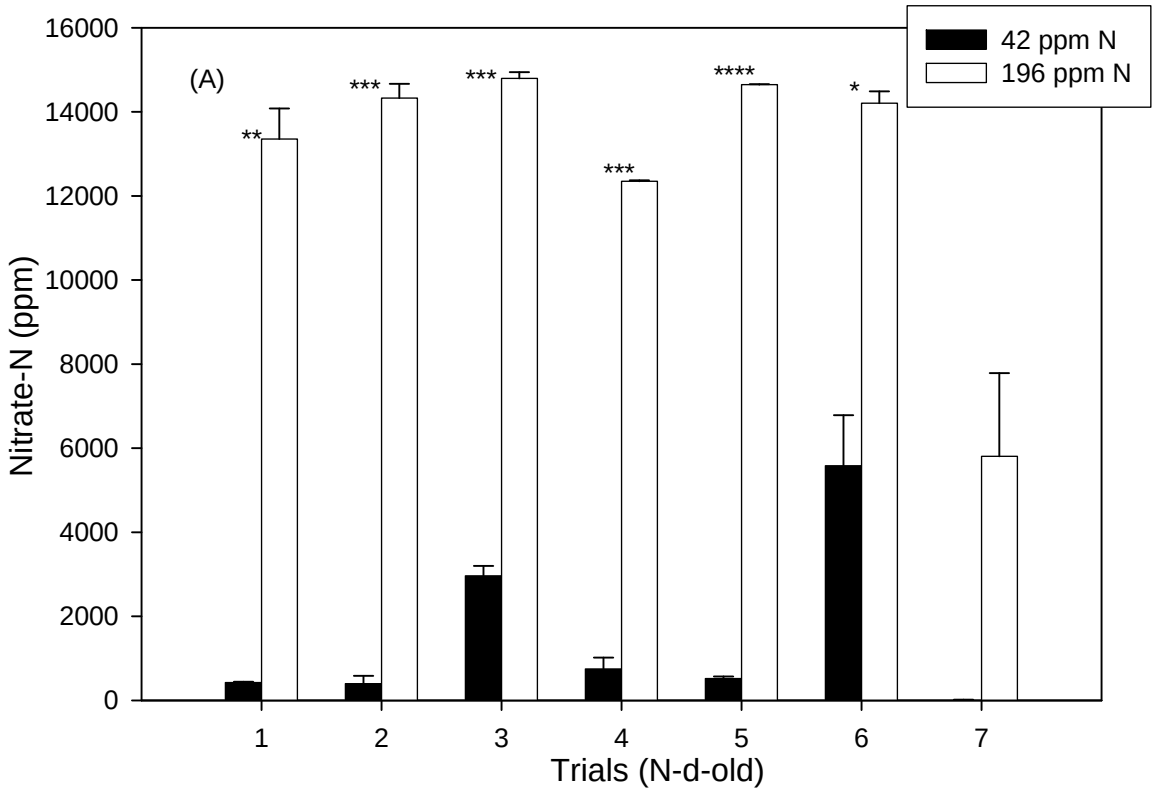

Fig. 5.4 Nitrate-N contents of leaf petioles from 2 nitrogen treatments (A) and weights of *S. exigua* larvae collected in 1- to 7-d-trials conducted in greenhouse cages (B). The weight of 1-d-old S. exigua larvae was not measured because of the small size. Larvae in 1-, 2-, 3-, 4-, 5-, 6-, and 7-d-old trials were 2-, 3-, 4-, 5-, 6-, 7-, 8-d-old at the time of recovery, respectively. Nitrate-N: 1-d-trial: $F_{1,2}$ = 317.87, P = 0.0031; 2-d-trial: $F_{1,2}$ = 1280.40, P = 0.0008; 3-d-trial: $F_{1,2}$ = 1833.87, P = 0.005; 4-d-trial; $F_{1,2}$ = 1821.28, P = 0.0005; 5-d-trial: $F_{1,2}$ = 69699.8, P < 0.0001; 6-d-trial: $F_{1,2}$ = 48.79, P = 0.02; 7-d-trial: $F_{1,2}$ = 8.56, P = 0.10). *S. exigua* weight: 2-d-trial: $F_{1,3}$ = 30.09, P = 0.01; 3-d-trial: $F_{1,3}$ = 8.72, P = 0.06; 4-d-trial; $F_{1,3}$ = 19.34, P = 0.02; 5-d-trial: $F_{1,3}$ = 6.81, P = 0.08; 6-d-trial: $F_{1,3}$ = 14.91, P = 0.03; 7-d-trial: $F_{1,3}$ = 22.83, P = 0.02).* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.

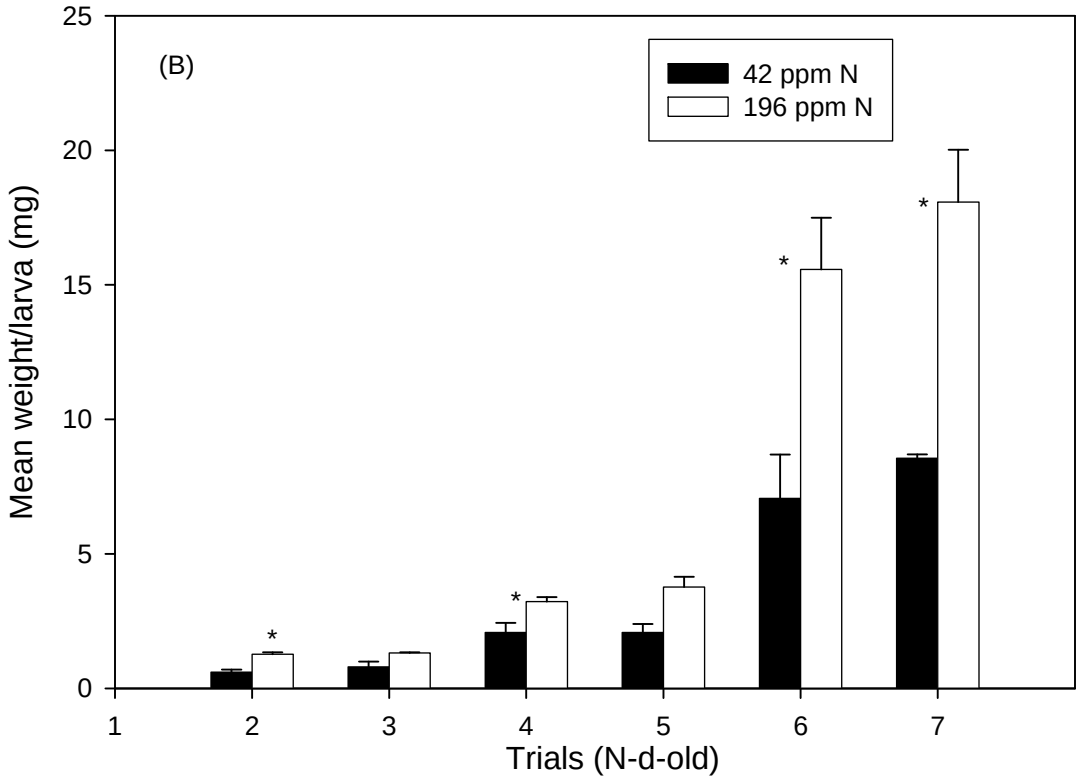

Fig. 5.4 Nitrate-N contents of leaf petioles from 2 nitrogen treatments (A) and weights of *S. exigua* larvae collected in 1- to 7-d-trials conducted in greenhouse cages (B). The weight of 1-d-old S. exigua larvae was not measured because of the small size. Larvae in 1-, 2-, 3-, 4-, 5-, 6-, and 7-d-old trials were 2-, 3-, 4-, 5-, 6-, 7-, 8-d-old at the time of recovery, respectively. Nitrate-N: 1-d-trial: $F_{1,2}$ = 317.87, P = 0.0031; 2-d-trial: $F_{1,2}$ = 1280.40, P = 0.0008; 3-d-trial: $F_{1,2}$ = 1833.87, P = 0.005; 4-d-trial; $F_{1,2}$ = 1821.28, P = 0.0005; 5-d-trial: $F_{1,2}$ = 69699.8, P < 0.0001; 6-d-trial: $F_{1,2}$ = 48.79, P = 0.02; 7-d-trial: $F_{1,2}$ = 8.56, P = 0.10). *S. exigua* weight: 2-d-trial: $F_{1,3}$ = 30.09, P = 0.01; 3-d-trial: $F_{1,3}$ = 8.72, P = 0.06; 4-d-trial; $F_{1,3}$ = 19.34, P = 0.02; 5-d-trial: $F_{1,3}$ = 6.81, P = 0.08; 6-d-trial: $F_{1,3}$ = 14.91, P = 0.03; 7-d-trial: $F_{1,3}$ = 22.83, P = 0.02).* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.

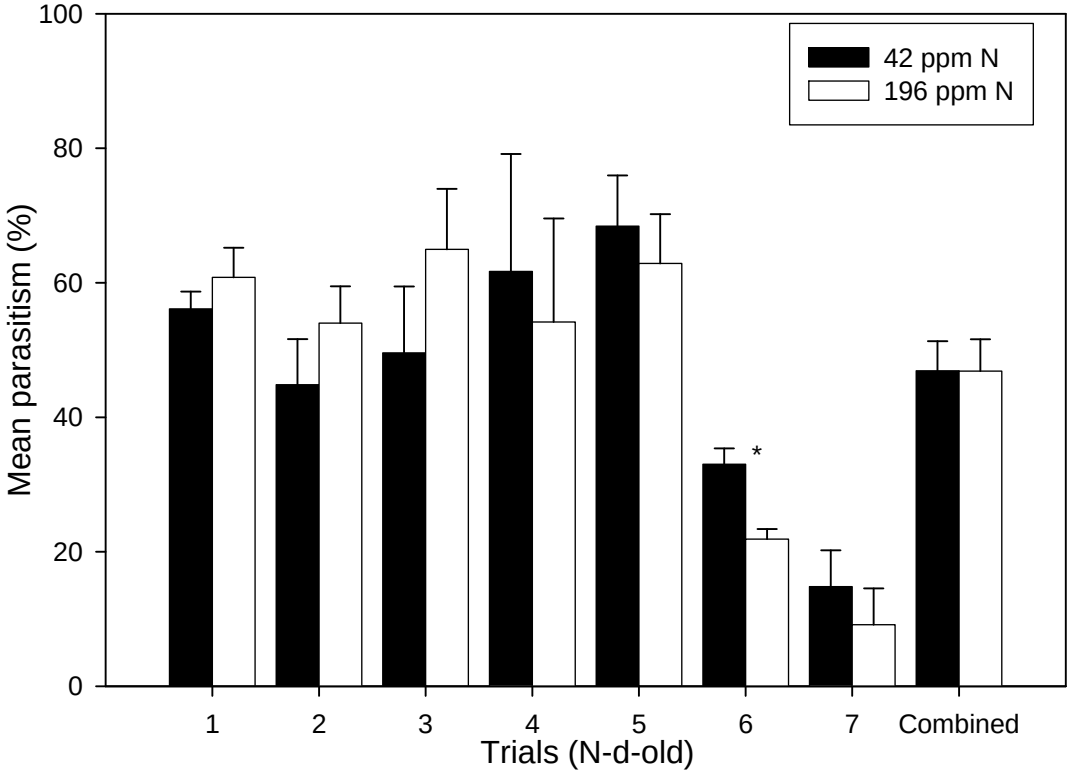

Fig. 5.5 Parasitism of *S. exigua* larvae collected in 1- to 7-d-trials conducted in greenhouse cages by *C. marginiventris*. Larvae in 1-, 2-, 3-, 4-, 5-, 6-, and 7-d-old trials were 2-, 3-, 4-, 5-, 6-, 7-, 8-d-old at the time of recovery, respectively. 1-d-trial: $F_{1,3} = 0.56$, $P = 0.51$; 2-d-trial: $F_{1,3} = 1.47$, $P = 0.31$; 3-d-trial: $F_{1,3} = 8.55$, $P = 0.06$; 4-d-trial; $F_{1,3} = 0.46$, $P = 0.55$; 5-d-trial: $F_{1,3} = 0.75$, $P = 0.45$; 6-d-trial: $F_{1,3} = 15.05$, $P = 0.03$; 7-d-trial: $F_{1,3} = 0.30$, $P = 0.62$). ** $P < 0.05$.

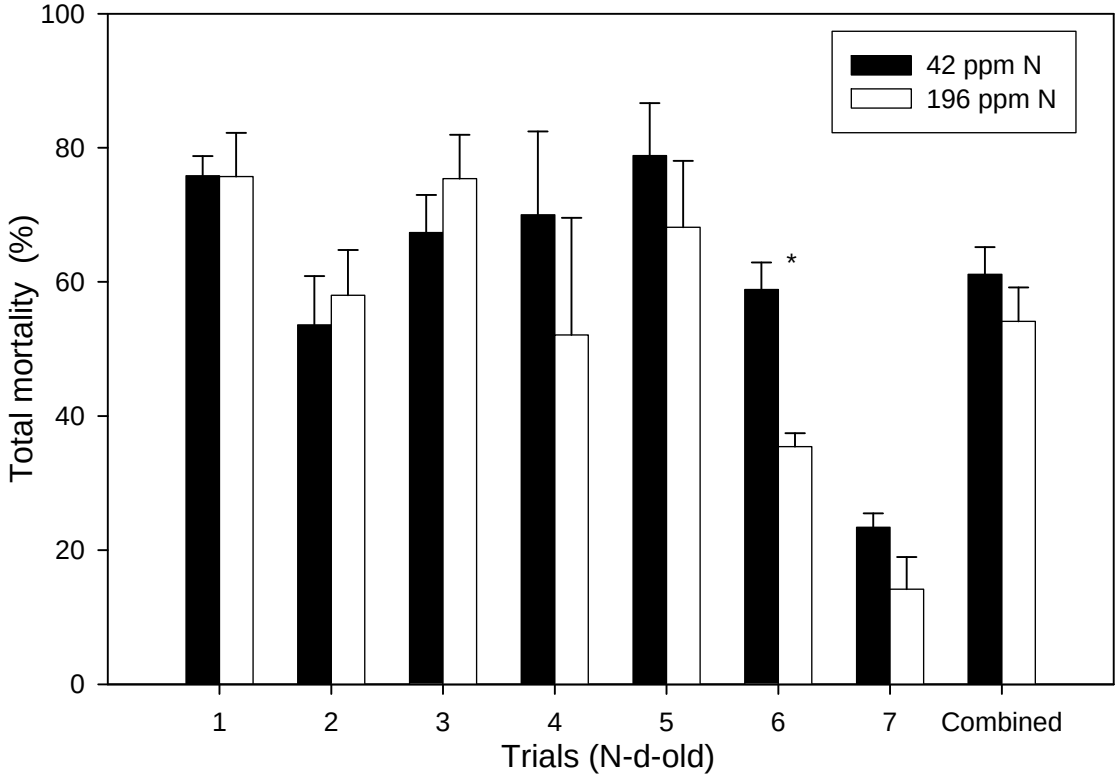

Fig. 5.6 Total mortality of *S. exigua* larvae collected in 1- to 7-d-trials conducted in greenhouse cages by *C. marginiventris*. Larvae in 1-, 2-, 3-, 4-, 5-, 6-, and 7-d-old trials were 2-, 3-, 4-, 5-, 6-, 7-, 8-d-old at the time of recovery, respectively. 1-d-trial: $F_{1,3} = 0.09$, $P = 0.79$; 2-d-trial: $F_{1,3} = 0.24$, $P = 0.66$; 3-d-trial: $F_{1,3} = 1.09$, $P = 0.37$; 4-d-trial; $F_{1,3} = 6.34$, $P = 0.09$; 5-d-trial: $F_{1,3} = 4.37$, $P = 0.13$; 6-d-trial: $F_{1,3} = 33.71$, $P = 0.01$; 7-d-trial: $F_{1,3} = 2.80$, $P = 0.19$. ** $P < 0.05$.

CHAPTER 6

IMPACT OF VARIABLE NITROGEN FERTILIZATION ON ARTHROPODS IN COTTON

[1]Chen, Y. and J.R. Ruberson. Submitted to *Agriculture, Ecosystem & Environment*, 10/20/07.

ABSTRACT: Reducing fertilizer applications can save production costs for cotton growers and lessen nitrogen (N) leaching into the soil and contamination of surface and ground water. But altered N fertilization may also affect pests and their natural enemies. In this study, plots with 4 different levels of fertilizer input (0, 45, 90, and 135kg/ha N) were used to investigate the influence of N on cotton pest and beneficial arthropod populations, and on cotton yield. We predicted that 1) N fertilization will enhance cotton plant growth; 2) cotton pest populations and mortality of insect herbivores due to parasitoid will be positively affected; 3) herbivore mortality due to predators will be negatively related to N fertilization; and 4) cotton yield may not be affect by N fertilization. Cotton plant growth was enhanced by N fertilization. N fertilization significantly affected some arthropods. Mirids were most abundant in the high N treatment in both years of the study. Arthropod predators also were generally more abundant in the high N treatment, but only spiders and Geocoris spp. were significantly affected by N treatment, with highest members present in the highest N treatment. The greatest mortality of sentinel pest egg due to predation occurred under low N conditions. N fertilization had no significant effects on parasitism of sentinel caterpillars. Cotton yield was not significantly affected by N fertilization in both seasons, and in 2005 the highest yield was observed in lowest N treatment while in 2006 the high yield was observed in high N fertilization treatments.

Key words: Resource availability; Nutrient; “bottom up” effects; “top down” effects; tri-trophic interactions; sustainable agriculture

1. INTRODUCITON

Two important components of sustainable agriculture are sound economic and environmental practices (Turner, 1993; White et al., 1993; Barnett et al., 1995). Fertilization entails significant economic and environmental costs. Among the fertilizers, nitrogen (N) is the most commonly applied nutrient in crop production (Weir et al., 1996). However, excess N input for crops not only adds costs to crop production, but can be environmentally disruptive and increase pest problems. Plant nutritional quality and plant defense that directly act on plant-consuming herbivores are altered by N fertilization and herbivorous insects can distinguish between plants receiving different N applications. For example, females of the butterflies Pieris rapae crucivora (L.) and P. canidia canidia (Sparrman) (Lepidoptera: Pieridae) (Chen et al., 2004) and the buckeye butterfly, Junonia coenia (Hübner) (Lepidoptera: Nymphalidae) (Prudic et al., 2005) prefer fertilized over unfertilized host plants for oviposition. The expression of direct plant defensive traits, such as digestibility reducers and toxins, are dependent on soil N availability. For instance, increased N reduced the total concentration of the carbon-based iridoid glycoside of Plantago lanceolata (Darrow and Bowers, 1999; Prudic et al., 2005) and the total phenolic content in tomato plants Lycopersicon esculentum (Stout et al., 1998).

Insect pest feeding on lower N host plants generally grow slower and attain less biomass within the same period of time in comparison to those feeding on higher quality foods (Lindroth et al., 1995; Chen et al., 2004). Therefore, predators may inflict higher mortality on slow-growing herbivores because predators need to consume a certain amount of prey mass to complete their development and smaller prey tends to be easier to subdue (Blackman, 1967; Loader and Damman, 1991; Williams, 1999). In contrast, parasitoids generally have a higher requirement for quality hosts. This is likely more important for idiobiont parasitoids that limit or

halt their hosts' development following parasitization. A variety of studies reported that parasitoids caused higher mortality under situations when hosts are feeding on plants of higher quality (Fox et al., 1990; Loader and Damman, 1991; Bentz et al., 1996).

Profitability is important in agricultural production. Based on the potential effect of N on crop-herbivore-natural enemy interactions, higher N input may not result in higher profitability, because the increased biomass due to greater N may be offset by increased biomass consumption of a larger pest population. Conversely, the negative effects of low N on plant biomass might be compensated for by increased direct and indirect defenses of plants.

In the study we investigated the effects of N fertilization on cotton (Gossypium hirsutum L.) growth, cotton yield, arthropod populations, and the possibility of sustainable cotton pest management through N management. Specifically, we tested the following hypotheses: 1) N fertilization will enhance cotton plant growth; 2) Increased N fertilization will increase pest populations; 3) Increased N fertilization will decrease pest mortality due to predators of pests; 4) Increased N fertilization will increase pest mortality due to parasitoids of pests; 5) N fertilization may not affect cotton lint yield. To the authors, this study is the first of its kind to comprehensively examine the effects of N fertilization on plant-herbivore-natural enemy interactions and plant fitness.

2. MATERIAL AND METHODS

2.1 Cotton field layout and management

The experimental fields were located on the Lang Farm, Tifton, GA. Cotton plants, G. hirsutum (c.v. FiberMax 989), were used in the experiments. In 2005, the field was 0.62ha in area and was divided into 20 plots. Each plot had 12 rows and was ca. 17m long. Plots were

arranged into 5 blocks. Four plots in each block were randomly assigned to 4 nitrogen treatments. The same randomized complete block experimental design was used in 2006. The field area in 2006 was 0.45ha and there were 6 blocks. Each plot in 2006 had 8 rows and was ca. 23m long. In both years, four treatments were used (1) no fertilizer application during the growing season; (2) one application of 45kg/ha of Royster Clark liquid N fertilizer (N-P-K=32-0-0, Bainbridge, GA) (1/2 to 1/3 of the standard amount applied in GA, USA, Hodges et al., 1989); (3) 2 applications of 45kg/ha (the standard amount of N fertilizer recommended in GA, USA); and (4) 3 applications of 45kg/ha. The cotton in 2005 was sown on 7 June, and the first N application was applied on 8 June, with second and third applications on 21 June and 1 July, respectively. The cotton in 2006 was sown on 15 May, and N was applied on 9, 19, and 29 June. Other cotton production practices followed agronomic practices for GA cotton production (UGA-CAES, 2004).

2.2 cotton plant growth characteristics

On 19 August 2005, cotton leaf chlorophyll levels were measured with a chlorophyll meter, the Minolta SPAD-502 (Konica Minolta Sensing, Inc., Japan). We randomly selected 10 plants in the middle of each plot were. For each plant 2 measurements were made on the basal part of the most recently matured expanded leaf. In September 2006, instead of measuring leaf chlorophyll, leaf petiole NO_3-N content was analyzed. Two petioles (ca. 6 nodes below the cotton terminal) from each of 10 plants per plot were collected. Samples were oven-dried at 65°C for 2d before being sent to the Soil, Plant, and Water Laboratory of the University of Georgia (Athens, Georgia) for N analysis. The plant tissue nitrate-N analysis utilizes the H_2O_2-H_2SO_4 mixture for digestion of plant material in the absence of heavy metals (McGill and Figureiredo,

1993). At the end of both the 2005 and 2006 growing season, 5 plants from each of the 2 middle rows of each plot were randomly selected for plant height measurements and to quantify the number of nodes per plant (cotyledon node 0). In 2005, total bolls on each plant were counted at the end of the season.

2.3 Insect pests and beneficial arthropods

Insect pests and beneficial arthropods were sampled with 1×1m drop cloths at 7d intervals. The drop cloth was positioned between 2 rows of cotton, and cotton plants within the length of the drop cloth on both sides were shaken. Specimens of common cotton arthropods were identified and counted. If identification of specimens could not be determined in the field, they were brought into the laboratory for further identification. In 2005, two samples each were taken approximately weekly between rows 5 and 6, and rows 8 and 9; while in 2006 rows 3 and 4, and 5 and 6 were used. Samples were taken toward the middle of plots to reduce edge effects.

2.4 Parasitism of field collected lepidopteran species

Caterpillars collected during drop cloth sampling were identified to species and kept individually in 5-ml diet cups filled with 3ml of modified Pinto bean diet (Burton, 1969), and checked regularly for parasitism. Diets were replaced as needed later. Parasitoids that emerged were identified at least to genus.

2.5 BAW egg predation rate and caterpillar parasitism rate

Laboratory-produced beet armyworm, Spodoptera exigua (Lepidoptera: Noctuidae), eggs and larvae were placed in the field to evaluate the influence of N on predation and parasitism rates. In

both years, about 50 neonate larvae were confined on a single leaf in the middle of a cotton plant using a 200-ml soft drink cup covered with a nylon sock tied at both ends. The cages were removed after 24h and larvae on the leaf were counted. Caterpillars were exposed to feral natural enemies for 48h, after which all remaining caterpillars were counted and placed in modified Pinto bean diet (Burton, 1969) in groups of 5 to 10 caterpillars per diet cup. Parasitism rate was calculated as the number of parasitoid cocoons plus those parasitoid larvae that emerged from hosts but failed to make cocoons, divided by the total number of caterpillars recovered. This may underestimate parasitism by not counting parasitoids that failed to emerge from hosts. Five cotton plants in the center of each plot were randomly picked as locations of replicates for the evaluation of parasitism rate on 9 August 2005 and on 4 and 25 August 2006.

Egg predation was determined only in 2006. One egg mass with ca. 40 beet armyworm eggs attached to paper tissue were stapled to one leaf in the middle of a cotton plant on 2 August. Eggs were frozen for 2d before experimentation to prevent hatching. The eggs were checked twice daily (once in the morning and once in the afternoon) and all remaining eggs were counted over a period of 2d. The plants were located in the middle of the plots. Four randomly selected cotton plants with one egg mass each in the center of each plot were used as replicates for the evaluation of predation rate.

2.6 Cotton lint yield

At the end of the cotton production seasons (November 9, 2005 and October 20, 2006) cotton from the two middle rows of each plot was machine-picked and weighed for yield and lint quality evaluation.

2.7 Statistical analysis

Analysis of variance (ANOVA, Proc GLM in SAS) (SAS Institute, 1999) was used to analyze plant growth characters (height, leaf chlorophyll content, and petiole nitrate-N levels), the number of cotton insect pests, and beneficial arthropods, cotton lint yield and quality in response to N regimes. For the cotton insect pest and beneficial arthropod analyses, sampling date was considered as a repeated measurement. Paired *t*-tests were used to separate means if the overall null hypothesis was rejected ($p<0.05$). Data were checked for model assumptions before analysis. Data were untransformed unless otherwise noted. Parasitism and predation rates were analyzed with a Kruskal-wallis non-parametric test (Proc NPAR1WAY Wilcoxon in SAS) (SAS Institute, 1999).

3. RESULTS

3.1 Cotton plant growth characteristics in response to N

N application significantly affected cotton plant growth (Tables 6.1 and 6.2). Cotton plants grown in high N treatments grew taller and had more nodes (Tables 6.1 and 6.2), although the number of bolls among treatments did not differ significantly in 2005 (Table 6.1).

N fertilization significantly increased leaf chlorophyll levels in 2005 (Table 6.1). Leaf petiole nitrate-N levels consistently increased with N addition and more than doubled in the two highest treatments T3 and T4 compared to the two lowest, although statistically no significant difference was detected (Table 6.2).

3.2 Insect pests and beneficial arthropods

The insect pests and predators observed during the 2 growing seasons are shown in Table 6.3.

N fertilization significantly increased fleahopper populations in 2005 (Table 6.4). Fleahopper populations were low throughout the season in the lowest N treatment, while those in the highest N treatment were significantly greater late in the growing season (Fig. 6.1). Sampling date had a significant effect on the insect pest populations in most cases (Table 6.4). The interactions between sampling date and N were not significant (Table 6.4).

Populations of spiders in 2005 were significantly affected by N treatment (Table 6.5). Spider populations were highest in the highest N treatment during the season (Fig. 6.2). Spider numbers in the three lower N treatments were not significantly different from one another and the difference between the two highest N treatments was also not statistically significant (Fig. 6.2). Sampling date significantly affected the abundance of all major natural enemies examined (Table 6.5). The interaction between sampling date and N on lady beetles was significant. Sampling date and N acted in opposite directions on lady beetle populations. No other significant interactions were observed (Table 6.5).

Basically the same patterns were observed in 2006 as in 2005. However, in 2006 the only insect pests and natural enemies significantly affected by N treatment were cotton aphid, Aphis gossypii Glover, (Table 6.4) and big eyed bugs, Geocoris spp., respectively (Table 6.5). Cotton aphids were abundant on the first two sampling dates (Fig. 6.3) but almost disappeared thereafter. Geocoris spp. (chiefly Geocoris punctipes) was abundant throughout the growing season, but populations peaked in the middle of the season (Fig. 6.4). As in 2005, sampling date in most cases had a significant impact on all arthropod populations, and interactions between sampling date and N treatment were only significant for L. lineolaris, coccinellids, and chrysopids (Table 6.4 and 6.5).

3.3 Parasitism of field collected lepidopteran species

The two most abundant groups of lepidopteran pests collected both years in weekly drop cloth samplings were heliothine and plusiine (chiefly Pseudoplusia includens) noctuids (Table 6.3). More caterpillars were collected in 2006 (n = 234) than in 2005 (n = 169). The parasitoids that emerged from field-collected caterpillars were exclusively hymenopteran and dipteran species [Cotesia marginiventris (Cresson), C. autographae (Meusebeck), Aleiodes sp., Meterous autographae Meusebeck (Hymenoptera: Braconidae), Euplectrus plathypenae Howard (Hymenoptera: Eulophidae), Copidosoma truncatellum (Dalman) (Hymenoptera: Encyrtidae), Archytas marmoratus (Townsend), Eucelatoria sp., and Lespesia sp. (Diptera: Tachinidae)].

Overall a higher percentage of caterpillars was parasitized in 2005 (25.44%) than in 2006 (9.83%). Parasitism of heliothine spp. in 2005 was low, while plusiines had high parasitism rates across all treatments (Table 6.6). In 2006, parasitism of heliothine spp. was again low, and that of the 2 species of loopers was higher than that of heliothine spp. (Table 6.6). In 2005 the highest parasitism of caterpillars was observed in 0kg/ha treatment, and the lowest in 45kg/ha treatment. In 2006, the highest parasitism was observed on 135kg/ha treatment, and the lowest in 0kg/ha treatment. All the 42 caterpillars collected in 0kg/ha treatment in 2006, none were parasitized.

3.4 Laboratory reared BAW caterpillar parasitism rate and eggs predation rate

The parasitoids reared from BAW caterpillars were exclusively the braconid endoparasitoid Cotesia marginiventris. BAW caterpillar parasitism rates are summarized in Table 6.7. In 2005 ca. 35-50% of the 1-instar BAW caterpillars were recovered, with no significant difference in recovery rates between treatments. The parasitism rate of recovered caterpillars ranged from 19-44%. It was relatively low in the 45 kg N treatment, but this was not significantly different from

the other treatments. In 2006 both recovery and parasitism rates were lower, compared to 2005. The recovery rate was 6-18% and parasitism rates 7-29%. No significant differences were observed among treatments, although parasitism rate in the no N treatment was less than half of that in the N treatments.

Predation on BAW eggs was rapid in the field (Table 6.8). N treatment significantly affected predation on BAW eggs in all sampling times. More eggs survived in the highest N treatment than in the other treatments on all sampling dates.

3.5 Cotton lint yield

N had no significant effects on cotton lint yield in either season (Table 6.9). In 2005 the highest yield was observed in the no N treatment, which was 300 to 400kg/ha higher than those in other treatments. But the variability was generally high. Overall the yield in 2006 was higher than that in 2005. Higher yields were found in the high N treatments (45, 90, and 135kg/ha) than in the low N treatment (0kg/ha), although the difference was not significant ($P = 0.23$).

4. DISCUSSION

4.1 Effects of N fertilization on cotton plant growth and pest populations

N fertilization in the study generally increased plant N levels and promoted cotton plant growth as shown in other studies (Dudt and Shure, 1994; Stiling and Moon, 2005). These changes can affect pest populations (Fox et al., 1990; Bi et al., 2001, 2003; Davies et al., 2004; Chau et al., 2005; Chau and Heinz, 2006). Many insect pest populations of low and high N treatments in this study were not significantly different from one other. However, one group of

insect pests – the mirids -- was more abundant in high N plots in both years. Aphis gossypii population in 2006 was also generally significantly and positively affected by N fertilization.

4.2. Effects of N fertilization on natural enemies

Parasitism of field-collected caterpillars across treatments was generally consistent in this study. N fertilization also did not have a significant effect on parasitism of laboratory-reared beet armyworm caterpillars that were put out in the field. However, the results were based on the assumption that there was no differential mortality of parasitized and unparasitized S. exigua larvae due to predation or fatal dislodgement across the treatments. As shown in Table 6.6, the recovery rates of beet armyworm caterpillars in both years were low. The majority of caterpillars were predated or lost, which may have affected the observed parasitism rates of recovered caterpillars. Additionally, parasitoids of phytophagous insects were shown to use different sources of cues to find their host/prey. Chemical cues in many cases are the dominant cues exploited during enemy foraging (Cortesero et al., 1997; Röse et al., 1998; Choh et al., 2004). N fertilization in different crops was exhibited to reduce the amounts of these volatile cues (van Wassenhove et al. 1990, Schmelz et al. 2003, but see Lou and Baldwin 2004). Therefore, the observed results may partly due to the poorer volatile cues when parasitoids are foraging in high N fertilization fields.

Predation of sentinel S. exigua eggs in the study was negatively affected by N fertilization. Insect herbivores feeding on host plants receiving low N fertilization typically grow slower and attain less biomass within the same period of time than those feeding on higher quality foods (Lindroth et al., 1995; Chen et al., 2004). Predators are known needing to consume a certain amount of prey mass to complete their development (Blackman, 1967; Loader and Damman,

1991; Williams, 1999). It's also likely that the increased predation is due to stronger herbivore-induced plant volatile cues produced in low N fertilization fields because many predators were shown to exploit these signals when searching for prey (Dicke et al., 1990; Aldrich et al., 2007).

4.2 Economic and environmental effects of N management

Sustainable agriculture requires both economic and environmental sustainability. One way to reduce agricultural production cost is to reduce applications of fertilizer (N). However, low N availability can translate to low crop biomass, which in turn may result in reduced crop quality and yield. Arthropod pests can consume large amounts of plant biomass, and the resultant damage can exceed resource needed for plant reproduction (Mooney, 1972). Low crop biomass and potential yield loss resulting from low N input can be offset if crops of low N suffer lower damage from pests. Lower pest damage on low N plants can arise from (1) pests' preference for high quality plants; (2) higher pest mortality by natural enemies (predators and parasitoids) of pests on low N plants; and/or (3) plant characters such as higher contents of defensive compounds that cause higher pest mortality.

In this study, higher N input did not translate into higher crop yield. The cotton yields in the highest N treatment (135kg/ha) did not differ from those in other treatments during the 2 experimental seasons. The yield had higher variance in the 2005 season, possibly because of the pre-experimental conditions of the field. The results were less variable in 2006, when higher yields were found in the 45kg/ha N treatment, than in the treatment with the highest N, and yields were lowest in the no fertilizer treatment. Therefore, both no fertilizer and too much N (135kg/ha in this case) did little to improve yields. Cotton growers in GA, USA normally apply 90-135kg/ha of N fertilizers in light sandy soil fields in which cotton was the crop grew in the

previous growing season (Hodges et al., 1989). N deficiency affects cotton growth mainly through the following three physiological responses: reduced photosynthetic rate, changed hydraulic conductivity which reduces leaf expansion, and changed response to water deficiency- which are collectively referred to the N stress syndrome (Radin and Mauney, 1986). As a result, lower N is generally associated with lower crop yield (Weir et al., 1996; Jones, 1997). However, according to Shear et al. (1946), plant growth is determined by both nutrition intensity and the balance between nutrients, given other environmental factors. As such, a rise or decrease in a single nutrient may not be reflected in the yield (Joham, 1986). N fertilizers do not persist well in soil (Ojeda et al., 2006; Kyllmar et al., 2006; Udawatta et al., 2006) and ammonia-N can be lost by volatilization. Nitrate forms of fertilizer also leach into the soil readily and can contaminate surface and ground water. With increasing concerns over environmental contamination, environmentally friendly crop production is and will increasingly be critical (Hamrick, 2002).

5. CONCLUSION

N fertilization decisions can affect pest and natural enemy populations, and appropriate fertilizer management may reduce crop production costs and environmental contamination for more sustainable agricultural production. Higher nutrient fertilization in the study did not translate into greater crop yield, and lower fertilizer applications tended to keep pest populations at reduced levels. In addition, as shown elsewhere, lower crop N level may enhance the control of pests by their natural enemies through releasing higher quantities of herbivore-induced volatile organic compounds that can be exploited by natural enemies for foraging. However, the effects of N on agricultural systems are complex. For instance, lower N application, though reducing costs, often leads to lower plant biomass and reduced yield. The ecological and

economic effects of N fertilization, particularly in field conditions, are so far poorly understood in any crop system. This issue may become increasingly important with increasing energy costs and elevated atmospheric carbon dioxide affecting the capacity of crop plants to acquire nitrogen. More field work with precision agronomic control must be undertaken before N manipulation can be utilized as a sustainable agriculture practice.

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Table 6.1 Plant growth characters in response to different N availability (Lang Farm, Tifton, GA 2005)[1]

N level (Kg/ha)	SPAD reading Mean±SEM (units)	Plant height Mean±SEM (m)	Node No. Mean±SEM	Boll No. Mean±SEM
0	41.88 ± 0.59a	0.77 ± 0.0035a	16.1 ± 0.06a	6.1 ± 0.08
45	46.43 ± 0.45b	0.85 ± 0.0040b	16.9 ± 0.05a	6.3 ± 0.08
90	48.56 ± 0.47c	0.95 ± 0.0055c	18.0 ± 0.06b	6.7 ± 0.09
135	51.48 ± 0.39d	0.92 ± 0.0053c	18.2 ± 0.07b	7.4 ± 0.06

Source	DF	F	*P*	DF	F	*P*	DF	F	*P*	DF	F	*P*
Block	2	7.97	0.0004	3	24.52	< 0.001	3	37.39	< 0.0001	3	1.78	0.22
Treatment	3	72.52	< 0.001	3	10.28	< 0.001	3	12.13	< 0.0001	3	1.44	0.30

[1]SPAD readings were determined on August 19; plant height and node and boll numbers were determined on September 16. Means followed by different low case letters within a column indicated significant difference. Data were analyzed with ANOVA. Means were separated with paired *t*-test if overall null hypothesis was rejected ($p < 0.05$).

Table 6.2 Plant growth characters in response to different N availability (Lang Farm, Tifton, GA 2006) [1]

N levels (Kg/ha)	Petiole NO_3-N Mean±SEM (ppm)	Plant height Mean±SEM (m)	Node No. Mean±SEM
0	151.25 ± 51.02	0.96 ± 0.0045a	22.1 ± 0.06a
45	219.80 ± 82.31	1.14 ± 0.0033b	24.8 ± 0.06b
90	427.22 ± 116.39	1.16 ± 0.0052b	24.7 ± 0.06b
135	446.49 ± 137.07	1.15 ± 0.0052b	24.3 ± 0.07b

Source	DF	F	*P*	DF	F	*P*	DF	F	*P*
Block	5	1.01	0.48	5	30.75	< 0.0001	5	10.36	< 0.0001
Treatment	3	0.58	0.15	3	11.92	< 0.0001	3	10.59	< 0.0001

[1]Leaf petioles were sampled on September 20 to determine petiole NO_3-N; Plant height and node data were determined on September 28. Means followed by different low case letters within a column indicated significant difference. Data were analyzed with ANOVA. Means were separated with paired *t*-test if overall null hypothesis was rejected ($p < 0.05$).

Table 6.3 Arthropods recorded in cotton in Lang Farm, Tifton, GA, 2005 and 2006 and total seasonal number

Taxon/Species	**2005**	**2006**	**Taxon/Species**	**2005**	**2006**
Pests			Predators		
Lepidoptera: Noctuidae			Araneae: Oxyopidae[1]	1360	565
Heliothis virescens (F.) and Helicoverpa zea (Boddie)	56	60	Oxyopes salticus		
Spodoptera exigua (Hübner)	9	4	Peucetia viridans		
S. frugiperda (J. E. Smith)	8	4	Araneae: Thomisidae[1]		
S. ornithogalli (Guenée)		18	Misumenops celer		
S. eridania (Stoll)	39		Araneae: Salticidae[1]		
Mocis spp.		52	Phidippus audax		
Pseudoplusia includens (Walker)	70	92	Hemiptera: Geocoridae		
Trichoplusia ni (Hübner)	11	2	Geocoris spp.	225	1246
Lepidoptera: Arctiidae			Hemiptera: Anthocoridae		
Estigmene acrea (Drury)		4	Orius insidiosus (Say)	350	793
Lepidoptera: Pyralidae			Hemiptera: Nabidae		
Achyra rantalis (Guene)	5		Nabis and Nabicula spp.	108	94
Hemiptera: Pentatomidae			Hemiptera: Pentatomidae		
Nezara viridula (L.)	53	30	Podisus maculiventris (Say)	10	20
Acrosternum hilare (Say)		11	Hemiptera: Reduviidae		41
Euschistus servus (Say)	44	34	Zelus spp.	7	
Hemiptera: Miridae			Neuroptera: Chrysopidae		
Pseudatomoscelis seriatus (Reuter)	21	219	Chrysoperla and Chrysopa spp.	104	78
Halticus bractatus (Say)	66	94	Neuroptera: Hemerobiidae		
Lygus lineolaris (Palisot de Beauvois)	24	52	Hemerobius and Micromus spp.	27	69
Neurocolpus nubilus (Say)		19	Diptera Syrphidae		
Hemiptera: Aphididae			Syrphus spp.		8
Aphis gossypii Glover	100	34166	Hymenoptera: Formicidae		

Solenopsis invicta	1154	4004
Coleoptera: Coccinellidae		
Coccinella septempunctata L.	80	610
Coleomegilla maculata (DeGeer)		2
Harmonia axyridis Pallas	112	230
Hippodamia convergens Guérin-Méneville	53	345
Scymnus spp.	455	2075

[1]Total no. of spiders from 3 orders.

Table 6.4 ANOVA results for insect pests in cotton field relative to N treatments (Lang Farm, Tifton, GA, 2005 and 2006)

Year		**Heliothine spp.**[1]		**Plusiine spp.**[2]		***Lygus lineolaris***		**Mirids**[3]		**Pentatomids**[4]		***Aphis gossypii***	
Source	**DF**	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
2005													
Block	4	2.33	0.06	1.23	0.30	1.03	0.40	2.45	0.05	0.93	0.45		
Date	6	9.02	<0.0001	6.52	0.0001	1.86	0.09	10.31	<0.0001	1.49	0.19		
Treatment	3	1.70	0.17	2.06	0.11	0.93	0.43	2.98	0.03	1.17	0.32		
Date*Treatment	18	1.64	0.06	1.00	0.46	0.86	0.62	0.97	0.50	0.69	0.82		
2006													
Block	5	1.78	0.12	2.63	0.03	0.39	0.86	1.58	0.17	0.80	0.55	1.89	0.10
Date	9	10.80	<0.0001	1.47	0.16	8.15	<0.0001	10.60	<0.0001	1.70	0.09	73.53	<0.0001
Treatment	3	0.50	0.68	1.19	0.31	2.04	0.11	0.73	0.54	1.98	0.12	3.09	0.03
Date*Treatment	27	0.73	0.83	0.85	0.68	1.97	0.0045	0.86	0.67	1.32	0.14	1.31	0.15

[1]Heliothis virescens and Helicoverpa zea; [2] Pseudoplusia includens and Trichoplusia ni; [3]Pseudatomoscelis seriatus and Halticus bractatus; [4]Nezara viridula, Acrosternum hilare, and Euschistus servus. Treatments: (1) no fertilizer throughout the growing season; (2) 1 application of 45 kg/ha during the season ; (3) 2 applications during the season; (4) 3 applications during the season. Data were analyzed with repeated measure ANOVA.

Table 6.5 ANOVA results for beneficial arthropods in cotton field in relation to N treatment (Lang Farm, Tifton, GA, 2005 and 2006)

Year		**Ants**		**Spiders**		***Geocoris* spp.**		**Ladybeetles**[1]		***Orius* spp.**		**Lacewings**[2]	
Source	**DF**	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
2005													
Block	4	0.30	0.88	2.13	0.08	4.82	0.0013	0.15	0.96	1.89	0.12	2.40	0.05
Date	6	8.99	<0.0001	9.90	<0.0001	11.21	<0.0001	71.35	<0.0001	15.77	<0.0001	16.14	<0.0001
Treatment	3	0.62	0.61	3.30	0.02	2.30	0.08	1.98	0.12	1.59	0.20	1.65	0.18
Date*Treatment	18	1.17	0.30	1.07	0.39	0.92	0.56	1.87	0.03	0.75	0.75	1.08	0.38
2006													
Block	5	0.36	0.87	3	0.01	1.20	0.31	4.55	0.0006	3.74	0.003	3.43	0.0054
Date	9	13.16	<0.0001	11.22	<0.0001	31.75	<0.0001	88.66	<0.0001	53.97	<0.0001	12.09	<0.0001
Treatment	3	0.90	0.44	1.76	0.16	7.33	0.0001	0.84	0.48	0.16	0.93	2.23	0.09
Date*Treatment	27	0.50	0.98	1.14	0.30	1.30	0.16	0.69	0.87	0.37	1.00	1.86	0.009

[1] Coccinellids: Coccinella septempunctata; Harmonia axyridis; Hippodamia convergens; Scymnus spp.;[2] Chrysopidae: Chrysoperla spp. and Chrysopa spp.; Hemerobiidae: Hemerobius spp. and Micromus spp. Treatments: (1) no fertilizer throughout the growing season; (2) 1 application of 45 kg/ha during the season; (3) 2 applications during the season; (4) 3 applications during the season. Data were analyzed with repeated measures ANOVA.

Table 6.6 Parasitism of caterpillars collected during weekly sampling (Lang Farm, Tifton, GA, 2005 and 2006).

N treatment (Kg/ha)	*Heliothis* spp. (%)	Plusiine spp. (%)	Others (%)	No. of caterpillars collected	Total (%)
2005					
0	0.00	50.00	66.67	22	36.36
45	0.00	42.86	11.11	42	16.67
90	13.64	47.37	33.33	62	30.65
135	0.00	40.00	8.33	43	20.93
Mean					25.44
2006					
0	0.00	0.00	0.00	42	0.00
45	4.55	30.77	12.50	67	13.43
90	0.00	5.56	12.12	69	7.25
135	0.00	27.27	13.04	56	16.07
Mean					9.83

Table 6.7 Parasitism and loss of sentinel BAW larvae (mean±MSE) (%) in cotton in relation to nitrogen treatment

N treatment	**2005**		**2006**	
(Kg/ha)	**Recovery rate**	**Parasitism rate**	**Recovery rate**	**Parasitism rate**
0	36.20 ± 1.68	43.97 ± 2.27	6.61 ± 0.60	7.69 ± 1.90
45	35.16 ± 1.77	19.44 ± 2.59	10.89 ± 1.74	24.76 ± 5.52
90	36.68 ± 3.12	32.74 ± 1.67	17.23 ± 1.29	18.25 ± 2.69
135	49.01 ± 2.66	42.00 ± 2.94	12.18 ± 1.04	28.39 ± 3.24
	N = 51	N = 48	N = 67	N = 38
	$\chi^2 = 2.15$	$\chi^2 = 4.81$	$\chi^2 = 2.71$	$\chi^2 = 2.11$
	DF = 3	DF = 3	DF =3	DF = 3
	$P = 0.54$	$P = 0.19$	$P = 0.45$	$P = 0.55$

Table 6.8 Predation/loss of sentinel BAW eggs (mean±MSE) (%) in cotton in relation to nitrogen application

N treatment	**Sampling time**			
(Kg/ha)	**6 h**	**24 h**	**30 h**	**48 h**
0	37.53 ± 1.64b	83.28 ± 1.43b	90.66 ± 1.10b	92.52 ± 0.94b
45	55.20 ± 1.82b	66.15 ± 1.73b	72.93 ± 1.54a	81.38 ± 1.45ab
90	43.23 ± 1.91b	70.18 ± 1.82b	75.02 ± 1.68a	90.48 ± 1.10b
135	16.71 ± 1.11a	36.90 ± 1.76a	52.00 ± 1.86a	61.64 ± 1.78a
	N = 24	N = 24	N = 24	N = 24
	$\chi^2 = 10.03$	$\chi^2 = 14.00$	$\chi^2 = 12.25$	$\chi^2 = 12.48$
	DF = 3	DF = 3	DF = 3	DF = 3
	$P = 0.0183$	$P = 0.0029$	$P = 0.0066$	$P = 0.0059$

Sampling time: hours after setting up of experiment. Data were analyzed with non-parametric Kruskal-wallis tests.

Table 6.9 Cotton yield in response to N availability (Lang Farm, Tifton, GA)

N treatment	**2005**	**2006**
(Kg/ha)	**Yield (kg/ha)**	**Yield (kg/ha)**
0	2059.19 ± 149.24	2447.09 ± 89.35
45	1645.76 ± 281.06	2776.13 ± 57.47
90	1661.67 ± 442.12	2691.91 ± 41.78
135	1765.02 ± 278.92	2771.74 ± 59.13

ANOVA

Source	**DF**	**F**	**P**	**DF**	**F**	**P**
Block	3	3.03	0.09	5	3.70	0.02
Treatment	3	0.59	0.64	3	1.62	0.23

Data were analyzed with ANOVA.

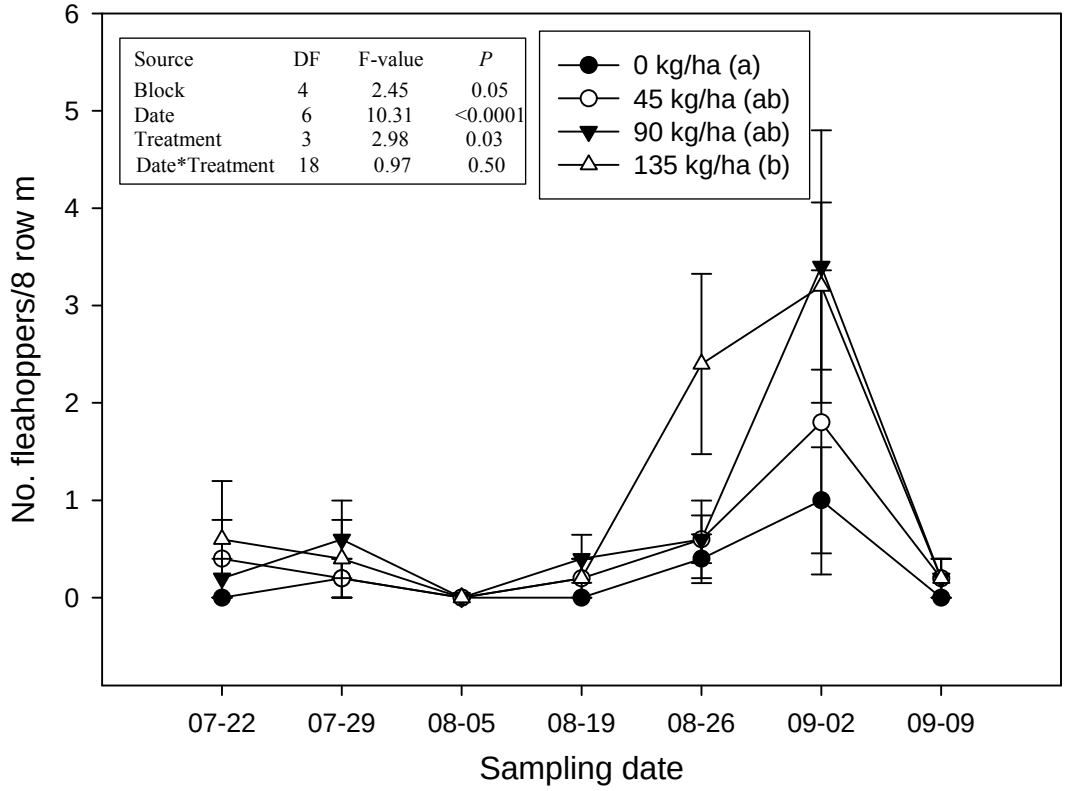

Fig. 6.1 Seasonal dynamics of the mirids P. seriatus and H. bractatus in cotton field in relation to 4 nitrogen treatments in 2005. Data were analyzed with repeated measures ANOVA.

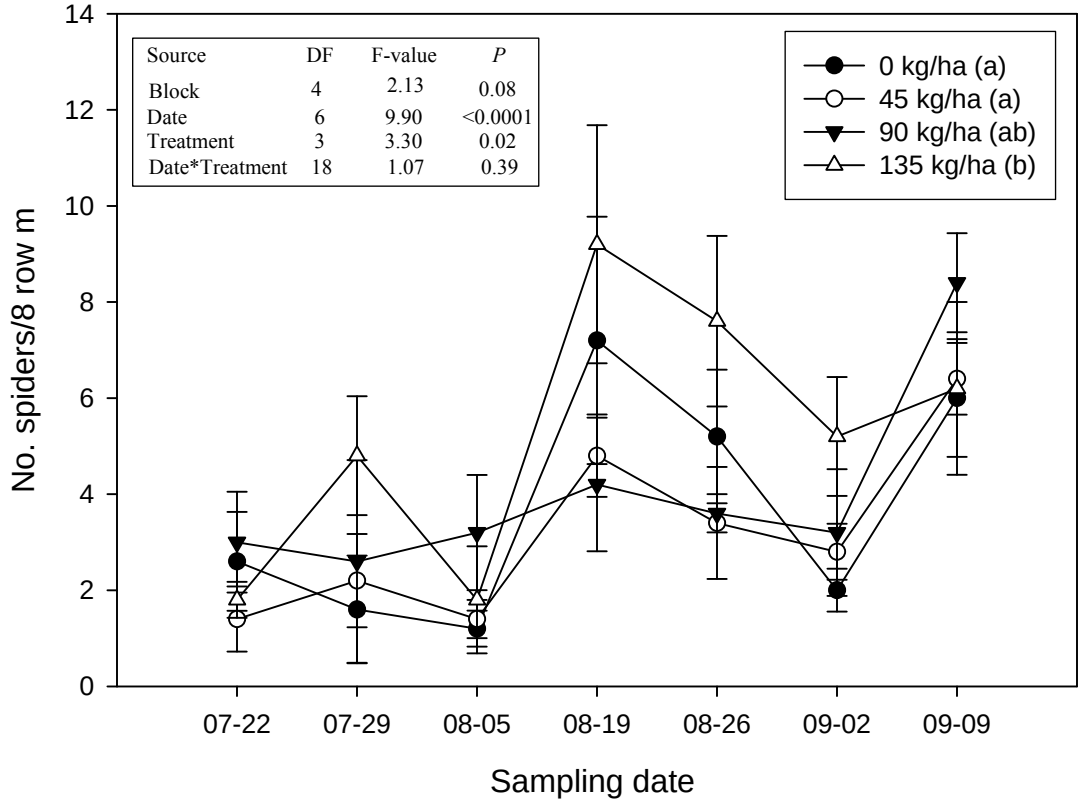

Fig. 6.2 Seasonal dynamics of spiders in cotton field, 2005 in relation to nitrogen treatments. Data were analyzed with repeated measures ANOVA.

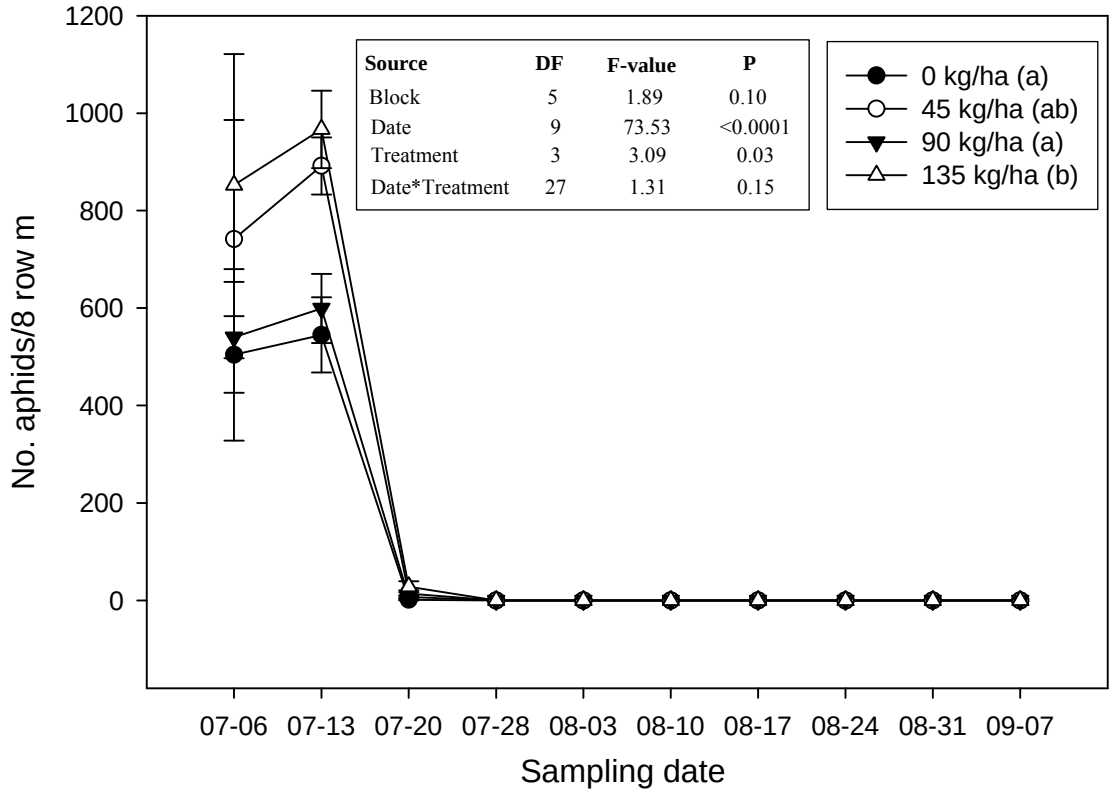

Fig 6.3 Seasonal dynamics of the cotton aphid, Aphis gossypii, in cotton in relation to nitrogen treatment in 2006. Data were analyzed with repeated measures ANOVA.

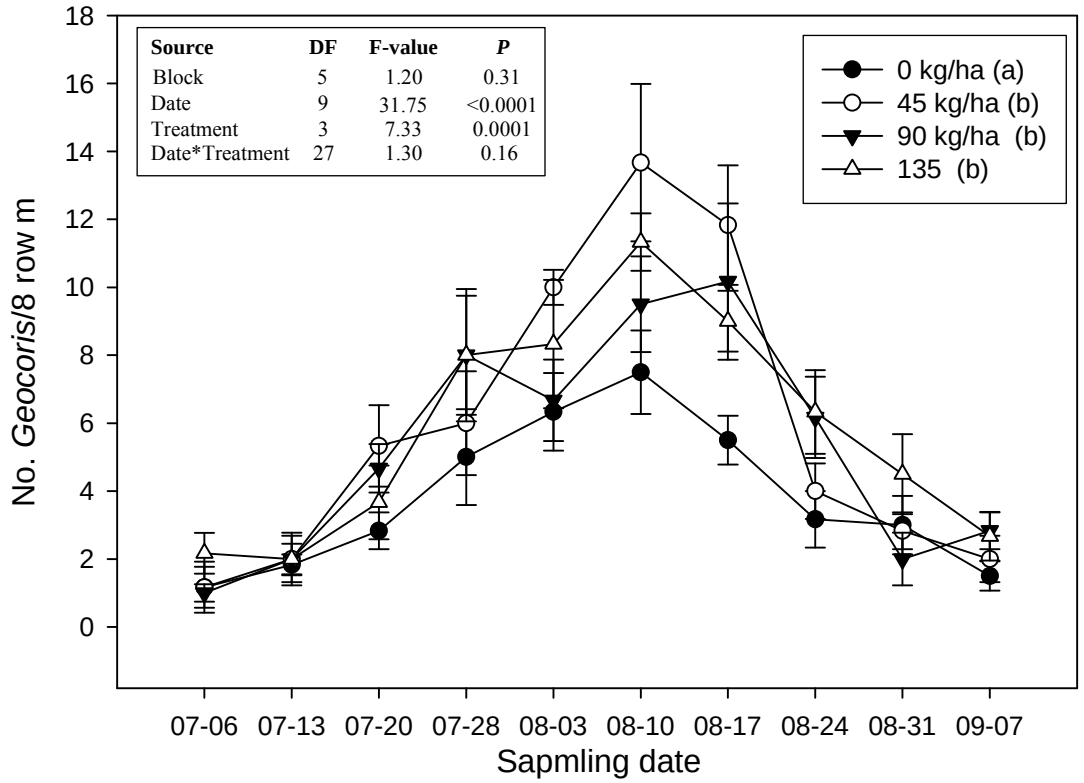

Fig 6.4 Seasonal dynamics of Geocoris spp. in cotton field, 2006 in relation to nitrogen treatment. Data were analyzed with repeated measures ANOVA.

CHAPTER 7

CONCLUSIONS

Nitrogen (N) application is one of the most common agronomic practices in crop production. Many problems, however, come with N fertlizaiton. From environmental perspectives, N application is associated with contamination. Ammonium forms of N evaporiate easily into air, and nitrate forms of N readily leach into soil, which contaminate our above- and under-ground water resources. From economic standpoints, increasing N application means rising production costs. Therefore, efficient utilization of N becomes urgent and promising, in particular, with the advent of sustainable agriculture and roaming energy crisis.

In the study we for the first time comprehensively investigated the effects of N on tritrophic interactions among cotton plants, beet armyworm, and the parasitoid *C. marginiventris*, aiming at finding the possibility of sustainable pest control strategies through N manipulation. Although the study could not directly provide solution of optimal N usage, it indicated that N fertilization could affect both bottom-up and top-down forces of pest management strategie, and cotton plants receiving both lower and higher N ferterlizaion have trade-offs.

Lower fertilization reduced plant biomass and negatively affected parasitoid development, which might ameliorate the efficiency of biological control. However, lower fertilization reduced the nutritional quality of cotton plants as food of herbivores, increased cotton plant direct defense to herbivore, and probably plant indirect defense through employing natural enemies because enhanced emanation of herbivore-induced volatile organic coumpounds. Lower nutritional quality and stronger defense might individually or collectively deter herbivore colonization.

Therefore, the detrimental effects of lower N application on cotton plants might be compensated by less damage due to lower herbivore populations.

On the contrary, higher N fertilization promoted cotton plant biomass production and parasitoid development. But it also increased the nutritional quality of cotton plants as food of herbivores, declined plant direct defense and probably plant indirect defense. Increased nutritional quality and weaker defense resulted in attractiveness of higher N plants for herbivore feeding and oviposition. As such, the positive effects of N addition might be offset by rising herbivore damages due to increasing herbivore populations.

The existence of trade-offs suggested that there could be an optimal N usage range. However, more studies are required before we can find out the range.