MICROPLITIS CROCEIPES (HYMENOPTERA: BRACONDIAE): A LIFE HISTORY STUDY AND *IN VITRO* REARING

A Thesis

by

LAURA ANN MCLOUD

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2011

Major Subject: Entomology

MICROPLITIS CROCEIPES (HYMENOPTERA: BRACONIDAE)*:* A LIFE HISTORY STUDY AND *IN VITRO* REARING

A Thesis

by

LAURA ANN MCLOUD

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Chair of Committee,	S. Bradleigh Vinson
Committee Members,	Julio S. Bernal
	Gary Wingenbach
Head of Department,	David Ragsdale

August 2011

Major Subject: Entomology

ABSTRACT

Microplitis croceipes (Hymenoptera: Braconidae): A Life History Study and *in vitro* Rearing. (August 2011)

Laura Ann McLoud, B.S., Clemson University

Chair of Advisory Committee: Dr. S. Bradleigh Vinson

Microplitis croceipes (Hymenoptera: Braconidae) is an endoparasitoid and potential biological control agent of the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae), an agricultural pest. The first objective of the following research was to amend current larval life history descriptions of *M. croceipes*. Larval head capsule width measurements were used to distinguish instar, and exuvium in abdominal cavities of post-egression hosts were indicative of a molt during parasitoid egression. Data revealed the larvae of *M. croceipes* pass through five instars, rather than three, as is indicated in the literature.

The second objective was to investigate the suitability of potential artificial diets to be used in *in vitro* rearing of *M. croceipes* larvae. Three concentrations each of glucose, trehalose, and protein, as well as a combination diet (derived from initial diet trials) were tested. Growth, molting, and death were noted for each diet, and data indicated that diet had a significant effect for each performance measure ($p = 0.0000$, $p < 0.0001$, $p < 0.0001$, respectively). Data also indicated that trehalose and protein were more vital to larval parasitoid development (growth and molting) than was glucose, but no larvae were reared passed the second instar on an artificial diet.

The final goals of the research were to evaluate the plausibility of rearing *M. croceipes* larvae to adulthood *in vitro* and to investigate post-egression host defensive behavior. Larvae were dissected from their hosts just prior to egression and placed in a cell culture plate in previously collected host hemolymph. Larvae were able to initiate pre-egression behavior in an *in vitro* environment, and a small percentage (6.67%) exhibited ecdysial splitting of the cuticle, however, no larvae were able to make the final molt *in vitro*. Post-egression hosts exhibited defensive behavior that may suggest they play a role in protecting pupating parasitoids. When the parasitoid exuvium was pulled from the egression wound in the host, hemolymph loss occurred and duration of the defensive behavior significantly decreased ($p < 0.0001$), indicating the exuvium acted to plug the egression wound, which prevented the host from bleeding to death and made it possible for the host to exhibit defensive behavior.

DEDICATION

My thesis is dedicated to my family, who supported me through this process.

ACKNOWLEDGEMENTS

I would like to thank my advising committee members, Dr. Bradleigh Vinson (PI), Dr. Julio Bernal, and Dr. Gary Wingenbach, for providing me with input and feedback throughout the duration of my master's degree program. I would also like to thank the members of Dr. Vinson's laboratory, particularly Dr. Indira Kuriachan and Dr. Asha Rao, for advising and mentoring me for the past two years. Additonally, I would like to thank Dr. Robert Wharton for encouraging me to investigate the life history of my study organism and for advice on how to proceed in that matter. Finally, I would like to thank Texas A&M University for providing me with funding during my master's degree program.

TABLE OF CONTENTS

Page

CHAPTER Page

CHAPTER Page

LIST OF FIGURES

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

Biological control is a viable pest control strategy that can be used to replace pesticide alternatives or be incorporated into integrated pest management (IPM) plans. In biological control, facilitated control of pest populations below the economic threshold is achieved through the release of natural enemies. Parasitoids are common biological control agents and are typically regarded as ideal for biological control because of a general efficiency in locating and attacking their hosts, in conjunction with a high degree of host specificity, which serves to reduce non-target effects associated with releasing large numbers of natural enemies.

Successful implementation of a biological control program relies on many factors, but hinges, in part, on the ability to rear, en mass, the biological control agent (Dindo et al., 2006). In the case of parasitoids, the agent, the parasitoid, and its host must be reared simultaneously. In many mass-rearing operations, the cost of rearing the host, in conjunction with rearing the parasitoid, is the limiting factor in producing a number of agents sufficient for use in biological control (Greany et al., 1989). Therefore, it is in the best interest of biological control implementers to devise an efficient mass-rearing operation that yields the highest number of fit individuals at the lowest cost.

This thesis follows the style of the Journal of Insect Physiology.

Microplitis croceipes (Braconidae) is a larval endoparasitoid of the tobacco budworm, *Heliothis virescens* (Noctuidae). Tobacco budworm is a major pest of cotton and other crops in the United States. As a parasitoid of *H. virescens*, *M. croceipes* has the potential to be utilized as a biological control agent of this pest (Powell and Elzen, 1989). Because mass rearing is often a necessary component of successful biological control programs, the development of an efficient and cost effect rearing protocol for *M. croceipes* is necessary. *In vitro* rearing will be investigated here as a potential alternative to traditional rearing methods for this parasitoid.

The proposed research addresses three broad objectives. The first objective is to amend current larval life history descriptions for *Microplitis croceipes*, with particular focus on the number of larval instars The second objective is to assess the dietary needs of larval *M. croceipes* (Braconidae), endoparasitoid of *Heliothis virescens* (Noctuidae), and the ability of the endoparasitoid larvae to survive and develop on an artificial diet in an *in vitro* rearing system. The third objective is to assess the plausibility of rearing these insects *in vitro* on an artificial diet by studying parasitoid dependence on the host for the final larval molt and ability of the larvae to molt *in vitro*.

CHAPTER II

LITERATURE REVIEW

Agricultural pest control has long been an issue of great debate into which much money has been invested. Traditionally, agricultural pest insects have been controlled with chemical pesticides. However, increasingly more stringent regulations on pesticide production, registration, and use, environmental and human dangers, and growing, widespread insect resistance to chemical pesticides, have motivated researchers to search for alternative means of pest management (Maredia et al., 2003).

Integrated pest management (IPM) seeks to moderate the use of chemical pesticides by employing sustainable and biological control measures that include farmer education, behavioral control, and planting pest resistant crops, to name a few (Maredia et al., 2003). IPM programs stress the importance of proactive rather than reactive control measures by encouraging farmers to monitor pest populations and implement control measures prior to growth of pest populations above the economic threshold. Among its arsenal of control tactics, IPM utilizes biological control of pest species via natural enemies. Biological control with natural enemies encompasses three categories of management techniques. Classical biological control hinges on the successful release and integration of an exotic enemy species to control pest populations. Conservation biological control focuses on maintaining healthy populations of natural enemies in the target environment and often includes habitat modifications to bolster local natural

enemy populations. Finally, augmentation biological control involves the release of natural enemies to increase conspecific populations. (Maredia et al., 2003).

Studies regarding the effectiveness, plausibility, and versatility of biological control methods have demonstrated that biological control is a viable option for both large and small scale farming operations, greenhouses and field crops (Maredia et al., 2003). Among the control agents commonly used for biological control are parasitoids. Parasitoids account for a large percentage of holometabolous, herbivorous insect mortality in the field (Hawkins, 1994) and thus are excellent candidates for many biological control programs. However, associated costs of rearing and maintaining colonies of both host and parasitoid detract from the practicality of implementing a large-scale insect biological control program using natural enemies. One solution is to develop a method by which the parasitoid can be reared independent of its host, an *in vitro* rearing method (Greany et al., 1989).

Grenier (2009) reviews the history of *in vitro* rearing of insects. According to Grenier, research into designing *in vitro* rearing systems for parasitoids and predators began in the 1950s with the express purpose of streamlining the rearing process for applications in mass rearing for laboratory research and field releases of beneficial insects. Early studies focused primarily on Hymenoptera, Diptera, Coleoptera, and Hemiptera. Diets designed for *in vitro* rearing may be generally categorized as either devoid of insect components (strictly chemical) or as containing insect components (Grenier, 2009).

Hymenoptera ectoparasitoids and egg parasitoids, as well as endoparasitic Diptera, represent the majority of parasitoids that can be, to date, reared to adulthood *in vitro* (Grenier, 2009). Hymenoptera successfully reared *in vitro* include species from the families Pteromalidae, Ichneumonidae, and Trichogrammatidae (Consoli and Parra, 1996; Ferkovich et al., 2000; Morales-Ramos et al., 1998; Rojas et al., 1996; Xie et al, 1997). In general, endoparasitoids present a unique challenge to *in vitro* rearing. Endoparasitoid larvae live and feed inside the host and often alter host physiology to meet their nutritional needs (Vinson and Iwantsch, 1980); the resulting dynamic environment may be difficult to mimic in an *in vitro* system (Greany et al., 1989). However, there have been studies demonstrating the potential to rear endoparasitoids *in vitro*. In the 1950s, according to Yazgen and House (1970), the endoparasitoid, *Pimpla turionellae* (Ichneumonidae) was reared *in vitro* on an artificial diet containing pig liver. Additionally, Yazgen and House (1970) report their research and successful rearing of a Hymenoptera endoparasitoid, *Itoplectis conquisitor* (Ichneumonidae), which was purportedly the first time a Hymenoptera endoparasitoid was reared *in vitro* on a strictly "chemically-defined" diet (Yazgen and House, 1970). More recently, Kuriachan et al. (2006) successfully reared the endoparasitoid, *Toxineuron nigriceps* (Braconidae) from second to third instar on an artificial diet that incorporated host hemolymph.

This study will focus on *Microplitis croceipes* (Hymenoptera: Braconidae: Microgastrinae), a solitary endoparasitoid of the larvae of the tobacco budworm, *Heliothis virescens* (Noctuidae). *M. croceipes* is a koinobiont, developing entirely within the confines of its host's body while the host remains active. Though parasitization does

not immediately kill the host, host food consumption is significantly decreased after parasitization (Powell and Elzen, 1989). Parasitization of *H. virescens* by *M. croceipes* results in what has been termed "developmental arrest" (Dahlman et al., 2003). Injection of teratocytes, extra-embryonic cells produced from the parasitoid egg (Dahlman et al., 2003), into healthy *H. virescens* results in developmental abnormalities even in the absence of a parasitoid larva (Zhang and Dahlman, 1989). After the parasitoid larva egresses from the host, the host may remain alive for a few days, up to a couple weeks (Webb and Dahlman, 1985), but does not feed (personal observation). Because *M. croceipes* primarily feeds on host hemolymph and fat body during its larval development, rather than also feeding on the host's major organs, the host is still able to function after parasitoid egression (Webb and Dahlman, 1985). However, the post-egression hosts do not pupate and eventually die, though death may not occur until a couple weeks after parasitoid egression (Web and Dahlman, 1985; Strand et al., 1988).

The strategy of feeding only on hemolymph and fat body, as opposed to consuming the entire host, allows *M. croceipes* to successfully develop in a wider range of host sizes. In parasitoid species that consume the majority of host tissues during development, death of the larval parasitoid may result from parasitization of larger hosts; the parasitoid attempts to consume all of its host resources, and, in doing so, essentially eats itself to death (observed in *Venturia canescens* (Ichneumonidae) (Harvey et al., 2008)). *M. croceipes*, however, because it consumes so little of the host, can develop within both smaller and larger hosts (Harvey et al., 2008), from second to fourth instar

(Strand et al., 1988), and even fifth instar under laboratory conditions (personal observation).

M. croceipes has been called "[o]ne of the most promising candidates for biological control of *Heliothis spp*. (Powell and Elzen, 1989)" Its narrow host range, primarily *Heliothis virescens* and *Helicoverpa zea* (Lewis, 1970; Strand et al, 1988) suggests that release of this parasitoid for biological control holds little risk of non-target affects on other Lepidoptera species. Additionally, parasitization of the host by *M. croceipes* results in developmental arrest in the host and the developmental biology of *M. croceipes* larvae as hemolymph feeders, allows for the parasitoids to successfully develop in a wide range of host sizes (Harvey et al., 2008; Strand et al., 1988). However, rearing large numbers of *M. croceipes* remains labor and time intensive; small-scale rearing efforts (production of 5-10 female parasitoids per week) require around 20 hours a week (personal experience). Additionally, Greany et al. (1989) listed the estimated cost of rearing *M. croceipes* for biological control at between $25-$250 per 1000 wasps.

In the 1980s, development of an artificial larval diet for *M. croceipes* was discussed and attempted (Greany, 1986), but to date, *M. croceipes* has not been reared to adulthood on an artificial diet. Greany (1986) successfully reared *M. croceipes* from egg to first instar *in vitro*; first instar larvae were maintained on a fetal bovine serum-based diet for up to two months, but did not molt into the second instar (Greany, 1986). Research described here was conducted in an attempt to rear *M. croceipes* in an *in vitro* environment, with the main objective being the development of an artificial diet on which to rear the parasitoid larvae. Additionally, the larval life history of the parasitoid was examined and revised, and the plausibility of rearing *M. croceipes in vitro* was investigated. In order to consider the use of *M. croceipes* as a biological control agent for *Heliothis spp*. it is necessary to understand its biology and nutritional needs during larval development.

CHAPTER III

LIFE HISTORY REVISION

Introduction

The larval life history of *M. croceipes* is perhaps best described by Lewis (1970), who recorded the duration and morphological characteristics of each larval instar. Because *M. croceipes* develops inside the host, it is difficult to track instar progression during larval development. Larval development cannot be observed directly because of the developmental nature of endoparasitoids, and dissection of the host in search of larval exuvia of early instars (indicating a molt) often yields no result (personal experience). Tracking changes in larval body size is not an adequate means of determining instar progression simply because the soft-bodied nature of the larvae does not allow for accurate measurements. Dyar (1890) demonstrated that larval head capsule size remains relatively constant within each instar and increases geometrically from one instar to the next. Therefore, the average head capsule size of larvae in a particular instar should be distinct from that of larvae in another instar. The life history of *M. croceipes*, in particular the number of larval instars, was investigated in the following study using Dyar's Law (1890) as a guideline in distinguishing instars. Particular focus was paid to the purported second and third instars described in Lewis' (1970) review of this parasitoid's life history.

Materials and Methods

Rearing Protocol

Heliothis virescens and *Microplitis croceipes* were reared according to standard methodology in the Vinson lab. *H. virescens* larvae were reared on Corn Earworm diet (Bioserv., Inc., Frenchtown, NJ) at 29˚C (12L:12D). Adult *M. croceipes* were maintained in a colony at 21˚C (12D:12L). *H. virescens* larvae were parasitized in the late fourth instar (marked by head capsule slippage) by mated, female *M. croceipes*. Each caterpillar was individually presented to mated wasps (3-6) held in a cage. The caterpillar was carefully observed and was promptly removed after a single sting from a single wasp. Penetration of host cuticle by the ovipositor of a wasp indicated a sting. Any caterpillar stung more than once was not used in the study. Parasitized *H. virescens* were placed on diet and incubated at 29˚C (12D:12L) until the time of parasitoid egression and pupation, at which point cocoons were transferred to another incubator (21˚C; 12D:12L) until adult emergence.

Life History Analysis

First Instar

H. virescens larvae were parasitized as described in the rearing protocol. On days 3-5 after parasitization, hosts were dissected in a saline solution (0.9g NaCl (Fisher Scientific, Fair Lawn, NJ, USA), 0.02g KCl (Sigma, St. Louis, MO, USA) 0.02g $CaCl_2$ (Sigma, St. Louis, MO, USA), 0.4g Glucose (Sigma, St. Louis, MO, USA) per 100ml of solution) by making an incision down the length of the abdomen, along the dorsal vessel. The abdominal cavity was flushed with the saline solution using a glass pipette, which

freed the parasitoid larva from the host. First instar parasitoid larvae flushed from the host were observed for head capsule slippage, evidence of an impending molt. Photographic record of the molt from first to second instar was kept.

Purported Second and Third Instars

H. virescens larvae were parasitized as described in the rearing protocol. On days 5-8 after parasitization (to account for variability in parasitoid development), hosts were dissected in a saline solution (0.9g NaCl (Fisher Scientific, Fair Lawn, NJ, USA), 0.02g KCl (Sigma, St. Louis, MO, USA) 0.02g $CaCl_2$ (Sigma, St. Louis, MO, USA), 0.4g Glucose (Sigma, St. Louis, MO, USA) per 100ml of solution) by making an incision down the length of the abdomen, along the dorsal vessel. The abdominal cavity was flushed with the saline solution using a glass pipette, which freed the parasitoid larva from the host. Parasitoid larvae were categorized as either second or third instar, according to the descriptive morphology of Lewis (1970)[1]. Sorted larvae were placed in 95% ethanol for 1 day, at which point the head capsule width of each larva was measured at 6.3x magnification using a micrometer adapted to the microscope eyepiece. The scale of the eyepiece micrometer was determined with a stage micrometer (20mm diameter disc micrometer reticle (Cambridge Instruments, Buffalo, NY, USA)). To measure the head capsule, the larvae were positioned dorsal side; width of the head capsule was measured at its widest point.

---

[1] Second and third instar larvae, as described by Lewis (1970) will hereby be referred to as '2nd' and '3rd' instars in this text. Instar designations based on data presented in this paper will be referred to as first, second, third, etc. instars.

Final Instar

H. virescens larvae were parasitized as described in the rearing protocol. Parasitized hosts were incubated at 29˚C (12L:12D) until the point of parasitoid egression. Fifty-five caterpillars (post-parasitoid egression) were dissected. The exit hole created by the parasitoid was located (internally). Presence or absence of a parasitoid exuvium extruding internally from the exit hole was noted and used as evidence of a final molt that occured during egression. Photographic record of the presence of the exuvium was kept. A control was not directly used for this particular section of experimentation. However, observations made in previous dissections (here, described in Chapter IV) were the basis of control for these exploratory dissections; hundreds of parasitized caterpillars were dissected during the *in vitro* rearing project described in the following chapter, Chapter IV, and never once was a parasitoid larval exuvium observed in the abdominal cavity of the host.

Statistical Analysis

Purported Second and Third Instars

Mean head capsule width for '2^{nd}' and '3^{rd}' instars was calculated. A two-sample *t*-Test was performed to indicate whether the means differed significantly between the '2^{nd}' and '3^{rd}' instars. Head capsule widths were graphed according to frequency of occurrence, and distinct peaks in the data were designated as distinct instars (histogram created with Microsoft® Excel® for Mac, Version 12.2.9 (2008)). A K-Cluster analysis was performed to indicate whether the peaks observed in the histogram were statistically distinct clusters, and a scatter plot of the clusters was generated. One-way ANOVA was

used to compare cluster means generated by the cluster analysis, and statistically significant means were compared *post hoc* using a Tukey test. Additionally, 95% confidence intervals were generated for each range produced by the cluster analysis. The *t*-test of instar means, K-Cluster analysis, one-way ANOVA, and Tukey test were performed using JMP® Statistical Discovery Software, Version 9.0.0 (2010, SAS Institute Inc. Cary, NC USA version 9.0.0).

Results

First Instar

Dissections of the host on days 3-5 after parasitization revealed the molt from first to second instar, during which the madibulate head capsule of the first instar is shed (Figure 1). The head capsule of the first instar larva is clearly mandibulate (Figure 2a). The first instar larva possesses notably sclerotized, sickle-shaped mandibles, as described by Lewis (1970). The mandibles of the second instar larva are highly reduced, compared to those of the first instar (Figure 2b), making the molt from first to second instar apparent. Lewis (1970) also described this significant difference in mandibular morphology between first and second instars of *M. croceipes*.

Purported Second and Third Instars

A histogram of head capsule widths from larvae identified as purported second instars (according to Lewis' (1970) morphological descriptions) revealed three distinct ranges of head capsule widths and indicated that the highest range contained representatives from both the '2nd' and '3rd' instars (Figure 3). A *t*-test assuming unequal variances indicated that the mean head capsule widths of '2nd' and '3rd' instars were significantly different ($p < 0.0001$) (Table 1).

K-cluster analysis was set to produce three clusters, based on the histogram representation of the data (two pure clusters of '2nd' instars and one mixed cluster of '2nd' and '3rd' instars—see Figure 3). A scatter plot was produced from the cluster analysis (Figure 4). Cluster analysis produced the following ranges of head capsule width: 0.29-0.46mm, 0.48-0.60mm, 0.62-0.67mm (Table 1). One-way ANOVA indicated a significant difference among the means of the head capsule clusters ($p < 0.0001$). A *post hoc* Tukey test indicated that the means of all three ranges were significantly different (Table 1). The confidence intervals (95%) for each range were generated (95% CI: 0.09-0.22; 0.29-0.47; 0.39-0.57). The 0.62-0.67mm range cluster contained a mixture of '2nd' and '3rd' instar larvae (Figure 3).

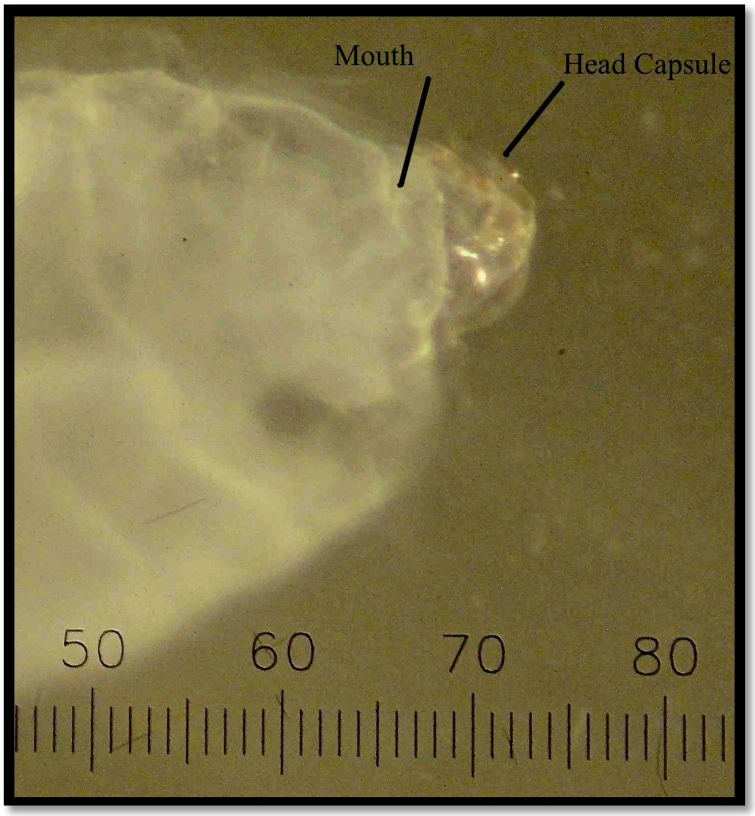

Figure 1
Photograph depicting the molt from first to second instar, as indicated by loss of the first instar head capsule. Measurements were determined with a micrometer fitted to a microscope. This photograph was taken at 6.3x magnification. In the scale depicted, 10 tick marks equals approximately 0.16mm.

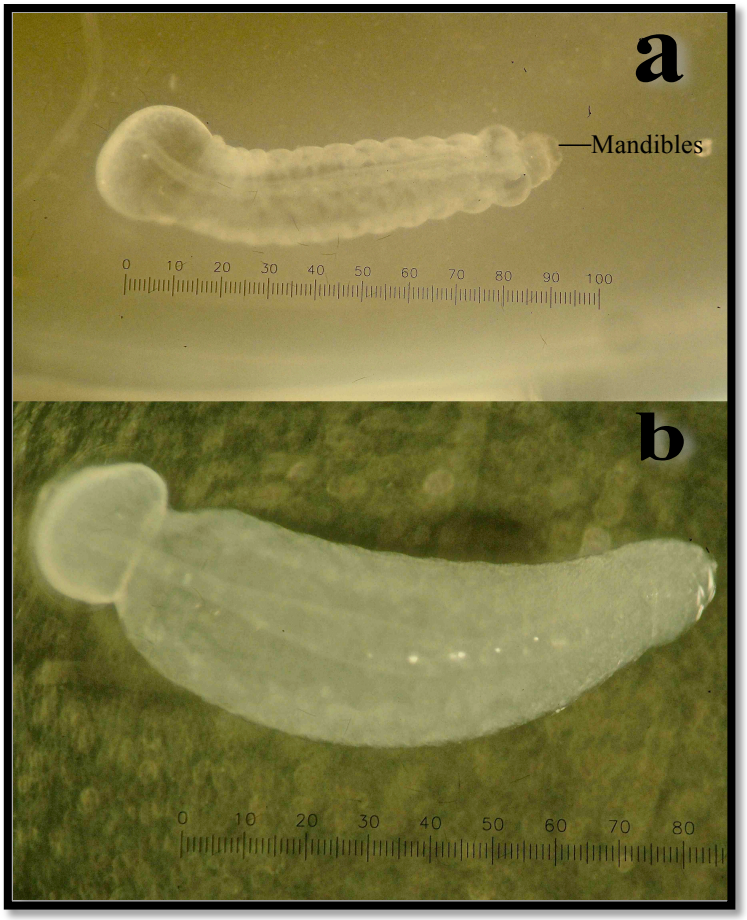

Figure 2
Photograph depicting first (a) and second (b) instar parasitoids. The loss of the mandibulate head capsule between the molt from first to second instar is apparent. Measurements were determined with a micrometer fitted to a microscope. In photograph 2a, 20 tick marks is equal to approximately 0.5mm; in 2b, 20 tick marks is equal to approximately 0.67mm.

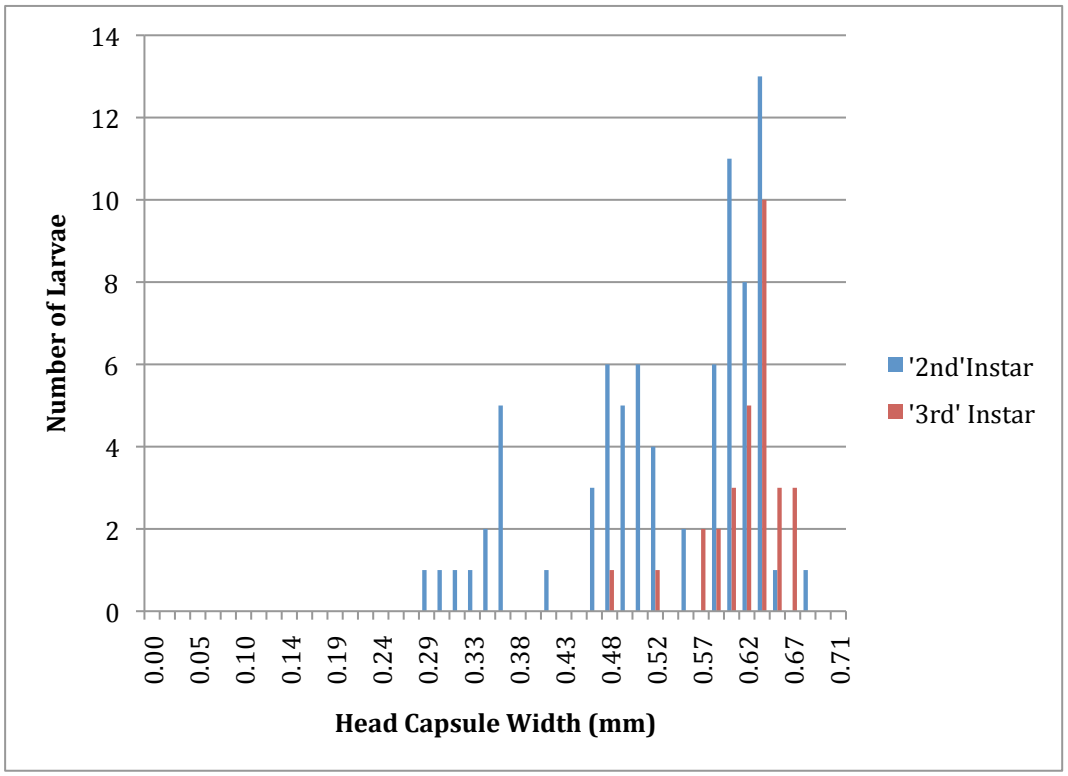

Figure 3
Graphical representation of discrete larval head capsule ranges. The terms '2nd' and '3rd' instar refer to how the larvae were characterized in accordance with morphological descriptions by Lewis (1970).

Table 1
Mean, range, and variance of head capsule widths as measured in larvae categorized as '2nd' and '3rd' instars. The number of '2nd' and '3rd' instar larvae comprising each cluster is also noted in the columns '2nd' Instar and '3rd' instar. Result from *t*-test and *post hoc* Tukey test, as well as results of one way ANOVA and *post hoc* Tukey test are also presented.

	Head Capsule Width*					
	Mean±Std Dev (mm)		**Range (mm)**	**Number of Larvae**	**Variance**	
Instar						
'2nd'	0.54±0.10 B		0.29-0.68	78	0.0100	
'3rd'	0.62±0.04 A		0.48-0.67	30	0.0017	
t-Test**	*P* <0.0001					
	Instar				**'2nd' Instar**	**'3rd' Instar**
Clusters	2nd	0.37±0.05 C	0.29-0.46	15	15	0
	3rd	0.54±0.02 B	0.48-0.60	41	40	1
	4th	0.63±0.05 A	0.62-0.67	52	23	29
One-way*** ANOVA	*P* <0.0001					

*Head capsules were measured dorsally at the widest point to determine width
** *t*-test conducted to indicate significant difference between head capsule width ranges for '2nd' and '3rd' instars.
*** One-way ANOVA conducted to indicate significant difference between head capsule width ranges of 2nd, 3rd, and 4th instars.

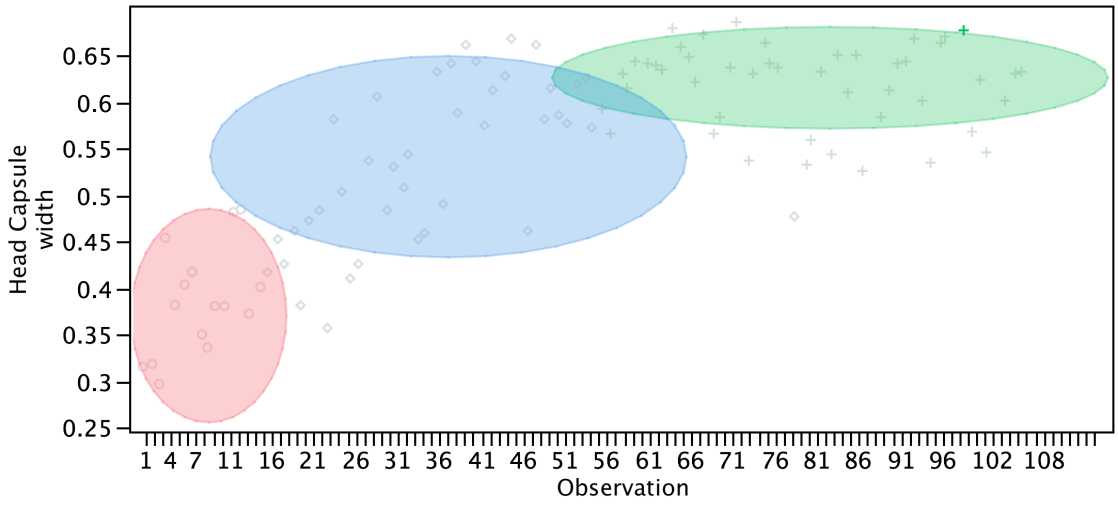

Figure 4
Scatter plot representation of K-cluster analysis of head capsule width measurements. Each cluster represents a statistically distinct range of head capsule widths. The horizontal axis represents the number of observations made, not the order in which the observations were made.

Final Instar

The exuvium from the egressed parasitoid was found internally attached to the emergence hole (Figure 5) in all 55 post-egression hosts that were examined. The structure extruding internally from the exit hole in the host was identified as the parasitoid's exuvium by the presence of the cuticle surrounding the anal vesicle, which still retained its shape (Figure 5). The lack of such both an exit wound and any tissue extruding from the host's body wall in the hundreds of previously dissected caterpillars indicated that the tissue found at the exit wound of post-egression caterpillars was left there by the egressing parasitoid. The exuvium passed through the exit wound and could be view externally with a microscope, where a small portion of it had stuck and dried to the host's body wall (Figure 6). The presence of a larval exuvium internally, extruding

from the exit wound, indicated that the parasitoid, upon egressing from the host, actually molted a final time, thus entering a new instar.

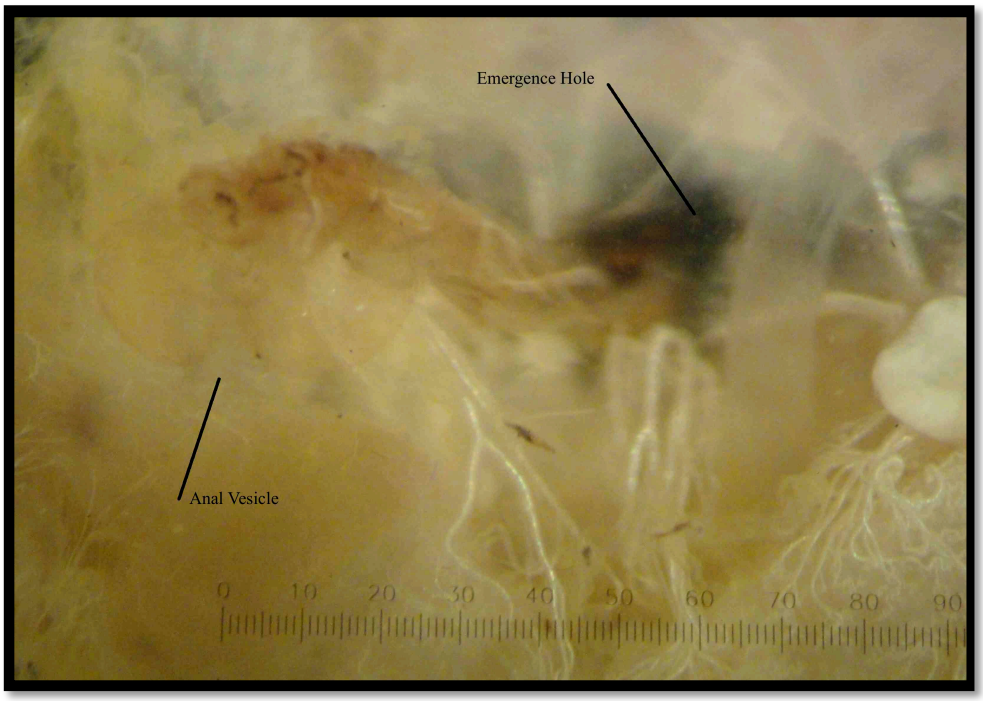

Figure 5
Internal view of the emergence hole through which the parasitoid egressed. The shed larval cast is visibly protruding from the wound, floating in the hemolymph of the host's abdominal cavity. The cuticle at the caudal end of the exuvium retains the shape of the anal vesicle. On the scale, 20 tick marks is equal to 0.5mm.

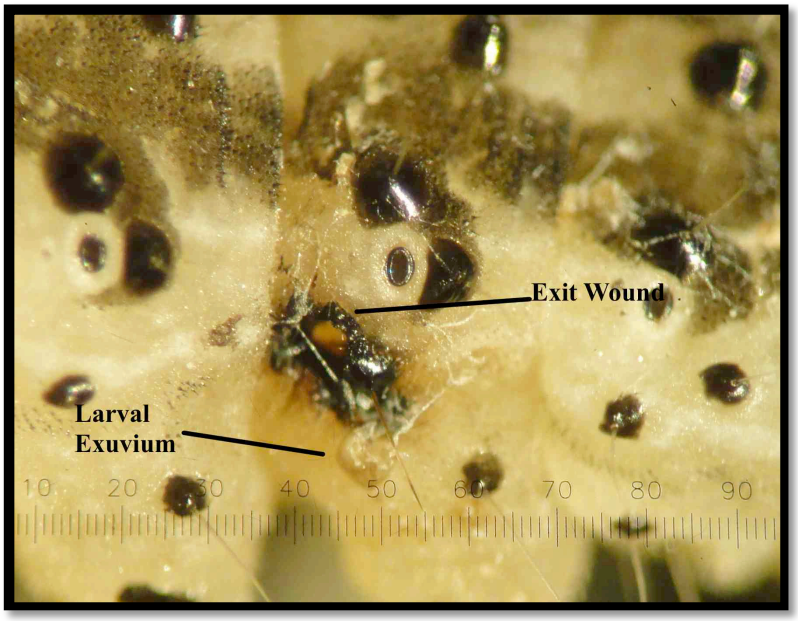

Figure 6
External view of the exit wound through which a parasitoid larva egressed from its host. A small portion of the larval exuvium is visibly sticking out of the wound. This photograph is a lateral view of the host in which the top of the photograph represents the dorsum. On the scale, 20 tick marks is equal to 0.5mm.

Discussion

Lewis' (1970) description of larval *Microplitis croceipes* life history and morphology makes note of three distinct instars. The first instar is characterized by large mandibles. During the molt from first to second instar, the head capsule is lost, and the mandibles are significantly reduced. According to Lewis (1970), the second instar molts around day 7 after parasitization, giving way to the third instar, which can be characterized by a gradual loss of the anal vesicle and increased definition of the spiracles. The third instar then egresses from the host, spins a cocoon, and pupates (Lewis, 1970).

As evidenced by the photograph in Figure 1, the molt from first to second instar was readily observed, and the reduction in mandible size between first and second instar was apparent (Figure 2). The molt from second to what Lewis (1970) describes as third instar was not observed during exploratory dissections of the host. Cluster analysis of head capsule measurements from larvae dissected from the host between days 5-8 after parasitization indicated the formation of three distinct clusters of head capsule widths: 0.29-0.46mm, 0.48-0.60mm, 0.62-0.67mm (Table 1). Overlap in the confidence interavals of two of the ranges (0.48-0.60mm and 0.62-0.67mm) can be explained by the presence of two outlining measurements taken from two '3rd' instars. The category of '2nd' instars (based on morphological descriptions by Lewis (1970)), contained larvae that were represented in all three clusters of head capsule width ranges (Figure 3). Larvae categorized as '3rd' instar were only present in the cluster range, 0.62-0.67mm, excluding a couple outliers. These data suggest that the second instar that Lewis (1970) describes is actually representative of three distinct instars, each with an average head capsule width occupying a distinct range (Figures 3 and 4). The highest range (0.62-0.67mm) included representatives from '2nd' *and* '3rd' instar larvae, suggesting that the larvae in this range, though described by Lewis (1970) as different instars, actually belong to the same instar. At this point, including the mandibulate first instar, four distinct instars of *M. croceipes* are identified: first (mandibulate larvae), second (0.29-0.46mm), third (0.48-0.60mm), and fourth (0.62-0.67mm). The exuvium found at the exit wound in the abdominal cavity of post-egression hosts (Figure 5), suggests that the

parasitoid larva molts from fourth to fifth instar as it egresses from the host. During this final (fifth) instar, the parasitoid larva spins the cocoon in which it pupates.

Literature regarding the natural history of larval *Microplitis spp*. indicates that the larvae pass through three or four instars (Khan and Ozer, 1988; Lewis, 1970; Luo et al., 2007; Puttler and Thewke, 1970; Song and Chen, 2008; Tawfik et al., 1980). Song and Chen (2008) make a brief mention of larval development in the genus *Microplitis*, and state that the number of larval instars varies between three and four, but they do not provide literature from which this information was gleaned, nor do they provide evidence of relevant studies they may have conducted. Khan and Ozer (1988), in their study of the larval life history of *Microplitis mediator*, state that the parasitoid has three instars, but provide head capsule measurements for the first instar only, which, like *M. croceipes*, possesses a distinctive mandibulate head capsule. The remaining instars are distinguished according to other morphological features. Khan and Ozer (1988) state that the final instar egresses from the host but make no mention of a molt occurring simultaneously with egression. Puttler and Thewke (1970) described the life history of *Microplitis feltiae* and indicated that its larvae pass through three instars as well. Methodology indicating the means by which the number of instars was examined is not made apparent. Head capsule measurements are provided for only the first and second instars, but are presented as single values (rather than ranges), and it is not indicated whether these values represent the measurements from single individuals or whether they are averages from a number of larvae. Puttler and Thewke (1970) do, however, indicate that *M. feltiae* molts as it egresses from the host. In this case, the second instar

molts into the third instar during egression. Luo et al., (2007) conducted a study on the life history of *Microplitis bicoloratus* in which they concluded the larvae pass through three instars. Instars in this study were distinguished by daily measurements of the width of the cephalic skeleton. However, it is not apparent *how* the measurements were used to indicate a molt from one instar to the next. In the data presented by Luo et al., (2007), head capsule widths between instars were not always significantly different, as was the case between a first instar range and a second instar range. Within the purported second instar, three ranges of cephalice skeleton widths were presented, one of which was significantly different from the other two ranges (Luo et al., 2007). Additionally, Luo et al. (2007) make no mention of a molt occurring during egression from the host. Finally, Tawfik et al. (1980) detailed the larval life history of *Microplitis rufiventris*, which they described as having three instars. Head capsule measurements were not taken, however, within each instar, measurements of the mandibles were made. Tawfik et al. (1980) do not mention a molt that occurs during egression, but rather state that the third instar larva egresses from the host and then spins its cocoon. Ambiguity in the literature regarding the number of instars in *Microplitis spp.* and sporadic mention of the occurrence of a molt during egression suggests the larval life history of this genus may not be well resolved. However, based on the data collected in the present study, it is suggested that *Microplitis croceipes* larvae pass through 5 instars.

CHAPTER IV

IN VITRO REARING AND ARTIFICIAL DIET DESIGN

Introduction

In vitro rearing on an artificial medium devoid of insect components represents a strategy by which parasitoids can be produced en masse, ideally at reduced cost, for the purpose of scientific research or release in biological control programs. In 1999, Consoli and Parra reported that 71 species of parasitoids had been reared, with some degree of success, on artificial diets; Hymenoptera represent 51 of those 71 species. The majority of *in vitro* rearing successes in Hymenoptera have involved the rearing of egg or pupal parasitoids (Grenier, 2009). Attempts at rearing endoparsitoids *in vitro* have produced less successful results. The following account details the development of a series of chemically defined diets and the attempted *in vitro* rearing of the endoparasitoid, *Microplitis croceipes*.

Materials and Methods

Rearing Protocol

Heliothis virescens and *Microplitis croceipes* were reared according to standard methodology in the Vinson lab. *H. virescens* larvae were reared on Corn Earworm diet, Bioserv., Inc., Frenchtown, NJ, USA) at 29˚C (12L:12D). Adult *M. croceipes* were maintained in a colony at 21˚C (12D:12L). Fourth instar *H. virescens* were individually parasitized and then incubated at 29˚C (12D:12L) until the time of parasitoid egression

and pupation, at which point cocoons were transferred to another incubator (21˚C; 12D:12L) until adult emergence.

Parasitizing

H. virescens larvae were parasitized in the late fourth instar (marked by head capsule slippage) by mated, female *M. croceipes*. Each caterpillar was individually presented to mated wasps in a closed container (never was more than one caterpillar in the container with the wasps). Each container housed 3-6 mated female wasps. The caterpillar was carefully observed and was promptly removed when the ovipositor a single wasp penetrated the caterpillar's cuticle. Any caterpillar stung more than once was not used in the study. Newly parasitized hosts were maintained at 29°C in 12D:12L conditions on an artificial diet. Twenty-four hours after parasitization, *H. virescens* larvae that had not molted to the 5^{th} instar were removed from the study. Parasitized hosts were then placed back in the incubator (29°C; 12D:12L) until dissection on day 4 after parasitization.

Diet Preparation

Stock Diet

A stock diet was prepared using HyClone TNM-FH (0.22µm filtered) insect medium (ThermoScientific, Logan, UT, USA) and was used as the base for all other diets. Development of the stock diet was largely based on the "artificial basic medium" developed by Kuriachan et al. (2006). The stock diet contained the following amino acids (added to the TNM-FH base) at concentrations of 1% w/v: L-asparagine (Gibco BRL, Grand Island, NY, USA), L-glutamine (Sigma, St. Louis, MO, USA), trans-4-

hydroxy-proline (Sigma, St. Louis, MO, USA), L-lysine (Sigma, St. Louis, MO, USA), L-serine (Sigma, St. Louis, MO, USA), L-threonine (Sigma, St. Louis, MO, USA), and L-valine (Sigma, St. Louis, MO, USA). Modifications to the Kuriachan et al. (2006) diet included the addition of 20ml (per 100ml of solution) fetal bovine serum albumin and the exclusion of trehalose and lacalbumin.

Diets

All diets were prepared from the stock diet. The stock diet was modified with the addition of glucose (Sigma, St. Louis, MO, USA), trehalose (Sigma, St. Louis, MO, USA), or protein (powdered chicken egg yolk, Sigma, St. Louis, MO, USA) to obtain the experimental diets. Nine artificial experimental diets were created and used in the *in vitro* rearing experiments: three glucose diets (12, 20, and 35µM/ml $*10^{-2}$), three trehalose diets (15, 26, and 35µM/ml), and three protein diets (0.5%, 1.0%, and 3.0%). Additionally, a positive control diet that lacked any sugar or protein additives was created from the stock. After evaluating the performance of larvae on each of the nine test diets, a combination diet was created using a mixture of additive concentrations used in the nine test diets. The methodology used in the creation of each type of diet (glucose, trehalose, protein, control, combination) is outlined in the sub-sections below. The test of each diet (the nine test diets, the control diet, and the combination diet) was replicated three times with 20 larvae in each replication. Trials consisted of single replications of two diets (e.g. a glucose and a trehalose diet). Trials did not consist of single replications for all diets simply because enough first instar parasitoids were not available to accomplish this ideal set up. Additionally, all three replications for each diet were

separated temporally to control for bias due to harvesting larvae from the same batch of parasitized caterpillars, and thus from the same batch of female parasitoids.

Glucose

Glucose concentrations were selected for testing based on known, daily hemolymph glucose levels of *H. virsecens* parasitized by *M. croceipes* (Dahlman and Vinson, 1975). The test concentrations selected were: 12, 20, and 35µM/ml $*10^{-2}$. These diets will be termed 12G, 20G, and 35G throughout the remainder of this paper. Glucose (Sigma, St. Louis, MO, USA) was dissolved in 100ml aliquots of stock diet to create each of the diets (12G, 20G, 35G). Following addition of glucose, the diet was sterilized by passing it through a Millipore 0.22µm GP Express Plus Membrane filter (Millipore, Billerica, MA, USA). Filtered diet was transferred to autoclave-sterilized glass jars. To each diet, 0.1% w/v powdered agar (Sigma, St. Louis, MO, USA) was added, and the solutions were heated under the hood and stirred with an ethanol (70%)-sterilized magnetic stirrer to dissolve the agar. Powdered agar was autoclaved in a tightly sealed glass jar before it was added to the diet. Finally, 2ml of penicillin-streptomycin (Invitrogen Corporation, Carlsbad, CA, USA) was added to each 100ml of diet. The diet was stored in a refrigerator until use.

Trehalose

Trehalose concentrations were also selected based on previous research investigating daily hemolymph trehalose levels in *H. virescens* parasitized by *M. croceipes* (Dahlman and Vinson, 1975). Concentrations tested were 15, 26, and 35µM/ml. These diets will be termed 15T, 26T, and 35T throughout the remainder of

this paper. Trehalose (Sigma, St. Louis, MO, USA) was dissolved in 100ml aliquots of stock diet to create each of the diets (15T, 26T, 35T). Following addition of trehalose, the diet was sterilized by passing it through a Millipore 0.22µm GP Express Plus Membrane filter (Millipore, Billerica, MA, USA). Filtered diet was transferred to autoclave-sterilized glass jars. To each diet, 0.1% w/v powdered agar (Sigma, St. Louis, MO, USA) was added, and the solutions were heated under the hood and stirred with an ethanol (70%)-sterilized magnetic stirrer to dissolve the agar. Finally, 2ml of penicillin-streptomycin (Invitrogen Corporation, Carlsbad, CA, USA) was added to each 100ml of diet. The diet was stored in a refrigerator until use.

Protein

The protein utilized in this study was powdered egg yolk (Sigma, St. Louis, MO, USA). Concentrations were tested at 0.5%, 1.0%, and 3.0% w/v. These diets will be termed 0.5P, 1.0P, and 3.0P throughout the remainder of this paper. The powdered egg yolk was dissolved in 100ml aliquots of stock diet to create the test diets (0.5P, 1.0P, and 3.0P). Following addition of the egg yolk powder, the diet was sterilized by passing it through a Millipore 0.22µm GP Express Plus Membrane filter (Millipore, Billerica, MA, USA). Filtered diet was transferred to autoclave-sterilized glass jars. To each concentration, 0.1% w/v agar (Sigma, St. Louis, MO, USA) was added, and the solutions were heated under the hood and stirred with an ethanol (70%) sterilized magnetic stirrer to dissolve the agar. Finally, 2ml of penicillin-streptomycin (Invitrogen Corporation, Carlsbad, CA, USA) was added to each 100ml of diet. The diet was stored in a refrigerator until use.

Control

The control diet was created by sterilizing 100ml aliquot of the stock diet, followed by the addition of 0.1% w/v agar (Sigma, St. Louis, MO, USA) and 2ml of penicillin-streptomycin. The diet was sterilized by passing it through a Millipore 0.22µm GP Express Plus Membrane filter (Millipore, Billerica, MA, USA). Filtered diet was transferred to autoclave-sterilized glass jars. To each concentration, 0.1% w/v agar (Sigma, St. Louis, MO, USA) was added, and the solutions were heated under the hood and stirred with an ethanol (70%) sterilized magnetic stirrer to dissolve the agar. Finally, 2ml of penicillin-streptomycin (Invitrogen Corporation, Carlsbad, CA, USA) was added to each 100ml of diet. The diet was stored in a refrigerator until use.

Combination Diet

A tenth experimental diet, termed 'combination diet,' was created by combining trehalose and protein concentrations that were selected on the basis of parasitoid performance on the original nine diets (12G, 20G, 35G, 15T, 26T, 35T, 0.5P, 1.0P, and 3.0P). Performance of the nine diets was evaluated mainly on the basis of larval growth, but larval mortality and molting were also considered. Methodology regarding evaluation of these performance measures is detailed in the next section. In deciding on a combination diet, glucose diets were excluded from consideration due to poor performance of larvae on the glucose diets, in terms of growth. Concentrations of 35µM/ml trehalose and 3.0% w/v protein were selected as the additive concentrations for the combination diet. These selections were made based on the results of a Tukey test (not shown in this report) comparing mean growth parameters (length and width) for

each of the nine test diets that indicated larval growth was highest on the 3.0P and 35T diets. The combination diet was prepared in accordance with the methodology used to prepare the original nine diets.

Experimental Set-Up

Parasitized hosts were dissected on day 4 after parasitization (with day 0 being the day of parasitization). Caterpillars were surface-sterilized for 10min in 70% ethanol, and were then transferred to a beaker filled with autoclave-sterilized, deionized water. Hosts were dissected in HyClone TNM-FH (0.22μm filtered) insect medium (Fisher Scientific, Fair Lawn, NJ, USA) with dissection tools that were flame-sterilized. All dissection tools were sterilized between each dissection.

Each host was individually dissected by making an incision in the abdominal cuticle, along the length of the dorsal vessel. Once the abdomen of the host was opened, the abdominal cavity was flushed with TNM-FH using an autoclave-sterilized glass pipette. Flushing of the abdominal cavity was done in order to free the parasitoid larva, which was often obscured by host tissues. Only first instar parasitoid larvae were used in the artificial diet experiments. The liberated parasitoid larvae were transferred from the dissecting dish to another dish containing HyClone TNM-FH with an ethanol (70%)-sterilized inoculating loop. Once deposited in the second dish of TNM-FH medium, the larvae were gently washed by pipetting the TNM-FH up and down several times with a sterilized glass pipette. The turbulence created by pipetting the medium aided in removing debris (particles from the host's abdominal cavity) from the parasitoid larva. Care was taken not to suck the larva up into the pipette to avoid damaging its cuticle.

After washing, the initial length and width of each parasitoid larva was measured before it was transferred from the washing dish to the diet. Length (dorsal) was measured from the tip of the head (rostrum) to the posterior end of the dorsal vessel (excluding anal vesicle). Width was measured dorsally at the widest part of the abdomen (excluding anal vesicle). Measurements were taken at 1x magnification using a micrometer adapted to a microscope. The eyepiece micrometer was scaled with a stage micrometer (20mm diameter disc micrometer reticle (Cambridge Instruments, Buffalo, NY, USA)). All larvae used in the artificial diet experiments were first instar. Larvae were classified as either mid- or late-first instars by observing the width of the head capsule, compared to the width of the body. If the head capsule and body were approximately the same width, the larva was categorized as a mid-first instar; if the body was notably wider than the head capsule, the larva was categorized as a late-first instar. Mid- and late-first instar classification will henceforth be termed Initial Size 1 and Initial Size 2, respectively.

After measurements, the larva was carefully removed from the washing dish with an ethanol (70%)-sterilized inoculating loop and transferred into a well of a sterile Nunclon Surface (Nalge Nunc International, Denmark, Europe) cell culture plate. Each plate contained 24 wells, 20 of which were filled with 125µl of a single artificial diet. Because there were three replications for each diet, and each replication contained 20 larvae, each culture plate constituted a single replication. Once a single larva had been placed in each of the 20, diet-filled wells, the cell culture plate was sealed with parafilm, then wrapped in aluminum foil (to simulate the dark environment inside the host) and

kept in a plastic box. The box containing the cell culture plate was placed in an incubator (29°C; 12D:12L) for the duration of the trial (9 days). Plates were removed from the incubator once a day. During this period (approximately every 24hrs), the larvae were transferred to fresh diet to escape the growth of mold (mold growth was observed on diet in preliminary trials 36-48hrs after placement of the cell culture plate in the incubator). Additionally, the larvae were observed for signs of molting and mortality (methodology in the next section).

A negative control was provided for each trial. Five parasitized caterpillars were selected (from the group of hosts being dissected) and set aside at the time when parasitoids were being dissected from their hosts for placement on the artificial diets (day 4 after parasitization). These caterpillars were placed in cell culture wells with a small amount of food (the artificial diet upon which the laboratory colony was reared) and placed in the incubator (29°C; 12D:12L) with the cell culture plate containing larvae on the artificial diet. Caterpillars used as a negative control were monitored for parasitoid egression and used as a reference for normal parasitoid developmental time.

Larval Performance

Performance of the larvae on each diet was evaluated in terms of growth (change in length and width), molting to a new instar, and survival. The larvae were observed once every day (approximately every 24 hours) during a 9-day trial period. Incidences of molting (from first to second instar) or death were noted each day for each larva. A larva molted if it shed its head capsule. Though the shed head capsule was not always visible in the diet, the notable difference in the mandibles between first and second instars of *M.*

croceipes was used to indicate a molt. A larva was determined to be dead if the dorsal vessel was no longer pumping hemolymph. Larvae that died before the end of the trial period were discarded and not counted in final length and width measurements, which were made on day 9 of each trial. Final length measurements (dorsal) were taken from rostrum to the base of the anal vesicle, and final width measurements were taken dorsally at the widest part of the abdomen.

Statistical Analysis

Software

Data were analyzed with either JMP® Statistical Discovery Software, Version 9.0.0 (2010, SAS Institute Inc. Cary, NC USA version 9.0.0) or Statistix 9 Analytical Software, Version 9 (1985-2008, Tallahassee, FL USA), as noted in each results section.

Larval Growth

Growth data were evaluated using Statistix 9 Analytical Software, Version 9. Two-way ANOVA (Type III SS) was performed to examine growth among the test diets (20G, 35G, 15T, 26T, 35T, 1.0P, 3.0P) and control diet; diets that produced 100% mortality were excluded from analysis (12G and 0.5P)). Effects of diet and initial size (and their interaction) on larval growth (change in length and width) were analyzed. Significant means were compared *post hoc* using a Tukey Test.

Mean growth (length and width) in Initial Size 1 and Initial Size 2 individuals was examined for each diet (20G, 35G, 15T, 26T, 35T, 1.0P, 3.0P, Control). Means were compared with a two-sample t-test, and significant means were compared *post hoc* with a Tukey Test.

Performance of the combination diet, in terms of larval growth, was evaluated in comparison to larval growth in the 35T and 3.0P diets. A Kruskal-Wallis one-way nonparametric ANOVA was performed to determine whether growth (length and width) differed significantly in the combination diet, compared to the 35T and 3.0P diets. Significant means were compared *post hoc* with a Tukey Test.

Larval Molting

Molting data were analyzed with JMP® Statistical Discovery Software. Diets were examined for significance in the mean number of individuals that molted to second instar using a two-way ANOVA. Effects of diet, initial size, and the interaction of diet and initial size were examined. Molting in the combination diet was analyzed (two-way ANOVA) separately in conjunction with the 35T and 3.0P diets. Significant means produced by each ANOVA were compared *post hoc* with a Tukey test.

Larval Mortality

Survival data were analyzed with JMP® Statistical Discovery Software. Survival was evaluated with a two-way ANOVA that examined the effects diet, initial size, and the interaction of diet and initial size on mean number of individuals that survived on each diet (20G, 35G, 15T, 26T, 35T, 1.0P, 3.0P, Control). Survival on the combination diet was tested (two-way ANOVA) separately in conjunction with survival in the 35T and 3.0P diets. For each ANOVA, significant means were compared *post hoc* with a Tukey test.

Results

Larval Growth

Initial length and width measurements were subtracted from final length and width measurements for each diet to obtain growth values for length and width. Mean change in length within diets differed significantly between Initial Size 1 and Initial Size 2 larvae only for diets 35G and 35T (Table 2). Mean change in width within diets differed significantly between Initial Sizes 1 and 2 for 35T only (Table 2).

Change in length between diets, was significantly influenced by diet and initial size (p =0.0000 and p = 0.0064, respectively), but not by the interaction of diet and initial size (p = 0.1692) (Table 3). *Post hoc* comparison of significant means revealed larvae on the 3P diet experienced the greatest mean increase in length (0.251mm±0.079); larvae on the 35G diet faired the worst (-0.396mm±0.058) (Table 4; Figure 7). The negative mean change in length resulted from what is termed here as 'shriveling' of the larvae. Larvae appeared to shrink and become malformed as a result of being placed on a poor diet. However, shriveled larvae were still alive, as indicated by pumping of the dorsal vessel, and thus were measured for final length and width.

Change in width between diets was influenced by diet and the interaction of diet and initial size (p =0.0000 and 0.0004, respectively), but not by initial size alone (p = 0.9695) (Table 5). *Post hoc* comparison of significant means indicated that the pattern of significance was not homogenous (Table 6 and Table 7). However, within the protein diets, there was a trend for increased widthwise growth with increasing concentration of protein (Figure 8).

Mean changes in both length and width between the combination diet and the 35T and 3.0P diets were significantly influenced by diet (p =0.0000 for length and p =0.0390 for width). *Post hoc* analysis of the means indicated that mean change in length of larvae on the combination diet was significantly larger than that of larvae on the 35T and 3.0P diets (Table 8). However, *post hoc* analysis of the mean changes in width indicated that change in width of larvae did not differ significantly between diets (Table 9).

Table 2

Mean change in length and width for each size class in each diet. Initial Size 1 indicates mid-first instar; Initial Size 2 indicates late-first instar. *Post hoc* Tukey test results are also listed. Means were compared *between* size classes for each diet.

		Mean Change In Size (mm)*		
	Diet	**Initial Size 1**	**Initial Size 2**	**P-value**
Length	Control	-0.14±0.31 A	-0.50±0.71 A	0.5978
	20G	-0.25±0.07 A	-0.10±0.15 A	0.4059
	35G	-0.28±0.06 A	-0.51±0.09 B	0.0438
	15T	-0.06±0.09 A	-0.40±0.20 A	0.1440
	26T	0.09±0.06 A	0.04±0.09 A	0.6603
	35T	0.38±0.11 A	-0.70±0.46 B	0.0300
	1.0P	0.23±0.04 A	0.12±0.10 A	0.1329
	3.0P	0.24±0.21 A	0.27±0.22 A	0.0744
Width	Control	0.01±0.11 A	-0.05±0.21 A	0.7515
	20G	0.09±0.04 A	0.30±0.09 A	0.0506
	35G	0.09±0.02 A	0.06±0.03 A	0.4948
	15T	0.08±0.03 A	0.10±0.06 A	0.7258
	26T	0.15±0.02 A	0.18±0.04 A	0.3992
	35T	0.29±0.04 A	-0.20±0.16 B	0.0047
	1.0P	0.11±0.03 A	0.18±0.06 A	0.3103
	3.0P	0.16±0.10 B	0.38±0.11 A	0.0014

* Means not connected with the same letter are significantly different (comparison *within* diet only)

Table 3

Summary of two-way ANOVA (Type III SS) results for mean change in length.

Source	**DF**	**Mean Squares**	**F-value**	**P-value**
Initial Size	1	10.0365	7.58	0.0064
Diet	7	12.3111	9.3	0.0000
Initial Size*Diet	7	1.9813	1.5	0.1692
Error	229	1.3235		

Table 4
Summary of Tukey test results showing the effects of diet and initial size on mean change in length. Levels not connected with the same letter are significant.

Source	Mean±SE	
Diet		
20G	-0.173±0.190	ABC
35G	-0.396±0.058	C
15T	-0.228±0.142	ABC
26T	0.065±0.063	AB
35T	-0.160±0.184	ABC
1P	0.174±0.089	AB
3P	0.251±0.079	A
Control	-0.318±0.136	BC
Initial Size		
1	0.026±0.029	A
2	-0.222±0.085	B

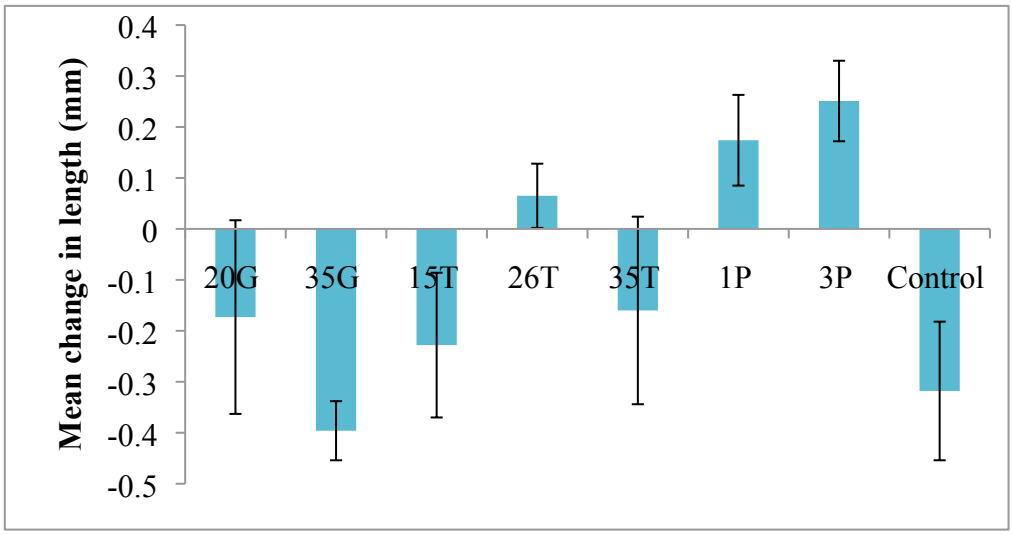

Figure 7 Graphical representation of the effect of diet on lengthwise growth. The graph is based on statistical significance indicated in Table 3. Error bars indicate standard error.

Table 5
Summary of two-way ANOVA (Type III SS) results for mean change in width.

Source	DF	Mean Squares	F-value	P-value
Initial Size	1	0.00029	0	0.9695
Diet	7	1.15329	5.78	0.0000
Initial Size*Diet	7	0.78709	3.95	0.0004
Error	229	0.1995		

Table 6
Summary of Tukey test results showing the effects of diet on mean change in width. Levels connected with an asterisk are significant.

Diet	Mean±SE							
		1.0P	**3.0P**	**Control**	**15T**	**20G**	**26T**	**35G**
1.0P	0.144±0.035							
3.0P	0.270±0.031	0.13						
Control	-0.019±0.053	0.16	0.29*					
15T	0.089±0.055	0.06	0.18	0.11				
20G	0.195±0.074	0.05	0.07	0.21	0.11			
26T	0.164±0.025	0.02	0.11	0.18*	0.08	0.03		
35G	0.071±0.022	0.07	0.20*	0.09	0.02	0.12	0.09	
35T	0.044±0.072	0.23	0.23	0.06	0.04	0.15	0.12	0.03

Table 7
Summary of Tukey test results showing the interaction between diet and initial size for mean change in width. Levels connected with an asterisk are significant.

Diet, Initial Size	Mean±SE						
		1.0P,1	**3.0P,1**	**Control,1**	**15T,1**	**20G,1**	**26T,1**
1.0P,1	0.108±0.028						
3.0P,1	0.157±0.021	0.05					
Control,1	0.012±0.034	0.10	0.14*				
15T,1	0.078±0.047	0.03	0.08	0.07			
20G,1	0.091±0.043	0.02	0.07	0.08	0.01		
26T,1	0.146±0.028	0.04	0.01	0.13	0.07	0.06	
35G,1	0.085±0.025	0.02	0.07	0.07	0.01	0.01	0.06
35T,1	0.289±0.024	0.18	0.13*	0.28*	0.21*	0.20*	0.14*
1.0P,2	0.180±0.063	0.07	0.02	0.17	0.10	0.09	0.03
3.0P,2	0.383±0.058	0.28	0.23*	0.37*	0.31*	0.29*	0.24*
Control,2	-0.05±0.100	0.16	0.21	0.06	0.13	0.14	0.20
15T,2	0.100±0.100	0.01	0.06	0.09	0.02	0.01	0.05
20G,2	0.300±0.141	0.19	0.14	0.29	0.22	0.21	0.15
26T,2	0.183±0.041	0.08	0.03	0.17	0.10	0.09	0.04
35G,2	0.057±0.038	0.05	0.10	0.05	0.02	0.03	0.09
35T,2	-0.200±0.141	0.31	0.36	0.21	0.28	0.29	0.35
Diet, Initial Size	**Mean±SE**	**35G,1**	**35T,1**	**1.0P,2**	**3.0P,2**	**Control,2**	**15T,2**
35G,1	0.085±0.025						
35T,1	0.289±0.024	0.2*					
1.0P,2	0.180±0.063	0.10	0.11				
3.0P,2	0.383±0.058	0.3*	0.09	0.20			
Control,2	-0.05±0.100	0.13	0.34	0.23	0.43*		
15T,2	0.100±0.100	0.02	0.19	0.08	0.28	0.15	
20G,2	0.300±0.141	0.22	0.01	0.12	0.08	0.35	0.20
26T,2	0.183±0.041	0.10	0.11	0.00	0.20	0.23	0.08
35G,2	0.057±0.038	0.03	0.23*	0.12	0.33*	0.11	0.04
35T,2	-0.200±0.141	0.28	0.29	0.38	0.58*	0.15	0.30
Diet, Initial Size	**Mean±SE**	**20G,2**	**26T,2**	**35G,2**			
20G,2	0.300±0.141						
26T,2	0.183±0.041	0.12					
35G,2	0.057±0.038	0.24	0.13				
35T,2	-0.200±0.141	0.50	0.38	0.26			

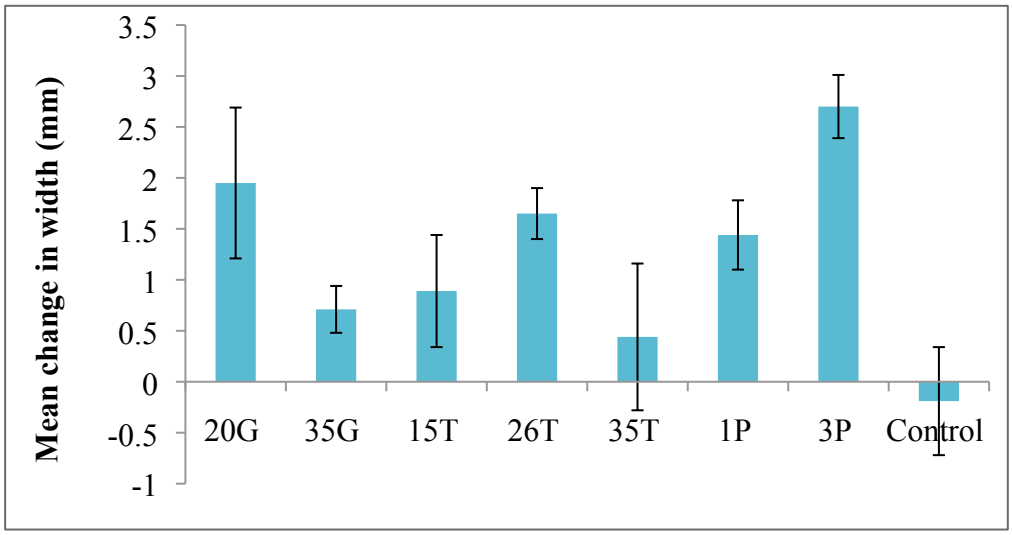

Figure 8
Graphical representation of the effect of diet on widthwise growth. The graph is based on statistical significance indicated in Table 5. Error bars indicate standard error.

Table 8
Summary of Kruskal-Wallis All-pairwise Comparison results showing the effects of diet on mean change in length for the combination diet, 35T, and 3.0P diets only. Levels not connected by the same letter are significantly different.

Source	Mean(mm)±SE	
Diet		
Combo	0.95±0.06	A
35T	0.63±0.11	B
3.0P	0.57±0.03	B

Table 9
Summary of Kruskal-Wallis All-pairwise Comparison results showing the effects of diet on mean change in width for the combination diet, 35T, and 3.0P diets only. Levels not connected by the same letter are significantly different.

Source	Mean±SE	
Diet		
Combo	6.80±0.02	A
35T	8.87±0.04	A
3.0P	6.92±0.02	A

Larval Molting

Mean number of larvae that molted from first to second instar was found to be significantly influenced by diet (p =0.0001), but not by either initial size or the interaction of diet and initial size (p =0.8164 and p =0.2733, respectively) (Table 10). Mean number of larvae that molted was highest on the 3.0P diet. No larvae molted on the 12G, 20G, or 0.5P diets (Table 11; Figure 9).

A comparison of molting in the combination diet and 35T and 3.0P diets indicated that neither diet, nor initial size, nor the interaction of diet and initial size significantly influenced the mean number of individuals that molted (p =0.1908; p =0.6995; p =0.4506, respectively) (Table 12).

Larval Mortality

Larval survival was significantly influenced by diet (p = <0.0001), but not by initial size or the interaction of diet and initial size (p =0.4929 and p =0.4706, respectively) (Table 13). Survival was highest on the 35G and 3.0P diets (Table 14; Figure 10).

A comparison of survival on the combination diet and the 35T and 3.0P diets showed that diet significantly influenced survival (p =0.0014), but that initial size and the interaction of diet and initial size did not (p =0.4273 and p =0.4932, respectively) (Table 15). Survival did not differ between the combination diet and the 3.0P diet, but was significantly higher for those two diets than larval survival on the 35T diet (Table 16).

Table 10
Summary of two-way ANOVA examining the effects of diet, size, and the interaction of diet and size on the mean number of individuals that molted to second instar.

Source	DF	Sum of Squares	F Ratio	*P*-value
Diet	9	2.123	4.004	<0.0001
Size	1	0.003	0.054	0.8164
Diet*Size	9	0.652	1.230	0.2733

Table 11
Summary of Tukey test results comparing the effects of diet on mean number of individuals that molted to second instar. Levels not connected by the same letter are significantly different.

Diet	Least Square Mean	
12G	0.000	B
20G	0.000	B
35G	0.079	B
15T	0.009	B
26T	0.081	B
35T	0.061	AB
0.5P	0.000	AB
1.0P	0.120	AB
3.0P	0.318	A
Control	0.000	B

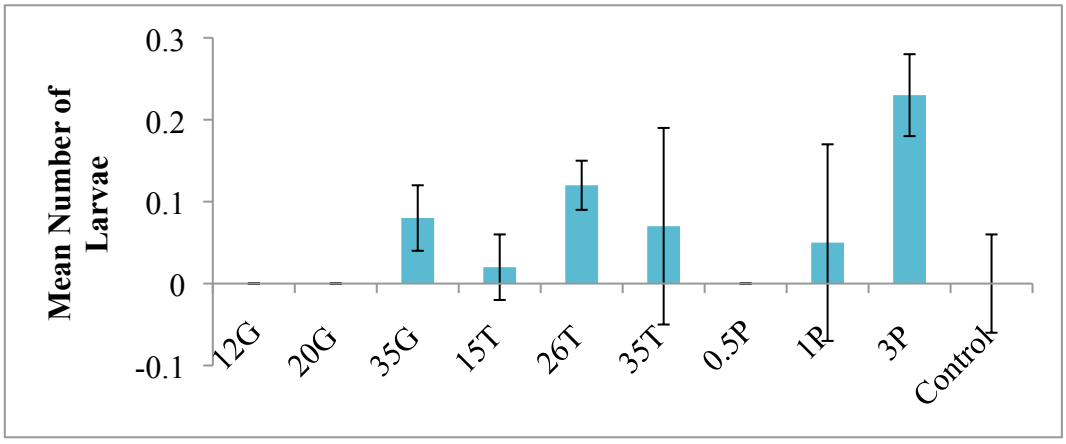

Figure 9 Graphical representation of the effect of diet on larval molting. The graph is based on statistical significance indicated in Table 10. Error bars indicate standard error.

Table 12
Summary of two-way ANOVA examining the effects of diet, initial size, and the interaction of diet and initial size on mean number of individuals that molted to second instar on the combination, 35T, and 3.0P diets *only*.

Source	DF	Sum of Squares	F Ratio	*P*-value
Diet	2	0.514	1.673	0.1908
Size	1	0.023	0.150	0.6995
Diet*Size	2	0.246	0.801	0.4506

Table 13
Summary of two-way ANOVA examining the effects of diet, initial size, and the interaction of diet and initial size on survival.

Source	DF	Sum of Squares	F Ratio	*P*-value
Diet	9	25.681	18.324	<0.0001
Size	1	0.073	0.471	0.4929
Diet*Size	9	1.348	0.962	0.4706

Table 14
Summary of Tukey test results comparing the mean number of individuals that survived on each diet. Levels not connected by the same letter are significantly different.

Diet	Least Square Mean	
12G	0.000	F
20G	0.160	EF
35G	0.859	A
15T	0.228	DEF
26T	0.655	ABC
35T	0.474	ABCDE
0.5P	0.000	CDEF
1.0P	0.553	BCD
3.0P	0.863	AB
Control	0.402	CDE

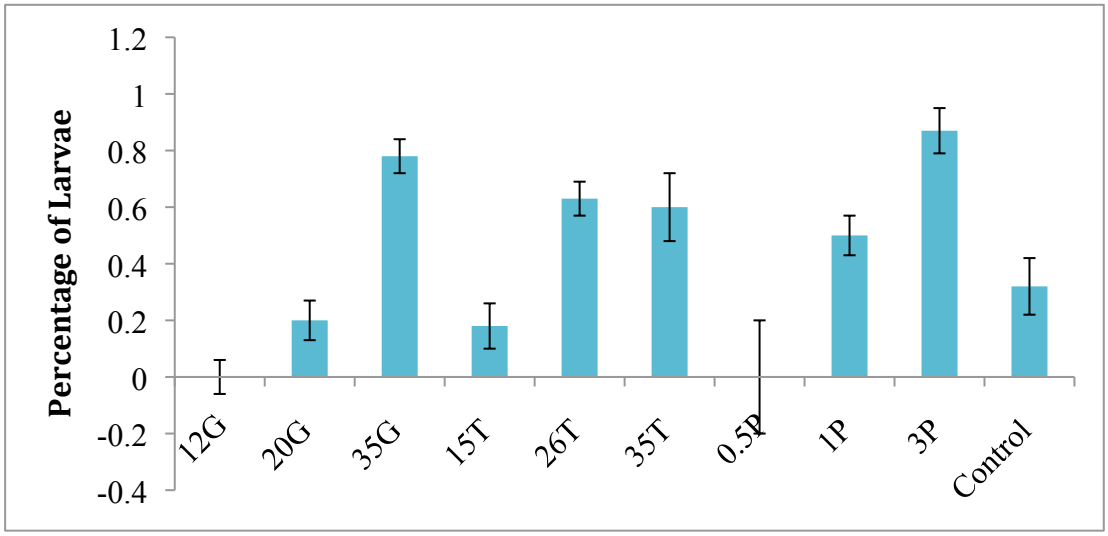

Figure 10 Graphical representation of the effect of diet on larval survival. The graph is based on statistical significance indicated in Table 13. Error bars indicate standard error.

Table 15
Summary of two-way ANOVA examining the effects of diet, initial size, and the interaction of diet and initial size on survival in the combo diet, 35T, and 3.0P diets *only*.

Source	DF	Sum of Squares	F Ratio	*P*-value
Diet	2	1.800	6.799	0.0014
Size	1	0.084	0.633	0.4273
Diet*Size	2	0.188	0.780	0.4932

Table 16
Summary of Tukey test results comparing the effect of diet on the mean number of individuals that survived on each diet. Levels not connected by the same letter are significantly different.

Diet	Least Square Mean	
35T	0.982	B
3.0P	0.862	A
Combo	0.474	A

Discussion

Diet Performance

Larval Growth

Larval growth on the test diets (20G, 35G, 15T, 26T, 35T, 1.0P, 3.0P, Control) was significantly influenced by diet type for both length and width. Length was also significantly influenced by initial size of the larvae (Initial Size 1 = mid-first instar; Initial Size 2 = late-first instar), but width was not. Mid-first instars exhibited greater change in length than did late-first instars. The influence of initial size on change in length of the parasitoids possibly suggests that lengthwise growth slows as the parasitoid progresses towards a molt; the cuticle of late-first instars (which are larger than mid-first

instars) may not be able to accommodate as great a change in length as was observed in mid-first instars.

Change in width was not significantly influenced by initial size of larvae, which could possibly indicate that larvae exhibit greater change in width earlier within the first instar (early first instars) and exhibit greater change in length later within the first instar (mid-first instars). Though width was not significantly influenced by initial size, it was significantly influenced by the interaction of diet and initial size. Comparison of mean change in growth with respect to the influence of diet and initial size indicated that the pattern of significance was not homogenous (Table 7). As a result, a discernable pattern of significance among mean changes in width was difficult to identify, indicating the influence of the interaction varied within and between diets.

The combination diet was prepared with concentrations of trehalose and protein identical to those concentrations used in the 35T and 3.0P diets. Growth of larvae on the combination diet was evaluated in comparison to growth of larvae on the 35T and 3.0P diets only. Diet significantly influenced change in both length and width in evaluation of larval growth on the combination diet, 35T, and 3.0P diets. In terms of mean change in length, the combination diet produced the best result. Though diet significantly influenced change in width for these diets, *post hoc* analysis indicated the influence was not strong enough to produce significantly different changes in width between the diets. Significantly greater lengthwise growth exhibited by parasitoid larvae on the combination diet may possibly suggest an interactive effect between the trehalose and

protein that produces a more optimal diet than either the 35T or 3.0P diets alone. However, possible interactions of these two additives were not examined directly.

Larval Molting

The molt from first to second instar was observed during the course of this study, but no larvae progressed further than the second instar. Molting was significantly influenced by diet, and the 3.0P diet had the highest mean number of individuals that molted. Mean number of molts appeared to increase with increasing concentration of sugar or protein for all of the diets (Figure 9). No larvae molted on the 0.5P diet, however, 1.0P and 3.0P produced more molts than any other diet.

A comparison of the mean number of larvae that molted on the combination, 35T, and 3.0P diets indicated that neither diet, nor initial size, nor the interaction of diet and initial size significantly influenced molting. The combination, 35T, and 3.0P diets did not differ significantly in terms of molting.

Larval Survival

Diet significantly influenced larval survival, and survival was highest on the 35G diet and lowest on the 12G diet. Generally speaking, larval survival appeared to increase as the concentration of sugar or protein increased. This trend however, was not true for trehalose, in which survival increased from 15T to 26T but decreased from 26T to 35T. Despite lower survival rates, larvae on the 35T diet exhibit greater lengthwise growth and more molts than did larvae on the 26T diet. In light of those data, lower survival on the 35T diet may have possibly resulted from experimenter error, such as mishandling of the larvae during transfer to new diet.

A comparison of the combination diet with the 35T and 3.0P diets indicated that diet also significantly influenced survival among these diets. Larval survival was significantly higher in the combination and 3.0P diets, compared to the 35T diet.

Experimental Errors

Design of this experiment was not ideal in several respects. First and foremost, a trial consisted of the testing of only a pair of diets (e.g. 12G and 35G or 12G and 20G), and the control diet was not included in every trial. The three control diet replications were used in the comparison for all diet results. Due to limitations in the number of first instar parasitoid larvae available and the time it took to set up a pair of diet replications (4-6 hours), an ideal experimental set-up, with each trial consisting of a single replication of all 9 tests diets and the control diet, was simply not feasible. Additionally, the number of mid- and late-first instars used in each diet trial was not equal. Because parasitoid development was variable, it was not possible to obtain equal numbers of mid- and late-first instars for each diet trial. Therefore, additional data are needed to understand potential success rate differences between mid- and late-first instars on the diets.

Conclusions

Overall, data indicated the importance of protein and trehalose in promoting larval development. Greatest mean increases in both length and width, as well as highest mean number of molts, were observed in larvae fed on diets supplemented with trehalose or protein. Larvae on the combination diet, which contained both trehalose and protein, also exhibited growth and molting.

In choosing test concentrations of glucose, trehalose, and protein, the goal was to mimic hemolymph conditions the parasitoid larva might experience *in vivo*. Glucose and trehalose values were selected on the basis of previous research detailing glucose and trehalose concentrations in the host hemolymph during various stages of larval parasitoid development (Dahlman and Vinson, 1975). The study by Dahlman and Vinson (1975) indicated that glucose concentrations in the hemolymph fell sharply from an average of 34.69 to an average of 13.46µM/ml (10^{-2}) between day 1 and day 3 after parasitization. Average concentration dropped again from 14.21 to 5.00µM/ml (10^{-2}) between day 6 and day 9. Glucose concentrations tested in this study were 12, 20, and 35µM/ml (10^{-2}) and were chosen as representatives of the range of glucose concentrations experienced by parasitoid larvae *in vivo*.

Endoparasitoids are known to manipulate the physiology of their host to accommodate their nutritional requirements during development (Vinson and Iwantsch, 1980). In light of this information it could be hypothesized that *M. croceipes* larvae manipulate the glucose production by the host. If that is the case, decreasing glucose concentration in the host hemolymph during parasitoid development could suggest that host resources are being allocated toward the production of other nutrients (such as trehalose), which may suggest the parasitoid does not require a lot of glucose during development. Larval performance, in the present study, however, appeared to improve with increasing concentration of glucose. This is perhaps contradictory to larval glucose-needs *in vivo*, as described by Dahlman and Vinson (1975), in which glucose concentration in the hemolymph of a parasitized caterpillar was found to decrease after

parasitization, possibly suggesting the parasitoid larva might require less glucose as it progresses developmentally. An alternative hypothesis, and one that better fits the data in this study (in which host performance was better with increasing glucose), is that parasitoid consumption of glucose may not be offset by increased host production of glucose. Under this hypothesis, glucose concentration in the host's hemolymph would decrease over time during parasitoid development as the parasitoid consumes glucose.

Trehalose concentrations were selected for testing in the same manner as glucose concentrations. At day 3 after parasitization, trehalose levels in the host hemolymph averaged 14.00μM/ml; between days 6 and 9, the average concentration fluctuated between 39.22 and 25.75 μM/ml. The concentrations tested in this study (15, 26, and 35μM/ml) were chosen as representatives of approximate host conditions during progression of parasitoid larval development. The general trend in this study was that larval performance increased as trehalose concentration increased. Considering the data collected in this study, along with the data presented by Dahlman and Vinson (1975), it may be hypothesized that the host increases production of trehalose during parasitoid development.

Protein concentrations tested were 0.5%, 1.0%, and 3.0%. Concentrations higher than 3.0% were not tested simply because the diet became too thick to adequately filter (see methodology). In the interest of uniformity, powdered chicken egg yolk (see methodology) was used as the protein source, which deviated from the use raw egg yolks in similar research (Kuriachan et al., 2006). Larval growth, survival, and molting were found to increase with increasing protein concentration in this study. Analysis of protein

composition of the hemolymph of *H. virescens* parasitized by *M. croceipes* showed a decrease in the concentration of conjugated protein in parasitized caterpillars, compared to non-parasitized caterpillars (Barras et al., 1972). Dahlman and Vinson (1975) suggest this shift in the protein profile of hemolymph in parasitized caterpillars occurs as a result of the consumption of conjugated proteins by the developing *M. croceipes* larva. Increased performance of *M. croceipes* larvae with increasing dietary protein concentration in the present study adds support to the hypothesis presented by Dahlman and Vinson (1975).

Microplitis croceipes larvae, in this study, successfully molted from first to second instar *in vitro*. Though number of individuals that made this molt was small (54 out of 661 larvae), the fact that molting did occur indicates that larvae are capable of making the molt from first to second instar *in vitro*. As of yet, *M. croceipes* has only been reared *in vitro* from egg to first instar. Greany (1986) successfully reared *M. croceipes* from post-germband eggs to late first instar on an artificial diet. Diet supplemented with host hemolymph and co-cultured host fat body promoted the highest hatching rate and the highest levels of growth and survival among larvae (Greany, 1986). However, larvae in Greany's (1986) study did not molt from first to second instar. Data collected in the present study shed light on the dietary needs of *Microplitis croceipes* larvae, but the attempt at rearing this species to adulthood *in vitro* was not successful.

CHAPTER V

PARASITOID LARVAL EGRESSION AND THE ROLE OF THE HOST IN PARASITOID PUPATION

Introduction

Endoparasitoid larvae develop inside the host but pupate external to the host. Typical of microgastrine parasitoids, *Microplitis croceipes*, egresses from the host by creating a hole in the host integument, from which it then emerges. After egressing from the host, the parasitoid larva begins spinning its cocoon a short distance from the host. Egression of the larval *M. croceipes* from the host is only briefly mentioned in the literature (Lewis, 1970). This section will focus on egression of the parasitoid from the host and the molt that occurs simultaneously. The means by which the parasitoid egresses (and molts) will be discussed, as well as implications of this final molt on the ability to rear *M. croceipes* to adulthood *in vitro*. Additionally, post-egression defensive behavior exhibited by the host is described, and the maintenance of this behavior due to the means by which the parasitoid egresses will be investigated and discussed relative to the host usurpation hypothesis (Brodeur and Vet, 1994).

Materials and Methods

Rearing Protocol

Heliothis virescens and *Microplitis croceipes* were reared according to standard methodology in the Vinson lab. *H. virescens* larvae were reared on Corn Earworm diet

(Bioserv., Inc., Frenchtown, NJ) at 29˚C (12L:12D). Adult *M. croceipes* were maintained in a colony at 21˚C (12D:12L). *H. virescens* larvae were parasitized in the late fourth instar (marked by head capsule slippage) by mated, female *M. croceipes*. Each caterpillar was individually presented to mated wasps (3-6) held in a cage. The caterpillar was carefully observed and was promptly removed after a single sting from a single wasp. Penetration of host cuticle by the ovipositer of a wasp indicated a sting. Any caterpillar stung more than once was not used in the study. Parasitized *H. virescens* were placed on diet and incubated at 29˚C (12D:12L) until the time of parasitoid egression and pupation, at which point cocoons were transferred to another incubator (21˚C; 12D:12L) until adult emergence.

Hemolymph Extraction

Hemolymph was extracted from hosts eight days after parasitization. Hosts were surface sterilized by immersion in 70% EtOH for 10 mins, after which they were transferred to 50ml of autoclave-sterilized deionized water. Hemolymph was extracted by cutting the proleg of a parasitized caterpillar and squeezing the hemolymph from the wound into an Eppendorff tube. A few crystals of phenylthiourea were placed in the each tube to prevent melanization of the extracted hemolymph.

Hemolymph Purification

Extracted hemolymph was purified according to the methods of Kuriachan et al. (2006) with the following modifications: Hemolymph was centrifuged for 10min at 10,000rpm. The supernatant was extracted and transferred to a water bath (62°C) for

2mins to inactivate phenoloxidases. The hemolymph was then stored at -80˚C in Eppendorff tubes until use.

Experiments

Egression Study

H. virescens caterpillars were individually parasitized during their fourth instar (in accordance with rearing protocol), and the parasitoid larvae were allowed to develop (29˚C; 12D:12L) until they egressed from the host. After egression, hosts were dissected, and the presence or absence of parasitoid larval exuvium in the host body cavity was noted. The success or failure of the corresponding parasitoid larva to pupate, as well as host bleeding was also noted. Ninety-three parasitized caterpillars were examined.

Pupation Study

Very late 3rd instar parasitoid larvae (day 9 after parasitization) were dissected from their hosts and placed in sterile Nunclon Surface (Nalge Nunc International, Denmark, Europe) cell culture wells containing 150µl hemolymph extracted from hosts 8 days after parasitization. Culture plates were sealed with paraffin, wrapped in aluminum foil, and placed in an incubator (29°C; 12D:12L) for 24 hours. Larvae were observed at 8 hours and 24 hours. Incidence of molting, ecdysial splitting of the larval skin, or death were recorded. Additionally, pre-egression behavior, termed ‘rasping’ was also recorded. Rasping was characterized by side-to-side movement of the head and simultaneous contraction of the muscles surrounding the mouthparts. In the host, the parasitoid larva exhibits this behavior as it scrapes at the host tissues and integument, forming a hole through which it egresses from the host (personal observation). The

experiment was replicated three times in three trials (one replication and one control per trial), with 15 larvae in each replication. As a control for each trial, 20 parasitized hosts (day 9 after parasitization) were selected to serve as a comparison for normal parasitoid development. Parasitoid egression from these hosts was recorded, and it was assumed that, by egressing from the host, the parasitoid had also molted. This assumption was based on the presence of the exuvium in 100% of post-egression hosts that were dissected in the 'Egression Study' portion of this set of experiments. By the end of 24 hours, if parasitoid egression had not occurred, the control host was dissected to determine why egression had not occurred.

Hemolymph Leakage and Defensive Behavior

H. virescens caterpillars were individually parasitized according to rearing protocol and were incubated (29˚C; 12D:12L) until parasitoid egression. Defensive behavior in post-egression hosts was elicited in response to prodding the host's abdomen with metal forceps. The behavior was characterized by a swift movement of the head towards the forceps and flaring of the mandibles. After egression of the parasitoids, the caterpillars were prodded, and those individuals eliciting the defensive response were separated. In defensive individuals, the parasitoid exuvium was carefully pulled (externally) from the egression wound by pulling on the portion of the exuvium that was stuck to the host's abdominal cuticle. Incidence of hemolymph leakage in these caterpillars was recorded. Caterpillars from which the exuvium was removed were then prodded periodically, and time until loss of the defensive response was recorded. The

experiment was replicated three times in three trials (one replication and one control per trial), with 20 larvae in each replication.

As a control for each trial, parasitized larvae were placed on filter paper and left there until parasitoid larvae egressed. Filter paper was checked for spotting, indicative of hemolymph leakage (Nakamatsu et al., 2006), and host status (dead or alive) was noted. After parasitoid egression, the caterpillars were observed daily for post-egression defensive responses. Each caterpillar was prodded daily for a week with metal forceps (up to three times), and elicitation (or failure thereof) of the defensive response was recorded.

Data Analysis

Software

Data were analyzed with JMP® Statistical Discovery Software, Version 9.0.0 (2010, SAS Institute Inc. Cary, NC USA version 9.0.0) and Microsoft® Excel® for Mac, Version 12.2.9 (2008) and Microsoft® Excel® 2008 for Mac, Version 12.2.9.

Egression Study

A pie chart was created to represent the percentage of post-egression hosts that contained parasitoid exuvia. Of those, the number of hosts that experienced hemolymph loss and the number of parasitoids that pupated are also represented.

Pupation Study

The frequency at which rasping, ecdysial splitting, ecdysis, pupation, and death were observed was determined for the experimental group. Additionally, the percentage of individuals in each of these categories was calculated. A Likelihood Ratio Chi Square

test was used to determine whether ecdysial splitting was contingent on rasping in the experimental group. Additionally, a two sample *t*-test was used to establish whether the mean number of individuals that underwent ecdysis differed significantly between the experimental and control groups. The two-sample *t*-test was conducted under the assumption that egression in the control group indicated ecdysis had occurred.

Hemolymph Leakage and Defensive Behavior

A two-sample *t*-test was used to determine whether mean time until loss of defense differed significantly between individuals that experienced hemolymph loss and those that did not. Significant means were compared with a Tukey test.

Results

Egression Study

Dissection of 93 parasitized hosts after parasitoid egression revealed the presence of the larval exuvium within the body cavity of 100% of the dissected hosts. Hemolymph loss was recorded in only two of the individuals (2.15%). The percentage of individuals that successfully spun cocoons was 93.54% (Figure 11). Of those six individuals that did not pupate, the exuvium was located in the host body cavity, and no associated host bleeding was noted.

Pupation Study

None of the 45 larvae in the three trials molted or pupated during the 24-hour test period. In the control group, 90% of caterpillars had parasitoids egress from them. Of the six caterpillars in which egression was not observed, dissection revealed the following

findings: no parasitoid (1/6), developmentally delayed parasitoid (4/6), dead parasitoid (1/6). Ecdysial splitting of the cuticle was observed in 2.22% of test subjects (Table 17) and is depicted in Figure 12. Rasping behavior was observed in 53.33% of the larvae (Table 17). Likelihood Ratio Chi Sqaure analysis indicated that ecdysial splitting was contingent on the rasping behavior (p =0.0425) (Table 17).

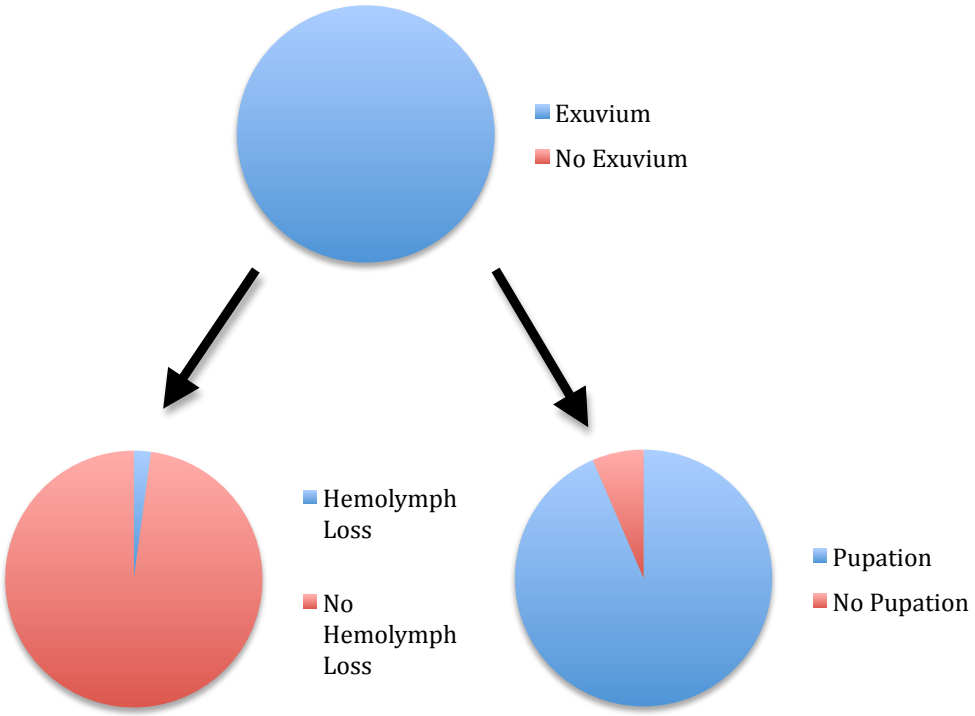

Figure 11
Graphical representation of the occurance of final exuvium of *M. croceipes* in post-egression host body cavity, host hemolymph loss resulting from egression, and pupation of parasitoid larvae.

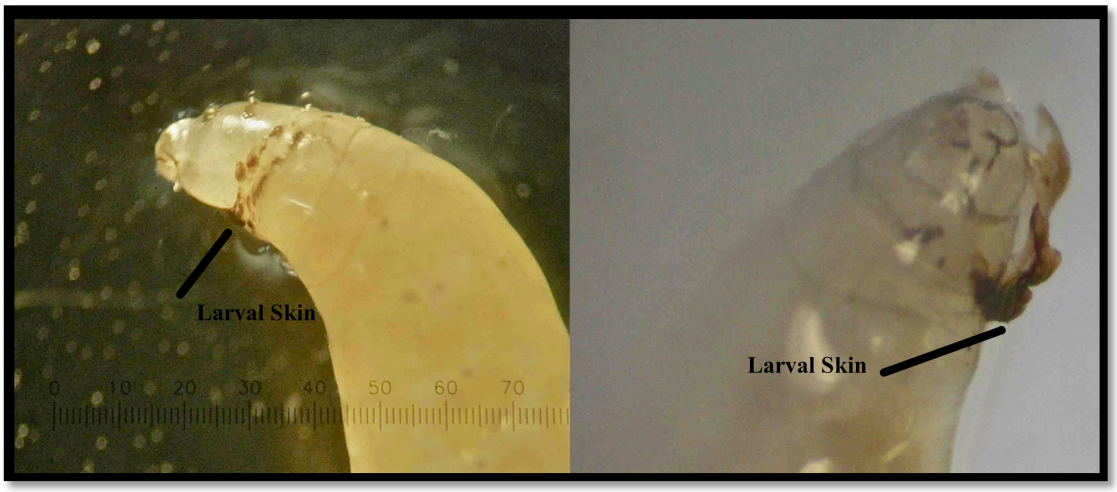

Figure 12
Ecdysial splitting of the cuticle in larvae removed from the host just prior to egression. Here, 10 tick marks is equal to 0.05mm.

Table 17
Frequency and percentage of individuals that exhibited ecdysis, ecdysial splitting of the cuticle, rasping behavior, or death the in pupation study (each trial was composed of 15 larvae, for a total of 45 larvae). Additionally, the result of the Chi Square test is listed. The test was performed to indicate a dependent relationship between ecdysial splitting of the cuticle and rasping. The two-sample *t*-test was conducted under the assumption that egression in the control group indicated ecdysis had occurred. Here, the *t*-test indicated that the mean number of individuals that underwent ecdysis in the experimental and control groups was significantly different.

Behavior	**Frequency**	**Percentage**
Rasping	24	53.33
Ecdysial Splitting	3	6.67
Ecdysis	0	0.00
Pupation	0	0.00
Death	1	2.22
Likelihood Ratio		
P -value	0.0466	
Control		
Egression	54	90.00
Two-Sample *t*-test	$P < 0.0001$	

Hemolymph Leakage and Defensive Behavior

Hemolymph leakage occurred in 100% of post-egression hosts from which the exuvium was removed. No hosts in the control group experienced hemolymph loss resulting from parasitoid egression. Hosts in the experimental group, on average, failed to exhibit defensive behavior after approximately 315s; hosts in the control group, on average, exhibited defensive behavior for approximately 3 days (Table 18). A two-sample *t*-test indicated that mean time until loss of defense differed significantly between individuals that experienced hemolymph loss and those that did not (p –value <0.0001) (Table 18). A Tukey test comparing significant means indicated that mean time until loss of defense was significantly longer in the control group, in which no individuals experienced hemolymph loss, compared to the experimental group, in which 100% of individuals experienced hemolymph loss (Table 18).

Table 18
Mean time until loss of defense and percentage of post-egression hosts that experienced hemolymph loss. Time is measured in seconds for the experimental group and in days for the control group. The *p*-value is the result of a two-sample *t*-test that compared the mean time until loss of defense in individuals that experienced hemolymph loss versus those that did not. Significant means were compared with a Tukey test.

	Mean±Stdev	**Hemolymph Loss**
Group		
Experimental	314.65±419.69 B	100%
Control	3.28±1.43 A	0%
P-value	<0.0001	

Discussion

Dissection of 93 post-egression hosts revealed the exuvium of the parasitoid larva attached to the parasitoid's emergence hole in all of the examined hosts (Figure 6). This data suggests that the parasitoid molts a final time as it egresses from the host (discussed in Chapter III). Because parasitoids pupate shortly after egression, presence of the exuvium in all of the post-egression hosts suggests the final molt (which occurs during egression) must be made prior to pupation. The placement of the exuvium (Figure 4), and the absence (except in two hosts) of hemolymph loss (Figure 6) also suggests that the shed skin of the parasitoid serves as a 'plug' that prevents the host from bleeding to death as the parasitoid emerges. This hypothesis was supported by hemolymph loss 100% of the time when the exuvium was removed (externally) by pulling it through the exit wound.

Shedding of the final exuvium as the parasitoid pushes its way through the host cuticle suggests that the body wall of the host may play a role in aiding the parasitoid in wriggling free of its shed cuticle. Whether the parasitoid larva requires an anchor to make its other molts (which occur inside the host) was not investigated. It is possible that the larva moves against the host's internal structures to aid in removal of the exuvium. However, it is suspected (by this researcher—personal observation) that the exuvium sloughs off slowly as the parasitoid feeds and increases in size. In observing second instar larvae that had recently shed the first instar head capsule, a membranous structure could often be observed trailing from the abdomen of the newly molted larva. It was suspected that this membrane was the sloughed cuticle of the first instar. Unlike the

cuticle of earlier instars, the cuticle of the egressing parasitoid appears to be more heavily sclerotized (Figure 12). If that is the case, it stands to reason that the parasitoid would not be able to 'break free' of this cuticle, as it possibly did in earlier instars, but would instead need to slip out of the exuvium, as it does during egression.

Data indicated that *M. croceipes* larvae molt a final time as they egress from the host, just prior to cocoon spinning and pupation. The hypothesis that the parasitoids cannot molt in the absence of the host (*in vitro*) was tested on the basis that the parasitoid appears to slide out of its larval exuvium as it pushes through the host's abdominal cuticle. Pre-egression parasitoids that were removed from the host and placed in hemolymph-filled cell culture wells were not able to molt. However, it was noted that some of the larvae (53.33%) initiated what was termed rasping behavior. Rasping was a behavior observed in parasitoids egressing from their hosts. This behavior involves a back and forth movement of the head and simultaneous contraction of the muscles surrounding the mouth. As the parasitoid performs this behavior *in vivo*, it appears to be scraping at the internal tissues of the host's abdomen. As the parasitoid continues to scrape the tissue, its head becomes visible through the cuticle of the host (allowing for observation of this behavior). Eventually, the parasitoid thins the cuticle to the point of being able to push its head through the cuticle.

In addition to rasping, ecdysial splitting of the cuticle was observed in a small percentage (2.22%) of the larvae. *In vivo*, ecdysial splitting of the parasitoid's cuticle occurs just before or just as the parasitoid pushes its head through the host integument. A

Likelihood Ratio Chi Square analysis indicated that, in the larvae removed from the host, ecdysial splitting was contingent on the rasping behavior (p =0.0425) (Table 17).

Though both rasping and ecdysial splitting of the cuticle were observed, no larvae molted or pupated, suggesting first, that the final molt cannot be made in the type of *in vitro* set-up used in this experiement, and second, that the parasitoid must make the final molt before it can pupate. Therefore, while creating an adequate artificial diet is crucial to successful *in vitro* rearing of *M. croceipes*, designing an *in vitro* rearing vessel that fulfills the role of the physical host is probably just as important. The parasitoid larva should be reared in a vessel in which it can complete its life history. Ideally, the parasitoid should be able to rasp and scrape a hole through the vessel material from which it can then molt as it egresses.

The final portion of this study investigated the role of the last larval exuvium as a 'plug' and the influence of this plugging mechanism on post-egression host defenses. Data indicated that parasitized caterpillars from which a parasitoid had egressed were still capable of exhibiting defensive behavior (although notably less aggressively). Defensive behavior here refers to swinging the head towards the stimulation (in this case, prodding with forceps) and flaring of the mandibles. Post-egression hosts exhibited this behavior for an average of 3.28 days (Table 18). In hosts in which the exuvium was removed through the exit wound, hemolymph leakage occurred, and defensive behavior was exhibited for an average of 314.56 seconds (Table 18). A two-sample *t*-test indicated that individuals that experienced hemolymph loss (the experimental group)

failed to exhibit defensive behavior significantly faster than individuals that did not lose hemolymph (the control group) (Table 18).

After the parasitoid egresses from the host, it spins its cocoon a short distance from the host. The host is relatively inactive after egression of the parasitoid but often curls around the parasitoid cocoon or rests atop it as the parasitoid pupates (personal observation). This 'guarding' behavior has been noted in other post-egression host species, and it has also been noted that these hosts will defend themselves when disturbed. Observations of this nature led to investigations of what has since been termed the "host usurpation hypothesis" (Brodeur and Vet, 1994). Host behavior of this nature was first studied in the *Pieris brassicae* (Lepidoptera: Pieridae)-*Cotesia glomerata* (Hymenoptera: Bracondiae) system, and has since been noted in other systems, including *Microplitis mediator* and its hosts (Harvey et al., 2008). *C. glomerata* is a gregarious parasitoid, and after the larvae egress and spin their cocoons, the host (which survives egression), spins silk over the mass of cocoons and often curls around it. The post-egression host in this host-parasitoid complex also responds aggressively when disturbed (Brodeur and Vet, 1994). *P. brassicae* is also parasitized by *Cotesia rubecula* (Braconidae). However, post-egression hosts parasitized by *C. rubecula* do not remain with the cocoons. A comparison of these two host-parasitoid systems (*P. brassicae*-*C. glomerata* and *P. brassicae*-*C. rubecula*) found that *C. glomerata* had greater survival rates, compared to *C. rubecula*, and Brodeur and Vet (1994) suggest that the presence or absence of the post egression host on top of the cocoon mass may be responsible for the differential survival rates. Brodeur and Vet (1994) also state that *C. glomerata* molts as

it egresses from the host, and egression wounds in the host cuticle are plugged with the shed exuvium to prevent host hemolymph loss. As discussed in this paper, Brodeur and Vet (1994) note that the prevention of hemolymph loss keeps the caterpillar alive and active after parasitoid egression.

Harvey et al. (2008) removed *Cotesia congregata* cocoons from their host (the parasitoids pupate on the host) and found that significantly fewer adult wasps emerged from these cocoons, compared to cocoons that remained attached to the host. In this case, the post-egression host, *Manduca sexta* (Sphingidae), is alive and active during parasitoid pupation. Harvey et al. (2008) do not specifically mention aggressive behavior elicited from post-egression *M. sexta*, but they do note observations in which hyperparasitoids avoided cocoons attached to *M. sexta*, which suggests the host provides the cocoons with some protection.

Specific study regarding the defensive behavior of post-egression *H. virescens* parasitized by *Microplitis croceipes* and the potential of that behavior to influence survival of pupating *M. croceipes* has not been directly studied. Data presented in this study demonstrated that *H. virescens* caterpillars are capable of eliciting defense responses for several days after parasitoid egression (Table 18). Though this behavior can be categorized as self-defense, it is possible that the caterpillar, in defending itself, is also providing some measure of protection to the parasitoid cocoon around which it is curled. Regarding the literature on this matter, it could be assumed that parastitoid survival is increased by the host's guarding behavior, but explicit tests are needed.

In conclusion, data suggest that the host plays a very important role during parasitoid egression and pupation. During egression, the parasitoid molts a final time as it pushes its way through the host cuticle and out of the host's body. Observations of the exuvium trailing from the exit wound made by the parasitoid suggests the body wall of the host acts as an anchor that holds the exuvium as the parasitoid wriggles out of it (as it egresses from the host's abdmonen). This hypothesis was supported by experiments that demonstrated that larvae were unable to make the final molt *in vitro*. Because the final molt occurs simulataneously with egression, the exuvium remains lodged in the exit wound and serves as a plug to prevents excessive hemolymph loss from the host. As a result, the host survives egression and may remain alive for several days. Post-egression hosts were observed to curl around the parasitoid cocoon and display defensive behaviors when disturbed. Host usurpation literature suggests aggression displayed by post-egression hosts may aid in protecting parasitoid cocoons, thus increasing parasitoid rate of survival.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Summary

Microplitis croceipes (Braconidae) is an endoparasitoid of tobacco budworm larvae, *Heliothis virescens* (Noctuidae). *H. virescens* is a major agricultural pest of cotton, tomatoes, and tobacco. As a parasitoid of the tobacco budworm, *M. croceipes* has potential as a biological control agent (Greany et al., 1989). However, current rearing practices, in which the host and parasitoid must be reared, make it impractical and costly to produce this parasitoid en mass. *In vitro* rearing could be used as an alternative to current mass rearing practices. This research encompassed three main goals in the study the biology of *M. croceipes* and the potential of rearing this parasitoid *in vitro*: to amend current descriptions of the larval life history of *M. croceipes*, to investigate suitability of potential artificial diets on which to rear the parasitoid larvae *in vitro*, and finally, to assess the plausibility of rearing *M. croceipes in vitro*, using current practices.

Conclusions

The first objective of this research was to amend current larval life history descriptions of *M. croceipes*. Larvae were dissected from hosts and categorized according to current morphological descriptions of the second and third instars (Lewis, 1970). Head capsule width measurement of each larva was recorded. Additionally, hosts from which a parasitoid had egressed were dissected in search of larval exuvia. Head capsule measurements revealed that the purported second instar (Lewis, 1970) larva

actually contained three distinct clusters of head capsule size, one of which coincided with the range of measurements gathered from purported (Lewis, 1970) third instar larvae. Dissections revealed a discarded exuvium at the mouth of the egression wound inside all post-egression hosts. These data indicated that *M. croceipes* larvae pass through five, rather than three instars, as is the current life history description for *M. croceipes* in the literature (Lewis, 1970).

The second objective was to investigate the suitability of potential artificial diets to be used in *in vitro* rearing. Larvae were dissected from the host during their first instar and place on an artificial diet. Three concentrations each of glucose, trehalose, and protein, as well as a combination diet (derived from initial diet trials) were tested. Growth, molting, and death were noted for each diet. Larval growth and survival were highest on trehalose and protein diets and tended to increase as dietary concentration of trehalose and protein increased. Molting from first to second instar was observed in both trehalose and protein diets, but not in glucose diets. Data indicated that trehalose and protein were more vital to larval parasitoid development than was glucose, but no larvae were reared passed the second instar on an artificial diet.

The final goal of the research was to evaluate the plausibility of rearing *M. croceipes* larvae to adulthood *in vitro* and to investigate post-egression host behavior. Larvae were dissected from their hosts just prior to egression and placed in a cell culture plate in previously-collected host hemolymph. Larvae were able to initiate pre-egression behavior in an *in vitro* environment, and a small percentage exhibited ecdysial splitting of the cuticle. However, no larvae were able to make the final molt *in vitro*. Failure of

the parasitoid to make its final molt *in vitro* indicates a low probability that *M. croceipes* could be reared to adulthood *in vitro* using current practices. Removal of the parasitoid exuvium from post-egression hosts resulted in host bleeding, which was not observed in hosts which retained the exuvium. Duration of elicitation of defensive behavior was significantly shorter in hosts from which the exuvium had been removed (and hemolymph subsequently lost). These data suggested that the parasitoid's final larval exuvium acts as a plug to prevent hemolymph loss during and after egression. That the host remains alive and active after parasitoid egression may suggest that the host plays a role during parasitoid pupation, particularly a defensive role (Brodeur and Vet, 1994).

REFERENCES

Barras, D.J., Kisner, R.L., Lewis, W.J., and Jones, R.L., 1972. Effects of the parasitoid, *Microplitis croceipes* on the haemolymph proteins of the corn earworm, *Heliothis zea*. Comparative Biochemistry and Physiology. 43B, 941-947.

Brodeur, J., and Vet, L.E.M., 1994. Usurpation of host behavior by a parasitic wasp. Animal Behavior 48, 187-192.

Consoli, F.L. and Parra, J.R.P., 1996. Biology of *Trichogramma galloi* and *T. pretiosum* (Hymenoptera: Trichogrammatidae) reared *in vitro* and *in vivo*. Arthropod Biology 89, 828-834.

Consoli, F.L., and Parra, J.R.P., 1999. Development of an artificial host egg for *in vitro* egg laying of *Trichogramma galloi* and *T. pretiosum* using plastic membranes. Entomologia Experimentalis et Applicata 91, 327-336.

Dahlman, D.L., Vinson, S.B., 1975. Trehalose and glucose levels in the hemolymph of *Heliothis virescens* parasitized by *Microplitis croceipes* or *Cardiochiles nigriceps*. Comparative Biochemistry and Physiology 52B, 465-468.

Dahlman, D.L., Rana, R.L., Schepers, E.J., Schepers, T., DiLuna, F.A., Webb, B.A., 2003. A teratocyte gene from a parasitic wasp that is associated with inhibition of insect growth and development inhibits host protein synthesis. Insect Molecular Biology 12, 527-534.

Dindo, M.L., Grenier, S., Sighinolfi, L., Baronio, P., 2006. Biological and biochemical differences between in vitro- and in vivo-reared *Exorista larvarum*. Entomologia Experimentalis et Applicata 120, 167-174.

Dyar, H.G., 1890. The number of molts of lepidopterous larvae. Psyche 5, 420-422.

Ferkovich, S.M., Sharpio, J., Carpenter, J., 2000. Growth of a pupal ectoparasitoid, *Diapetimorpha introita*, on an artificial diet: stimulation of growth rate by a lipid extract from host pupae. Biological Control 45, 401-413.

Greany, P., 1986. *In vitro* culture of hymenopterous larval endoparasitoids. Journal of Insect Physiology 32, 409-419.

Greany, P.D., Ferkovich, S.M., Clark, W.R., 1989. Progress towards development of an artificial diet and an *in vitro* rearing system for *Mircoplitis croceipes*. Southwestern Entomologist Suppl. No. 12, 89-94.

Grenier, S., 2009. *In vitro* rearing of entomophagous insects—past and future trends: a minireview. Bulletin of Insectology 62, 1-6.

Harvey, J.A., Bezemer, T.M., Gols, R., Nakamatsu, Y., Tanaka, T., 2008. Comparing the physiological effects and function of larval feeding in closely-related endoparasitoids (Braconidae: Microgastrinae). Physiological Entomology 33, 217-225.

Hawkins, B.A., 1994. Patterns and Process in Host-Parasitoid Interactions. Cambridge University Press, New York.

Kellogg, V.L., 1903. Studies for students III—Elementary studies in insect histology. Psyche, 118-122.

Khan, S.M., and Ozer, M., 1988. Biology of *Microplitis mediator* [Hym.: Braconidae] a gregarious endoparasite of *Agrotis segetum* [Lep.: Noctuidae]. Entomophaga 33. 211-217.

Kuriachan, I., Consoli, F.L., Vinson, S.B., 2006. *In vitro* rearing of *Toxoneuron nigriceps* (Hymenoptera: Braconidae), a larval endoparasitoid of *Heliothis virescens* (Lepidoptera: Noctuidae) from early second instar to third instar larvae. Insect Physiology 52, 881-887.

Lewis, W.J., 1970. Life history and anatomy of *Microplitis croceipes* (Hymenoptera: Braconidae), a parasite of *Heliothis* spp. (Lepidoptera: Noctuidae). Annals of the Entomological Society of America 63, 67-70.

Luo, K., Trumble, J.T., Pang, Y., 2007. Development of *Microplitis bicoloratus* on *Spidoptera litura* and implications for biological control. Biological Control 52, 309-321.

Maredia, K.M., Dakouo, D. and Mota-Sanchez, D., 2003. Integrated Pest Management in the Global Arena, CABI Publishing, Cambridge, MA.

Morales-Ramos, J.A., Rojas, M.G., Coleman, R.J., and King, E.G., 1998. Potential use of *in vitro*-reared *Catolaccus grandis* (Hymenoptera: Pteromalidae) for biological control of the boll weevil (Coleoptera: Curculionidae). Journal of Economic Entomology 91, 101-109.

Nakamatsu Y., Tanaka T., and Harvey J.A., 2006. The mechanism of the emergence of *Cotesia kariyai* (Hymenoptera: Braconidae) larvae from the host. European Journal of Entomology 103, 355-360.

Powell, J.E., and Elzen, G. W., 1989. Biological relationships of *Microplitis croceipes*. Southwestern Entomologist Suppl. No. 12, 11-16.

Puttler, B., and Thewke, S.E., 1970. Biology of *Microplitis feltiae* (Hymenoptera: Braconidae), a parasite of the black cutworm, *Agrotis ipsilon*. Annals of the Entomological Society of America 63, 645-648.

Rojas, M.G., Morales-Ramos, J.A., and King, E., 1996. *In vitro* rearing of the boll weevil (Coleoptera: Curculionidae) ectoparasitoid *Catolaccus grandis* (Hymenoptera: Pteromalidae) on meridic diets. Journal of Economic Entomology 89, 1095-1104.

Song, D., and Chen, J., 2008. Five new species of the genus *Microplitis* (Hymenoptera: Braconidae: Microgastrinae) from China. The Florida Entomologist 91, 283-293.

Strand, M.R., Johnson, J.A., Culin, J.D., 1988. Developmental interactions between the parasitoid *Microplitis demolitor* (Hymenoptera: Braconidae) and its host *Heliothis virescens* (Lepidoptera: Noctuidae). Annals of the Entomological Society of America 81, 822-830.

Tawfik, M.F.S., Hafez, M., and Ibrahim, A.A., 1980. Immature stages of *Microplitis rufiventris* Kok. Deutsche Entomologische Zeitschrift 27, 39-50.

Vinson, S.B., and Iwantsch, G.F., 1980. Host regulation by insect parasitoids. The Quarterly Review of Biology 55, 143-165.

Webb, B.A., and Dahlman, D.L., 1985. Developmental pathology of *Heliothis virescens* larvae parasitized by *Microplitis croceipes*: parasite-mediate host developmental arrest. Archives of Insect Biochemistry and Physiology 2, 131-143.

Xie, Z.N., Wu, Z.X., Nettles, W.C. Jr., Saldana, G., Nordlund, D.A., 1997. *In vitro* culture of *Trichogramma* spp. on artificial diets containing yeast extract and ultracentrifuged chicken egg yolk but devoid of insect components. Biological Control 8, 107-110.

Yazgen, S., and House, H.L., 1970. An Hymenopterous insect the parasitoid *Itoplectis conquisitor*, reared axenically on a chemically defined synthetic diet. Canadian Entomologist 102, 1304-1306.

Zhang, D., and Dahlman, D.L., 1989. *Microplitis croceipes* teratocytes cause developmental arrest of *Heliothis virescens* larvae. Archives of Insect Biochemistry and Physiology 12, 51-61.

VITA

Name:	Laura Ann McLoud
Address:	Department of Entomology Texas A&M University TAMU 2475 College Station, TX 77843-2475
Email Address:	lauramcloud@gmail.com
Education:	B.S., Biological Sciences, Clemson University, 2009 M.S., Entomology, Texas A&M University, 2011