THE LATE IMMATURE DEVELOPMENT OF TOXONEURON NIGRICEPS, A KOINOBIONT ENDOPARASITOID, AND STEPS TOWARD AN IN VITRO REARING SYSTEM

A Dissertation

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ABSTRACT

Toxoneuron nigriceps is a koinobiont larval endoparasitoid of the tobacco budworm, *Heliothis virescens*. No koinobiont parasitoid (which develop in active, growing hosts) has been reared completely in vitro. However, *T. nigriceps* has been partially reared in vitro, from egg stage through 2nd larval instar and from 2nd to 3rd larval instar. Despite reaching its final larval instar, *T. nigriceps* failed to develop past the larval stage. In the following study, two aspects of *T. nigriceps* development, post-egression tissue feeding and cocoon formation through pupation, were examined to determine why *T. nigriceps* failed to develop in vitro beyond the larval stage and how in vitro rearing might be improved to rear *T. nigriceps* to adulthood. The importance of post-egression feeding and the possibility of developing an artificial post-egression diet were examined by manipulating the post-egression feeding behavior of *T. nigriceps*. The tissues of pre- and post-tissue feeding *T. nigriceps* larvae were analyzed to investigate the macronutritional benefit of post-egression feeding. A preliminary artificial post-egression diet was tested, and the putative quality of *T. nigriceps* reared to adulthood using this diet was examined. The formation of subterranean pupation chambers by healthy and parasitized *H. virescens* larvae was examined to investigate potential behavioral manipulation by *T. nigriceps* during this process. Finally, the potential use of artificial pupation chambers by *T. nigriceps* was investigated.

Post-egression feeding was determined to be a vital part of *T. nigriceps* development, contributing to both parasitoid size and survival to adulthood. A

preliminary artificial post-egression diet was developed, which facilitated rearing of *T. nigriceps* from 3rd larval instar to adulthood. The dimensions and burial depth of chambers formed by parasitized *H. virescens* were significantly different from those of chambers formed by healthy *H. virescens*, suggesting that *T. nigriceps* manipulated host behavior during excavation. *Toxoneuron nigriceps* was able to use a number of common laboratory items as substitute pupation chambers during cocoon formation, and the merits of several artificial chambers were discussed. With provision of an adequate post-egression and artificial pupation chamber, in vitro rearing of *T. nigriceps* from the larval stage to adulthood may be possible.

DEDICATION

To all of my family and friends, whose support I could not live without.

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CHAPTER I

INTRODUCTION

A parasitoid is a specialized parasitic insect that derives nutrition from the body of only a single host during larval development, ultimately killing the host in the process, and is free living as an adult (Godfray 1994). Parasitoids have arisen independently in the orders Hymenoptera, Diptera, Coleoptera, Lepidoptera, and Strepsiptera, but have the largest representation in Hymenoptera. Parasitoids can be divided into ectoparasitoids, which develop and feed externally, and endoparasitoids, which develop and feed within the cuticle of the host. They can also be divided into idiobionts and koinobionts. Idiobionts are those parasitoids that utilize sessile, non-feeding hosts. This can include hosts that are either in an inactive stage, such as eggs or pupae, or hosts that have been permanently paralyzed via venom injected by the mother when it selected the host. Koinobionts, in contrast, develop on or in hosts that are still active, feeding, and developing. These parasitoids most commonly develop as endoparasitoids of larvae of holometabolous species, but can also attack nymphs and adults or, in some cases, may utilize several host stages (Godfray 1994).

Besides having fascinating life-history strategies, many parasitoids have great potential for use as agents of biological control. Biological control is the practice of using natural enemies, rather than chemicals, to control populations of pest species. This practice can reduce the need for pesticides, which not only reduces the exposure of

consumers, agricultural workers, and the environment to potentially harmful chemicals, but also can be economically efficient. While monetary costs have been difficult to measure precisely, in some cases biological control has been estimated to save hundreds of thousands of dollars in chemical control and prevent millions of dollar's worth of damages (Gutierrez et al. 1999). Although parasitoids and predators have both been used for the biological control of insect pests, hymenopteran parasitoid species account for the majority of cases, mostly due to their higher degree of host specificity and, thus, decreased risk of non-target effects (Gordh et al. 1999, van Lenteren 2012).

One of the most common uses of parasitoids for pest regulation is their use in augmentative biological control, in which the population of natural enemies naturally present in an area is increased by release of a number of individuals reared in an insectory. This can be carried out as an innoculative release, in which the natural enemies and their offspring are expected to offer control of the pest population, or as an innundative release, in which large numbers of natural enemies are released such that they suppress the pest population below levels at which the biological control agent can persist for multiple generations (Elzen and King 1999). A major limitation to use of mass-reared parasitoids in augmentative biological control, however, is the fact that in order to rear a parasitoid one must also rear its host, which in many cases requires rearing plants to feed the hosts. There are two steps that can be taken to shorten the production line, and thus reduce the cost of rearing a parasitoid. The first is to eliminate the need for growing plants by developing artificial diets on which the host species can be reared. The second is to eliminate the need for the host by developing artificial in

vitro rearing systems for the parasitoids (Grenier 2009). In once case, rearing egg parasitoids from the family Trichogrammatidae on artificial media, rather than on natural hosts, could potentially reduce production costs by as much as 50% (Cônsoli and Grenier 2010).

One of the earliest attempts to rear a hymenopteran parasitoid in vitro was carried out by Simmonds (1944), who attempted to rear a number of pupal ectoparasitoids on raw beef. Although the parasitoid eggs hatched, and the first instar larvae apparently grew, only one larva molted and none survived to adulthood. Later, Bronskill and House (1957) successfully reared eggs of the pupal endoparasitoid, *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae), to eclosion in a saline solution then fed the resulting larvae a diet of pureed pork liver and saline. Out of 152 eggs collected, 147 hatched, and out of these ten larvae reached adulthood. Since then, more sophisticated artificial rearing methods have been attempted, with varying degrees of success.

As of 2011, 61 hymenopteran parasitoid species have been reported as having being reared partially or completely in vitro, and half of these, 30 species, are egg parasitoids (Cônsoli and Grenier 2010).

Out of the 30 species of egg parasitoids reared in vitro, 21 belong to the family Trichogrammatidae (Cônsoli and Grenier 2010). Thus far, these egg parasitoids are the greatest success story in in vitro rearing of hymenopteran parasitoids. In 1975, Hoffman et al. reported the first successful rearing of an egg parasitoid from egg to adult in an artificial medium (Hoffman et al. 1975). Eggs of *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) were dissected out of host eggs, rinsed, and placed

in a medium derived from hemolymph plasma from 5th instar *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) larvae. Parasitoids reared in this medium were able to reach both the pupal and adult stages, though development was delayed, and some adults had wings that failed to fully expand (Hoffman et al. 1975). Some species of Trichogrammatidae have been reared entirely in vitro for multiple generations, with female wasps ovipositing directly into artificial eggs, eliminating the need for parasitoid eggs to be dissected out of host eggs. One of these is *Trichogramma minutum* Riley (Hymenoptera: Trichogrammatidae), which was reared for ten generations in wax artificial eggs filled with a diet composed of chicken egg yolk, chicken embryo extract, milk, yeast extract, FreeAmine III, and extract from *Manduca sexta* L. (Lepidoptera: Sphingidae) eggs (Nordlund et al. 1997).

Although many species of Trichogrammatidae have been reared completely in vitro, most diets have included one or more ingredients derived from Lepidoptera species (Thompson 1999). In China, where the majority of Trichogramma in vitro rearing has taken place, extracts from silk moth [*Bombyx mori* L. (Lepidoptera: Bombycidae)], pupae are commonly used in artificial diets. In this case, use of insect extract is economical, as pupae are a byproduct of a massive silk industry (Grenier et al. 1995). In the United States and Europe, however, the use of insect extracts is likely uneconomical because a large, insect-based industry is unavailable.

Even when parasitoids can be completely reared on artificial diets these diets are often still suboptimal. When reared on a series of artificial diets composed of fetal bovine serum, lactalbumin, chicken egg yolk, and pupal holotissue from either a natural

host, *Anagasta kuehniella* Zeller (Lepidoptera: Pyralidae), or from *Diatraea saccharalis* F. (Lepidoptera: Crambidae), larval development time of the larval ectoparasitoid *Bracon hebetor* Say (Hymenoptera: Braconidae) was significantly longer than for parasitoids reared in vivo on *A. kuehniella*, though pupal development time did not differ significantly (Magro and Parra 2004). Additionally, *B. hebetor* reared on certain artificial diets failed to form cocoons significantly more often than those reared on hosts, and parasitoid viability was much higher on the natural host (74%) than on even the best performing artificial diet (43%) (Magro and Parra 2004).

Artificial diets used to rear most idiobiont parasitoids have included one or more undefined nutrient sources, such as lactalbumin, host holotissue, or chicken egg yolk, which makes determining the exact nutritional requirements of these parasitoids difficult (Cônsoli & Grenier 2010). A small number of parasitoids, however, have been reared on defined diets. One such parasitoid is *Exeristes roborator* F. (Hymenoptera: Ichneumonidae), a generalist larval ectoparasitoid. The nutritional requirements of this parasitoid, which are likely to be similar to those of other ectoparasitoids, were investigated in a series of experiments carried out by S. N. Thompson. When reared on a series of holidic diets, each with one or more amino acids deleted, *E. roborator* failed to develop past the 3rd larval instar if one or more of the 10 essential amino acids were absent. This indicates that this parasitoid, like nearly all animals, has an absolute requirement for these amino acids in its diet (Thompson 1976a). Deletion of non-essential amino acids from the diet did not significantly reduce survival, though *E. roborator* did exhibit some increases in development time, likely owing to increased

synthesis of these amino acids by *E. roborator* to make up for the deficiency (Thompson 1976a). In another study, *E. roborator* was reared on a series of defined diets, each containing all 19 L-amino acids, but in concentrations varying from 1 to 6% and with some diets having the carbohydrate source, D-glucose, deleted. Parasitoids reared on diets containing 6% amino acids developed to completion at rates similar to those reared on natural host insects, even in the absence of glucose. When reared on diets containing 1% and 3% amino acid concentrations, larval survival was similar to that of larvae reared on 6% amino acid diets only when glucose was included; however, development time in these treatments was significantly increased (Thompson 1976b). For *E. roborator*, and likely other parasitoids, it seems that although carbohydrates can be used as an energy source during larval development, amino acids are a vital nutritional source. Another defined diet study revealed that at least some dietary carbohydrate is necessary for *E. roborator*. In contrast to the Thompson 1976b experiment, less than 50% of parasitoids reared on diets lacking glucose completed development to adulthood. However, when 2-8% glucose was included in diets, survival rates ranged from 83 to 94%. Increased dietary glucose also correlated with increased larval mass, at least partially due to increased lipid synthesis in larvae reared on higher-carbohydrate diets (Thompson 1979). Dietary lipids did not appear to be vital to the development of *E. roborator*, and addition of free fatty acids to diets appeared to have toxic effects. Adding triglycerides to the diet, while not detrimental, did not appear to improve larval development (Thompson 1977).

A dietary requirement for primarily amino acids and carbohydrates, with amino acids being the more important factor, has also been demonstrated in the rearing of another idiobiont parasitoid, *Itoplectis conquisitor* Say (Hymenoptera: Ichneumonidae), a parasitoid of pupal Lepidoptera. When this parasitoid was reared on defined artificial diets, the proportion of parasitoids that reached adulthood increased with increasing concentrations of dietary amino acids. When diets contained 2% glucose, development was not significantly effected by amino acid concentration. However, when diets contained 1% glucose, parasitoid development time was increased at lower amino acid concentrations. Once again, high levels of lipids in diets appeared to have toxic effects, as the number of parasitoids that completed development was reduced in lipid-rich diets (Yazgan 1971).

To date, all hymenopteran parasitoids reared completely in vitro have been idiobionts; no koinobiontic larval endoparasitoid has been fully reared on an artificial medium (Thompson 1999, Grenier 2009). This is likely due mainly to the higher degree of integration between host and parasitoid physiologies in these relationships. Idiobiont parasitoids feed either on a single non-growing stage or on a host that has been permanently paralyzed, which means that their food source does not drastically change over time. Koinobiont parasitoids, in contrast, have a food source that changes not only in size, due to continued host growth, but also in quality, due to changes in host nutritional physiology.

Although the concentrations of various nutrients in the hemolymph fluctuate naturally during the development of insects, koinobiont parasitoids have been shown to

manipulate the nutritional physiology of their hosts in a number of ways to make them more suitable for the parasitoid. Parasitization of *M. sexta* by the koinobiont endoparasitoid *Cotesia congregata* Say (Hymenoptera: Braconidae) causes increased synthesis of trehalose in the host hemolymph (Thompson 2001). A carbon labeling study revealed that this was the result of increased gluconeogenesis, which was induced by parasitization (Thompson 2001). Elevation of host trehalose concentration is known to be induced by a number of other parasitoids, including *Microplitis croceipes* Cresson (Hymenoptera: Braconidae) (Dahlman and Vinson 1975) and *Campoletis sonorensis* Cameron (Hymenoptera: Ichneumonidae) (Dahlman and Vinson 1976). *Costesia flavipes* F. (Hymenoptera: Braconidae), in contrast, appears to cause a reduction in carbohydrate concentration in the host hemolymph in the late stages of parasitism (Salvador and Cônsoli 2008).

The concentration of proteins in the host hemolymph can also be altered by koinobiont parasitoids, as demonstrated in the relationship between *C. flavipes* and its host *D. saccharalis*. The concentration of proteins in the hemolymph of parasitized *D. saccharalis* larvae began to drop significantly 4 d after parasitization, in contrast to healthy larvae, in which protein concentrations remained relatively static (Salvador and Cônsoli 2008). This may have been due to consumption of these proteins by the gregarious *C. flavipes* larvae, but could have also been the result of down-regulation of the synthesis of some proteins, in order to make the environment within the host hemolymph more favorable to the developing parasitoids. Lipid concentration in the

hemolymph also decreased in the late stages of parasitism in this system (Salvador and Cônsoli 2008).

Host-parasitoid relationships among koinobionts can be further complicated by the presence of polydnaviruses, viral or virus-like sequences that have become integrated into the genomes of certain koinobiont parasitoids (Vinson 2011). Polydnaviruses have been divided into two main groups: ichnoviruses, which occur in the subfamily Campopleginae of Ichneumonidae, and bracoviruses, which occur in members of the microgastrine complex of Braconidae (Drezen and Beckage 2012). A potential third strain of polydnavirus, associated with the ichneumonid subfamily Banchinae are currently under investigation (Drezen et al. 2012). Despite having arisen independently, the polydnavirus strains operate in a similar manner. The polydnaviruses are incorporated into the genomes of wasps in the form of proviruses, and lack the segments of genome needed to replicate on their own. Instead, viruses are replicated within specialized calyx cells in the reproductive tracts of female parasitoids, packaged into virions in the calyx fluid, and injected into hosts along with the wasp egg upon parasitization. The virus genome itself is passed vertically between wasps from parent to offspring. Once within the host, the viral genes are expressed for the first time and act on the host cells to favor survival of the immature parasitoid that will carry and pass on the viral genome (Burke and Strand 2012).

One of the most important effects polydnavirus expression is the suppression of host immune responses against the immature parasitoid. The main threat presented to developing parasitoids by host immune defenses is encapsulation of the egg, which

occurs when, upon recognition of the egg as a foreign object, two types of hemocytes, granulocytes and plasmatocytes, begin binding to the egg surface. These cells will eventually build a thick layer of overlapping cells and form a capsule sufficiently impermeable to cause the death of the parasitoid egg through asphyxiation (Strand 2012). Polydnaviruses prevent encapsulation from occurring by altering the properties of hemocyte cells. The bracovirus of *M. croceipes*, for example, has been shown to permanently alter key cytoskeletal proteins within granulocytes and plasmatocytes, causing them to lose their ability to bind to and spread over surfaces (Strand 2012). The bracoviruses of *Cotesia* species also alter the adhesive properties of host hemocytes; however, these alterations are not permanent, and the duration of the effect varies among species (Strand 2012).

Another effect of polydnaviruses involves the developmental arrest of hosts, which permanently remain in larval or prepupal form, and so are prevented from undergoing a pupal molt while parasitoids are developing internally. The *C. congregata* bracovirus causes an absence of JH esterase in the host larva, along with deficiency in edysteroids and continual synthesis of JH by the corpora allata, which permanently arrests the host in the larval stage (Beckage 2012). The ichnovirus carried by *C. sonorensis* causes the degeneration of the host larva's prothoracic gland, leaving the host unable to produce the ecdysteroids needed to molt (Beckage 2012). Polydnaviruses have also been implicated in regulating the nutritional physiology of hosts. In the *Chelonus inanitus* L. (Hymenoptera: Braconidae)-*Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) system, the polydnavirus was linked to an increase in glycogen concentration

in the hemolymph of larvae infected with polydnavirus but no parasitoid, while glycogen concentration was lower in the hemolymph of hosts containing a live parasitoid larva, indicating that glycogen is up-regulated by the polydnavirus and consumed by the parasitoid itself (Kaestlin et al. 2005). Polydnaviruses and polydnavirus-bearing parasitoids represent a tightly coevolved interaction. The parasitoids require the polydnaviruses to make the host suitable for survival and development, while the polydnavirus depends on the parasitoid to carry its genome to subsequent generations. Thus, rearing of a polydnavirus bearing koinobiont in the absence of its polydnavirus and resulting polydnavirus-induced factors could pose a new level of difficulty in the development of artificial rearing systems for these parasitoids.

Despite the difficulties inherent to complete in vitro rearing of koinobionts, a number of species have been partially reared, from egg to hatching or through one or more larval instars. One such koinobiont is *C. sonorensis*, which was reared from egg to 3rd larval instar by Hu and Vinson (1997) in a medium with no host-derived factors, though this required extensive experimentation. TNM-FH medium was used as a base medium and supplemented with additional amino acids and varying amounts of trehalose, lactalbumin, bovine serum albumin, and a number of other proteins to form a series of experimental diets. A number of additives, including trehalose, bovine serum albumin, lactalbumin and threonine appeared to improve parasitoid hatch rate; however, only addition of lactalbumin resulted in larvae that molted from 1st to 2nd larval instar. Combinations of bovine serum albumin, trehalose, and lactalbumin were then added to TNM-FH in varying concentrations, generating another series of experimental diets. One

of these diets, containing 1% trehalose, 0.1% bovine serum albumin, and 0.5% lactalbumin, resulted in 100% of larvae molting from 1st to 2nd instar, and 22.7% further molting to 3rd instar. This diet was further supplemented with fresh egg yolk, which further improved molting to 3rd instar when added in low (1%) concentration. Additional supplementation of this diet with various combinations cholesterol, juvenile hormone, and 20-hydroxyecdysone showed that combinations of these three factors could further improve molt to 3rd instar, though if added alone only 20-hydroxyecdysone improved development (Hu and Vinson 1997).

For *C. sonorensis*, as is likely to be the case with other parasitoids, nutrition was not the only consideration for artificial rearing. In vitro rearing of *C. sonorensis* was further improved by altering the physical properties of the rearing environment. When the amount of artificial diet added to rearing containers was reduced, leaving the cuticle of the parasitoid exposed to air, larvae were able to reach the 5th, and final, larval instar with some developing to the prepupal stage. Rearing of larvae in increased O_2 concentrations, as well as with the addition of an agar layer beneath the diet, which gradually removed liquid from the substrate, improved larval growth. However, no *C. sonorensis* prepupae successfully formed cocoons or pupated (Hu and Vinson 1998). Another koinobiont endoparasitoid, *Venturia canescens* Gravenhorst (Hymenoptera: Ichneumonidae), has been reared in vitro from pre-germband egg through pupation; however, the diet used in this case was composed of 50% host pupal extract (Nakahara et al. 1997).

Toxoneuron nigriceps Viereck (Hymenoptera: Braconidae), the subject of this dissertation, is a solitary koinobiont endoparasitoid. Formerly known as *Cardiochiles nigriceps*, it was moved to the genus *Toxoneuron* following this genus's removal from synonymy with *Cardiochiles* (Dangerfield et al. 1999). *Toxoneuron nigriceps* is a specialist on the larval stage of the tobacco budworm, *Heliothis virescens* F. (Lepidoptera: Noctuidae), which is a bud-damaging pest in a number of crops, including cotton, soybean, tobacco, and tomato (Fitt 1989). In the past, *T. nigriceps* offered a fair amount of natural control of this pest. In one early report, the parasitism rate of *T. nigriceps* in tobacco fields averaged between 50 and nearly 100% (Chamberlin & Tenhet 1926). A two year study in cotton fields in Georgia generated estimated parasitization rates of 96% one year and 76% parasitism the next (Lewis et al. 1972). *Toxoneuron nigriceps* has also proven to be a useful model organism in the research of parasitoid physiology, especially with regard to host-parasitoid interactions (Vinson 1972). The *H. virescens*-*T. nigriceps* system was used to uncover possible functions of teratocytes, specialized cells derived from the serosal membrane of some parasitoids (Vinson 1970), and it was in the calyx fluid of *T. nigriceps* that a polydnavirus was first observed and described (Vinson and Scott 1975).

The life cycle of *T. nigriceps* begins when a female wasp locates a suitable host and deposits an egg. Wasps will typically attack 2nd through 4th instar *H. virescens* larvae, though 1st and 5th instar larvae are occasionally attacked as well (Lewis and Vinson 1968). The parasitoid egg is injected into the host hemocoel along with venom and calyx fluid. Though *T. nigriceps* will inject its eggs at various points of host body,

eggs tend to end up in the posterior end of the larva, an area known as the tokus region, regardless of oviposition site. This is beneficial to *T. nigriceps* because of the higher concentration of oxygen in this area, due to the presence of a pair of dense tracheal tufts known as the "tokus lung" (Rao et al. 2009). Immediately after oviposition, the *T. nigriceps* egg is slender, but widens toward one end throughout embryogenesis. Mature eggs average a length of 0.78 mm and average width of 0.0236 mm at the widest point. Eclosion generally occurs between 36 to 48 h after oviposition, but can take longer depending on the stage of the host (Lewis and Vinson 1968). Hatching of the egg does not occur until the host reaches its 5th larval instar, at a time that coincides with naturally increased protein concentration in the host hemolymph (Pennacchio et al. 1993).

The 1st instar larva of *T. nigriceps* is small and slender upon hatching, measuring approximately 0.67 mm in body length and 0.236 in head width (Lewis and Vinson 1968). The head capsule is dorsoventrally flattened and bears a pair of large, sickle-shaped mandibles, which 1st instar larvae use in physical combat against competing larvae (Vinson and Iwantsch 1980). The 1st larval instar lasts approximately 5 to 6 d, during which time the larva grows in size, becomes slightly green in color, and develops a visible anal vesicle. Second instar larvae, which measure approximately 3.67 mm in length and 0.90 mm at their greatest width shortly after molting, have head capsules that are generally similar in shape to those of 1st instar larvae, but are not as heavily sclerotized and thus are less suitable for combat (Lewis and Vinson 1968). Larvae spend 2 to 3 d in the 2nd larval instar before molting to the 3rd and final instar. Larvae at this stage are approximately 9.0 mm in length and measure 2.6 mm at their greatest width.

The anal vesicle is still present at the beginning of this stage, but is later shed (Lewis and Vinson 1968).

Third instar larvae remain inside the host for approximately 2 d after molting, and then begin egression by chewing a hole in the host cuticle, usually on the lateral mesothoracic region, and egressing through this hole. Rather than detaching entirely from the host, the *T. nigriceps* larva leaves its posterior end anchored within the host, then curls around to chew a second hole in the host cuticle, through which it will feed on remaining host tissues until only the head capsule and exocuticle of the host remain (Lewis and Vinson 1968). This external feeding phase generally takes place in a pupation cell, or chamber, formed by the host prior to parasitoid egression. Healthy *H. virescens* larvae in the field form these chambers below the surface of the soil in preparation for the pupal molt. Larvae parasitized by *T. nigriceps* are permanently arrested at the prepupal stage and do not undergo pupation even if the parasitoid fails to develop, yet they form pupation chambers just as their healthy counterparts do. After egression and tissue feeding, *T. nigriceps* larvae spin cocoons within these chambers by producing silk and rolling in place within the chamber (Chamberlin and Tenhet 1926). Parasitoids developing in the field may enter diapause and overwinter in these chambers as prepupae, when temperatures are unfavorable (López 1982).

Like a number of other koinobionts, *T. nigriceps* has not been completely reared in vitro, but has been artificially reared through most of its immature stages. Pennacchio et al. (1992) reared *T. nigriceps* from egg to 2nd larval instar in an artificial liquid medium composed of 21 amino acids, glucose, trehalose, vitamins, inorganic salts, and a

number of additional supplements. In the base diet, only eggs that had been in the host for 21 h or more before dissection hatched. Eggs that had been dissected out of a host 9 h after parasitization had a 56% eclosion rate when the medium was supplemented with fetal bovine serum. When eggs were dissected out 15 h or more after parasitization, 80% eclosed in this supplemented medium. No eggs that had been within the host for less than 7 h, a time that coincides with the germ band anlage formation, successfully developed in vitro (Pennacchio et al. 1992).

In both the base diet and the fetal bovine serum-supplemented diet, none of the larvae hatched in vitro were able to obtain the critical weight needed to molt to the 2nd larval instar. Growth of parasitoids improved when bovine albumin and lactalbumin were added to the diet containing fetal bovine serum, though larvae still failed to molt. With further supplementation with chicken egg yolk, however, 10% of larvae molted to the 2nd larval instar. Development to 2nd instar was delayed, taking nearly three times as long as normal, in vivo development, and no larvae molted to 3rd instar. However, this study clearly showed that *T. nigriceps* required no exogenous hormone sources to develop from post-germ band egg to 2nd larval instar (Pennacchio et al. 1992).

Toxoneuron nigriceps was further reared in vitro from early 2nd to 3rd larval instar by Kuriachan et al. (2006). A base artificial diet composed of TNM-FH insect medium supplemented with additional asparagine, glutamine, hydroxyproline, lysine, serine, threonine, and valine, as well as 1% trehalose, 1% bovine serum albumin, and 0.5% lactalbumin was tested against diets additionally supplemented with hemolymph extracted from both healthy and parasitized *H. virescens* larvae at various ages. Fresh

chicken egg yolk was also added to each diet. Diets of each formulation were tested in both a liquid and semisolid form, thickened with agar. Although only the composition of the diet appeared to affect larval size, with larvae reared on diets containing hemolymph from parasitized *H. virescens* larvae growing larger than those fed on diets containing unparasitized hemolymph, the viscosity of the diet—liquid or semisolid—had a significant effect on larval molting. The highest rate of molting, 100%, occurred among *T. nigriceps* larvae reared on semisolid diets containing hemolymph from *H. virescens* larvae parasitized 5 d previously or earlier. Similar diets in liquid form yielded a maximum of 50% molt to 3rd instar (Kuriachan et al. 2006).

Toxoneuron nigriceps larvae reared to 3rd instar exhibited some of the characteristics of mature larvae. Most larvae reared on semisolid diets containing late parasitized hemolymph shed the anal vesicle, and many voided the meconium. Some also secreted a white substance from the anterior oral area, interpreted as an attempt at producing silk, and many exhibited rolling, expanding, and contracting motions, which could be interpreted at attempts to form a cocoon and enter the pupal stage. None of the larvae in the experiment of Kuriachan et al. (2006) survived for more than 3 d after molting to the 3rd larval instar, however, and none formed cocoons or entered the pupal stage.

Toxoneuron nigriceps appeared very close to reaching pupation in the in vitro rearing effort of Kuriachan et al. (2006), though pupation ultimately failed to take place. At this stage in development, *T. nigriceps* larvae reared naturally would normally have already egressed from their hosts. In this context, an important question is why did

larvae that had thus far been successfully reared in the absence of their hosts fail to survive past this point. The answer may lie in two aspects of this parasitoid's late immature development. First, *T. nigriceps* larvae initiate destructive tissue feeding upon egressing from the host: Although they have developed on nutrients extracted from the hemolymph up to this point, post-egression 3rd instar larvae consume the liquefied remains of all remaining host tissues including the fat body, a major nutrient storage organ. Thus, while the artificial diet used to rear *T. nigriceps* larvae apparently was adequate for development to the 3rd larval instar, it may have lacked a number of factors present in host tissues that are necessary for the parasitoid's development beyond the larval stage. Second the physical environment that *T. nigriceps* larvae normally experience after egression from the host seems relevant. When developing within a host, larval egression does not begin until after the host, arrested at a precocious prepupal stage, has excavated a pupation chamber either in the soil (in the field) or in the diet used for rearing the host (when reared in the laboratory). It is within this chamber that immature *T. nigriceps* normally commence destructive tissue feeding, spin cocoons, and pupate. Previous observations of mature *T. nigriceps* larvae suggest that while they form cocoons readily within host-formed chambers, if left on a flat surface, such as on top of the soil, larvae fail to form cocoons and will simply lay down a layer of silk and attempt to pupate naked on top of it (Chamberlin and Tenhet 1926). The cocoon is an important structure for developing prepupae and pupae, offering not only protection against physical damage, but also from desiccation (Tagawa 1996). It is possible that, when reared to 3rd instar in vitro, *T. nigriceps* larvae were not provided with an environment

that allowed formation of a cocoon. This could account for the failure of these larvae to survive long enough to begin pupal development.

In the following studies, two periods of the late immature development of *T. nigriceps* were examined: post-egression tissue feeding, and cocoon formation through pupation. In the first set of studies, the importance of post-egression feeding to *T. nigriceps*, in terms of contribution to development and contribution to macronutritional state, was examined, and the possibility of developing an artificial post-egression diet for *T. nigriceps* was assessed (Chapter II). Next, based on the results of this first set of experiments, a base artificial post-egression diet was developed and tested to compare the development of *T. nigriceps* fed this diet after egression to that of larvae either fed host tissues or not fed at all (Chapter III). A number of further experimental diets, with various other nutrients added, were further tested (Chapter III). The relationship between the size and fitness of *T. nigriceps* adults was also examined, to determine how best to evaluate the fitness of any adults produced from artificial post-egression feeding treatments (Chapter III). In the final set of experiments, pupation chamber formation by both healthy *H. virescens* larvae and those parasitized by *T. nigriceps* were compared to look for evidence of either exploitation or manipulation of host behavior by the parasitoid at this step, which may result in alteration of chamber architecture or burial depth (Chapter IV). Additionally, a number laboratory items were tested for use as artificial pupation chambers for *T nigriceps* being reared in the absence of a host-formed chamber (Chapter IV).

Like other koinobiont parasitoids, *T. nigriceps* naturally develops in a highly dynamic environment. If complete in vitro rearing of *T. nigriceps*, or any other koinobiont endoparasitoid, is ever achieved it will most likely require a series of different artificial media, with a progression of varied nutritional and physical properties. Because of this, rearing of *T. nigriceps* in vitro would not likely be economical enough to improve its potential for use as a biological control agent, at least not without development of an automated system. It could, however, serve as a model organism, as it has asking other questions. Not only could it serve as a model for in vitro rearing parasitoids so difficult to rear as polydnavirus-bearing koinobiont endoparasitoids, but it could also reveal how such parasitoids would respond to being reared without the influence of their host, their polydnavirus, and the factors produced by the interactions of the two.

CHAPTER II

POST-EGRESSION FEEDING

Introduction

Endoparasitoids are often divided into two informal groups, "hemolymph feeders" and "tissue feeders," based on their feeding habits during larval development. Hemolymph feeders are those endoparasitoids that feed non-destructively by filtering nutrients from host hemolymph as larvae, though some may consume varying amounts of the fat body before egression, and then pupate outside of the host (Harvey et al. 2008). This non-destructive feeding style generally leaves the host alive, though moribund, at the time of larval egression. The hemolymph feeding strategy appears to be prevalent in the braconid subfamilies Microgastrinae, Cheloninae, and Cardiochilinae (Gauld and Bolton 1988, Harvey et al. 2000).

Tissue feeders begin their development in a similar fashion, feeding non-destructively on hemolymph; however, late in their development, they will make a behavioral switch and begin a destructive tissue-feeding phase. The timing of the switch from hemolymph to tissue feeding can be critical. If the switch occurs too early, the host will be killed before it is large enough to provide sufficient resources for maximal parasitoid growth. Letting the host grow too large, however, can also be fatal to the parasitoid, as many tissue feeders pupate within the host cuticle, and excess tissue can impede pupation (Hemerik and Harvey 1999). Tissue feeding is the more common strategy by far, occurring in most ichneumonid endoparasitoids, as well as many

braconids (Gauld & Bolton 1988). Tissue feeding appears to be a plesiomorphic trait (Harvey et al. 2008).

Toxoneuron nigriceps Viereck, a member of subfamily Cardiochilinae (Hymenoptera: Braconidae), begins its development in a manner similar to that of hemolymph feeding parasitoids. From 1st to early 3rd larval instar, *T. nigriceps* feeds non-destructively on hemolymph. During its late 3rd instar, however, it begins a destructive tissue-feeding phase. *Toxoneuron nigriceps* is somewhat unusual in this respect, as destructive feeding occurs not within the host, as it does among most tissue feeders, but externally, after parasitoid egression. As the mature *T. nigriceps* larva egresses, it does not separate completely from the host but leaves its posterior end anchored within the hole in the cuticle through which it egressed. The larva then curves around to form a second hole in the host cuticle through which it feeds on the remaining host tissues, which by this point have been liquefied into a viscous pulp. External feeding can last for several hours, during which time the parasitoid alters drastically in appearance, changing from the bright green of its host's hemolymph to a dull whitish color, and nearly doubling in length and girth.

As many members of Cardiochilinae are strictly hemolymph feeders, and *T. nigriceps*, until egression, develops in a manner very similar to that of hemolymph feeders, it appears that in this case the tissue feeding strategy may have arisen secondarily from an ancestrally hemolymph feeding group. If this is the case, then the tissue-feeding phase of *T. nigriceps* is likely an important component of larval development, and deprivation of such feeding could have significant developmental

consequences. Such deprivation may have been relevant in the failure of *T. nigriceps* to develop past the 3rd larval instar when reared by Kuriachan et al. on a preliminary artificial diet (Kuriachan et al. 2006). Third instar parasitoid larvae reared on artificial media by Kuriachan et al. (2006) had an opaque, fragile appearance, unlike in vivo-reared counterparts, perhaps owing to a lack of some tissue components that are normally taken up upon egression from the host.

In this study, newly-egressed *T. nigriceps* larvae were subjected to five different post-egression feeding treatments in order to investigate the importance of post-egression tissue feeding. To evaluate the effects of this feeding phase, some larvae were allowed to feed naturally on their *Heliothis virescens* F. (Lepidoptera: Noctuidae) hosts, while others were prevented from any post-egression feeding. To determine whether or *T. nigriceps* will commence feeding after having their normal post-egression behavior disrupted and having tissue presented to them in an unfamiliar form, *T. nigriceps* larvae were fed experimentally with tissue scraped out of the host after parasitoid egression. To investigate whether or not *T. nigriceps* will feed on non-host tissue and, if so, whether the parasitoid can benefit from this unparasitized tissue, other *T. nigriceps* larvae were fed tissues scraped from healthy *H. virescens* prepupae.

The nutritional benefits of post-egression feeding were also evaluated by extracting and quantifying total proteins, lipids and carbohydrates from tissue of both newly-egressed *T. nigriceps* larvae and from larvae that had completed post-egression feeding. Comparison of the basic nutritional states of *T. nigriceps* larvae before and after tissue-feeding may help elucidate what macronutritional benefit larvae gain from this

step of larval feeding. This information could valuable for formulating an artificial post-egression diet, if it appears that *T. nigriceps* will accept an artificial substitute for host tissue, even while the behavioral components of post-egression feeding have not been studied in detail.

Materials and Methods

Rearing Practices. *Heliothis virescens* larvae were reared on artificial corn earworm diet (Corn earworm diet, BioServ Inc., Frenchtown, NJ) under controlled conditions (29 ± 1ºC; 60 ± 10% RH and a photoperiod of 14:10 [L:D] h) (Vanderzant et al. 1962). *Toxoneuron nigriceps* was reared in a laboratory culture according to Vinson et al. (1973). Hosts were parasitized by placing a late 4^{th} instar larva with a single mated female wasp under the lid of a 35 by 5 mm plastic petri dish. The wasp and host larva were observed until the wasp oviposited once, at which time the larva was removed and placed in a rearing vial with diet. Parasitized hosts were removed from the diet approximately 10 days after parasitization and placed individually in 16 by 100 mm glass test tubes for observation.

Post-Egression Feeding Treatments. As mature *T. nigriceps* larvae egressed from their hosts, they were subjected to one of four feeding treatments. In the first treatment, the control, larvae were allowed to feed normally on host tissues for 3 h before being removed from the host remains, weighed, and placed individually in 0.5 ml gelatin capsules (Electron Microscopy Sciences, Fort Washington, PA). The second treatment served as a negative control, in which larvae were removed from the host

remains immediately upon egression, weighed, and placed in gelatin capsules with no post-egression feeding. In the third treatment, parasitoid larvae were removed from the host remains, which were then opened by cutting down the dorsal midline. The remaining internal tissues were scraped out of the cuticle using the spatula end of a Spoonula (Thermo Fisher Scientific, Waltham, MA) and placed on a piece of filter paper in a 35 by 10 mm plastic Petri dish. The *T. nigriceps* larva was placed on the filter paper, with its mouthparts and posterior end just touching the collected host remains. The Petri dish was then closed and placed in the rearing incubator, where the larva was allowed to feed on the tissues for 3 h before being weighed and placed in a capsule. After 3 h, the host tissue became melanized and hardened, and thus unsuitable for feeding by the parasitoid.

Larvae in the fourth treatment were handled similarly to those in the previous treatment, but were presented with tissue scraped from an unparasitized *H. virescens* larva at the prepupal stage. This stage was chosen because the tissue is somewhat homogenized and viscous, and is more similar to the liquefied tissue in parasitized larval remains than the tissue of other larval stages. Again, *T. nigriceps* larvae were allowed to feed on tissue for 3 h before being removed from any remaining tissue, weighed, and placed in a gelatin capsule. After the feeding treatment, each larva in its gelatin capsule was placed individually in a labeled glass test tube for observation. These test tubes were held in a test tube rack in the rearing incubator and observed for cocoon formation and emergence of adult wasps.

The masses of larvae (post-feeding treatment), cocoons, and adults from each treatment were logarithm transformed to normalize, then compared using a repeated measures MANOVA followed by Bonferroni corrected pairwise comparisons (JMP 7, SAS Institute Inc., Cary, NC). To look for evidence of larval feeding on scraped host tissues and scraped unparasitized tissues, initial larval mass was subtracted from final larval mass to calculate the mass gained through feeding in each of these treatments. One sample *t*-tests were used to determine if mass gain was significantly greater than zero in the scraped, parasitized and scraped, unparasitized tissue treatments (SPSS, SPSS Inc., Chicago, IL). Mass gain was compared between these two treatments using an independent samples *t*-test (SPSS, SPSS Inc., Chicago, IL). The proportions of parasitoids in each treatment that went on to form cocoons and to emerge as adult wasps were compared using *G*-tests, followed by a Tukey-type multiple comparison among proportions (Zar 2010).

Quantification of Proteins, Lipids and Carbohydrates. Mature *T. nigriceps* larvae were collected from hosts that had been removed from diet containers approximately 10 d after parasitization—at which point the hosts no longer feed—and placed individually in 16 by100 mm glass test tubes for observation. The wasp larvae were collected either immediately after they finished egression, before the initiation of tissue feeding, or immediately after they had completed tissue feeding and removed their head from the host remains. Larvae from both treatments were then massed, placed individually in microcentrifuge tubes, and flash-frozen in liquid nitrogen. The frozen larvae were stored in a freezer at -80°C until the extractions were carried out, at which

time larvae were crushed and homogenized within the tube using a glass rod coated in clean parafilm.

Total carbohydrates in larval samples were measured following a procedure modified from van Handel (1985a) using anthrone reagent, which changes from yellow to blue-green in the presence of carbohydrates. Anthrone reagent was prepared by measuring 150 ml of de-ionized water into a 1 liter Erlynmeyer flask After placing the flask in an ice bath, 380 ml concentrated (97%) sulfuric acid was added. Approximately 750 mg anthrone (J. T. Baker, Phillipsburg, NJ.) was then added to this solution and mixed until it dissolved completely. The complete anthrone reagent was then stored in a refrigerator, where it could be kept stable for a number of weeks.

A small sample of homogenized tissue (1 to 5 mg) was taken from each larva and placed in a 12 by 75 mm glass culture tube. To measure the total carbohydrate content of each sample, 5 ml of anthrone reagent was added directly to each culture tube. Each tube was vortexed to mix it, and then placed in a test tube heater and held at 90-92°C for 17 minutes. After cooling to room temperature, the absorbance of each sample was read at 625 nm in a USB4000 Fiber Optic Spectrometer (Ocean Optics, Inc., Dunedin, FL). Carbohydrate content was calculated from absorbance using a standard curve generated by measuring three sets of 25, 50, 100, 150 and 200 µl portions of 1 mg/ml solution of sucrose in 25% ethanol.

Total lipids in larval samples were measured following a procedure modified from van Handel (1985b) using a vanillin-phosphoric acid reagent, which changes from clear to pink when it reacts with lipids. The vanillin-phosphoric acid reagent was

prepared by adding 600 mg vanillin ReagentPlus (Sigma-Aldrich Co., St. Louis, MO) to 100 ml hot de-ionized water then mixing until the vanillin dissolved. This solution was then cooled to room temperature before 400 ml concentrated (85%) phosphoric acid was added. The reagent was stored at room temperature in a sealed glass bottle covered in aluminum foil, to keep the light-sensitive reagent from being degraded.

Small samples of larval tissue (1 to 10 mg) were measured into 12 by 75 mm glass culture tubes, to which 0.5 ml of 1:1 (v:v) chloroform-methanol solution was added and mixed to extract lipids. Each tube was then heated until the solvent evaporated completely. Next, 0.2 ml of concentrated (97%) sulfuric acid was added and the samples were placed back in the heating block and held at approximately 95°C for 10 minutes. Samples were then removed from the heating block and cooled to room temperature before vanillin-phosphoric acid reagent was added up to a previously-determined 5 ml mark on each culture tube. Samples were carefully poured back and fourth between two test tubes four times to mix thoroughly. Absorbance of each sample was measured at a wavelength of 525 nm, or at 490 nm for samples with particularly high lipid concentrations. Standard curves at 525 nm and 490 nm were generated using three sets of 50, 100, 200 and 400 µl samples of a standard solution of 1 mg/ml vegetable oil in chloroform.

Total protein in larval samples was measured using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). With this kit, protein assays are carried out using a solution in which Cu^{+2} is reduced to Cu^{+1} by proteins, causing a color change in a bicinchoninic acid reagent. The procedure was carried out following kit instructions.

For each sample, a small amount of homogenized *T. nigriceps* larval tissue (between 1 and 10 mg) was measured into a microcentrifuge tube. Following the procedure of Wheeler and Buck (1996), one ml of 1 M NaOH was added to each sample to extract non-water soluble proteins. Each sample was then agitated on a vortex machine for one minute to thoroughly mix the solution. A 0.1 ml aliquot of each sample was added to a 12 by 75 mm glass culture tube, to which 2 ml of the BCA reagent was then added. Each test tube was briefly vortexed again, to mix the sample and reagent then incubated in a heating block for 30 minutes at 37°C. After cooling to room temperature, each sample was measured in the spectrometer at a wavelength of 562 nm. A protein standard curve was generated using three sets of 2.5, 12.5, 25, 50, 75, 100, 150 and 200 μg samples of bovine serum albumin standard.

The proportion of larval tissue composed of protein, lipid and carbohydrate in the tissues of pre- and post-tissue feeding larvae was calculated by dividing the estimated mass of each macronutrient in a sample by the total mass of the sample it was taken from. These proportions of carbohydrate, lipid and protein—measured as mg macronutrient per mg sample (wet mass)—were compared between pre- and post-tissue feeding larvae. Because all three assays were carried out using samples from the same 60 larvae, all three parameters were compared using a MANOVA (JMP 7, SAS Institute Inc., Cary, NC).

Results

Post-Egression Feeding Treatments. In a repeated measures MANOVA, post-treatment larval mass, cocoon mass, and adult mass formed a single canonical axis (Fig. 1). Feeding treatment had a significant effect on parasitoid size (Table 1), with parasitoids from the control treatment being significantly larger than those fed scraped, parasitized tissue ($F = 21.654$, Bonferroni corrected $P = 7.55\text{x}10^{-5}$), fed unparasitized *H. virescens* ($F = 75.544$, Bonferroni corrected $P = 1.94\text{x}10^{-12}$), or not fed at all ($F = 48.889$, Bonferroni corrected $P = 4.19\text{x}10^{-9}$). Parasitoids fed scraped host tissue were also significantly larger than those fed unparasitized *H. virescens* tissue ($F = 15.366$, Bonferroni corrected $P = 0.0011$) and those that were not fed ($F = 9.036$, Bonferroni corrected $P = 0.0211$). The masses of larvae fed unparasitized *H. virescens* tissue and those not fed after egression did not differ significantly ($F = 0.106$, Bonferroni corrected $P = 4.475$). The raw mean masses of parasitoids in each treatment are summarized in Table 2. While there was an effect of parasitoid stage on mass, it was expected as pupae generally weigh less than larvae, and adults less than pupae. This effect was likely magnified by measuring wet mass of larvae and dry mass of. However, this factor did not significantly affect the model.

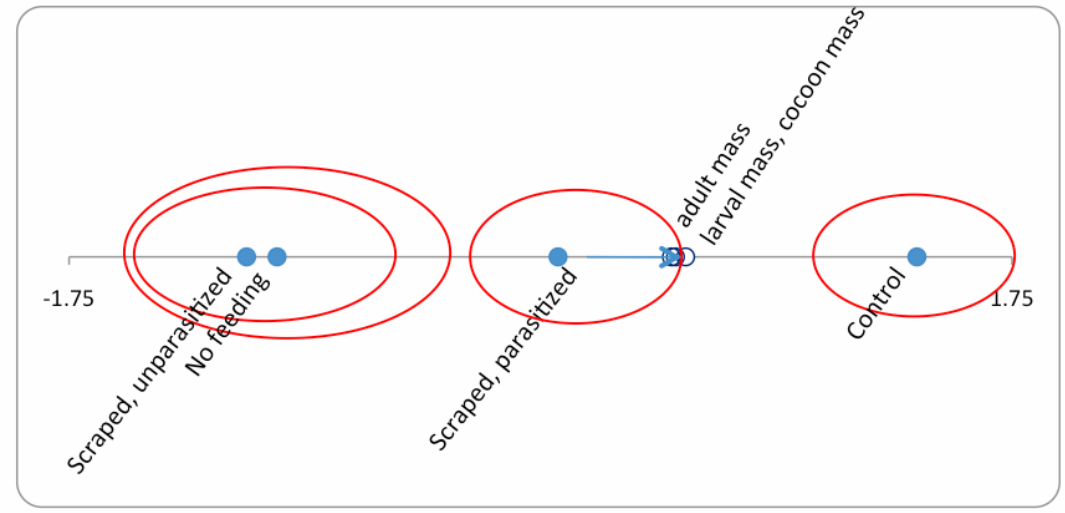

Fig. 1. Canonical centroid plot of parasitoid mass. Solid dots represent mean, and ellipsoids represent the 95% confidence interval. Non-overlapping ellipsoids indicate significantly different groups. Post-treatment larval mass, cocoon mass, and adult mass form a single canonical axis, with all three measures of mass effected similarly by the treatments. Larvae from the control treatment were largest, with larvae fed scraped, parasitized tissues reaching intermediate sizes, and larvae fed unparasitized tissues or not fed at all forming one group, representing the smallest parasitoids (MANOVA [JMP 7, SAS Institute Inc., Cary, NC]).

Table 1. Statistical results of a repeated measures MANOVA of post-treatment larval mass, cocoon mass, and adult mass from each feeding treatment (JMP 7, SAS Institute Inc., Cary, NC).

Repeated measures MANOVA				
Between subjects	F	DFnum	DFdenom	P
Treatment	30.2169	3	81	<.0001
Within subjects				
Stage	10829.7537	2	80	<.0001
Treatment × stage	0.6225*	6	160	0.7121
	*approximated			

Table 2. Mean masses (± SE) of larvae, cocoons, and adult parasitoids (in mg). Larvae and cocoons were measured in fresh mass, and adults in dry mass.

Treatment	Final larval mass	Cocoon mass	Adult dry mass
Control	66.2 (4.2)	59.2 (2.8)	7.2 (0.3)
No feeding	51.0 (1.3)	31.1 (1.1)	4.5 (0.3)
Scraped, parasitized	63.1 (2.2)	43.3 (1.6)	5.4 (0.3)
Scraped, unparasitized	52.1 (1.6)	33.3 (1.0)	4.3 (0.2)

Larvae presented with scraped, parasitized host tissue gained significant mass during the 3 h feeding period ($t = 10.0878$, df = 29, $P < 0.0001$; one sample *t*-test [SPSS, SPSS Inc., Chicago, IL]), as did larvae presented with tissues scraped from unparasitized *H. virescens* prepupae ($t = 6.6581$, df = 29, $P < 0.0001$; one samples *t*-test [SPSS, SPSS Inc., Chicago, IL]). This indicated that larvae in both treatments consumed the tissues presented to them (Fig. 2). However, larvae fed parasitized tissue from gained significantly more mass than those fed unparasitized tissue ($t = 5.7705$, df = 58, $P < 0.0001$; independent samples *t*-test [SPSS, SPSS Inc., Chicago, IL]), with those from the scraped parasitized tissue treatment gaining (mean ± SE) 15.4 ± 1.5 mg and those from the scraped unparasitized tissue treatment gaining 5.4 ± 0.8 mg.

Feeding treatment did not have a significant effect on the proportion of *T. nigriceps* larvae in each treatment that formed cocoons ($G = 3.527$, $P = 0.317$; *G*-test [Zar 2010]) (Fig. 3). However, feeding treatment significantly affected the proportion of parasitoids that emerged as adult wasps (Fig. 4). Parasitoids that were not fed after egression survived to adulthood in 50% of cases, a significantly smaller proportion than in the control treatment ($q = 5.5097$, $P < 0.005$), the scraped, parasitized tissue treatment ($q = 4.3635$, $P < 0.025$), and the scraped, unparasitized tissue treatment ($q = 4.3635$, $P < 0.025$). The proportions of parasitoids that emerged as adults in the control treatment, as well as in the two scraped tissue treatments, did not differ (Tukey-type multiple comparison among proportions [Zar 2010]).

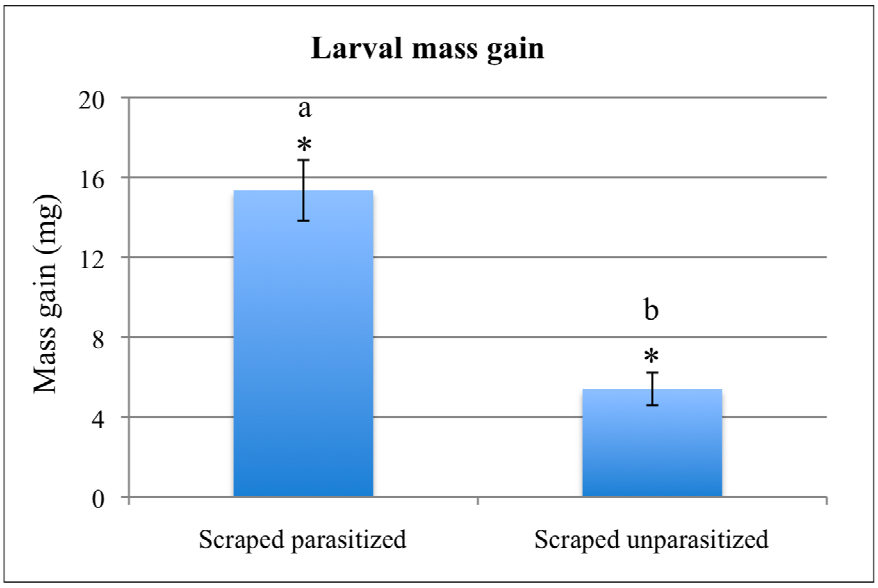

Fig. 2. Larval mass gain (mg). Error bars represent SE. An asterisk indicates a gain significantly different from zero ($P < 0.0001$ one sample *t*-tests [SPSS, SPSS Inc., Chicago, IL]). Different letters above columns indicate a statistical difference between treatments ($P < 0.0001$; independent samples *t*-test [SPSS, SPSS Inc., Chicago, IL]).

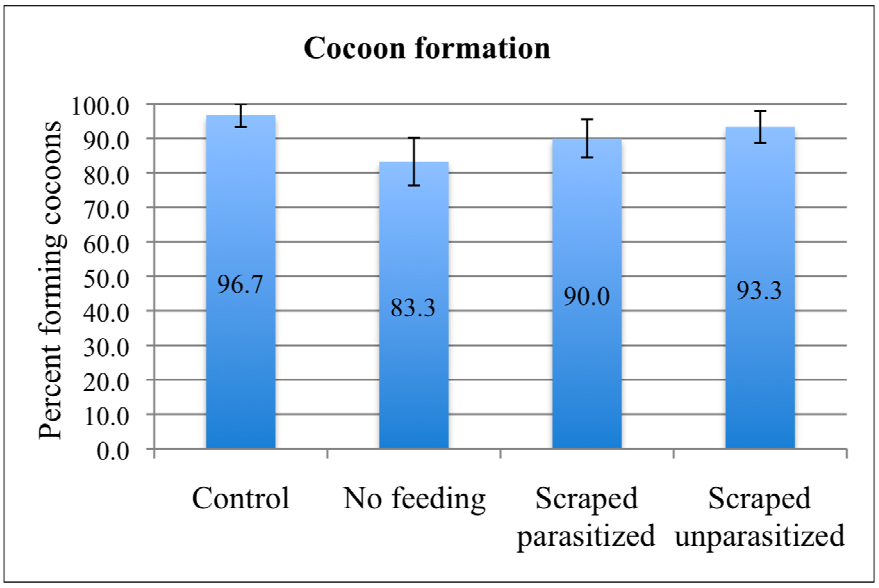

Fig. 3. The percent of parasitoids in each treatment that formed complete cocoons. Error bars represent SE. No significant differences were found ($G = 3.527$, $P = 0.317$; G-test [Zar 2010]).

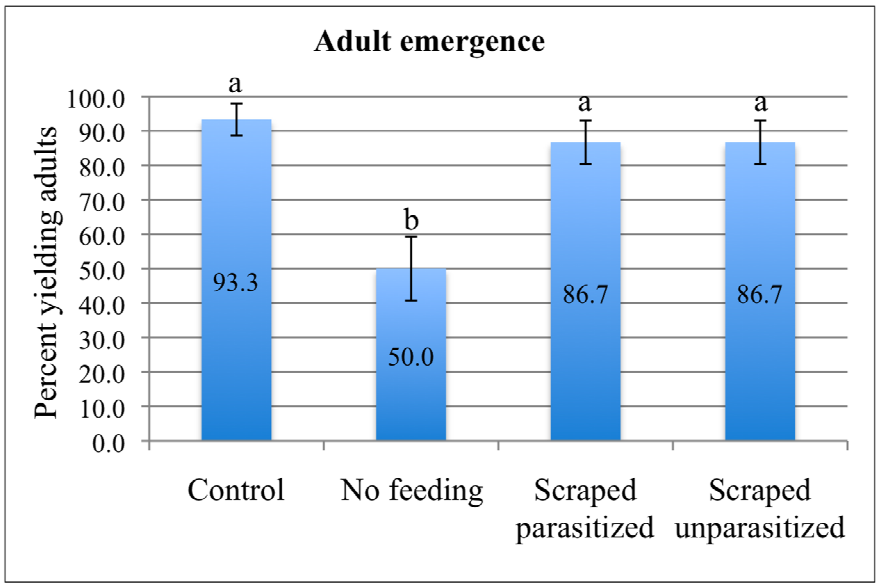

Fig. 4. The percent of parasitoids in each treatment that emerged as live adult wasps. Error bars represent SE. The same letters above columns indicate numbers that are not significantly different ($P < 0.05$; Tukey-type multiple comparison among proportions [Zar 2010]).

Quantification of Proteins, Lipids and Carbohydrates. Concentrations of proteins, lipids and carbohydrates were significantly higher in the tissues of *T. nigriceps* larvae that had completed post-egression tissue feeding than in the tissues of those that had not experienced tissue feeding (Fig. 5) ($F = 52.147$, $P = 3.2\text{x}10^{-16}$; MANOVA ([JMP 7, SAS Institute Inc., Cary, NC]). Raw concentrations of proteins, lipids, and carbohydrates are displayed in Table 3. The increase in tissue concentration of carbohydrates and proteins were similar, with an approximately 1.5-fold increase in

both. The difference in lipid concentration was much greater, however, with the tissues of post-feeding larvae containing nearly 3-fold greater concentration.

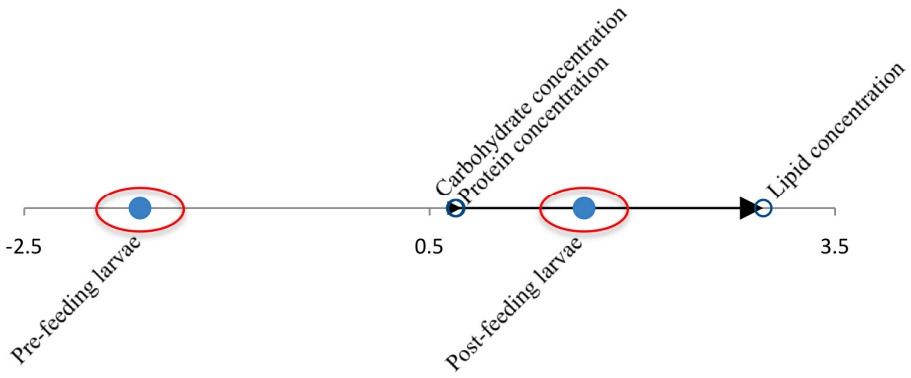

Fig. 5. Canonical centroid plot of macronutrient concentration (mg macronutrient/mg tissue sample [wet mass], log transformed) in the tissues of pre- and post-tissue feeding *T. nigriceps* larvae. Solid dots represent mean, and ellipsoids represent the 95% confidence interval. Non-overlapping ellipsoids represent statistically significant difference between groups. Protein, lipid, and carbohydrate concentration formed a single canonical axis. Macronutrient concentrations were higher in the tissues of post-feeding larvae than in those of pre-feeding larvae ($F = 52.147$, $P = 3.2\text{x}10^{-16}$; MANOVA ([JMP 7, SAS Institute Inc., Cary, NC]).

Table 3. The mean raw macronutrient concentration of, and difference (%) between, homogenized tissue from pre- and post-tissue feeding *T. nigriceps* larvae.

Treatment	Protein concentration (±SE)	Lipid concentration (±SE)	Carbohydrate concentration (±SE)
Pre-feeding	0.1547 (0.0102)	0.0471 (0.003)	0.0253 (0.0024)
Post-feeding	0.2097 (0.0103)	0.1279 (0.006)	0.0365 (0.0035)
Difference	35.56%	171.51%	44.27%

Discussion

Post-egression tissue feeding is vital to the late immature development of *T. nigriceps*. Although a lack of tissue feeding did not prevent mature larvae from forming cocoons, there was a clear impact on the ability of parasitoids to pupate and emerge as adult wasps. Parasitoids that were not offered any sort of tissue after egression had only a 50% rate of survival to adulthood, compared to the 93% survival rate of those that were allowed to carry out normal host feeding behavior. The importance of post-egression tissue feeding was also demonstrated in a recent study by Kuriachan et al. (2011). In their study, larvae prevented from tissue feeding after egression went on to form significantly fewer cocoons than those that had been allowed to carry out normal tissue feeding: Only 58% of larvae prevented from tissue feeding in the study emerged from cocoons as adult wasps, compared to the 100% emergence rate of larvae allowed to tissue feed. Additionally, many adults that emerged from the non-feeding treatment were

deformed and had incomplete wing development, and generally suffered from a reduction in longevity, compared to those that had fed on tissue.

In the post-egression feeding experiment carried out here, provision of *H. virescens* tissue, including tissue from unparasitized larvae, improved *T. nigriceps* survival, bringing adult emergence up to levels similar to those seen in control conditions. Parasitoids in both the scraped parasitized and scraped unparasitized tissue treatments reached adulthood in nearly 87% of cases, a significantly higher proportion than reached adulthood what not fed tissues after egression. However, while larvae fed on tissue scraped out of both parasitized and unparasitized *H. virescens* larvae, those that were given unparasitized tissue gained significantly less mass than those given parasitized tissue (Fig. 2). When compared to parasitoids from the fed tissue from unparasitized *H. virescens* prepupae did not significantly differ in mass from those provided with no tissue, and yet their survival to adulthood was improved dramatically.

Toxoneuron nigriceps larvae fed on tissue that was presented to them. In both the scraped parasitized tissue and scraped unparasitized tissue treatments, larvae gained significant mass during the 3 h feeding window (Fig. 2). *Toxoneuron nigriceps* that were given unparasitized tissue after egression differ statistically in mass from those that were not fed after egression (Fig. 1, Table 1), but nonetheless did feed after egression. That *T. nigriceps* parasitoids fed on scraped tissue, both parasitized and unparasitized, is promising, as it shows that even if they are disrupted from their normal post-egression behavioral pattern, they will initiate feeding on at least some substances that are presented to them. In particular, feeding on tissue from unparasitized *H. virescens*

suggests that they will consume non-host matter. That developing *T. nigriceps* benefit from this tissue by reaching adulthood in similar proportions to parasitoids that underwent natural post-egression feeding, despite little tissue being consumed, offers hope that *T. nigriceps* may also feed on and benefit from some type of artificial post-egression diet. When deciding what type of artificial diet might best benefit newly-egressed *T. nigriceps* larvae, the results of the second half of this study may offer insight.

That proportions of carbohydrates, proteins, and lipids were all higher in the tissues of post-tissue feeding larvae than in those of pre-tissue feeding larvae is not surprising in and of itself; however the rate at which these macronutrients differ from each other pre- and post-tissue feeding offers insight into the macronutritional benefit *T. nigriceps* gains during post-egression feeding. Not only do larvae allowed to tissue feed consume more matter overall, the matter that they consume during this stage is likely to have a very different composition to the hemolymph that they have been feeding on in their previous developmental stages.

While hemolymph can contain relatively high concentrations of proteins, carbohydrates (in the form of trehalose) and lipids (bound in lipophorins) are found in lower concentrations (Wyatt 1961). What draws attention is which of these macronutrients showed the largest increase. The largest difference in macronutrient concentration was seen in lipids, which were 2.7-fold higher in the tissues of post-feeding larvae than in pre-feeding larvae. At least some of this difference is likely due to lipogenesis during post-egression feeding. However, larvae feeding on tissues are

feeding, in part, on the liquefied remains of the fat body, which is where the majority of the host's lipid supply is stored in the form of triglycerides. This is likely to be an important nutritional source for developing *T. nigriceps*, especially since a number of adult wasps (including several members of Braconidae and Ichneumonidae) are not capable of lipogenesis, and must rely on stores of lipids acquired during larval development (Visser and Ellers 2008).

If *T. nigriceps* can be induced to feed on an artificial post-egression diet, this diet should reflect the difference in macronutrient concentration exhibited by pre- and post-tissue feeding larvae. The diet will need to contain carbohydrates, but will likely need to have proportionally greater protein and, perhaps most importantly, a proper lipid composition and concentration. Another vital factor will be a source of sterols as *T. nigriceps*, like other insects, are incapable of synthesizing these de novo (Clark and Bloch 1959). Another factor to consider, apart from nutrition, will be the consistency of this diet. By the time mature larvae egress from the host, the remaining tissues inside have been partially liquefied, turning them into a viscous, pulpy substance. This is likely important for mature *T. nigriceps* larvae, which do not have mandibles that are well suited for chewing solid foods. A diet with too liquid a consistency, however, would pose the problem of larvae drowning in it, rather than being able to feed. Taking these two factors, as well as preliminary experimentation, into consideration fresh chicken egg yolk was chosen as a base for development of an artificial post-egression diet for *T. nigriceps*.

CHAPTER III

AN ARTIFICIAL POST-EGRESSION DIET

Introduction

Koinobiont parasitoids appear to have dynamic nutritional requirements that change throughout their development within a living, growing host. This is in contrast to idiobionts, which feed on a relatively static nutrition source: a single, non-growing host stage or a permanently paralyzed host. In the *Heliothis virescens-Toxoneuron nigriceps* system, early larval development of the parasitoid takes place during the 5th, and final, larval instar of the host as it prepares for a pupation that will not occur. As such, protein and glycerol concentrations in the hemolymph are elevated at this time (Pennacchio et al. 1993). Parasitization of *H. virescens* by *T. nigriceps* has also been linked to increases in the concentrations of proteins and several free amino acids in the host hemolymph (Cônsoli and Vinson 2004). This indicates that the nutritional needs of *T. nigriceps* larvae likely change as the parasitoids develop, and as the nutritional physiology of the host changes.

An even greater change in nutritional intake takes place after *T. nigriceps* egresses from the host and its feeding behavior switches from hemolymph feeding to destructive tissue feeding. The post-egression feeding study described in Chapter II demonstrated that post-egression tissue feeding is vital to the development of *T. nigriceps*. It not only significantly increased parasitoid mass, but also contributed greatly

to survival of *T. nigriceps* to adulthood. Even parasitoids fed a suboptimal post-egression diet—tissue from unparasitized *Heliothis virescens* prepupae—fared far better than those starved after egression, despite showing no significant difference in post-treatment mass. This indicates that provision of an adequate post-egression diet is fundamental to complete in vitro rearing of *T. nigriceps*.

The macronutrient quantification of pre- and post-tissue feeding larvae in Chapter II suggested that a post-egression diet for *T. nigriceps* larvae perhaps should be rich in lipids, but should also provide proteins and some carbohydrates. A source of sterols is also likely to be necessary, as insects cannot synthesize these de novo (Clark and Bloch 1959). Observations of *T. nigriceps* larvae as they feed on host tissues further suggest that this diet should be a viscous, semi-solid medium that would allow larvae to feed easily without risk of drowning in the diet. After preliminary testing of a number of potential media, a preliminary artificial diet composed of fresh chicken egg yolk and TNM-FH media was selected for further experimentation. This mixture has a consistency that is similar to that of the partially digested host tissues that *T. nigriceps* feed on normally, as well as a putatively suitable nutritional makeup. Per 100 g, fresh chicken egg yolk contains on average 16.86 g protein, 26.54 g lipid, 3.59 g carbohydrate, and 1.085 g cholesterol (USDA 2011). Chicken egg yolk has been successfully used as a supplement in artificial diets for a number of parasitoids, including *Campoletis sonorensis* Cameron (Hymenoptera: Ichneumonidae) (Hu and Vinson 1997), *Bracon hebetor* Say (Hymenoptera: Braconidae) (Magro and Parra 2004), and earlier stages of *T. nigriceps* itself (Pennacchio et al. 1992, Kuriachan et al. 2006).

In order to evaluate the likely quality of *T. nigriceps* reared to adulthood on an artificial diet, an assessment of fitness proxies of resulting adult wasps would be informative. Rather than measuring indices of quality (such as longevity and potential fecundity) directly, many past studies have used adult size as a proxy or surrogate measure for parasitoid fitness. Among many species, the size and quality of the host determine the amount and the quality of the food that immature parasitoids consume. This in turn is a main determinant of adult parasitoid size (Godfray 1994). It is often presumed that adult size is closely tied to parasitoid quality, in terms of both longevity and fecundity. A number of studies have shown correlations between adult size and egg load, as well as between size and adult longevity, among a wide variety of parasitoid species (Godfray 1994). However, a number of species, in contrast, have shown no correlation between body size and these measures of quality. In some cases, body size may have a greater impact on one sex than the other. Among adult male wasps of *Aphidius nigripes* Ashmead (Hymenoptera: Aphidiidae), for example, size did not appear to have a significant effect on longevity, mating success, fertility, or flight ability. Among female wasps, however, increased body size was linked to higher innate capacity to increase, though longevity did not show a similar correlation (Cloutier et al. 2000). Therefore, it may be better to measure components of parasitoid quality directly, rather than relying on parasitoid size to make assumptions about quality.

In the present study, the relationship between *T. nigriceps* adult size and two proxies of quality, longevity and female potential fecundity, were examined. Longevity was measured as the number of days between adult emergence and starvation, when

wasps were provided with only water but no food. Female potential fecundity was measured as ovary mass 5 d after adult emergence, as *T. nigriceps* is intermediate on the ovigeny index, with females having some mature eggs upon emergence, but with further ovary maturation throughout their adult lifespan. Based on the findings, the best method of assessing the fitness of adult wasps that had been fed various post-egression diets as larvae could be determined. This was used as part of the process for evaluating the suitability of the preliminary artificial post-egression diet for *T. nigriceps* larvae. The development and putative quality of *T. nigriceps* fed this diet after egression from the host was compared to that of parasitoids fed tissue scraped out of hosts after egression and to that of parasitoids that were not fed at all after egression. The base artificial diet was then compared to other experimental diets that had been further supplemented with additional sources of carbohydrate, protein, or lipid to see if an increase in any of these three macronutrients improved the growth and development of *T. nigriceps* fed these diets after egression.

Materials and Methods

Rearing Practices Rearing of *H. virescens* and *T. nigriceps* was carried out as described in Chapter II, with the exception of the parasitization process. For the purposes of this study, groups of 30, late-4th instar *H. virescens* larvae were placed in plastic cages with two mated female *T. nigriceps* wasps for 2 h to allow for parasitization.

Body Size and Fitness. Adult wasps for this study were collected by isolating cocoons, reared under standard laboratory practices, in glass test tubes plugged with

cotton. This allowed newly emerged wasps to be easily identified, and prevented mating between male and female wasps. To examine the relationship between body size and longevity, 30 male wasps and 30 females wasps were collected and the date of their emergence was recorded. Wasps were then kept individually in cotton-plugged test tubes with de-ionized water, held behind a cotton ball. No food was provided. Wasps were held in an incubator at controlled conditions (29 ± 1ºC; 60 ± 10% RH and a photoperiod of 14:10 [L:D] h) and monitored daily. When wasps died, the date of death was recorded and used to calculate longevity. Dry mass, hind tibia length, and forewing length of each wasp were measured and recorded. Hind tibia length, forewing length, and the cube root of dry mass were log transformed to normalize, and the correlations between the three measures of body size were examined using a multivariate analysis (JMP 7, SAS Institute Inc., Cary, NC). Based on the conclusions of this test (see Results) the relationship between dry mass and adult longevity was examined separately for male and female wasps using a linear regression analysis (JMP 7, SAS Institute Inc., Cary, NC).

To examine the relationship between body size and potential fecundity, an additional 25 female wasps were collected and their date of emergence recorded. *T. nigriceps* is neither completely synovigenic nor pro-ovigenic, with females emerging with some mature ovarioles, but continuing to mature more throughout their adult lifetime. To allow for some further egg maturation, female wasps were held in containers within the incubator and provided with de-ionized water and a honey-water diet (to prevent re-absorption of eggs due to starvation). Five days after emergence, wasps were killed by freezing, and then placed in sealed microcentrifuge tubes and held in the

freezer. Each wasp was later thawed briefly then dissected under a microscope. The ovaries were removed from each wasp and placed on a pre-weighed piece of weighing paper to be dried and weighed. The initial mass of the weighing paper was subtracted from the mass of the paper plus ovaries to determine the dry mass of each set of ovaries. Ovary mass was used rather than a count of mature ovarioles due to time limitations. The dry mass of the remaining the wasp bodies (minus the ovaries) were measured and recorded. Body and ovary dry mass were both cube root and log transformed to normalize. The relationship between these two parameters was examined using linear regression (JMP 7, SAS Institute Inc., Cary, NC).

Evaluation of the Base Artificial Diet. A base artificial diet was prepared by first separating the yolk from the white of a fresh chicken egg. Portions of yolk were then carefully extracted using a 1 cc disposable syringe. Egg yolk was added up to the 1.5 ml mark of microcentrifuge tubes into which 0.2 ml of TNM-FH (HyClone Laboratories Inc., Logan, UT) had previously been added. The microcentrifuge tubes were then closed and the solution mixed on a vortex machine. The diet was kept refrigerated in sealed microcentrifuge tubes. The performance of *T. nigriceps* fed this base diet was evaluated by comparing between three post-egression feeding treatments: a negative control with no post-egression feeding, a positive control with newly-egressed larvae fed host tissue, and a treatment in which larvae were fed the base diet.

Toxoneuron nigriceps larvae from the no post-egression feeding treatment were removed from their hosts' remains immediately after egression, before tissue feeding could take place, weighed, and then placed in a gelatin capsule. Larvae in the host tissue

treatment were also removed from their hosts' remains and weighed immediately after egression. The host remains were then opened by cutting along the dorsal midline with microscissors, and the tissue inside was carefully scraped out using the spatula end of a Spoonula (Thermo Fisher Scientific, Waltham, MA). This scraped tissue was then placed on a piece of No. 5 filter paper inside of a 35 by 10 mm plastic Petri dish. The parasitoid larva was then placed next to the host tissue, with its posterior end and mouthparts barely touching the tissue. The Petri dish was then closed and placed in an incubator at controlled conditions (29 ± 1ºC; 60 ± 10% RH and a photoperiod of 14:10 [L:D] h) for 3 h (during photophase), after which point the tissue would begin to harden and melanize. The larva was then removed from any remaining tissue, weighed, and placed in a gelatin capsule. Larvae being fed the base artificial diet were handled similarly to those being fed host tissue, except that they were placed next to an approximately 0.1 ml droplet of the base diet. These larvae were also limited to 3 h of feeding. After 3 h, larvae were removed from any remaining diet, weighed, and placed in gelatin capsules.

In all three treatments the gelatin capsules, with larvae inside, were placed individually in labeled glass test tubes, which were then plugged with cotton and placed in a test tube rack. The developing parasitoids were held at controlled incubator conditions and monitored for the formation of cocoons. Only complete cocoons—those that fully enclosed larvae—were counted and recorded. Cocoons were then monitored daily for emergence of adult wasps. Date of emergence was recorded, and each wasp was then placed in a test tube and provided with de-ionized water, but no food, and

placed back in the incubator. Wasps were checked twice per day, in the morning and afternoon, and the date of death recorded. Longevity was recorded in half-day intervals.

Post-treatment larval mass was compared between each treatment using an analysis of covariance, with pre-feeding larval mass used as a covariate (JMP 7, SAS Institute Inc., Cary, NC). Mass gains in the host tissue and base diet feeding treatments, calculated by subtracting pre-treatment mass from post-treatment mass, were analyzed using one-sample *t*-tests to test for gains significantly greater than zero (SPSS 16.0, SPSS Inc., Chicago, IL). The mass gained by larvae in the host tissue feeding treatment and the base artificial diet feeding treatment were compared using an independent samples *t*-test (SPSS 16.0, SPSS Inc., Chicago, IL). The proportions of parasitoids in each treatment that formed cocoons and that emerged as adult wasps were compared using *G*-tests (Zar 2010). Adult wasp longevity was compared between treatments using a one-way analysis of variance (SPSS 16.0, SPSS Inc., Chicago, IL). Cocoon mass and adult mass were not analyzed in this study, as results for a similar experiment in Chapter II showed that final larval mass is highly predictive of both cocoon and adult mass, and that these factors do not appear to be separate from one another in a MANOVA.

Additional Supplements to the Artificial Diet. In addition to the base artificial diet, three modified artificial diets were tested. A diet with increased carbohydrate content (carbohydrate diet) was formulated by supplementing with glucose. The normal glucose content of TNM-FH (0.7 g/liter glucose) was doubled by adding 0.021 g of additional D-glucose to 30 ml of TNM-FH. The diet was then prepared similarly to the

base diet, with 0.2 ml of this glucose-enhanced TNM-FH mixed with 1.3 ml of fresh chicken egg yolk.

A diet with increased protein content (protein diet) was created by adding 4.5 ml of a lactalbumin solution to 30 ml of TNM-FH, which contains 3.33 g/liter lactalbumin normally. As in the previous two diets, 0.2 ml of this modified TNM-FH was mixed with 1.3 ml chicken egg yolk.

To boost lipid content in the final test diet, 0.03 ml linolenic acid was added to 0.2 ml TNM-FH and 1.3 ml chicken egg yolk (lipid diet). Attempts were made to dissolve the linolenic acid using chloroform before addition to the diet, but as all of these attempts failed as soon as TNM-FH was added to the mixture, it was instead added directly into the diet and mixed vigorously on a vortex machine before each use.

Each of these four artificial diets was tested on 20 newly-egressed *T.* nigriceps larvae. Again, larvae were weighed immediately after egression, placed on filter paper with 0.1 ml of diet inside of a petri dish, then kept in the rearing incubator for approximately 3 h. After 3 h had elapsed, larvae were removed from the artificial diet. In the previous experiment using the base diet, larvae usually rolled into the center of the droplet of diet at some point and ended up coated in the mixture. In this experiment, larvae were gently washed in Pringle's saline solution to remove any diet residue before being weighed and placed in gelatin capsules. Parasitoids were monitored for formation of a complete cocoon and for emergence of adult wasps. Any adults that emerged were placed individually in test tubes, provided with water, and observed until death. Date of emergence and date of death were recorded to calculate adult longevity.

Again, post-treatment larval mass was compared between each treatment using an analysis of covariance, with pre-feeding larval as a covariate (JMP 7, SAS Institute Inc., Cary, NC). Larval mass gains in each treatment were tested for significant differences from zero using one sample *t*-tests (SPSS 16.0, SPSS Inc., Chicago, IL) and compared among treatments using a one-way analysis of variance (SPSS 16.0, SPSS Inc., Chicago, IL). The proportions of parasitoids in each treatment that formed cocoons and that emerged as adult wasps were compared using a *G*-test (Zar 2010). Adult wasp longevity was compared between treatments using a one-way analysis of variance (SPSS 16.0, SPSS Inc., Chicago, IL).

Results

Body Size and Fitness. The three measures of body size were highly correlated with each other (Table 4) (multivariate analysis [JMP 7, SAS Institute Inc., Cary, NC]). As dry mass had the highest correlation with longevity of the three (Table 3), this alone was used as a measure of body size (Fig. 6). Among male wasps, there was no significant correlation between adult dry mass and longevity ($P = 0.2514$). Among female wasps, dry mass and longevity were weakly, but significantly, correlated (adjusted $R^2 = 0.2840$, $P = 0.0012$) (Fig. 6). No significant correlation was found between body dry mass and ovary dry mass among female *T. nigriceps* (Fig. 7).

Table 4. Correlations between adult longevity and three measures of body size (multivariate analysis [JMP 7, SAS Institute Inc., Cary, NC]).

	Longevity	Dry mass	Forewing length	Hind tibia length
Longevity	--			
Dry mass	0.29419697	--		
Forewing length	0.26655099	0.8960587	--	
Hind tibia length	0.20839428	0.86098844	0.82591939	--

Dry mass, forewing length, and hind tibia length were all highly correlated with each other. Dry mass was the measure of body size with the highest correlation with longevity.

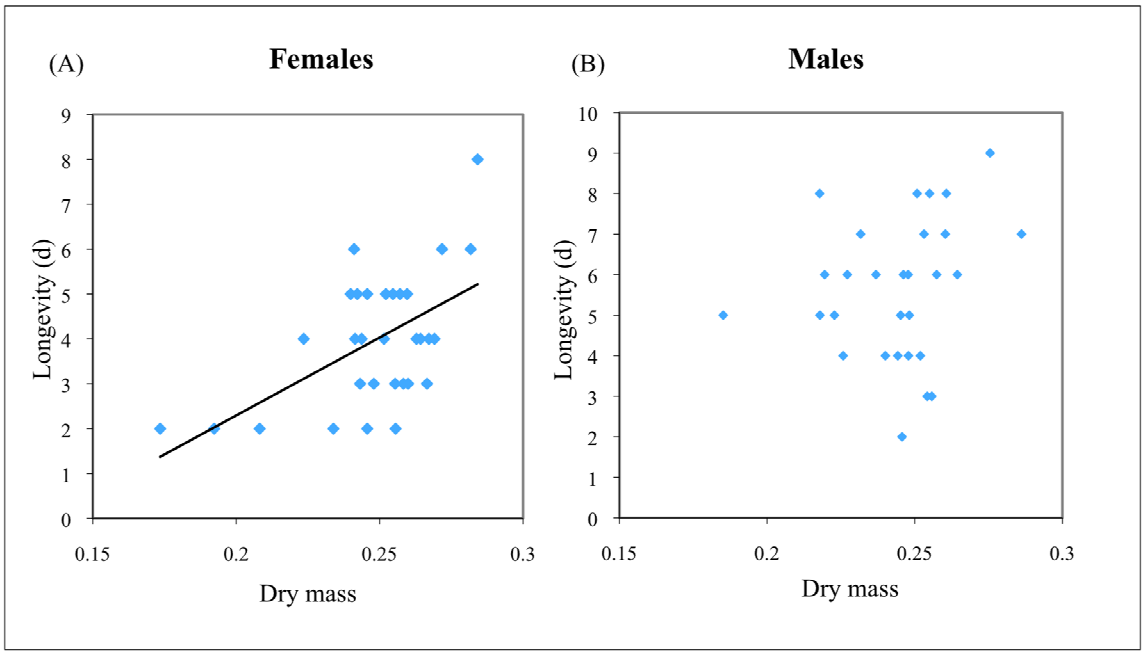

Fig. 6. Longevity (d) versus dry mass (cube root and log transformed) for (A) female wasps and (B) male wasps. A significant correlation was found for female wasps (adjusted $R^2 = 0.2840$, $P = 0.0012$). No significant correlation was found for male wasps (adjusted $R^2 = 0.0132$, $P = 0.2514$; linear regression [JMP 7, SAS Institute Inc., Cary, NC]).

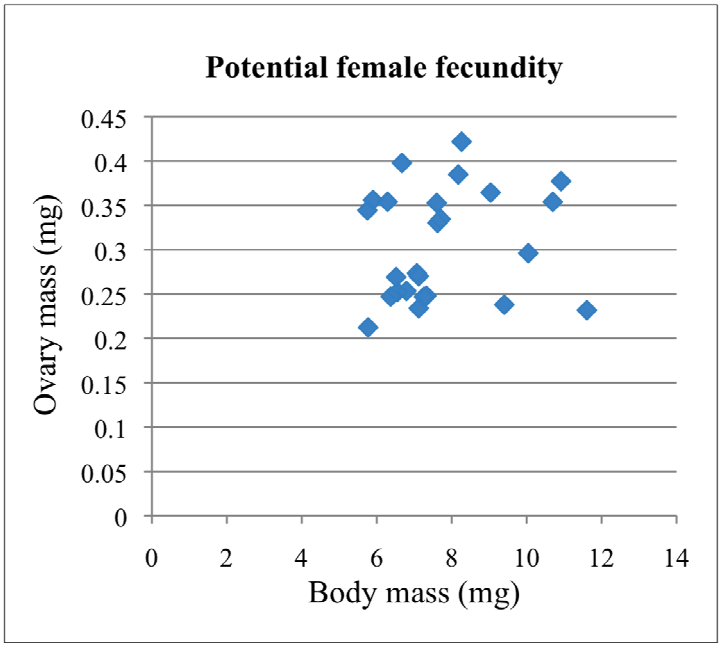

Fig. 7. Ovary dry mass versus body dry mass. No significant correlation was found (adjusted R^2 = -0.0206, P = 0.4800; linear regression [JMP 7, SAS Institute Inc., Cary, NC]).

Evaluation of the Base Artificial Diet. Because body size was found to not be predictive of longevity among males and only weakly predictive of longevity among females, final larval mass was the only mass compared between treatments (Fig. 8). Although adult dry mass may have been informative when considering the longevity of adult female *T. nigriceps*, too few of the adults that emerged from any treatment were female to draw statistically meaningful conclusions. To compare adult quality, longevity of any resulting wasps was directly measured and compared among treatments (Fig. 9). Both feeding treatment (F = 30.9250, $P = 7.61 \times 10^{-11}$) and pre-feeding larval mass (F =

173.6407, $P = 2.42x10^{-22}$) had significant effects on post-feeding larval mass (analysis of covariance [JMP 7, SAS Institute Inc., Cary, NC]). Larvae fed host tissue and those fed the base artificial diet had significantly greater post-feeding treatment mass than larvae that were not allowed to feed after egression ($P < 0.05$; Tukey HSD [JMP 7, SAS Institute Inc., Cary, NC]), but these masses did not differ significantly from each other ($P > 0.05$; Tukey HSD [JMP 7, SAS Institute Inc., Cary, NC]).

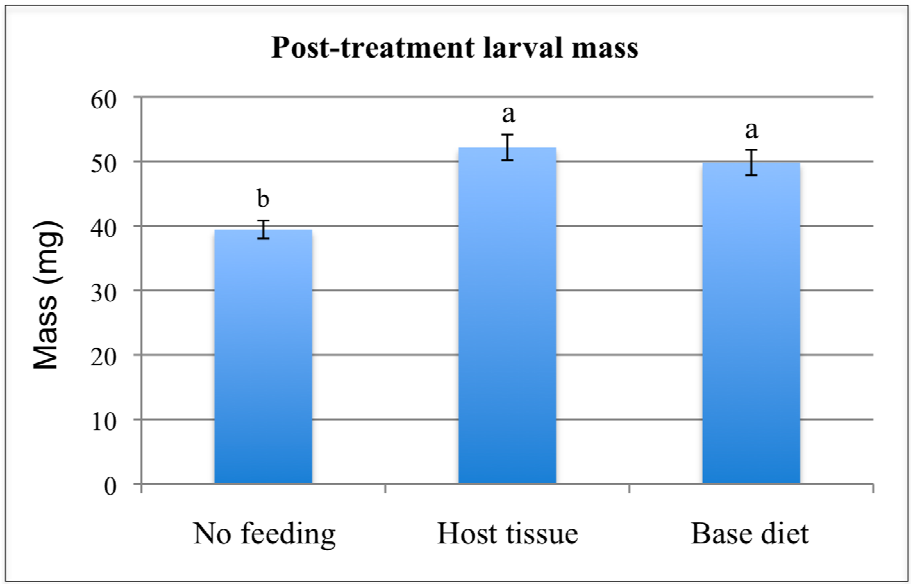

Fig. 8. The mean post-feeding mass of *T. nigriceps* larvae. Error bars represent SE. Columns marked with the same letter are not significantly different ($P < 0.05$; Tukey HSD [JMP 7, SAS Institute Inc., Cary, NC]).

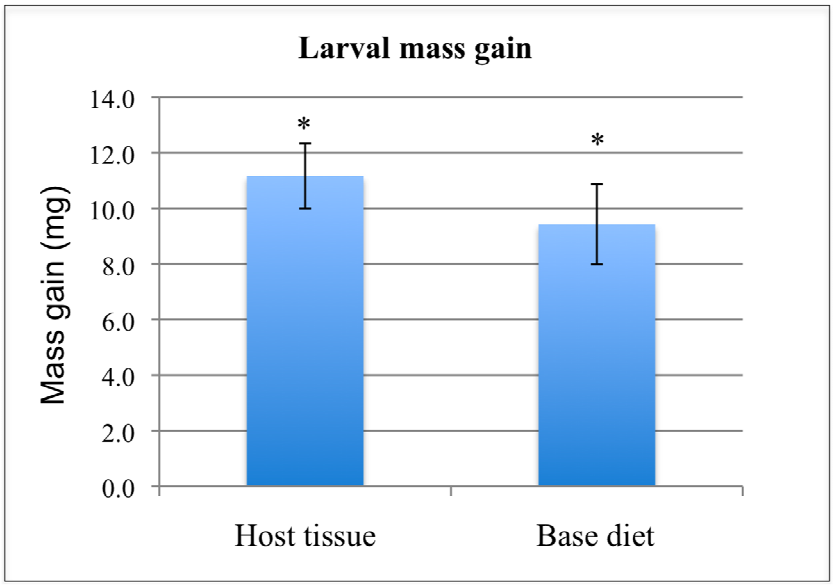

Fig. 9. Mean larval mass gained during feeding in the host tissue and base artificial diet feeding treatments. An asterisk indicates a gain significantly greater than zero ($P < 0.0001$; one-way *t*-test [SPSS 16.0, SPSS Inc., Chicago, IL]). Larval mass gain did not differ between the host tissue and base artificial diet treatments ($P = 0.3520$; independent samples *t*-test [SPSS 16.0, SPSS Inc., Chicago, IL]).

Larvae fed both host tissue (one sample $t = 9.5684$, df = 29, $P < 0.0001$) and the base artificial diet (one sample $t = 6.5413$, df = 29, $P < 0.0001$) gained significant mass during the 3 h feeding treatment (Fig. 9) (one sample *t*-test [SPSS 16.0, SPSS Inc., Chicago, IL]). The mass gained by *T. nigriceps* larvae in the host tissue and base artificial diet treatments did not differ significantly ($t = 0.3983$, df = 58, $P = 0.3520$; independent samples *t*-test [SPSS 16.0, SPSS Inc., Chicago, IL]).

No statistically significant differences were found in the proportions of parasitoids in each treatment that formed complete cocoons ($G = 5.99$, $P > 0.05$). Of *T. nigriceps* larvae not fed any diet after egression, 83.3% formed complete cocoons, while 93.3% of larvae fed host tissue after egression formed cocoons, and 86.7% of larvae fed the base artificial diet formed cocoons. More *T. nigriceps* that had been fed host tissue after egression emerged as adult wasps (66.7%) than did larvae fed the base artificial diet (43.3%) or not fed at all (43.3%); however, this difference was not statistically significant ($G = 4.23$, $P > 0.05$; G-test [Zar 2010]). Treatment had a significant effect on the longevity of the adults that emerged, however ($F = 5.6709$, $P = 0.0069$; one-way analysis of variance [SPSS 16.0, SPSS Inc., Chicago, IL]) (Fig. 10). Adult parasitoids that were not fed after egression were significantly shorter-lived than those that had been fed host tissue ($P = 0.0133$) and those that had been fed the base artificial diet ($P = 0.0148$). The longevity of adults from the host tissue and base diet treatments did not differ significantly ($P = 0.9691$).

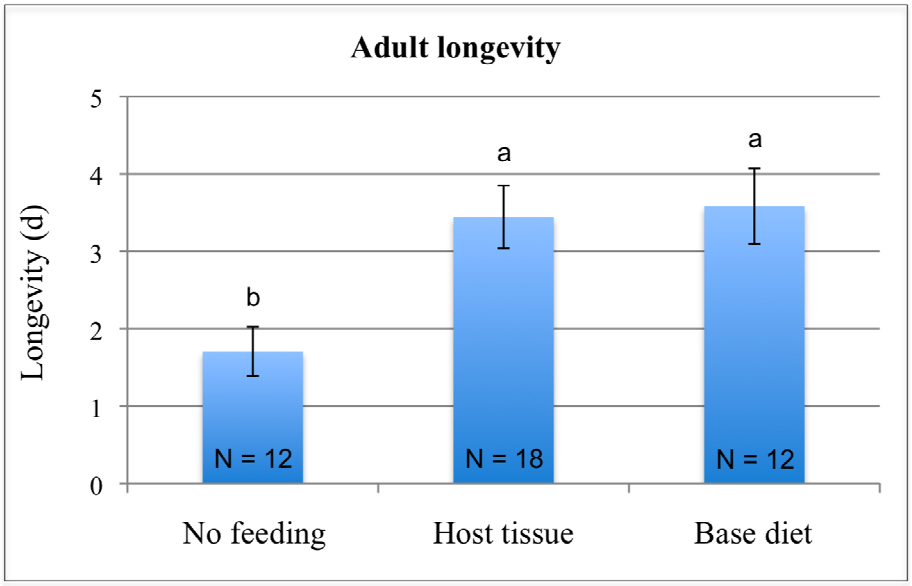

Fig. 10. Longevity (in d) of adult *T. nigriceps* reared from no feeding, host tissue, and base artificial diet treatments. Columns marked with the same letter are not significantly different ($P < 0.05$; Tukey HSD [SPSS 16.0, SPSS Inc., Chicago, IL]).

Additional Supplements to the Artificial Diet. Larvae fed the base diet reached a final mass (mean ± SE) of 50.6 ± 2.8 mg, those fed the increased carbohydrate diet reached 46.5 ± 2.9 mg, those fed the increased protein diet reached 43.7 ± 1.7 mg, and those fed the increased lipid diet reached 44.4 ± 2.2 mg. The only significant effect on post-treatment larval mass came from pre-treatment mass ($F = 33.4840$, $P = 1.57 \times 10^{-7}$) Treatment did not have a significant effect on post-treatment larval mass ($F = 1.8645$, $P = 0.1486$) (analysis of covariance [JMP 7, SAS Institute Inc., Cary, NC]). Larvae in each treatment gained significant mass (Fig. 11, Table 5) (one-sample *t*-test [SPSS 16.0, SPSS Inc., Chicago, IL]). Larval mass gain did not differ significantly

between treatments (F = 1.7374, P = 0.1665; one-way analysis of variance [SPSS 16.0, SPSS Inc., Chicago, IL]).

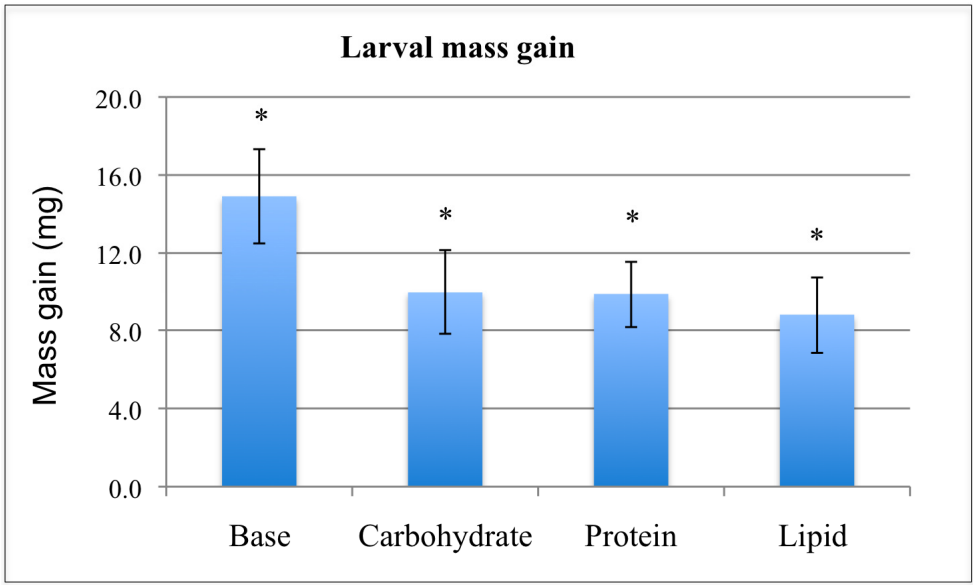

Fig. 11. Mean larval mass gain (mg) in each supplemented diet treatment. Error bars represent SE An asterisk indicates a gain significantly greater than zero (P < 0.0001; one-sample t-test [SPSS 16.0, SPSS Inc., Chicago, IL]). No significant differences between treatments were found (P = 0.1665; one-way analysis of variance [SPSS 16.0, SPSS Inc., Chicago, IL]).

Table 5. Statistical values of one-sample *t*-tests for mass gains in each of the four supplemented diet treatments (test value = 0) (SPSS 16.0, SPSS Inc., Chicago, IL).

Treatment	*t*	df	*P*
Base	6.1756	19	< 0.0001
Carbohydrate	4.6068	19	0.0002
Protein	5.8130	19	< 0.0001
Lipid	4.4999	19	0.0002

The diet treatment did, however, have a significant effect on cocoon formation ($G = 20.8986$, $P < 0.001$; *G*- test [Zar 2010]) (Fig. 12). While cocoon formation occurred in similar proportions among larvae fed the base diet, increased carbohydrate diet, and increased protein diet, those fed the increased lipid diet fared poorly, with only 35% forming cocoons. This was a significantly smaller proportion than was seen in the base diet ($q = 5.2503$, $P < 0.001$), protein diet ($q = 4.6259$, $P < 0.001$), and carbohydrate diet ($q = 3.5644$, $P < 0.01$) treatments (Tukey-type multiple comparison among proportions [Zar 2010]). Of the parasitoids fed the lipid diet after egression, 65% died as larvae, without having produced any silk.

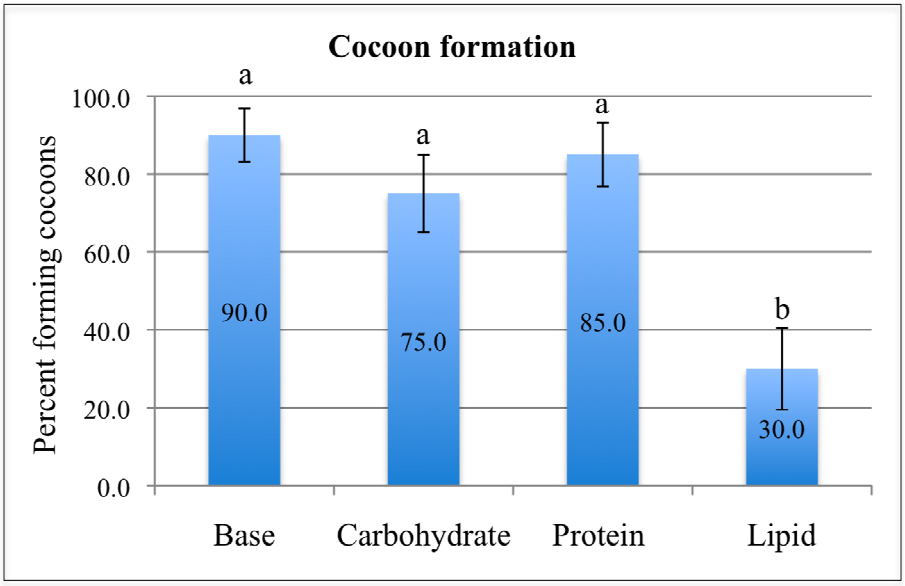

Fig. 12. The percent of *T. nigriceps* larvae in each supplemented diet treatment that formed complete cocoons. Columns marked with the same latter are not significantly different ($P < 0.01$, Tukey-type multiple comparison among proportions [Zar 2010]).

No adult wasps emerged from the lipid diet treatment. Twenty percent (n = 4), 35% (n = 7), and 15% (n = 3) of *T. nigriceps* reached adulthood from the base diet, carbohydrate diet, and protein diet treatments, respectively. These three proportions did not differ significantly ($G = 2.3704$, $P > 0.25$; G-test [Zar 2010]). The longevities of these adults (mean ± SE) were 2.9 ± 1.2 d for those fed the base diet, 2.6 ± 0.5 d for those fed the carbohydrate diet, and 2.2 ± 0.4 d for those fed the protein diet. The longevities of these adults were not significantly different ($F = 0.1411$, $P = 0.8699$; one-

way analysis of variance [SPSS 16.0, SPSS Inc., Chicago, IL]), and varied between 0.5 and 6 d.

Discussion

The relationship between adult size and the quality proxies, longevity and potential fecundity, did not appear to be as definite for *T. nigriceps* as it is in other parasitoids. No significant relationship was found between size and longevity for male wasps, though a weak correlation was seen in females. There was no apparent relationship between body size and potential female fecundity, as measured in ovary mass. Investigations of the relationship between body size and certain fitness parameters from beyond the order Insecta also suggest that large size does not always correlate with fitness proxies, such as reproductive ability. In some taxonomic groups, small size may be favorable; for example, small males may be able to compete better for mates, due to greater agility (Blackenhorn 2000). In some cases, body size may be positively correlated with increased fecundity at some sizes, but reach an asymptote at the largest sizes (Blackenhorn 2000). In light of these observations, it would seem prudent to suggest that, in future studies, conclusions about the fitness of *T. nigriceps*, as well as other parasitoids for which the size-fitness hypothesis has not been tested, not be drawn simply from measures of adult size, but rather from direct measurements of fitness parameters.

This does not necessarily indicate that there is no relationship at all between adult *T. nigriceps* size and indicies of quality other than female longevity, however. It is

possible that the measure of female fecundity used in the present study was simply not an accurate measure for this parameter. *Toxoneuron nigriceps* is a somewhat pro-ovigenic species, with females having some mature ovarioles upon adult emergence, but continuing to mature eggs throughout their adult life spans. When measuring potential female fecundity, the assumption was that the greater the ratio of developed to undeveloped eggs, the greater total ovary mass would be. As all female wasps were kept alive and fed for 5 d after emergence, the mass of ovaries was presumed to give some indication at what proportion of eggs each female had been able to mature at this point in time. This proportion, in turn, would indicate how many eggs she would be capable of laying at 5 d old. Perhaps a measure of how many hosts a female could successfully parasitize in her adult lifetime would have revealed some relationship between body size and fecundity. A count of the total numbers mature and immature eggs in the ovaries of each wasp may have also been a better indicator of potential fecundity. Unfortunately, time was a limiting factor at the time this experiment was conducted.

The base artificial diet, made from fresh chicken egg yolk and TNM-FH cell culture medium, appears to be an improvement over no post-egression feeding at all. Final larval mass among parasitoids fed this diet was greater than that of larvae not fed after egression, and did not differ significantly from that of larvae fed scraped (parasitized) host tissues. As larval mass is highly predictive of adult mass (Chapter II), this suggests that *T. nigriceps* fed this diet after egression would also have larger masses as adults than those that were starved after egression. This would indicate that female *T. nigriceps* emerging from these treatments would have longer adult longevity than those

that experienced no post-egression feeding. Larval mass gain was also similar between larvae fed scraped host tissues and those fed the base artificial diet, indicating that the artificial diet is no less palatable to *T. nigriceps* than host tissue. No differences were found in the proportions of parasitoids that formed cocoons in each treatment, which matches the findings reported in Chapter II, where none of the four feeding treatments (natural tissue feeding, starvation after egression, feeding on tissue scraped from the host, and feeding on tissue scraped from a healthy *H. virescens* larva) had a statistical effect on cocoon formation. No differences were found in the proportion of parasitoids that survived to adulthood in each treatment, however, even between parasitoids fed host tissues and those not fed at all. This appears to be due to a decrease in survival among parasitoids fed host tissues from what was seen in a similar treatment in Chapter II. In Chapter II, 86.7% of parasitoids fed scraped host tissues after egression later emerged as adult wasps, in contrast to the 66.7% that emerged as adults here, a 35% decrease in adult yield. Perhaps some aspect of the conditions under which larvae in this experiment were reared differed from those in the previous post-egression feeding experiment. The percent of parasitoids fed the base artificial diet that reached adulthood, 43.3%, was the same as that of larvae not fed after egression, yet still not significantly lower than the emergence rate of larvae fed host tissue. In light of this, it is difficult to conclude how well this artificial post-egression diet prepares *T. nigriceps* larvae for pupation and adult emergence. The longevity of those adults that did emerge from each treatment, however, paints a clearer picture, with parasitoids fed the base artificial diet living significantly

longer as adults than those that were not fed, and not differing in longevity from those that were fed host tissue.

Addition of further glucose (carbohydrate diet), lactalbumin (protein diet), or linolenic acid (lipid diet) to the base artificial diet had no apparent effect on either final larval mass or larval mass gain, compared to the base artificial diet. This suggests that no one diet was any more palatable to *T. nigriceps* larvae than the others, and all diets allowed larvae to reach similar sizes. Supplementation with linolenic acid, however, proved toxic to larvae. Increased glucose and lactalbumin did not increase the proportion of parasitoids that formed cocoons after feeding when compared to the base diet, nor did it have any statistical effect on the proportion that reached adulthood. Adult emergence was low in all three of these treatments, lower even than the proportion that reached adulthood in the initial evaluation of the base artificial diet, with many wasps having died as pharate adults within their cocoons. Additionally, during the time period in which this study took place, even *T. nigriceps* being reared under normal laboratory practices had low emergence rates, suggesting that this was due to an unknown problem within the *T. nigriceps* culture, rather than being an effect of post-egression feeding treatment.

The addition of linolenic acid to the artificial post-egression diet was detrimental to developing *T. nigriceps*, as the majority of larvae fed this diet died within a day of feeding. No parasitoids from this treatment emerged as adults. Excess free fatty acids appeared to have a toxic effect on *T. nigriceps*, as has been the case with a number of

other parasitoids reared partially or fully in vitro (Thompson 1977, Nakahara et al. 2002).

When macronutrients were extracted from tissue samples of pre- and post-tissue feeding larvae in Chapter II, the greatest difference in macronutrient concentration was seen in lipids, which were nearly three-fold greater in the tissues of post-tissue feeding larvae than in pre-tissue feeding larvae. While this may have been due entirely due to lipogenesis following ingestion of other, non-lipid nutrients, this seems unlikely, as one of the major host tissues remaining after egression of the parasitoid larva is the fat body, in which lipids are stored. Perhaps *T. nigriceps* is simply only able to digest lipids in a form other than free fatty acids. A whole body analysis of *H. virescens* tissue showed that the majority of neutral lipids, 48 to 60%, are triglycerides, and that free fatty acids made up less than 10% of total lipids (Barras et al. 1970). Triglycerides are the form in which lipids are stored within the insect fat body. Lipids that have been mobilized, and are present in the hemolymph, are bound within lipophorins. Lipophorin and other lipoproteins, both insect and vertebrate in origin, have been used as vital sources of lipids in rearing of another larval endoparasitoid, *Venturia canescens* Gravenhorst (Hymenoptera: Ichneumonidae) (Nakahara et al. 2002), and would perhaps be a beneficial, rather than detrimental, addition to a post-egression diet for *T. nigriceps*.

An egg yolk and TNM-FH diet appears to function as an adequate preliminary artificial post-egression diet for *T. nigriceps*. However, examining the nutritional content of *H. virescens* tissues remaining after parasitoid egression could allow for development of a better, artificial post-egression diet, either through further supplementation of the

egg yolk based diet, or through formulation of a new diet altogether. Although the macronutrient contents of *T. nigriceps* larvae immediately before and after post-egression feeding were examined, this revealed only the macronutritional state of the larvae themselves, rather than the nutritional value of the host tissues they were feeding on. A study of the overall protein, carbohydrate, and lipid content of the liquefied host tissues, as well as identification of the specific substances that make up the majority of such tissues, would aid improving upon the preliminary artificial diet. This would reveal not only the proportions of major macronutrients consumed by *T. nigriceps* during the tissue feeding phase, but also what form these macronutrients take.

Another consideration for artificial post-egression feeding is how the diet is presented to *T. nigriceps* larvae. Here, larvae were placed next to a droplet of the diet upon filter paper. Although *T. nigriceps* larvae were able to feed this way, they nearly always became partially engulfed in the diet during the feeding process, after having apparently rolled into the middle of the droplet. Even after larvae were removed from the remaining diet, a film was left over the cuticle that in time could form a hard crust. Many larvae appeared to have sloughed this layer of dried diet off before pupation, but it is not known if this film would contribute to the death of some larvae in each artificial diet treatment if it were not removed. During the evaluation of supplemented artificial diets, this problem was eliminated by washing each larva in saline solution after feeding, to remove the layer of diet, a process that is not at all practical for any sort of large-scale rearing effort. Considering this, it would be prudent, in future studies of artificial post-egression feeding, to partially envelop the artificial diet in a membrane, leaving an

opening large enough for *T. nigriceps* larvae to insert their heads for feeding, but not large enough to allow the diet to leak out and cover the larvae. This would also slow down the drying of the diet, allowing for longer feeding times and, potentially, greater larval growth.

CHAPTER IV

PUPATION CHAMBERS[*]

Introduction

Parasitoids depend on their hosts for nutrition during larval development, but reliance on host-related factors does not end at egression. A parasitoid also requires a suitable environment for pupation, a vulnerable stage of development. Some endoparasitoids remain within the cuticle of their hosts' after larval development, while others egress from the host late in larval development to pupate externally. Whether they pupate with in the host remains or outside, immature parasitoids are not highly mobile. The parasitoid itself cannot choose a location for pupation, but it must rely on the host to do this. A number of endoparasitoids have been known to modify the behavior of their hosts in ways that benefit the parasitoid during pupation, either outside of the host or within the host remains. Thus, the solitary endoparasitoid, *Aphidius nigripes* Ashmead (Hymenoptera: Aphidiidae) pupates within the hollowed out exocuticle of its host, *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae). These aphid "mummies" are attached to plant surfaces and immobile and thus are vulnerable to attack by predators and hyperparasitoids. However, before mummification, hosts of *A. nigripes* tend to move away from aphid colonies, typically found on the lower surfaces of leaves, and onto the

[*] Part of this chapter is reprinted with permission from "The Suitability of Various Artificial Pupation Chambers for Use by the Koinobiont Parasitoid, *Toxoneuron nigriceps* (Hymenoptera: Braconidae) by R. E. Henderson, I. Kuriachan, and S. B. Vinson. 2011. Annals of the Entomological Society of America, 104, pp 1355-1359, Copyright 2011 by the Entomological Society of America.

upper surfaces of apical leaves. A study on the survival of pupating *A. nigripes* showed that parasitoids suffered significantly lower mortality when mummies were located on upper leaf surfaces than when they were on lower leaf surfaces, mainly due to a reduction in the rate of hyperparasitism (Brodeur and McNeil 1992). This indicates that this parasitoid manipulates host behavior to induce them to move to locations that may be unfavorable for the host, but are favorable for the parasitoid developing inside. *Hymenoepimecis* spp. (Hymenoptera: Ichneumonidae) that are near completion of larval development will induce their hosts, the orb-weaving spider *Plesiometa argyra* Archer (Arachnida: Tetragnathidae), to form a specialized web structure called a cocoon web from which the parasitoid larva will suspend its cocoon after it kills and consumes its host. The structure of cocoon webs is very consistent and differs greatly from normal orb webs formed by healthy spiders. The altered behavior of the spiders appears to be chemically induced by parasitoids shortly before the formation of the cocoon web. This specialized structure appears to offer pupating parasitoids increased protection from being washed away in heavy rains (Eberhard 2000). Some parasitoids will induce their hosts to physically protect them after egression. When gregarious *Cotesia glomerata* L. (Hymenoptera: Braconidae) larvae egress from their host, they leave their host larva, *Pieris brasisicae* L. (Lepidoptera: Pieridae), alive but moribund. The host will not feed or develop further, but remains alive for several days and will often remain coiled on top of the cocoons formed by the parasitoids that emerged from it. These hosts will sometimes also produce silk, spinning an additional layer of silk over the cocoons, and will also react with violent thrashing movements and regurgitation if touched. The silk

layer and this reaction likely serve to protect the vulnerable parasitoid pupae from attack by predators and hyperparaitoids (Brodeur and Vet 1994). Similar manipulation is seen in the relationship between *Glyptapanteles* spp. (Hymenoptera: Braconidae) and *Thyrinteina leucocerae* Rindge (Lepidoptera: Geometridae), with moribund hosts staying near parasitoid cocoons and thrashing their heads in response to physical touch. In a laboratory study, 17 out of 20 hosts from which parasitoids had emerged reacted to the presence of the predatory bug, *Supputius cincticeps* Stäl (Hemiptera: Pentatomidae), by violently swinging their heads, while only one of 20 unparasitized larvae reacted to the predators in this way. The thrashing behavior of the parasitized larvae had the effect of causing half of the predators to give up their search for *Glyptapanteles* pupae, supporting the hypothesis that this is an adaptive usurpation of host behavior by the parasitoid (Grosman et al. 2008).

Toxoneuron nigriceps Viereck (Hymenoptera: Braconidae) is an endoparasitoid o *Heliothis virescens* F. (Lepidoptera: Noctuidae) that egresses from the host during late larval development and pupates externally. Modification of the host behavior following the egression of the parasitoid larva is not possible, because the internal contents of the host are entirely consumed upon egression, killing the host. However, *T. nigriceps* may manipulate the behavior of the host shortly before egression, while the host is excavating a pupation chamber in soil. Modification to how deep under the soil surface the chamber is formed may be adaptive. Because *T. nigriceps* in the field typically overwinter as prepupae in these chambers (López 1982), a chamber's depth below the surface of the soil could determine the protection afforded to parasitoids from harsh environmental

condition and attack by predators and hyperparasitoids. Variations in the internal dimensions of the chamber itself could also be adaptive. Preliminary observations suggest that *T. nigriceps* prepupae require an enclosed space in which to form their cocoons. If this is true, then the internal dimensions are likely to influence cocoon formation in *T. nigriceps*.

The parasitoid cocoon not only offers physical protection to vulnerable prepupae and pupae as they develop, but may also protect them from desiccation. Pupae of *C. glomerata* that had been removed from their cocoons suffered increased mortality during pupation when reared at lower relative humidity, while those developing inside intact cocoons did not suffer increased mortality (Tagawa 1996). The cocoon may also aid in shedding of the pupal cuticle. In the previously mentioned study, 86% of *C. glomerata* that reached adulthood after pupating naked had difficulty fully shedding the pupal cuticle, with portions remaining attached to the wingtips and posterior extremities, while those that emerged from cocoons did not appear to have this difficulty (Tagawa 1996). A similar phenomenon has been observed in the *T. nigriceps* laboratory culture. In the rare cases in which a parasitoid reaches adulthood after pupating naked, they often are unable to detach portions of the pupal cuticle from their wingtips, antennae, and legs, and often cannot seem to free themselves from the voided meconium, which remains attached to the end of the abdomen.

Toxoneuron nigriceps spin their cocoons in pupation chambers formed by their hosts, but will only lay down a mass of silk and attempt to pupate naked if left on a flat surface (Chamberlin and Tenhet 1926, Chapter I). If *T. nigriceps* requires a small,

enclosed space, and if there are particular dimensions that best suit cocoon-forming larvae, then there may be parasitoid-related differences between the dimensions of a pupation chamber formed by a healthy *H. virescens* larva and one formed by a larva parasitized by *T. nigriceps*. Thus, knowledge of the dimensions of pupation chambers formed by parasitized *H. virescens* larvae is likely important. For example, knowing the appropriate dimensions would facilitate identification of common laboratory items that might replace host-formed chambers, allowing cocoon formation be parasitoids reared in the absence of their hosts.

In the present study, evidence of host manipulation during pupation chamber formation was sought, along with potential adaptive significance of parasitoid-induced differences in chamber architecture, and the potential of substituting common laboratory items for a host-formed pupation chamber. This was done by comparing the internal dimensions of chambers formed by healthy *H. virescens* larvae to those parasitized by *T. nigriceps* were compared, along with the depth below the surface at which the chambers were formed and the amount of silk used to construct them. The potential adaptive significance of altering chamber for *T. nigriceps* was examined by evaluating the ability of *T. nigriceps* to form cocoons in artificial chambers constructed based on the dimensions of pupation chambers formed by healthy and parasitized *H. virescens* larvae. The potential of substituting an artificial pupation chamber for host-formed ones during rearing of *T. nigriceps* was examined by placing mature parasitoid larvae in one of several common laboratory items (see Materials and Methods) that formed a small, partially- to fully-enclosed space. Larvae were also placed in chambers that had been

formed by hosts in the *H. virescens* diet (control) and in Petri dishes—a wide, flat space in which *T. nigriceps* larvae were not expected to form cocoons—for comparison. The ability of *T. nigriceps* larvae to form cocoons in these chambers, the mass of any resulting cocoons, and the emergence of adult wasps were recorded and compared between treatments. The suitability of each chamber type for pupation, as well as the practicality of its use in an in vitro rearing system, were also explored.

Materials and Methods

Chambers Formed by *H. virescens*. To obtain pupation chambers for depth, length and diameter measurement, late 5th instar *H. virescens* larvae were placed individually in plastic rearing vials filled with approximately 3 cm of topsoil. Fifty of these larvae were parasitized by *T. nigriceps*, while an additional 50 were healthy. The larvae were allowed to excavate pupation chambers in the soil, part of which was usually visible from the outside of the tube, allowing for some observation. When all *H. virescens* from the healthy treatment had pupated, those from the parasitized treatment were checked. Those that remained in the larval stage were considered to be successfully parasitized, while those that had pupated were presumed to not be parasitized and were removed from the experiment. All remaining tubes containing pupation chambers were then placed in a freezer overnight to kill the insects inside. Chambers were then filled with plaster of Paris mixture, administered through a large syringe that was used to gently probe the soil surface until a passage to the chamber could be found. After being filled with the plaster of Paris mixture, samples were left to dry for one week or more.

Dried chamber molds were removed from the soil, gently brushed to remove debris, and covered in a thin layer of water-based sealant to protect them from damage (Mod Podge waterbase sealer, Plaid Enterprises Inc., Norcross, GA) This process was repeated until 100 intact chamber molds—50 formed by healthy larvae and 50 formed by parasitized larvae—were obtained. The depth below soil surface, and the length, width, and height of each chamber (Fig. 13) were measured using electronic calipers (General Tools Manufacturing Co., LLC, New York, NY). The size and shape of chambers were separated for analysis by using the length, width, and height of each chamber to calculate a geometric mean, then dividing each of these dimensions by the geometric mean. This removed the size component from each of these dimensions, generating relative length, relative width, and relative height for each chamber. Data were then log-transformed to normalize distribution. A principal components analysis was applied to the three log relative measures of chamber size (length, width, and height) to reduce these measures to two dimensions. The shape, size, and depth below soil of chambers formed by healthy and parasitized larvae were then compared using a MANOVA (JMP 9, SAS Institute Inc., Cary, NC).

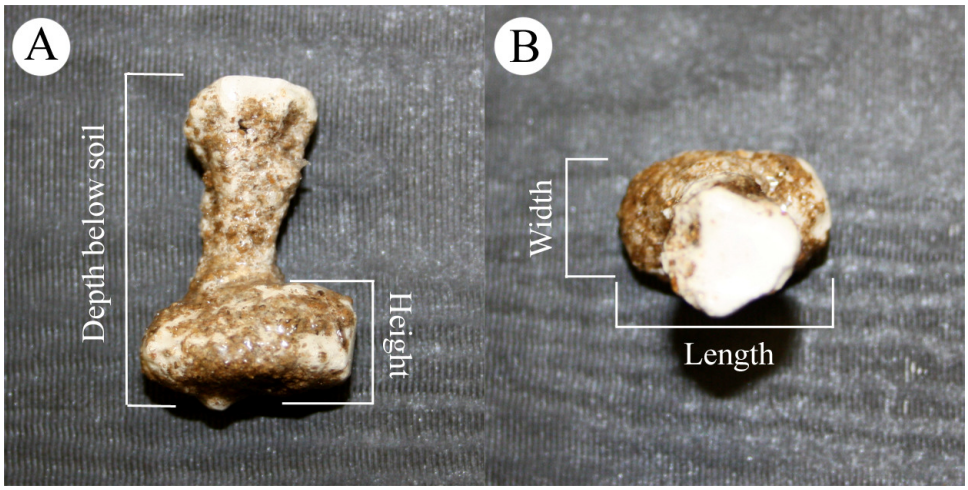

Fig. 13. A cast of a typical *H. virescens* pupation chamber formed in soil. Views from the side (A) and from the top down (B). The dimensions measured as depth below soil, chamber length, chamber width, and chamber height are marked.

Because soil particles and other material become embedded in the silk lining the pupation chambers, an additional 50 each healthy and parasitized *H. virescens* larvae were placed in tubes containing approximately 3 cm of granulated table sugar, a substrate that could be easily removed from silk by dissolving it in water. After chambers were formed, remaining pupae, larvae, exuvia, and frass were removed from the samples. Each sample was then transferred to a beaker, to which approximately 40 ml of hot de-ionized water was added. The mixture was then stirred until all sugar dissolved, then poured gradually through a funnel containing pre-weighed filter paper to catch the silk. Remaining matter in the beaker was rinsed into the funnel with additional de-ionized water, and then an additional 15 ml of water was poured through the filter

paper to rinse through any remaining sugar residue. Each filter paper circle was allowed to dry for 24 h then re-massed. The difference between the final and initial mass of each filter paper circle was determined to be the mass of silk contained in the pupation chamber. Silk mass from chambers formed by healthy and parasitized larvae were compared using an independent samples *t*-test (SPSS 16.0, SPSS Inc., Chicago, IL).

Importance of Chamber Size. Cylindrical artificial chambers constructed from paper, and based on the mean lengths and widths of chambers formed by parasitized and healthy *H. virescens* larvae, respectively, were used to test for the potential adaptive significance of manipulation of pupation chamber size by *T. nigriceps*. Artificial chambers emulating those formed by parasitized larvae were 16 mm in length and 11 mm in diameter, while those emulating chambers formed by healthy larvae were 18 mm in length and 12 mm in diameter. Five chambers of each type were constructed, with each chamber used six times each during the course of the experiment. Thirty *T. nigriceps* larvae per treatment (chamber type) were placed in the paper chambers immediately after completing issue feeding. Chambers, with larvae inside, were kept in an incubator under controlled incubator conditions (29 ± 1ºC; 60 ± 10% RH and a photoperiod of 14:10 [L:D] h), and at 3 d the success or failure of the larvae inside to form a cocoon was recorded. Cocoons and naked prepupae were removed from the chambers and placed individually in wells of 24-well clear plastic assay plates (Thermo Fisher Scientific, Waltham, MA) to be monitored for adult emergence. The proportions of *T. nigriceps* in each treatment that formed cocoons and that reached adulthood were compared using a one-tailed Z-test for difference between two proportions (Zar 2010). A

subset of nine larvae from each treatment were weighed prior to placement in chambers and the masses compared with an independent samples *t*-test to rule out bias in the size of larvae being placed in each chamber type (SPSS 16.0, SPSS Inc., Chicago, IL).

Artificial Pupation Chambers. To evaluate the potential of various common laboratory items for use as artificial pupating chambers in the rearing of *T. nigriceps*, 25 larvae per treatment were collected immediately after completion of post-egression tissue feeding and placed in one of six chambers: 1) host-formed pupation chambers in *H. virescens* diet, 2) 35 by 10 mm plastic Petri dishes (BD Biosciences Discovery Labware, Bedford, MA), 3) 0.5 ml gelatin capsules (Electron Microscopy Sciences, Fort Washington, PA), 4) wells of a polypropylene microcentrifuge tube storage rack (Thermo Fisher Scientific, Waltham, MA), 5) medium-sized cotton balls (U.S. Cotton Co., Lachine, Québec, ON, Canada), and 6) 16 by 100 mm glass test tubes (Thermo Fisher Scientific, Waltham, MA).

Host-formed pupation chambers, which served as a positive control, were obtained by allowing parasitized *H. virescens* larvae to form chambers in the larval diet within rearing vials, then carefully removing the larvae and setting aside the diet, and the chamber within, for later use. Mature 3rd instar *T. nigriceps* larvae were later placed individually in these chambers and the vials plugged with cotton. Petri dishes were used as a negative control, as they only provided a flat, open space in which parasitoid larvae were not expected to form cocoons. Larvae were placed in the center of the Petri dish, and the lid was placed over top and held closed with tape. In the gelatin capsule treatment, larvae were carefully placed inside of capsules, which were then closed and

stored individually within test tubes. Larvae held in microcentrifuge tube racks were placed in individual wells within a rack, and each well plugged was with a small amount of cotton. Cotton balls used as pupation chambers were prepared by pressing on the center of the ball with one finger, forming a pocket in the middle into which a larva would be placed. The edges of the cotton ball were then carefully closed over top of the pocket and held closed with tape. In the test tube treatment, larvae were placed at the bottoms of test tubes, which were plugged with cotton and held upright in a test tube rack.

Parasitoids in all treatments were held at controlled conditions in the rearing incubator and left for 7 d. After 7 d, each chamber was opened to check for the presence or absence of a cocoon. Only complete cocoons, which fully-enclosed the larva inside, were counted as present. Cocoons were then carefully removed from the pupation chambers and weighed, with mass being recorded as a measure of cocoon quality. Rather than being placed back in their chambers, cocoons were placed individually in labeled glass test tubes for observation. In the laboratory culture, cocoons are regularly removed from diet chamber and placed together in a clear plastic box for further rearing. This does not appear to have any negative impact on pupation and subsequent adult emergence. Parasitoids that did not form full cocoons were left in their pupation chambers, as they were considered too fragile to be handled without injury. Naked larvae and pupae were recorded as having failed to form cocoons, but were still monitored for development to adulthood. All *T. nigriceps*, regardless of cocoon formation, were monitored for adult emergence.

Twenty-five replicates were used in this experiment, with each replicate consisting of one larva per chamber type. The proportion of *T. nigriceps* larvae that formed complete cocoons in each treatment, and which emerged as adults, were compared using a Tukey-type multiple comparison among proportions (Zar 2010). The masses of complete cocoons were compared using a one-way analysis of variance (SPSS 16.0, SPSS Inc., Chicago, IL).

Results

Chambers Formed by *H. virescens*. Statistically significant differences were found between the dimensions of pupation chambers formed by healthy *H. virescens* larvae and those formed by larvae parasitized by *T. nigriceps*, as well has how deeply the chambers were buried beneath the soil surface (Fig. 14). Parasitized larvae formed chambers at 23.33 ± 0.64 mm (mean ± SE) under the surface of the soil, significantly deeper than those formed by healthy larvae, 19.43 ± 0.56 mm under the soil surface. Also, chambers formed by parasitized larvae were also significantly smaller than those formed by healthy larvae. Chambers formed by parasitized larvae had smaller relative widths than those formed by healthy larvae, but had greater relative heights (Wilks' Λ = 0.6633, F = 15.7541, P = 6.3578E-10; MANOVA [JMP 9, SAS Institute Inc., Cary, NC]). The mass of silk (mean ± SE) extracted from chambers was 4.40 ± 0.60 mg from chambers formed by healthy *H. virescens* larvae and 3.97 ± 0.22 mg from chambers formed by parasitized larvae. These masses were not significantly different (t = 0.6674, df = 98, P = 0.5061; independent samples t-test [SPSS 16.0, SPSS Inc., Chicago, IL]).

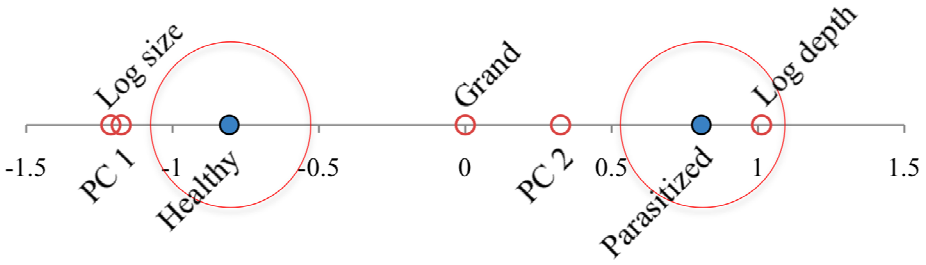

Fig. 14. Canonical centroid plot of the size and depth of pupation chambers formed in soil by healthy and parasitized 5th instar *H. virescens* larvae, as well as two principal components (PC 1 and PC 2) of relative chamber shape. Circles represent 95% confidence intervals, and non-overlapping circles indicate statistically different groups. Chambers formed by healthy and parasitized larvae differed significantly, with chambers formed by parasitized larvae being smaller overall than those formed by healthy larvae, and also being located deeper beneath the soil surface (Wilks' $\Lambda = 0.6633$, $F = 15.7541$, $P = 6.3578E\text{-}10$; MANOVA [JMP 9, SAS Institute Inc., Cary, NC]).

Importance of Chamber Size. Of all *T. nigriceps* larvae ($N = 30$) placed in "parasitized type" paper chambers (based on the dimensions of chambers formed in soil by parasitized *H. virescens*) 90% formed complete cocoons, while only 63%, formed complete cocoons when placed in the larger, "healthy type" paper chambers (based on the dimensions of chambers formed in soil by healthy *H. virescens* larvae) ($Z = -2.44$, P

< 0.05; one-tailed *Z*-test [Zar 2010]) (Fig. 15). Although more adult wasps emerged from the "parasitized type" chamber treatment than from the "healthy type" treatment, the corresponding emergence rates did not differ significantly ($Z = -0.83$, $P > 0.05$; one-tailed Z-test [Zar 2010]). Approximately 63% of *T. nigriceps* from the "healthy type" chamber treatment emerged as adults, while approximately 73% emerged from the "parasitized type" treatment.

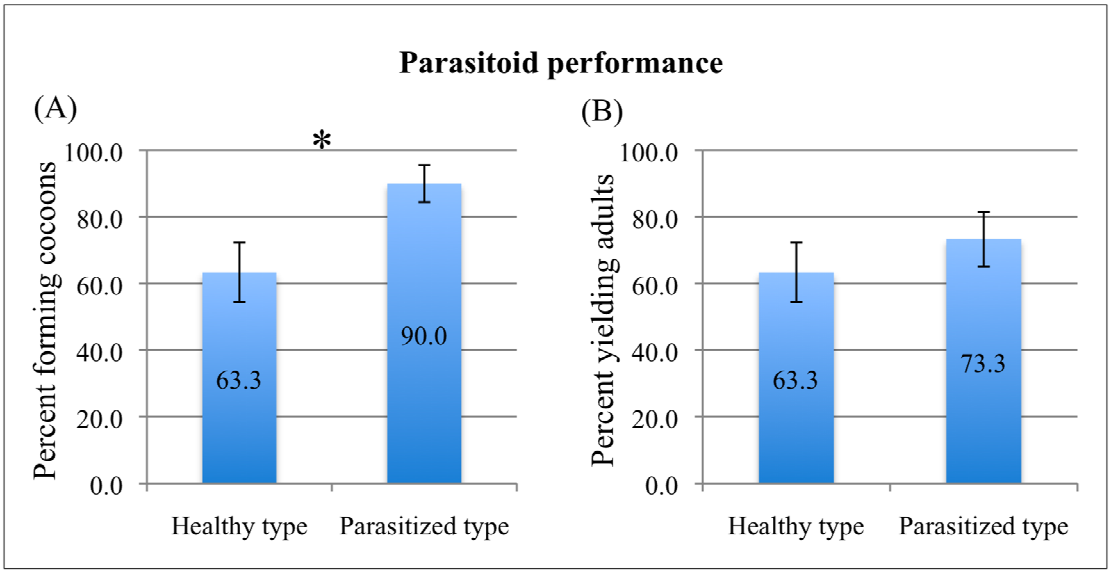

Fig. 15. The proportion of *T. nigriceps* that (A) formed cocoons and (B) emerged as adult wasps after placement in paper chambers (based on the dimensions of chambers formed in soil by healthy or parasitized *H. virescens* larvae). Error bars represent SE. An asterisk indicates a statistically significant difference ($P < 0.05$; one-tailed Z-test for difference between two proportions [Zar 2010]).

Artificial Pupation Chambers. *Toxoneuron nigriceps* larvae formed cocoons at variable rates in all chamber types, except for Petri dishes. Thus the proportion of cocoons formed by larvae in this treatment was smaller than in all other treatments, while the highest rate of cocoon formation occurred among larvae placed within indentations in cotton balls, in which 100% of larvae formed complete cocoons ($P < 0.001$) (Fig. 16). This was not statistically different from cocoon formation within host-formed control chambers, in which 96% of larvae formed full cocoons, but was significantly higher than the proportion of larvae that formed cocoons in microtube rack wells (76%, $q = 4.288$, $P < 0.05$) and upright test tubes (68%, $q = 5.149$, $P < 0.01$). In gelatin capsules, 84% of larvae formed cocoons, which was not significantly different from any treatment other than Petri dishes. Overall, cocoon formation was highest in the diet chamber and cotton ball treatments, lowest in the Petri dish treatment, and intermediate in gelatin capsule, microcentrifuge tube rack well, and test tube treatments.

The masses of cocoons formed (mean ± SE) were 79.96 ± 1.92 mg in host-formed chambers ($N = 22$), 75.12 ± 2.44 mg in gelatin capsules ($N = 21$), 73.84 ± 2.18 mg in microcentrifuge tube rack wells ($N = 19$), 74.22 ± 2.40 mg in cotton balls ($N = 25$), and 78.29 ± 2.66 mg in test tubes ($N = 16$). No significant differences in cocoon mass were found between any of the treatments in which cocoons were formed ($F = 1.360$, $P = 0.254$; one-way analysis of variance [SPSS 16.0, SPSS Inc., Chicago, IL]).

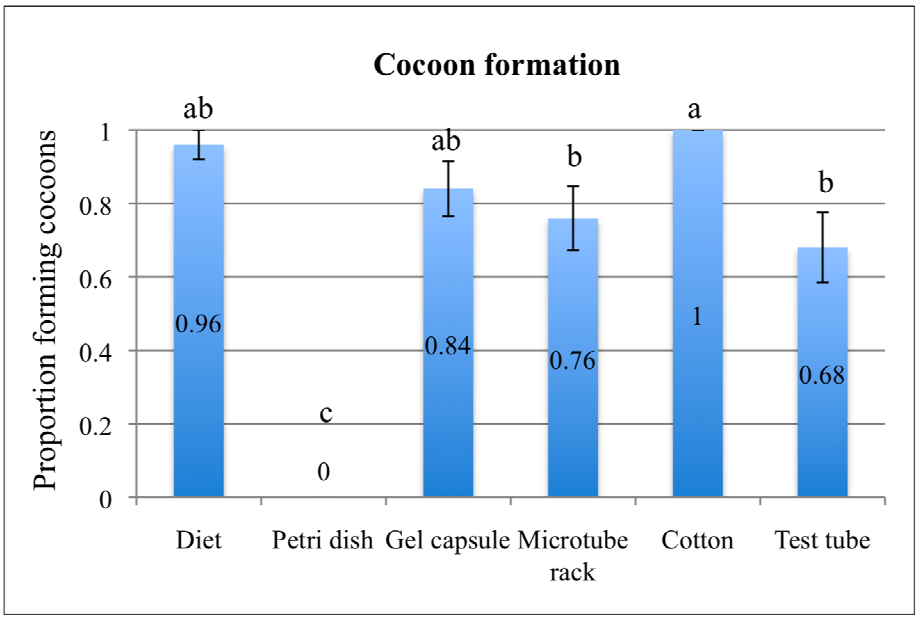

Fig. 16. The proportion of *T. nigriceps* larvae in each chamber type that formed complete cocoons. Error bars represent SE. Columns labeled with the same letter are not significantly different ($P < 0.05$; Tukey-type multiple comparison among proportions [Zar 2010]).

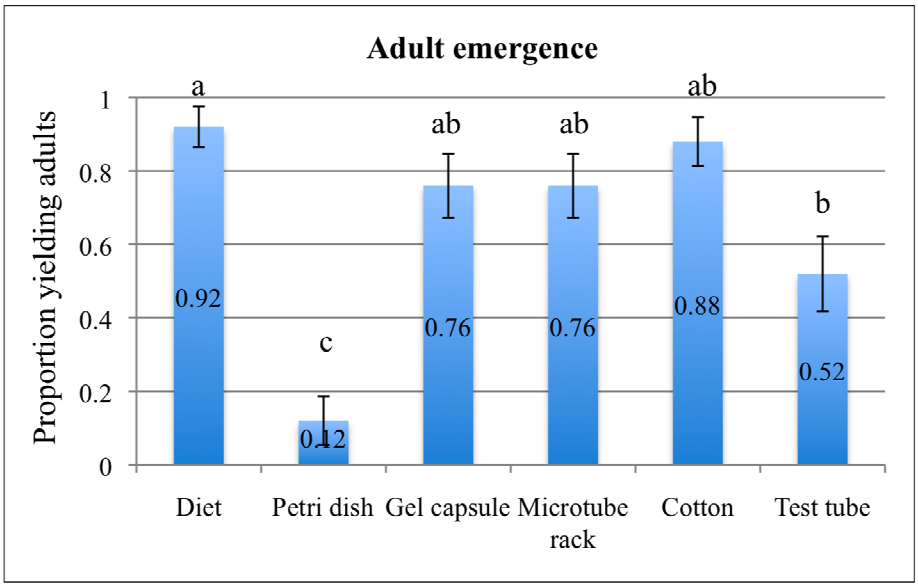

Fig. 17. The proportion of *T. nigriceps* from each chamber type that emerged as adult wasps. Error bars represent SE. Columns labeled with the same letter are not significantly different ($P < 0.05$; Tukey-type multiple comparison among proportions [Zar 2010]).

Some adult wasps emerged from every treatment (Fig. 17). Although no cocoons were formed in Petri dishes, three of the parasitoids placed in these chambers as larvae (12%) pupated naked and survived to adulthood. Overall, adult emergence was highest in the control, cotton ball, gelatin capsule, and microcentrifuge tube rack well treatments, lowest in the Petri dish treatment, and intermediate in the test tube treatment. Of the larvae placed in host-formed control chambers, 92% emerged as adults. The proportions of parasitoids reaching adulthood in other treatments were 76% in gelatin capsules, 76% in microcentrifuge tube rack wells, 88% in cotton balls, and 52% in test

tubes. The proportion of *T. nigriceps* that reached adulthood after being place in Petri dishes was significantly smaller than all other treatments ($P < 0.05$ versus test tubes, $P < 0.001$ versus all other treatments). A smaller proportion of parasitoids from the test-tube test tubes as larvae emerged as adults than from the control ($q = 4.568$, $P < 0.05$). No other significant differences were found.

Discussion

The comparison between the pupation chambers formed in soil by healthy and unparasitized *H. virescens* larvae showed that there are significant differences in size, relative width, relative height, and depth below the soil surface. Although the smaller size of pupation chambers excavated by parasitized *H. virescens* larvae could be explained by these larvae simply having less energy to expend in digging (given the late stages of parasitization by this point), this does not explain the increased depth of burial, as excavating further below the soil surface before forming a chamber requires the use of more energy. Having a chamber deeper underground is may be adaptive for *T. nigriceps*. A deeply buried chamber may serve to protect *T. nigriceps* prepupae in the field from unfavorable temperatures and weather as they overwinter in these chambers (Lopez 1982). A more deeply buried chamber may also offer vulnerable pupae and prepupae better defense against detection and attack by natural enemies of. *Toxoneuron nigriceps* overwintering in the field have reportedly suffered mortality due attack by the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae) (Lopez 1982). Although a survey of natural enemies of *T. nigriceps* is not available, hyperparasitism

may also be a source of mortality in the field. *Brachymeria pomonae* Cameron (Hymenoptera: Chalcididae), a parasitoid imported into the quarantine in the United States for testing as potential biological control agent of the pink bollworm (*Pectinophora gossypiella* Saunders [Lepidoptera: Gelechiidae]), was shown to facultatively hyperparasitize *T. nigriceps* pupae in the laboratory, causing 76% mortality in pupae that were exposed to it, though actual hyperparasitism rates were low, with most *T. nigriceps* mortality due to trauma caused during probing by *B. pomona* (White et al. 1998). Although this facultative hyperparasitoid did not appear to develop well on *T. nigriceps*, it was indeed attracted to it, and closely related wasps may show similar attractions. Manipulation of host behavior by parasitoids has been linked to avoidance of hyperparasitsm in other host-parasitoid systems. The parasitoid-induced movement of hosts away from aphid colonies and toward apical leaves appeared to reduce hyperparasitism in the *M. euphorbiae-A. nigripes* system (Brodeur and McNiel 1992). *Euphydryas phaeton* Drury (Lepidoptera: Nymphalidae) larvae parasitized by *Cotesia* spp. also tend to be located higher on host plants than unparasitized larvae (Stamp 1981). These *Cotesia* parasitoids, which pupate in cocoons attached to the outside of the host cuticle, suffered hyperparasitism levels in excess of 50% in the field; however cocoons attached to hosts located higher on the host plant suffered lower levels of hyperparasitism than those located lower on plants (Stamp 1981). Deeper excavation by *H. virescens* parasitized by *T. nigriceps* may also be a means of reducing hyperparasitism, by potentially making deeply buried parasitoid cocoons more difficult for hyperparasitoids to detect or to reach.

Small pupation chamber size may also be adaptive for *T. nigriceps*, rather than being simply a byproduct of parasitism-induced fatigue in *H. virescens*. Significantly more *T. nigriceps* larvae formed complete cocoons when placed in chambers of similar size to those formed by parasitized *H. virescens* than when placed in those similar to the larger chambers formed by healthy *H. virescens*. This suggests that pupation chamber size may influence the ability of *T. nigriceps* to form cocoons, which are an important protective structure. Although the proportion of *T. nigriceps* reaching adulthood after being placed in the "healthy type" or "parasitized type" chambers did not differ in the present experiment, absence of a cocoon appeared to have severe consequences for survival to adulthood in the Petri dish treatment of the following artificial pupation chamber experiment, with only three out of 30 naked parasitoids having emerged as adults. Thus, *T. nigriceps* may be manipulating the behavior of *H. virescens* during pupation chamber formation, causing them to form pupation chambers that are smaller than usual, but greater different relative width and height and are deeper under the surface of the soil. While this manipulation of the host may be less obvious than the sight of a moribund host larva standing over and protecting the pupating parasitoids that egressed from it, it is likely equally significant. If natural selection has lead to *T. nigriceps* manipulating the characteristics of the chambers they pupate in, then such manipulation must be adaptively significant. This is further supported by the results of the experiments using artificial pupation chambers.

Provision of an artificial pupation chamber, in all cases, was an improvement over a flat open surface in terms of both formation of a complete cocoon and survival to

adulthood. No *T. nigriceps* larvae were able to from cocoons when left in Petri dishes. The majority laid down silk in a haphazard fashion and attempted to pupate naked on top of it; however, most of these died as prepupae. Out of 25 larvae placed in Petri dishes for pupation, only three managed to develop to adulthood. This clearly demonstrates that pupation within a cocoon is far more likely to allow for complete development than naked pupation. Furthermore, a small, at least partially enclosed space appears to be necessary for *T. nigriceps* to be physically able to form a cocoon. Perhaps as larvae turn, secreting silk, they use nearby surfaces to anchor silk to. Or perhaps they need a narrow space to guide their movements as they turn, to keep them from simply rolling away form where they started. Thorough observation of *T. nigriceps* larvae as they form their cocoons, perhaps through the use of time-lapse filming, would elucidate this.

No differences were found in the masses of the cocoons formed within any of the artificial pupation chambers, indicating that none required the use of significantly more silk to form a cocoon, nor did any cause stunting of the parasitoids within them. There were, however, differences in how readily cocoons could be formed within various artificial chambers and how many parasitoids were able to develop to adulthood after forming their cocoons in these chambers. As such, it is apparent that some of the items used in this experiment serve better as substitute pupation chambers for *T. nigriceps* than others.

Of the artificial pupation chambers tested, the most effective type was the 0.5 ml gelatin capsule. The proportion of parasitoids that formed cocoons in this chamber type was similar to that in control chambers and cotton balls, and the proportion of parasitoids

emerging as adults from cocoons formed in gelatin capsules also did not differ significantly from the control. Gelatin capsules are inexpensive and practical. For example, they come ready to use, unlike cotton balls, which require some manipulation before use as pupation chambers. Although cocoons were removed from the gelatin capsules before adult emergence in this experiment, adult wasps have been observed chewing through the capsules and emerging from them without assistance. The capsules are also disposable and take up little space. However, placement of parasitoids in these capsules must be done carefully to avoid injuring them when the capsule is closed. As this process can be time consuming, gelatin capsules would serve best as pupation chambers in small-scale rearing, as in the case of a short experiment, rather than being used in large-scale, long term rearing efforts.

On a larger scale, microcentrifuge tube racks may be better candidates for artificial pupation chambers. Although a significantly smaller proportion of parasitoids formed cocoons in the wells of these racks than in cotton balls, this proportion was not significantly lower than those forming cocoons in host-formed chambers. Emergence of adult wasps also occurred at rates similar to those of the control treatment. The use of wells of these racks as pupation chambers is fairly simple and requires little effort. Larvae need only be placed one per well, with no further manipulation necessary. Although the wells were plugged with cotton in this experiment, simply covering the wells with a paper towel may suffice. The microcentrifuge tube racks are solid and sturdy, offering good physical protection to the developing parasitoids inside. They are also space-efficient, as each rack can hold 76 larvae, and the racks themselves can be

stacked on top of one-another. Washing the racks thoroughly between uses, however, would be recommended.

The highest proportion of cocoon formation occurred in cotton balls, in which all larvae used in the experiment formed cocoons. This was a higher proportion than in even the host-formed control chambers, though not statistically significantly so. However, though they serve well for allowing immature parasitoids to form cocoons, cotton balls would not make for a practical replacement for host-formed pupation chambers even on a small scale. The formation of indentations in individual cotton balls is time consuming and the cotton balls themselves offer little physical protection to the delicate larvae and pupae developing inside. If the cotton ball chambers are dropped, or if an even moderately heavy object is accidentally placed on top of them, the fragile immature parasitoids would most likely be killed.

Performance was comparatively poor in test tubes. Although the proportion of parasitoids that formed cocoons in this treatment was not significantly smaller than in the control chambers, the proportion of parasitoids that emerged as adults in this treatment was approximately half of that which emerged from host-formed control chambers. This may be due to the fact that test tubes offer only a partially enclosed space, rather that a fully enclosed one. It also could be related to the size of the test tubes used. As demonstrated by the comparison among differently sized paper chambers, the size of the space in which parasitoid larvae are enclosed before pupation makes a difference in how well they form cocoons.

Any in vitro rearing system developed for *T. nigriceps* must include some form of pupation chamber to ensure successful cocoon formation and pupation. Depending on the scale of the rearing operation, either gelatin capsules or microcentrifuge tube racks may be most practical. However, other artificial pupation chambers, perhaps even more efficient and practical, could be developed specifically for this use. The study of natural pupation chambers formed by parasitized *H. virescens* larvae certainly offers a clue as to what conditions best suit *T. nigriceps* during cocoon formation. The difference in the ability of parasitoid larvae to form cocoons in paper chambers based on the dimensions of chambers formed by either healthy or parasitized *H. virescens*, a difference of mere millimeters, attests to the importance of these dimensions.

CHAPTER V

CONCLUSION AND FUTURE DIRECTIONS

Post-egression tissue feeding is a vital step in the development of *Toxoneuron nigriceps* Viereck (Hymenoptera: Braconidae) larvae. Deprivation of this feeding impacted both the size and survival of parasitoids. Provision of a post-egression diet improved the late immature development of *T. nigriceps*, when reared in vivo to its final larval instar. Although it was difficult to conclude how well parasitoids developed when fed artificial diet relative to host tissue, due to low overall emergence rates of *T. nigriceps* in all feeding treatments, the preliminary artificial diet appeared to be an improvement over no post-egression feeding at all. Whether larvae reared in vitro, either from egg stage or from 2nd larval instar, would also benefit from an artificial tissue substitute remains to be seen. It is possible that the preliminary artificial diet used by Kuriachan et al. (2006) to rear *T. nigriceps* from 2nd to 3rd larval instar was deficient in one or more nutrients that are usually acquired by *T. nigriceps* before egression from the host. If this is the case, larvae may not develop far enough into the final larval instar to benefit from an artificial post-egression diet.

Provision of an artificial pupation chamber, such as a gelatin capsule, also improved the development of mature *T. nigriceps*, in comparison to leaving them on a flat, open surface. Larvae that were placed in small, enclosed spaces after post-egression feeding formed complete, functional cocoons and, successfully pupated and emerged as

adult wasps more frequently than those placed in an open space. In most post-egression feeding experiments, diet did not affect the ability of *T. nigriceps* larvae to form cocoons. The only exception to this was larvae that were fed a linolenic acid-enriched diet, which caused larvae to die before they began producing silk. As such, even if larvae were deprived of an artificial substitute for post-egression tissue feeding, an artificial pupation chamber could improve the ability of *T. nigriceps* larvae to form cocoons during in vitro rearing. Although gelatin capsules and wells of microcentrifuge tube racks provided adequate conditions for mature *T. nigriceps* larvae to form cocoons, more suitable artificial pupation chambers could be developed. Evidence of behavioral manipulation by *T. nigriceps* during the formation of pupation chambers by *H. virescens* larvae suggested that this parasitoid has specific requirements for the dimensions of its pupation chamber. A thorough investigation of all dimensions of these chambers, accounting for both size and shape, as well as other plausibly relevant variables, such as the texture of chamber walls, could provide the information necessary to design ideal, custom-fabricated pupation chambers for *T. nigriceps*. If such chambers could be created easily and inexpensively, then mass rearing of these parasitoids, even when done in vivo, could be improved.

The best approach to future attempts at in vitro rearing of *T. nigriceps*, at least from 2nd instar onward, may be to integrate everything that has been learned about its late immature development in this project. An artificial post-egression diet, enclosed in a membrane with an opening large enough to allow *T. nigriceps* larvae to insert their heads into the diet without allowing it to leak, would allow for prolonged larval feeding. If this

encapsulated diet and the parasitoid larva were both placed in a gelatin capsule, then larvae could be simply left to feed until satiation, then spin their cocoon in the confines of an artificial pupation chamber. These steps could allow, for the first time, development of *T. nigriceps* from larva to adult in an artificial rearing system. If the methods used by Pennacchio et al. (1992) also put into practice, then complete rearing of *T. nigriceps* from egg to adult may even be possible. If so, *T. nigriceps* would be the first koinobiontic larval endoparasitoid to be reared through all immature stages in an artificial system, though this rearing would not yet be complete.

There are, of course, aspects of *T. nigriceps* development that require further investigation before a complete in vitro rearing system can be developed for. For one, further studies would need to be undertaken to improve rearing of newly laid eggs in artificial media. Pennacchio et al. (1992) saw eclosion only from *T. nigriceps* eggs that were dissected out of hosts 7 h or more after oviposition. There may be nutrition and/or hormonal requirements that are provided either by the host, by maternal factors injected during oviposition, or from some combination of the two, that induce germ band formation in *T. nigriceps* eggs. Rearing of *T. nigriceps* eggs in vitro was improved, however, through addition of insect derived factors, so that freshly-laid eggs, dissected from hosts immediately after oviposition, were able to undergo embryonic development in an artificial medium containing *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) cell lysate, though these larvae showed some signs of deformity (Cônsoli and Vinson 2004). To create a complete, multi-generation, in vitro rearing system, female *T. nigriceps* wasps would also need to be induced to oviposit into an artificial medium.

Parasitoids searching for hosts use a number of cues to determine host suitability, including shape, size, texture, chemicals (both olfactory cues and contact cues), and perhaps even color and movement (Vinson 1976). Some or all of these cues will need to be taken into account when developing an artificial host that *T. nigriceps* will be willing to oviposit into.

Another remaining potential difficulty is the possibility that *T. nigriceps* larvae go through an additional, currently undescribed, molt as they egress from the host. Some microgastrine braconids, such as *Cotesia kariyai* Watanabe (Hymenoptera: Braconidae), molt immediately prior to egression, leaving the exuvium behind to plug the hole in the host cuticle through which the parasitoid egressed (Nakamatsu et al. 2006). A similar larval molt was also described in *Microplitis croceipes* (Hymenoptera: Braconidae) as it egresses from it host, *H. virescens*, leaving its exuvium in site of egression (McLoud 2011). When the remains of *H. virescens* hosts were dissected immediately after egression of *T. nigriceps*, the exit wounds often contained material that may have been exuvia of *T. nigriceps* larvae, though this has not been confirmed (R. E. Henderson, personal observation). If *T. nigriceps* undergoes an additional larval molt as it egresses from the host, an artificial membrane may be needed to allow for similar behavior in larvae being reared in an artificial medium. However, when *T. nigriceps* was reared in vitro by Kuriachan et al. (2006), some of the resulting larvae were observed voiding the meconium, a step which does not usually take place until the end of larval development. This indicates that although they failed to pupate, these parasitoids may indeed have reached the final larval instar, without need of a membrane to molt through.

Another remaining concern in the artificial rearing of parasitoids is the quality of parasitoids reared in vitro, as compared to in vivo reared counterparts. Although standards for evaluating the quality of parasitoids reared in vitro have not been established, some authors have suggested measures including assessment of morphological characteristics (e.g., size of last instar larvae or pupae), development and reproductive abilities (e.g., development time, survival rate of each stage, sex ratio of adults, and fecundity), biochemical (e.g., hormone titers and protein, lipid and carbohydrate content of parasitoids), behavioral (e.g., parasitization efficiency, flight activity, and host location ability), and genetic aspects (e.g., genetic variability and homozygosity rate) (Grenier and De Clercq 2003).

Existing studies comparing the quality of idiobiont parasitoids reared completely on artificial diets to those reared in vivo show mixed results. Two egg parasitoid species, *Trichogramma galloi* Zucchi and *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae), showed reduced female longevity and fecundity when reared in artificial diets (Cônsoli and Parra 1996). When *Trichogramma minutum* Riley was reared in vitro for ten consecutive generations, parasitoids had longer development times and a higher percentage of deformities, compared to those reared on eggs of *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae); however, female wasps reared in vitro were larger, lived longer, and parasitized more *H. zea* eggs than their in vivo reared counterparts (Nordlund et al. 1997). *Catolaccus grandis* Burks (Hymenoptera: Pteromalidae), an ectoparasitoid of the boll weevil *Anthonomus grandis grandis* Boheman (Coleoptera: Curculionidae), reared on an artificial diet showed no reduction in fecundity compared to

those reared on hosts within the first five generations of artificial rearing, though fecundity was significantly reduced after ten generations (Morales-Ramos et al. 1998). When tested in the field, higher parasitism rates were obtained in plots where *C. grandis* reared in vivo had been released than in plots where in vitro reared parasitoids had been released (Morales-Ramos et al. 1998).

If fully reared in vitro, koinobiont parasitoids such as *T. nigriceps* could be more economical as biological control agents than those reared in natural hosts. However, this goal is likely quite distant, as complete in vitro rearing of *T. nigriceps* would require either multiple diets or one diet supplemented with additional factors a number of times during early larval development, as well as transfer from an early larval diet to a post-egression diet and pupation chamber. This amount of labor is not likely to be more practical than simply rearing *T. nigriceps* in *H. virescens*. Additionally, the preliminary diets used to successfully rear *T. nigriceps* from 2nd to 3rd larval instar all contained hemolymph extracted from parasitized *H. virescens* (Kuriachan et al. 2006). As such, rearing of these host insects would still be necessary even in an artificial rearing system, at least until non-insect substitutions for host hemolymph can be found.

A more immediate benefit to complete in vitro rearing of *T. nigriceps* would be the research opportunities it could offer as a model organism. Not only could *T. nigriceps* demonstrate how in vitro rearing of koinobiont larval endoparasitoids can be achieved but, more importantly, also allow insight into the consequences of rearing such a parasitoid in the absence of the host with which it is so tightly coevolved. Additionally, *T. nigriceps* is a polydnavirus-bearing parasitoid, and the consequences of rearing a

polydnavirus-bearing wasp in the absence of its polydnavirus, as well as the associated influences, have yet to be investigated. A parasitic wasp and its polydnavirus undergo constant mutualistic coevolution with each other and constant antagonistic coevolution with the host. A parasitoid would not lose its polydnavirus entirely, as it is integrated into the wasp's own genome and passed vertically from parent to offspring. However, if reared in an artificial environment for many generations without the need for the polydnavirus to fight the host immune response, the mutualism between the wasp and polydnavirus could break down, resulting in a parasitoid that is unable to survive the immune response of a living host larva. Until a polydnavirus-bearing parasitoid such as *T. nigriceps* can be fully reared in an artificial, in vitro system for multiple generations, this problem will remain unexplored.

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