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Influence of host plant nitrogen fertilization on haemolymph protein profiles of herbivore *Spodoptera exigua* and development of its endoparasitoid *Cotesia marginiventris*

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HIGHLIGHTS

► *Cotesia marginiventris* is a larval endoparasitoid of *Spodoptera exigua*.

► *Cotesia margiventris* development was prolonged by low nitrogen fertilization of cotton plants.

► Two proteins with molecular weights of ca. 84 and 170 kDa dominated *S. exigua* haemolymph proteins.

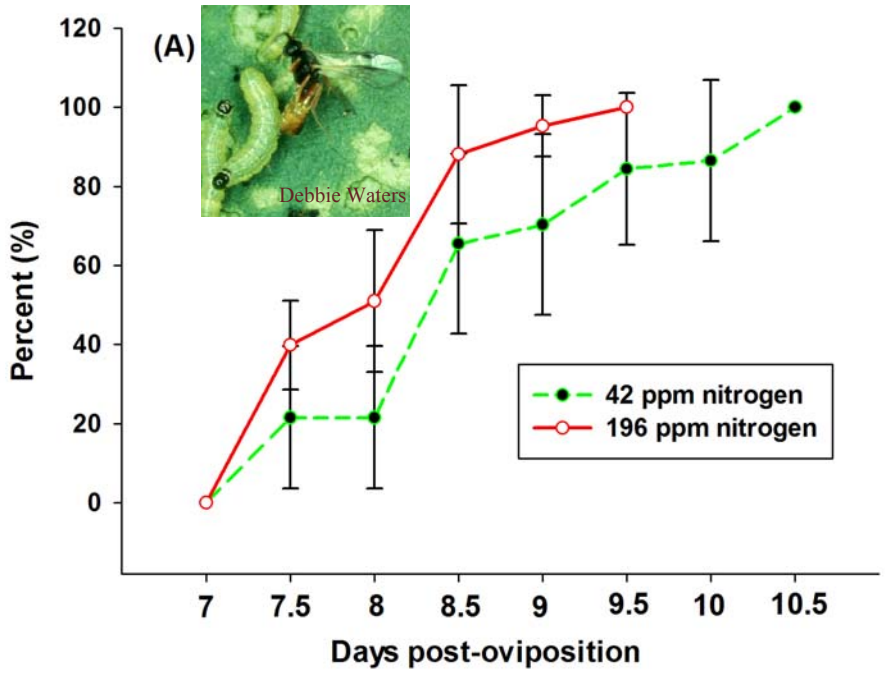
► Parasitism reduced some haemolymph protein concentrations in *Spodoptera exigua*

► Nitrogen treatment and parasitism status interacted to alter concentration of an 84 kDa protein.

► The prolonged development of *C. marginiventris* in hosts provided with nitrogen-poor diets can have profound ecological consequences.

GRAPHICAL ABSTRACT

Nitrogen effects on cumulative percentage (mean ± 95% CI) of *C. marginiventris* forming cocoons due to fertilization levels of cotton leaves in the host's (*Spodoptera exigua*) diet.



ABSTRACT

Nitrogen has complex effects on plant-herbivore-parasitoid tri-trophic 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 varying nitrogen fertilization (42, 112, 196, and 280 ppm) on cotton plants (*Gossypium hirsutum* L.) 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 low host plant nitrogen fertilization through the herbivore *S. exigua*. The percentage of *C. marginiventris* offspring developing to emerge and spin a cocoon, and total mortality of parasitized *S. exigua* larvae were unaffected by nitrogen level. The developmental time of *C. marginiventris* larvae in *S. exigua* larvae feeding on low (42 ppm) nitrogen cotton plants was approximately 30% longer than that of those feeding on high (112, 196, and 280 ppm) nitrogen plants. Parasitoid size (length of right metathoracic tibia), a proxy for fitness, of *C. marginiventris* males was positively affected by nitrogen level. Total amounts of *S. exigua* haemolymph proteins were not affected by nitrogen level, but were reduced by parasitism by *C. marginiventris*. Two proteins with molecular weights of ca. 84 and 170 kDa dominated the *S. exigua* larval haemolymph proteins. Concentrations of the 170 kDa haemolymph protein were unaffected by nitrogen treatment, but parasitism reduced concentrations of the the 170 kDa protein. Concentrations of the 84 kDa protein, on the other hand, were interactively affected by parasitism and nitrogen treatment: higher nitrogen fertilization (112, 196, and 280 ppm) increased protein concentrations relative to the 42 ppm treatment for unparasitized *S. exigua larvae*, whereas nitrogen treatment had no effects on

parasitized larvae. For *S. exigua* larvae feeding on 42 ppm nitrogen plants, parasitism increased concentration of the 84 kDa protein, while for those feeding on 112, 196, and 280 ppm nitrogen plants, parasitism decreased concentrations of the protein. Possible mechanisms and ecological consequences for the extended development of *C. marginiventris* on *S. exigua* hosts grown on low-nitrogen plants are discussed.

Keywords: Biological control, *Gossypium hirsutum*, Tri-trophic interactions

1. Introduction

Nitrogen has profound effects through plants that can extend across trophic levels. In plant-herbivore interactions, low nitrogen availability decreases plant quality as a food resource for herbivores in many cases, which can be further exacerbated by increases in plant defensive compounds (Stout et al., 1998; Chen et al., 2008a,b). Herbivores fed on host plants with limited nitrogen access consequently tend to suffer detrimental effects (Loader and Damman, 1991; Kaneshiro and Johnson, 1996; Glynn et al., 2003). The negative effects can further extend to natural enemies of these herbivores (Campbell and Duffey, 1979; Duffey and Bloem, 1986; Kester and Barbosa, 1991; van Emden, 1995; for a review, see Turlings and Benrey, 1998). For example, predacious stink bugs (*Podisus maculiventris* Say) reared on caterpillars fed on diets incorporating powdered young leaves of *Plantago lanceolata* L. grew faster compared with conspecifics reared on caterpillars of the same species fed on powdered mature leaves (Strohmeyer et al., 1998). The higher growth rate on young-leaf diet was attributed to higher nutrient levels, despite higher concentrations of iridoid glycosides. The antibiotic effect of nicotine absorbed in tobacco hornworm, *Manduca sexta* (L.), haemolymph on survival of the gregarious parasitoid *Cotesia congregata* (Say) provides another example (Morgan, 1910; Gilmore, 1938; Thurston and Fox, 1972). Increased nitrogen fertilization in tobacco, *Nicotiana attenuata* Torr. ex S. Watson, increased nicotine content in the plants (Lou and Baldwin, 2004). *Manduca sexta* is a specialist herbivore of tobacco that can process tobacco's nicotine effectively, mostly through excretion. However, some nicotine is sequestered in the *M. sexta* haemolymph without any negative effect on the herbivores (Self et al., 1964). The parasitic wasp *C. congregata*, however, is more sensitive to nicotine than its host, which reduces parasitoid

53 survival when nicotine levels in the plant are elevated (Parr and Thurston, 1972; Thorpe and
54 Barbosa, 1986; Barbosa et al., 1991).

55 The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is an important
56 crop pest and a generalist herbivore with over 90 known host plant species (Pearson, 1982). Its
57 populations in the southeastern United States are often suppressed by the parasitoid *Cotesia*
58 *marginiventris* (Cresson) (Hymenoptera: Braconidae) and other natural enemies (Ruberson et al.,
59 1994; Mohaghegh et al., 2001; Bianchi et al., 2002). For example, mortality incurred by feral *C.*
60 *marginiventris* in the field can reach up to 45 % for *S. exigua* larvae exposed only 2 d in the field
61 (Chen and Ruberson, 2008). *Cotesia marginiventris* is a koinobiont larval endoparasitoid,
62 although it can also function as a facultative egg-larval parasitoid (Ruberson and Whitfield,
63 1996). *Cotesia marginiventris* undergoes three larval instars before emerging through posterior
64 abdominal segments of the host and spinning a cocoon (Boling and Pitre, 1970). The parasitoid
65 can complete larval development in 6-10 d at 30 ºC, with most emergence of parasitoid larvae
66 from the hosts occurring 7 d after oviposition (Boling and Pitre, 1970).

67 Studies directly linking plant nitrogen effects to parasitoid development, in particular from
68 mechanistic perspectives, are limited. The current study addressed this issue by examining the
69 impact of the nitrogen fertilization of cotton plants, *Gossypium hirsutum* (L.), on *S. exigua* and
70 its endoparasitoid *C. marginiventris*. The objectives of this experiment were: 1) To examine the
71 impact of nitrogen treatments of cotton plants on *C. marginiventris* development, as mediated by
72 its herbivore host *S. exigua*; and 2) to investigate changes in haemolymph protein profiles of *S.*
73 *exigua* larvae fed on cotton plants treated with different levels of nitrogen as indicators of
74 possible mechanism(s) for the developmental differences, if development of *C. marginiventris*
75 development is affected by host plant nitrogen fertilization.

2. Methods

2.1. Plants

Cotton plants (cv. FiberMax 989) were grown using the method described elsewhere (Chen et al., 2008a). Briefly, cotton plant seedlings were fertilized daily with 100 ml of 112 ppm nitrogen nutrient solution for ca. 2 wk, at which time four cotton plants of the same height and with similar-sized leaves at the same leaf positions were assigned to a block. The four plants within each block were each randomly assigned to four nitrogen levels (42, 112, 196, and 280 ppm nitrogen). Cotton plants were fertilized with corresponding nitrogen solutions daily for ca. 2 wk, until the initiation of the experiment. Leaching (watering without nutrients) followed every fourth nitrogen solution application in order to reduce salt (salinity) buildup. All experimental plants were at the 3- to 5-mature-leaf stage when experiments were initiated.

2.2. Insects

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

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

Two trials were conducted. In Trial 1, groups of 50 neonate *S. exigua* larvae (less than 16-h old) were placed in 5-ml diet cups filled with 3ml of modified Pinto bean diet (Burton, 1969) and maintained in an environmental chamber at 25 ± 1ºC and L14:D10 for 2 d before exposure to parasitoids. A 3- to 4-d-old *C. marginiventris* female prepared as described by Chen (2007) was allowed to parasitize 2-d-old larvae. The use of 2- or 3-d-old (in later experiments) *S. exigua* larvae as hosts was because *C. marginiventris* females prefer early instar larvae to oviposit and

host suitability is greatest in young hosts (Beckage et al., 2003). Parasitoid oviposition was visually verified and only one stinging event was allowed per host larva to avoid confounding effects due to parasitoid competition, superparasitism and/or excess physical injury that might cause the death of the hosts. Ten parasitized larvae (one replicate) were then placed in a Petri dish (d = 50 mm, h = 9 mm; Becton Dickinson and Company, Franklin Lakes, NJ, USA) provided with excised leaves of one of the four nitrogen levels. We assumed that each observed stinging event would result in egg deposition and that successful egg deposition rates across nitrogen treatments were the same. Cotton leaves were changed twice daily. The leaves used on each change were from the same nodes of plants receiving four nitrogen levels. *Spodoptera exigua* larvae were examined twice daily (early morning and late afternoon) for emergence of *C. marginiventris* larvae, cocoon formation, and adult emergence from cocoons. The lengths of the right metathoracic tibiae of all *C. marginiventris* emerged were measured with an ocular micrometer, as a direct measure of parasitoid size and an indirect measure of fitness. Because almost all emerged parasitoid adults were males, only the data of male tibia length were evaluated. Each nitrogen level was replicated 8 times. Because leaf chlorophyll content is a good, non-destructive indicator of nitrogen status for cotton (Wood et al., 1992; Chen and Ruberson, 2008), the leaf chlorophyll levels were determined between 1000 and 1200 h with a chlorophyll meter (SPAD-502;Konica Minolta Sensing, Inc., Japan) on the leaf blades of true leaves 1-4 immediately before their use for rearing *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 and their averages were used in statistical analyses. SPAD readings were later converted to leaf chlorophyll equivalents using the formula $Y = 10^{X^{0.265}}$, where Y is leaf chlorophyll content (μmol m^{-2}) and X is SPAD

121 reading (Markwell et al 1995). The experiment was a randomized complete block design with
122 eight blocks and four treatments (i.e., nitrogen levels) in each block.

123 Because the developmental time of *C. marginiventris* was significantly affected by nitrogen
124 level, a dissection study (Trial 2) was conducted to further delineate nitrogen effects on
125 individual developmental stages of the parasitoid. To simplify the experiment, only 42 and 196
126 ppm nitrogen levels were used. Cotton plants and *S. exigua* larvae were prepared as above, and
127 parasitized *S. exigua* larvae were reared in groups of 10 larvae in Petri dishes. *Spodoptera exigua*
128 larvae were first dissected 24h after parasitism, and were thereafter dissected twice (12h apart)
129 daily until pupation (cocoon spinning). The developmental stages of *C. marginiventris* were
130 classified as egg, first, second, and third instars based on Boling and Pitre (1970). At each
131 dissection a Petri dish from each nitrogen level was randomly selected and all 10 *S. exigua* larvae
132 in the dish were dissected. After the appearance of the first cocoons dissection was replaced by
133 cocoon monitoring. Dates, times, and numbers of cocoons were recorded. Percentage of *C.*
134 *marginiventris* offspring in each developmental stage were calculated by dividing the number of
135 *C. marginiventris* at that stage by 10 and then multiplying 100. Only data for cocoon stages are
136 presented here because the data for the second and third instar stages did not have enough
137 replicates due to their short durations and considerable overlap and nitrogen having no effects on
138 the first instar. Developmental time from oviposition to cocoon formation was also computed.
139

140 *2.4. Quantification of total haemolymph proteins of* S. exigua *larvae*

141 Because development of *C. marginiventris* in *S. exigua* larvae feeding on cotton plants with low
142 nitrogen (42 ppm) was significantly prolonged, and because *C. marginiventris* larvae are
143 exclusively haemolymph feeders (Gauld and Bolton, 1988; Wharton, 1993; Strand, 2000), the

144 total host hemolymph proteins were determined to grossly assess possible changes that might
145 affect parasitoid development.

146 The experiment was a 2 (stung and unstung) x 4 (nitrogen levels) factorial design. Neonate
147 *S. exigua* larvae were reared on excised leaves from one of the four nitrogen levels for 48 h.
148 Larvae were then stung by *C. marginiventris* as described in the previous experiment. Stung
149 larvae were thereafter reared on corresponding leaf tissues for six days, when total haemolymph
150 proteins of *S. exigua* larvae were determined with the Pierce® original BCA™ protein assay kit
151 (Rockford, IL), using bovine serum albumin (BSA) as the protein standard. Control (unstung)
152 larvae of the same age cohort as the parasitized larvae also were assessed. To collect *S. exigua*
153 larval haemolymph, a larva was pinned down through both the head and the last segment of
154 abdomen. A proleg on the second or the third segment of abdomen was cut off and 1 µl of
155 haemolymph was collected from individual larvae with a micropipette and diluted into a
156 microcentrifuge tube containing 49 µl of Ringer's solution (Farquharson, 1974). The tube was
157 briefly vortexed to achieve a homogeneous solution. A volume of 25 µl of homogenate was
158 pipetted into one well of a 96-well microplate. A volume of 200 µl of working reagents from the
159 Pierce kit was then added to the well. The whole sample preparation procedure was conducted at
160 a low temperature (on top of ice) environment. Each treatment was replicated 8 times (1
161 individual larva/replicate). The samples in the microplate were shaken on a plate shaker for 30
162 sec and incubated at 37 ºC for 30 min before cooling down to room temperature. The sample was
163 read with a Packard FluoroCount™ fluorescent plate reader (Packard Instrument Company,
164 Meriden, CT) at the wavelength of 562 nm.

165

166 *2.5. Quantification of protein profiles of* S. exigua *larval haemolymph*

To further delineate possible protein differences among *S. exigua* larvae reared on four nitrogen treatments, we determined relative amounts of selected individual haemolymph proteins by staining selected 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 (ca. 1 µl from each larva) 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 40 µl PAGE-prep elution buffer. One microliter of the elution was pipetted into a microcentrifuge tube containing 29 µl of Ringer's solution for total protein assay as in the preceding experiment. The remaining sample (ca. 39 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,

190 and myosin with a molecular weight of 18.3, 28, 39.2, 60, 84, 120, and 215 K, respectively).
191 BIO-RAD mini-protein® II dual vertical slab gel electrophoresis cell (Bio-Rad Laboratories,
192 Hercules, CA) was used. The running buffer was Pierce® Tris-HEPES-SDS buffer containing
193 100 mM Tris, 100 mM HEPES, and 3 mM SDS with a pH of 8 ± 0.25. Pierce® Coomassie
194 brilliant blue G-250 was used to stain the gels to reveal the proteins. The de-stained gels were
195 then digitally recorded with an Olympus camera (CAMEDIA C-5060, Olympus, Japan) and the
196 amounts of the main proteins quantified with BIO-RAD universal Hood II (BIO-RAD
197 Laboratories, Segrate, Italy). The quantities of the proteins were calculated as stained density
198 (intensity·mm^2 μl^{-1} haemolymph).
199
200 *2.6. Statistical analysis*
201 All statistical analyses were conducted in SAS v. 9.2 (SAS Institute, Inc., Cary, NC, U.S.A.). An
202 $\alpha = 0.05$ was used in all hypothesis testing. Data were checked for normality with Kolmogorov-
203 Smirnov's *D* statistic and variance homogeneity with Levene's test before being subjected to
204 further tests. If data, either transformed or untransformed, met model assumptions, they were
205 analysed by ANOVA (PROC GLM in SAS) (SAS Institute 2010). Multiple mean comparisons
206 were conducted with Tukey's tests. Otherwise data were either analysed by non-parametric
207 Kruskal-Wallis tests with multiple mean comparisons following Elliott & Hynan (2011), or by
208 using generalized estimating equations (GEE) with dissection time (n = 7) as repeated
209 measurements (PROC GENMOD in SAS) (SAS Institute Inc. 2010).
210 Leaf chlorophyll data were analyzed by a one-way ANOVA, separately for each leaf
211 position. In Trial 1 of the *C. marginiventris* developmental study, total mortality of *S. exigua*,
212 percentage of *C. marginiventris* reaching the pupal stage in cocoons, and percentage of *C.*

213 *marginiventris* cocoons yielding adults in the first parasitoid developmental study were not
214 normally distributed and were analyzed by Kruskal-Wallis tests. Data on developmental time (in
215 days) from oviposition to cocoon were transformed with the Box-Cox method ($\lambda = -4.4$) and data
216 on developmental time from oviposition to adult were logarithm transformed. Transformed data
217 were then analyzed by one-way ANOVA. Data on male metathoracic tibia length were analyzed
218 by one-way ANOVA. The male right metathoracic tibia length was also regressed against
219 nitrogen levels (PROC REG in SAS).

220 In Trial 2 of the the *C. marginiventris* developmental study, developmental time from
221 oviposition to cocoon formation was analyzed by one-way ANOVA. Cumulative percentage of
222 *C. marginiventris* forming cocoons was analyzed by GEE. The distribution of errors was
223 modeled as multinomial and the percentages were linked to their expected values with a
224 logarithm function.

225 Data on total protein content, and stained densities for 84 and 170 kDa proteins were
226 analyzed by a two-way ANOVA with nitrogen (4 levels: 42, 112, 196, and 280 ppm nitrogen)
227 and parasitism status (2 levels: parasitized and unparasitized) as two factors.

228

229 **3. Results**

230 *3.1. Development of* C. marginiventris *in* S. exigua *hosts*

231 Nitrogen treatment significantly affected cotton plant leaf chlorophyll levels, regardless of leaf
232 position (true leaf 1: $F = 19.90$; $df = 3, 21$; $P < 0.0001$; true leaf 2: $F = 56.01$; $df = 3, 21$; $P <$
233 0.0001; true leaf 3: $F = 115.60$; $df = 3, 21$; $P < 0.0001$; true leaf 4: $F = 63.06$; $df = 3, 21$; $P <$
234 0.0001; Fig. 1). The difference in chlorophyll content of the cotton leaves confirmed the four
235 levels of nitrogen treatment used in this study were adequate for examining the host

plant–herbivore–parasitoid interactions. Nitrogen treatment did not affect percentage of stung hosts in which *C. marginiventris* offspring successfully completed larval development ($\chi^2 = 4.22$; $df = 3$; $P > 0.05$) or total mortality of *S. exigua* larvae ($\chi^2 = 2.33$; $df = 3$; $P > 0.05$; Table 1). The percentage of *C. marginiventris* cocoons yielding adults was affected by nitrogen treatment ($\chi^2 = 10.64$; $df = 3$; $P < 0.05$), but with no obvious relationship to nitrogen level. The percentage of *C. marginiventris* offspring yielded by stung *S. exigua* larvae reared on 112 and 280 ppm nitrogen treatments and developing to adulthood was twice as high as in those reared on the 42 and 196 ppm nitrogen treatments (Table 1). High nitrogen treatment significantly reduced the developmental time of *C. marginiventris* offspring from oviposition to pupation and adult emergence (from oviposition to cocoon formation: $F = 21.46$; $df = 3, 21$; $P < 0.0001$; from oviposition to adult: $F = 4.49$; $df = 3, 21$; $P < 0.05$; Table 1). Male parasitoid size, as indicated by the proxy of right metathoracic tibia length, was not significantly influenced by nitrogen treatment ($F = 2.89$; $df = 3, 12$; $P > 0.05$; Table 1). However, there was a positive and significant correlation between nitrogen treatments and tibia length ($P < 0.01$; Fig. 2).

In the dissection study, high nitrogen level significantly hastened *C. marginiventris* reaching the cocoon stage ($\chi^2 = 5.69$; $df = 1$; $P < 0.05$) (Fig. 3A). The average developmental times of *C. marginiventris* reared in 42 and 196 ppm nitrogen treatments were 8.8 ± 0.27 and 8.1 ± 0.08 d, respectively. The difference was statistically significant ($F = 5.9$; $df = 1, 10$; $P < 0.05$) (Fig. 3B).

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

There was no significant interaction between nitrogen treatment and parasitism status ($F = 0.85$; $df = 3, 56$; $P > 0.05$) (Table 2). Nitrogen treatment did not affect *S. exigua* haemolymph total

259 protein contents (F = 0.56; df = 3, 56; P > 0.05). However, parasitism by *C. marginiventris*
260 decreased *S. exigua* haemolymph total protein concentrations (F = 13.96; df = 1, 56; P < 0.001)
261 (Table 2).
262
263 *3.3. Quantification of protein profiles of* S. exigua *larval haemolymph*
264 Haemolymph of parasitized and unparasitized *S. exigua* contained the same proteins. Two
265 proteins dominated the haemolymph of *S. exigua*, with molecular weights of ca. 170 (Protein 1)
266 and 84 (Protein 2) kDa (Table 3). Nitrogen treatment and the interaction between nitrogen
267 treatment and parasitism status did not affect content of Protein 1 (Nitrogen treatment: F = 0.14;
268 df = 3, 16; P > 0.05; Interaction: F = 0.12; df = 3, 16; P > 0.05).
269 In contrast, nitrogen and parasitism status interacted in determining the density of
270 heamolymph Protein 2 (F = 12.40; df = 3, 16; P < 0.001; Table 3). For unparasitized *S. exigua*
271 larvae, nitrogen treatment influenced the density of Protein 2 (F = 14.21; df = 3, 8; P < 0.01),
272 with larvae in higher nitrogen treatments (112, 196, and 280 ppm) having greater density of
273 Protein 2 than those in the lower nitrogen treatment (42 ppm) (Table 3). For parasitized *S. exigua*
274 larvae, nitrogen treatment did not affect the density of Protein 2 (F = 1.01; df = 3, 8; P > 0.05).
275 Parasitized larvae feeding on 42 ppm nitrogen plants contained more of Protein 2 than
276 unparasitized *S. exigua* larvae fed on 42 ppm nitrogen cotton plants (F = 31.16; df = 1, 4; P <
277 0.01). In contrast, unparasitized *S. exigua* larvae feeding on 112, 196, and 280 ppm nitrogen
278 cotton plants contained higher density of Protein 2 than their parasitized counterparts feeding on
279 plants of the same nitrogen treatments (112ppm: F = 13.87; df = 1, 4; P < 0.05; 196 ppm: F =
280 52.62; df = 1, 4; P < 0.01; 280 ppm: F = 48.71; df = 1, 4; P < 0.01).
281

4. Discussion

Negative effects of host plants on insect herbivores in many cases can be translated to higher trophic levels. For example, the survival, developmental times and larval weights of *Spodoptera littoralis* (Boisduval) larvae feeding on transgenic maize (*Zea mays* L.) expressing endotoxin gene of *Bacillus thuringiensis* Berliner (*Bt*) were significantly lower compared to larvae feeding on normal maize diets, and *C. marginiventris* offspring that developed in *S. littoralis* larvae fed on *Bt* maize exhibited reduced survival rates, 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).

Development of *C. marginiventris* in *S. exigua* larvae fed on low nitrogen (42 ppm) cotton plants was extended in our study relative to the other nitrogen treatments. Presence of certain 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 to interact with carbohydrates (Thompson, 1981). In the current study, the total concentrations of haemolymph proteins or peptides between nitrogen treatments of unparasitized *S. exigua* larvae were not significantly different from each other, excluding total protein concentration as a primary cause for the slowed *C. marginiventris* development. The reduced concentrations of total proteins in parasitized *S. exigua* larvae, however, might suggest a utilization of proteins by *C. marginiventris*. The protein profiles of *S. exigua* larvae feeding on host plants of various nitrogen treatments tended not to differ across treatments, and two proteins

with molecular weights of ca. 84 and 170 kDa were the most abundant proteins in *S. exigua* larval haemolymph. The amounts of the l70 kDa protein in *S. exigua* larvae were not significantly affected by nitrogen treatment, indicating that this protein was likely not a significant factor in slowing *C. marginiventris* development in the low-nitrogen treatment. In contrast, levels of the 84kDa protein were significantly reduced in unparasitized larvae in the 42 ppm nitrogen treatment, demonstrating an effect of food nitrogen on this haemolymph protein. However, there were no significant differences in levels of the 84 kDa protein across nitrogen treatments for parasitized larvae, and in all treatments greater than 42 ppm the amounts of the protein present were reduced by one half to two thirds in parasitized relative to unparasitized larvae. Thus, parasitism contributed to reductions in this protein at higher nitrogen levels, whether by direct effect or indirectly by modifying development of the host. In contrast, levels of the 84 kDa protein in parasitized larvae in the 42 ppm treatment were twofold higher than in unparasitized larvae. These protein differences may be a result of the developmental delays incurred by the host through parasitism, and simply reflect an earlier developmental physiology in parasitized hosts than in the unparasitized hosts. To address this question, comparative study between parasitized and unparasitized *S. exigua* on changes of this protein over time needs to be conducted.

Another explanation for the prolonged development of *C. marginiventris* might be a shift in nutrient ratios. 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 the endoparasitoid *Exeristes roborator* (F.) (Hymenoptera: Ichneumonidae) (Thompson, 1981). Therefore, the imbalance of P:C in host

328 haemolymph due to feeding on host plants with varying nitrogen fertilization and their
329 interactions may be a cause for the protracted development of *C. marginiventris* offspring. The
330 graded response in parasitoid size to changes in nitrogen levels may reflect a shift in nutrient
331 ratios or quality in response to available nitrogen.

332 Possible changes in relative concentrations of allelochemicals, such as gossypol and tannins,
333 in haemolymph of *S. exigua* larvae feeding on cotton plants with low nitrogen fertilization might
334 also delay the development of *C. marginiventris* larvae, because a variety of plant defensive
335 compounds have been reported to be enhanced by nitrogen deficiency in host plants (Stout et al.,
336 1998; Darrow and Bowers, 1999; Chen et al. 2008b), and the developmental time of male *C.*
337 *marginiventris* was observed to be significantly affected by host plant species of *S. exigua* that
338 differed in glucosinolate content, which function as feeding deterrents or toxins against
339 herbivores (Sznajder and Harvey, 2003). The development of *Diadegma terebrans* (Grav.)
340 (Hymenoptera: Ichneumonidae), an endoparasitoid of *Ostrinia nubilalis* (Hübner) (Lepidoptera:
341 Pyralidae) was slowed when developing on hosts fed diet containing the allelochemical 2,4-
342 dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a hydroxamic acid which confers
343 resistance of crops, such as maize, to herbivores (Campos et al., 1990). *Cotesia marginiventris* is
344 a generalist endoparasitoid, and is more susceptible to plant chemistry than some specialist
345 endoparasitoids (Barbosa et al., 1991; Sznajder and Harvey, 2003; Harvey et al., 2005). Plant
346 defensive chemicals sequestered in host haemolymph may not only directly and detrimentally
347 affect parasitoid performance, but may also interact with specific nutrients and make them
348 unavailable to parasitoid larvae. For example, tomatine can directly cause cytolysis and can
349 intervene with many β-sterols in host haemolymph and impede the utilization of these critical
350 nutrients by larvae of *Hyposoter exiguae* (Viereck) (Hymenoptera: Ichneumonidae) (Campbell

and Duffey, 1979). Thus, host chemistry may interact with plant chemistry in complex ways to influence parasitoid development.

A critical life history trade-off faced by parasitoids is to grow larger at the expense of longer developmental time or to grow faster at the expense of smaller adult size (Strand, 2000). The relative values of these trade-off options can be affected by the feeding ecology of the host insects – for example, 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 exophytic leaf feeders and are exposed hosts for *C. marginiventris*. The longer developmental time of *C. marginiventris* offspring developing in *S. exigua* larvae fed on 42 ppm nitrogen plants and smaller resulting male size subverts the tradeoff, affecting both development and fitness. Prolonged developmental time increases exposure time of the parasitoid to predation and parasitism, because immature parasitoids generally suffer the same mortality as their hosts (Hawkins, 1994), and protracted exposure time of hosts and exposed parasitoid cocoons may lead to higher mortality due to abiotic and biotic factors as the slow growth-high mortality hypothesis predicts (Clancy and Price, 1987). Thus, reductions in nitrogen availability to plants, whether through reduced fertilization or increased atmospheric carbon dioxide decreasing the capacity of plants to acquire nitrogen (Cotrufo et al., 1998), may have important consequences for survival of parasitoids, particularly those of exophytic hosts, and on their subsequent movement and reproduction.

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Table 1 Development (at 25 ± 1°C, L:D 14:10; mean ± SE) of *C. marginiventris* on *S. exigua* larvae reared on cotton plants receiving various levels of nitrogen.

Nitrogen 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	36.8 ± 4.70	79.9 ± 5.94	30.6 ± 14.03	11.6 ± 0.53b	17.3 ± 0.14b	0.72 ± 0.02
112	52.5 ± 7.01	91.3 ± 3.50	74.5 ± 12.66	8.5 ± 0.21a	14.6 ± 0.26a	0.75 ± 0.02
196	34.6 ± 4.52	85.2 ± 4.76	33.1 ± 12.68	8.1 ± 0.17a	14.0 ± 0.22a	0.77 ± 0.02
280	41.3 ± 7.43	83.8 ± 5.65	61.8 ± 11.91	8.3 ± 0.19a	15.1 ± 0.56a	0.80 ± 0.01

Means within a column followed by different low-case letters denote significant difference at $\alpha < 0.05$; n = 8.

Table 2 Haemolymph total proteins (mean ± SE μg μl^{-1}) of unparasitized *S. exigua* larvae and larvae parasitized by the parasitoid *C. marginiventris* and reared on cotton plants receiving various nitrogen levels; n = 8.

Nitrogen levels (ppm)	Unparasitized *S. exiuga* larvae	Parasitized *S. exigua* larvae
42	19.7 ± 3.18	13.5 ± 1.00
112	17.5 ± 0.90	15.2 ± 0.99
196	19.5 ± 1.61	13.8 ± 0.75
280	19.4 ± 1.34	16.9 ± 1.44

Table 3 Stained densities (mean ± SE) (intensity·mm^2 µl^{-1}) of two abundant haemolymph proteins of *S. exigua* larvae reared on cotton plants receiving various nitrogen levels. Larvae were either parasitized by *C. marginiventris* (stung) or were unexposed to parasitoids (unstung).

Nitrogen level (ppm)	Unparasitized		Parasitized	
	Protein 1 (170 kDa)	Protein 2 (84 kDa)	Protein 1 (170 kDa)	Protein 2 (84 kDa)
42	883.4 ± 254.68	267.4 ± 40.20b**	571.1 ± 104.53	571.9 ±36.86
112	942.7 ± 262.42	1977.4 ±361.54a*	484.7 ± 88.40	624.3 ± 36.43
196	886.1 ± 288.01	1318.1 ± 104.40a**	434.7 ± 63.26	541.3 ± 23.81
280	922.6 ± 267.24	1738.2 ± 133.00a**	652.3 ± 75.90	651.2 ± 81.05

Means within a column followed by different lower-case letters denote significant differences at $\alpha < 0.05$. * and ** represent significant difference of protein quantity between haemolymph Protein 2 of unparasitized and parasitized *S. exigua* larvae reared on the same nitrogen levels at $\alpha < 0.05$ and 0.01, respectively; n = 6.

Figure caption

Fig. 1 Leaf chlorophyll levels (mean + 1 SE μmol m^{-2}) of cotton plants receiving various nitrogen levels. Low-case letters above the bars denote significant difference among treatments within the same leaf position at $P < 0.05$; n = 8.

Fig. 2 Linear regression of nitrogen level against male right metathoracic tibia length; n = 74 *C. marginiventris*. Some data points overlap so that fewer than 74 points were shown.

Fig. 3 Nitrogen effects on cumulative percentage (mean ± 95% CI) of *C. marginiventris* forming cocoons (A) and developmental time (mean ± SE) of *C. marginiventris* from oviposition to cocoon (B). Four hundred 3-d old *S. exigua* larvae were allowed to be parasitized by the parasitoid on 19 November, 2007. *S. exigua* larvae were randomly and equally assigned to 42 and 196 ppm nitrogen levels. Two hundred larvae in each nitrogen level were randomly grouped into 10. A group from each nitrogen level was randomly selected for dissection, twice daily. The percentage of *C. marginiventris* forming cocoon in (A) was the number of cocoon divided by 10 and then multiplied by 100. The development time in (B) was the average of observed *C. marginiventris* cocooned. n = 6.

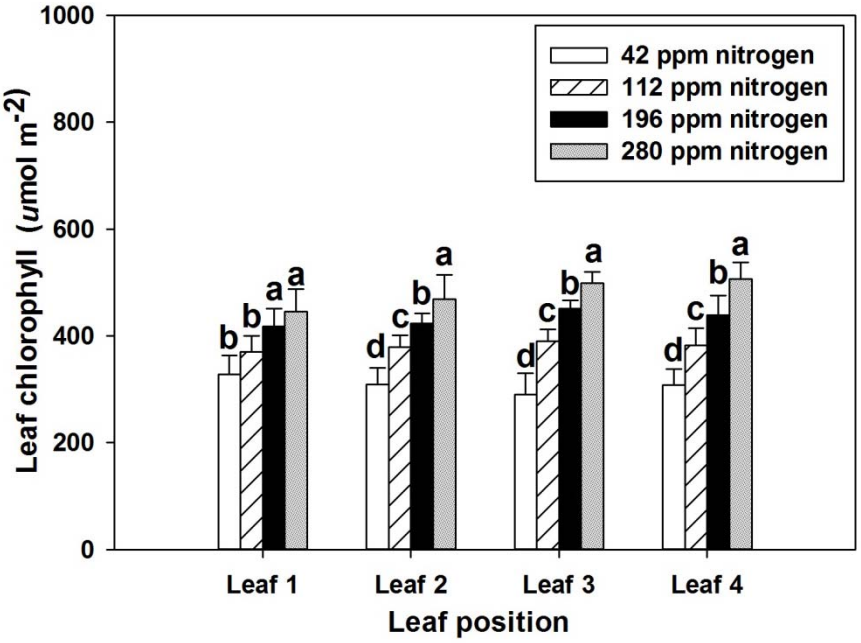
1000
800
600
400
200
0
Leaf chlorophyll (umol m-2)
42 ppm nitrogen
112 ppm nitrogen
196 ppm nitrogen
280 ppm nitrogen
b
b
a
a
d
c
b
a
d
c
b
a
d
c
b
a
Leaf 1
Leaf 2
Leaf 3
Leaf 4
Leaf position

Fig. 1

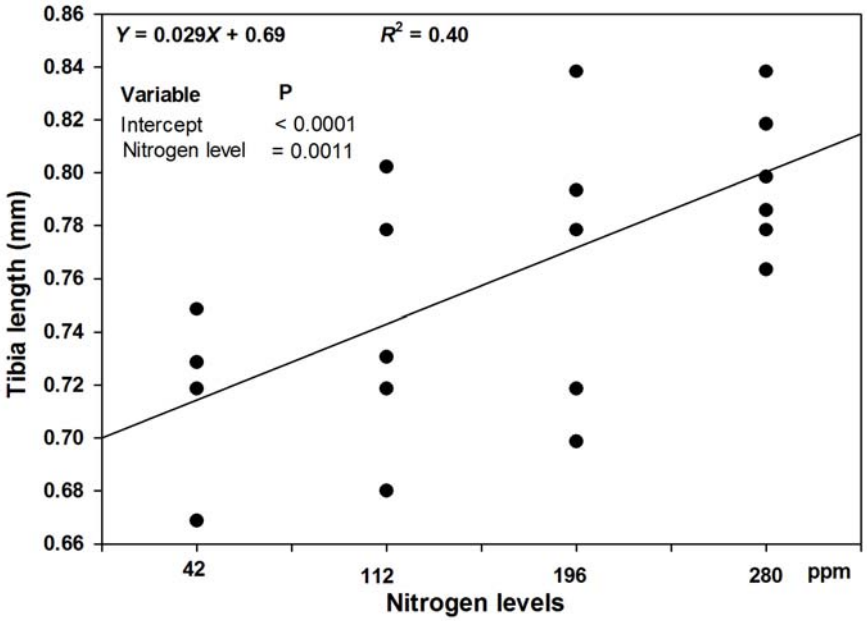
Y = 0.029X + 0.69
R2 = 0.40
Variable
P
Intercept
< 0.0001
Nitrogen level
= 0.0011
Tibia length (mm)
0.86
0.84
0.82
0.80
0.78
0.76
0.74
0.72
0.70
0.68
0.66
42
112
196
280
ppm
Nitrogen levels

Fig. 2

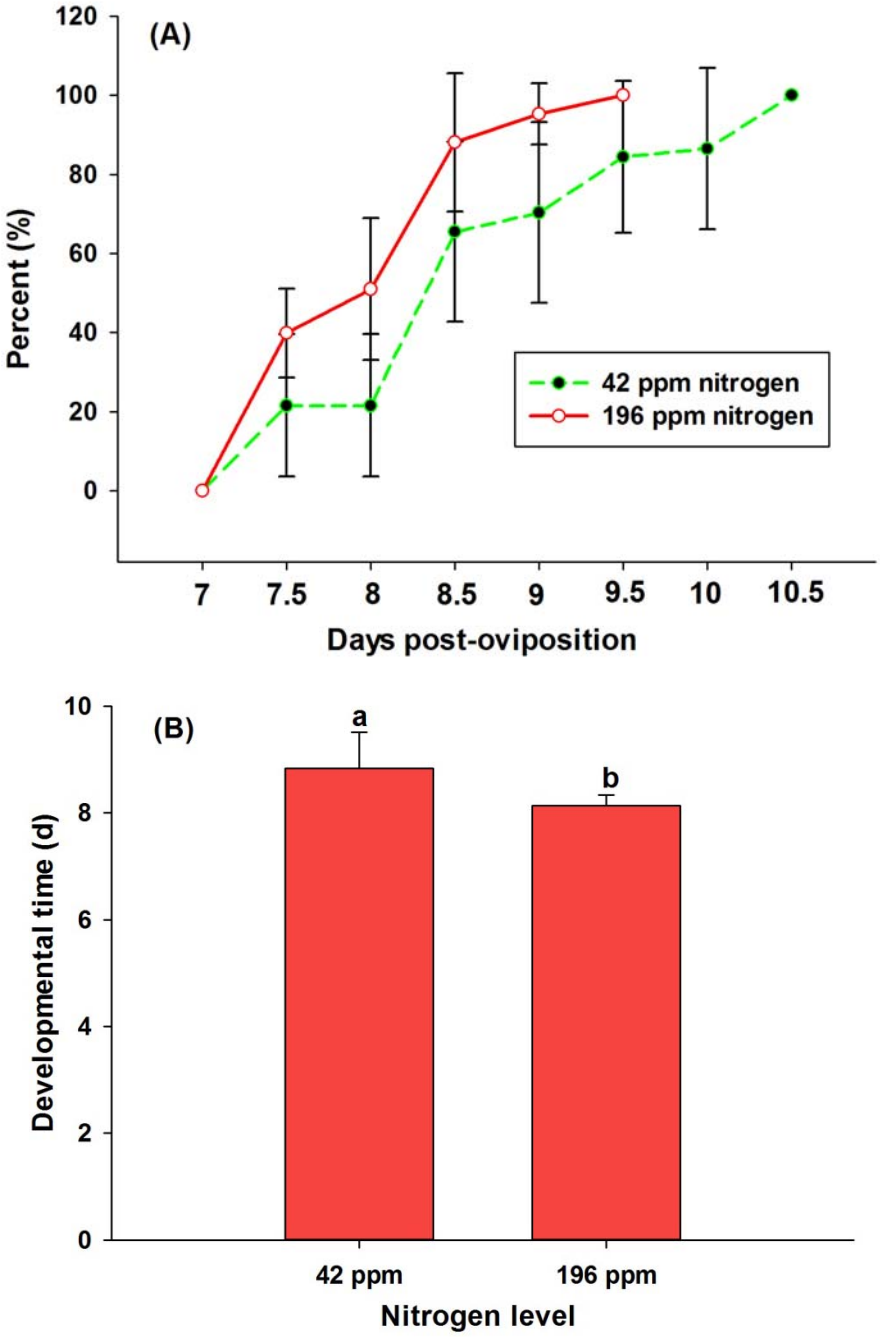
(A)
Percent (%)
120
100
80
60
40
20
0
42 ppm nitrogen
196 ppm nitrogen
7
7.5
8
8.5
9
9.5
10
10.5
Days post-oviposition
(B)
Developmental time (d)
10
8
6
4
2
0
a
b
42 ppm
196 ppm
Nitrogen level

Fig. 3