DEVELOPMENT OF SALIVARY GLAND STRUCTURES WITHIN THE FIRST THREE LARVAL INSTAR STAGES OF ENDOPARISITOID WASP *TOXONEURON NIGRICEPS* (HYMENOPTERA: BRACONIDAE)

A Scholars Thesis

by

SOPHIA NORISHAH DANIELS

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Entomology

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Approved by:

Research Advisor: Bradleigh Vinson
Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

Development of Salivary Gland Structures Within the First Three Larval Instar Stages of Endoparasitoid Wasp *Toxoneuron nigriceps* (Hymenoptera: Braconidae). (May 2012)

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This study focused on determining which larval instar stage of the endoparasitoid wasp, *Toxoneuron nigriceps* (Hymenoptera: Braconidae), harbors the presence of salivary gland structures. The development of the salivary glands indicates the ability of the larvae to penetrate and emerge from the cuticle of the host, *Heliothis virescens* (Lepidoptera*:* Noctuidae), after which re-insertion of the head into the host initiates further feeding. This post-egression feeding is a rare trait among Hymenoptera parasitoid species. *H. virescens* larvae were parasitized using *T. nigriceps* female wasps and dissected to obtain samples of first, second and third endoparasitoid larval instars. Once obtained, the endoparasitoid larvae were dissected in order to isolate and characterize the presence of salivary gland structures, identifying the exact larval instar stage(s) in which the salivary glands were developed and the extent of their development. It was discovered that the presence of salivary glands occurred within *T. nigriceps* larvae during the late first, early second, late second, early third and late third instars.

DEDICATION

A little girl's view of the world is vast; in order to gain the confidence she needs to carry on her dreams, she must have the strength of her family's faith behind her. I would like to dedicate all the hard work, long hours, and drive to my amazing family. My parents never hesitated to sacrifice their time and wisdom in order to allow me to accomplish everything that at one point seemed so daunting. They believed in me, filling my soul with lessons over acceptance, patience and perseverance. Likewise, my grandparents were extraordinarily strengthening in their support and encouragement, always eager to listen to my experiences and dreams. I am proud of my work ethic, but I accredit all of the values incorporated with that ethic to having been raised and influenced by exceptionally hardworking and dedicated individuals whose achievement I can only aspire to. More importantly, in the wake of success they taught me to maintain a humble outlook; respect comes from defining yourself by the goodness of your heart. My sister still has a whole life ahead of her, so through the enrichment of exposure I dedicate to her the encouragement to pursue a future with higher education. Having stood by me through the darkest days, I dedicate my continued will to Michael. Lastly, I dedicate this endeavor of research to my revered mentors Dr. Bradleigh Vinson and Dr. Indira Kuriachan. I am grateful beyond expression for the opportunities and guidance they have provided.

ACKNOWLEDGMENTS

No great endeavor is ever accomplished alone. I am deeply grateful for all of the help and support I have received over the duration of this project. First I would like to acknowledge and express my gratitude towards my mentor Dr. Vinson for allowing me to use his laboratory in order to conduct research. It was Dr. Vinson who first proposed this project, and his belief in me has been a driving force in its completion. I would like to acknowledge Dr. Kuriachan for her help and support in ensuring the quality and direction of my writing. Dr. Kuriachan has always been more than a mentor to me, and I am indebted to her wise, understanding and nurturing character. I thoroughly enjoyed learning from her, and I shall continue to do so. I would like to express a heartfelt thanks to Dr. Woolley for allowing me to utilize his laboratory equipment for taking photographs. In his wise words, “lighting is everything,” and the beauty of the specimens would not have been illuminated without his help. I would also like to thank my dear friend Roxanne Ramirez for helping me with taking and editing the photographs. Altogether these individuals have helped me to accomplish a thesis I am proud of. I shall never forget their generosity and kindness.

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CHAPTER I

INTRODUCTION

Toxoneuron nigriceps is a natural koinobiont endoparasitoid species of the tobacco budworm *Heliothis virescens,* an economically significant agricultural pest. A koinobiont organism kills its host at the end of its own development, which in this case takes place inside of the body of the host. The host-seeking female *Toxoneuron nigriceps* wasp will oviposit eggs directly into the *Heliothis virescens* larvae, after which the larvae show signs of arrested development and immunosuppression. This modification in host larval physiology is likely a result of induction by the obligate symbiotic polydnavirus (TnPV) associated with *Toxoneuron nigriceps* (Drezen et al. 2007). Substances secreted by parasitoid wasps such as venoms, calyx fluids, and teratocytes can also influence physiological changes when injected by females during oviposition (Consoli et al. 2007). Further, it has been discovered that the last instar larvae of *Heliothis virescens* fail to pupate and have little 20-hydroxyecdysone as well as an increase in juvenile hormone after being parasitized by *Toxoneuron nigriceps*, indicating a mechanism of endocrine control that prohibits metamorphosis. (Borst et al. 2003) Studies have been conducted to prove that *Toxoneuron nigriceps* endoparasitoid eggs require nutrients from the host hemolymph to initiate and complete embryogenesis.

This thesis follows the style of Annals of the Entomological Society of America.

Changes in the amino acid composition and protein profile of the host hemolymph are directly correlated with the developing endoparasitoid's nutritional demands (Pennacchio et al. 1994, Consoli and Vinson 2004).

Upon the female parasitoid's oviposition into the host, the egg remains free in the hemolymph. Exactly 36 to 48 hours after oviposition, the first instar endoparasitoid larva hatches from the egg and suspends within the body cavity of the host. The first instar larva lasts five to six days and is characterized by a typical caudal-type larval form averaging 0.67 mm in length. The head is heavily sclerotized and dorsoventrally flattened amid smooth larval integument lacking setae but possessing a well-developed posterior anal vesicle (later stage). The second instar lasts two to three days and lacks the heavily sclerotized head capsule of the first instar. The body, averaging 3.67 mm in length, is translucent green with a posterior caudal horn ventral to the anal vesicle. The development of spiracles coincides with the presence of a distinct closed tracheal system. The midgut is solid with ingested hemolymph and occupies a large proportion of the body cavity. Second instar endoparasitoid larval development parallels that of the host's molt into its fifth instar; the larvae cannot fully develop unless the host passes this critical point of development, regardless of which host stage is being parasitized (Pennacchio et al. 1993). The third instar, averaging 9.00 mm in length, lasts two days prior to emergence with loss of the caudal horn and presence of visible setae. Larval color becomes more opaque and the spiracles are clearly defined along with a characteristic lateral ridge.

All developing instars that remain in the body of the host possess simple falcate mandibles and feed freely on host hemolymph (Lewis and Vinson 1968). A preliminary study conducted in Dr. Vinson's laboratory found that as the endoparasitoid enters into the third instar, the host's internal organs become liquefied, and the cuticle becomes very thin unlike the unparasitized (control) host larvae. It is hypothesized that the secretions by the endoparasitoid larvae may cause the digestion of the host cuticle, which is comprised of fibrous chitin embedded in a complex protein matrix. Another study confirmed the role of *Toxoneuron nigriceps* parasitism in the endocrine alterations of the larval host physiology pertaining to temporal alterations in the gut replacement process during fifth instar host development. It was found that the whole gut replacement process is significantly delayed in parasitized larvae, with complete differentiation of the new gut epithelium being observed four days later than in unparasitized controls; similar temporal profiles of cell death and metabolic activity were still maintained between the unparasitized and parasitized larvae (Eguileor et al. 2008). As aforementioned, the host larvae are highly susceptible to the critical and detrimental demands posed by endoparasitoid larval development.

A unique behavioral trait of the koinobiont *Toxoneuron nigriceps* larva is post-egression re-insertion of the head into the host cuticle through creation of a hole for tissue feeding (Kuriachan 2011). Egression happens by way of the larva piercing a hole big enough to first push the head out and then slowly push the entire body out of the metathoracic region of the host. As soon as the head and part of the body is out of the host, the

endoparasitoid larva curls and reinserts its head into the host a few millimeters posterior to its emergence hole and begins to feed on the host tissue. Preceding parasitoid pupation, the host has been wholly consumed in part from a retained head capsule and integument (Lewis and Vinson 1968).

Toxoneuron nigriceps is a potential biological control agent for *Heliothis viriscens* due to its host specificity. The tobacco budworm (*Heliothis virescens)* is an economically important pest insect that targets high-profile crops including tobacco and cotton. This particular species is found throughout the eastern and southwestern United States and in California (Davidson and Lyon 1979, Metcalf and Metcalf 1993). The parasitized host larvae will not survive to pupation even if the endoparasitoid larvae die before their final instar, seeing as physiological alterations and suppressed immunity progress the host ultimately towards death. The initial toxins released into the host larvae upon oviposition by the parasitoid female can also lead to immediate death if overexposure has occurred from excess stinging.

Due to the increased concern over the use of pesticides, biological control has become one of the more important components of integrated pest management programs (IPM). Studies are underway to rear *Toxoneuron nigriceps* in vitro, and successful attempts were reported in rearing *Toxoneuron nigriceps* from egg to second instar (Pennachio et al. 1992), from second instar to third instar (Consoli et al. 2006) and from post egression to pupation (Kuriachan 2011).

In order to achieve an understanding of the complete development of the parasitoid from egg to adult, it is imperative to understand the morphological and physiological changes occurring in the parasitoid larvae. This project focuses on determining the changes happening in the *Toxoneuron nigriceps* larvae towards the final developmental stages inside the host. The egression behavior points towards secretion by the parasitoid larvae that aids in the digestion of host cuticle for emergence and subsequent re-insertion. Seeing as the parasitoid larvae lack strong enough mandibles for mechanical cutting and tearing, both the egression and re-insertion behavior could only be possible through cuticle digestion. The secretion of enzymes capable of cuticle digestion is hypothesized to arise from the salivary glands. The objective of the study is to determine whether or not *Toxoneuron nigriceps* larvae possess salivary glands, and if so determine at what stage they appear.

CHAPTER II

METHODS

Rearing

Rearing of both T. nigriceps and H. virescens

An important aspect of rearing parasitoids is to ensure the maintenance of the colony, consisting of provision for the rearing of hosts to sustain the natural koinobiont life cycle. In the case of *T. nigriceps* parasitoid wasps, *H. virescens* caterpillars were reared on artificial diet until the appropriate early fourth instar larval stage was obtained for parasitizing. Another set of *H. virescens* larvae were also reared into adulthood for the cultivation of eggs. In order to parasitize, early fourth instar *H. virescens* larvae were placed in a cage along with two mated T. *nigriceps* female wasps (15:1) for a two-hour period, induced to oviposit their eggs through use of an incubator. The constant temperature (30°C) and light in the incubator stimulated effective oviposition (laying eggs). The parasitized larvae were then placed in vials and allowed to incubate for three weeks at 28°C in order to permit the parasitoid *T. nigriceps* larvae to develop, emerge and pupate. The parasitoid cocoons were then pulled and placed in the same incubator to allow for the emergence of the adult *T. nigriceps* wasps.

For the replication of each larval target stage of *T. nigriceps*, 15 *H. virescens* larvae were individually parasitized and allowed to incubate over a course of three weeks. In order to target each specific larval instar stage, the parasitoid *T. nigriceps* larvae were allowed

to incubate inside the *H. virescens* host larvae according to the specific days of development yielding each of the three instars. The first instar, lasting 5-6 days, was obtained at least 48 hours or two days after initial parasitization and upon hatching of the egg; the early second instar was obtained 8-9 days succeeding the hatching of the egg; the late second instar was obtained around 10 days succeeding the hatching of the egg; the early third instar was obtained 11-12 days succeeding the hatching of the egg; the late third instar was obtained by the 12th day succeeding the hatching of the egg, with two days from the beginning of the third instar stage marking the time of emergence (egression) from the metathoracic region of the host. Each parasitoid larval instar stage was obtained with a replication of no less than five trials to insure the validity of the findings.

Dissection

Dissection of H. virescens host larvae

The parasitized *H. virescens* host larvae, whose development was arrested by the fifth instar, were dissected under a light stereomicroscope in a solution of ten percent ethyl alcohol. This was done in order to extract the *T. nigriceps* parasitoid larvae. A longitudinal cut was made with microdissection shears along the dorsal side of the body starting beneath the head capsule. Second and third instar parasitoid larvae were extracted with blunt forceps from either the median section or caudal end lengthwise alongside the gut organ; first instar parasitoid larvae remained free in the body cavity of the host and were flushed out with a glass pipette. After extraction the parasitoid larvae

were separated and identified as either first, second or third instar based on morphological characteristics outlined in Lewis and Vinson (1968).

Dissection of T. nigriceps parasitoid larvae

The dissection of the first instar *T. nigriceps* parasitoid larval stage was based on the following characteristics: average 0.67 mm length and 0.24 mm width (after first molt), 13 segments with the last modified into a tail-like organ projecting downwards, large heavily sclerotized head segment, white color, smooth integument, lack of setae and visible gut only in later development. The later stages of the first instar were also evident with the initial development of the small caudal horn ventral to the anal vesicle. The first instar larvae were dissected under a light stereomicroscope in a solution of ten percent ethyl alcohol. A posterior to anterior longitudinal cut was made with microdissection shears along the ventral side of the larval body towards the apex of the head capsule.

The dissection of the second instar *T. nigriceps* parasitoid larval stage was based on the following characteristics: average 3.67 mm length and 0.90 mm width (after first molt), 13 segments with the tail-like structure modified into a small caudal horn ventral to the anal vesicle, smooth larval integument, translucent green color, distinct midgut and developing spiracles. The late second instar larvae were larger by about two millimeters and possessed a thickened integument and gut. The second instar larvae were dissected under a light stereomicroscope in a solution of ten percent ethyl alcohol. A cut was made with microdissection shears from the posterior caudal end longitudinally along the

ventral midline of the body to the apex of the head. The larval skin was folded out on both sides laterally from the midline cut in order to display the gut and interior surface of skin running just underneath the ventral folds along which the salivary glands were initially identified.

The dissection of the third instar *T. nigriceps* parasitoid larval stage was based on the following characteristics: average 9.00 mm length and 2.60 mm width (after first molt), smooth integument, a row of setae on the dorsum of the sixth segment, distinct spiracles on the first and fourth through eighth segments, shed anal vesicle and caudal horn (later stage), solidified tan gut transition from the translucent green of the second instar (later stage) and a distinct sublateral ridge with a retracted head capsule. It should be noted that the late third instar larval stage was obtained post-egression. Both the early third instar and late third instar larvae were dissected under a light stereomicroscope in a solution of ten percent ethyl alcohol. A cut was made with microdissection shears from the posterior caudal end longitudinally along the ventral midline of the body to the apex of the head capsule. The larval skin was folded out on both sides laterally from the midline cut in order to display the massive gut and interior surface of skin and tissues along which the salivary glands were identified and partially extracted for view from both the early and late third instar stages. The partial extraction of the salivary glands was performed by use of blunt forceps as well as a 0.15 mm minuten pin probe to gently tease one of the branches of the salivary gland structure forward and away from the tissues. This isolation allowed for a closer examination and comparison of the structure.

Photography

All dissections and photography were performed using a Leica® MZ16 stereomicroscope attached to a mounted Zeiss® AxioCAm MRC5 digital camera. The Zeiss® AxioVision software program was used in order to create a montage of the specimens consisting of serially focused images taken in multiples. The exposures were compiled into the montage using the Helicon® Image software program. The pictures were then edited to adjust for brightness and contrast with formatting performed using the Photoshop® CS5 program, Adobe Lightroom 3 and Indesign CS5.

CHAPTER III

RESULTS

First instar

The late first instar larvae of *T. nigriceps* (Fig. 1) were found to have the presence of translucent, thin and initially developing salivary gland structures surrounding the diminutive gut organ; these were first identified before dissection. The glands were tubiform and visibly contrasted against the translucent body, originating at the base of the labium within the heavily sclerotized head capsule. The labium is the posterior-most fused mouthpart. From the base of the labium, the slender mass of glands extended to just above the anterior tip of the gut organ and was separated into two lateral extensions (branches) traveling to narrowly flank the median gut organ on its left and right side. The branches encompassed roughly less than one fourth of the area surrounding the gut organ. These extensions were divided along the left and right side of the larval body. Each extension consisted of a subdivided and forked ventral and dorsal arm running parallel and ending near the apex of the posterior end of the larval body towards the developing anal vesicle (Chapman 1998). It was affirmed that the salivary gland development occurred towards the later stages of the first instar and were not present within the early stages of the first instar.

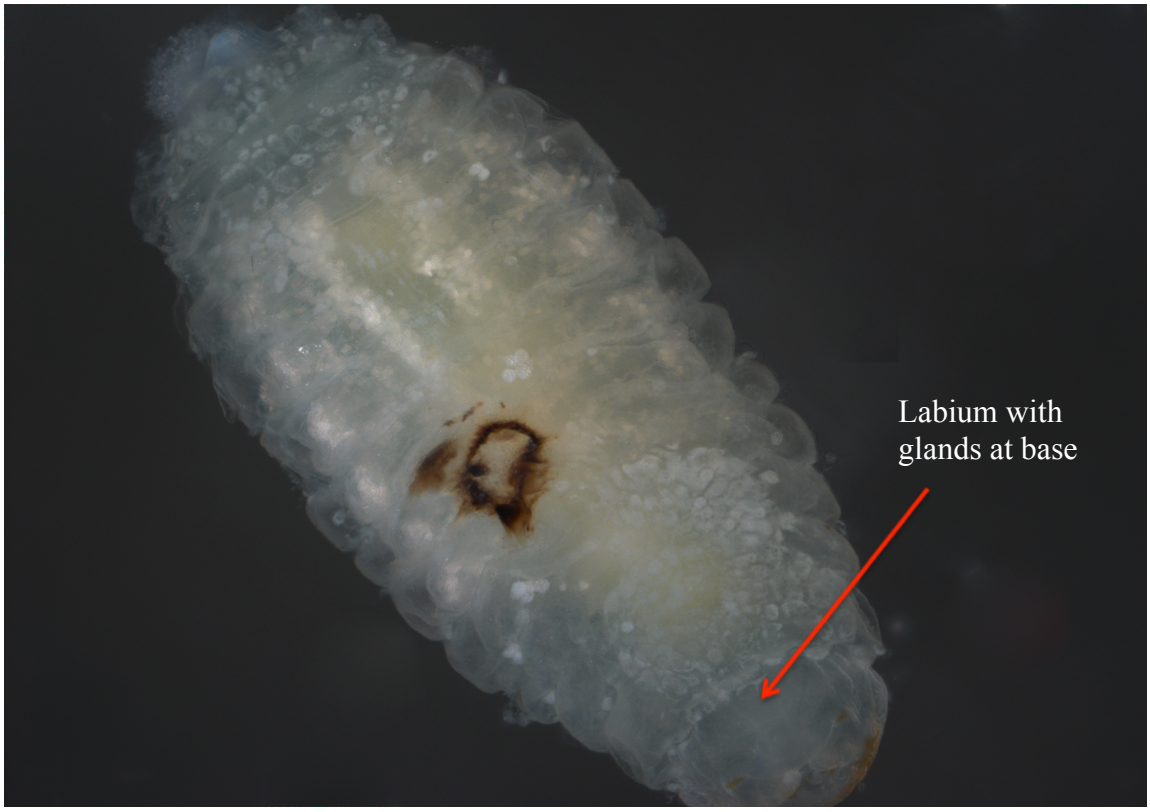

Fig. 1. Late first instar larva of *T. nigriceps* with salivary glands.

Second instar

The second instar larvae of *T. nigriceps* were found to have a more developed gut from the first instar larvae. It was observed that there were well defined and tubiform salivary gland structures seen clearly through the transparent larval integument preceding dissection within early second instar larvae (Fig. 2); these structures originated at the base of the labium, unobstructed by a sclerotized head capsule. The narrow mass of glands was divided from the labium into two branches narrowly flanking the median translucent green gut organ on its left and right side; they encompassed roughly less than one third of the area surrounding the gut organ. These extensions were divided along the

left and right side of the larval body. Each extension consisted of a subdivided ventral and dorsal arm running parallel and ending near the apex of the posterior end of the larval body. As the early second instar matured into the late second instar (Fig. 3A), the difference in gut organ size was evident; the gut organ took up a majority of the body cavity in later development. As well, the integument and tissues of the late second instar were greatly thickened, obstructing the external view of salivary gland structures. Upon dissection (Fig. 3B), the salivary glands were visible and appeared identical in their morphology to the morphology of the early second instar. The main difference between the early second instar and the late second instar was that the salivary glands comprised a larger area surrounding the gut in the latter and were thicker in circumference.

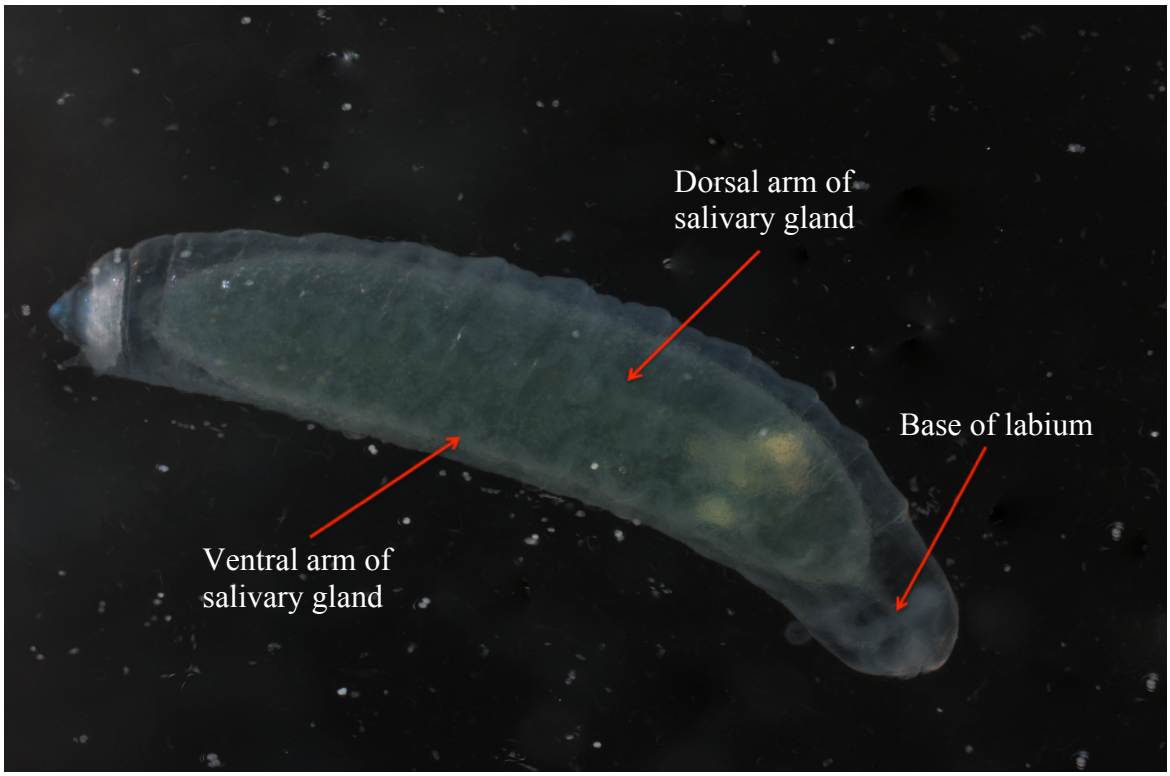

Fig. 2. Early second instar larva of *T. nigriceps* with salivary glands.

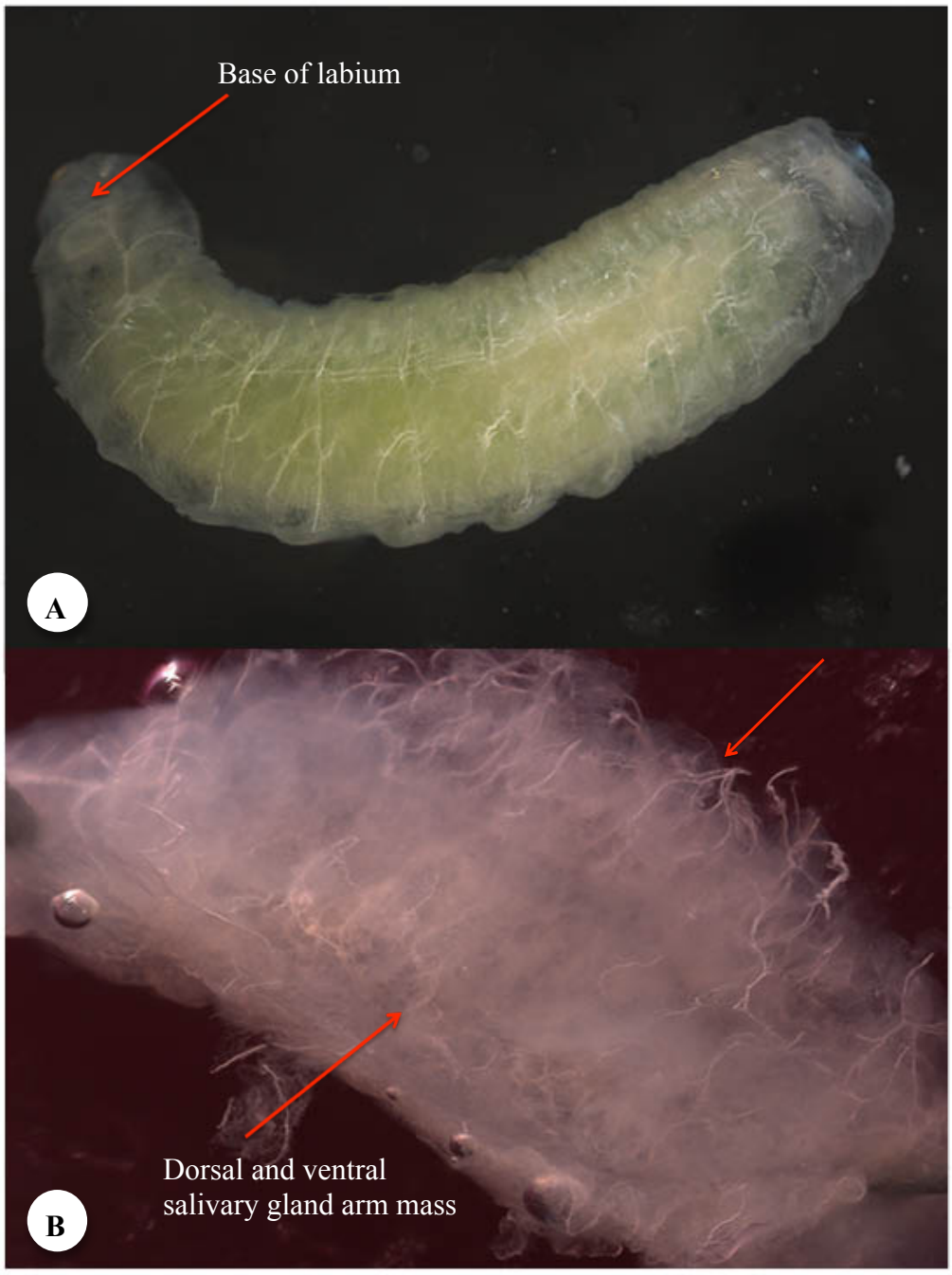

Fig. 3. (A) Late second instar larva of *T. nigriceps* with salivary glands before dissection. (B) Late second instar larva of *T. nigriceps* with salivary glands after dissection.

Third instar

The early third instar larvae of *T. nigriceps* (Fig. 4A) contained an enlarged solid green central gut organ surrounded by a mass of fat bodies and thickened translucent tissue lining the interior of the larval integument. Once dissected (Fig. 4B), salivary gland structures were found imbedded between these tissues, often hard to differentiate. The appearance of these salivary gland structures was markedly different from their appearance in the second instar larvae of *T. nigriceps*. Within the early third instar larvae, the tubiform salivary gland structures were characterized by a translucent color and thickened circumference from the late second instar. These salivary gland structures encompassed a majority of the region surrounding the gut organ. The elongation of the dorsal and ventral arms amounted in the expansion of the salivary glands widthwise along the body. The strands of the dorsal and ventral arms appeared to be configured in a transverse series of hairpin loops. Although physically larger in size, their formation and orientation within the larval body was assumed to be identical to the earlier instar morphology. The late third instar larvae of *T. nigriceps* (Fig. 5A) contained a larger, solid tan colored gut as a result of host tissue consumption. The presence of fat bodies was also proliferated. Upon dissection (Fig. 5B) it was discovered that the morphology of the salivary glands within the late third instar was similar to the salivary glands found within the early third instar, however they were opaque and comparatively larger in circumference.

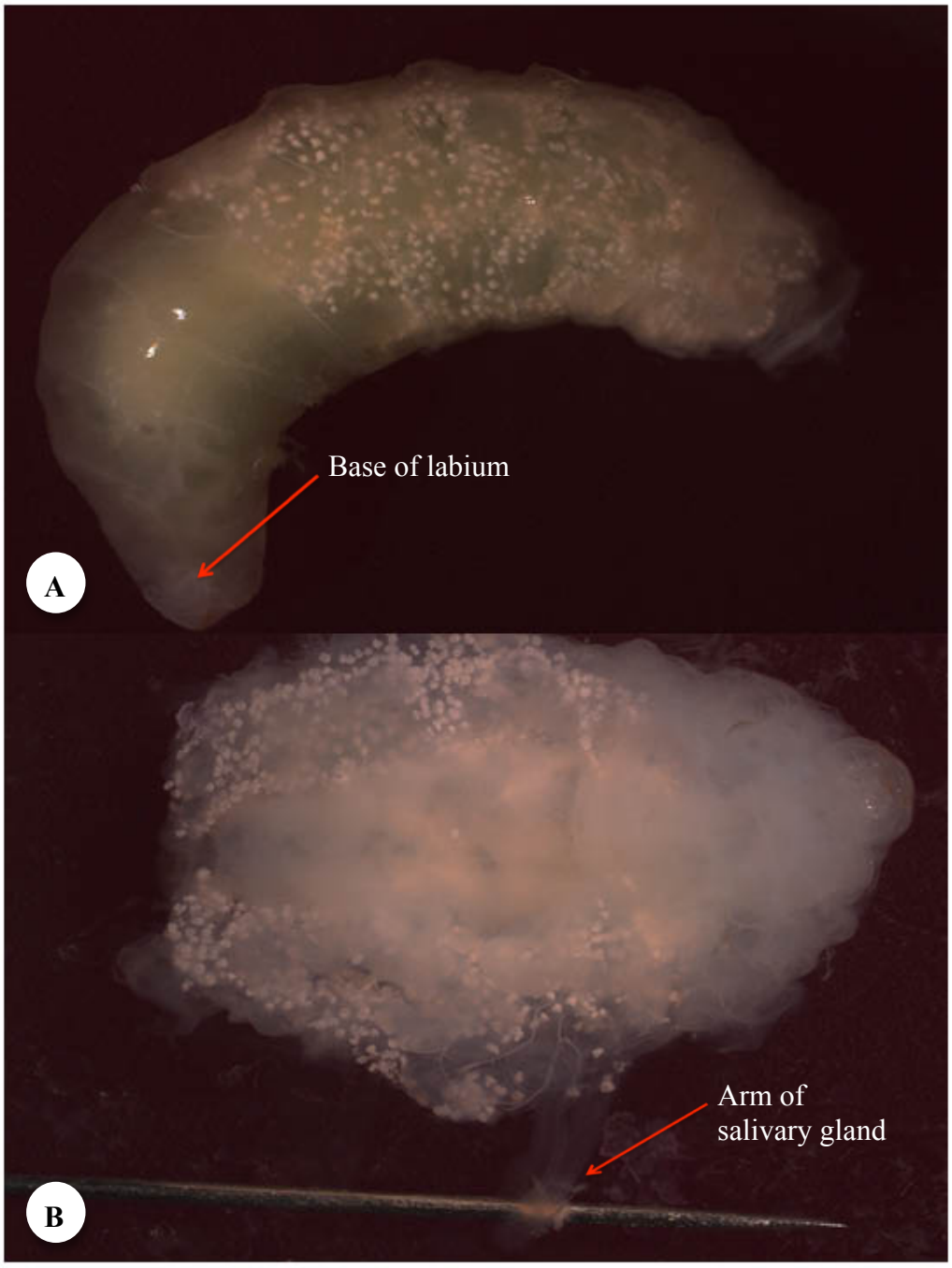

Fig. 4. (A) Early third instar larva of *T. nigriceps* with salivary glands before dissection. (B) Early third instar larva of *T. nigriceps* with salivary glands after dissection.

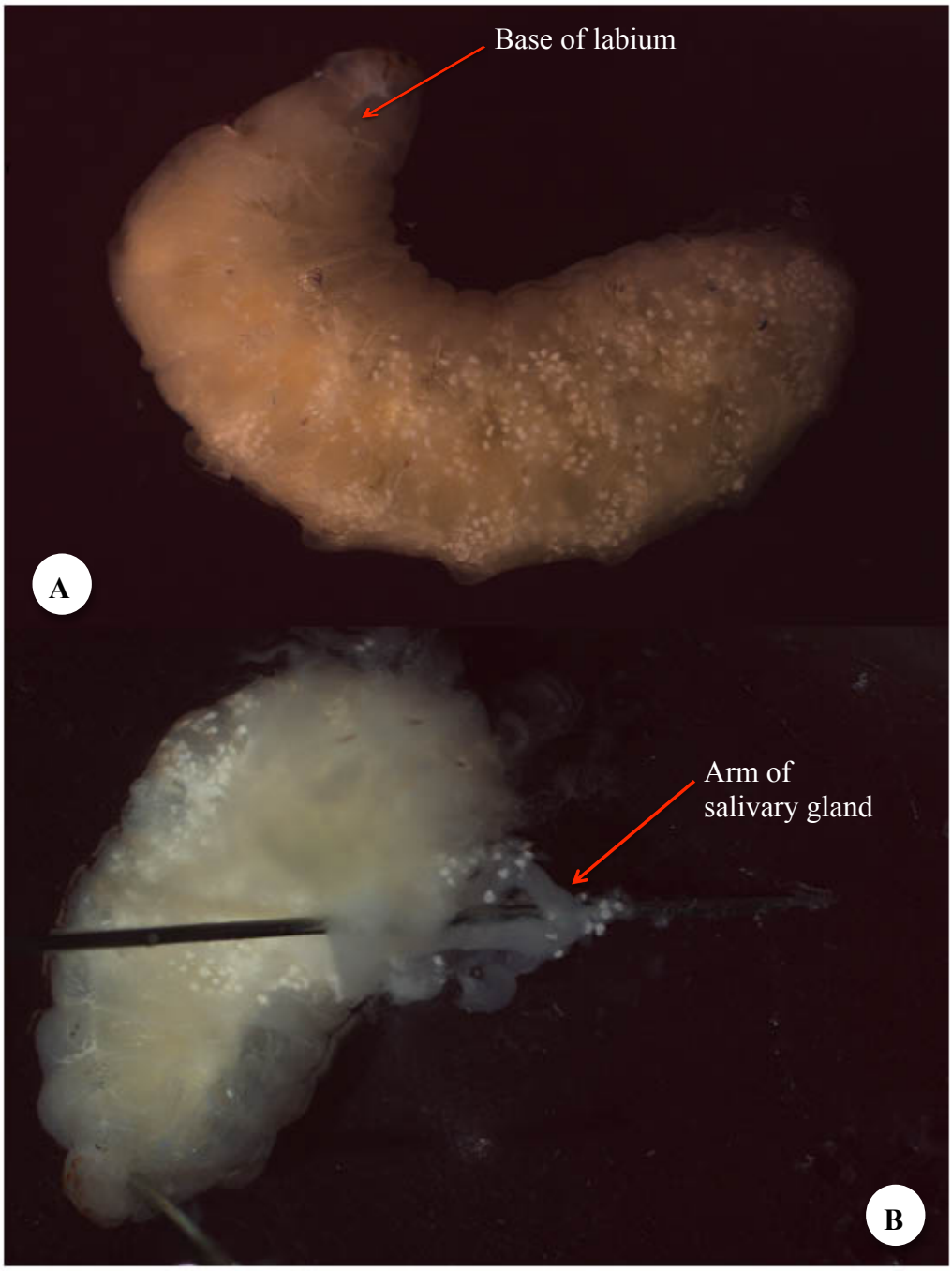

Fig. 5. (A) Late third instar larva of *T. nigriceps* with salivary glands before dissection. (B) Late third instar larva of *T. nigriceps* with salivary glands after dissection.

CHAPTER IV

SUMMARY AND CONCLUSIONS

It was confirmed that each developmental instar stage of *T. nigriceps* larvae possessed salivary gland structures. The presence of salivary gland structures within the early third instar developmental stage of *T. nigriceps* assumes the possibility that their utility provides for the capability of egression from chitinous host cuticle. This is practical considering the falcate mandibles of the early third instar larvae, although functionally adapted for their size, do not possess the mechanical strength required to tear through fibrous cuticle. Therefore, the salivary glands could provide the larvae a chemical means of excretion (salivary enzymes) for the digestion of host cuticle during the behavior of egression and later re-insertion.

The development of the salivary glands within the late first instar was not an expected find when reflecting upon the initial expectations at the beginning of the project. The presence of salivary glands within the earlier instars could indicate an added utility alongside egression, which was the utility previously discussed and first hypothesized. This could include aspects such as providing aid in the efficient digestion of host hemolymph. The reasoning behind the idea of additional utility stems from the fact that tissue generation requires a great amount of energy, which is a valuable and easily depleted resource. Typically, energy during development would only be expended if the

organism required the formation of tissues for use, preferably immediate. This is only an insight, and further research would have to be conducted.

The presence of the salivary glands within all three larval instars of *T. nigriceps* provides another step linked into further research regarding the biology of these parasitic Hymenoptera. Continuing effort should focus on the histology of the salivary gland tissues as well as the egression and re-insertion behavior exhibited by the third instar larvae of *T. nigriceps* with emphasis on the production of secretions. It is through the endeavor of future research that the true use of the salivary glands will be discovered and expanded upon.

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