Monochamus alternatus (Coleoptera: Cerambycidae) Hemolymph Saccharide Content and Carbohydrase Activity in Response to Parasitism by *Dastarcus helophoroides* (Coleoptera: Bothrideridae)[1]

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J. Entomol. Sci. 53(1): 1–10 (January 2018)

Abstract *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) is the main borer of several pine species (e.g. *Pinus massoniana* Lamb) and is also the primary vector of the pest pine wood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle (Aphelenchida: Parasitaphelenchidae). *Dastarcus helophoroides* (Fairmaire) (Coleoptera: Bothrideridae) is a natural enemy and an ectoparasitoid of *M. alternatus*. We studied the effects of parasitism by *D. helophoroides* on the hemolymph saccharide content in parasitized *M. alternatus* larvae. Our results showed that hemolymph saccharide content was altered by parasitism. For example, in comparison to unparasitized insects, the total sugar content increased in larvae 12 h after parasitism, the reducing sugar content declined at 24 h after parasitism but increased at 72 h after parasitism, and the trehalose content was inhibited at 6 h and 24 h after parasitism. These results indicate that *D. helophoroides* larvae may regulate saccharide metabolism in *M. alternatus* larvae, resulting in the changes of saccharide content in host hemolymph.

Key Words *Dastarcus helophoroides*, *Monochamus alternatus*, saccharide content, carbohydrase activity

Parasitoids have been shown to alter the physiology and metabolism of host species (Bischof and Ortel 1996, Dahlman 1975, Dahlman and Vinson 1975, 1976, 1980, Ji et al. 2013, Kaeslina et al. 2005, Lü et al. 2000, Thompson and Binder 1984, Thompson and Yamada 1984, Tan et al. 2003, Wang et al. 2015). Compared to unparasitized controls, total sugar and trehalose content in the hemolymph of overwintering *Pieris rapae* L. pupae parasitized by *Pteromalus puparum* L. were elevated from 12 h to 48 h after parasitism and declined thereafter (Lü et al. 2000). Total sugar content in the hemolymph of *Spodoptera exigua* Hübner parasitized by *Microplitis pallidipes* Szepligeti began to increase at 24 h after parasitization until it was significantly greater than controls at 48 h and 120 h (Ji et al. 2013). Glycogen concentration in the total body tissue of *Lymantria dispar* L. parasitized by *Glyptapanteles liparidis* Bouche was significantly elevated and exceeded the concentration of the nonparasitized larvae by a factor of approximately 3, and the levels of trehalose, glucose, and sorbitol were significantly reduced in parasitized

[1]Received 23 December 2016; accepted for publication 01 May 2017.
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individuals (Bischof and Ortel 1996). Hemolymph trehalose levels from *Heliothis virescens* F. larvae parasitized by *Microplitis croceipes* Cresson and *Campoletis sonorensis* Cameron were greater than the controls (Dahlman and Vinson 1975, 1976). Glycogen and trehalose content in *Tenebrio molitor* L. parasitized by *Scleroderma sichuanensis* Xiao decreased sharply on the 3rd to 6th day after parasitism, reaching a minimum on the 10th day, while reducing sugar content increased, reaching its maximum concentration on the 10th day (Tan et al. 2003). In *Bemisia tabaci* Gennadius parasitized by *Encarsia sophia* Girault & Dodd, trehalose content peaked at 24 h after parasitism and then decreased (Wang et al. 2015).

The activity of α-amylase and trehalase in *T. molitor* increased rapidly on the 1st to 3rd day after parasitism by *S. sichuanensis*, reaching maximum levels on the 3rd day, and then declining slowly to the level observed on the 1st day after parasitism (Tan et al. 2003). Parasitism by *P. puparum* caused an increase of trehalase activity in overwintering pupae of *P. rapae*, reaching its highest level on the 5th day after parasitism, while trehalase activity in the hemolymph was not observed in unparasitized overwintering pupae (Lü et al. 2000). In *B. tabaci* parasitized by *E. sophia*, the trehalase activity was low until 24 h after parasitism and increased thereafter (Wang et al. 2015).

Pine sawyer beetle, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), is the main stem borer pest of several pine species (e.g. Pinus massoniana Lamb) and causes serious damage to its host. It is also the primary vector of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle (Aphelenchida: Parasitaphelenchidae) (Mamiya and Enda 1972). *Monochamus alternatus* exhibits one generation per year and the larvae bore into dead or dying pine trees, where they develop through five instar stages, overwintering as larvae in the galleries in xylem. Adults begin to emerge in mid-April the following year (Xiao 1992).

Dastarcus helophoroides (Fairmaire) (Coleoptera: Bothrideridae) is an important natural enemy and an ectoparasitoid of *M. alternatus* (Inoue 1993, Taketsune 1982, Yang et al. 2014). It exhibits one to two generations annually, and overwinters as adults (Qin and Gao 1988). Larvae have six instars and feed on the host for 5–7 d before pupating (Wang and Ogura 1999). Neonates of *D. helophoroides* use their six legs to move towards the habitat and search for hosts by gustatory and olfactory sensilla (Wei et al. 2009). Larvae are able to parasitize *M. alternatus* larvae, pupae, and young adults (Yang et al. 2014). Their legs degenerate after parasitizing the host (Wei et al. 2009). Based on studies of its biology (Qin and Gao 1988) and development of artificial diets (Lei et al. 2005, Ogura et al. 1999, Wang and Ogura 1999), *D. helophoroides* is now mass-produced in laboratory.

The mechanism of parasitism of this parasitoid of its host is little known. Therefore, our objective herein was to identify the effects of parasitism by *D. helophoroides* on the physiology and metabolism of *M. alternatus* larvae by characterizing the hemolymph saccharide content and carbohydrase activity in response to parasitism. Our study of saccharide content included hemolymph total sugars, reducing sugars, glycogen, trehalose, and glucose. Carbohydrase activity included α-amylase and trehalase.

Materials and Methods

Host insects. *Monochamus alternatus* larvae were collected in the Huanglishu Forest Farm (N 32°11′, E 118°06′) of Quanjiao County, Anhui Province, China, during March 2015. They were removed from the xylem of infested *Pinus massoniana* Lamb, and placed individually into small sterile tubes (1.2 cm in height, 5.0 cm in diameter). Larvae were transported to the Anhui Provincial Academy of Forestry and maintained at 8 ± 0.5°C and 50 ± 5% relative humidity (RH) until used in the assays. We chose 5th instars, according to the head capsule measurements described by Liu et al. (2008), for the assays in order to segregate age effect from parasitism effect on saccharide content and carbohydrase activity. Timing of collection of the larvae coincided with the recommended release of *D. helophoroides* adults during late March for improved parasitism efficiency, when most *M. alternatus* larvae are 5th instars at that time (Li et al. 2013).

Parasitoid rearing. *Dastarcus helophoroides* were supplied by the Institute of Forest Protection, Anhui Provincial Academy of Forestry, Hefei, China, and were reared on *M. alternatus* larvae and adults, as well as an artificial diet. We used the fifth generation of the parasitoid reared in laboratory for the trials. They were the laboratory-reared descendent of the female *D. helophoroides* given by the Research Institute of Forest Ecology, Environment and Protection, Chinese Academy of Forestry, Beijing, China. The predecessors were gathered in the tunnels of *M. alternatus* in *Pinus massoniana*, while they were parasitizing larvae or pupae of their hosts.

Parasitism. A small brush was used to inoculate newly hatched *D. helophoroides* larvae to *M. alternatus* larvae, with 6 parasitoids per host. The parasitized *M. alternatus* larvae were maintained at 25 ± 0.5°C and 50 ± 5% RH for 6, 12, 24, 48, or 72 h. The parasitized larvae were placed individually in a sterile tube (1.2 cm in height, 5.0 cm in diameter) that had a small hole (1.5 mm in diameter) drilled in its cover. Unparasitized *M. alternatus* larvae were handled in the same manner and served as controls. There were three replicates (insects) for each assay with seven hemolymph components (i.e., total sugar, glycogen, reducing sugar, glucose, trehalose, α-amylase activity, trehalase activity) over five time periods after parasitism (6, 12, 24, 48, and 72 h), as well as three replicates of untreated controls, resulting in a total of 210 *M. alternatus* larvae used in the assays.

Assays. Prior to hemolymph extraction, larvae were surface-sterilized with 75% ethanol, rinsed with sterile water, and air-dried. One hundred microliters hemolymph was obtained by cutting their posteriors with sterile scissors and placed in a 1.5-ml sterile centrifuge tube with a few phenylthiourea crystals, and then centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant was used for saccharide content assays.

Total sugar content was assayed by methods of Roe (1955) in which 5 μl hemolymph and 100 μl anthrone reagents (0.2%) were mixed and held in boiling water for 15 min, and then serially diluted 10 times. Total sugar content was monitored by measuring absorbance at 620 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA). One unit of total sugar content was defined as $\Delta A_{620} = 1$.

Glycogen content was measured using methods adopted from Roe (1955) and Lorenz (2003) in which 5 μl hemolymph was combined with 15 μl anhydrous ethyl alcohol and 5 μl saturated solution of Na_2SO_4 and stored at 4°C for 1 d, and then centrifuged at 4,000 rpm for 15 min at 4°C. The supernatant was discarded and the precipitate was incubated at 70°C for 10 min after which 5 μl ultrapure water and 100 μl anthrone reagent were added to the precipitate. That mixture was held in boiling water for 15 min and serially diluted 10 times. The absorbance at 620 nm was measured. One unit of glycogen content was defined as $\Delta A_{620} = 1$.

A 3,5-dinitrosalicylic acid (DNS) method was used to measure reducing sugar content as per Lü et al. (2000) in which 10 μl hemolymph and 100 μl DNS reagents (0.04 M DNS, 0.4 M NaOH, 1.06 M $C_4O_6H_4KNa$) were mixed and held in boiling water for 5 min, and then cooled to room temperature. The absorbance at 540 nm was measured, with 1 unit of reducing sugar content defined as $\Delta A_{540} = 1$.

A glucose test kit (glucose oxidase) (Changchun Hui Li Biological Technology Co. Ltd., Jilin, China) was used to measure glucose content. Ten microliters hemolymph and 100 μl glucose test reagents were mixed and incubated at 37°C for 15 min. The absorbance at 510 nm was measured, with 1 unit of glucose content defined as $\Delta A_{510} = 1$.

Trehalose content was measured by the method of Tan et al. (2003) with slight modifications. Ten microliters hemolymph and 20 μl 0.075 M H_2SO_4 were mixed and held in boiling water for 10 min. After cooling, 20 μl 30% KOH was added to the mixture, which was then held in boiling water for 20 min after which 50 μl of the solution was removed and mixed with 100 μl anthrone reagents and held in boiling water for 15 min, and then cooled to room temperature. The absorbance at 620 nm was measured, with 1 unit of trehalose content defined as $\Delta A_{620} = 1$.

Activity of α-amylase was measured using the methods of Tan et al. (2003) in which 50 μl hemolymph and 250 μl 1/15 M phosphate buffered solution (PBS) (pH 6.9) were mixed and centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant was collected as α-amylase extract, and 50 μl of the enzyme extract was combined with 50 μl 10% amylum and 200 μl PBS were mixed and incubated at 35°C for 30 min. The amylum was replaced by 50 μl H_2O in the controls. One hundred microliters of this reaction solution and 100 μl DNS were mixed and held in boiling water for 5 min, and then cooled to room temperature. The absorbance at 540 nm was obtained, and 1 unit of α-amylase activity was defined as $\Delta A_{540} = 1$.

Trehalase activity was determined by methods of Tan et al. (2003) where 50 μl hemolymph and 250 μl 1/15 M PBS (pH 5.4) were mixed and centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant was collected as trehalase extract, and 50 μl of that enzyme extract was mixed with 50 μl trehalose (1.33×10^{-3}) and 200 μl PBS and incubated at 35°C for 30 min. The trehalose was replaced by 50 μl H_2O in the controls. One hundred microliters of this reaction solution was mixed with 100 μl DNS and held in boiling water for 5 min and then cooled to room temperature. Absorbance was measured at 540 nm.

Statistical analyses. Differences in total sugar content, glycogen content, reducing sugar content, glucose content, trehalose content, α-amylase activity, and trehalase activity among the five time periods were examined using analysis of variance (ANOVA) followed by Tukey's honestly significant diference (HSD) for comparisons among significantly different treatment means (Li et al. 2015). Content and activity also were compared between parasitized and unparasitized larvae at

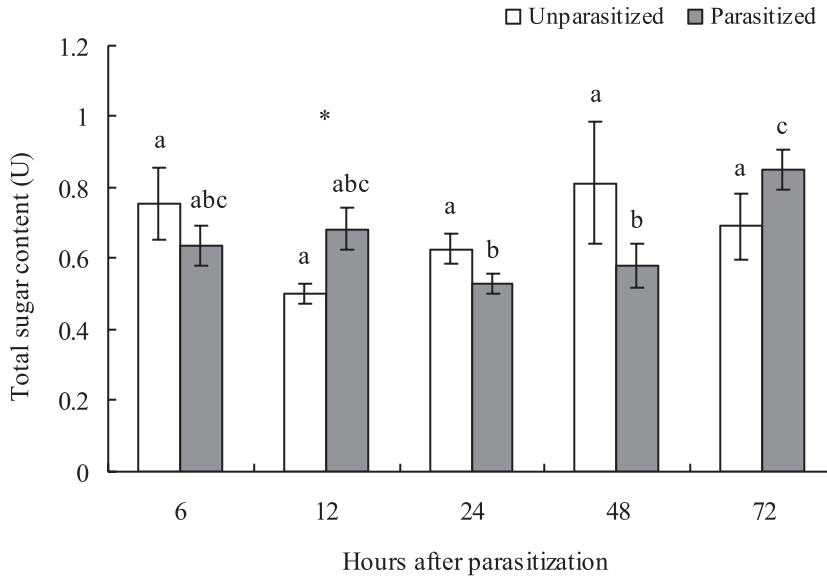

Fig. 1. Total sugar content in hemolymph of *M. alternatus* larvae parasitized or unparasitized by *D. helophoroides.* (The error bars indicate SE. The different letters labeled above the bars in same color indicate significant differences between time points at the $P = 0.05$ level by ANOVA, while the same letters indicate no difference. An asterisk (*) indicates significant differences between parasitized and unparasitized samples at $P < 0.05$ by pairwise *t* test. $n = 3$ replicates for each time point).

each time interval for each hemolymph component using the pairwise *t* test (Li et al. 2015). SPSS 19.0 software was used to analyze all data (Li et al. 2015).

Results

Total sugar content in the hemolymph of unparasitized *M. alternatus* larvae did not change during the 72-h period ($F = 1.436$, df = 14, $P = 0.292$, ANOVA), while in parasitized larvae, the total sugar content measured at 72 h after parasitism was significantly higher than the levels observed at 24 h and 48 h after parasitism ($F = 5.057$, df = 14, $P = 0.017$, ANOVA) (Fig. 1). Total sugar content of hemolymph from parasitized larvae was significantly higher ($T = -4.449$, df = 2, $P = 0.047$) than that of unparasitized larvae at 12 h after parasitism; however, no significant differences were detected between parasitized and unparasitized larvae at other time intervals (Fig. 1).

Reducing sugar content in parasitized larvae was significantly lower at 6 h, 12 h, and 24 h after parasitism than at 48 h and 72 h ($F = 20.171$, df = 14, $P < 0.001$, ANOVA); whereas, reducing sugar content did not change significantly over time in unparasitized larvae ($F = 1.262$, df = 14, $P = 0.347$, ANOVA) (Fig. 2). In comparison

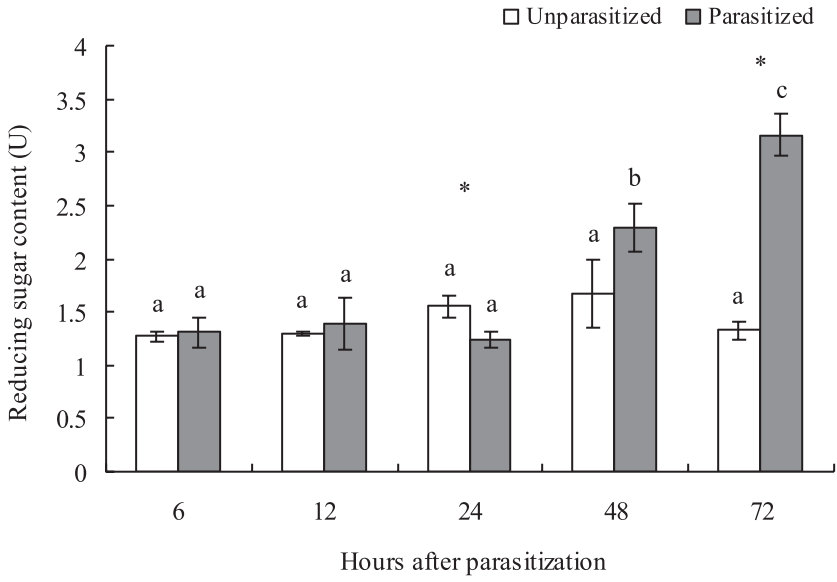

Fig. 2. Reducing sugar content in hemolymph of *M. alternatus* larvae parasitized or unparasitized by *D. helophoroides.* (The error bars indicate SE. The different letters labeled above the bars in same color indicate significant differences between time points at the $P = 0.05$ level by ANOVA, while the same letters indicate no difference. An asterisk (*) indicates significant differences between parasitized and unparasitized samples at $P < 0.05$ by pairwise *t* test. $n = 3$ replicates for each time point).

to unparasitized controls, reducing sugar content in parasitized larvae was significantly inhibited at 24 h after parasitism ($T = -4.449$, df $= 2$, $P = 0.047$) but was significantly elevated at 72 h after parasitism ($T = -6.992$, df $= 2$, $P = 0.020$) (Fig. 2).

Trehalose content was significantly higher in the hemolymph of parasitized larvae at 72 h after parasitism than observed at earlier sampling times ($F = 11.453$, df $= 14$, $P = 0.001$, ANOVA), while trehalose content in unparasitized larvae remained significantly unchanged over the sampling period ($F = 2.216$, df $= 14$, $P = 0.140$, ANOVA) (Fig. 3). In comparing trehalose content between parasitized and unparasitized larvae at the sample intervals, significantly lower levels were detected in parasitized larvae at 6 h and 24 h after parasitism (6 h, $T = 4.406$, df $= 2$, $P = 0.048$; 24 h, $T = 16.143$, df $= 2$, $P = 0.004$) (Fig. 3).

Glycogen content did not differ significantly among the sample times for either unparasitized or parasitized larvae. Furthermore, no significant differences in glycogen content were detected among the sample times for unparasitized or parasitized larvae. And, likewise, no significant differences in glucose content were detected between parasitized and unparasitized larvae at any of the sample time

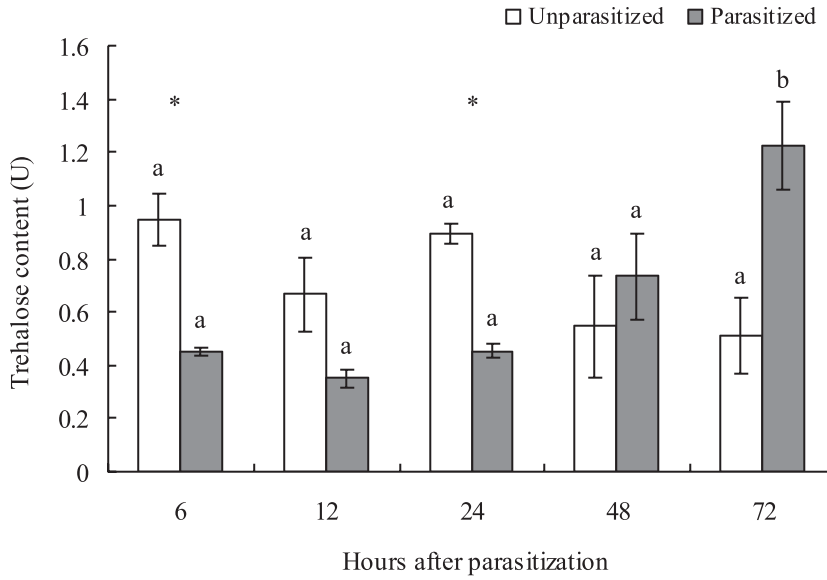

Fig. 3. Trehalose content in hemolymph of *M. alternatus* larvae parasitized or unparasitized by *D. helophoroides.* (The error bars indicate SE. The different letters labeled above the bars in same color indicate significant differences between time points at the $P = 0.05$ level by ANOVA, while the same letters indicate no difference. An asterisk (*) indicates significant differences between parasitized and unparasitized samples at $P < 0.05$ by pairwise *t* test. $n = 3$ replicates for each time point).

intervals, and no differences were detected over time in either parasitized or unparasitized larvae.

No significant differences in α-amylase activity occurred over time in parasitized larvae, while significant fluctuations were observed over time in unparasitized larvae ($F = 4.842$, df = 14, $P = 0.020$, ANOVA) (Fig. 4). The α-amylase activity in the hemolymph of parasitized larvae was consistently lower than that observed in unparasitized larvae, although the differences between parasitized and unparasitized larvae were not significantly different. Furthermore, we detected extremely low trehalase activity in the hemolymph of parasitized and unparasitized larvae regardless of sample time.

Discussion

These results are the first reported on the effects of parasitism by a coleopteran parasitoid on the physiology and metabolism of its hosts. Based on our results, parasitism by *D. helophoroides* larvae impacts the content of saccharides in the hemolymph of parasitized *M. alternatus* larvae. Inhibition or stimulation of saccharide physiology in the host larvae differed among sampling times. For

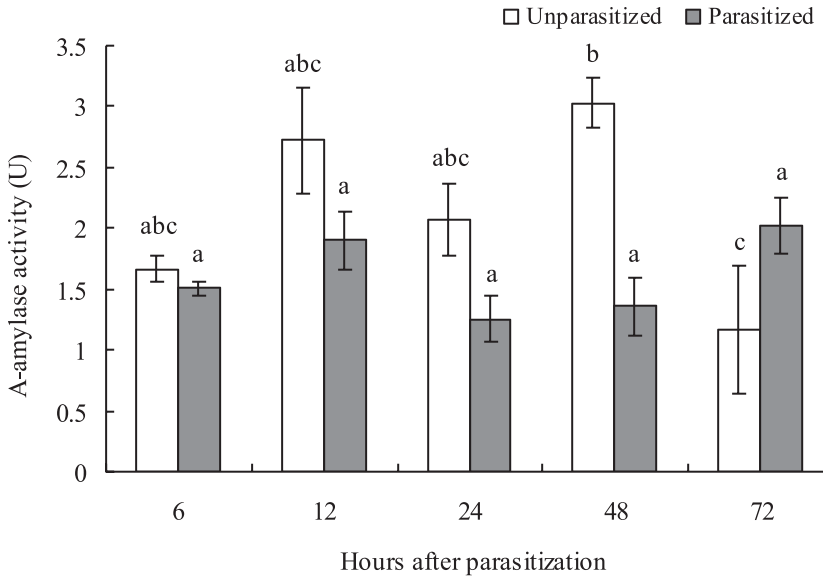

Fig. 4. Alpha-amylase activity in hemolymph of *M. alternatus* larvae parasitized or unparasitized by *D. helophoroides.* (The error bars indicate SE. The different letters labeled above the bars in same color indicate significant differences between time points at the $P = 0.05$ level by ANOVA, while the same letters indicate no difference. $n = 3$ replicates for each time point).

example, total sugar content in parasitized larvae was significantly higher than in unparasitized larvae 12 h after parasitism, while no significant differences were found between parasitized and unparasitized larvae at other sample times. Reducing sugar content appeared to be significantly inhibited in parasitized larvae 24 h after parasitism but stimulated at 72 h after parasitism. Trehalose content in parasitized larvae was significantly inhibited at 6 h and 24 h after parasitism. These findings reflect the findings of others with these three saccharides in other host–parasitoid systems, such as *H. virescens–Microplitis croceipes*, *H. virescens–C. sonorensis*, *L. dispar–G. liparidis*, *Pieris rapae–Pteromalus puparum*; *T. molitor–Scleroderma sichuanensis*; *Spodoptera exigua–M. pallidipes*, and *B. tabaci–E. sophia* (Bischof and Ortel 1996, Dahlman and Vinson 1975, 1976, Ji et al. 2013, Lü et al. 2000, Tan et al. 2003, Wang et al. 2015).

Changes in the concentrations of reducing sugar and trehalose in the hemolymph of *M. alternatus* larvae parasitized by *D. helophoroides* suggest that these are important for the parasitoid development or the immune response of the host. On the other hand, glycogen and glucose levels remained unaffected and apparently played little to no role in the parasitic process or immune response of the host. However, we postulate that *D. helophoroides* larvae can modulate the nutrition status of the host to fulfill their own nutritional and developmental demands. Although saccharide metabolism is affected by the parasitic association, we found

no changes in the enzymatic activity in the host hemolymph, thus, leaving the regulatory mechanisms for the observed saccharide metabolism unclear.

Host metabolism in the host–parasite system depends largely on the host immune response to the parasitoid. We previously reported both humoral immune responses and cellular immune responses of *M. alternatus* larvae to parasitism by *D. helophoroides* (e.g., hemolymph melanization reaction, phenoloxidase activity, antibacterial activity, total hemocyte number) (Li et al. 2015). However, the interactions between immune responses and metabolism regulation are unknown and require further research.

Our results provide a basis for understanding the physiological mechanisms occurring in *M. alternatus* when parasitized by *D. helophoroides*. Such information may provide a means for exploiting novel methods of *M. alternatus* control methods dealing with saccharide metabolism regulation as well as serving as an important resource for developing artificial diet for rearing diets and methods for *D. helophoroides* larvae. However, further functional studies are needed to characterize the impact of altered carbohydrate homeostasis in this insect species.

Acknowledgments

We thank Biliu Lin and Lin Zhang from the Huanglishu Forest Farm of Quanjiao County, Anhui Province, for their diligent works in collecting samples. This research was sponsored by the National Natural Science Foundation of China (Project NO 31300547) and the Anhui Provincial Natural Science Foundation (Project NO 1208085QC72).

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