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Evolutionary Conservation of a Modified Spermatogenesis Program in Rhabditis Nematodes with Skewed Sex Ratios

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Evolutionary Conservation of a Modified Spermatogenesis Program in *Rhabditis* Nematodes with Skewed Sex Ratios

A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honors in Biology at the College of William & Mary.

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Abstract:

The cellular divisions in *Rhabditis* sp. SB347 male spermatogenesis have been modified to re-purpose an asymmetric division. This results in the dramatically skewed sex ratios observed in the progeny of males of this species. Here we confirm this asymmetry in the division of tubulin and major sperm protein (MSP). MSP, a necessary sperm component, is segregated exclusively into spermatids bearing the X chromosome while tubulin is segregated into the nullo-X sperm. Timing of the partitioning events reveals that MSP migration is not directly dependent on tubulin spindle asymmetry. Additionally, the endoplasmic reticulum is also segregated asymmetrically to the nullo-X spermatid during the partitioning phase of spermatogenesis. This results in only the X-bearing spermatids being functional, and thus the exclusive production of feminine offspring.

This pattern of asymmetry appears to not be isolated to SB347. We investigated several other species in the *Rhabditis* clade including SB372, JU1809, and JU1782. Using MSP and tubulin as markers, each species was noted to display asymmetric divisions very similar to those in SB347. This corresponds with data suggesting that these species demonstrate similar skews in the sex ratios of offspring (Pires daSilva, unpublished).

Not only do these species display unusual cellular divisions, but the size of the spermatids is greatly diminished compared to other closely related nematodes that have been studied. These two features make the species in the *Rhabditis* clade interesting subjects of study. They may serve as models to bolster our current understanding of cellular polarization in spermatogenesis and the mechanisms of distinction between residual body and spermatid. They may also yield important insights into the evolution of sex and gamete size.

Lastly, evidence suggests that the non-functional sperm, lacking the X chromosome, produced by *Rhabditis* sp. SB347 form large clusters in the male gonad. The fate of these aggregates is unknown, but they appear to be removed from the spermatid population. This draws some parallels to the apoptotic fate of the residual body in *C. elegans*. Study of the *Rhabditis* nematodes is just beginning, but it promises some interesting findings and novel insights into nematode biology.

Section 1: Introduction

Nematode Diversity

The phylum Nematoda is a remarkably diverse group of organisms. Despite a superficially similar body plan, there is great genetic distance and rich variation in the biology of nematodes. Nematodes include parasitic species as well as free-living organisms and all variations in between. They seem to inhabit every possible habitat on earth, ranging from desert environments to deep-sea sediments (De Ley, 2006). Nematodes have evolved to make use of almost any available food source. There are species that represent herbivores, carnivores, omnivores, and those that feed on microscopic bacteria, fungi, or algae (Wharton, 1986).

Nematodes even inhabit the most prohibitive environments. Two species of the genus *Cryonema* live their entire lives below the freezing point in arctic ice (Tchesunov & Riemann, 1995). These may be extreme examples, but it illustrates how proliferative the members of the phylum have been in evolving to fit into novel habitats. Amid this profound diversity, some researchers have developed a bias toward viewing *C. elegans* as the archetypal nematode. While *C. elegans* continues to serve as an excellent model organism, significant biological diversity likely goes overlooked in the countless other nematode species.

The phylum Nematoda is divided into three major groupings, the Chromadoria, Enoplia, and Dorylaimia (De Ley, 2006). The model organism *Caenorhabditis elegans* falls within the Chromadoria lineage, as do all of the nematodes we will discuss here.

Caenorhabditis elegans as a Model Organism

The nematode *Caenorhabditis elegans* is a free-living soil nematode. It has a short generation time of about three days during which it passes through four larval stages (designated L1-L4) before reaching adulthood and sexual maturity. Adult hermaphrodite worms are roughly 1.5mm in length, and can produce 300-350 progeny through self-fertilization (Riddle et al., 1997). Under conditions of environmental stress, the regular developmental pathway can be diverted at the L1 stage through an alternative form called the dauer stage. Dauer worms display environmental resilience, low metabolism, and dispersive behavior.

C. elegans was largely popularized by the work of Sydney Brenner. He desired an organism in which to study the effects of genetics on complex systems, such as the nervous system (Brenner, 1974). Other systems such as *Drosophila* were so complex as to be nearly impossible to begin studying, bearing roughly 10^5 neurons. In an attempt to study genetic effects on complex systems, he proposed that beginning with the simplest available form of the complex system would be the most informative. *C. elegans*, with roughly 300 neurons proved a much more desirable organism.

Brenner saw *C. elegans* as an ideal model organism for many reasons. It has large genetic units, bearing 5 pairs of autosomes and one pair of sex chromosomes (Nigon, 1949). Individuals with a single X chromosome are males while those with two X chromosomes are self-fertile hermaphrodites, somatic females that produce ~300 sperm before switching over to oogenesis (Ellis & Schedl, 2006). This chromosomal pattern of sex determination appears to be common among nematodes (Pires-daSilva, 2007). It bears

resemblance to familiar systems of sex determination in humans and other mammals, but lacking the additional Y chromosome in addition the single X in males.

Thanks to the presence of hermaphrodites, mutant lines can be propagated through either selfing or through male-hermaphrodite out-crossing. Brenner also noted the short generation time as being crucial for ease of study, especially in genetics. Lastly, significant work had already established the nutritional needs of *C. elegans*, making laboratory culture fairly simple (Dougherty et al., 1959). These properties make *C. elegans* a powerful tool for laboratory work. Not only is it good for genetic work, but has also proved critical in developmental studies and many other fields of biology.

Extensive developmental study has led to the mapping of cellular divisions, and the discovery that the somatic cellular divisions during both embryonic development and in the developing larvae are invariant (Sulston & Horvitz, 1977; Deppe et al., 1978). This is incredibly powerful information when applied to developmental studies in the organism. Alterations in the division of a single cell can now be determined based on this work.

Incredibly powerful genetic tools have been developed in *C. elegans.* Mutant lines have been used to understand many cellular processes, including spermatogenesis. It was the first multicellular organism to have its genome sequenced. Mutagenesis and subsequent deep sequencing has yielded many mutant strains that can be used to study gene function. The method of RNA interference (RNAi) was first discovered and developed in *C. elegans* (Fire et al., 1998). This provided a targeted means to probe gene function without having to develop mutant lines. More recently, methods utilizing the CRISPR-Cas9 system have been developed for targeted genome editing in *C. elegans* (Friedland et al., 2013). This allows for production of mutant lines for genes of the researcher's choice.

Spermatogenesis in *C. elegans*

The ultimate goal of spermatogenesis is to produce male gametes in order to fertilize oocytes. To be competitive, sperm must be able to move quickly. Therefore, there is a need to discard cellular components that are not necessary for sperm function. In *C. elegans*, this is accomplished through the formation of a residual body, a cytoplasmic compartment produced from the spermatocytes following divisions in meiosis II. It serves as a receptacle to discard unnecessary cellular components and excess cytoplasm. This cytoplasmic shedding as a mechanism to "streamline" the motile gamete is a common feature of spermatogenesis in diverse organisms, though it typically occurs much later in the process of sperm development (Kato et al., 1996).

The sexual dynamics of *C. elegans* are interesting in that normal populations of *C. elegans* are predominantly hermaphrodites. A rare X-chromosome nondisjunction event during hermaphrodite meiosis results in male progeny appearing as approximately 0.1% of offspring (Hirsh et al., 1976). When males mate with a hermaphrodite, they sire progeny that are 50% male, increasing their number slightly in the population.

In *C. elegans* males, spermatogenesis occurs continuously throughout adulthood. In hermaphrodites, spermatogenesis occurs during the fourth larval stage before the gonad switches over to exclusively produce oocytes. Oocyte production continues throughout adulthood, so all hermaphrodite-produced sperm that remain in the spermatheca will be able to fertilize. Here we will focus on spermatogenesis in the males. Progression of meiosis occurs linearly along the length of the gonad, beginning at the distal tip (Figure 1-1a). Near the distal tip, a number of germline stem cells are maintained by signaling from a somatic cell known as the distal tip cell (DTC) (Kimble & Crittenden, 2007). These cells are

maintained through mitotic divisions. They remain as syncytia, attached to a central canal of cytoplasm known as the rachis (Riddle et al., 1997).

As the germline stem cells migrate away from the DTC, losing the localized signaling, they enter into the transition zone. At this point they leave mitosis and begin specification and entry into meiosis. During an extended pachytene stage, the paired homologs undergo crossing-over events (Goldstein, 1982). Following synapsis, the cells enter into a karyosome stage in the condensation zone. During this phase, the chromosomes detach from the nuclear envelope and form a single compacted mass (Shakes et al., 2009). At the end of the karyosome stage, the individual spermatocytes separate from the rachis and rapidly enter into meiotic divisions (diagrammed in Figure 1-1b).

Based on work by Ward and coworkers (1981), we know that meiotic divisions in *C. elegans* yield four spermatids from each primary spermatocyte (Figure 1-2). Primary spermatocytes contain homologous pairs of each of the chromosomes and their sister chromatids. During the first meiotic division, homologous pairs separate, and the unpaired X bivalent segregates to one of the two secondary spermatocytes produced. Following meiosis I, the cytoplasmic division is often incomplete, leaving a cytoplasmic bridge between the secondary spermatocytes. During meiosis II, the sister chromatids separate. Following completion of anaphase II, the transient cleavage furrow recedes and two secondary furrows form establishing the central residual body. Cellular components necessary for sperm function partition to the spermatids and components unnecessary for further sperm development and excess cytoplasm remain in the residual body.

The fibrous body-membranous organelle (FB-MO) is an organelle unique to nematode spermatogenesis. The MO is a membranous structure derived from the Golgi

apparatus and the FB develops in close association (Roberts et al., 1986). The FB is largely comprised of polymerized major sperm protein (MSP). The FB-MO complex appears to serve as a vehicle for the segregation of necessary cellular components to the spermatids.

Unlike in flagellated sperm, which often use tubulin for motility, nematode sperm use MSP as the key motility protein (Roberts & Stewart, 2000). MSP is therefore necessary for sperm function, and would therefore be expected to segregate into the spermatids. Tubulin is critical for the segregation of chromosomes during meiosis, but serves little function in nematode sperm after. Tubulin in *C. elegans*, apart from the centrioles, ultimately segregate to the residual body. This makes these components ideal for use as markers in these cellular divisions.

The tubulin spindles provide the motive force to separate the chromosomes during meiotic divisions (Figure 1-2a). Following anaphase II, entering the phase we refer to as partitioning, tubulin detaches from the poles, and is segregated into the residual body. Since tubulin in not necessary for sperm motility in *C. elegans*, it is discarded. This ultimately results in residual bodies that stain brightly for tubulin. Conversely, spermatids lack any significant staining for tubulin, although like all sperm, they do contain a centriolar pair.

We can see that MSP localized to the fibrous bodies remains distributed throughout the cytoplasm during meiosis I and metaphase II (Figure 1-2b). However, in anaphase II, we see that the fibrous bodies begin to get excluded from the center of the spermatocyte. A distinct residual body is not apparent until late in anaphase II. In partitioning, the FB-MOs are segregated exclusively into the spermatids. Once spermatids have separated from the residual body, the fibrous bodies depolymerize resulting in diffuse MSP staining

throughout the spermatids. The residual bodies can be seen devoid of significant MSP staining.

Rhabditis sp. SB347

Most study of the *Rhabditis* clade has been focused on *Rhabditis* sp. SB347. This species was first isolated by the lab of Walter Sudhaus at the Freie Universität Berlin. The particular strain was isolated from the back of a deer tick, *Ixodes scapularis*. The species is curious in that it is trioecious, featuring male, female, and hermaphrodite sexes (Félix, 2004). Much like in *C. elegans*, males appear to be distinguished from the feminine sexes by an XX:XO system (Shakes et al., 2011),

The feminine worms are further divided into female and hermaphrodite worms. Female worms can only reproduce by mating with males. Adult hermaphrodites are somatically identical to females, but they produce a number of sperm during a transient developmental stage before switching to oogenesis (Félix, 2004). Hermaphrodite worms are therefore able to fertilize themselves with their limited supply of sperm to produce progeny. While they appear to be chromosomally identical, the female-hermaphrodite designation in SB347 is coupled to a divergence in their developmental pathways. Feminine worms that pass through the dauer phase develop into hermaphrodites, and this occurs even without the stressful conditions needed for *C. elegans* dauering (Chaudhuri et al., 2011). When conditions are held constant, their developmental pathway appears to be determined somewhere prior to or during the L1 phase. Some data suggests that this specification, while still maintaining plasticity, is made during embryonic development within the parent (Shakes, unpublished). L1 worms that would be females, when cultured

in dauer-inducing conditions (cholesterol depleted medium and antibiotic-killed food), will alter their fate to become hermaphrodites. Conversely, hermaphrodite-destined L1 larvae treated with the dauer-inhibitor dafachronic acid, a sterol-derived hormone, will develop into females (Chaudhuri et al., 2011). Therefore, passage of feminine SB347 worms through the dauer phase appears to be both necessary and sufficient to produce hermaphrodite adults.

Another curiosity of *Rhabditis* sp. SB347 is the fact that male worms only sire small numbers of male offspring. In crosses with females, male worms only produced 1.6% male offspring (Félix, 2004). This pattern appears to conflict with Mendel's Law of Segregation. Half of gametes produced by males would be expected to carry the X chromosome, producing feminine worms, and half should carry no X chromosome, producing males. Also of interest is the fact that hermaphrodite selfing yields low numbers of males, but significantly higher than the numbers seen in *C. elegans* (Figure 1-3). Male crosses with hermaphrodites produce similar proportions of male progeny to selfing hermaphrodites, but male crosses with females produce fewer numbers. This suggests that the production of male offspring in crosses may not be dependent on the male contribution of nullo-X gametes, but may instead result from X-chromosome loss during oocyte meiosis.

The mechanism for the lack of male progeny produced by male crosses has been determined to be an asymmetric cell division during meiosis II of male spermatogenesis (Figure 1-4; Shakes et al., 2011). No residual bodies are produced, with the nullo-X spermatid receiving the tubulin. The MSP-containing FBs also segregate exclusively to the X-bearing spermatids. This uneven division of cellular components results in each primary spermatocyte producing only two functional spermatids, rather than the canonical four.

These two functional sperm produced each bear an X chromosome, which will produce feminine progeny upon fertilization of an X-bearing oocyte.

Other *Rhabditis* Nematodes

Rhabditis sp. SB347 has been an interesting subject of study. It has several unique characteristics that make it a fascinating subject. While it may be interesting because of its novelty, the question remains whether or not it has broader significance among nematodes. Animal parasitic nematodes are thought to be evolutionarily derived from free-living soil nematodes (Anderson, 1983). Some nematodes, such as SB347, display a form of dispersive behavior known as phoresy, the utilization of other organisms (e.g. bettles and ticks) as vehicles for long-distance transportation. This is hypothesized to be an intermediate behavior between free-living and parasitism. In addition to their usual dispersive behavior, dauer larvae of some species display head waving behavior, thought to be an adaptive behavior in phoretic worms to increase chances of contacting another organism (Kiontke & Sudhaus, 2006). This behavior is notable in *C. elegans* and *Rhabditis* sp. SB347. This combined with the fact that SB347 was originally isolated from the back of a tick seems to suggest that phoresy is part of its ecological niche. Therefore, these nematodes may serve as a good model organism for parasitic nematodes which often prove very difficult to culture in the laboratory, making scientific study challenging.

In order to make sense of the characteristics noted in SB347, we must look at the context of the species. Currently, very little study has been done on nematodes in the *Rhabditis* clade. Several related nematodes (Figure 1-5) appear to be promising candidates. Species SB372, JU1809, JU1782, and JU1783 appear to display skewed sex ratios

reminiscent of the patterns seen in SB347 (Pires daSilva, unpublished). Study of these worms will help to better understand SB347 and the significance of its unusual traits.

The scope of this study

The investigational scope of this study is tripartite. The first goal of this study is to confirm and further characterize the asymmetric divisions that have been previously noted in *Rhabditis* sp. SB347. More specifically, I aimed to characterize the relative timing of known events and to use additional markers to study the extent of the asymmetry. My second aim was to investigate male spermatogenesis in several closely related species of nematodes with the goal of determining whether the SB347 isolate is an outlier, or if the patterns previously documented are characteristic of the entire clade. Thirdly, observations have shown that the sperm produced by several of the *Rhabditis* species are greatly diminished in size. Here I have compared the size of spermatids in several species in an attempt to understand this pattern of cellular miniaturization.

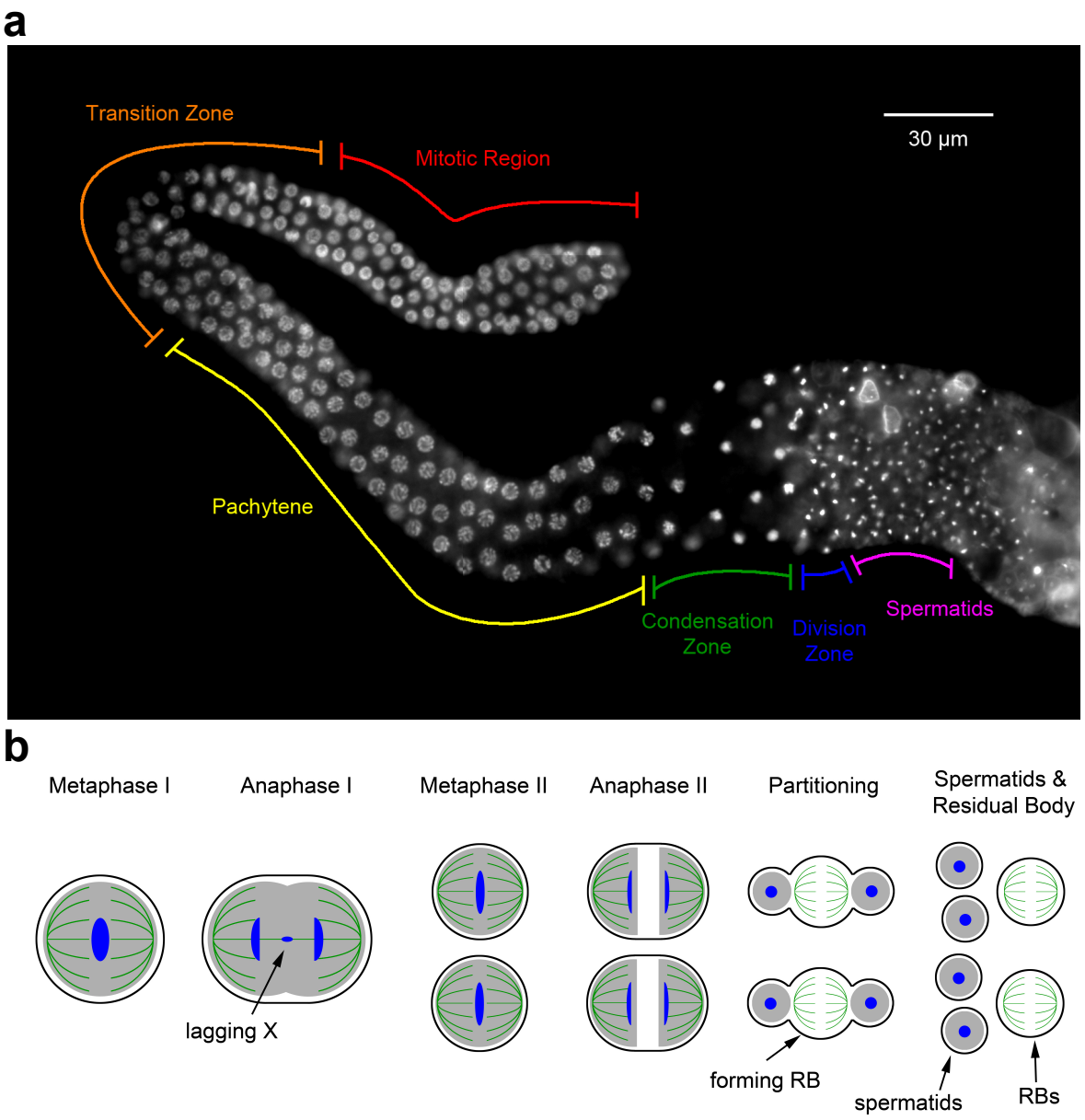

Figure 1-1: *Caenorhabditis elegans* Male Germ Line and Meiotic Divisions. (a) The germline development progresses along the length of the gonad beginning at the distal tip. Germline stem cells are maintained through mitosis in the mitotic zone. As cells progress away from the distal tip, they transition to meiotic programs. Synapsis occurs during the pachytene stage, and then the chromatin condenses through a karyosome stage. Meiotic divisions occur rapidly in the division zone producing a collection of spermatids. These will not be activated until mating has occurred. **(b)** Diagram showing localization of several cellular components during the meiotic divisions that occur within the division zone, progressing from left to right. Blue represents DNA, green lines represent microtubules, and gray shading represents MSP contained within FB-MOs. A lagging X chromosome is apparent in anaphase I, and MSP begins segregation during anaphase II. During partitioning, tubulin is partitioned to the forming residual body.

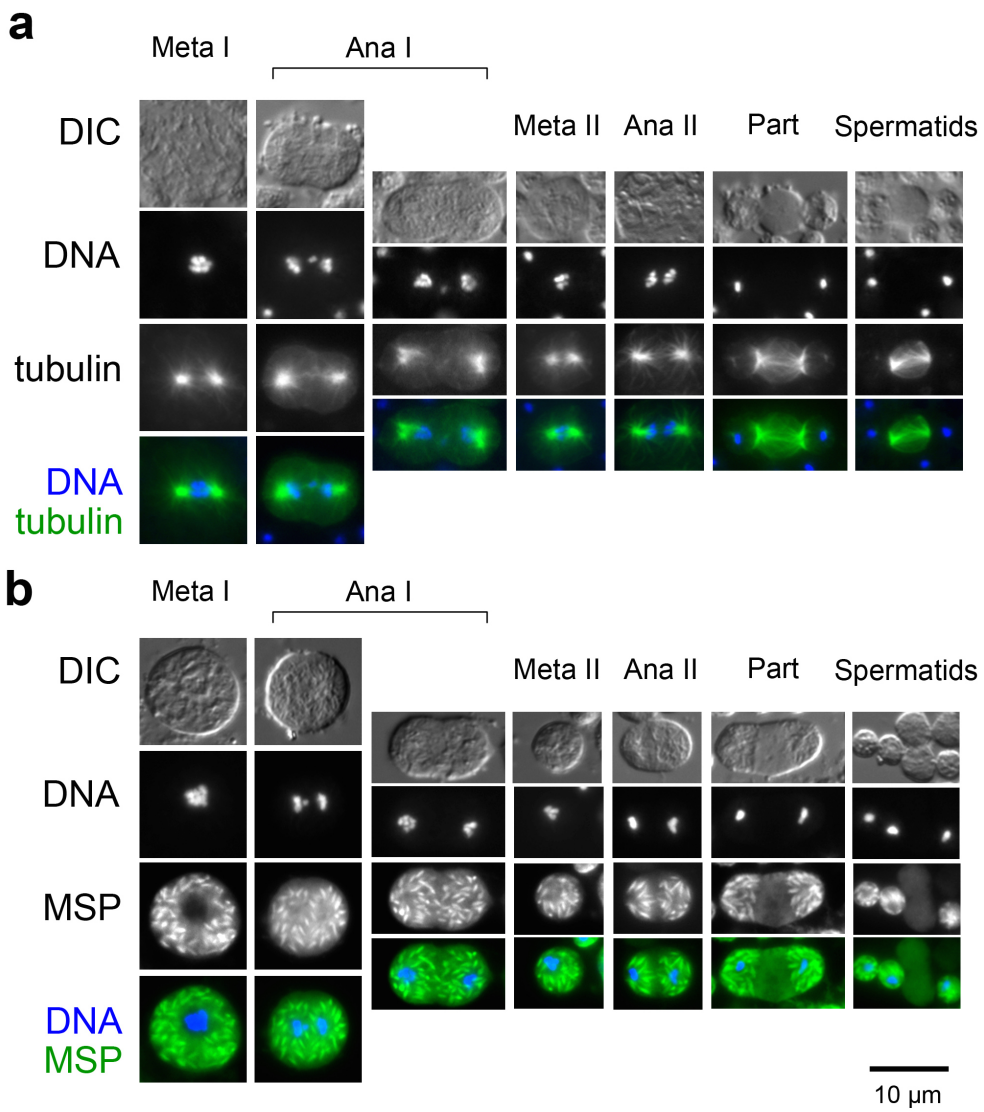

Figure 1-2: Male Spermatogenesis in *C. elegans.* DIC and immunocytology of methanol-fixed sperm spreads of approximately 80 male gonads for tubulin and 160 for MSP Meiosis progresses from left to right through metaphase and anaphase of meiosis I and II followed by a partitioning event resulting in spermatids. A lagging X-chromosome is apparent in early anaphase I DNA staining. **(a)** Immunostaining of α-tubulin reveals that when chromatin reaches the poles in anaphase II, the spindles disassemble and migrate toward the center. It gets partitioned to the residual body, visible as the round mass between the spermatids with bright tubulin staining. **(b)** Immunostaining of MSP reveals that in anaphase II, the fibrous bodies containing MSP are segregated to the two poles. The MSP is ultimately partitioned into the spermatids to the exclusion of the residual body, seen as the elongated mass in the center of the spermatid panel.

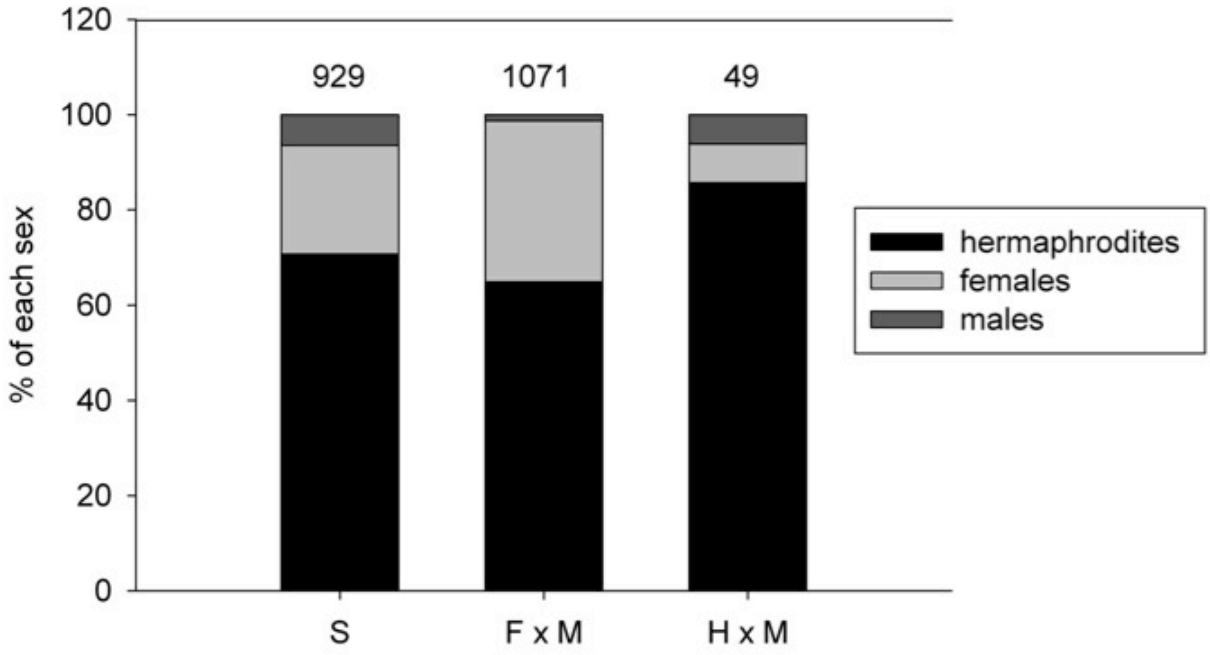

Figure 1-3: Sexes of Offspring in *Rhabditis* sp. SB347. The sexes of the offspring of SB347 vary depending on the nature of the reproductive event. Hermaphrodite selfing (S) curiously produces low numbers of males, significantly more than in selfing *C. elegans*. Hermaphrodite crosses with males (H x M) yield offspring with similar numbers of males, but with an increased proportion of hermaphrodites within the feminine worms. Female crosses with males yield low numbers of males as well. (Pires daSilva, unpublished data).

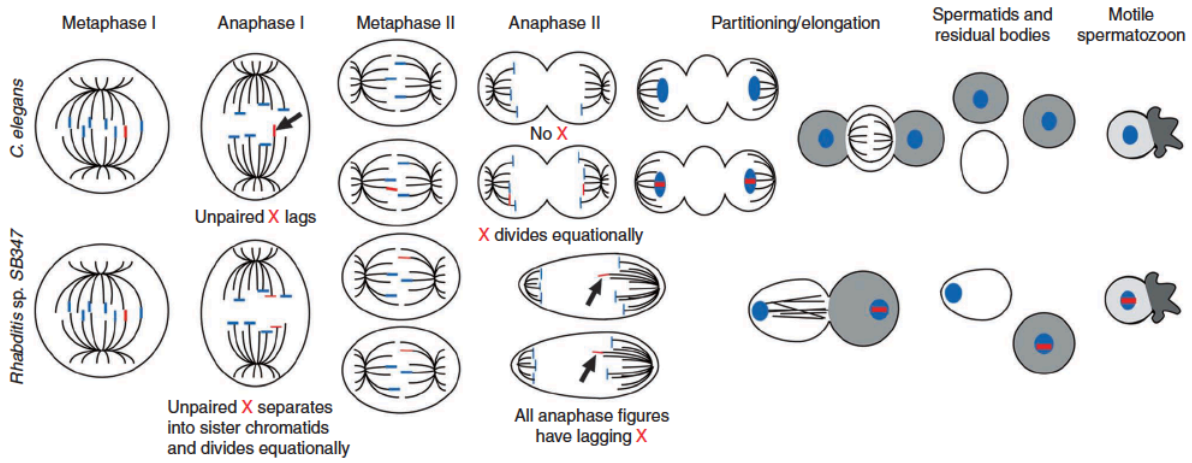

Figure 1-4: Schematic of Spermatocyte Divisions. This schematic outlines the divisions seen in *C. elegans* and *Rhabditis* sp. SB347. Chromosomes line up at the metaphase plate during meiosis I. In anaphase I, the homologous chromosomes separate. In *C. elegans*, the X chromatids segregate to one side. The unpaired nature results in a lagging X chromosome visible. In SB347, the X chromatids appear to separate, and no lagging X is noted. However, the lagging X chromosome appears in anaphase II because the chromatid does not have a sister. The tubulin spindle also becomes asymmetric, larger on the side with the X chromosome. When anaphase II completes, the chromatin condenses, and the cells enter the partitioning phase. *C. elegans* produces four spermatids containing MSP (shown as gray shading) and a residual body. No residual bodies are noted in SB347, but the nullo-X spermatids appear to take on the function of the residual body. (Figure from Shakes et al., 2011).

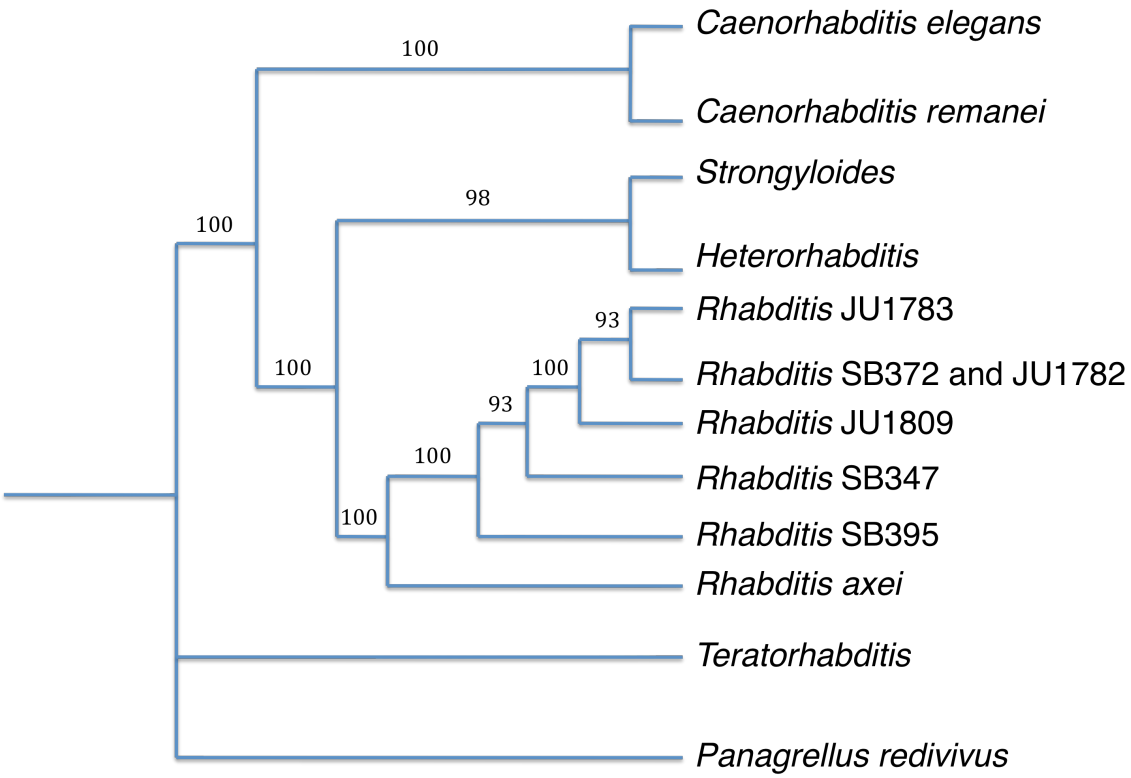

Figure 1-5: Molecular Phylogeny of Several Nematode Species. This molecular phylogeny was constructed using 18S rRNA sequence data from the respective species. It demonstrates the relatedness of several of the studied species and shows their relation to other clades within Nematoda. (Kiontke & Fitch, unpubished data)

Section 2: Experimental Results

Methods and Materials:

Maintenance of Strains

All nematode strains were maintained on plates of MYOB agar (Church et al, 1995). The surface of each plate was seeded with the *E. coli* uracil auxotroph mutant strain OP50 as a food source (Brenner, 1974). The mutant is used to prevent bacterial overgrowth. Strains were maintained at room temperature.

The *C. elegans* mutant strain *him-8 (e1489)* was used because of increased prevalence of males (Hodgkin et al., 1979). The *Rhabditis* species with the SB strain designation (SB347 and SB372) were isolated by the lab of Walter Sudhaus at the Freie Universität Berlin in Berlin, Germany. Species bearing the JU strain designation (JU1809 and JU1782) were isolated by the lab of Marie-Ann Félix at the Institut Jacques Monod in Paris, France.

Dissection and Tissue Fixation

For dissection, male worms were isolated from culture plates within ~24 hours following the L4-adult transition, approximately 4 days after embryos were laid. Worms were transferred to unseeded MYOB agar plates for several minutes before proceeding with dissection in order to remove excess bacteria.

Dissection was carried out to remove the gonad from the body on ColorFrost Plus slides (Fisher Scientific) using a 30-gauge syringe needle in 5 μL of Edgar's buffer (Edgar, 1995) containing 0.25 mM levamisole as an anesthetic. After the gonad was dissected out and separated from the intestines and remainder of the worm body, a coverslip with four

small dots of silicone grease was placed on top of the dissected gonads with slight pressure in order to prepare a sperm spread. Due to smaller gonad size, more pressure is necessary for the preparation of sperm spreads in the *Rhabditis* species than in *C. elegans* or *R. axei*. Gentle movement of the coverslip was used to aid the separation of individual spermatocytes from the mass of cells, though care must be taken not to lyse the cells. Following dissection, sample slides were immediately frozen in liquid nitrogen. The coverslips were removed by freeze-cracking, then immediately placed in Coplin jars containing 100% methanol cooled to -20°C containing 3Å molecular sieves to maintain dryness. Methanol fixation was carried out for a minimum of 24 hours at -20°C before proceeding to immunostaining procedures.

Immunostaining

After completion of fixation, the slides, containing approximately 10 dissected worms each, were washed three times in Phosphate Buffered Saline (PBS) for 10 minutes each wash. The PBS washes were followed by a 20 minute wash in blocking solution (PBS containing 0.5% Bovine Serum Albumin, 0.04% sodium azide, and 0.1% Tween-20) to reduce non-specific antibody binding.

Tubulin staining:

A dilution of 1:80 was prepared using FITC conjugated anti-α-tubulin monoclonal antibody DM1A (Sigma) in antibody buffer (PBS containing 0.5% BSA and 0.04% sodium azide). 25μL of the solution were applied to each slide. They were then incubated at room temperature in a humidity chamber for 1.5-2 hours in darkness. Incubation was followed

by a dip wash in PSB and application of a cover slip with 7μL of Fluoro Gel II mounting medium with DAPI (Electron Microscopy Sciences).

MSP Staining:

A dilution of 1:300 was prepared using mouse anti-MSP monoclonal 4D5 clone N2 (provided by David Greenstein). 25μL of the dilution was applied to each slide, which were then incubated for 2 hours in a humid chamber. A 1:80 dilution of the secondary antibody was prepared with DyLight 488-conjugated affinity-purified goat anti-mouse IgG (Jackson ImmunoResearch). Following a 2 minute PBS wash, 25μL of the secondary dilution were applied to each slide followed by a 1.5 hour incubation in darkness. Following secondary incubation, slides were washed in PBS for 3 minutes and a cover slip was applied with 7μL of the DAPI-containing mounting medium.

Cyp-33E1 staining:

Cyp33-E1 is a member of the cytochrome P450 superfamily which are localized to the endoplasmic reticulum (Chakrapani et al., 2008). 25μL of undiluted CYP33E1 antibody (Developmental Studies Hybridoma Bank, University of Iowa) (Hadwiger et al., 2010) solution were applied to each slide. Slides were incubated for 2 hours in a humid chamber. Following a 3 minute wash in PBS, 25μL of a 1:80 dilution of DyLight 488-conjugated affinity-purified goat anti-mouse IgG (Jackson ImmunoResearch) were applied. Slides were incubated for 2 hours in darkness followed by a 3 minute PBS wash. Slides were dipped in dH_2O and a coverslip was applied with 7μL of DAPI-containing mounting medium.

Imaging of Stained Tissue

All slides were imaged on an Olympus BX60 epifluorescence microscope using a QImaging EXi Aqua CCD camera. An Olympus PlanApo 60x objective lens was used to take all images. Nomarski optics were used for the differential interference contrast (DIC) imaging. Photos were taken, merged, and exported for analysis using the program iVision. Minimal processing in Adobe Photoshop was used to produce figures.

Spermatid Size Data

The sperm size data was obtained from unfixed sperm spreads prepared from young adult worms. Male worms were transferred to unseeded MYOB agar plates to remove excess bacteria. Dissection was carried out as before, but with the addition of Hoechst DNA stain to the dissecting buffer. Minimal pressure was used upon application of the coverslip to preserve the native shape of the cells. Images were taken using Nomarski DIC with the same 60x objective lens. Spermatids were identified based on their relative size to other germ cells and their chromatin morphology. Analysis of spermatid size was carried out in Adobe Photoshop using analytic tools to determine the cross-sectional area of the cells. Approximately five dissected gonads, with roughly 20-30 spermatids per gonad, were used to obtain the data for each species, and no significant variations in spermatid size were noted between individuals.

Results:

Confirmation of Asymmetry in *Rhabditis* sp. SB347 Male Spermatogenesis

Since sex appears to be chromosomally determined in the species, *Rhabditis* sp. SB347 is curious in that mating events between male and female worms only produce around 1.6% (4/253) male offspring (Félix, 2004). This inheritance pattern appears to violate patterns of Mendelian inheritance. The surprising lack of male progeny suggests that something unusual is happening in spermatogenesis. Previous work has shown that this effect is accomplished through asymmetric cell divisions in spermatogenesis (Shakes et al., 2011).

As has been previously documented, *Rhabditis* sp. SB347 demonstrates a dramatic asymmetry in the secondary spermatocyte divisions (Figure 2-1). Metaphase and anaphase of meiosis I proceed symmetrically across a plane orthogonal to the spindle axis. The division of the primary spermatocyte lacks the clear lagging X chromosome that is present in *C. elegans* spermatogenesis (Figure 1-2). In *C. elegans*, this lagging X chromosome is thought to be due to the fact that it is unpaired to a homologue (Albertson & Thomson, 1993).

In *C. elegans*, homologous chromosomes separate during meiosis I, with a pair of sister chromatids present in the secondary spermatocytes. Without a homologous pair, the X chromatids lag during anaphase I, but are ultimately separated into one of the secondary spermatocytes. In SB347, the lagging X is displaced to meiosis II, suggesting that there is a symmetric division involving the X chromosomes during anaphase I. This seems to suggest that the sister chromatids split during meiosis I while the autosomes separate from their

homologues (Shakes et al, 2011). During meiosis II, the X chromatids are now unpaired resulting in the lagging behavior.

Based on the immunocytology, metaphase and early anaphase of meiosis II begin symmetrically in SB347, with tubulin spindles being evenly sized (Figure 2-1a). Early anaphase II reveals an X chromosome lagging significantly behind the other chromosomes in the division.

Approximately 80 stained gonads were scored to determine the progression of events in anaphase II and partitioning using chromatin morphology as the landmark. Partitioning is identifiable by formation of a cleavage furrow, separation of the tubulin spindle from the centrosome, and supercondensation of chromatin into circular masses. 100% (11/11) cells with the lagging X chromosome in the center of the two chromatin masses showed symmetric tubulin spindles. Once the X chromosome began to segregate to one side 98% (39/40) showed spindle asymmetry with the larger spindle on the side receiving the X chromosome. Only one cell demonstrated symmetric spindles with a migrating chromosome. When the X chromosome reaches a pole, the chromatin condenses and the cell enters what we refer to as the partitioning phase. Of the cells showing supercondensed chromatin (circular shape without distinct chromosomes, as seen in partitioning in Figure 2-1) 5% (2/43) retained clear asymmetry in the tubulin distribution favoring the larger chromatin mass, indicative of the X chromosome. 33% (14/43) had tubulin distributed evenly throughout the cytoplasm and 63% (27/43) showed tubulin concentrated at the smaller chromatin mass, the nullo-X pole. We propose that immediately upon entry into partitioning, the tubulin remains concentrated at the X-bearing pole. However, it quickly begins migrating and ultimately becomes concentrated at the nullo-X

pole. The percentages of cells noted at each stage potentially provide a rough estimate of the relative rate of each step in the migration.

We expect to see FB-MOs displaying opposite polarization, ultimately segregating to the X-bearing side. Looking at MSP staining as a marker (Figure 2-1b), 100% (11/11) cells with the lagging X chromosome positioned in the center of the chromatin masses showed even distribution of MSP throughout the cytoplasm. 100% (32/32) of cells with the X chromosome displaced to one side also showed an even distribution of MSP throughout the cytoplasm despite the cell shape quickly becoming asymmetric. Upon chromatin condensation, 11% (4/37) cells displayed even distribution of MSP and 89% (33/37) showed asymmetry in distribution favoring the larger chromatin mass.

From these data, a rough series of events can be constructed. During meiosis I, the sister chromatids of the single X chromosome split such that one X chromatid segregates to each of the two secondary spermatocytes. During meiosis II, the singlet X chromatid of each secondary spermatocyte is unpaired and thus lags during anaphase II. Segregation of this X chromatid is concurrent with establishment of asymmetry in the tubulin spindles. Upon chromatin condensation, the tubulin begins migrating toward the nullo-X sperm pole. Likewise, the FBs quickly begin migration toward the X-bearing pole. These separations are completed before cytoplasmic cleavage finishes. From these data, FB-MO segregation appears to be independent of tubulin spindles, since the segregation of the MSP marker does not correspond with the initial tubulin asymmetry. It is not until the centrosome turns off and the tubulin relocalizing towards the non-X pole that MSP polarizes. This differs from the timing of events in *C. elegans*, where FB-MO migration begins during the anaphase II division.

Endoplasmic Reticulum is Segregated During Partitioning in SB347

Based on patterns of division in MSP and tubulin, it appears that the nullo-X spermatid in SB347 takes on the role of the residual body. Actin, a residual body component in *C. elegans*, is also known to partition to the nullo-X spermatid in SB347 (Shakes et al., 2011). In order to further study the extent of this RB character, I decided to look at the partitioning of the endoplasmic reticulum. Based on previous work in the Ward lab, we know that ER segregates into the residual body in *C. elegans*. Cyp33E1 is a member of the cytochrome P450 superfamily that localizes to the endoplasmic reticulum (Hadwiger et al., 2010). In *C. elegans* (Figure 2-2a), there appear to be two patterns of staining visible in spermatocytes. There is diffuse staining that appears to represent the endoplasmic reticulum. However, there is also punctate staining that resembles patterns seen for FB-MOs. This is potentially a result of the fact that the MOs are derived from the Golgi body, which is contiguous with the ER (Roberts et al., 1986). Curiously, the punctate staining seems to disappear quickly after budding of the spermatids. The staining seems to vanish, but this could be the result of the signal being diluted as it disperses from its foci or it may be degraded. The more diffuse staining, presumably representative of the ER, segregates into the central residual body during anaphase and partitioning. This makes sense, as the ER is not necessary for the proper function of a sperm, and would likely be discarded.

Staining for cyp33E1 in SB347 (Figure 2-2b) displays the familiar asymmetry we have previously seen. Punctate staining is not apparent as it is in *C. elegans*, and at some stages there is apparent concentration of the ER around chromatin masses. It displays a segregation pattern that is the opposite of that of FBs (Figure 2-1b). The ER staining

remains distributed throughout the cytoplasm until entry into the post anaphase II partitioning phase as indicated by the state of chromatin condensation. At that point, the cyp33E1 staining quickly segregates towards the nullo-X pole while MSP staining segregates towards the X-bearing pole. Upon completion of the cellular division, two distinct sets of spermatids exist, those displaying cyp33E1 staining, and those without cyp33E1 staining, bearing the X chromosome.

Meiotic Divisions Display Asymmetry in Other Species

While we have shown that *Rhabditis* sp. SB347 displays an asymmetric division during spermatogenesis as a means for producing the noted sex skew, the question arises as to whether this is an isolated case, within the species or even within this specific isolate. To determine whether the observed pattern is an isolated oddity or a characteristic feature of this clade, we investigated the sub-cellular distribution of tubulin and MSP during spermatogenesis in several closely related species. Tubulin and MSP were chosen as markers for the asymmetry because they play a critical role in chromosome segregation and sperm motility, respectively. Additionally, MSP acts as a marker for the functional X-bearing sperm and tubulin serves as marker for the non-functional nullo-X sperm in SB347.

The species *Rhabditis* sp. SB372, *Rhabditis* sp. JU1809, and *Rhabditis* sp. JU1782 were chosen for investigation because they also displayed unusual sex ratios in crosses (Pires daSilva, unpublished). Molecular data also suggests a close relation to *Rhabditis* sp. SB347, making these interesting subjects for study. Analysis was carried out much the same way that it was in SB347. Fixed sperm spreads were immunostained and imaged to

reveal meiotic patterns. From the prepared sperm spreads, the patterns of the divisions were determined.

What was found appeared to be patterns very similar to that of SB347. In SB372 (Figure 2-3), JU1809 (Figure 2-4), and JU1782 (Figure 2-5) the first meiotic division appears to progress symmetrically. In both cases, the lagging X chromosome is distinctly absent from anaphase I. While it is less apparent, there is a lagging X during meiosis II divisions. It never achieves the same level of separation from the other chromatin masses as it does in SB347, potentially due to the smaller size of the cells. However, it is possible to distinguish the X-bearing pole based on the size of the anaphase chromatin mass. Following the X-chromosome, we can see that tubulin spindles quickly become asymmetric in early anaphase II.

Spermatid Size Varies Greatly Between Species

In *C. elegans*, male sperm preferentially fertilize oocytes over hermaphrodite sperm upon mating. This is not due to differences in activation or contents of the seminal fluid, but because the male sperm are competitively superior to the consistently smaller hermaphrodite sperm (LaMunyon & Ward, 1995). The male sperm are therefore able to outcompete the hermaphrodite-produced sperm for fertilization. The driving force behind this competitive advantage is the size of the sperm produced (LaMunyon & Ward, 1998). Larger sperm are able to crawl at a faster rate. physically displace smaller sperm, and have an adhesion advantage in the spermatheca in order to achieve competitive superiority.

It makes sense that producing larger numbers of sperm would give the statistical advantage of fertilizing over competing males, however most sperm contained within the

spermatheca will eventually fertilize an oocytes (Kadandale & Singson, 2004; Ward & Carrel, 1979). In crosses, there is a limited amount of space in the spermatheca, and therefore limited sperm that may fit. As sperm are regularly swept out by passing oocytes, there is competition to re-enter the spermatheca and to exclude competitors. In *C. elegans*, the superiority of larger sized sperm is an important characteristic. This may result in temporary or permanent displacement of competing sperm from spermatheca. The ideal strategy would be to produce large numbers of large sized sperm. Due to nutritional, energetic, and time constraints, a tradeoff must be made between size of sperm and number of sperm produced. Larger sperm take more energy, material, and time to manufacture while smaller sperm are cheap and quick to produce. Additionally, there may be pressure for hermaphrodites to produce smaller sperm in an effort to maximize the number of gametes produced during the transient spermatogenic phase (Baldi et al. 2011). This pressure may carry over to male spermatogensis via shared regulatory pathways (Baldi et al., 2011).

The level of sperm competition in a species appears to be a determinant of the size of male sperm produced (LaMunyon & Ward, 1999). Male members of gonochoristic species must compete with a large number of other males, comprising half of the population, in order to fertilize female worms. Hermaphroditic species, on the other hand, typically have very few males, so there is little sperm competition. Gonochorism was shown to correspond with large male sperm while hermaphroditic species displayed much smaller sizes, even when adjustments were made for phylogeny (LaMunyon & Ward, 1999).

In our experiments, *C. elegans* was used as a reference point due to its prevalence in the literature and its inclusion in previous relevant studies. *Rhabditella axei* was chosen as a gonochoristic species which is closely related to the other *Rhabditis* species. By measuring cross-sectional area of the spermatids, it was determined that *R. axei* produced much larger sperm (60.1 ±7.3μm^2) than *C. elegans* (15.2 ±2.5μm^2). These values were comparable with the data presented in the LaMunyon & Ward (1999) paper. The striking variation in the size of primary spermatocytes can be easily seen in Figure 2-6.

Rhabditis sp. SB347, SB372, and JU1809 present spermatids with significantly diminished size (Figure 2-7). SB347 spermatids were the largest of the three (6.7 ±1.6μm^2) followed by SB372 (4.6 ±0.9μm^2) and JU1809 (4.6 ±0.6μm^2). These species do not fit cleanly into gonochorism or hermaphroditism. Instead they are trioecious, having three sexes: male, female, and hermaphrodite. The prevalence of hermaphrodites and females vary between species under normal culture conditions (Pires da Silva, personal communication). However, they still display dramatic skews in the sex ratios during crosses. The smaller size of the spermatids corresponded with a reduced spread of the size distribution (Figure 2-8).

Nullo-X Spermatids Aggregate After Completion of Spermatogenesis

In *C. elegans*, the residual bodies act as waste receptacles for the unnecessary cellular components. Similar cytoplasmic shedding is a common feature of spermatogenesis in many organisms. A recent study suggests that the residual bodies formed during *C. elegans* spermatogenesis undergo a process that resembles apoptosis and are ultimately engulfed by the surrounding gonadal sheath cells (Huang et al., 2012).

In SB347, SB372, JU1809, and JU1782 the nullo-X spermatids appear to take on the role of the residual body. They receive the components that the sperm do not need for proper functioning, shown here to include the ER and tubulin. Furthermore, although our studies indicate that these nullo-X sperm are produced in equal numbers to X-bearing sperm, we consistently observed larger numbers of X-bearing spermatids accumulating within male gonads. If the nullo-X sperm have taken on the general roles of the residual bodies, then we predict that they may be undergoing a similar apoptosis and reabsorption process. The nullo-X sperm in SB347 appear to aggregate into large clusters (Figure 2-9), a phenomenon previously observed with actin-positive nullo-X spermatids (Shakes, personal communication). Spermatids displaying cyp33E1 staining and chromatin masses indicative of nullo-X sperm appear to form large clusters by some means, potentially involving selective expression of cell-adhesion molecules. It currently remains to be investigated what the function of these clusters are, or if they are followed by apoptotic events like in *C. elegans* residual bodies.

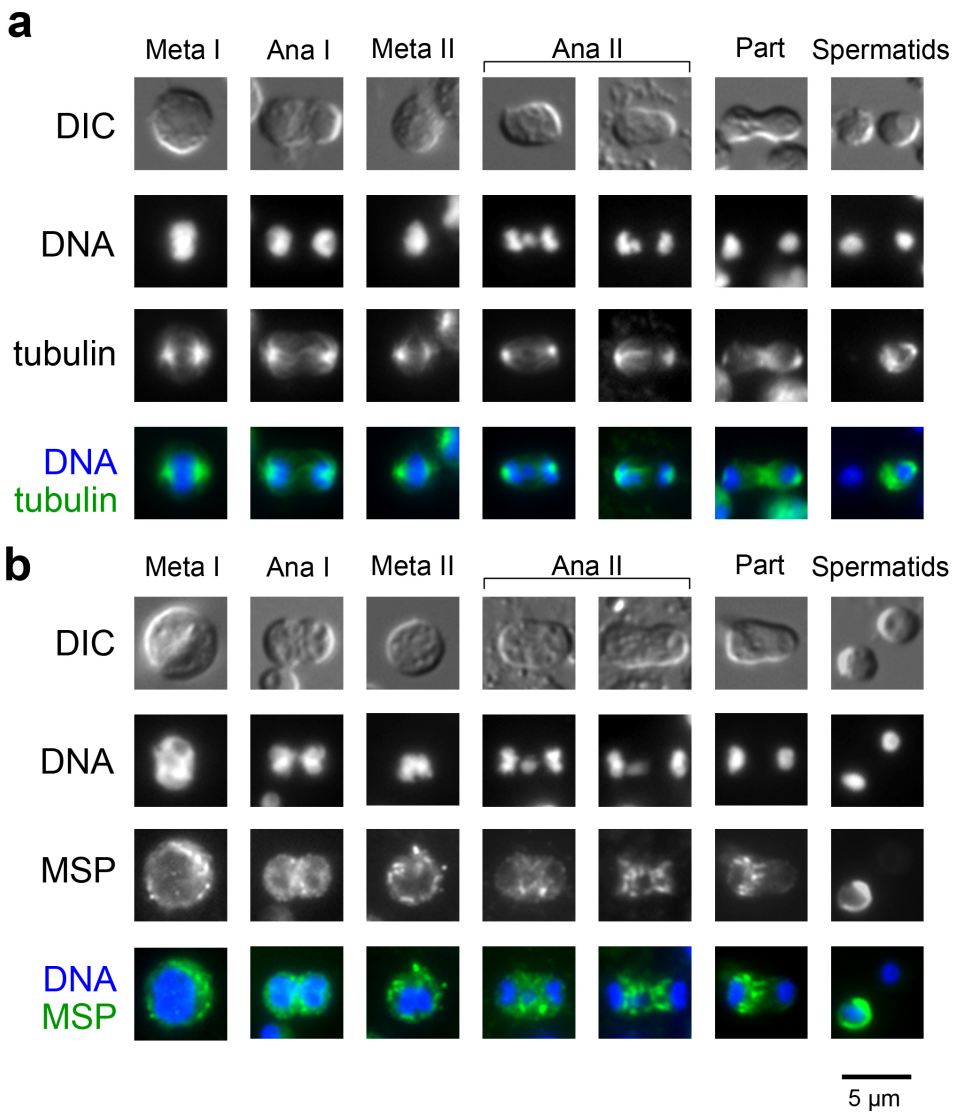

Figure 2-1: Male Spermatogenesis in *Rhabditis* sp. SB347. DIC and immunocytology of methanol-fixed sperm spreads. The lagging X-chromosome is displaced to meiosis II in this species. **(a)** Images compiled from approximately 160 dissected gonads. Tubulin spindles quickly become asymmetric during anaphase II, with the larger spindle associated with the X-chromosome. In the partitioning phase, the spindles detach and are segregated exclusively into the nullo-X sperm. No residual body is formed, but the nullo-X sperm contain the tubulin. **(b)** Images compiled from approximately 80 dissected gonads. Though the cell is asymmetric in shape, MSP remains evenly distributed in the cytoplasm until the partitioning phase. At that point, the MSP rapidly segregates to the pole bearing the X chromosome.

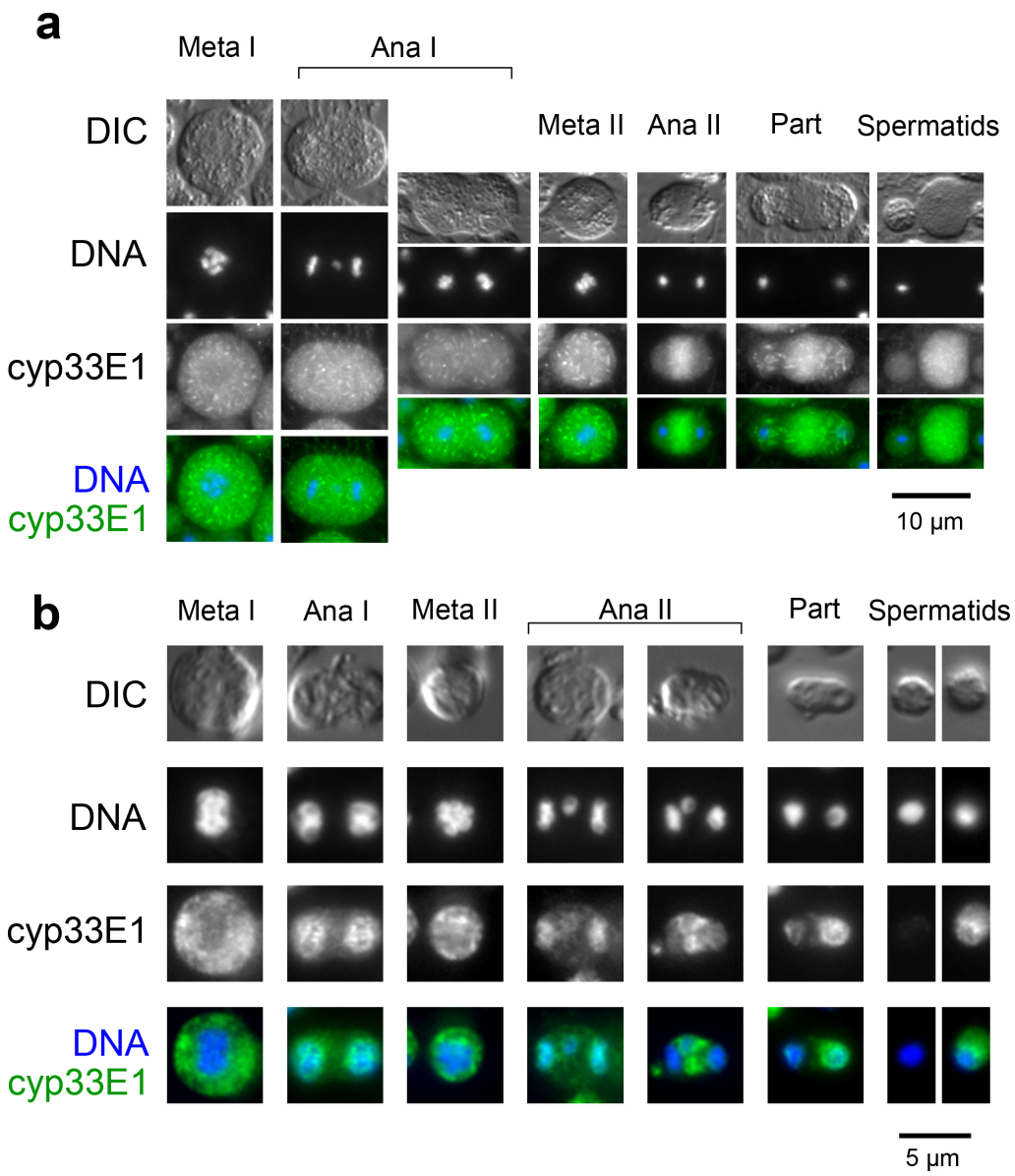

Figure 2-2: Cyp33E1 Division During Spermatogenesis. Cyp33E1 is a member of the cytochrome P450 superfamily that serves as a marker for the endoplasmic reticulum. **(a)** In *C. elegans* spermatogenesis, uneven distribution of cyp33E1 becomes apparent during anaphase II. This carries through the partitioning event, resulting in cyp33E1 concentrated in the residual body with little staining in the spermatids. **(b)** *Rhabditis* sp. SB347 demonstrates the familiar asymmetry during meiosis. Following anaphase II, there is asymmetric distribution of cyp33E1 favoring the nullo-X pole.

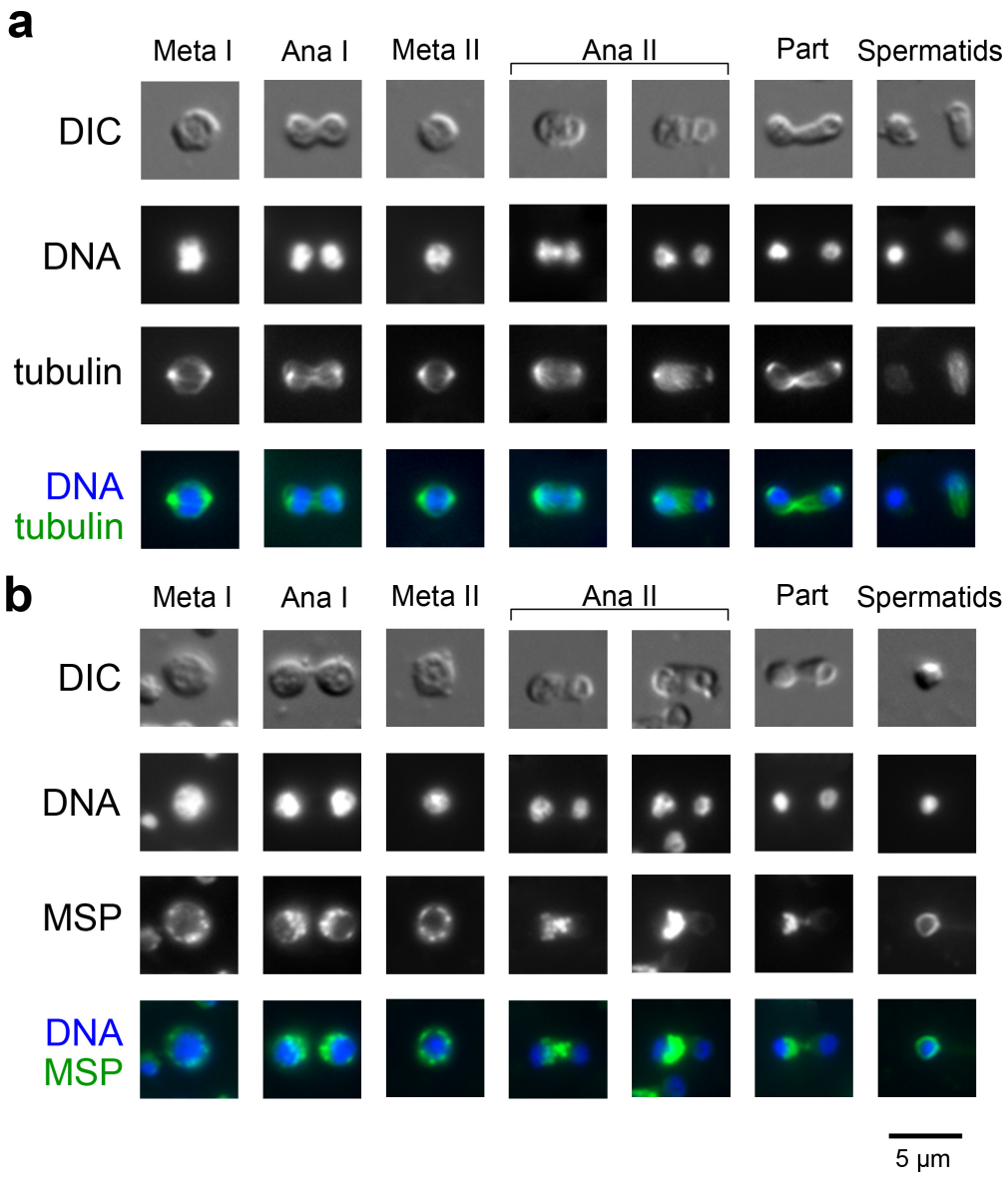

Figure 2-3: Male Spermatogenesis in *Rhabditis* sp. SB372. DIC and immunocytology of methanol-fixed sperm spreads. Images compiled from approximately 320 dissected and stained gonads for each MSP and tubulin. The lagging X-chromosome is less obvious in this species than in SB347, though asymmetry is still present in tubulin **(a)** and MSP **(b)** during meiosis II and partitioning. Only the functional, X-bearing spermatid is displayed in the MSP panel.

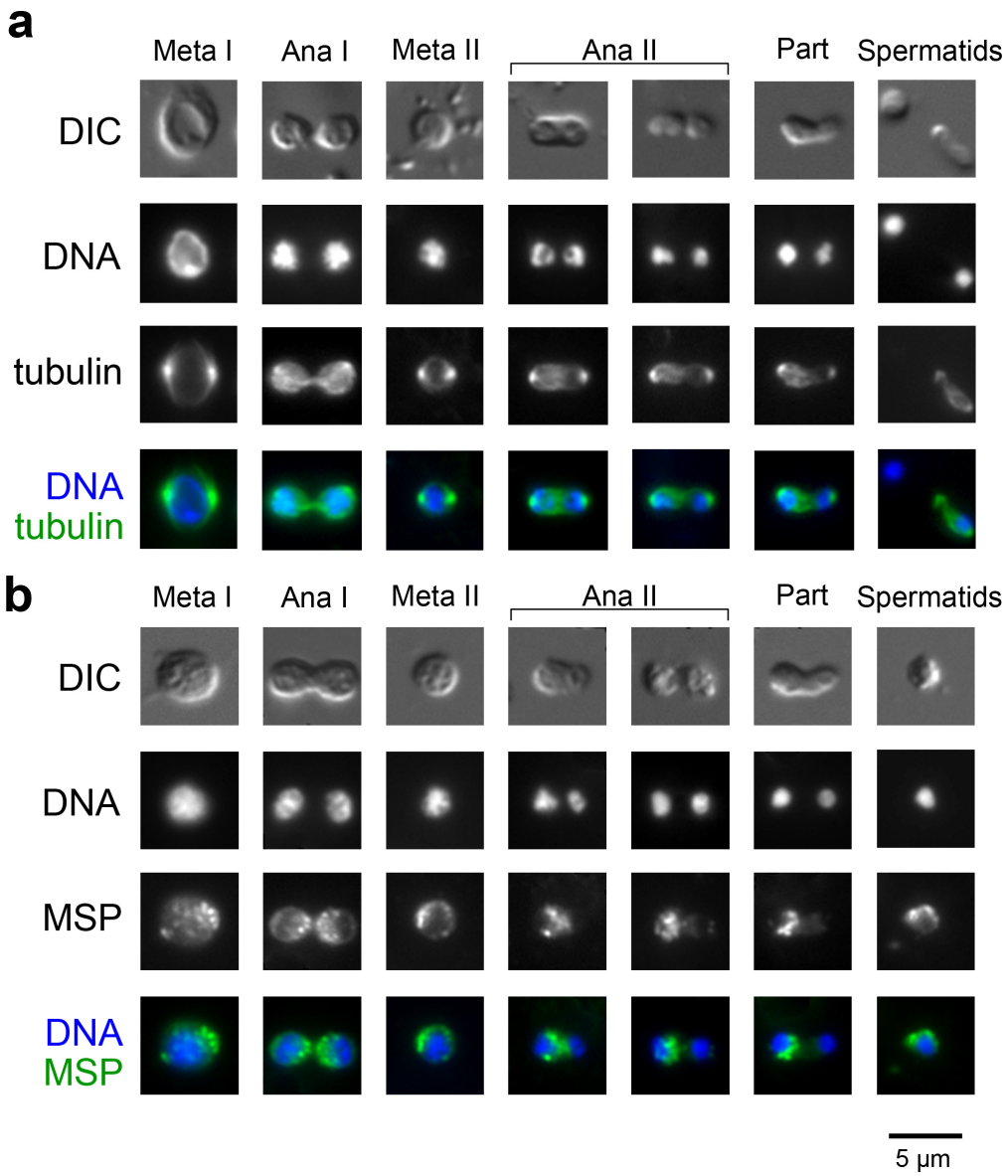

Figure 2-4: Male Spermatogenesis in *Rhabditis* sp. JU1809. DIC and immunocytology of methanol-fixed sperm spreads. The pattern displayed is very similar to that in *Rhabditis* sp. SB372 (Figure 2-3). Figure assembled from approximately 160 prepared gonads each for tubulin and MSP.

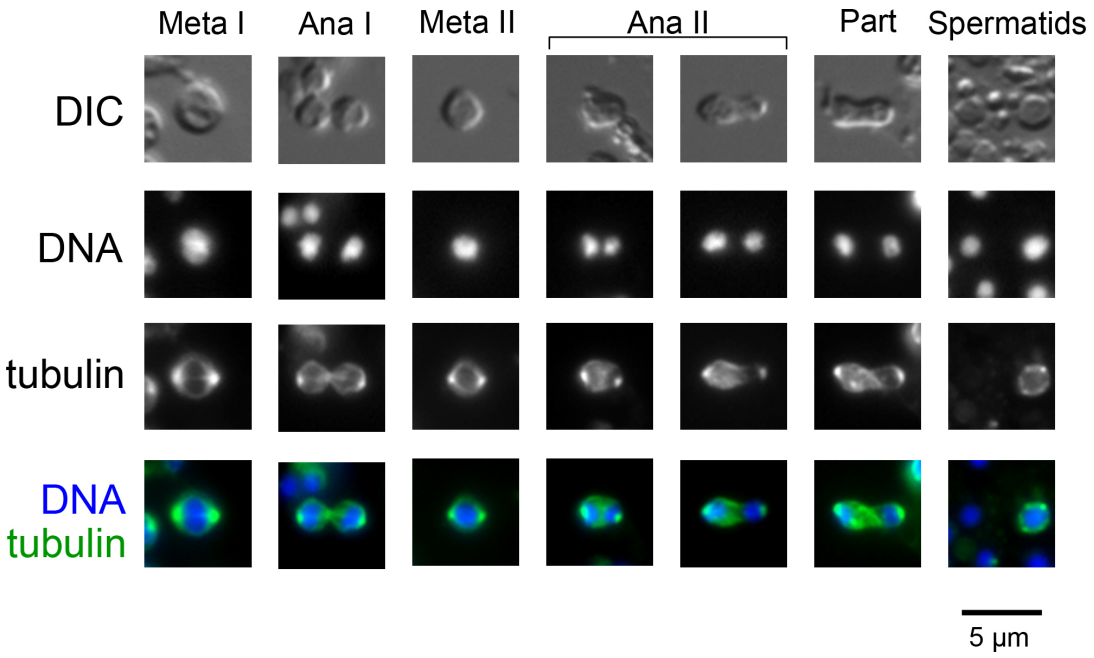

Figure 2-5: Male Spermatogenesis in *Rhabditis* sp. JU1782. DIC and immunostaining of α-tubulin in methanol-fixed sperm spreads. Images compiled from approximately 80 dissected male gonads. The division patterns displayed are very similar to those previously presented.

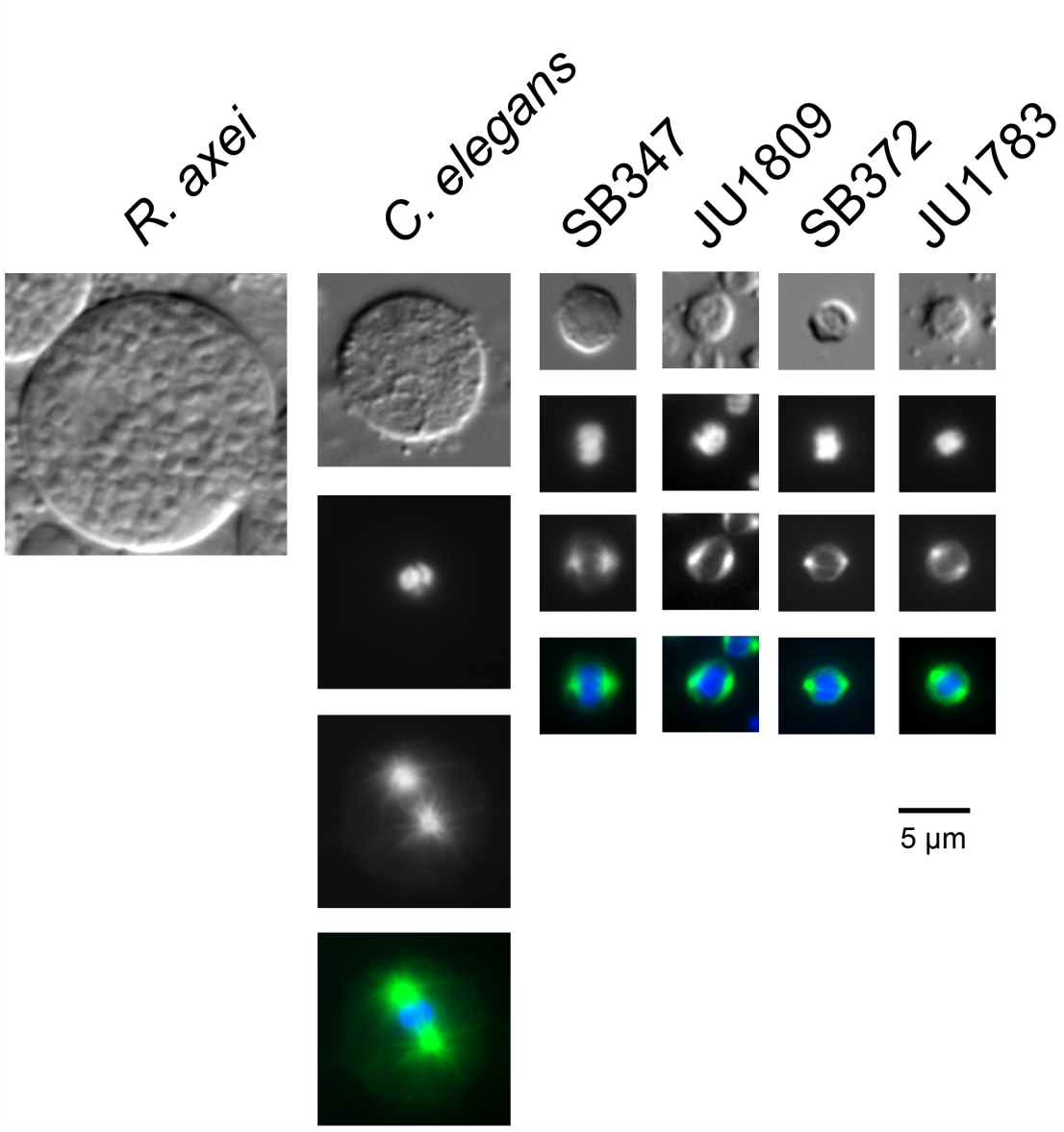

Figure 2-6: Size Comparison of Primary Spermatocytes. A visual comparison of the size of primary spermatocytes in *R. axei, C. elegans,* and four *Rhabditis* species. The gonochoristic *R. axei* presents the largest spermatocytes, followed by the hermaphroditic *C. elegans*. The *Rhabditis* species present dramatically reduced spermatocyte sizes.

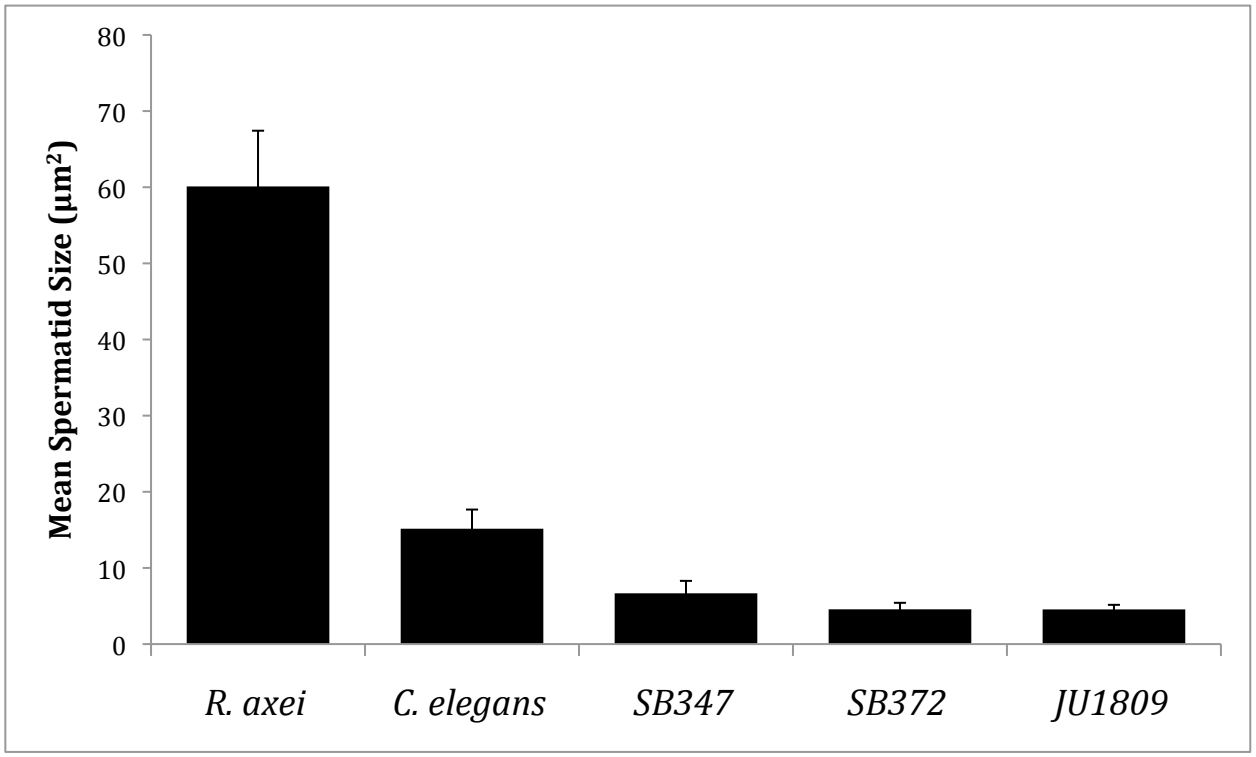

Figure 2-7: Mean Spermatid Sizes Across Species. Spermatid sizes were measured as cross-sectional area in several species. The means spermatid size for each species is displayed with the standard deviation as the error bar. *R. axei* size was 60.1 (±7.3)μm², *C. elegans* was 15.2 (±2.5)μm², SB347 was 6.7 (±1.6)μm², SB372 was 4.6 (±0.9)μm², and JU1809 was 4.6 (±0.6)μm².

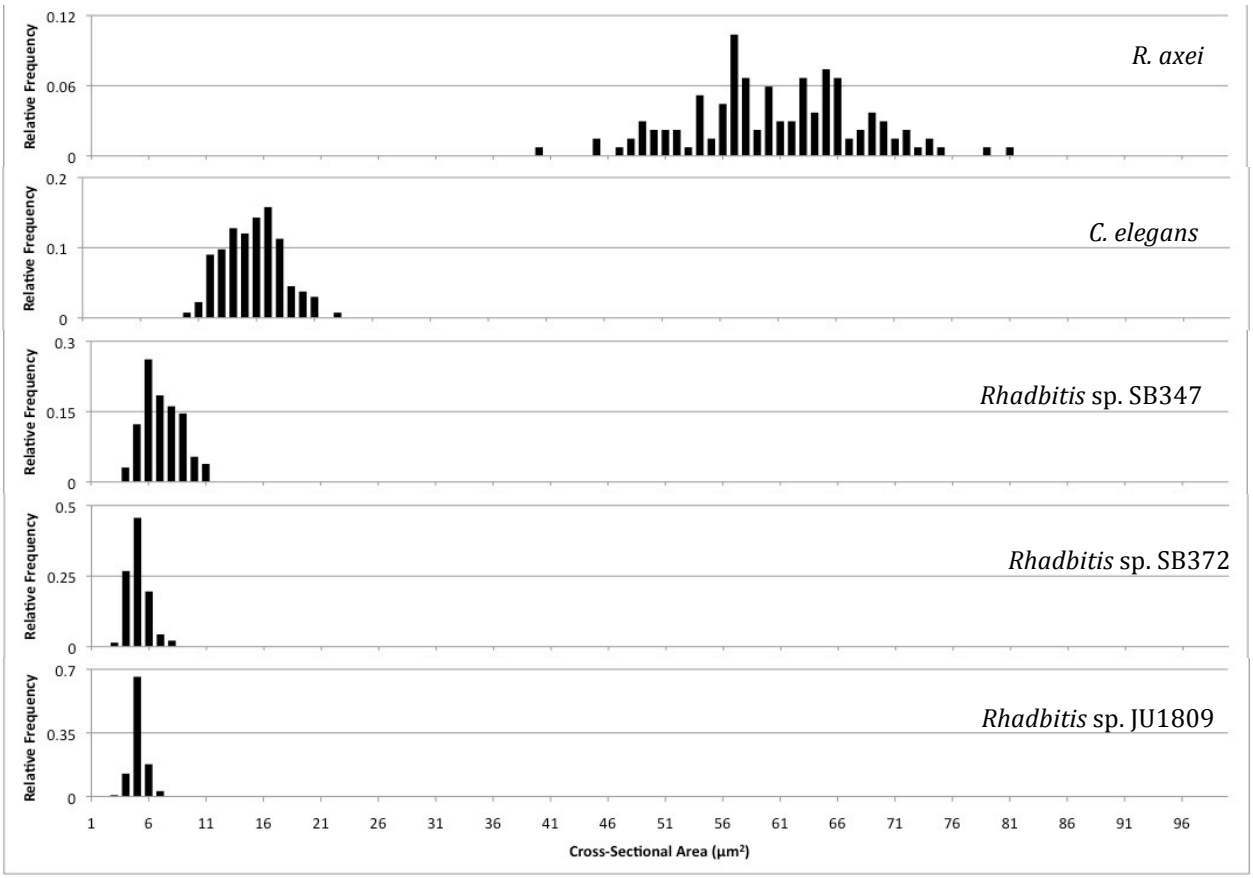

Figure 2-8: Distribution of Male Spermatid Sizes. Cross-sectional area of spermatids were measured from DIC images. Data was obtained for *R. axei* (n=135), *C. elegans* (n=133), *Rhabditis* sp. SB347 (n=130), *Rhabditis* sp. SB372 (n=138), and *Rhabditis* sp. JU1809 (n=135). Large variation in spermatid size can be seen across related species.

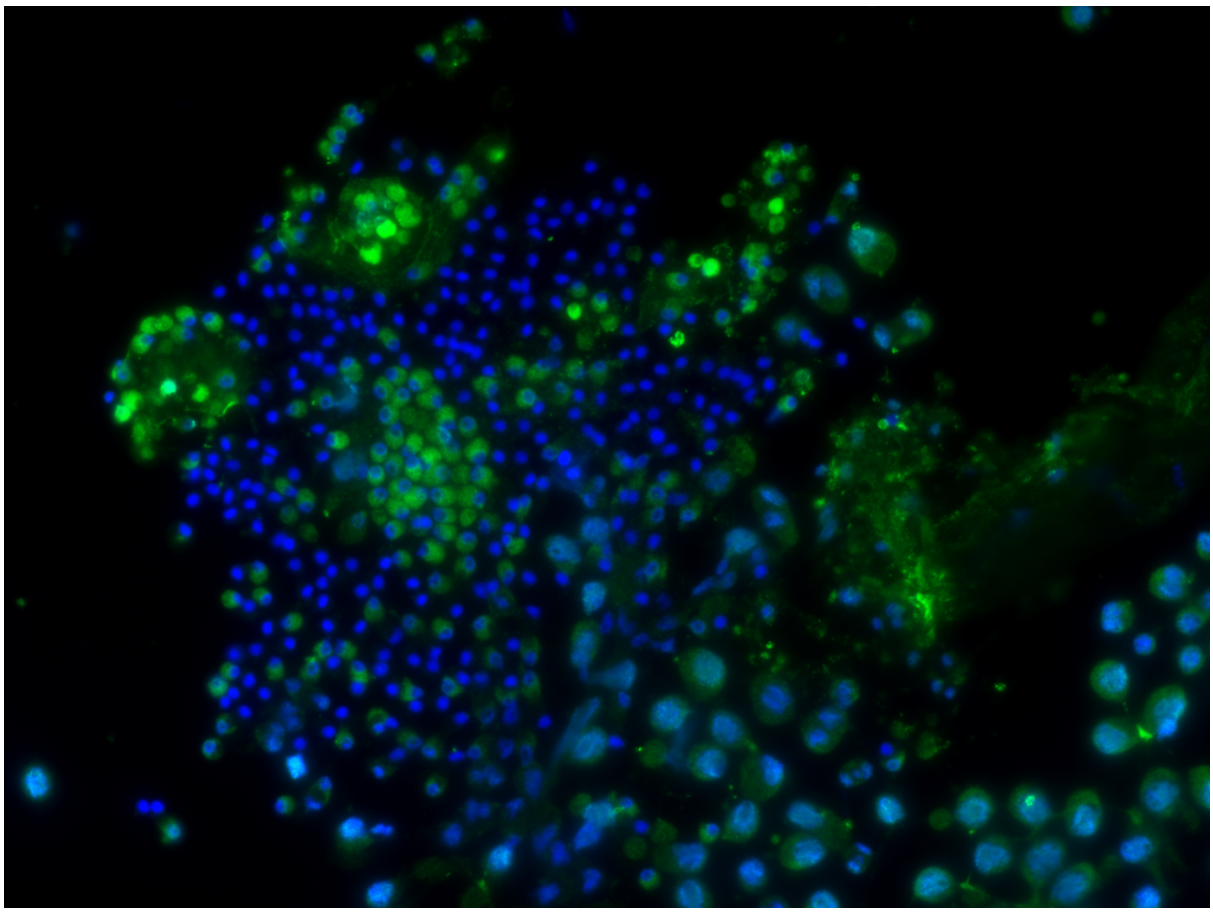

Figure 2-9: Clustering of nullo-X Spermatids in SB347. After the partitioning of cellular components during meiosis, the number of nullo-X sperm appears to decrease significantly from the 50% that would be expected to be present. Occasionally, slides such as the one above reveal a pattern of aggregation of the nullo-X sperm (here stained green with anti-cyp33E1 antibody). What happens to the cells after clustering is still unknown.

Section 3: Discussion and Future Directions

Discussion:

Here we confirm and extend previous characterization of the asymmetric cell divisions in *Rhabditis* sp. SB347 male spermatogenesis that lead the unusual sex skews produced in progeny sired by male worms. We present more detailed analysis of the timing of partitioning of tubulin and MSP as well as introducing an endoplasmic reticulum marker. This asymmetric cell division includes partitioning of tubulin and the endoplasmic reticulum to the nullo-X spermatids and partitioning of MSP contained within the FB-MOs to the X-bearing spermatids. Using tubulin and MSP as markers, we also document a similar progression of events during the process of spermatogenesis in a number of related species including the *Rhabditis* species SB372, JU1809, and JU1782 each of which demonstrates similar sex skews. We also show that the spermatids in these species are greatly diminished in size compared to *C. elegans*, other nematodes that have been previously studied, and eukaryotes in general. Lastly, we present evidence of the aggregation of nullo-X spermatids within the gonad following spermatogenesis.

One of the goals of this work was to investigate the nature of the spermatocyte divisions and post-meiotic partitioning process in several species of the clade of SB347. The unusual phenotype of asymmetric cell divisions only producing functional X-bearing sperm in SB347 males was previously noted (Shakes et al., 2011). However, it was previously unknown if this was an isolated case of a strange spermatogenic program or if this was a feature with broader relevance in Nematoda. Potentially, it could have been a feature of just this species or even a strange mutation in the original isolated line. The results here suggest that the spermatogenesis-dependent sex skew is conserved across

several species closely related to *Rhabditis* sp. SB347. Though slight variations may occur, similar cell division patterns are displayed in SB372, JU1809, and JU1782. Similar mechanisms may be a feature of other nematode species displaying alternating generations. Some species, including parasitic worms, are known to have male-female generations that give rise exclusively to self-fertile hermaphrodite or parthenogenic generations.

In SB347, the patterning of the ER during divisions is interesting. In anaphase I and early anaphase II, the cyp33E1 staining appears to slightly concentrate around the chromatin. It would be interesting to look at a marker of the nuclear membrane, such as the nuclear pore, to see if there is a transient association or reassembly during meiotic divisions. The asymmetric partitioning also adds to the growing data suggesting the residual body function of the nullo-X spermatid in SB347.

In *C. elegans* and the more closely related *R. axei*, male spermatogenesis yields four functional gametes per primary spermatocyte, two of which bear X chromosomes. However, the *Rhabditis* species that we have studied appear to produce only two functional sperm. This is achieved through asymmetric division during meiosis II, and results in only X-bearing spermatids receiving the cellular contents required for functional gametes. As X-bearing sperm produce feminine progeny, this phenomenon accounts for the dramatic sex skews observed in male progeny of these species

This study also attempted to elucidate the precise order of events during the spermatogenic cell divisions in SB347 (Figure 3-1). The order of some events within the asymmetric division are difficult to distinguish from the cytology work. It is unclear what drives the asymmetry during anaphase II. It is possible that the segregation of the X

chromosome signals for the developing asymmetry in the spindle or that the spindle asymmetry determines the pole to receive the X chromosome. Within the spermatogenesis program of the SB347 clade, it is not until the chromatin becomes supercondensed, forming small circular masses, that the MSP-containing FB-MOs begin to segregate. Additionally at this point, the centrosome deactivates and tubulin migration toward the nullo-X pole begins. This suggests that FB-MO migration is not directly dependent on the tubulin spindle asymmetry. Based on the numbers counted for cells with this chromatin morphology, these events occur rather quickly following the transition.

The evidence leaves some room for interpretation. It is unclear what the driving force of spindle asymmetry is in anaphase II. Potentially, the migration of the X chromosome to one pole is stochastic and the spindle polarity follows. It is also possible that some polarity is established through another means and the enlarged spindle on one pole provides the necessary force to overcome the opposing spindle forces. It is clear that entering into partitioning, the centrosome deactivates to allow migration of tubulin. Evidence suggests that actin forms rings at the junction of the two cells during partitioning (Shakes, unpublished). It then separates to the nullo-X spermatid before complete division. This combined with evidence from *C. elegans* may suggest that actin cytoskeleton plays a role in the partitioning of FB-MOs.

The fact that asymmetric meiosis is a feature of all of these closely related species studied suggests that this feature likely arose before an evolutionary branch point. However, all of these species appear closely related. If this feature was noted in more distant species, it might suggest it is an ancestral trait or that there was some reason for convergent evolution on the trait.

The reasons for the conservation of this feature across the species studied here, if any exist, are unclear though speculation is possible. From what we know of free-living nematode ecology, some species may spend most of their lives in isolated patches of food, such as on rotting fruit that have fallen to the ground (Félix & Duveau, 2012). When these food supplies become depleted, they likely enter the dauer phase as a means of dispersing and locating new reservoirs of food. In *Rhabditis* sp. SB347, only the feminine worms appear to be able to enter the dauer phase. Additionally, dauering appears to be necessary and sufficient for the development of a young feminine worm into a hermaphrodite (Chaudhuri et al., 2011). In the nutritional oasis model of nematode ecology, an incredibly important ecological role is placed on the hermaphrodites of SB347. They are critical to the survival of lines because they can disperse and locate new food sources. Upon maturation into adult hermaphrodites, these worms are able to populate the new location on their own.

This necessity of feminine worms for the continuation of the species may suggest that this feature is an adaptational one. However, under lab culture conditions, the sex skews sometimes begin to take on the appearance of an illness. Particularly in *Rhabditis* sp. JU1809, it seems to have led to instability in the line. Crosses between males and females resulted in large numbers of female progeny, constituting 89% (435/489) of the feminine offspring produced (Pires daSilva, A., unpublished data). In a population where self-fertile hermaphrodites and males are both scarce, reproduction becomes slow and inefficient. In species where hermaphrodites are more prevalent, this is less of an issue. As environmental conditions can have a significant effect on the development of feminine worms in SB347 (Chaudhuri et al., 2011), it is possible that the laboratory culture

conditions altered the numbers of hermaphrodites developing from what they would be in the wild. However, the case does lead to speculation.

In the gene-centered view of evolution, a gene's success is determined solely by its ability to replicate itself. Therefore, it may be possible that a selfish genetic element arose on the X chromosome in some of the *Rhabditis* nematodes. This element could distort meiosis in males so that it only produces functional sperm that contain the X chromosome, and therefore copies of itself. This would eliminate competition against the nullo-X sperm produced by the individual, increasing the likelihood of this genetic element being passed on. It would therefore be successful at replicating itself, but possibly detrimental to the organism and the population as a whole. The presence of hermaphrodites in the population could alleviate some of the strains that this meiotic distortion would put on the species. It would delay or prevent the population from crashing due to lack of mating partners for the female worms, permitting the genetic element to persist. Curiously, hermaphrodites retain the ability to produce small numbers of males through a suspected loss of X chromosomes during oogenesis. As hermaphrodites require additional time to develop through dauer, production of males and females may allow faster maturation and thus an advantage in the rate of gene propagation.

This sort of meiotic distortion is not unprecedented. In *Drosophila melanogaster*, the Segregation Distorter (SD) system is a well-studied meiotic driver. It exists in wild populations of *Drosophila* at a rate of about 1-5% (Palopoli & Wu, 1996). Males heterozygous for the autosomal gene SD produce >90% offspring carrying SD. The gene cluster renders the wild-type sperm that would be produced as non-functional. It does so by preventing activation through spermiogenesis, bearing a close resemblance to what we

have noted in these nematodes. SD was also shown to have evolved very rapidly, allowing very little time for neutral evolution in the genes in carried along with it. This suggests that it is not unfeasible that a similar distorter could arise in nematodes.

Another well-documented example of a selfish genetic element occurs in mice. The t-locus is known to harbor alleles that result in meiotic distortion (Silver, 1993). The percentage of offspring carrying the distorting allele is higher than would be predicted by Mendelian genetics. Homozygosity for some of these alleles is lethal, so they are detrimental to the population. Yet, they are able to persist because the number of times they gain transmission is greater than the number of times they are lost through lethality.

Several cases of intragenomic conflict are known. It is therefore possible that such an event occurred for an element held on the X chromosome in these *Rhabditis* species. It seems unlikely that such a feature would arise multiple times in all of these closely related species, so appears to have occurred in an ancestral line. Additionally, some distortion effects have been noted in *Caenorhabditis briggsae*. In these worms, male-derived sperm bearing the X chromosome show increased competitiveness over those that lack it. This results in initial progeny from crosses being almost exclusively feminine, but the proportion of males increases over time (Lamunyon & Ward, 1997). It seems likely that some sort of asymmetric division during spermatogenesis results in less competitive nullo-X sperm, though not nearly to the same extent as seen in *Rhabditis* species. This late appearance of males may help to replenish dwindling supplies of self-produced sperm in the hermaphrodites of the species.

The adaptive and the selfish genetic hypotheses are not necessarily mutually exclusive. An adaptive skew in the functionality of sperm, if the responsible genetic

element were located on the X-chromosome, would also serve to enhance its own replication.

The results on the size of sperm in these species were also quite striking. Previous research indicates that the level of sperm competition within a species is a good predictor of average sperm size. From the relatively small number of males present in their populations, we could hypothesize that their sperm would be small. However, the extent of their miniaturization far exceeds that of other nematode species that have been previously reported in the literature. With such small size, there may be physical limitations to the number of functional spermatids produced from each spermatocyte. The spermatids are diminished to the point that there is very little cytoplasm remaining besides what is taken up by the chromatin. It is remarkable that they are able to maintain their function with such drastic reduction in size. The average cross sectional area for the *Rhabditis* species that we report on here was 5.3 μm^2. This compares to 15.2 μm^2 for the well-studied *C. elegans*. For comparison, human sperm have a head measuring roughly 15 μm^2 and human red blood cells are approximately 40 μm^2. The sperm of the *Rhabditis* species, therefore, are remarkably small.

It is unclear why the sperm produced are so small. In the absence of large amounts of sperm competition, there are not significant selective pressures to make larger sperm. However, this is true of hermaphroditic species such as *C. elegans*, which does not display this level of spermatid reduction. It is possible that since two spermatids produced from a primary spermatocyte are rendered non-functional in these species, the effective nutritional and energetic costs of producing the X-bearing sperm are increased significantly. This could lead to pressure to further reduce the sperm size, resulting in the

dramatic results reported. Additionally, pressures in the hermaphrodites to produce the largest number of sperm possible during the developmental window of spermatogenesis may lead to small sperm, a characteristic that may carry over to male spermatogenesis in the species because of a largely shared spermatogenic program (Baldi et al. 2011).

Future Directions:

This study has highlighted several other species displaying asymmetric spermatocyte divisions as a means to achieve skewed sex ratios. However, this is only a small number of species. There have been enormous amounts of research into *C. elegans* as a model organism. However, in some cases this has led to the, perhaps incorrect, assumption that it is the prototypical nematode. There are many nematodes that are difficult or impossible to culture in the lab, and few of the culturable ones have come under significant scientific scrutiny. There is still much to be learned about nematode biology.

Studying the *Rhabditis* nematodes could provide valuable information for future studies regarding the evolution of sex, a notoriously difficult subject to study. This study only covered a few of the identified *Rhabditis* strains. More characterization is necessary to understand how the asymmetric spermatogenic divisions are distributed among species in this and other nematode clades. Extensions of this study can provide a picture of when this pattern arose and perhaps something about its relevance in their ecology.

We only have a small glimpse at the nature of the division itself. SB347 appears to serve as a good model for this type of division due to its slightly larger spermatocytes and the larger body of literature currently published. We have only studied the partitioning patterns of a few proteins and cellular components, but a more complete view would be

helpful. Segregation of the mitochondria, a necessary sperm component, is of interest. *C. elegans* residual bodies appear to be able to undergo apoptotic events while lacking mitochodria, a phenomenon which remains to be studied. Markers of the centrosome, kinetochore, as well as chromatid remodeling are interesting subjects for study.

The mechanisms driving the segregation of cellular components are not fully understood. In *C. elegans* and most studied nematodes, residual bodies are produced during spermatogenesis. This helps to reduce the size of the sperm and aid in motility. In asymmetric-dividing *Rhabditis* species, the nullo-X sperm appears to take on the role of the residual body, receiving the unnecessary components of the cell. It seems to make sense that instead of developing a completely new mechanism of cellular polarization, the *Rhabditis* nematodes have simply developed a modified version of the program used to differentiate between spermatid and residual body in *C. elegans* and other nematodes.

Based on the timing of events, the fibrous body segregation in SB347 does not appear to be tied to the movement of the tubulin spindles. *Spe-15*, an unconventional minus-end directed myosin protein, appears to be necessary for proper partitioning of *C. elegans* FB-MOs, mitochondria, and actin filaments between the spermatids and the residual body (Kelleher et al., 2000). Chromosome sorting remains unaffected in *spe-15* deletion mutants. However the defective partitioning of these other cellular components suggests a key role for the actin cytoskeleton. We suspect that this actin-dependent sorting of FB-MOs may be conserved as the predominant force in the *Rhabditis* species.

In addition to the mechanism of partitioning, the means by which cells establish polarity remains elusive. Understanding the basic mechanism of polarity in *C. elegans* would likely inform how the *Rhabditis* species appear to achieve asymmetric polarization.

Conversely, Understanding how the X-concurrent polarization works in *Rhabditis* species could give crucial insight into similar mechanisms in *C. elegans* and other organisms.

Curiously, the nullo-X sperm appear not to be present in equal proportions among populations of spermatids in the seminal vesicle. As they are produced in a 1:1 ratio to the X-bearing sperm, this indicates that they are somehow being removed from the population before fertilization occurs. We have noted clustering of the non-functional spermatids in the gonad, but the mechanism and purpose of this clustering requires investigation. In *C. elegans,* residual bodies produced in spermatogenesis are cleared from the male germline via processes dependent on apoptotic pathways and engulfment by gonadal sheath cells (Huang et al., 2012). Due to the fact that the nullo-X sperm appear to take on the role of the residual body in the species studied here, we hypothesize that they are cleared through a similar mechanism. The authors of the paper on *C. elegans* made use of fluorescently labeled annexin V as a marker for phosphatidylserine being presented on the outer leaflet of the cell membrane. This appears to be an early sign of this apoptotic process, and potentially a signal for engulfment by the sheath cells. We would like to study the behavior of the nullo-X sperm after completion of spermatogenesis. Annexin V appears to be a good marker to begin research on this subject.

The sperm size data provides some interesting results. The male spermatids produced are dramatically diminished in size compared to most other nematode species that have been studied. Though sperm competition is known to be a factor related to size, the sperm are diminished beyond many other species with comparable prevalence of male worms (LaMunyon & Ward, 1999). By broadening the scope of species studied, an obvious

correlation between size and another factor may become apparent. This unknown factor may be what has led to the significant reduction in size seen.

Another interesting question is how the male sperm size compares to that of hermaphrodite-derived sperm. Male sperm in *C. elegans* are known to consistently outcompete hermaphrodite sperm when both are present in the spermatheca (Ward & Carrel, 1979). This appears to be largely due to the increased size of male sperm compared to hermaphrodites of the same species (LaMunyon & Ward, 1998). We have not yet thoroughly investigated the nature of hermaphrodite sperm in the *Rhabditis* nematodes. The level of miniaturization in these sperm does not appear to leave much room for variation between sexes, as there must be a lower limit to what can remain functional as a motile sperm. It remains to be seen if male sperm have a competitive advantage over hermaphrodite sperm in these species. Additionally, investigating hermaphrodite spermatogenesis could reveal more about the nature of the asymmetric division. If the asymmetric partitioning is preserved, then it suggests that the polarity of anaphase II divisions may not be dependent on the X chromosome as both poles should receive an X chromatid.

Though interesting in their own right, the *Rhabditis* nematodes may serve as a good model for evolutionary studies. Species with a wide variety of characteristics occur in close phylogenetic relation. The gonochoristic *R. axei* with large sperm are within evolutionary spitting distance of the trioecious *Rhabditis* sp. SB347 with dramatically diminished sperm. Meiotic division appears to be symmetric in *R. axei* while they display marked asymmetry in SB347. What curious characteristics the other *Rhabditis* nematodes display remains to be discovered.

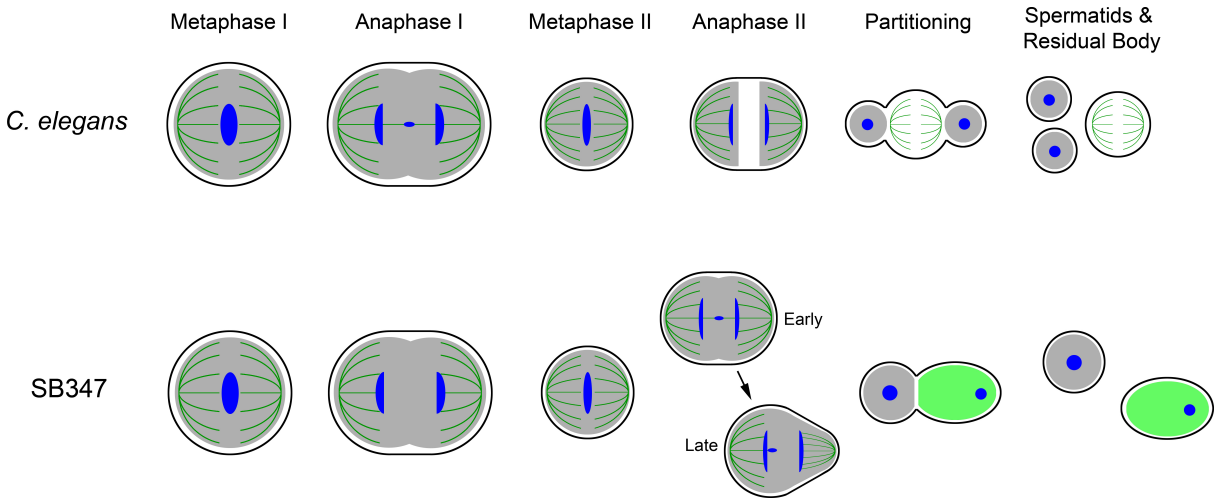

Figure 3-1: Spermatogenesis in *C. elegans* and *Rhabditis* sp. SB347. A modified schematic of spermatogenic divisions in *C. elegans* and *Rhabditis* sp. SB347 based on our new data. All events following anaphase II occur twice for each primary spermatocyte, once for each secondary spermatocyte produced. Tubulin is displayed as green, DNA is blue, and FB-MOs are gray shading. In *C. elegans* FB-MO migration to the poles begins during anaphase II. In SB347, this event does not occur until entry into the partitioning stage. *C. elegans* produces spermatids and a residual body containing tubulin and the endoplasmic reticulum. SB347 produces no residual bodies, but the nullo-X sperm, depicted with a smaller chromatin mass, contain the tubulin and ER while lacking FB-MOs.

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