POLYEMBRYONIC PROLIFERATION: FROM 2 TO 2000

by

SHIRA D. GORDON

(Under the Direction of Michael R. Strand)

ABSTRACT

Polyembryonic wasps are parasitoids that lay their eggs inside the bodies of other insects. These eggs then undergo a clonal phase of development that results in production of multiple genetically identical offspring. *Copidosoma floridanum* produces up to 3000 morula stage embryos that form an assemblage called a polymorula. Many aspects of *C. floridanum* development are well studied but comparatively little is known about the proliferation phase of embryogenesis. In the present studies, spatio-temporal patterns of mitosis are characterized using cell cycle markers during the proliferation phase. Proliferation involved both mitosis of embryonic cells and partitioning of morulae by the inner embryonic membrane. All embryonic cells incorporated the S-phase marker bromodeoxyuridine (BrdU) but mitosis varied spatially and temporally. Additional observations indicated that the polymorula becomes associated with the tracheal system of the host; potential recruitment of the host's tracheae to the parasitoid also is discussed.

INDEX WORDS: Polyembryony, Proliferation, Branchless, Trachea, Cell cycle

POLYEMBRYONIC PROLIFERATION: FROM 2 TO 2000

by

SHIRA D. GORDON

BA, The University of Colorado, Boulder, 2002

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2006

POLYEMBRYONIC PROLIFERATION: FROM 2 TO 2000

by

SHIRA D. GORDON

Major Professor: Michael R. Strand

Committee: Scott Dougan
Ken Ross

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2006

DEDICATION

I dedicate this work to the thousands of insects I have killed.

I hope for all that died, this thesis makes their death less ill-willed.

I would like to tell their story about polyembryonic proliferation.

Little is known about how the animals make their clonal nation.

We understand division, but who is dividing?

What controls the growth while they’re multiplying?

Are all the embryos equal?

Do all share the same fate?

Who produces sequels, when they proliferate?

Looking at growth as the polymorula expands,

how can it survive while left in the caterpillar’s hands?

Is there a signal that all embryos listen to?

Is this what helps the embryos decide what to do?

This thesis helps answer some,

But of course there is still more to be done.

ACKNOWLEDGEMENTS

I would like to thank all of the many people that helped me get here today. Most importantly, Mike Strand has given me guidance and insight on how to become a good scientist. I also appreciate his funding and supporting me through my years here. I would like to thank my committee for their support and encouragement that has helped me get through the degree. I would like to thank my parents and brother for listening to all of my “science talk.” Additionally, I would like to thank my father and brother for their late night editing help. I would like to thank my mom for being the emotional support whenever I need it. Finally, I would like to thank my boy friend, Ryan, and all of my friends for giving me support and good excuses to get out of the lab and have fun.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Chapter 1: *Copidosoma floridanum*: an Example of Polyembryonic Development

Introduction

Organismal biology aims to understand the "what," "how" and "why" of any specific organism. Understanding unique features of one organism may help explain related features for other organisms. This comparative concept is most readily used in developmental biology. To be able to understand and compare different organismal developmental processes, one needs to understand the evolutionary heritage between the two. However, while ancestry plays a critical role in how animals develop, thereby shaping the life history of the organism, environmental factors are also important for influencing the animal's characters (Wray, 2000).

Organisms such as endoparasitoids are good examples of how ancestry and environment interact to affect developmental traits. Endoparasitoids are insects that develop as internal parasites of other insects during their immature stages but are free-living as adults (Strand, 2000; Whitefield, 2003). Most insects are terrestrial and lay eggs packaged with a rich source of yolk that provides the nutrients necessary for the embryo to develop. Terrestrial insect eggs are usually surrounded by a rigid chorion that protects the embryo from desiccation. In contrast, many endoparasitoids lay eggs that lack yolk and that are surrounded by a very thin chorion. These alterations of parasitoid egg morphology likely arose because the developmental environment inside their arthropod host allowed for adaptations from terrestrial eggs. The host provides a

nutrient-rich, aquatic environment that physically protects the egg making the ancestral traits of a large yolk and strong chorion not needed, changing the requirements of the egg.

An extreme evolutionary alteration of parasitoid embryogenesis is polyembryony--a form of clonal development in which a single egg divides into two or more genetically identical offspring (Iwabuchi, 1991). While polyembryony loosely includes instances where occasional twins occur, the term is more commonly used with organisms that must produce multiple embryos to survive. Polyembryonic animals include some parasitic invertebrates (cestodes, trematodes and insects), some colonial and aquatic invertebrates (oligochaetes, bryzoans) and a few mammals (armadillos) (Strand and Grbic, 1997). Polyembryony has evolved independently at least five times within insects: once in the Strepsiptera (Ivanova-Kasas, 1972) and in four families of Hymenoptera (Braconidae, Platygasteridae, Encyrtidae and Dryinidae) (Strand and Grbic, 1997; Strand, 2003). The term “polyembryony” first came into use in 1898, by Marchal, in reference to parasitic Hymenoptera (Ivanova-Kasas, 1972). The most studied examples of polyembryony in the animal kingdom are in Hymenopteran wasps, particularly in *Copidosoma floridanum* (Patterson, 1921; Ivanova-Kasa, 1972; Ode and Strand, 1995; Grbic et al, 1996; Grbic et al, 1998; Harvey et al, 2000; Giron and Strand, 2004; Corley et al, 2005).

One of the largest polyembryonic Hymenopteran broods produced is in *C. floridanum* (Hymenoptera: Encyrtidae), producing up to 3,000 offspring per host (Ode and Strand, 1995). As the embryos clonally divide to produce the total brood, each embryo must retain the components needed to produce its own progeny. Therefore,

each embryo needs to receive a full complement of germ cells. Germ cells are the cells that become gametes, initially originating from germ line stem cells (Extavour and Akam, 2003). *C. floridanum* is a good system for understanding the basics of polyembryonic proliferation, which may lead to insights into germ line stem cells. The goal of this thesis is to better understand the proliferation stage of *C. floridanum* development.

Development of **Copidosoma floridanum**

Copidosoma floridanum is one of the most common and widely distributed species of Hymenoptera in the world (Guerrier and Noyes, 2005). It is an egg-larval parasitoid ovipositing into eggs of moths in the subfamily, Plusiinae. For these studies *C. floridanum* parasitized the host *Trichoplusia ni* (Fig 1.1A). Egg-larval parasitoids oviposit into the egg stage of a host arthropod with the offspring completing their development in the host's larval stage (Kaeslin et al, 2005). Like most Hymenoptera, *C. floridanum* is haplo-diploid, where fertilized eggs develop into females and unfertilized eggs become males. Thus, unmated females can only lay male eggs, which are haploid, while mated *C. floridanum* females can lay both male and female eggs dependent on the environmental conditions. Mated females oviposit one or two male or female eggs, based on the quality of the host egg (Ode and Strand, 1995). Quality is dependent on the age of host egg or if the egg has been previously parasitized.

C. floridanum starts its development inside the host egg. C*. floridanum* eggs contain no yolk and are surrounded by a thin chorion (Fig. 1.1B). In the first 24 hours after oviposition, the wasp egg undergoes complete (holoblastic) cleavage (Fig. 1.1C) to

form a single embryo with multiple cells. Some of these cells contain the germ plasm, initially prepackaged into the oocyte (Donnell et al., 2004). Unlike monoembryonic animals, the developing embryo ruptures out of its chorion therefore continuing development unconstrained. At this stage the embryo is called a “primary morula” and consists of approximately 200 embryonic cells surrounded by a multinucleated extraembryonic membrane of polar body origin (Fig. 1.1D) (Grbic et al, 1998). The syncytial extraembryonic membrane aids the developing embryo in avoiding encapsulation by the host (Corley and Strand, 2003). In addition, the extraembryonic membrane protects the developing brood from intra-specific competition in hyperparasitized hosts (Giron and Strand, 2004).

Development continues as the host hatches from its egg to a larval stage. About 48 hours after oviposition into the host, the primary morula begins to proliferate, splitting into genetically identical “secondary morulae,” which together make up “polymorulae” (Fig 1.1E & F). The enveloping membrane surrounds all secondary morulae. Each morula is an embryo made up of a solid ball of cells. This proliferation stage lasts approximately two weeks as the host molts through 5 instars. During the proliferation stage, two castes are formed. Only polyembryonic encyrtid wasps form two castes from the genetically identical egg. In one caste, a small number of embryos lack germ cells and begin morphogenesis prematurely, forming precocious larvae (Fig 1.1G) (Patterson, 1921; Ivanova-Kasas, 1972; Donnell et al, 2004). These larvae already are present starting in the host's first instar and continue to develop through the fourth instar. The numbers of these precocious larvae progressively increase as the host caterpillar ages, resulting in up to 200 precocious larvae being present when the host

molts to its final (fifth) instar. The role of the precocious larvae--sometimes called soldier larvae--is to defend the embryos in the other caste, the reproductive caste, from both inter- and intra-specific competition (Cruz, 1981; Grbic et al., 1992; Harvey et al., 2000; Giron et al, 2004).

The second caste, the reproductive caste, contains the remaining majority (>1000) of embryos. To help differentiate between the two castes during the proliferation stage, proliferating morulae can be detected by a marker against the germ cells, anti-Vas, which is absent in soldier morulae (Donnell et al, 2004). By the end of the host's fourth instar, embryos of the reproductive caste synchronously initiate morphogenesis (Grbic et al., 1996). On the second day of the fifth instar these embryos eclose to reproductive larvae (Fig. 1H). The larvae go through two instars: first living in the host's hemolymph obtaining oxygen through diffusion, and then consuming the entire interior of the caterpillar, breathing via their own trachea. The reproductive caste larvae pupate while remaining in the host's cuticle as the soldier caste larvae eventually die. Free-living adults emerge about two weeks later (Fig 1.1I).

A deeper look at the proliferation stage of development

An essential aspect of proliferation is obtaining oxygen. Most parasites obtain their oxygen through diffusion from the host's hemolymph or through their own feeding tubes (Fisher, 1963). By contrast, during the proliferation stage, polyembryonic morulae obtain their oxygen through the host's tracheal system, which supplies oxygen to the host's tissues. Encyrtid polyembryonic wasps have infiltrated the host's tracheal system uniquely obtaining oxygen directly from the host (Doutt, 1947; Ivanova-Kasas, 1972;

Baehrecke and Strand, 1990). The trachea is the only host tissue to stay in direct contact with the polymorula throughout development (Ivanova-Kasas, 1972). One hypothesis for the tracheal attachments to the polymorula is that the tracheae are attracted to the high levels of carbon dioxide in the area of the polymorula (Doutt, 1947). While there is a growing understanding of insect tracheal growth, little is actually known about the tracheal attachments to polyembryonic Encyrtids and the role(s) they may play in proliferation.

The development of *C. floridanum* embryos was well described in the early 1900s (Patterson, 1921). The core of each morula consists of embryonic cells (Fig 1.2). The embryonic cells (EC) are made up of both primordial germ cells and somatic cells and can vary greatly in number from 4 to 50 (Patterson, 1921; Donnell et al, 2004). Surrounding the embryonic cells is a layer of cells called the inner envelope (IE) (Fig 1.2). Together the embryonic cells and the inner envelope cells make up an individual morula. Surrounding all the morulae is an enveloping membrane of polar origin (EM) (Fig 1.2). The terminology describing this enveloping polar membrane is not consistent among all the descriptions; various names include trophamnion, involucrum, polar membrane and enveloping membrane. The term “enveloping membrane” will be used here. The enveloping membrane is the same membrane that encapsulates the 24 hour embryo mentioned earlier. The ploidy of the nuclei in this syncytial enveloping membrane increases throughout development (Baehrecke et. al, 1992). During the onset of morphogenesis, the enveloping membrane constricts the developing embryo, creating a new layer called the outer envelope (OE), isolating the embryos from the other morulae (Fig 1.2). During the period of proliferation, the formation of all morulae

results from a surrounding membrane invagination to the morula, subsequently partitioning the embryonic cells into new morulae (Fig 1.1F). The process of membrane invagination and cell partitioning repeats an indeterminate number of times during the first through fourth instars of the host caterpillar creating up to 3000 morulae (Ode and Strand, 1995).

There is some disagreement about which membrane--inner envelope or enveloping membrane--actually invaginates and partitions the embryonic cells. Early *Copidosoma* work suggests the inner membrane is responsible for the partitioning of a morula into new morulae (Patterson, 1921), whereas more recent studies suggest the morula partitioning is a result of the enveloping membrane (Grbic et al, 1996; Strand and Grbic, 1997; Grbic et al, 1998; Donnell et al, 2004; Extravour, 2004; Corley et al, 2005). Some of the confusion may be attributed to the difference in the terminology and descriptions of the polymorula layers. Recent descriptions do not mention either the outer envelope (Baehrecke and Strand, 1990) or the inner envelope (Baehrecke et al, 1992).

Specific Aims

Polyembryony is a very unique form of development that has inspired many studies (Patterson, 1921; Doutt, 1947; Ivanova-Kasas, 1972; Cruz, 1981; Strand, 1989; Baehrecke et al, 1992; Ode and Strand, 1995; Grbic et al, 1998; Giron et al, 2004; Corley et al, 2005). Studies focus on various aspects of polyembryony especially in *Copidosoma*, such as the life cycle of *C. floridanum* (Baehrecke et al., 1992; Strand and Grbic, 1997; Giron and Strand, 2004), the genes that regulate morphogenesis of

individual embryos into larvae (Baehrecke et al., 1993; Grbic et al., 1996; 1998, Donnell et al, 2004) and the functions of the two castes (Cruz, 1981; Grbic et al., 1992; Harvey et al., 2000; Giron et al., 2004). However, there are some major unknown areas in the proliferation of the morulae. First of all, while studies explain the actual partitioning of the embryos (Patterson, 1921; Baehrecke and Strand, 1990, Grbic et al., 1998), little is known whether each morula has equal participation in proliferation or if a subset of morulae participate more. Research suggests that proliferative morulae are capable of producing a continuous range of clone morulae from only one to many morulae, dependent on both the host's age and to a greater extent the parasite's age (Corley et al, 2005). The older the parasite morula, the less it will proliferate. However, the cell cycle stages of the morulae are unknown. Commercially available markers for mitosis and DNA synthesis can identify cell cycle stage. Another area needing clarification is the confusion in the literature of which layer is partitioning the morulae: the inner envelope or the enveloping membrane. Finally, while references have been made to tracheal attachments from the host to the polymorulae, there are minimal descriptions about the role(s) the trachea may play in controlling polymorula proliferation.

The specific aims are to:

1. To identify patterns involved in morula proliferation characterizing the cellular activity of the cells in the morulae through mitosis and DNA synthesis;
2. To decipher which membrane is responsible for morula partitioning, thereby clarifying the literature; and
3. To characterize the host's tracheal associations to the polymorula.

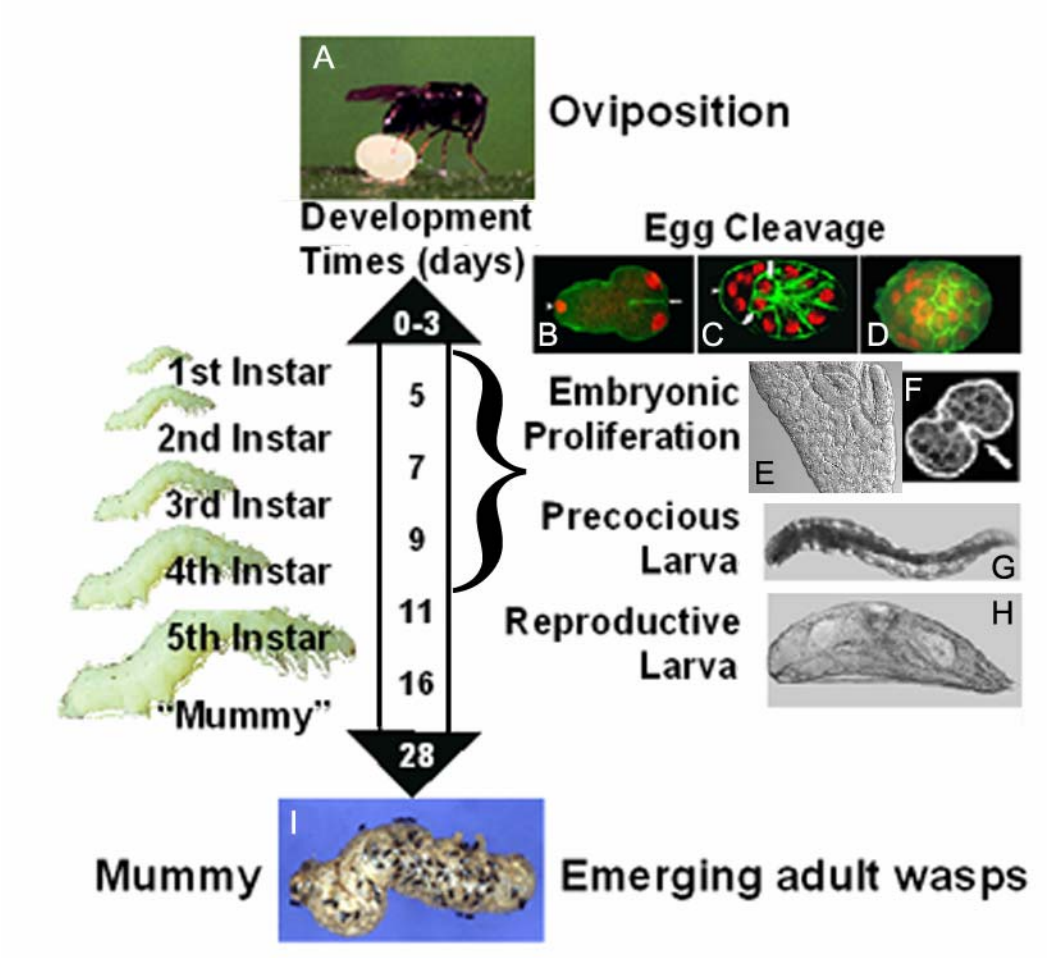


Figure 1.1 Development of *Copidosoma floridanum* in its host *Trichoplusia ni*.

The parasite's stages (right) correlate with the host's stages (left) over approximately 28 days (indicated center). The initial *C. floridanum* egg is laid into the egg of the host, *Trichoplusia ni* (A). During the *T. ni*'s egg development, the *C. floridanum* egg begins development (B,C), reaching a primary morula stage within 24 hours (D). After the host egg hatches (day 3), proliferation continues producing a polymorula (E) made up of many morulae formed from membrane invagination (F). A precocious, soldier caste ecloses throughout embryonic proliferation (G). The reproductive caste ecloses during the host's 5th instar, approximately the 12th day. Reproductive larvae consume the caterpillar and pupate in the remaining cuticle, emerging about 28 days after initial oviposition.

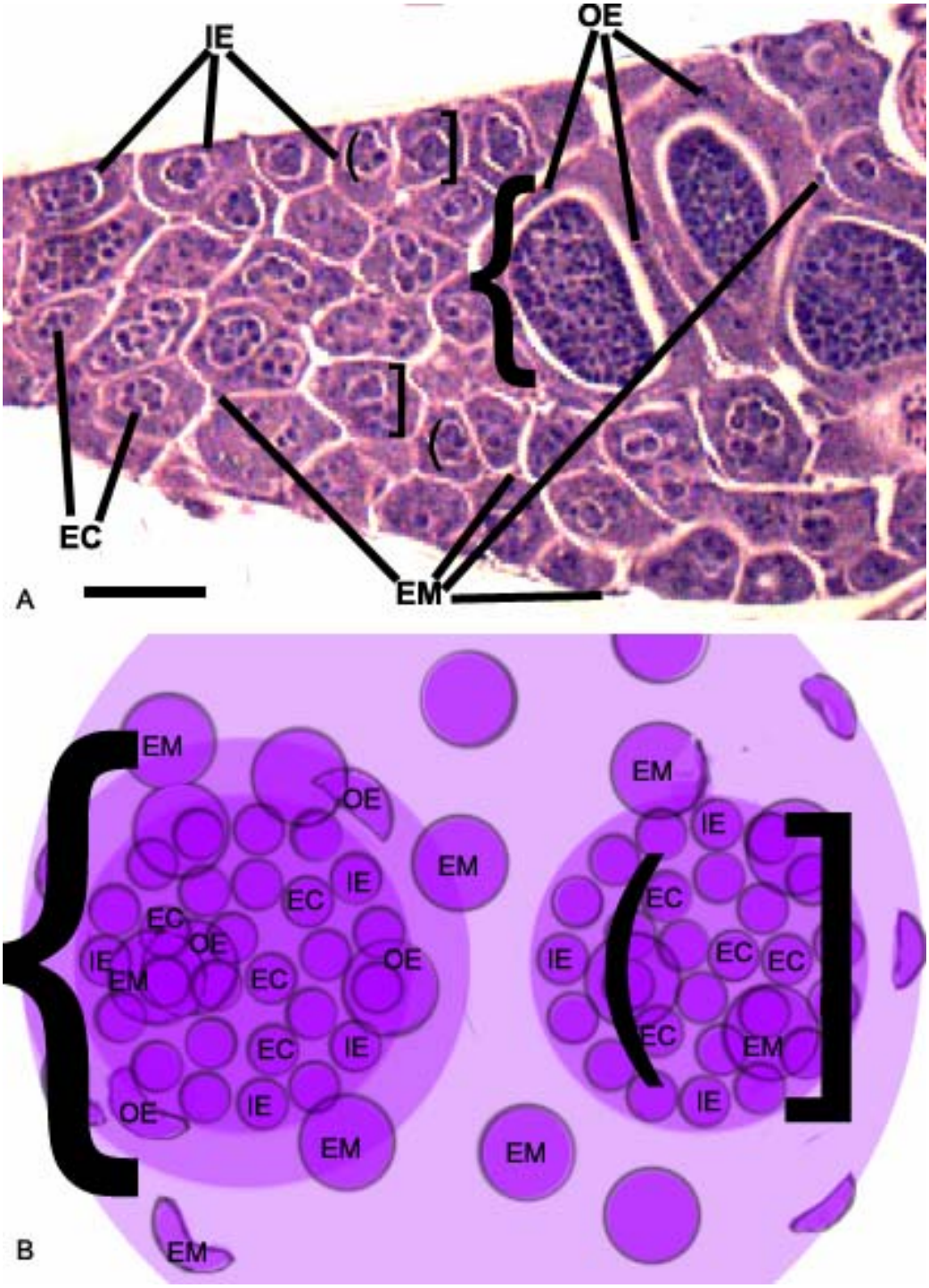


Figure 1.2 Understanding The Morula Environment

A hematoxylin-eosin stained section (A) and a model (B) of a polymorula. Open parenthesis indicates the core of a morula, closed bracket indicates a proliferative stage morula and open brace indicates a morphogenic morula. The nuclei associated with each layer are labeled: EC for Embryonic Cell, IE for Inner Envelope, OE for Outer Envelope and EM for Enveloping Membrane. Scale Bar equals 80 μm

Chapter 2: Characterization of cell cycle patterns in embryonic proliferation polyembryonic wasps *C. floridanum*

Introduction

Descriptions of polyembryony in parasitic wasps began around the turn of the century, first by Marchal in 1889 (Ivanova-Kasas, 1972). One species of polyembryonic wasps, C. floridanum, has been reported to produce broods of up to 3,000 individuals (Ode and Strand, 1995). A recent study suggests that all *S. floridanum* morulae do not proliferate equally (Corley et al, 2005). This was determined by injecting single morulae into unparasitized hosts and allowing the morulae to proliferate. The range of morulae produced from the initially injected morula ranged from only one to over 100. While these data suggest morulae are not equal in their proliferative potential, spatio-temporal patterns of proliferation within a polymorula are unknown. The main aim of this study is to characterize patterns of morulae proliferation as measured by cell cycle stages (M-phase and S-phase).

Morulae proliferate through membrane invagination and subsequent partitioning of embryonic cells. However, there is some confusion whether the inner envelope or the enveloping membrane partition the morula (Patterson, 1921; Grbic et al, 1996; Strand and Grbic, 1997; Grbic et al, 1998; Donnell et al, 2004; Extravour, 2004; Corley et al, 2005). Accordingly, a second aim of these studies is to resolve this confusion by describing the cellular environment of the morulae.

Polymorulae are attached to the host's trachea. The tracheal attachments could very likely play a large role in mediating proliferation. For example, morulae always grow in a sheet only a few morulae thick, which may facilitate tracheal attachment. The final aim of this study is to better understand the host's tracheal attachments to the polymorulae by descriptive studies of development. This aim will be addressed in Chapter 3.

Material & Methods

Insect rearing

Copidosoma floridanum was reared in *Trichoplusia ni* as described by Strand (1989). Newly emerged, mated adult wasps parasitized fresh, host eggs the majority of which were less then 24 h old. Parasitized host larvae were transferred to 30 ml plastic cups with paper lids and fed an artificial diet. The animals lived at 27 ± 1°C and a 16 h light-8 h dark photoperiod regime.

Dissection and dissociation

Parasitized *T. ni* were physiologically staged and polymorula were collected at specific times during the first-fifth instars as previously described (Baehrecke et al., 1993). Once reaching the proper stage, host larvae were dissected along the dorsal midline in phosphate-buffered saline (PBS). Wasp embryos were then collected and fixed in 4% paraformaldehyde in PBS. In some cases, embryos were disassociated prior to fixing in 0.25% trypsin in water (Sigma) for 15 min. After rinsing in PBS, samples were either used immediately or stored in methanol at –20°C.

Paraffin sections

To create sections of proliferating polymorulae, whole second and third instar parasitized caterpillars were fixed in formalin and sent to the University of Georgia Histology Laboratory for embedment in paraffin. Sections were cut 7 μM thick and placed on glass slides. The laboratory sent back one section stained with hematoxylin-eosin. The slides were deparaffinized by washing two times in xylenes for 5 min. The slides were rinsed in 100% ethanol, followed by rehydration in 70% ethanol and deionized water. To break aldehyde bonds, the slides were treated with 0.25% trypsin in water (Sigma) for 10 min.

Immunocytochemistry

Immediately prior to the initiation of mitosis, serine 10 (ser10) of histone H3 is phosphorylated throughout the condensing chromatin (Hendzel et al., 1997). This phosphorylation event is required for proper chromosome condensation and is highly conserved across the metazoa. As a result, antibodies generated against an H3 oligopeptide phosphorylated at ser10 recognize M-phase cells in organisms ranging from *Tetrahymena* to mammals. Similarly antibodies against histone H1 can be used to label all nuclei. M-phase cells in polymorulae were visualized by incubating fixed embryos in anti-H3 (upstate) and all nuclei were visualized by anti-H1 (Santa Cruz). Germ line cells in embryos were distinguished from somatic cells using an antibody to the germ cell marker Vasa (Donnell et al., 2004). Samples stored in methanol (MeOH), were rehydrated in a graded series MeOH/PBS (75%, 50%, 25% MeOH). Samples were then washed in PBS and permeablized in 0.2% PBS-Tween (PBT) followed by

blocking with 1.0% bovine serum albumen (BSA) in PBT. Samples were then incubated with each primary antibody diluted 1:200 in BSA for 4h (at RT) to overnight (at 4°C). After rinsing several times in PBT, primary antibodies labeling was visualized using goat anti mouse or goat anti rabbit secondary antibodies, conjugated to Texas red or FITC (Jackson Labgs). Secondary antibodies were applied at a 1:1000 ratio for 2hr (at RT) to overnight (at 4°C).

Bromodeoxyuridine (BrdU) labeling

S-phase cells were identified by the thymidine analog, bromodeoxyuridine (BrdU). As cells duplicate their DNA, BrdU is incorporated into the DNA, in place of thymidine. The BrdU is then diluted in daughter cells as chromosomes replicate and segregate. After 4 cycles of the cell cycle the signal becomes undetectable (Lauderdal and Mastick, personal communication). Anti-BrdU antibodies bind the DNA in the nuclei and the fluorescent tags are excited by fluorescence. Two methods were used to deliver BrdU to the hosts: feeding and injecting. For feeding, hosts were fed a diet of 2.0mg/ml BrdU from the beginning of the instar of choice. For injections, the hosts were injected with 2.5 μL of 2.0 mg/ml per g of the host's body weight. After the desired time elapsed, the animals were dissected.

Samples processed for BrdU were denatured by a 20 min incubation with 3 N HCl, then neutralized with sodium borate for 3 min. The processing then followed the same steps as histone labeling using a mouse anti-BrdU primary antibody (Biomedia) at a 1:1000 ratio. Methods for double labeling BrdU and histone H3 were similar to Newmark and Sanchez Alvarado (2000) with the primary anti-H3P antibody applied first.

Samples were then rinsed in PBS for at least 8 hours and fixed with 4% paraformaldehyde in PBS for 15 minutes. BrdU processing followed as described above except both secondary antibodies were added simultaneously. To label samples for both BrdU and histone H1, the samples were processed sequentially, starting with anti-BrdU.

Phalloidin labeling

To visualize the actin filaments between cells, fixed samples were labeled with phalloid. 5 µL of Alexa Fluor 488 Phalloidin (Molecular Probes) were left to air dry and diluted with 1000 µL of PBS. Samples were incubated with phalloid at 4°C for overnight.

Microscopy

All samples were mounted in 50% glycerol-PBS and imaged using a Leica TCS confocal microscope filtered with Nomarski optics.

Results

Histone H3 revealed proliferation of morulae was not synchronous

Morulae within a polymorula did not go through mitosis in a synchronous fashion from the first through fourth host instars. This was determined by labeling polymorulae with a mitosis marker, histone H3, which identified mitosis at the moment of fixation. Individual morulae could be divided into three groups of mitotic activity: synchronously mitotic (SM) morulae, occasionally mitotic (OM) morulae and no mitotic activity (NMA)

morulae (Fig 2.1). A morula that was considered *synchronously mitotic (SM)* had all its inner envelope cells going through mitosis at the same time (Fig 2.1C). An *occasionally mitotic (OM)* morula contained anywhere from one cell to fewer than all the cells in mitosis (Fig 2.1D). A morula that contained *no mitotic activity (NMA)* had no cells undergoing mitosis (Fig 2.1E). A limitation of measuring M-phase with histone H3 was that anti-H3P indicates the mitotic state only at the time of fixation with no reference to an earlier or later time.

Polymorulae collected at different times of the hosts first-fourth instars usually contained all three types of morulae. However, most polymorulae were made up of more than 50% OM morulae and only a small percentage of SM and NMA morulae. On average, SM morulae comprised about 5.4% of the polymorula. Some polymorulae were composed entirely of OM morulae, fewer polymorulae consisted of only NMA morulae. Polymorulae never consisted of only SM morulae.

SM morulae were used to study the mitotic stages of morulae because in SM morulae, there was no confusion as to the cellular state of all the inner envelope cells. For example in both OM and NMA morulae the non-mitotically active cells could be resting in a G-phase or could be duplicating DNA in S-phase. Also, SM morulae have a strong, positive signal using antibody labeling which was easy to quantify. For the rest of the mitotic studies, proliferation analyses were based on the frequency and distribution of SM morulae.

Understanding the Synchronously Mitotic (SM) Morulae

In SM morula all the inner envelope cells underwent mitosis at the same time (Fig 2.2A). The distinction that only the inner envelope cells, and not the embryonic cells, were the mitotically active cells is critical. Initially, optical sections using confocal microscopy revealed the mitotic state, via fluorescent labeling of histone H3, of the cells in the morulae. Only cells on the periphery of the morula, inner envelope cells, expressed the mitotic label while the embryonic cells in the center of the morula remained unlabeled (Fig 2.2B). Histone H1 is a nuclear marker that should label all nuclei, however, the signal from the constitutive histone H1, was extremely weak and in some cases not detectable. The variability of the H1 signal, suggests the lack of an H3 label in the embryonic cells potentially could have be an artifact. To test whether the SM morulae really lacked mitosis signal in the embryonic cells or if the labeling technique was constrained by inadequate permeablization, samples were embedded in paraffin and cut into sections 7µM thick. Sections of over 10 SM morulae verified that SM morulae only had a mitotic H3 label in the inner envelope cells (Fig 2.2C) and did not have any mitotic activity in the embryonic cells, as seen by double labeling with H1 (Fig 2.2D).

Spatial patterns of proliferation

SM morulae were more likely to have clustered together and remain contiguous with each other than be isolated. For example, in Fig 2.1B, one pool of SM morulae clustered in a group of 5 and another in a group of 2. The SM morulae for all polymorulae collected were analyzed to determine if there was any significance in their

location within a single polymorula. The observed frequency distribution of SM morulae examined differed significantly from an expected frequency for the Poisson distribution (X^2 = 74.3; d.f.=6; $p<0.01$) indicating that the SM morulae were non-randomly distributed within polymorulae. Also, the calculated coefficient of dispersion for these data was greater than 1, again indicating that SM morulae have a clustered distribution in polymorulae.

In support of the SM morulae distribution data, pulse BrdU incubations showed morulae in S-phase occurred in distinct regions (Fig 2.3A). Regions of BrdU incorporation indicated morulae in the same region went through S-phase at a similar time, compared with those morulae that contained no label. Because the BrdU label stays with cells for a few rounds of the cell cycle, the regions of BrdU morulae could have indicated a single morula was producing all the surrounding morulae; however, BrdU labeled morulae were not always contiguous (Fig 2.3B), implying multiple morulae can be in S-phase at the same time. Both the histone H3 data and the BrdU data showed no consistent spatial relationship to the center or the sides of the polymorula, nor any relationships to the positioning next to morulae undergoing precocious morphogenesis (data not shown).

Temporal patterns of proliferation

Polymorulae sampled throughout each instar contained morulae in all three of the mitotic groups with slight variations. Within an instar the percentages of SM morulae were assessed over three time windows: early (0-10 hrs), intermediate--end of feeding through beginning of apolysis (12-30 hrs), and late (40 hrs-molt) (Table 2.2). The

average percentage of SM morulae was pooled for each time window because the tendency of SM morulae to cluster would not be evenly distributed in fragmented polymorulae inside the host. At least five animals were used for each time window, with the exception of the first time window in the first instar.

The percentage of SM morulae progressively increased with later instars (Table 2.1). In the first instar average SM morulae stayed relatively constant at 3.3%. In the second instar the average percentage SM morulae rose to 5.0%, however was highest at the beginning of the instar (6.0%) and lowest at the end of the instar (4.2%). The average percentage SM morulae rose again for the third instar to 6.2%. In this instar the middle time window had the largest percentage SM morulae, 9.5%. The average percentage of SM morulae in the fourth instar was not calculated over the whole instar because in the last time window of the instar embryos synchronously begin morphogenesis (Grbic et, al., 1998). For the first two time windows of the fourth instar, the average percentage of SM morulae rose again to 7.1% but dropped significantly in the last portion to 3.3%. This drop is due to the beginning of the onset of morphogenesis of the reproductive caste. During the fifth instar the SM morulae were very rare representing less than 0.01%. The fifth instar SM morulae were very small, younger embryos that were most likely slower to develop.

Within an instar, all cells in the polymorula went through S-phase.

A thymidine analog, BrdU, was permanently incorporated into the DNA, labeling S-phase, to understand if all morulae proliferated at least once within a host's instar (Truman and Bate, 1988; Ladurner et al., 2000). Feeding a diet containing 2.0 mg/ml

BrdU to the hosts, is a way to continually label cells in S-phase. At the end of each of the first three instars, anti-BrdU was detected in all the cells of all the morulae (Fig 2.4 A-C). Double labeling the polymorulae with anti-H1P showed the signal was easily observed in the enveloping membrane, outer envelope and inner envelope nuclei (Fig 2.4D). The signal was much fainter in the embryonic cells. To be sure the embryonic cells were going through S-phase, morulae were disassociated and double labeled for anti-BrdU and anti-Vas. Vas is present in the embryonic cells and so is a good marker for the inner core of morulae (Donnell et al, 2004). Double labeling with anti-Vas and anti-BrdU revealed the embryonic cells incorporated BrdU, and therefore went through S-phase too (Fig 2.4E). Feeding BrdU to the caterpillars for only the first half the instar, 24 hours, still exhibited most of the morulae with incorporated BrdU (Data not shown).

Injections of BrdU exhibit many cells in S-phase at any given moment.

Injections of BrdU over short time intervals revealed BrdU incorporated into cells quickly, therefore cells spent long portions of time in S-phase. BrdU feeding experiments showed that most the morulae went through S-phase at least once over 24 hours, however, gave no reference to when only some morulae were in S-phase. Injections of 2.5 μL of 2.0 mg/ml per g of the host's body weight exposed the developing polymorulae to a one time exposure of BrdU, which inevitably was depleted over time. Once the BrdU is depleted the cells with BrdU only can divide about 4 times before the signal is diminished (Lauderdale and Mastick, personal communication). BrdU injections under 7 hours revealed regions of incorporation and non-incorporation (Fig 2.3). To identify when only a few morulae, not large regions, were in S-phase, hosts

were injected with BrdU and dissected/fixed in less then half an hour. However, these injection data revealed high levels of BrdU incorporation in the inner envelope cells of many morulae (Fig 2.5). With so many cells incorporating BrdU so quickly, cells must spend a large portion of time in S-phase. This quick incorporation period of BrdU also has been described in human lymphoblast cells, which can incorporate BrdU after only 6 minutes of exposure (Gratzner, 1982).

Waiting 24 hours after injections yielded variable results

BrdU injections, followed by a 24-hour waiting period resulted in a range of incorporation of the polymorulae from a strong signal to a weak signal or practically no signal with some cases a mixture of all three. A weak BrdU signal implied multiple rounds of the cell division occurred without the presence of free BrdU and the cell's signal was slowly being diluted between daughter cells. Feeding experiments exhibited most cells went through S-phase at least once in 24 hours, so cells with no signal means either the cells went through S-phase after the free BrdU was depleted or the cells duplicated so many times that the BrdU was no longer detectable. The data cannot clearly distinguish which of the two scenarios is correct. Interestingly, in polymorulae that showed practically no signal, the enveloping membrane nuclei often contained a signal (Fig 2.6). Cells with a strong signal went through S-phase phase at least once in the 24 hours but did not go through many rounds of the cell cycle. Studies testing the length of time to go from S-phase to M-phase may help answer the question of what the morulae did over a longer time.

The minimum transition time for morulae to go from S-phase to M-phase is 4 hours, but many morulae take more time

The length of time cells take to go from S-phase to M-phase was variable. A common method for determining the length of the cell cycle is to pulse cells with BrdU, wait different lengths of time and then label against anti-H3P and anti-BrdU, (Newmark and Sanchez Alvarado, 2000). After 24 hours of feeding on a BrdU diet, morulae were double labeled (data not shown). These data indicated that morulae can go from S-phase to M-phase in less then 24 hours, but did not identify the minimal length of time needed for morulae to transition from S-phase to M-phase. To resolve shorter time windows, third and fourth instar hosts were one time injected and dissected incrementally later. BrdU injections under 4 hours revealed no double labeling of S- and M-phase (Fig 2.7A). Single BrdU injections, followed by a waiting period of over 8 hours contained some morulae double labeled (Fig 2.7B). In the third instar, after waiting 8 hours after the pulse of BrdU, only 10.1% of the morulae were labeled. However, after waiting 19.5 hours, the percentage peaked to 53.4%, but went down to 11.6% at 25 hours. The reduced percentage of double-labeled morulae in the third instar suggests that the signal was diminished. The range in time for double labeling during the third instar suggests that while some morulae can go through S- to M-phase in 8 hours, most morulae cycled more slowly peaking close to 20 hours after injection. In contrast, during the fourth instar, more morulae cycle much more quickly reaching close to 50% after only 8 hours of injection. These results suggest the cell cycle changes among instars and more morulae are participating in proliferation during the fourth instar.

SM Morulae May Not Be a Signal of Proliferation

In SM morula, all the inner embryonic cells were duplicating, and therefore the morula was doubling in size, potentially indicating one stage of morula proliferation. However, the morulae from the second half of the host's fourth instar were in the early stages of morphogenesis and should have stopped proliferation and so should not be SM morulae. Nonetheless, some fourth instar SM morulae were going through the early stages of morphogenesis, embryo compaction (Fig 2.8A-C) (Grbic et al, 1998). To assess whether SM morulae were an early signal of morphogenesis, third instar proliferating morulae were double-labeled for anti-histone H3P and anti-Vas. The Vas signal was present in the third instar SM morulae (Fig 2.8D & E). Since Vas is only present in the reproductive caste, an SM morula was not a precursor to morphogenesis. Interestingly, cells positive against both Vas and H3 were not located in SM morulae (Fig 2.8F). An additional reason why SM morulae are not a precursor to morphogenesis is because there was no large increase of SM morulae when morphogenesis synchronously begins in the reproductive morulae during the fourth instar. Under careful examination, embryos in the fourth instar embryo compaction stage sometimes partitioned into two new embryos, and therefore were still proliferating (Fig 2.8I & J).

SM morulae did not indicate morulae that are currently partitioning

SM morulae did not indicate morulae that partitioned into new morulae. While most SM morulae were completely round (Fig 2.9A), some SM morulae exhibited signs of morula partitioning (Fig 2.9B1-7). SM morulae could have included the partitioning stage as well as either (or both) before or after partitioning of the morulae. However,

many partitioning morulae existed as OM or NMA morulae (Fig 2.9B5-12, B13) so SM morulae cannot be correlated with encompassing part or all steps of morulae partitioning. Likewise, isolation and partitioning of the embryonic cells was independent of if morulae were SM morulae (Fig 2.9C & D).

Discussion

Proliferation is not synchronous in a polymorula

The main aim of this study--identifying patterns in proliferative morulae--depends on the assumption that morulae proliferation does not occur synchronously among all morulae. This assumption stems from studies of *Copidosoma* that recognize the morulae do not all partition to new morulae at the same moment (Patterson, 1921; Grbic et al., 1996; Grbic et al., 1998). This study shows morulae proliferation is not synchronous. The mitotic state morulae falls in one of 3 groups (SM, OM, NMA) (Fig 2.2). Similarly, morulae do not go through S-phase at the same time but incorporate BrdU in groups of morulae (Fig 2.3, 2.5). Together, these data highlight an asynchrony among morulae proliferation. However, combined all these studies do not resolve if over time all the morulae are participating in morulae production equally. A recent study suggests some morulae have a higher proliferative capacity than others (Corley et al, 2005). In these experiments individual morulae were injected into new hosts to determine how many progeny can result from a single morula. Some morulae were able to produce large numbers of progeny while others did not proliferate and therefore only gave rise to single larva.

Proliferation occurs by regions

Observing S-phase and M-phase of the cell cycle reveals that a polymorula proliferates heterogeneously by regions (Fig 2.1, 2.3, 2.5, 2.6). The molecular mechanism dictating the regional proliferation is not known, but the synchronous clusters suggest a local signal diffusing between the morula. These regional differences may be responsible for determining precocious larvae, which is variable between hosts, dependent on host conditions (Harvey et al., 2000).

All regions of a polymorula take part in proliferation

Since monitorig M-phase cells with immunocytochemistry can only mark the cells at the moment of fixation, BrdU is used to monitor cells involved in S-phase over a window of time. In each of the proliferative instars, all the cells go through S-phase at least once (Fig 2.4). These data suggest all morulae aid some in proliferation, because all cells are duplicating their DNA. While the DNA could be duplicated to supply a soon-to-be morphogenesis caste morulae with more genetic information, too many morulae quickly incorporate BrdU to become soldiers (Fig 2.5). Because proliferative morulae do not range greatly in size (Baehrecke and Strand, 1990), DNA synthesis is indicative of a proliferation event that will occur.

The rate of proliferation changes over time

As the polymorula progresses to the fourth instar, a greater percentage of morulae take part in proliferation (Table 2.2). The number of SM morulae in the last time interval of the fourth instar drops dramatically because most proliferation ceases and

morphogenesis begins (Grbic et al, 1998). Within an instar, prolferation is not constant (Table 2.1). Cell cycling variability of the morulae can result from the changing environment within a single instar. Studies show certain stages of *C. floridanum* development are synchronized with the developmental stages of the host (Strand 1989). For example, a caterpillar eats during the first half of the instar and wanders during the second half preparing to molt. Also, the host's ecdysteroid levels influence the reproductive caste to begin morphogenesis (Baehrecke et al., 1993).

The time it takes for morulae to transition from S-phase to M-phase varies among instars. Pulse experiments of BrdU estimate 8 hours are needed for the minimum transition time from S-phase to entry into M-phase. However, the time needed for 50% of the morulae to be double labeled by BrdU and anti-H3 is not constant. In the third instar, 50% of the morulae are double labeled after 19.5 hours, while 50% of morulae from the fourth instar hosts are labeled after 8 hours. The fourth instar became 50% double-labeled morula more quickly, implying a higher percentage of morulae are taking part in proliferation sooner. These data fit with the percentage of SM morulae data (Table 2.2). In contrast, injection experiments of single morulae suggest morulae in the fourth instar cannot proliferate to as many morulae as those in the third instar (Corley et al, 2004). Together, these data suggest more morulae in the fourth instar are proliferating but the capacity of daughter morulae per morula is lower. This quick, large increase of morulae during the fourth instar may be due to a brief increased level of participation among the morulae. One reason why the injection studies of a fourth instar morula only resulted in one morula, not reflecting the higher level of morula participation in proliferation, is because the injections only used morulae from the second day of the

fourth instar (Corley et al., 2004), which is when proliferation is already greatly reduced (Table 2.2). If the early and middle were tested, one would expect more larvae to result compared with the late time window. Injections of earlier instar morulae resulted in many more larvae, these results may be because earlier instars contain smaller pools if morulae proliferating more actively. For example, at 8 hours after BrdU injection 10.1% of the morulae transitioned from S-phase to M-phase. This number is greater than the average number of SM morulae in the middle time window of the instar. By 19.5 hours after BrdU injection over 50% showed the transition, but 5.5 hours later the signal was gone for the cycling morulae, indicating a fast turnover in a small population of morulae.

Morula proliferation does not stop during the early stages of morphogenesis--embryo compaction

Based on the literature, synchronous morphogenesis of the reproductive caste morulae begins during the second half of the fourth instar (Grbic et al., 1998). While, the percentage of SM morulae does drop significantly during the last part of the fourth instar (Table 2.1), the initial stages of morphogenesis are still capable of proliferating (Fig 2.8). Contrary to published literature while most morulae do begin morphogenesis synchronously, notably some, 3.3%, do not (Baehrecke et al., 1993; Grbic et al., 1997; Grbic et al., 1998).

Two very distinct cell types in morulae confirm that inner envelope cells are responsible for partitioning morulae

Using the mitosis marker, histone H3, two very different pools of cells in the morulae become clear: inner envelope cells in the periphery of each morula and embryonic cells at the core (Fig 2.2, 2.8D-H). SM morulae highlight the fact that the inner envelope cells often go through mitosis at the same time, which is at a different time than the embryonic cells. Early description studies mention these two layers based only on morphological observations (Patterson, 1921). However, later work does not clearly address the inner envelope and outer envelope layers (Baehrecke and Strand, 1990; Baehrecke et al., 1992). A greater confusion in the literature occurs when trying to explain which layer invaginates to partition the morulae. Initial studies describing morulae proliferation suggest the inner envelope is responsible for morulae partitioning. By contrast, later studies explain the partitioning is a result of the enveloping membrane (Grbic et al, 1996; Strand and Grbic, 1997; Grbic et al, 1998; Donnell et al, 2004; Extravour, 2004; Corley et al, 2005). The focus of these recent studies had little if anything to do with morulae partitioning, yet they are not consistent with the studies from the turn of the century.

The mitotic analysis shows the layers indeed have different functions. The data in this study support the morulae partitioning as a result of the inner envelope. Morula partitioning first starts with embryonic cell segregation (Fig 2.9C & D; 2.10-Dashed circle) (Patterson, 1921; Donnell et al., 2004). Next, the inner envelope cells migrate between the embryonic cells, eventually splitting the morula into two (Fig 2.9B, 2.10-Circle). The enveloping membrane follows the splitting of the morulae (Fig 2.10-

Square) (Patterson, 1921). If the enveloping membrane were responsible for morulae partitioning one would expect to see the enveloping membrane to have corresponding cellular activities with morulae, in addition to invaginating morulae before the inner envelope cells surround groups of embryonic cells.

SM morulae do not directly correlate to morula partioning

Morula partitioning is independent of the SM morulae (Fig 2.9). The expectation is that by synchronously doubling the cells of a morula, the morula is going to divide. However, morula partitioning occurs in morulae from all three mitoic groups: SM, OM and NMA morulae (Fig 2.9). An SM morula indicates all the inner envelope cells are going through mitosis, creating twice as many inner envelope cells. However, during the proliferative stage, the morula diameter can range from 30μM to 75μM while the cell diameter stays approximately the same size, 4-5μm (Baehrecke and Strand, 1990). One explanation for the change in morula size is that a partitioning morula can divide into a round morula and a morula that is getting ready to partition and divide again (Fig 2.10-Dashed square) (Patterson, 1921).

Actively partitioning morulae being either SM, OM or NMA morulae can have a couple of explanations. In one explanation the mitotic state of the inner envelope may be uncorrelated to morulae partitioning so that the frequency of which mitotic type of morula (SM, OM or NMA) involved in partitioning is completely independent. In an alternative explanation, partitioning morulae may not have enough inner envelope cells for a complete partitioning event and therefore need to double the cell number during partitioning. In a final explanation, the partitioning SM morulae may be getting ready to

partition again soon after the first partitioning is complete, therefore needing twice as many cells. The latter two explanations suggest the inner envelope responds to the embryonic cells' needs. Since transitioning from S-phase to M-phase takes at least 4 hours either the process of partitioning is very slow or the inner envelope cells have an altered cell cycle. To resolve these hypotheses, it would be helpful to confirm what drives proliferation.

A modification of the cell cycle to suit the needs of a cell is not a new idea. The traditional sequence of the cell cycle is Growth phase (G1) to DNA Synthesis phase (S) to a Growth phase (G2) and to Mitosis phase (M) (Lee and Orr-Weaver, 2003). The cell grows during the two growth stages, G1 and G2; during S phase the DNA duplicates so that the cell will be able to allocate equal copies of DNA during mitosis, when the nucleus actually splits. The length of G1 and G2 phases affects growth and ultimate cell size. However, cells can modify and vary the elements included in the cell cycle enabling cells to account for specialized needs. For example, in embryos from organisms that go through a rapid embryogenesis, such as insects, amphibians, and marine invertebrates, G1 and G2 phases are initially completely skipped and the cell cycles from S-M-S-M multiple times, utilizing the maternal stockpiles deposited during oogenesis (Lee and Orr-Weaver, 2003). Another example of an altered cell cycle is in cells that are polyploid. These metabolically active cells have more than one set of DNA per cell, focusing on synthesizing larger quantities of specific products (Smith and Orr-Weaver, 1991). There are several of ways cells become polyploid, including, blocked cytokinesis, no cytokinesis or nuclear division, or missing chromosome segregation. An extreme version of polyploid cells occurs in most larval tissues of *D.*

melanogaster, which become polytene. A polytene cell is a polyploid cell where the chromosomes remain next to each other (Alberts et al, 1989). These cells follow a cell cycle pattern where they go from G-S-G-S skipping the M phase all together (Smith and Orr-Weaver, 1991). A final type of alteration to the cell cycle is during the second phase of meiosis. These cells divide without duplicating DNA in an S-phase, creating cells with half as many chromosomes.

Data in this study suggest cells spend a long time in S-phase (Fig 2.5). For example, after only a 10 minute exposure to BrdU a large number of cells are labeled; to become labeled these cells are in S-phase. Perhaps, cells that are in S-phase for long time periods may be becoming polyploid, preparing for rapid rounds of mitoses during morula division. During morphogenesis, a polyploid membrane surrounds each embryo (Baehrecke et al., 1992). That study only measured the diameters of morphogenic embryos and so there is no information indicating if earlier stages can become polyploid or even polytene. In one hypothesis, polyploid or polytene cells may explain how the inner envelope cells potentially divide without a new S-phase, like in meiosis, and can achieve rapid partitioning of the embryonic cells. Perhaps, this is how some partitioning morulae can be SM morulae as well, preparing to partition again.

Conclusions of proliferation and implications to stem cell research

Understanding that all morulae play some role in proliferation has important implications for proliferation, implying proliferation is not limited to a small population of morulae, rather all morulae continue to proliferate during development. During the

earlier instars, a smaller percentage of morulae divide into new morulae relative to the fourth instar.

Morula partitioning may rely on a number of elements. The inner envelope cells play an important role in proliferation, aiding the developing morulae for when they split (Patterson, 1921). Without an adequate number of inner envelope cells, new morulae cannot be formed. As discussed earlier, proliferation of polymorulae by regions may be due to a local signal diffusing between morulae, suggesting an external signal controls morulae partitioning. A second element that may be involved in proliferation is an inter morula signal from the germ cells. Recent studies suggest the germ cells play a role in proliferation (Donnell et al, 2004). The embryonic cells can range in number from 4 to 50 per morula (Patterson, 1921); while not all the embryonic cells are germ cells, a high number of embryonic cells probably corresponds to higher numbers of germ cells. When morulae have large numbers of embryonic cells the morula divides and morulae with fewer numbers of embryonic cells make more embryonic cells (Patterson, 1921). If the inner envelope is responding to the number of germ cells as recently suggested (Donnell et al, 2004), then the inner envelope is responding to the local environment, uncoupling the mitotic activity with morula partitioning. In a similar scenario, *D. melanogaster* cells respond to their local germline stem cells. In *D. melanogaster* testies differentiated germ cells can revert back to germline stem cells if the niche is damaged (Wong et al, 2005). These cells are able to sense their environment to fill in the necessary role. Perhaps in stem cell research, a stronger emphasis should be placed on the environment that the stem cells are actually proliferating, rather than the stems cells themselves.

Future Directions

This study suggests that among instars, participation in proliferation of morula changes. In the first instar the percentage of proliferation is low. During the later instars the proliferation increases, however the percentage participation by the morulae changes among the instars. For example, the highest percentage participation is seen within the third instar. However, more morulae are participating in the fourth instar as seen by how quickly morulae transition from S-phase to M-phase compared to how fast this occurs in the third instar. These studies indicate that a more careful time course of cell cycle rounds is needed to fully understand morulae proliferation. BrdU injections need to be conducted while being conscious of the age within an instar, not just which instar. Additionally, the windows of time following injections needs to be narrower to identify more smoothly the percentages of morulae that have transitioned from S-phase to M-phase.

Steps involved in morulae partitioning are unclear. This study showed the morulae are partitioned from an invagination of the inner envelope, but the partitioning is not obviously linked to the mitotic stage of the cells in the inner envelope. One way to help clarify what is going on during partitioning is through studies counting the number of embryonic cells in partitioning morulae in relation to what mitotic state the partitioning morulae are in (SM, OM or NMA). Additionally, understanding the ploidy of the nuclei in partitioning morulae may clarify why some partitioning morulae are SM morulae and some are not. Finally, a careful consideration needs to be made as to what stage embryo proliferation and partitioning stops. This study shows that during the embryo

compaction stage, embryos still partition. Proliferation was thought to have stopped by the onset of morphogenesis.

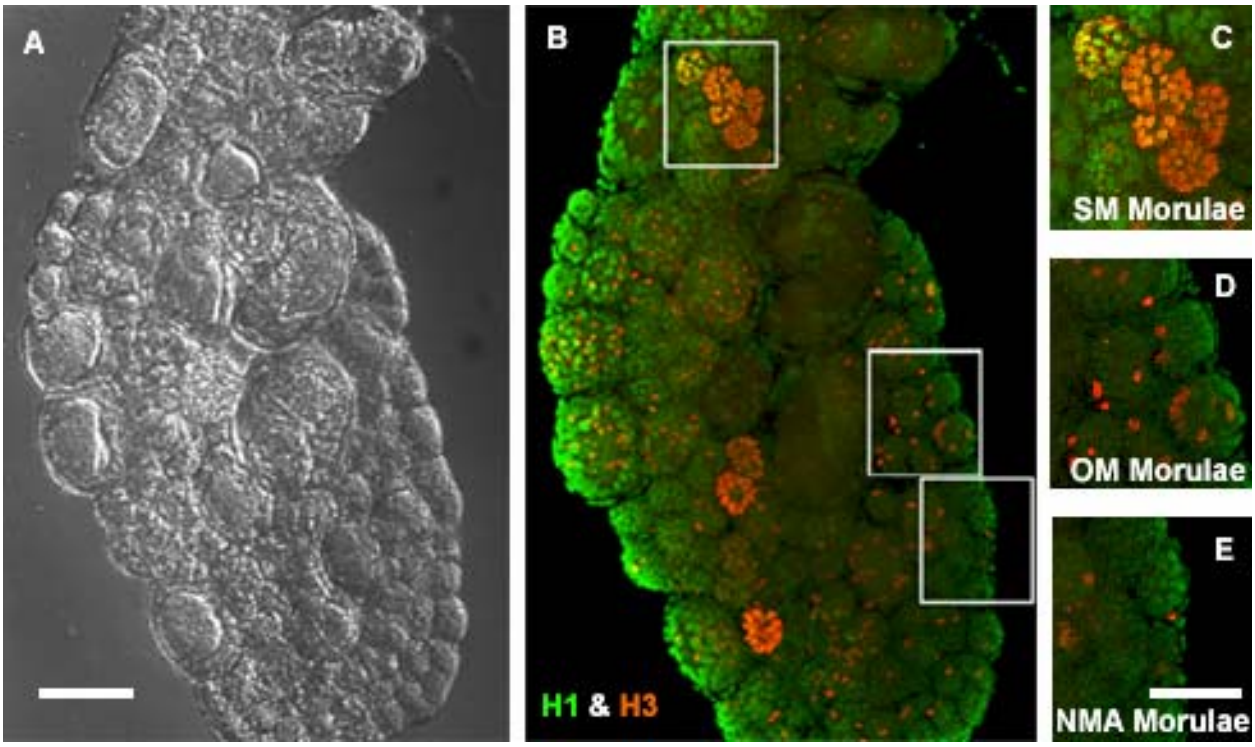


Figure 2.1 Histone H3 and H1 Labelling of Morulae in a whole mount polymorula
A third instar polymorula viewed under Nomarski optics (A) and with nuclear labeling of histone H1 (green) and histone H3 (red). Three mourla mitotic states were observed: Synchronously Mitotic (SM), Occasionally Mitotic (OM) and No Mitotic Activity (NMA). Embryos are boxed in (B) and shown at a higher magnification in C-E. Scale bars in A and B equal 200 µm. Scale bars in C-E equal 100 µm.

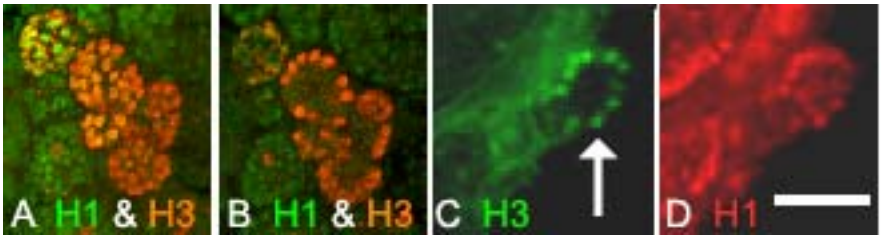


Figure 2.2 Comparison of Histone H3 and H1 labeling of optically sectioned (A, B) and Paraffin sectioned (C, D) morulae
In (A) three dimensional optical reconstruction from SM morulae while (B) shows a single 2µm optical section through the center of each embryo. Only the inner envelope cells are labeled by anti-H3. (C) shows that only the inner envelope cells are anti-H3 labeled in an SM morula from a paraffin section, whereas (D) shows that both the inner envelope and embryonic cells are labeled by anti-H1. Scale bar equals 100 µm.

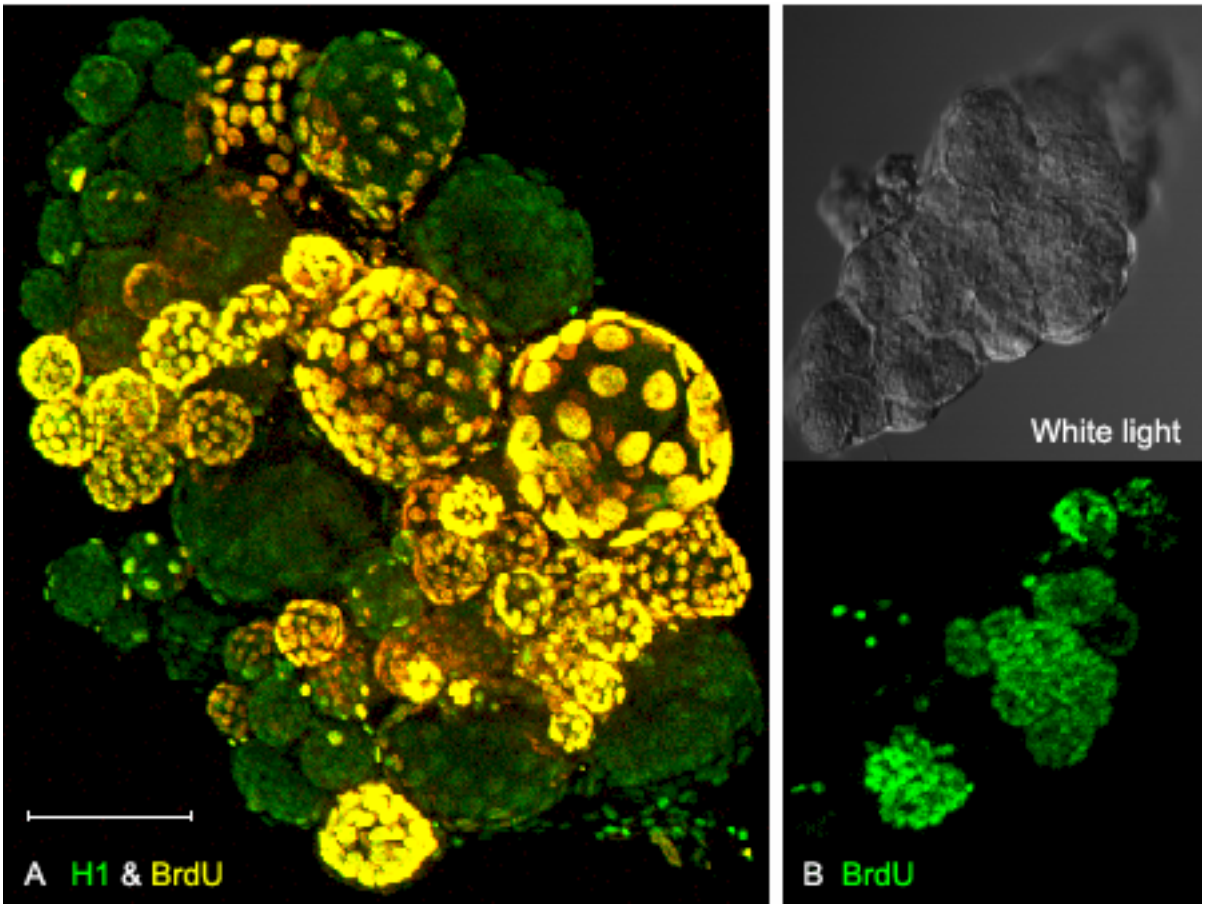


Figure 2.3 Spatial Incorporation of Injected BrdU

Polymorulae injected with BrdU show regional incorporation. Anti-histone H1 is green and anti-BrdU red, double labeled as yellow (A). Note the unattached regions of BrdU of the polymorula viewed with white light Nomarski optics (B) and anti-BrdU (green) (C). Scale bar equals 200 µm.

Table 2.1 SM Morulae Within an Instar

Host Instar	Age in instar	Total # of Morulae	SM Morulae	% SM morulae
1	early	8	0	0
	middle	555	18	3.2
	late	1331	46	4.5
2	early	614	37	6
	middle	587	34	5.8
	late	1138	48	4.2
3	early	437	35	8
	middle	528	50	9.5
	late	1709	82	4.8
4	early	3635	207	5.7
	middle	13838	1026	7.4
	late	7863	261	3.3

Table 2.2 SM Morulae Among Instars

Host Instar	Total # of Morulae	SM Morulae	% SM morulae
1	1944	64	3.3
2	2339	118	5
3	2674	167	6.2
4*	17473	1233	7.1
4**	7863	261	3.3
5	4026	4	1 X 10^-4

*Indicates the proliferative portion of the fourth instar;
**Indicates the beginning of reproductive caste morphogenesis in the fourth instar

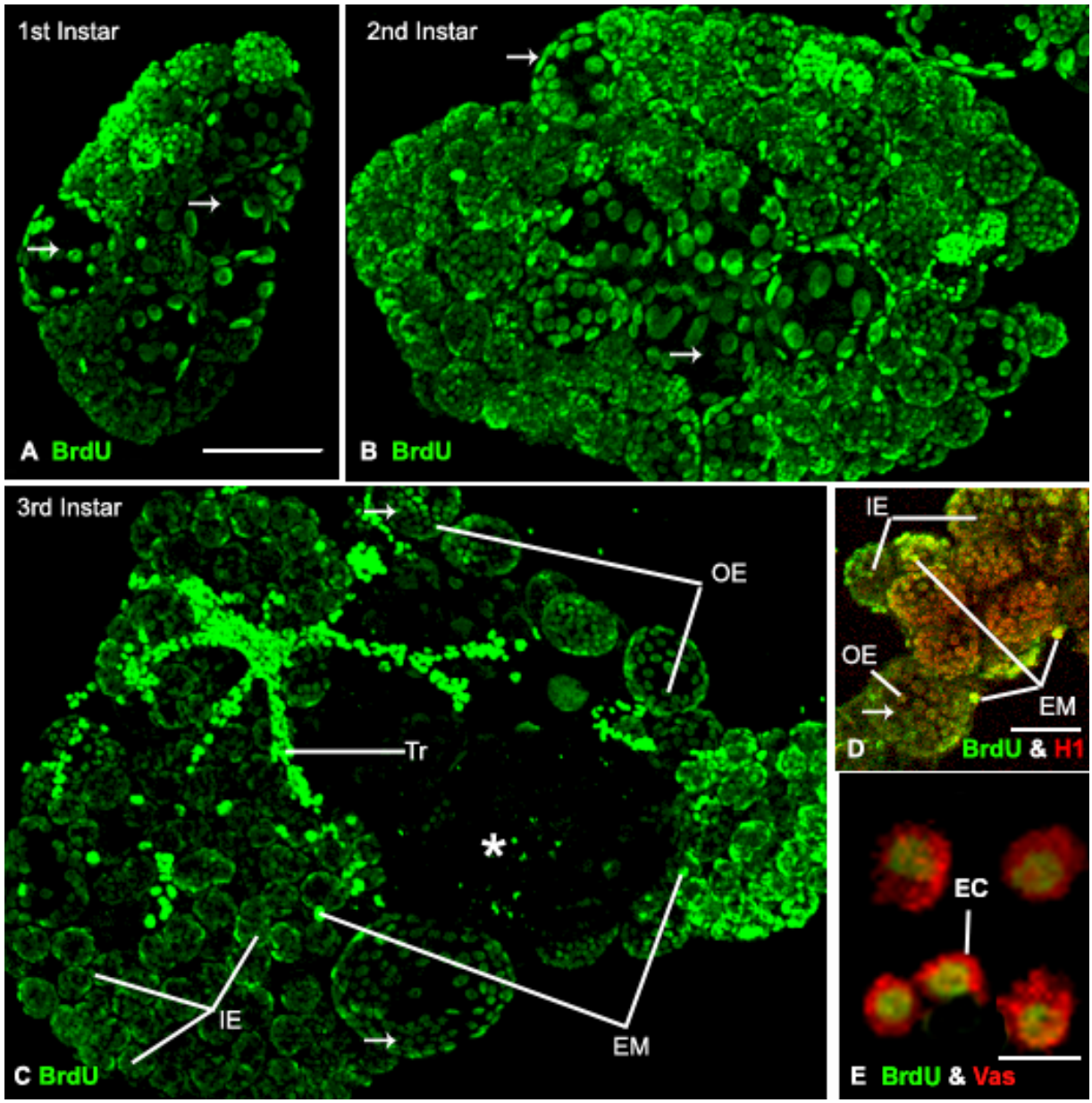


Figure 2.4 BrdU Incorporation into Polymorulae

Polymorulae dissected from the end of the host's instar after the host was fed on a BrdU (green) diet from the beginning of the 1st (A), 2nd (B) and 3rd instars (C). A sample double-labeled for BrdU (green) and H1 (red) appearing yellow in most cells helps identify cell types (D). Dissociation of cells reveal a BrdU signal in EC as verified by an internal marker of Vas (red) (E). Arrows indicate position of precocious soldier; asterisk indicates a very advanced soldier where label does not penetrate cuticle; Tr:trachea; EM: Enveloping Membrane; OE:Outer Envelope; IE:Inner Envelope; EC:Embryonic cells. Scale bar for A-C equals 200 µm. Scale bar for D equals 100 µm. Scale bar for E equals 10 µm.

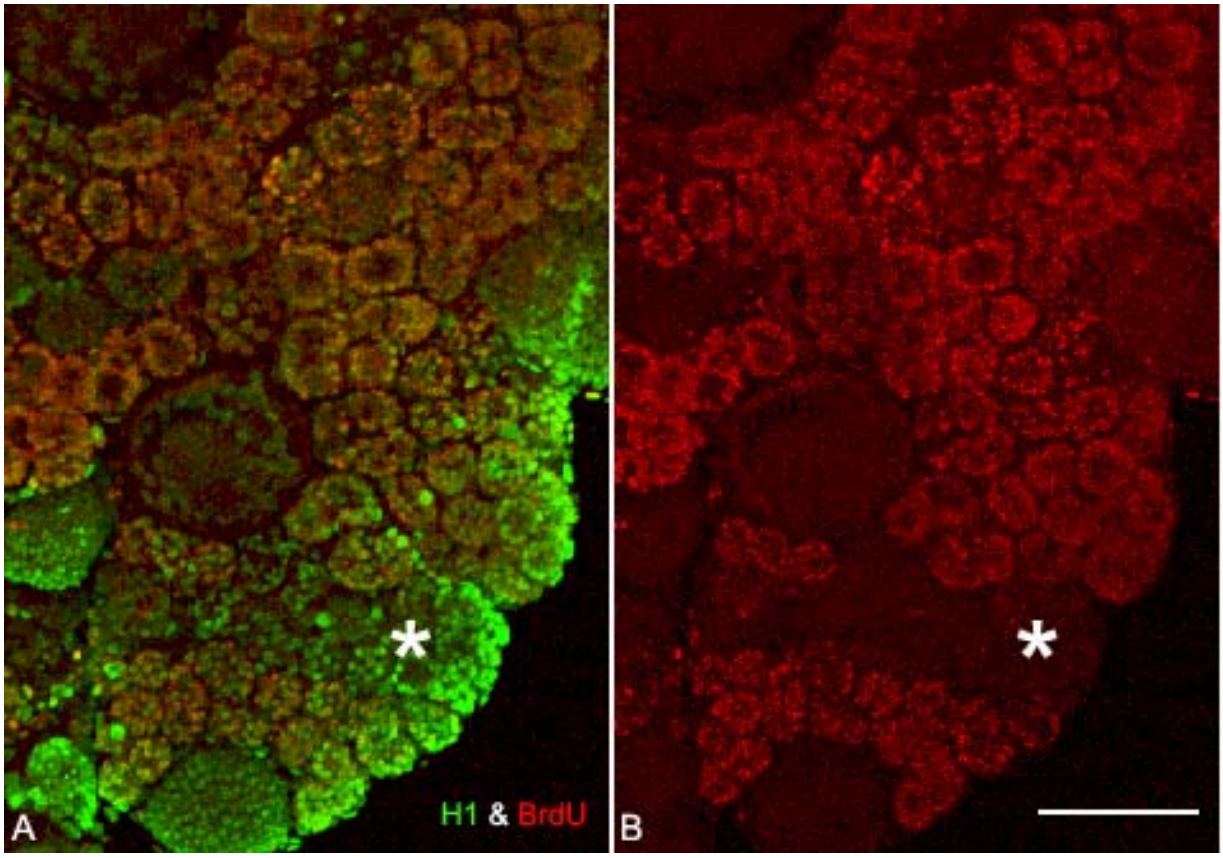


Figure 2.5 BrdU Incorporation after 10 Minutes of Injection
A short, 10 minute incubation with BrdU reveals quick incorporation of BrdU into the outside embryonic cells in a double label merged image of Histone H1 (green) and BrdU (Red) (A). Just BrdU is shown (B). The asterisk highlights a region of no BrdU incorporation. Scale bar equals 200 µm.

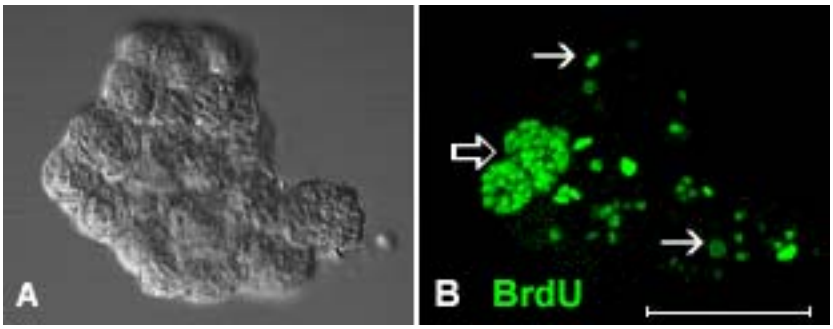


Figure 2.6 Limited signal in inner envelope cells of a polymorula 25h after injection with BrdU
A Nomarski image (A) and an anti-BrdU (green) image (B) show BrdU incorporation 25 hours after injection of BrdU. Arrows indicate enveloping membrane BrdU incorporation; block arrow indicates three morulae with inner envelope incorporation. Scale bar equals 200 µm.

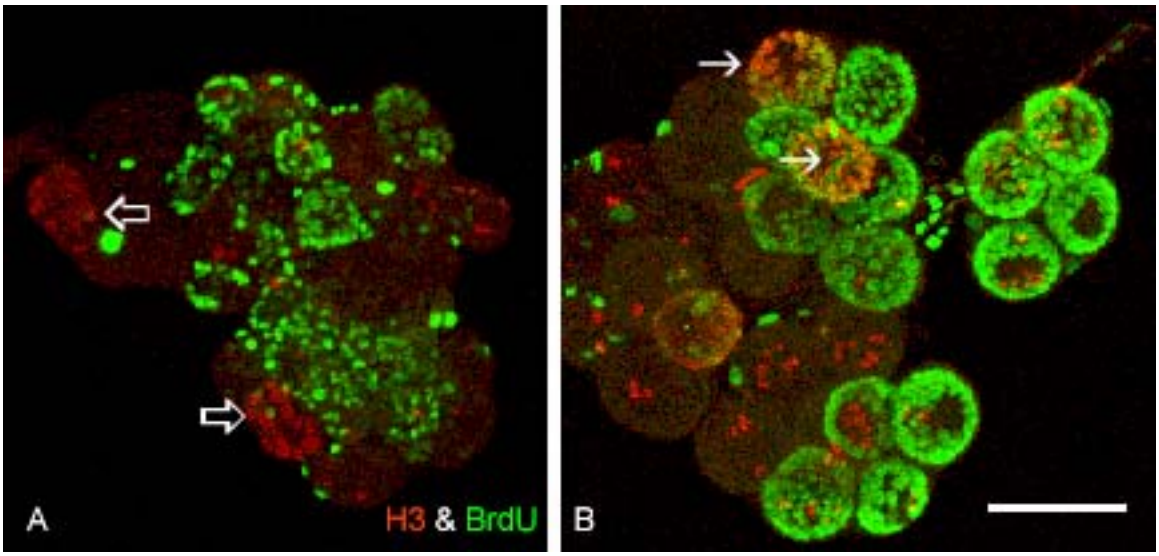


Figure 2.7 Double label of H3 and BrdU
Polymorulae dissected from a BrdU-injected host double-labeled with anti-BrdU (green) and anti-H3 (red). In (A) morulae were collecte from third instar hosts 4h post-injection with BrdU. No cells were double labeled. In (B) embryos were collected 25H post-injection with BrdU. Most cells in specific morulae (arrows) were double-labeld, whereas cells in the other morulae were not. Scale bar equals 80 µm.

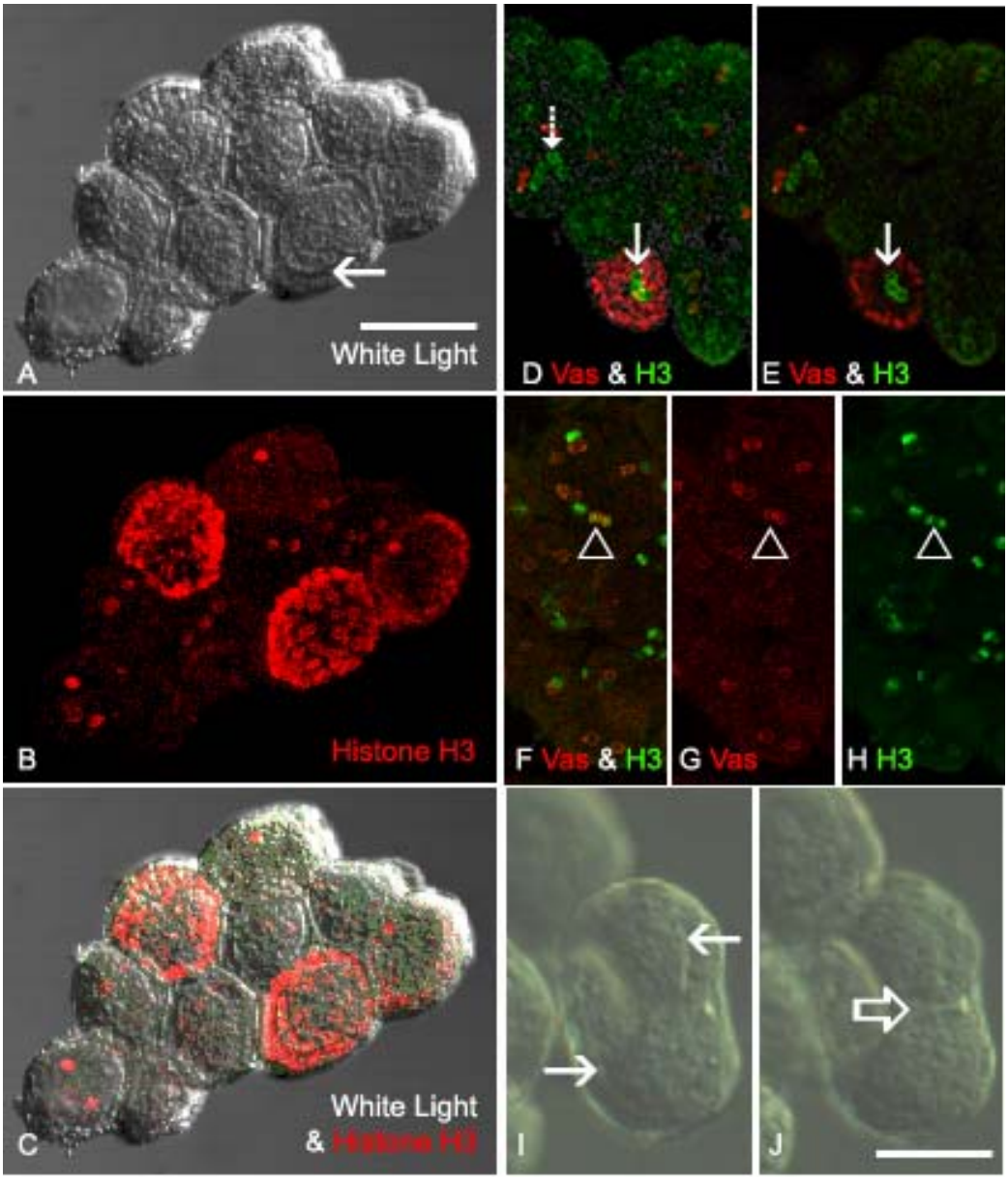


Figure 2.8 SM Morulae can be in the initial stages of morphogenesis
Nomarski (A), anti-H3 (red) (B) and composite image (C) show SM embryos during the compaction stage of morphogenesis. A double labeled merged image of Vas signal (green) with anti-H3P (red) (D) and a section (E) of a third instar SM morulae show SM morulae are not an early stage of morphogenesis. Composite Vas (red) and anti-H3P (green) (F) with individual sections of Vas (G) and anti-H3 (red) show an H3 positive signal in Vas positive cells. Two compaction stage embryos still share a single outside embryonic layer (I) while are in the process of partitioning (J). Horizontal arrows indicate locations of embryonic thickening and compaction; block arrow indicates the location of the embryo splitting; solid vertical arrow indicates *vasa* label inside a SM morula; dashed vertical arrow indicates a *vasa* label inside a non-SM morula. Scale bar for A-H equals 80 µm. Scale bar for I & J equals 60 µm.

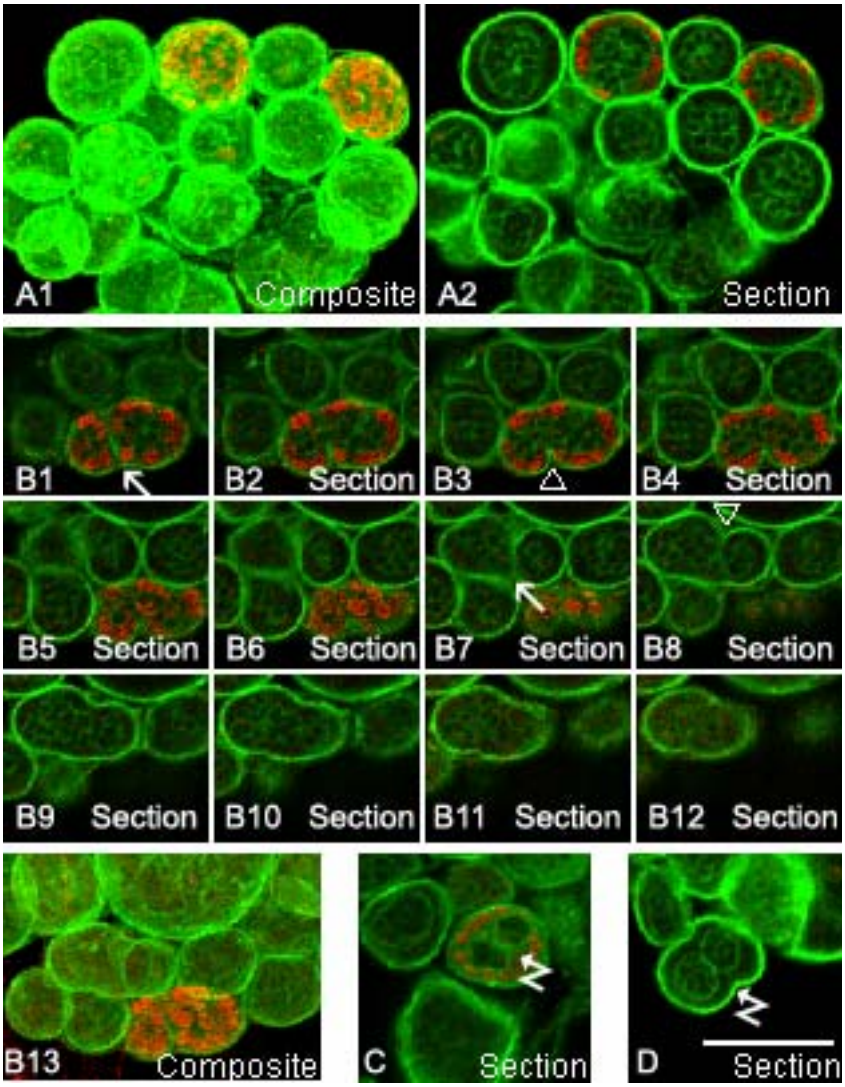


Figure 2.9 Morulae Splitting

Phalloidin labeled morulae (green) double labeled with anti-histone H3P (red). Most SM morulae are round in shape (A), however some SM morulae exhibit signs of splitting (optical sections B1-B7; composite merged image B13; C). Non-SM morulae also exhibit signs of splitting (optical sections B5-B12 and composite B13; D). Arrows indicate phalloidin separation of morulae; arrow heads indicate morulae splitting; zig-zag arrows indicate two sets of inner embryonic cells contained in one set of outer embryonic cells, early stages of splitting. Scale bar equals 80 µm.

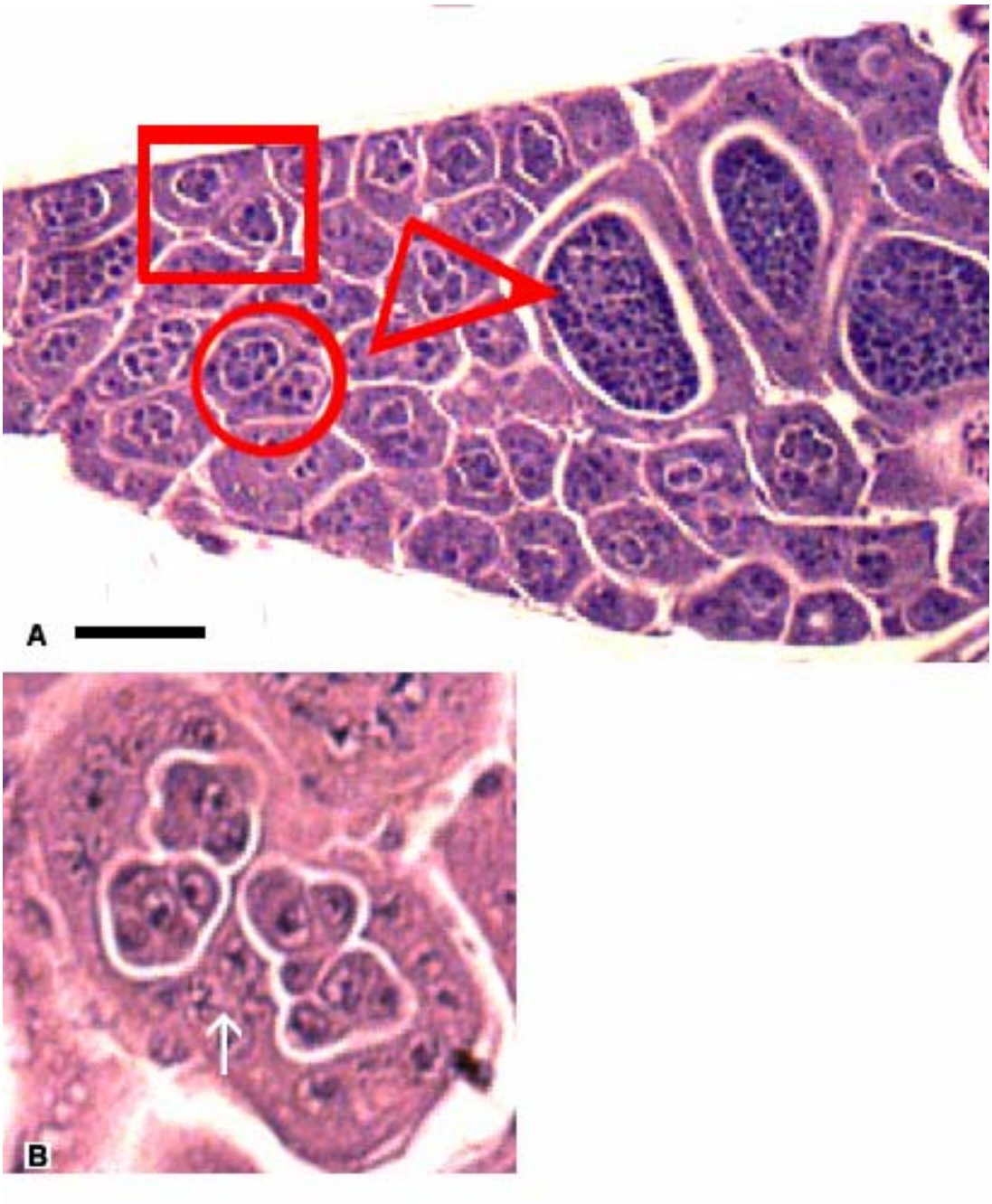


Figure 2.10 Understanding partitioning morulae

A hematoxylin-eosin stained section indicating the phases of morula partitioning. First there is embryonic cell segregation (triangle) followed by the inner envelope partitioning the embryonic cells (circle) and finally followed by the enveloping membrane partitioning (square) (A). Sometimes the partitioning morulae are preparing to partition again (B). Arrow indicates where the inner envelope cells are beginning to invaginate between two groups of embryonic cells each of which are already partitioned into two groups.

Chapter 3 Development of polymorulae along with its host's trachea

Introduction

Endoparasites, such as polyembryonic encyrtid wasps are the only parasites known to actually use the host's oxygen supply directly (Doutt, 1947; Ivanova-Kasas, 1972; Baehrecke and Strand, 1990). Early descriptions report that the developing polymorula develop in direct contact with the host's tracheal system (Doutt, 1947). However, the tracheal attachments to polymorulae have never been described. After a general review of insect tracheal systems, the types of polymorulae found in the host will be described in addition to the various tracheal attachments to these polymorulae. These data lay the groundwork to test if factors from the polymorula impact the growth of the host's trachea to the parasite. Therefore, the discussion of this chapter will elucidate the potential directions for studying proliferation in association with host tracheal growth.

Insect Trachea

The role of the insect tracheal system is to supply oxygen to all of the cells. The development of the insect tracheal system is best understood in *Drosophila melanogaster.* Tracheae derive from specialized cells, called placodes, that are specified during embryogenesis (Affolter and Shilo, 2000). By stage 12 of embryogenesis in *D. melanogaster* ten paired placodes divide to form approximately 80

cells each. The placode cells are then differentiated by the activity of three signaling pathways: Decapentaplegic, Wingless/WNT and Hedgehog (Petit et al., 2002). After the cells in the placodes are determined, tracheae continue to grow throughout larval development but only through elongation and rearrangement, not through cell division (Samakovlis et al., 1996). Cell division begins again at the end of the larval stages, just before morphogenesis to the pupal stage (Madhavan and Schneiderman, 1977).

After the tracheae initially form, oxygen deficient cells throughout the host tissues release a diffusible ligand, from the protein product of the *branchless (bnl)* gene, which attracts and binds its receptor *breathless (btl)* which localizes at terminal tracheal tips (Sutherland et al, 1996; Zelzer and Shilo, 2000). The oxygen levels regulate expression of Bnl thereby regulating the terminal growth of the tracheae. As the cell's oxygen need is met, the Bnl signal stops. In studies conducted with *D. melanogaster* overexpression of Bnl induces more terminal branching of the tracheae (Jarecki et al, 1999).

Branchless: an FGF Family Member

bnl is a member of the fibroblast growth factor (FGF) gene family. The FGF family is distinguished by a conserved 120 amino acid motif known as the FGF domain (Sutherland et al., 1996). The FGF domain is conserved from *C. elegans* through humans; however, humans encode more than 20 FGF family members while *D. melanogaster* encodes only three (Popovici et al., 2005). All FGF family members studied to date bind the receptor belonging to the receptor tyrosine kinase (RTK) family; FGFs also have a strong affinity for heparin (Thisse and Thisse, 2005). The role of *bnl* in directing the growth of trachea is analogous to the role of *fgf10,* which directs the

growth of mammalian lungs (Huang and Stern, 2005). The other two insect FGFs, *pyramus* and *thisbe*, pattern the mesoderm (Stathopoulos et al, 2004).

Materials and Methods

Methods, such as animal maintenance, sample collection and sample processing were identical to Chapter 2. The descriptions made in the results section are from observations of over 1000 dissected hosts.

Results

Characterization of polymorula growth

Polymorulae development differed qualitatively in features such as caste ratios, numbers and shape. One shared trait of all polymorulae was that all were only a few morulae thick. However, no two polymorulae were the same, which complicated the characterization of growth, development and proliferation. The characterization of polymorulae was more uniform within a single cohort. Cohorts consisted of similar aged moth eggs parasitized by a single brood of female wasps at the same time. Because the variation of polymorulae depended to a large degree on the cohort, cross-cohort analyses were less informative than intra-cohort analyses.

The quality of polymorulae in a single host was less variable. The different elements of host quality (caste ratios, shape and size) were independent. The caste ratios making up a polymorula ranged from having a high ratio of soldier larvae (Fig 3.1A), as measured by precocious development, to large levels of proliferative morulae (Fig 3.1B-D). Previous studies show male broods have a lower ratio of soldier larvae

develop (Grbic et al., 1996). A second measure of polumorulae quality was the number of morulae in each polymorula. Sometimes the polymorulae fragmented (Fig 3.1B), with many small clusters of morulae located throughout the host's hemolymph and fat bodies. However, the polymorulae also remained in sheets with well over 500 morulae. In addition the final number of adult wasps per host varied from 600-3000, which could have reflected how many morulae were present in each polymorula (Ode and Strand, 1995). The quality of these sheets ranged from over 70 morulae long and 1-2 to 40 morulae wide (Fig 3.1C & D). Together this variation made the growth of polymorulae difficult to quantify.

Physical evidence for C. floridanum *interactions with* T. ni *tracheae*

Tracheal associations with wasp morulae were found as early as the caterpillar's first instar. In all cases the trachea attached to the extraembryonic membrane that surrounded the polymorula. Upon dissection the trachea often could not be pulled off of the polymorula without fragmenting the polymorula. The first and second instar polymorulae were characteristically in the host's thorax; trachea attached at the anterior end of the polymorulae (Fig 3.2). Despite which type of polymorula, the trachea remained connected and interdigitated with the embryos throughout development (Fig 3.3). As the polymorula grew larger and fragmented, as discussed in the previous section, the tracheal associations became more complex. In some instances, the tracheal network became much thicker with more branches (Fig 3.3A); in other cases, the trachea attached at additional locations (Fig 3.3B). In polymorulae that fragmented, the smaller ends of trachea loosely attached to the fragments of polymorulae (Fig 3.3C).

As the morulae began morphogenesis, very large tracheal trunks attached to the polymorula (Fig 3.3D), probably to match the increased oxygen need. As the polymorula fragmented into individual embryos, every single morula had an attachment as well (Fig 3.3E).

Discussion

Characterization of polymorulae and tracheal growth

Polymorulae do not follow a consistent pattern of growth among hosts (Fig 3.1). The only consistent feature among all polymorulae is how thick the polymorula becomes, never exceeding a few morulae thick. There are a few explanations why the polymorula have a physiological limit in thickness. First, the precocious soldier larvae must be able to break free when they develop so that they are not trapped and can defend the brood without disrupting it. However, all male broods also grow only a few morulae thick, yet they typically do not produce soldiers until the host's fourth instar (Grbic et al., 1996). Therefore, a more likely constraint on polymorulae thickness is the need for oxygen. Perhaps in a thick polymorula the trachea cannot be in physical contact with the inner morulae, because every embryo connects to the host's trachea (Fig 3.3). The tracheae are so closely attached, that attempts to remove the tracheae from the polymourla usually disrupted the polymorula, breaking it apart. In addition, as the polymorula develops and becomes larger, the tracheal attachments increase in complexity by becoming more numerous and having thicker branches (Fig 3.2). Because tracheae grow towards a *bnl* signal, to achieve a host-polymorula tracheal connection, the parasitoid morulae may be releasing one or more extracellular ligands,

such as Bnl, that stimulate host's tracheal growth. This explains why older, larger polymorulae have so many different types of tracheal attachments. In a separate study, small clusters of morulae were dissected from one host and injected into a new, unparasitized host resulting in a close association between the morulae and the host's trachea (Strand unpublished).

Future Directions

In *D. melanogaster* the *bnl* must be expressed in cells in need of oxygen to direct the growth of trachea. Bnl is a chemoattractant guiding tracheae over long distances to cells in need of oxygen (Jarecki et al, 1999). This implies the parasite is most likely attracting the host's cells through a *C. floridanum* Bnl. The next step in understanding the role and relationship of tracheal development to the parasite is identifying both the host's and the parasite's *branchless (bnl)* and *breathless (btl)* genes. Both genes have high homology of the FGF (bnl) and FGF-Receptor (btl) domains. Once sequenced, in situ mRNA hybridizations will show their expression patterns. If the hypothesis is true that the parasite co-opts the tracheal development signals of the host, one might expect to see a C. floridanum bnl released by the polymorula with no btl signal, in addition to no host bnl signal around the polymorula, but with a host btl signal in the tracheae attached to the polymorula (Fig 3.4).

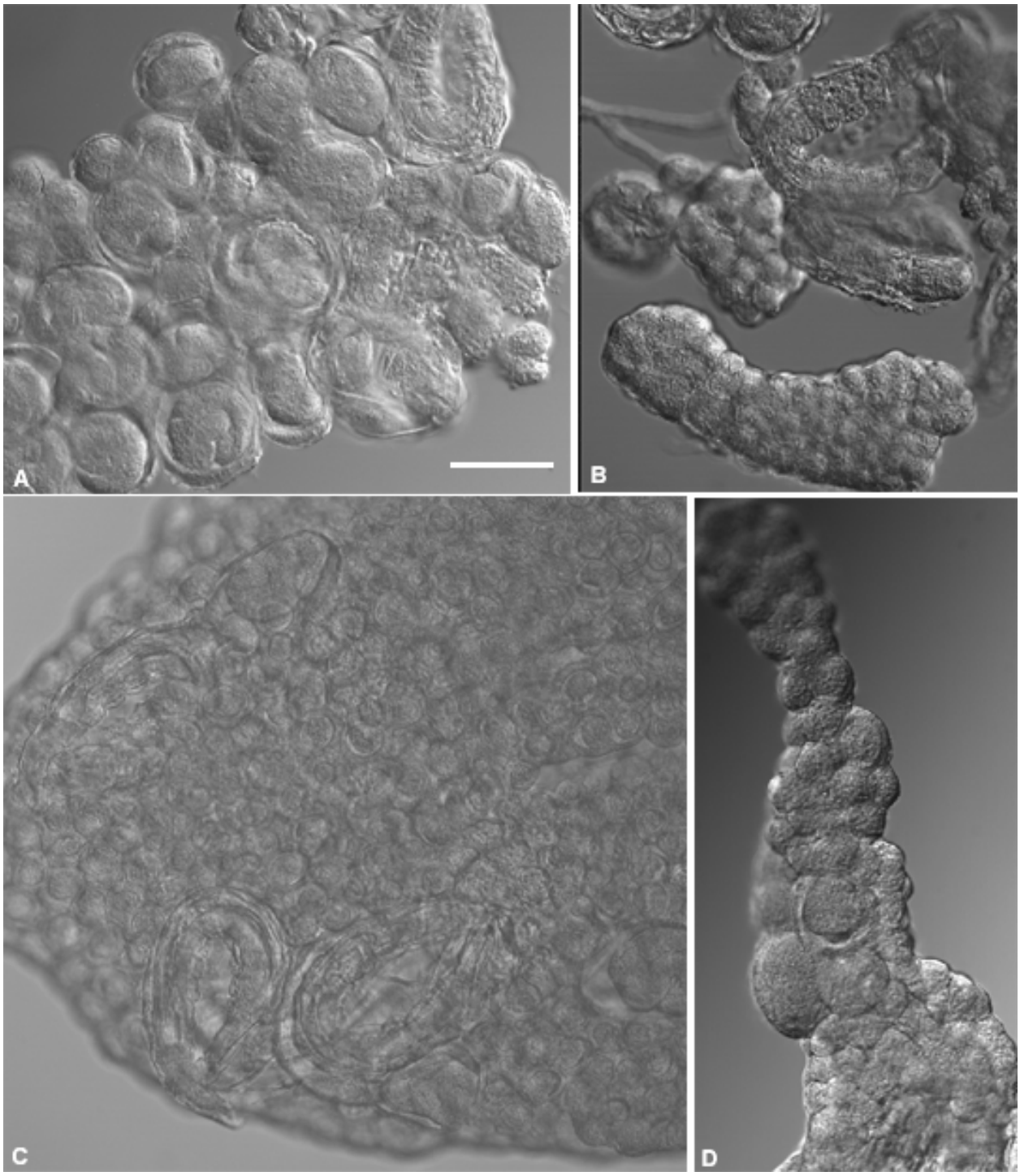
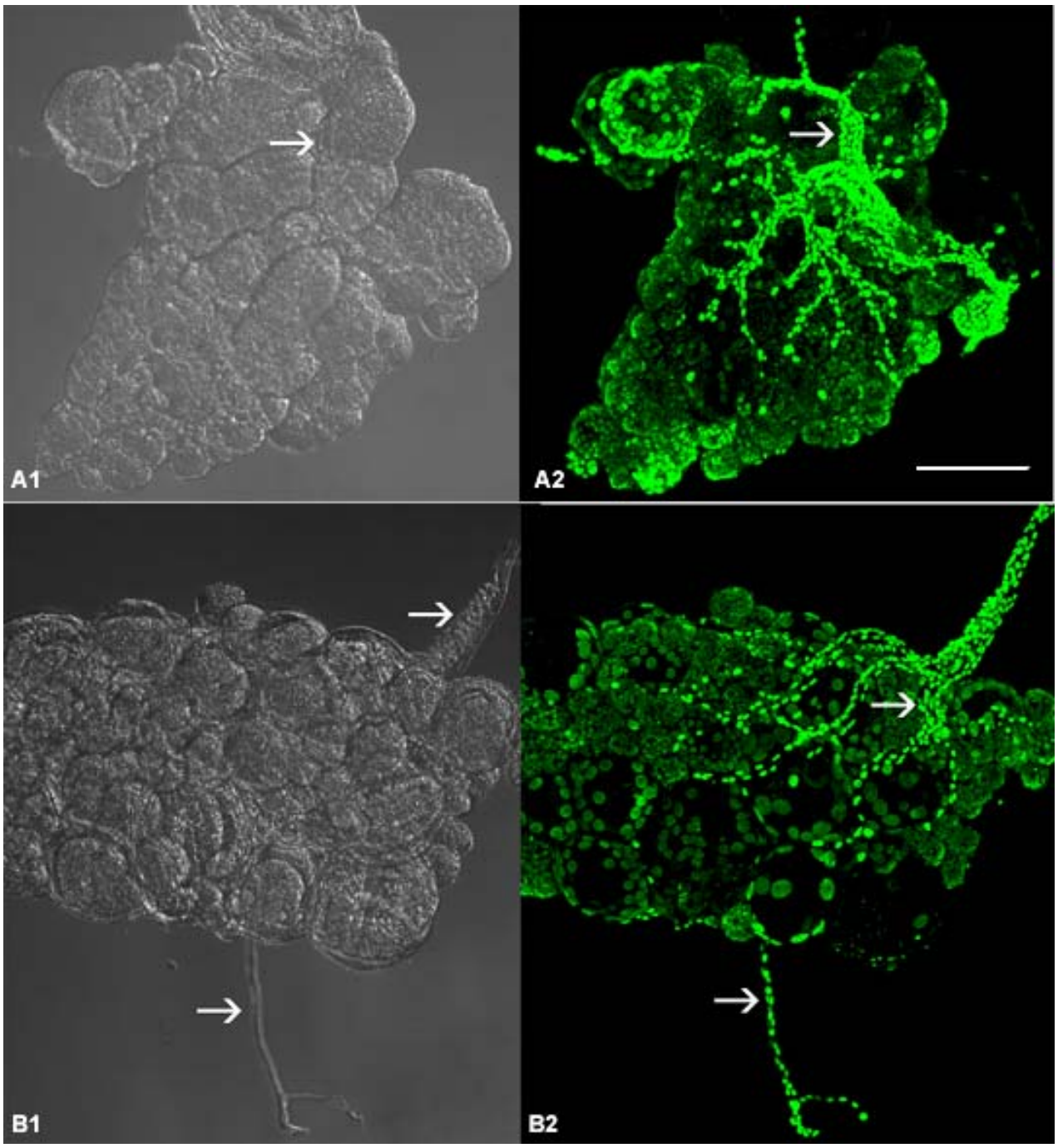
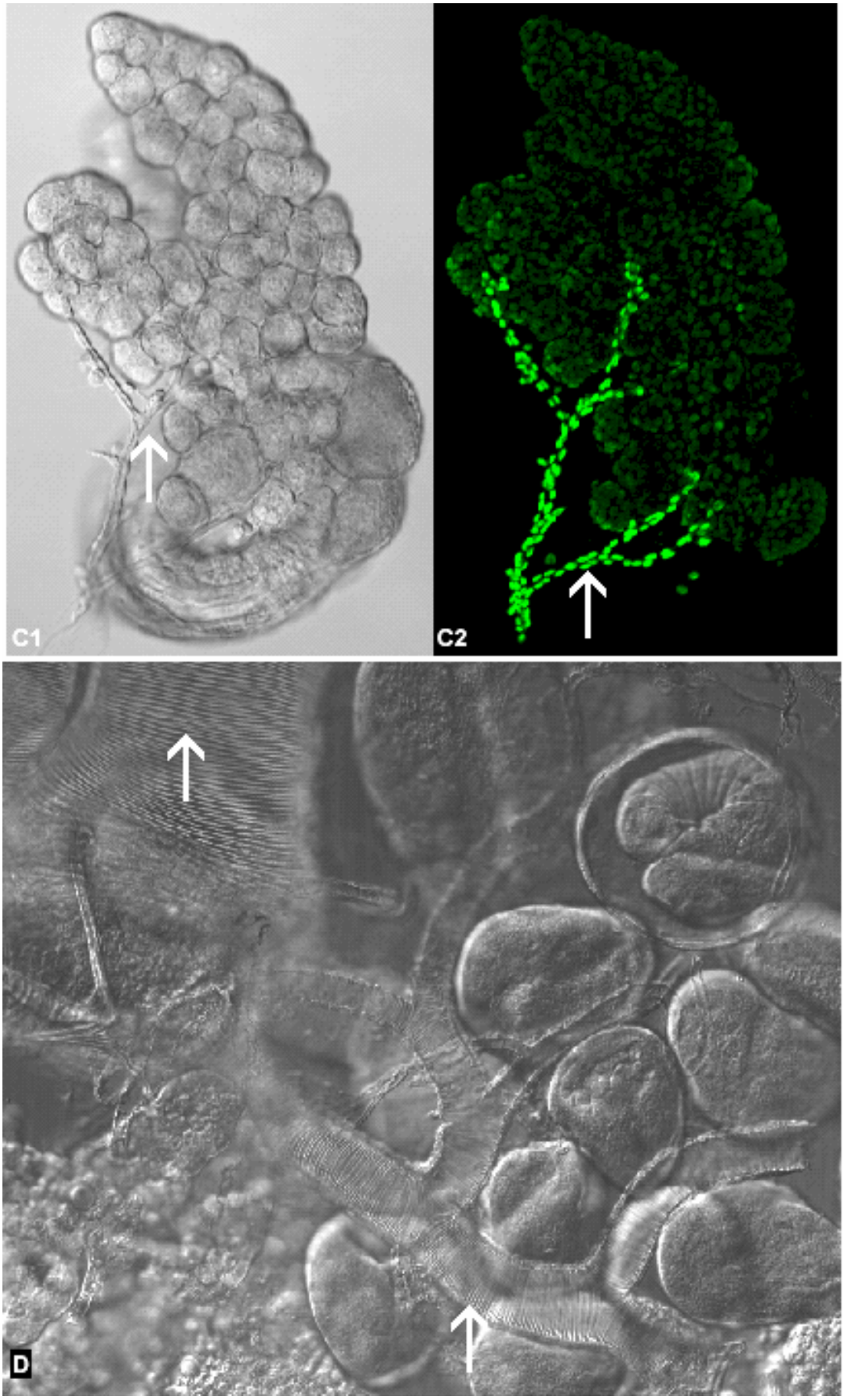


Figure 3.1 Variable Polymorulae Growth
Some polymorulae have high soldier levels (A), some fragment (B), some are in wide sheets (C) and some are in skinny sheets (D). All images are viewed under Nomarski optics. Scale bar represents 200 μm.

Figure 3.2 A Model of Early Polymorula Placement
Polymorulae (black) are characteristically in the thorax of the fist two instars, with a close and tight tracheal attachment (green).


A1
A2
B1
B2


C1
C2
D

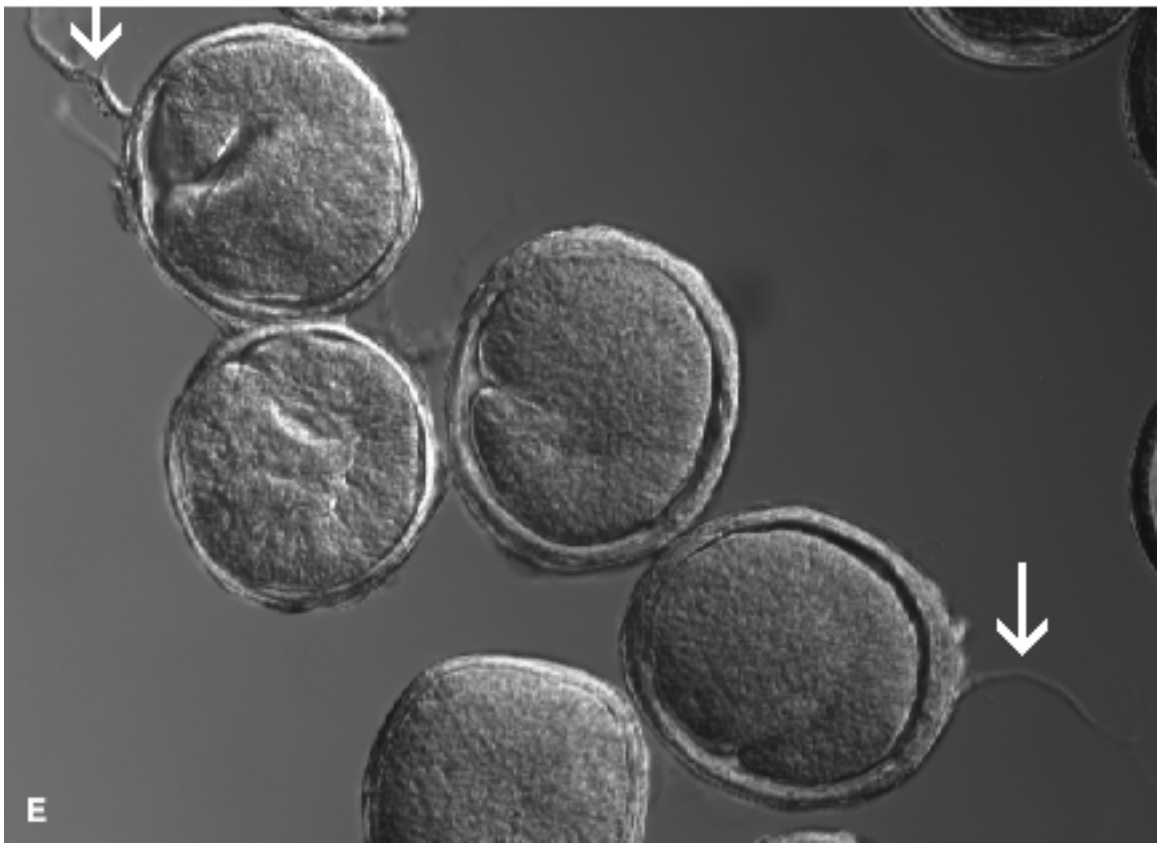


Figure 3.3 Tracheal Attachments
Various types of tracheal attachments seen through Nomarski optics (A1, B1,C1, D & E) and with a nuclear stain to help see the tracheal extensions (A2, B2 & C2). Tracheae attachments can range from very thick and branched (A), to multiple connections (B) or to a loose connection (C). Older morphogenic morulae can be attached to very thick tracheal trunks (D). When morulae split into individual morulae each morula has its own trachea attached (E). Arrows indicate trachea.

Chapter 4: Conclusions

Conclusions from the Data

The main generalization that can be drawn from this study is that all morulae aid in proliferation, producing more morulae, though not to the same extent. As the host becomes older, through four instars, a progressively higher percentage of morulae participate in proliferation of new morulae at a given moment. In addition, the progression through the cell cycle occurs the fastest in the fourth instar.

Within an instar, all the cells go through DNA synthesis, S-phase, at least once (Fig 2.5). However, S-phase occurs in the polymorula by region. In addition, mitosis occurs asynchronously throughout the polymorula. This is exemplified by the morulae existing in three different mitotic groups: synchronously mitotic (SM) morulae, occasionally mitotic (OM) morulae and no mitotic activity (NMA) morulae (Fig 2.1). The difference between these groups is how many cells in the inner envelope of the proliferative morula label positively for mitosis. The relative percentage of these three morula types changes among and within instars. The SM morulae most readily represent proliferation because all the inner envelope cells are duplicating producing twice as many cells. However, the SM morulae do not indicate morulae splitting and partitioning to new morulae because splitting and partitioning morulae can be in any of the three types of morulae (SM, OM or NMA).

The results in this study now clarify which layer--inner envelope or enveloping membrane--of the polymorula is responsible for partitioning the morulae. The inner envelope is a morula-specific layer, surrounding the embryonic cells within a given morula, while the enveloping membrane surrounds all the morulae. These results suggest the inner envelope cells are responsible for partitioning the morulae. The cells in the inner envelope often act in a synchronous fashion, both with mitosis and DNA synthesis. Additionally, both the embryonic cells partition and the inner envelope forms between them before the enveloping layer begins to invaginate into the morulae (Fig 2.10).

Observations in this study characterize polymorulae growth as well as the tracheal affiliations to them. Polymorulae do not grow in a consistent manner. However, a cohort of moth eggs parasitized by a brood of wasps does tend to grow in similar fashion. Across several cohorts, polymorulae vary by caste ratios, numbers of morulae and shape of the polymorula (Fig 3.2). The ratios of the two castes, precocious larvae and proliferative reproductive morulae, range from very few precocious larvae in the developing brood to seemingly very few proliferative morulae. In any given polymorula, the numbers of morulae range from very few to over 500; this is due to the polymorula fragmenting inside the host, for unknown reasons. Finally the shape of the morula is variable from narrow--only a few morulae wide--to over 40 morula wide and over 70 morulae long. The tracheal attachments are equally variable, potentially because the tracheae connect to each of the different types of polymorulae. Initially tracheae usually attach closely to the anterior end of the polymorula. As the animals age, the number of tracheal attachments increases, the tracheal system thickens in diameter, and

sometimes tracheae become loosely attached to the polymorula fragments (Fig 3.3). One trait shared by all the tracheae was that all attachments were very strong, such that physically removing them would disrupt the integrity of the polymorula architecture.

Pitfalls of the Research

To try to resolve the unclear issues in characterizing the development of polymorulae, many new techniques were used, each with its own limitations. One technically hard procedure was figuring out how to process the slides with the sections. After many attempts of processing standard slides from the University of Georgia Histone Laboratory, thicker (7 μM) sections produced better results. However, the final methods used for this processing were still mediocre in obtaining good anti-H3 labeling. Another method that took many trials to overcome is counterstaining the cells for anti-H3P and anti-BrdU. While the primary antibodies are conjugated to different animals, the primary antibodies cannot be incubated at the same time. However, the secondary antibodies must be applied at the same time for good results.

Another problem was counting the morulae. The different staining techniques and white light images did not always give the same number of morulae. For example, one morula under white light appeared as 4 morulae using anti-H3P. Additionally, morulae that were in the process of splitting were hard to quantify. Another problem with counting the morulae is when the polymourla was a few morulae thick, the focus had to constantly be adjusted to count the center morulae. For this work, all of the counting was done in a uniform fashion with every effort to be consistent in counting the embryos, so the quality of the counting should be normalized.

The largest difficulty in completing this research was the quality and nature of the animals. As discussed in Chapter 3 the quality of the polymorulae ranged greatly. Ideally, an individual host animal takes five minutes to dissect; these broods are the broods that are beautiful sheets of embryos. However, often brood fragmentation and high soldier numbers made the dissections very lengthy if even worth conducting at all, after a large time investment, the yield would be minimal. Soldier number could usually be reduced with only a single wasp ovipositing into each egg. Because of the variability, BrdU experiments were only conducted when the host was of worthy quality.

Future Directions

The work in this thesis characterizes the cell cycling behaviors of the cells during the proliferation stage of development. While the work explains new phenomena that occur during proliferation (regional proliferation behavior, morula transition time from S- to M-phase and morulae partitioning independent from mitotic state of the cells), it still does not answer the question of what drives a morula to split into two. Future studies should determine if there is any correlation in partitioning morulae between the cell number of inner envelope cells, in addition to embryonic cells, compared with the mitotic state of the inner envelope cells. Future studies also need to carefully consider the timing within an instar of how long morulae take to transition from S- to M-phase.

Now that the basics of polymorula-trachea affiliations have been described, more precise methods need to be used to understand the relationship between the two. The hypothesis is that the polymorula is sending a diffusible ligand, Branchless, to attract the host's trachea received by *breathless*. Molecular studies need to identify the predicted

genes involved in the interaction between the two. *in situ* mRNA hybridizations should identify the expression patterns of the genes identifying if the parasite signal the host's trachea to obtain their oxygen.

References

Affolter M and Shilo B-Z. 2000. Genetic control of branching morphogenesis during *Drosophila* tracheal development. Current Opinion in Cell Biology. 12:731-735.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. 1989. Molecular Biology of the Cell. Second Edition. Garland Publishing, Inc.

Baehrecke EH and Strand MR. 1990. Embryonic morphology and growth of the polyembryonic parasitoid *Copidosoma floridanum* (Ashmead) (Hymenoptera:Encyrtidae). International Journal of Insect morphology and Embryology. 19: 165-175.

Baehrecke EH, Grbic M, Strand MR. 1992. Serosa ontogeny in two embryonic morphs of *Copidosoma floridanum*: The influence of the host hormones. The Journal of Experimental Zoology. 262:30-39.

Baehrecke EH, Aiken JM, Dover BA, Strand MR. 1993. Ecdysteroid induction of embryonic morphogenesus in a parasitic wasp. Developmental Biology. 158:275-287.

Corley L and Strand MR. 2003. Evasion of encapsulation by the polyembryonic parasitoid *Copidosoma floridanum* is mediated by a polar body-derived extraembryonic membrane. Journal of Invertebrate Pathology. 8:86-89.

Corley LS, White MA, Strand MR. 2005. Both endogenous and environmental factors affect embryo proliferation in the polyembryonic wasp *Copidosoma floridanum*. Evolution and Development. 7:115-121.

Cruz YP. 1981. A sterile defender morph in a polyembryonic hymenopterous parasite. Nature. 294:446-447.

Donnell DM, Corley LS, Chen G, Strand MR. 2004. Caste determination in a polyembryonic wasp involves inheritance of germ cells. Proceedings of the National Academy of Science. 101:10095-10100.

Doutt RL. 1947. Polyembryony in *Copidosoma koehleri* Blanchard. The American Naturalist. 81:435-453.

Extravour CG. 2004. Hold the germ cells, I'm on duty. BioEssays. 26:1263-1267.

Extavour CG and Akam M. 2003. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development. 130:5869-5883.

Fisher RC. 1963. Oxygen requirements and the physiological suppression of supernumerary insect parasitoids. Journal of Experimental Biology. 40:531-540.

Giron D, Dunn D, Hardy ICW, Strand MR. 2004. Aggression by polyembryonic wasp soldiers correlates with kinship but not resource competition. Nature 430: 676-679.

Giron D and Strand MR. 2004. Host resistance and the evolution of kin recognition in polyembryonic wasps. Proceedings of the Royal Society of Biology (Suppl.) Biolology Letters. 271:S395-398.

Gratzner HG. 1982. Monoclonal antibody to 5-Bromo- and 5-Iododeoxyruridine: a new reagent for detection of DNA replication. Science. 218: 474-475

Grbic M, Ode PJ, Strand MR. 1992. Sibling rivalry and brood sex ratios in polyembryonic wasps. Nature 360:254-256.

Grbic M, Nagy LM, Carroll SB, Strand MR. 1996a. Polymebryonic development: insect pattern formation in a cellularized environment. Development. 122:795-804

Grbic M, Nagy LM, Strand MR, 1996b. Pattern duplications in larvae of the polyembryonic wasp *Copidosoma flridanum*. Dev Genes Evol. 206:281-287

Grbic M, Rivers D, Strand MR. 1997. Caste formation in the poleymbryonic wasp *Copidosoma floridanum* (Hymenoptera: Encyrtidae): *in vivo* and *in vitro* analysis. J. Insect Physiol 43:553-565

Grbic M, Nagy LM, Strand MR. 1998. Development of polyembryonic insects: a major departure form typical insect embryogenesis. Dev Genes Evol 208:69-81

Guerrier E and Noyes J. 2005. Revision of the European species of *Copidosoma* Ratzeburg (Hymenoptera: Encyrtidae), parasitoids of caterpillars (Lepidoptera). Systematic Entomology. 30:97-174

Harvey JA, Corley LS, Strand MR. 2000. Competition induces adaptive shifts in caste ratios of a polyembryonic wasp. Nature. 406:183-186

Hendzel MJ, Wei Y, Mancini MA, Van Hooser A, Ranalli T, Brinkley BR, Bazett-Jones DP, Allis CD. 1997. Mitosis-Specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma. 106:348-360

Huang P and Stern MJ. 2005. FGF signaling in flies and worms: More and more relevant to vertebrate biology. Cytokine & Growth Factor Reviews. 16:151-158.

Ivanova-Kasas OM. 1972. Polyembryony in Insects. In *Developmental Systems, Insect.* Eds. Counce SJ and Waddington CH. Academic, New York 243-271.

Iwabuchi K. 1991. Early embryonic development of a polyembryonic wasp, *Litomastix maculata* Ishii, *in vivo* and *in vitro*. Appl. Ent. Zool. 26(4):563-570

Jarecki J, Johnson E, Krasnow MA. 1999. Oxygen regulation of airway branching in *Drosophila* is mediated by Branchless FGF. Cell. 99:211-220.

Kaeslin M, Wehrle I, Grossniklaus-Burgin C, Wyler T, Guggisberg U, Schittny JC, Lanzrein B. 2005. Stage-dependent strategies of host invasion in the egg-larval parasitoid *Chelonus inanitus*. Journal of Insect Physiology. 51:287-296.

Ladurner P, Rieger R, Baguna J. 2000. Spatial distribution and differentiation potential of stem cells in hatchlings and adults in the marine Platyhelminth *Macrostomum* sp.: a Bromodeoxyuridine analysis. Developmental Biology. 222:231-241

Lee LA and Orr-Weaver TL. 2003. Regulation of cell cycles in *Drosophila* development: intrinsic and extrinsic cues. Annual Review of Genetics. 37:545-78.

Madhavan MM and Schneiderman HA. 1977. Histological analysis of the Dynamics of growth of imaginal discs and histoblast nests during the larval development of *Drosophila melanogaster*. Wihelm Roux's Archives. 183:269-305.

Newmark PA, Sanchez Alvarado A. 2000. Bromodeoxyuridine specifically labels the regenerative stem cells of Planarians. Developmental Biology. 220:142-153

Ode PJ, Strand MR. 1995. Progeny and sex allocation decisions of the polyembryonic wasp *Copidosoma floridanum*. Journal of Animal Ecology. 64:213-224

Patterson JT. 1921. The development of *Paracopidosomopsis*. J. Morph. 36:1-69

Petit V, Ribeiro C, Ebner A, Affolter M. 2002. Regulation of cell migration during tracheal development in *Drosophila melanogaster*. Int. J. Dev. Biol. 46:125-132.

Popovici C, Roubin R, Coulier F. 2005. An evolutionary history of the FGF superfamily. BioEssays. 27:849-857.

Samakovlis C, Hacohen N, Manning G, Sutherland DC, Guillemin K, Krasnow MA. 1996. Development of the *Drosophila* tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. Development. 122:1395-1407.

Smith AV and Orr-Weaver TL. 1991. The regulation of the cell cycle during *Drosophila* embryogenesis: the transition to polyteny. Development. 112:997-1008.

Stathopoulos A, Tam B, Ronshaugen M, Frasch M, Levine M. 2004. pyramus and thisbe: FGF genes that pattern the mesoderm of *Drosophila* embryos. Genes and Development. 18:687-699.

Strand MR. 1989. Developmental synchrony between the polyembryonic parasitoid *Copidosoma floridanum* and its host *Trichoplusia ni*. Entomol. Exp. Appl. 50:37-46

Strand MR. 2000. Developmental traits and life-history evolution in parasitoids. In *Polulation Biology of Parasitoids*. Eds. Hochberg CM and Ives AR. Princetion University Press. 139-162.

Strand MR. 2003. Polyembryony. In: Encyclopedia of Insects (R. Carde and V. Resch, eds.). Academic Press. 928-932.

Strand MR and Grbic. 1997. The Development and Evolution of Polyembryonic Insects. Current topics in Developmental Biolgy. 35:121-157.

Sutherland D, Samakovlis C, Krasnow. 1996. *branchless* encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. Cell. 87:1091-1101.

Thisse B and Thisse C. 2005. Functions and regulations of fibroblast growth factor signaling during embryonic development. Developmental Biology. 287:390-402.

Truman JW and Bate M. 1988. Spatial and temporal patterns of neurogenesis in the central neverous system of *Drosophila melanogaster.* Developmental Biology. 125:145-157.

Whitefield JB. 2003. Phylogenetic insights into the evolution of parasitism in Hymenoptera. Advances in Parasitiology. 54:69-100.

Wong MD, Jin Z, Xie T. 2005. Molecular mechanisms of germline stem cell regulation. Annual Review of Genetics. 39:173-195.

Wray GA. 2000. The evolution of embryonic patterning mechanisms in animals. Cell & Developmental Biology. 11:385-393

Zelzer E and Shilo B-Z. 2000. Cell fate choices in *Drosophila* tracheal morphogenesis. BioEssay. 22:219-226.