Changes in the Hemolymph Metabolites of *Epiphyas postvittana* parasitized by *Meteorus ictericus*

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ABSTRACT

When the endoparasitoid, *Meteorus ictericus*, parasitizes the light brown apple moth (LBAM), it induces changes in the concentrations of its host's metabolites. It is believed that the changes caused over time correspond to the changing needs of the developing parasitoid; making a more stable internal environment for he developing parasitoid egg. To determine what metabolic changes occur over time, the concentrations of proteins, lipids, and carbohydrates were measured in the hemolymph of the parasitized LBAM at three time points: one-day, four-days, and seven-days after parasitization. There was an initial increase in the hemolymph proteins of the parasitized larvae compared to the controls, but at the latter two time points the parasitized larvae had significantly less protein than the controls. The hemolymph carbohydrates did not appear to differ significantly until the seventh day when the parasitized larvae had a lower concentration. The control larvae had a higher concentration in the hemolymph lipids in both the one- and seven-day groups, but no change was observed in the four-day larvae. In conclusion, the parasitoid female injects proteins and lipids into the host which are then utilized by the developing parasitoid.

KEYWORDS

host regulation, biological control, nutritional ecology, parasitization, parasitoid nutrition

INTRODUCTION

The light brown apple moth (*Epiphyas postvittana*, LBAM) is classified as an invasive threat to California agriculture with the capability of spreading across the country. LBAM has a broad range of host plants, used for oviposition and larval nutrition, which could allow for spread across much of the southern United States (Venette et al. 2003). It is a tortricid leafroller that consumes foliage and then pupates within a rolled up leaf, effectively shielding the pupa from insecticides (Bürgi and Mills 2010). This leaf rolling activity kills crops that are important to California's economy, such as pome fruits and berry fruits (Suckling and Brockerhoff 2010, Varela et al. 2008). California has a zero tolerance policy for live larvae in exports making the economic impact of LBAM significant (Bürgi and Mills 2010). Determining the best possible method of regulation of LBAM is integral to preventing further damage to California's agricultural sector (Gutierrez et al. 2010).

When LBAM was first recognized as a threat to agriculture in the 1960s, insecticides were used as a preventative measure, but developed resistance brought biological control to the forefront of eradication options (Varela et al. 2010). A native species of Australia, LBAM was first found to be an invasive pest to New Zealand before moving to California in 2007 (Suckling and Brockerhoff 2010). In New Zealand, LBAM quickly developed a resistance to the broad-spectrum insecticides used to eradicate it (Varela et al. 2010). Alternative methods of control such as releasing pheromones to disrupt mating were attempted, but biological control, a method of using natural enemies of a pest to control it, was found most effective (Varela et al. 2010). Populations of the leafroller significantly decreased after biological control methods using natural predators were implemented (Varela et al. 2010). Specifically, parasitoid wasps are the predator of choice for many similar insect species in need of control, and have proven beneficial in eradicating pests (Unruh and Lacey 2000, Unruh et al. 2003).

High parasitoid reproductivity is an essential component for a biological control program's success. One factor which can determine reproductive success in parasitoids is the nutrient transfer from the host to the parasitoid after egg deposition (Salvador and Consoli 2007). In parasitoid wasps, egg deposition can occur either on the outside of the host (ectoparasitoids) or inside the host larvae (endoparasitoids) (Jervis et al. 2001). Previous research has primarily focused on nutrient transfer in ectoparasitoids, leaving much to be discovered in endoparasitoids

(Salvador and Consoli 2007). One potential model for nutrient transfer in endoparasitoids is *Meteorus ictericus*, a wasp which controls LBAM (Pluciennik and Olszak 2009). Native to California, *M. ictericus* is the parasitoid that causes the highest and most consistent parasitism rates of LBAM in the field (Linda Bürgi, personal communication). For these reasons, *M. ictericus* is a logical model for understanding the potential for endoparasitoids as a mechanism of biological control.

The transfer of nutritional reserves from *M. ictericus* to LBAM will elucidate what biochemical changes occur during parasitization of the host. In this study I examine the potential transfer of nutritional content by measuring the concentrations of proteins, carbohydrates, and lipids before and after parasitization. I seek to determine if the parasitoid injects nutritional materials with the eggs to create a more stable environment in the larva, and what metabolic changes are induced. I will measure these concentrations at three different time points after parasitization to observe the effects over time. I expect to find increased protein, carbohydrate, and lipid levels in the hemolymph after parasitization, followed by a decrease in all levels as the parasitoid larvae develop. With this added information about *M. ictericus*, biological control researchers will be able to determine the impact of parasitoids on populations of LBAM.

METHODS

Study Organisms

I reared the host species, LBAM, on a diet of pink bollworm under controlled laboratory conditions (21º C ± 1º C). I raised the parasitoid, *M. ictericus*, on other LBAM that had been reared on the pink bollworm diet. I used four- to seven-day-old *M. ictericus* to parasitize the experimental larvae.

Sample Selection and Treatment

To ensure the experimental larvae had recently molted to the fifth instar, I selected late fourth instar larvae 24 hours before dissection. *M. ictericus* parasitizes early fifth instar larvae due to the potential access to nutrients in the later host instars (Lawrence 1990). I made all

measurements and dissections in a sterile environment. I considered the larva to be in its late fourth instar if it had a head capsule width of 0.375mm which had separated from its previous head capsule. I put each larva into a separate glass vial with a plantain leaf and 1:1 honey: water. The plantain leaf served as the food for the larva while the honey water was food for the parasitoid. It was necessary to have both diets in all vials to control for any effect they may have posed to the control group.

For each treatment, I added a four- to seven-day-old *M. ictericus* parasitoid to the vial. I removed all parasitoids from the vials after 24 hours, which I determined to be an appropriate length of time to expect parasitization (Linda Bürgi, personal communication). The three treatment groups were one-day, four-day, and seven-day, differing by the number of days left between parasitization (or not, for control larvae) and dissection. On the day of dissection, I selected larvae that had molted to the fifth instar (0.5mm head capsule width). For one-day larvae, I only used larvae that were of larval weight between 11 and 21mg to control for confounding that may have occurred due to a larger weight range.

Sample Collection

Post treatment, I removed the larvae from their containers for hemolymph collection, which was used to measure protein, carbohydrate, and lipid content (Salvador and Consoli 2007). First, I weighed the larvae, surface sterilized them in a separate dish, and blotted them on a clean paper towel. I then collected 2µL of hemolymph in a 2µL micropipette from the larva's proleg and immediately added the collected hemolymph to 20µL of anticoagulant buffer (98mM NaOH; 0.19M NaCl; 1.7mM EDTA; 41mM citric acid, pH 4.5). After, I centrifuged the mixture (1000g x 2 min), collected the supernatant, and stored at -20º C until analysis.

Metabolite Quantification

Protein Quantification

To measure the protein concentration in the hemolymph, I used Bradford's assay (Bradford 1976). I added 2µL of the hemolymph sample solution to 7µL anticoagulant buffer in

duplicate to a 96-well plate. I then added 270µL of the Coomassie reagent (Coomassie Plus Protein, Pierce Biotechnology, Inc., Rockford, IL) to each sample. I shook the plate for 10 seconds at low speed, and let it sit for 10 minutes before analysis. I read the absorbance on a microplate absorbance reader (EL 808 Ultra Microplate Reader) at 595nm after shaking. I compared the samples to a Bovine Serum Albumin standard curve to determine the protein concentration of each of the samples.

Carbohydrate Quantification

To measure carbohydrate concentration in the hemolymph, I used an anthrone reagent (Salvador and Consoli 2007). I added 232µL of diluted anthrone reagent (0.05% anthrone, 1% thiourea, 66% hydrogen sulfate) to 4µL of each sample duplicate. I immediately vortexed and treated the samples at 100º C for 15 minutes while keeping the samples in the dark. I read the absorbance of the samples on a microplate absorbance reader (EL 808 Ultra Microplate Reader) at 625nm. I used glucose as a standard.

Lipid Quantification

To quantify lipid concentration, I used the vanillin reagent assay (Van Hendel 1985). I extracted the lipids by adding 200µL of $CHCl_3$: CH_3OH (1:1) to the samples and evaporating them at 100º C. I then added 30µL of sulfuric acid and incubated the samples at 90º C for 10 minutes. Next, I added the vanillin reagent and immediately vortexed the samples. Finally, I read the absorbance on an EL 808 Ultra Microplate Reader at 525 nm. I compared samples to a vegetable oil standard curve to determine final lipid concentration.

Statistical Analysis

To determine if there was a difference in the concentrations of the metabolites corresponding to parasitism level, I used t-tests by analyzing the data with R (R Development Core Team 2011) and R Commander (Fox 2005). I separated the information into proteins, carbohydrates, and lipids, and compared the control larvae to the parasitized larvae at each of the

time points (one-, four-, and seven-day). I considered the difference between the control and experimental groups to be statistically significant at a p-value < 0.05.

Additionally, I compared the weights of the larvae in the control group to the parasitized group at each time point to determine if there was a significant difference in the weights. To accomplish this, I again used a t-test. Finally, I used a generalized linear model (GLM) to determine whether or not the larval fresh weight contributed to the association between treatment and metabolic concentration (R Development Core Team 2011).

RESULTS

Larval Weight

By weighing the larvae at the time of hemolymph collection, I was able to determine if the difference in metabolite concentrations was impacted by the larval weight. I found a significant difference in the larval weights of the control and parasitized groups at all time points. The weight of the one-day parasitized group was higher than the control group, while the control group had a higher weight at the four-day and seven-day levels (Fig. 1).

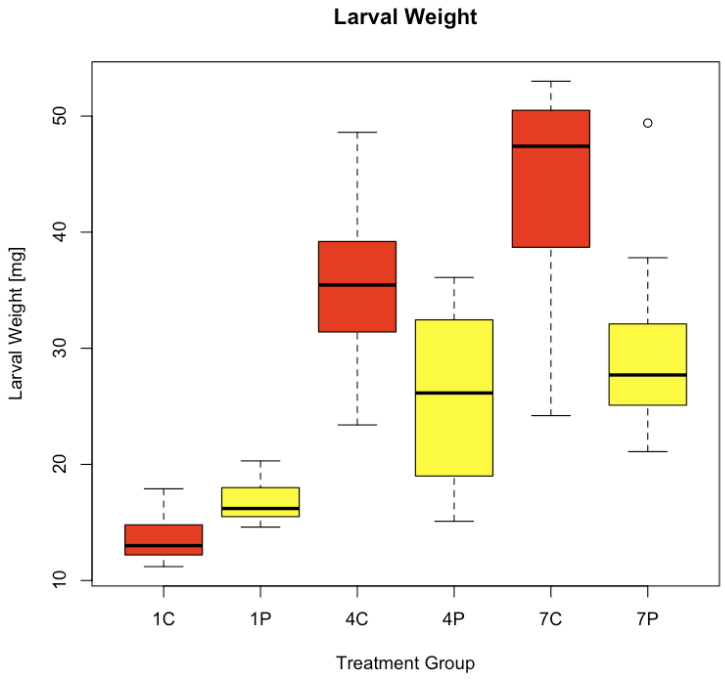

Figure 1. Larval fresh weight (mg) of LBAM larvae in the control group (C), and one, four, and seven days after parasitization by *M. ictericus* (P).

Protein Quantification

I found that the concentration of protein stored in the hemolymph was significantly higher in the parasitized group when compared to the control group in the one-day group (Fig. 2, p-value <0.001, effect size = 21.36 µg/µL). In the four-day and seven-day groups, the concentration of protein stored in the hemolymph of parasitized larvae was significantly greater than that in the control group (Fig. 2, p-value <0.001, effect sizes = 32.88 µg/µL and 54.39 µg/µL respectively). A GLM indicated an effect of larval weight on the association between the seven-day control and parasitized larvae.

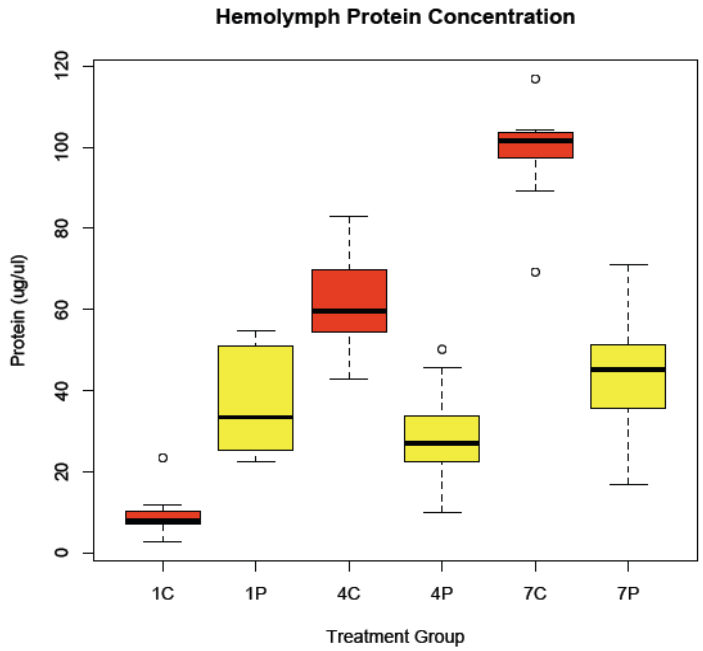

Figure 2. Concentrations of protein in the LBAM hemolymph in the control group (C), and one, four, and seven days after parasitization by *M. ictericus* (P): Measured by spectrophotometry using Bradford's assay with BSA as a standard.

Carbohydrate Quantification

I measured a statistically significant difference in the hemolymph carbohydrates after parasitization in the seven-day group. In this group, the parasitized larvae had a higher carbohydrate concentration than the control group (Fig. 3, p-value <0.05, effect size = 0.05 µg/µL). In the four-day and seven-day groups, the change in the hemolymph carbohydrate

concentration was not statistically significant (Fig. 3). A GLM indicated that neither mass nor treatment affected the carbohydrate concentration in the four-day groups, and that mass did not affect the one-day or seven-day groups.

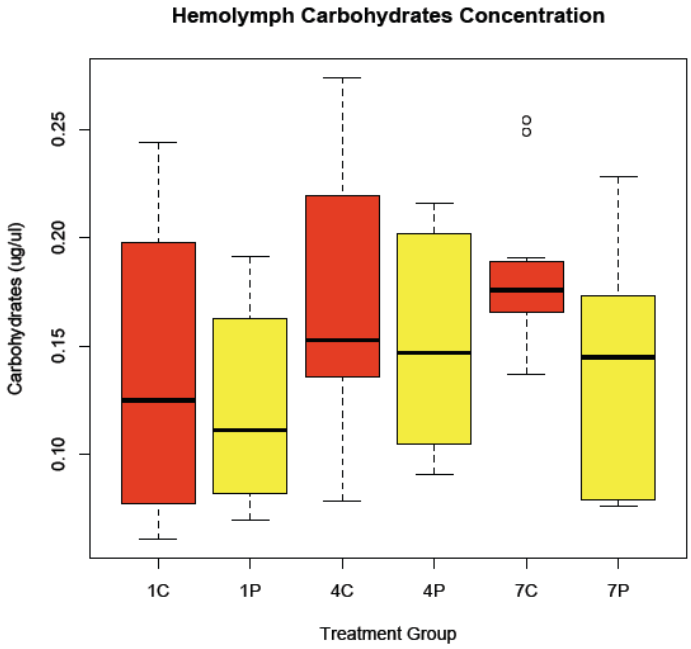

Figure 3. Concentrations of carbohydrates in the LBAM hemolymph in the control group (C), and one, four, and seven days after parasitization by *M. ictericus* (P): Measured by spectrophotometry using the anthrone reagent with glucose as a standard.

Lipid Quantification

I found the change in lipid concentration in the one-day larvae hemolymph to be statistically significant, with the control group having a lower concentration than the parasitized group (Fig. 4, p-value <0.001, effect size = 34.04 µg/µl). In the seven-day group, I found the change to also be statistically significant, and to be less concentrated after parasitization (Fig. 4, p-value <0.001, effect size = 24.56 µg/µL). A GLM showed no interaction with weight in the association between treatment group and lipid concentration.

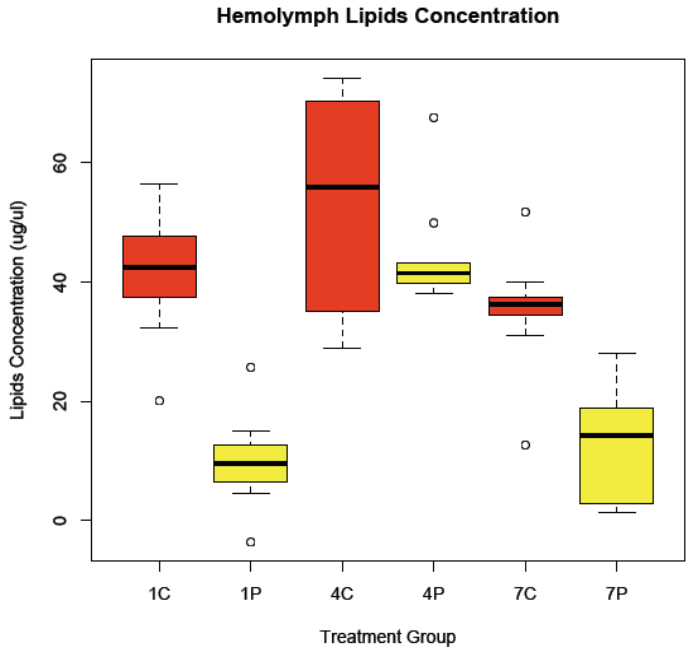

Figure 4. Concentration of lipids in the LBAM hemolymph in the control group (C), and one, four, and seven days after parasitization by *M. ictericus* (P): Measured by spectrophotometry using the vanillin reagent with vegetable oil as a standard.

DISCUSSION

To determine metabolic changes resulting from parasitization of LBAM by *M. ictericus*, I measured and compared host metabolite concentrations in unparasitized larvae and those parasitized by the endoparasitoid at three different time points after parasitization. Through hemolymph extracts from the host larvae, I used spectrophotometry to quantify the amounts of proteins, carbohydrates, and lipids in the host. I predicted that the concentration of these three metabolites would initially increase as a result of the injection of metabolites during oviposition, and then decrease over time as the parasitoid larvae continued to develop inside the host. This prediction was mostly supported by the results. The concentration of proteins in the hemolymph did decrease over time, with an initial increase in the one-day group, and the changes in the carbohydrates and lipids showed a similar trend with less statistical significance.

Proteins

The results support my hypothesis, suggesting that the parasitoid initially injects nutrients into the host which the developing parasitoid larva then utilizes for its growth. I observed an initial concentration of hemolymph protein which was higher in the parasitized larvae, followed by a higher concentration in the control groups. The same trend was found in a similar study comparing the metabolites in a moth species, *Diatraea saccharalis,* parasitized by a parasitoid wasp, *Cotesia flavipes*. This study observed a lower concentration of hemolymph protein in the parasitized larvae when compared to the unparasitized control group over time (Salvador and Consoli 2007). In the host-parasitoid relationship between *Toxoneuron nigriceps* and *Heliothis virescens* the proteins in the parasitized larval group were relatively equal in concentration to those in the control group (Consoli et al. 2005), however it is difficult to determine what mechanism accounts for the disparity between the studies. In LBAM, I observed an eventual increase in the hemolymph proteins around the seventh day after an initial decrease. A decrease in protein concentration over time could suggest protein uptake regulation by the parasitoid (Consoli et al. 2005) or even a direct consumption of the hemolymph by the parasitoid (Salvador and Consoli 2007, Nakamatsu et al. 2001). The higher protein concentration in the one-day parasitized compared to the control group could be caused by the significant difference in weight between the two groups, however a GLM did not support this relationship.

Carbohydrates

I did not find a significant change in the hemolymph carbohydrates of the host larvae after parasitization until the seventh day at which point the control larvae had a higher concentration than the parasitized larvae. There was an initial increase in hemolymph carbohydrates in the parasitized *D. saccharalis* and *T. Nigriceps,* followed by a steady decline over time (Salvador and Consoli 2007, Consoli et al. 2005). However, the majority of this decline occurred after the seventh day, which may account for the lack of overall decrease found in my study which only extended to the seventh day. The eventual reduction observed could be caused by a regulation process by the parasitoid that aims to keep the host larva from reducing its caloric intake (Salvador and Consoli 2007).

Lipids

The concentration of lipids in the LBAM larvae hemolymph was relatively variable over the course of the study, but was always lower in the parasitized larvae than in the control larvae. This difference was significant in both the one-day and seven-day groups. A decrease was observed after the fifth day in other studies, which is comparable to my own findings (Salvador and Consoli 2007). This is likely caused by the ability of the parasitoid larvae to gain access to the fat body content after a destructive enzyme is released onto the fat body (Nakamatsu et al. 2002). The fat body, rather than the hemolymph, is the main storage site for lipids in the host larvae, which may also support the variability in lipid concentration in my study (Salvador and Consoli 2007).

Reasons for Metabolic Changes

Parasitoids have been shown to alter the host organism's internal environment to make it more supportive of the parasitoid larvae's development (Consoli et al. 2005). Some parasitoid females produce a larger quantity of yolk-poor eggs to conserve their own energy resources, resulting in the need for the growing parasitoid to obtain nutrients from its host. These eggs have an outer membrane that has evolved to allow the transfer of nutrients to the developing parasitoid larva from the host (Consoli and Vinson 2004). As the parasitoid grows within the host larva, it consumes nutrients originating from the host, causing a decrease in the metabolic content of the host.

Limitations

There are some limitations to my study design which make it difficult to compare to other research studies. First, in order to observe a statistical significance in all of my results, a retrospective power analysis indicates that between 50 and 400 replicates would have been necessary, given the spread of the metabolite concentrations I found. I also was unable to confirm the parasitization of several parasitized replicates, especially in the one-day group, because the parasitoid larvae were still too small to observe at this stage. Additionally, the

variability in the concentration of hemolymph lipids, and the lack of any distinct trends, may be due to an error in my methods in which I failed to mix the sulfuric acid and vanillin reagent before analysis. This study will later be repeated to determine if there was an error in the lipid analysis. Finally, my results cannot be extrapolated to describe the interaction between ectoparasitoids and their host species, as they often inject paralyzing venom when parasitizing their hosts which may further change the metabolic content (Asgari and Rivers 2010).

Future Directions

In future studies, it would be beneficial to compare changes in metabolites in a host larva when parasitized by an endoparasitoid to similar studies of an ectoparasitoid. This comparison could determine whether the results of this study of *M. ictericus* could be extrapolated to observation of ectoparasitoids or other endoparasitoids. This project should also be expanded to include up to 400 replicates as suggested by the retrospective power analysis. As many of the significant changes in metabolic concentrations were not observed until after the seventh day in other relevant studies, additional data should be collected at later time points to determine the overall trends of metabolic concentrations. Finally, SDS-PAGE analysis of the proteins in the LBAM host larvae would help to determine which proteins specifically are changing with parasitization. Some other studies have used this method to further support their claims regarding the changes induced by the parasitoid on the host larva (Salvador and Consoli 2007, Consoli and Vinson 2004, Nakamatsu et al. 2002, etc.).

Conclusions

There is an exchange of metabolites during the process of parasitization of LBAM by *M. ictericus*. Overall, the metabolic effects of parasitization were not evident until after the first day, but seem to suggest an injection of proteins and a subsequent ingestion and utilization of metabolites. The concentration of proteins in the hemolymph did decrease with parasitization over time, with an initial increase in the one-day group, as predicted. The changes in the carbohydrates and lipids were variable, with the parasitized group tending to have a lower metabolic concentration than the unparasitized larvae. These results confirm that parasitization

does have an effect on the host metabolic content of the LBAM larvae. This information can help inform researchers of the precise effects of parasitoid involvement in the broader spectrum of biological control practices.

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