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An Investigation of Hermaphroditism in R sp SB 347

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ABSTRACT

Rhabditis sp. SB347 is a triecious species with three sexes: males, females, and self-fertile hermaphrodites. This study aims to characterize the patterns and cytological characteristics of reproduction in *R.* sp. SB347 hermaphrodites. The first chapter investigates the sex ratios and brood structure of self-fertilizing *R.* sp. SB347 hermaphrodites. This study finds that hermaphrodites produce 10.5% males in total broods and often have several bursts (>10.5%) of male production within individual broods. This analysis of complete broods is the first of its kind in *R.* sp. SB347 and one of the few reports of population studies in a triecious nematode species with skewed sex ratios.

The second chapter explores patterns of chromosome segregation during oocyte meiosis as a potential mechanism for enabling self-fertilizing XX hermaphrodites to produce XO male progeny. This study reveals that during hermaphrodite oogenesis, aberrant X-chromosome segregation patterns may contribute to X-chromosome loss specifically during meiosis I. During metaphase I, I observed 6 paired autosomes and two X chromosomes in hermaphrodite oocytes. The X chromosomes were variably paired in a bivalent (paired homologs) or unpaired as univalents (unpaired homologs). My data suggests that the unpaired X chromosomes lag during anaphase I and are either segregated reductively to yield a haplo-X ovum or are both extruded into the first polar body to yield a nullo-X ovum. Fertilization with haplo-X sperm would yield an XX feminine or XO male, respectively. Importantly, no dead embryos were observed during brood studies, suggesting that meiotic segregation defects are X-chromosome specific. These findings suggest that X-chromosome loss serves as a mechanism for XO male production in *R.* sp. SB347 self-broods.

In the final chapter, we investigate spermatogenesis in *R.* sp. SB347 hermaphrodites using *Caenorhabditis elegans* as a comparative model. As in the well-studied model nematode *C. elegans*, most hermaphroditic nematodes are assumed to make a one-time switch from producing spermatocytes to producing oocytes. However our analysis of older *R.* sp. SB347 hermaphrodites revealed not only the expected presence of spermatozoa, but also the continued production of spermatocytes and spermatids. Furthermore, we documented the novel presence within a nematode species of mitotically-dividing spermatogonia that give rise to spermatocytes. Spermatogonia have not been previously reported among nematodes, and thus this study contributes to the understanding of hermaphroditism in a triecious species.

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This M.S. is dedicated to my parents.

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Introduction

Evolution has provided many different methods of sex determination (Gamble and Zarkower 2012). Of these methods, chromosomal sex determination is of particular interest because it is used by many different species, including humans (Sinclair 1998). In humans, sex determination is mediated through an XX/XY system in which females are XX and males are XY. Other species use an XX/XO system of sex determination in which females or hermaphrodites are XX and males have a single X chromosome, but no second sex chromosome. Because there is no Y chromosome, XX/XO systems are more labile than the XX/XY systems and lend themselves to sex ratio distortion (John 1957). One phylum that exhibits sex skews in many different species is Nematoda (Dix et al. 1994; Harvey et al. 2000; Shakes et al. 2011, Shinya et al. 2014; Yoshida et al. 2009). This group contains many species with diverse modes of reproduction, including gonochorism, hermaphroditism, heterogony, and parthenogenesis (Kiontke and Fitch 2005). Within the *Caenorhabditis* genus alone, hermaphroditism has evolved independently at least three times (Kiontke et al. 2004).

Caenorhabditis elegans is a well-studied nematode species composed of XX hermaphrodites and XO males (Nigon 1949). *C. elegans*

hermaphrodites have two gonad arms. Within each arm, approximately 150 sperm are produced during the fourth larval stage, before the gonad switches over to only producing oocytes (Hodgkin 1986; Ward and Carrell 1979). Sperm are stored in the spermathecal, and through the process of self-fertilization, individual *C. elegans* hermaphrodites can produce ~300 progeny (Hodgkin 1986). The total number of progeny produced during self-fertilization is limited by the number of sperm generated during the L4 stage. Alternatively, hermaphrodites can produce up to 1400 progeny when crossed with males as a secondary sperm source is provided (Hodgkin 1983). As expected, outcrossing between XX hermaphrodites (oocytes) and XO males (sperm) yields 50/50 sex ratios of hermaphrodite and male progeny. However, because XX hermaphrodites produce X-bearing sperm and X-bearing oocytes, hermaphrodite self-fertilization yields almost all XX hermaphrodite progeny. In *C. elegans,* the rare (0.2%) males produced through hermaphrodite self-fertilization are associated with mis-segregation of the X chromosome during spermatogenesis (Hodgkin et al. 1979; Meneely et al. 2002).

Early genetic studies of meiotic chromosome segregation in *C. elegans* yielded two major subsets of mutants (Hodgkin et al. 1979). Although all of these mutants were called *him* (*h*igh *i*ncidence of *m*ales), one subset is associated with general defects in meiotic chromosome segregation. Affected hermaphrodites produce large proportions of both males (loss of an X) and dead embryos (loss or non-disjunction of one or

more autosomes). Another subset, which includes *him-5* and *him-8*, are specifically defective in X-chromosome segregation; affected hermaphrodites produce large proportions of male offspring (loss of an X) and morphologically distinct, triplo-X "dumpy" hermaphrodites (Brenner 1974; Hodgkin et al. 1979). Importantly, affected hermaphrodites produce few dead embryos (Hodgkin et al. 1979). Molecular studies suggest that *him-8* encodes a zinc-finger, DNA binding protein involved in X-chromosome pairing and synapsis (Meneely et al. 2012; Phillips et al. 2005). HIM-8 binds to pairing centers on the X-chromosome that associate with the nuclear envelope during meiotic prophase (Phillips et al. 2005). Cytological studies have confirmed that *him-8* mutants preferentially deposit unpaired X chromosomes into the first polar body to yield nullo-X ova (oocytes with a haploid complement of all autosomes, but no sex chromosomes) (Cortes et al. 2015).

Recent studies of another nematode species, *Rhabditis* sp. SB347, have allowed scientists to investigate skewed sex ratios and population dynamics in a trioecious species. As a trioecious species, *R.* sp. SB347 is comprised of XO males, XX females, and XX hermaphrodites (Felix 2004). Hermaphroditism is linked to passage through an obligate dauer larval stage; during which the non-feeding animals are able to disperse to new environments by way of invertebrate carriers as well as survive extreme temperature, chemicals, and starvation (Felix 2004; Chaudhuri et al. 2011). One study suggests that the female/hermaphrodite phenotype is

specified during early development as future females can be distinguished from their hermaphroditic siblings by their gonad size during the mid-L1 (first larval) stage (Felix 2004). However this female/hermaphrodite phenotype also seems to be plastic as it can be secondarily adjusted by worm-produced pheromones (Chaudhuri et al. 2011). Chaudhuri et al. used the steroidal endocrine hormone, Δ7-dafachronic acid (DA) to demonstrate that, by inhibiting dauer formation, hermaphrodite-fated (small gonad) L1 larvae will develop into females (2011).

R. sp. SB347 populations are composed predominantly of XX hermaphrodites, which unexpectedly produce 10% XO male progeny during self-fertilization (Felix 2004). Furthermore, these hermaphrodites have been reported to produce the majority of both their XO males and XX females early in the brood (Felix 2004; Chaudhuri et al. 2011). Even more striking non-Mendelian sex ratios occur in the context of *R.* sp. SB347 male spermatogenesis with males siring <5% male progeny (Felix 2004). This paternally-based sex skew has been linked to a cellular mechanism whereby only the X-bearing sperm of males are functional (Shakes et al. 2011). In this first chapter, I begin to address the complementary question of how selfing XX hermaphrodites produce ~10% male offspring, by analyzing the pattern of male production by individual hermaphrodites over the duration of their reproductive period.

Results

To date, published studies of *R.* sp. SB347 self-broods have been restricted to either overall sex ratios or analysis of brood structure within the first 30 hours of reproduction (Felix 2004; Chaudhuri et al. 2011). In the current study, I found that hermaphrodites were reproductively capable for 139 $\pm$ 3.6 hours (mean + S.E.) with a range of 96-168 hours. The prior studies only assessed the early portion of self-broods (Felix 2004; Chaudhuri et al. 2011).

To verify and extend these studies, I conducted complete brood studies on time-synchronized, celibate adult hermaphrodites; each hermaphrodite generates one complete brood. Consistent with the literature, my brood studies revealed that hermaphrodites produced 10.5 $\pm$ 0.4% males (mean $\pm$ S.E.) in self-broods (n= 27,933 total progeny from 112 complete broods). To more precisely analyze the brood structure over time, a subset of these 112 brood studies were tightly synchronized by initiating the brood counts at the L4/adult molt (Figure 1; n=36 complete broods). Consistent with published studies (Felix 2004), hermaphrodites produced a larger proportion of males early in the brood. However in addition to this early burst of males, my studies revealed that most hermaphrodites produced a second burst of males later in the brood, approximately 84 hours after reaching sexual maturity (Figure 1 and Table 1; n=36 complete broods). During both the initial (0-12) interval and the later (72-84) interval, adult hermaphrodites produced higher proportions of

male progeny than in the rest of the brood (Student's t test, $p<0.05$). Importantly, we failed to detect any dying embryos or dead larvae in these brood studies (n= 112 broods); suggesting that male production by selfing hermaphrodites involves a regulated mechanism for specifically altering the pattern of X-chromosome inheritance rather than random errors in meiotic chromosome segregation.

To determine if there was variation among individual hermaphrodites with regard to the order of male production, I evaluated trends of male production in each brood (n=36 complete broods, Supplemental Tables 1 and 2). I found there to be several distinct patterns of male production within self-broods. In the most common pattern (19/36), males were produced in high (>10.5%) proportions in two different intervals (Figure 2, Worm 8), consistent with the overall trend of the average brood graph (Figure 1). In other broods, males were produced in high proportions (>10.5%) over one (n=6/36, Figure 2, Worm 25), three (n=9/36), four (n=1/36, Figure 2, Worm 10), or five (n=1/36) different intervals (Figure 2, Supplementary Tables 1 and 2). In 5/36 of these broods, high proportions (>10.5%) of males were limited to the first 24 hours of laying, indicating that the majority of complete broods were not consistent with published literature (Figure 2C, Worm 10, Felix 2004).

HIM-8 Brood Studies

HIM-8 is a protein involved in pairing and segregation of X-chromosomes during oocyte meiosis in *C. elegans* (Phillips et al. 2005). Improper segregation of X chromosomes during oocyte meiosis causes *him-8* hermaphrodites to produce nullo-X ova, which develop into males when fertilized by X-bearing spermatozoa (Phillips et al. 2005; Hodgkin et al. 1979; Cortes et al. 2015). To determine whether the self-broods of *him-8* hermaphrodites would exhibit similar fluctuations in male production throughout the brood, I conducted complete brood studies on time-synchronized, celibate adult *him-8* hermaphrodites. Results showed that male production is relatively constant across *him-8* self-broods (Figure 3). Male proportions ranged from 39.6 $\pm$ 2.9% in the earliest time intervals down to 30.9 $\pm$ 1.9%, 60 hours after reaching sexual maturity (Figure 3). After 60 hours of reproduction, standard error of the data points becomes increasingly large as brood sizes rapidly decline (Table 1 and Figure 3 and Supplemental Tables 3 and 4).

The apparent ability of *R.* sp. SB347 hermaphrodites to modulate the proportion of male progeny throughout the brood has important implications for the availability of potential mating partners for both the female and presumably sperm-depleted hermaphrodite siblings. The production of males is also important for increasing genetic diversity within the larger population (Anderson et al. 2010). Studies of mating between several *Caenorhabditis* species showed that outcrossing between species resulted in hermaphrodite sterilization by heterospecific male sperm (Ting

et al. 2014). Heterospecific sperm displace conspecific sperm and migrate ectopically to damage developing oocytes within the oviduct (Ting et al. 2014). To examine how the absolute number of males were distributed within a given self-brood, we examined patterns of relative male production by individual hermaphrodites (males produced within interval/total males produced in a single brood). By this measure, the proportions of male progeny did not vary greatly during time intervals (<96 hrs) when the hermaphrodites were still producing large numbers of progeny (Figure 4). (Total progeny counts and total male counts are also presented in Supplementary Tables 1 and 2.)

Effect of Maternal Development on Male Production

It has been shown that there is sexual plasticity in XX animals of *R.* sp. SB347 based on environmentally induced activation or repression of the dauer pathway (Chaudhuri et al. 2011). Specifically, hermaphrodite-fated L1 larvae could be redirected to become females in the presence of dafachronic acid, a steroid hormone. Alternately, female-fated L1 (first stage) larvae could be redirected to hermaphrodite development when food was limited and cholesterol was absent (Chaudhuri et al. 2011). Based on these findings, we wondered if, through epigenetic mechanisms, nutrition of hermaphrodite parents during pre-dauer development would affect XX/XO sex ratios within self-broods. To test whether the nutrition

status of hermaphrodite parents altered the proportion of male progeny within total broods, we selected two groups of hermaphrodites as the P_0 generation. One group of hermaphrodites developed to the dauer stage in the presence of ample food (n=33), while the other subset of hermaphrodites developed with limited food and were maintained in the dauer state for >8 days in the absence of food (n=24). For the actual brood counts, the two types of dauers were placed on fresh plates with ample nutrition and transferred twice daily until they expired. Results show that hermaphrodites undergoing pre-dauer development in starvation conditions produced 9.8 $\pm$ 0.9% (mean $\pm$ S.E.) male progeny while hermaphrodites undergoing pre-dauer development in high food conditions produced 12.2 $\pm$ 0.7% (mean $\pm$ S.E.) male progeny (Student's t test, p=0.04, Figure 5 and Table 2). To test whether these differences were associated with differences in overall brood sizes, we compared the number of total progeny produce by starved and fed hermaphrodites. However, brood sizes in the two populations did not significantly vary, as worms from hermaphrodites starved during pre-dauer development produced 220.5 $\pm$ 15.3 (mean $\pm$ S.E.) progeny, while their fed counterparts produced 211.6 $\pm$ 13.1 (mean + S.E., p=0.662) progeny (Table 2).

Discussion

To determine when and under what conditions self-fertilizing *R.* sp. SB347 hermaphrodites produce male XO male progeny, I conducted brood studies on synchronized celibate adult hermaphrodites. Self-broods of individual hermaphrodites showed variability in patterns of male production over time (Figure 1, Table 1), but had similar proportions of male offspring for the total brood. Results indicate that male production typically occurs in either one or two distinct peaks and is largely correlated with time (Figure 1).

I hypothesized that male production in *R.* sp. SB347 self-broods may be related to X-chromosome loss as in self-broods of *C. elegans him-8* hermaphrodites (Cortes et al. 2015). Results showed variation in the proportion of males produced over time within *R.* sp. SB347 self-broods (Figure 1). In contrast, within *him-8* self-broods, the proportion of males produced was not only higher overall, but the proportion of males also remained relatively constant throughout the brood (Figure 3).

I observed two types of variability with regard to male production in self-broods: (1) There is variability within overall brood patterns (Figure 1), and (2) there is variation between individual self-broods (Figure 2). The second burst of male production is intriguing and suggestive of bet-

hedging strategies with genetic underpinnings that may be selected for in wild populations.

Males are important in populations to prevent inbreeding depression and to increase genetic diversity (Charlesworth et al. 1993; Heller and Maynard Smith 1979; Lande and Schemske 1985; Lynch et al. 1995; Schultz and Lynch 1997). The latter allows adaptation to stochastic environments (Crow 1992; Maynard Smith 1978; Stebbins 1957). Because nutritional resources vary widely due to the relatively quick production of large offspring populations (Table 1), animals often produce different types of progeny so that some of them will survive should the environment drastically change (Maynard Smith 1978; Williams and Mitton 1973). The following theoretical model will serve as an example. As dauers, hermaphrodites display a characteristic tube waving behavior in which they stand on their tails (Felix 2004; Kiontke 1999; Sudhaus 1976). This behavior, known as phoresy, allows them to search for an invertebrate carrier (e.g., tick, Rhode Island isolate and beetle, Virginia isolate) (Felix 2004). The carrier will allow them to travel to a new food patch where a single hermaphrodite can reproduce through self-fertilization. Early in the reproductive period of an *R.* sp. SB347 dauer, it is most beneficial to make progeny that reach sexual maturity quickly. Males and females require ~60-72 hours for development from egg to adult, while hermaphrodites require ~ 96 hours to reach sexual maturity under optimal conditions (Figure 6). The additional 24-36 hours required

during hermaphroditic development is significant because their total reproductive lifetime is only 5-6 days (139 ± 3.6 hours, mean ± SE). Because males and females do not dauer, they can start mating sooner than hermaphrodites. The males can cross either with females or with hermaphrodites that may have their supply of self-sperm depleted (Hodgkin 1986). In natural populations, resources (nutrition) dissipate as competition from offspring increases. As a response to this environmental change, perhaps it has been advantageous for hermaphrodites to shift from producing males/female to hermaphroditic progeny. Hermaphrodites have the ability to dauer and can withstand the increased resource competition for months or display phoretic behavior to disperse. The dauers display phoretic behavior toward a non-parasitic host, like a tick or beetle, which gives them additional modes for dispersing to an environment with more favorable conditions (Baird 1999; Felix 2004; Kiontke 1997; Kiontke et al. 2002). These animals will require more time to reach sexual maturity, but can survive well in an environment with limited resources and other potential hazards.

Evolutionary theory suggests that although males confer benefits to hermaphrodite populations, their production and maintenance within the population is generally considered to be costly. Firstly, while males do not produce self-progeny of their own, their production requires valuable sperm and eggs from another individual(s) (Smith 1978; Stearns 1988). For *C. elegans* XX hermaphrodites, production of XO males may be

particularly costly because self-fertilizing hermaphrodites are limited to ~300 self-sperm (Hodgkin 1986). Furthermore, once present in the environment, XO males of *C. elegans* will out-cross with hermaphrodites to yield high proportions of male progeny (Hodgkin et al. 1979). Thus males serve to further increase the number of males in the population (Hodgkin et al. 1979). Lastly, the stress of copulation in nematodes is known to reduce hermaphrodite lifespan (Gems and Riddle 1996).

Despite the large cost of males, self-fertilizing *R.* sp. SB347 XX hermaphrodites produce high proportions of male progeny overall (10%) and even higher proportions during specific intervals (Felix 2004; this work). The main issue with standard arguments outlining the high cost of males, is that, in *R.* sp. SB347, male outcrossing does not result in high proportions (50%) of males. Instead, *R.* sp. SB347 males actually sire <5% XO male progeny and >95% XX feminines (Shakes et al. 2011). Furthermore, most of these XX animals are actually self-fertile hermaphrodites (Shakes et al. 2011, Pires da-Silva, personal communication). Thus, the benefits of male production in self-broods outweigh the cost to produce them and have likely been selected for over evolutionary time.

Published studies show that environmental factors, like nutrition, alter sex skews of progeny in the parasitic nematode, *Heterorhabditis bacteriophora* (Kahel-Raifer and Glazer 2000; Strauch et al. 1994). Kahel-Raifer and Glazer found that low food conditions resulted in an increase in

the proportion of hermaphrodite progeny (2000). To determine whether pre-dauer nutrition of hermaphrodite adults would alter self-brood compositions in *R.* sp. SB347, I conducted brood studies on worms from conditions with ample food and those from starved plates. I hypothesized that broods of hermaphrodites that experienced starvation may have lower proportions of male progeny, while broods of hermaphrodites that developed to the dauer stage with ample food may have higher proportions of male progeny. The parental hermaphrodites experienced starvation during larval development, and possibly even earlier as their parents may have been starving as well. Results showed that a life history of starvation (during early development) is associated with a slightly increased proportion of males (12.2 vs. 9.7% Student's t test, $p=0.04$), but does not have an effect on total brood size (Student's t test, $p=0.66$)(Figure 5 and Table 2). These results suggest that there may be epigenetic factors that influence the proportion of males in self-broods, but that total brood size may be genetically programmed and not subject to modification by epigenetic mechanisms.

The higher proportion of male progeny associated with starving parental hermaphrodites may be beneficial to the population for several reasons. Firstly, the production of males may coincide with a higher proportion of female progeny (Chaudhuri et al. 2011; Felix 2004) that would be necessary for reproduction. Secondly, by nature, outcrossing increases genetic diversity among offspring populations and may confer

advantages that would not be present in progeny populations of selfing hermaphrodites. Finally, male progeny may be beneficial in populations of hermaphrodites that have experienced a prolonged dauer stage because male (and female) progeny will reach sexual maturity more quickly than their hermaphrodite siblings (Felix 2004; Figure 6) and will therefore begin reproduction sooner.

Future Directions

The current study has uncovered patterns of male production in self-broods of *R.* sp. SB347 hermaphrodites. Because this was one of the first studies to examine reproduction throughout the total brood, many questions remain. It will be important to determine if the patterns of male production in self-broods are heritable across generations. What characteristics may be passed down? Will the trends in male production be conserved? Will the total brood sizes be comparable? As an extension of these questions, if these phenotypes are determined to be heritable, can they be selected for over time or are they immutable? To address these questions, I would conduct brood studies on individual hermaphrodites selected during their final larval molt and track their progeny outputs over time. I would then repeat this experiment using the F1 progeny as adult hermaphrodites. Based on preliminary brood study data, I would expect that brood sizes might be conserved across generations, but that trends in male production may be variable.

In light of the epigenetic studies conducted as part of this chapter, it would be important to determine how maternal condition might affect total brood size or male proportions. As shown in recent studies in *R.* sp. SB347 hermaphrodite self-broods, steroid hormones can modulate sexual plasticity in XX animals mediated by the dauer pathway. The dauer pathway in *C. elegans* is homologous to the insulin-like growth factor receptor in humans that has implications for longevity due to metabolism

and oxidative stress (Murphy and Hu 2013). To determine what environmental conditions may affect lifespan or reproductive patterns in *R.* sp. SB347 hermaphrodites, it would be important to assay self-broods with different nutritional conditions. In the current study, I evaluated the self-broods of hermaphrodites that had been starved during their early larval stages and possibly earlier, before the gametes that generated their zygotes came together. It would be interesting to observe effects of limited nutrition on the F_1 generation after P_0 hermaphrodites reached sexual maturity. To do this, I would select hermaphrodites as dauers from plates with high food. Then, I would transfer them to plates in which food was limited through poor quality or low amounts. From this study, I would be able to determine the effects that nutrition has on developing oocytes as manifested through offspring production.

Chapter 2: Investigation of oocyte meiosis in *Rhabditis* sp. SB347 hermaphrodites

Introduction

Meiosis is the multi-step process by which diploid germ cells give rise to haploid gametes (sperm and eggs). This chromosome-centered process involves the pairing and recombination of homologous chromosomes as well as the "reductive" segregation of these chromosomes during two rounds of cell division (Figure 7). During the first division, homologs segregate to the two daughter cells, effectively decreasing the genetic information by half. During the second division, sister chromatids segregate in a process that is very similar to mitosis.

Faithful meiotic segregation of chromosomes during the formation of gametes is critical for the viability of the resulting embryo. When chromosomes mis-segregate, the resulting embryo or fetus typically dies *in utero* or shortly after birth. Individuals that survive past this stage often have severe developmental consequences. In humans, the best-known disorder that results from mis-segregation of chromosomes during meiosis is Trisomy 21 or Down syndrome. The mis-segregation of this tiny autosome is familiar, because this is the only known autosomal trisomy in humans that allows individuals to live to middle age.

While meiotic mis-segregation of autosomes can often result in embryonic lethality, conditions associated with mis-segregation of sex chromosomes are less severe. Triple-X Syndrome (XXX) and Turner

Syndrome (XO) are two examples of sex chromosome aneuploidies in humans. Triple-X females often do not show adverse phenotypes and are usually fertile, but females with Turner Syndrome fail to display secondary sex characteristics and are sterile (Campbell 1996; Griffith et al. 1993; Kowles 1985).

Caenorhabditis elegans is a powerful genetic system for studying meiotic segregation defects as the animals have short lifespans, large broods, and obvious phenotypes associated with meiotic defects. Naturally occurring instances of X chromosome mis-segregation in wild type self-fertilizing *C. elegans* hermaphrodites (N2 strain) result in the production 0.2% XO male progeny (Hodgkin et al. 1978). In contrast, self-fertilizing *C. elegans* hermaphrodites with mutations in *him (h*igh *i*ncidence of *m*ales*)* genes produce high frequencies of XO male progeny, and, depending on the nature of the mutation, high frequencies of dead embryos (Hodgkin et al. 1979). Some *him* mutations can increase male production by self-fertilizing hermaphrodites as much as 150 fold (Hodgkin et al. 1978). Mutations in two him genes (*him-5* and *him-8*) specifically affect X chromosome segregation. In these strains, affected hermaphrodites produce high proportions of males, but few or no dead embryos. In *him*-8 mutants, the X chromosomes are specifically lost through nondisjunction events during the reductive (meiosis I) division in oocyte meiosis (Cortes et al. 2015). The high frequency of X-chromosome mis-segregation in *him-8* ova results in selfing *him-8*

hermaphrodites producing 36.7% XO male progeny and 6.4% triplo-X dumpy hermaphrodites in addition to normal XX hermaphrodites (Hodgkin et al. 1979). The observation that there are fewer triplo-X dumpy progeny than XO male progeny suggests that there is a bias towards depositing unpaired X univalent into the first polar body of oocyte meiosis (Hodgkin et al. 1979); a prediction that has been subsequently confirmed cytologically (Cortes et al. 2015; Figure 7C and D).

One nematode species that exhibits striking sex skews in non-mutant populations is *Rhabditis* sp. SB347. *R.* sp. SB347 is especially interesting to study because it is a triecious species with three distinct sexes: XX hermaphrodites, XX females, and XO males. Two distinct types of skewed sex ratios have been observed; selfing hermaphrodites produce an unexpected large number of males (Felix 2004; Chapter 1) and males sire almost exclusively feminine progeny (Felix 2004; Shakes et al. 2011). Cytological studies of male spermatogenesis have revealed two notable features of spermatogenesis in *R.* sp. SB347. During the first meiotic division that is normally associated with the unlinking and segregation of homologs, the sister chromatids of the X univalent split and segregate (Shakes et al. 2011). Then, during anaphase of meiosis II, key sperm motility proteins specifically segregate to the X-bearing spermatids (Shakes et al. 2011). The end result of this process is the production of viable X-bearing spermatozoa and non-viable nullo-X spermatids that causes cross progeny to be >95% XX feminine (Felix 2004; Shakes et al.

2011). These findings suggest that there is special handling of the X-chromosome as part of a modified meiotic program during male spermatogenesis.

How selfing *R. sp.* SB347 XX hermaphrodites produce XO male progeny remains poorly understood. Importantly, *R.* sp. SB347 hermaphrodites produce high proportions (~10%) of male progeny without producing dead embryos (Felix 2004; this study – Chapter 1). Thus wild type *R.* sp. SB347 hermaphrodites have evolved a mechanism to specifically alter X-chromosome segregation during the meiotic divisions of one or both sets of gametes, analogous to the *him-8* strains of *C. elegans*. In terms of the underlying mechanism, two pieces of preliminary data suggest that oogenesis may be the source of nullo-X gametes. First, XX hermaphrodites produce ~10% XO male progeny during both self-fertilization and in outcrossing with XO males (Pires-da Silva, personal communication). If hermaphrodite spermatogenesis contributed to male production by generating nullo-X sperm, we would expect to see larger proportions of male progeny during selfing than in outcrossing with males as almost all male sperm bear an X chromosome (Shakes et al. 2011). Second, mating sperm-depleted *R.* sp. SB347 hermaphrodites with males (n=39 complete broods) generated similar proportions of male progeny as female-male crosses (n=24 broods) (Shakes et al. 2011), suggesting that hermaphrodite sperm, like male sperm, are haplo-X.

Based on these findings, we hypothesize that the X chromosome may also be governed by separate meiotic mechanisms during hermaphrodite oocyte meiosis, resulting in nullo-X ova during hermaphrodite oogenesis and the large number of males observed in self-broods (Chapter 1, Felix 2004). In Chapter 2, we use cytological approaches to explore X-chromosome loss in hermaphrodite oogenesis. Results show that X homologs may be unpaired during metaphase I, lag during anaphase I, and be preferentially deposited into the first polar body during oocyte meiosis.

Results

Similar to *C. elegans, R.* sp. SB347 has XX hermaphrodites that reproduce by self-fertilization or outcrossing (Pires da-Silva 2007; Felix 2004). However, while *C. elegans* hermaphrodites produce nearly 100% XX progeny, *R.* sp. SB347 hermaphrodites only produce ~90% XX progeny, suggesting selection favoring production of a nullo-X gamete (Felix 2004; Chapter 1.) To investigate the underlying cause of the distorted sex ratio among the self-progeny of *R.* sp. SB347 hermaphrodites, we examined oogenesis in celibate *R.* sp. SB347 and *C. elegans* hermaphrodites using cytological approaches. We dissected celibate adult hermaphrodites at various developmental time-points and analyzed their meiotic embryos using immunofluorescence microscopy (Figure 8A). *C. elegans* N2 (wild type strain) served as our control.

Consistent with published literature, we found that meiosis involves the formation of an acentriolar spindle that shortens and rotates to segregate homologous chromosomes in meiosis I, followed by segregation of sister chromatids in meiosis II (Albertson and Thomson 1993). In each division, the meiotic spindle is initially parallel to the embryo cortex, but then rotates to become perpendicular to the cortex (Figure 8A, Albertson and Thomson 1993). The spindle forms a mid-body between the dividing homologs and the first polar body is extruded (asterisk in Figure 8B, Albertson and Thomson 1993; McNally and McNally 2005). This process repeats as sister chromatids segregate in meiosis II (McNally and McNally 2005). After each meiotic division, we observed the formation of a polar body.

Oogenesis in hermaphrodite self-embryos of *R.* sp. SB347 follows a similar process. After fertilization, the spindle forms around the chromatin in prometaphase of meiosis I (Figure 9A). The spindle forms parallel to the cortex, shortens, and rotates to become perpendicular to the cortex (Figure 9A). The microtubules come between homologous chromosomes to form a mid-zone during anaphase, and half of the genetic material is extruded into the polar body in a highly asymmetric cell division (Figure 9B). A similar process occurs during meiosis II to segregate sister chromatids (Figure 9B), yielding a haploid ovum and a second polar body (not shown). In contrast to our analysis of oocyte meiosis in *C. elegans*, our analysis in *R. sp.* SB347 revealed the frequent presence of lagging chromosomes during anaphase I (n=24/58, Figure

10). Notably, we did not observe lagging chromosomes in the meiotic embryos of R. sp. SB347 during anaphase II (n=0/45, Figure 10).

To verify our assessment, we examined oocytes of *C. elegans him-8* mutants as these have been specifically shown to exhibit unpaired X chromosomes during diakinesis (Phillips et al. 2005) and lagging X chromosomes during anaphase I (Cortes et al. 2015). Consistent with this published study, we also observed lagging chromosomes during oocyte meiosis I in *him-8* (Figure 10). At the same time, the absence of dead embryos on *R.* sp. SB347 brood plates suggests that the molecular mechanism underlying male production is specific to the X chromosome. Given the similarity to our *R. sp.* SB347 results, we hypothesized that X chromosomes may also be unpaired during oocyte meiosis in *R.* sp. SB347.

To determine if X univalents are unpaired during oocyte meiosis, we imaged fixed embryos stained with DAPI and anti-α-tubulin to determine the number of chromosomes (DAPI-staining bodies, either two unpaired univalents or a single bivalent) present at metaphase plates. As a control, we imaged metaphase plates from meiotic embryos of *C. elegans* hermaphrodites and saw the expected five paired autosomal bivalents and one set of paired X homologs in meiosis I and unpaired univalents in meiosis II (Figure 11). Based on preliminary studies of metaphase plates during male spermatogenesis, we hypothesized that *R.* sp. SB347 metaphase plates would have 7 or 8 DAPI-staining bodies,

representing 6 paired autosomes (bivalents) and X homologs that were either paired (a single bivalent) or unpaired (2 univalents), respectively. Results (Figure 11) show metaphase plates with either seven (5/17) or eight (12/17) DAPI-staining bodies during metaphase I, and either six (n=16/19) or seven (n=3/19) DAPI-staining bodies during metaphase II.

To independently confirm the number of autosomes present in *R.* sp. SB347 animals, we conducted a separate analysis of male spermatogenesis. Since males have a single X chromosome that segregates into sister chromatids during meiosis I, we expected to see metaphase I plates with seven DAPI-staining bodies (6 autosomal bivalents and an unpaired X univalent). During metaphase II, we also expected to see seven DAPI-staining bodies (6 autosomal univalents, and a single X chromosome). Our results show that all spermatocytes contained 7 DAPI-staining bodies (Figure 12).

Based on our brood studies (Chapter 1), we hypothesize that during periods of high male production (>15%) 0-12 hours and 72-84 hours after the final hermaphrodite molt, we should observe higher frequencies of lagging X-chromosomes and metaphase plates containing 8 DAPI-staining bodies. However, this approach did not yield stage-specific differences in the frequency of meiotic figures with mis-segregating X chromosomes (data not shown).

Discussion

Recent studies have shown that *R.* sp. SB347 XX hermaphrodites produce large proportions (10%) of XO male progeny. Our results suggest a cellular mechanism that helps explain this phenomenon. First, X chromosomes are often unpaired during metaphase I (Figure 11); then, they lag during anaphase I (Figure 10), and, finally, they are deposited into the polar body to yield a nullo-X ovum.

In comparing oocyte meiosis in *C. elegans* and *R.* sp. SB347 hermaphrodites, we observed a lagging chromosome during meiosis I of *R. sp.* SB347, that was notably absent in *C. elegans* wild type controls. In contrast, we did observe lagging X chromosomes during meiosis I in *him-8* hermaphrodite self-embryos (Figure 10), as previously reported by others (Cortes et al. 2011). Presuming that, as in *him-8* hermaphrodites (Cortes et al 2015; Phillips et al. 2005), the unpaired univalents observed at metaphase I (Figure 11) are X chromosomes, which subsequently lag on the metaphase I spindle (Figure 10), our results suggest that these events are responsible for nullo-X oocyte production among *R.* sp. SB347 hermaphrodites.

Interestingly, although we observed 7 (6 paired autosomes plus paired X homologs) or 8 (6 paired autosomes plus 2 unpaired X univalents) DAPI-staining bodies at metaphase I, we never observed 8 DAPI-staining bodies (6 autosomal univalent and two X univalent) in metaphase II (Figure 11). This finding suggests that unpaired X-

univalents may segregate equally into the first polar body and secondary oocyte, or may both segregate into the first polar body.

As part of our analysis we attempted to correlate periods of high male production (Chapter 1) with frequencies of X-chromosome loss (Chapter 2). We expected to observe similar frequencies of male progeny and X-chromosome loss throughout 12 hour intervals in self-broods. Results showed similar frequencies of X-chromosome loss across self-brood intervals (not shown). Perhaps the unpaired univalents or lagging X chromosomes triggered cell cycle checkpoint delays. These delays in the cell cycle would prolong these phases of meiotic division in male-fated embryos and would account for the inflated frequency of lagging X-chromosomes observed. The unpaired X chromosomes may segregate to opposite spindle poles to yield a haplo-X ovum, both be retained within the oocyte to yield a diplo-X ovum, or both extruded to yield a nullo-X gamete. Fertilization of these ova by X-bearing sperm would yield an XX feminine animal, triplo-X feminine animal, or XO male as shown in *him-8* hermaphrodites (Figure 7D, Cortes et al. 2015). In *C. elegans,* triplo-X animals have a characteristic dumpy phenotype and are uncoordinated (Hodgkin et al. 1979). We did not observe dumpy animals in *R.* sp. SB347 self-broods in Ch 1. or metaphase II figures with 8 DAPI-staining bodies. These observations suggest that X chromosomes are not retained within oocytes, but either segregate equally or are both deposited into the first polar body.

We encountered several challenges in attempting to correlate results from Chapters 1 and 2. Firstly, the relatively large proportion of males produced in *R.* sp. SB3467 self-broods is actually a small (10%) proportion of total reproductive output. For comparison, *C. elegans him-8* hermaphrodites produce almost four times more male progeny. Additionally, the brood intervals associated with high (15%) or low (10%) male production do not present dramatically different male proportions, necessitating a large sample size to demonstrate a similar comparison on a cytological level. As a final point, the transgenic technology has not yet been developed for *R.* sp. SB347 as it is in *C.* elegans, so I am unable to construct fusion proteins to observe meiotic progression in real time. For this study, I was confined to immunofluorescence microscopy of fixed embryos, which only provides a small snapshot of meiotic events.

To put these results into context, similar reports of X-chromosome loss as a driving force for distorted sex ratios have been described in other nematode species. In one such example, XX hermaphrodites of *Rhabdias ranae* demonstrate X-chromosome specific meiotic segregation defects during hermaphrodite spermatogenesis. It has been shown that during meiosis I, unpaired X univalents lag on the metaphase plate and segregate away from each other into secondary spermatocytes (Runey et al. 1978). During meiosis II, X chromosomes lag again and one sister chromatid is deposited into a small cellular body, known as a cytophore to yield one X-bearing spermatid and one nullo-X spermatid (Runey et al.

1978). Upon fertilization of X-bearing oocytes, these sperm products would yield a 1:1 ratio of XX and XO progeny. As a parasitic nematode, this altered meiotic mechanism may be advantageous by allowing hermaphrodites to produce free-living male and female offspring to alternate between generations (Gregory 1966; Runey et al. 1978). Similarly, *R.* sp. SB347 XO males have altered X-chromosome segregation patterns that result in almost no (<5%) male progeny (Felix 2004; Shakes et al. 2011). In this mechanism, males produce equal numbers of nullo-X and X-bearing spermatids, but the nullo-X spermatids are not capable of fertilizing oocytes (Shakes et al. 2011). Although each of these examples involves X-specific chromosome segregation defects during spermatogenesis, they share several interesting features with the skewed sex ratio we describe during oogenesis of *R.* sp. SB347 hermaphrodites. In each situation, chromosomes are unpaired during metaphase and lag during anaphase. The reason that the chromosomes are unpaired may vary, but each situation resulted in lagging during anaphase.

In Chapter 2, we hypothesized that the sex ratio distortion observed among hermaphrodite self-progeny is be caused by nullo-X ova. Our results show that X univalents are unpaired during metaphase I, lag during anaphase I, and are deposited into the first polar body to yield nullo-X ova. Although further studies will be required to determine the molecular factors involved with X chromosome pairing in *R.* sp. SB347 hermaphrodites, this

study is one of the first to describe a meiotic mechanism related to a distorted sex ratio on a cytological level.

Future Directions

To further test our model that X-chromosome loss during anaphase I of oocyte meiosis serves as the mechanism for male production in *R.* sp. SB347 self-broods, I would employ several different types of microscopy along with cytological tools. Based on my hypothesis that cell cycle checkpoint delays are prolonging anaphase in situations where X-chromosomes lag, I could use differential interference contrast microscopy on self-embryos to time meiotic events from fertilization to pronuclear stages that precede cleavage. Tracking these embryos through development would allow me to determine the phenotype: XO male or XX feminine. I would expect that embryos that give rise to male progeny would have longer times from fertilization to pronuclear stages because of the lagging X chromosome that causes a delay at the M phase cell cycle checkpoint.

In my studies, I attempted to use a laser scanning confocal microscope generate 3-D reconstructions of metaphase plates within meiotic embryos. This technique resulted in photobleaching and poor quality images. In future studies, a spinning disc confocal microscopy may provide better results and quantifiable data as this technique uses a series of pinholes and lower excitation energy to generate an image.

Chapter 3: Cytological analysis of sperm production in hermaphrodites of *Rhabditis* sp. SB347

Introduction

Hermaphroditism is a reproductive strategy in which an organism can have both sexes during their life-time (Munday et al. 2006). This strategy is advantageous for organisms living at low population densities, which may not encounter a suitable mating partner (Tomlinson 1966). There is great variation among hermaphrodites. Some species change sex completely in a sequential switch of both germline and somatic structures (e.g., clownfish). Within this classification, animals can change from female to male or male to female. Other species possess both male and female somatic and germline structures and require another hermaphrodite to reproduce (e.g., earth worm). These animals cannot self fertilize. Another group of hermaphrodites have female somatic tissue, but produce both sperm and eggs. These animals are exclusively or mostly self-fertile (e.g., banana slug), and either experience a one-time switch between spermatogenesis and oogenesis (Ghiselin 1969; Smith 1970; Warner et al. 1975; Williams 1975) or produce both gametes simultaneously (Ghiselin 1969).

One phylum that contains many economically relevant species with diverse modes of reproduction, including several different versions of hermaphroditism is Nematoda (Kiontke and Fitch 2005). Among

nematode researchers, there is a common assumption that most sequential hermaphrodites are like the well-studied "model" nematode *Caenorhabditis elegans*; producing a small number of sperm before switching over to oocyte production (Bell 1982). However, the literature does contain several reports of hermaphroditic species with complex life cycles and alternative reproductive strategies including alternation of generations, parthenogenesis, and either simultaneous or alternating sperm and egg production.

Rhabdias ranae provides an example of a nematode species that alternates between parasitic hermaphroditic generations and free-living male and female generations. While some investigators have classified these hermaphrodites as protandrous (Hyman 1951), others have documented the production of sperm simultaneously along with oocytes (Runey et al. 1978; Schleip 1911). Like hermaphrodites of *R. ranae*, hermaphrodites of *Meloidogyne hapla* produce oocytes simultaneously with spermatozoa (Triantaphyllou 1993). However, in the absence of sperm, hermaphrodites can can also reproduce by parthenogenesis (Triantaphyllou 1993). A third example of hermaphroditic reproduction is represented by *Rhabditis gurneyi,* a hermaphroditic species that alternates between sperm and egg production (Potts 1910). Taken together, these examples illustrate the complexity of hermaphroditic reproductive mechanisms among nematodes.

The rhabditid clade provides an interesting model for studying hermaphroditism as hermaphroditism has independently evolved more than ten times, with three occurrences in the *Caenorhabditis* genus alone (Kiontke and Fitch 2005; Kiontke, et al. 2004). The most extensive cytological studies in any hermaphroditic nematode have been conducted in *C. elegans*. The *C. elegans* germline is oriented in a linear, temporal array of germ cells that allows easy observation of mitotic and meiotic progression (Crittendon et al. 1994). In *C. elegans,* hermaphrodites make an initial pool of ~300 sperm to be used for self-fertilization during adulthood (Brenner 1988; Figure 13). Spermatogenesis is limited to the L4 stage, after which, the germline switches over to the exclusive production of oocytes and no more sperm are produced (Kimble and White 1981). In the course of the cytological study exploring oocyte meiosis in *Rhabditis* sp. SB347 hermaphrodites (Chapter 2), meiotically dividing spermatocyte-like cells were observed in older animals. This peculiar and unexpected observation raised the question of how sperm are produced in hermaphrodites of this species. We found that *R.* sp. SB347 hermaphrodites are not sequential hermaphrodites, like *C. elegans*, but produce spermatozoa simultaneously along with ova utilizing spermatogonia-like sperm precursor cells as progenitors. These cells are located outside of the traditional mitotic proliferative zone, but contain mitotic potential and do not express differentiated sperm cell markers. In

Chapter 3, we use cytological approaches to investigate these unexpected, but fascinating observations in *R.* sp. SB347 hermaphrodites.

Results

Cytological comparisons between germlines of XX animals in *C. elegans* and *R.* sp. SB347 show vastly different structures (Figure 15A). Specifically, *C. elegans* has a much larger mitotic proliferative zone, but not nearly as many oocytes as *R.* sp. SB347 (Figure 15A). Preliminary characterization of the *R.* sp. SB347 hermaphrodite germline revealed the presence of pHisH3 (ser 10) positive cells of unknown function (K. Rehain Honors Thesis 2012; Pablo Ordonez and Lakshmi Nagarajan, unpublished observations). These cells could also be recognized by their distinctive pattern by DAPI staining. In these early studies, our lab group hypothesized that these cells were part of the somatic gonad, perhaps functioning as nurse cells to provide proteins or mRNAs to the developing oocytes. To further explore the nature of these "mystery cells" of unknown function, I examined the germlines of *R.* sp. SB347 XX females for the presence of these cells. I expected that if they were involved in oogenesis, that I would also see them in XX females. Results show that hermaphrodite germlines contained "mystery cells" but female germlines (n=31/31) did not (Figure 15B, white arrow), indicating that they were specific to hermaphrodites. Because hermaphrodites and females are somatically identical (Felix 2004; Chaudhuri et al. 2011), the fact that these "mystery cells" are present in hermaphrodites, but not females suggests that these cells may have a role in sperm development.

In the separate context of analyzing older (48 hour post final molt) self-fertile *R.* sp. SB347 hermaphrodites for my cytological studies (Chapter 2), I observed a cluster of condensed DAPI-staining nuclei with prominent tubulin spindles, that were never observed in older celibate *C. elegans* hermaphrodites or in *R.* sp. SB347 females (Figure 15B, white double arrow). These cells appeared very similar in size and pattern to dividing spermatocytes in *R.* sp SB347 males (Shakes et al. 2011; E. Winter Honors Thesis 2014). Additionally, these cells were located near clusters of spermatids within the spermatheca of older, celibate hermaphrodites (Figure 15B, red arrow). Given these results, I hypothesized that the proximal spermatocyte-like cells (Figure 14B, white double arrow) may actually be spermatocytes.

To determine whether the "mystery cells" were involved in spermatogenesis, I used the major sperm protein (MSP) as a marker for sperm fate and stage. I expected that if these cells are involved in spermatogenesis, they would stain with MSP in a fibrous body morphology characteristic of dividing primary and secondary spermatocytes of *C. elegans* (Figure 16A) and later in a pseudopod-localized pattern within activated spermatozoa (Smith 2005; Figure 16B). Examination of *R.* sp. SB347 hermaphrodite germlines revealed that some clusters of "mystery cells" are MSP positive ("B" in Figure 17) whereas others were MSP negative ("A" in Figure 17). Additionally, within the MSP positive clusters, the MSP was organized into distinct fibrous bodies and cells were often

dividing ("B" in Figure 17), suggesting that these cells are meiotically dividing spermatocytes. These MSP positive clusters were always observed near the most proximal region of the germline near the -1 and -2 oocytes (data not shown).

Further analysis of MSP negative and MSP positive cell clusters revealed that within both types of clusters, cells are synchronized within the cell cycle (Figure 18A and B). The MSP negative cells may be precursors to the MSP positive cells, and the MSP positive cells may be spermatocytes or spermatozoa depending on the MSP morphology. The MSP positive cells had two distinct MSP patterns. In one pattern, MSP formed punctate structures, most closely resembling organization of MSP polymers in fibrous bodies (Figure 14B, 17B and 18B). Within and adjacent to the spermathecae, I observed spermatozoa with hypercondensed haploid chromatin masses and localization of MSP to pseudopods (Figure 18C). Within MSP positive cells clusters, I also observed cells with similarly condensed haploid chromatin masses that labeled positive for tubulin, but lacked MSP, suggesting that, as in males (Shakes et al. 2011) some hermaphrodite spermatids are inviable (18B blue arrow).

Discussion

It is widely accepted that most hermaphroditic nematodes are like *C. elegans* in that they produce a small number of sperm before switching to oocyte production (Bell 1982). However, several examples of hermaphrodites that produce spermatozoa and ova simultaneously have been reported (Runey et al. 1978; Triantaphyllou 1993). In the current work, I demonstrate a novel mechanism of sperm production among nematodes in which the germline uses spermatogonia to generate spermatocytes.

My studies reveal the presence of meiotically dividing spermatocytes in older celibate XX hermaphrodites in *R.* sp. SB347. These cells are absent from XX female germlines (15B, red arrow) and similar in size and morphology to dividing spermatocytes observed in *C. elegans* L4 hermaphrodites (data not shown), *C. elegans him-8* males (Figure 16), and *R.* sp. SB347 males (data not shown). These clusters are located in the proximal region of the gonad are MSP positive (Figure 17B), while more distal clusters are MSP negative (Figure 17A). My findings suggest that the MSP positive cells with tubulin spindles (17B) are meiotically dividing spermatocytes, while the MSP negative clusters (17A) are dividing mitotically. Based on this model, young L4 hermaphrodites of *R.* sp. SB347 begin sperm production slightly before oocyte production (Diane Shakes and Caitlin McCaig, unpublished observations) but then begin to produce both sperm and oocytes throughout the remainder of

their reproductive period(Figure 15B). Taken together, these findings suggest that *R.* sp. SB347 hermaphrodites are not protandrous, but rather simultaneous hermaphrodites. Similar reports have been found in *R. ranae*, a parasitic nematode in which older hermaphrodites have been shown to produce sperm along with ova (Runey et al. 1978). The experimental manipulation of the sperm to oocyte switch in temperature sensitive sex-determination mutants of *C. elegans* suggests that, even in this typically "sperm-first" system, germ cells in the distal mitotic zone retain the potential to develop into either spermatocytes or oocytes (Barton et al. 1987).

My studies suggest that the origin of spermatocytes in older, celibate hermaphrodites may be the previously reported "accessory somatic cells of unknown function" (K. Rehain Honors Thesis 2012). I have observed clusters of cells along the length of the hermaphrodite gonad that are MSP negative (Figure 17A and 18A) and have prominent tubulin spindles (Figure 18A). Cells within each cluster are in the same stage of the cell cycle (Figure 18A). In addition, I have found that the proximal clusters, closest to the spermatheca, have more cells, suggesting that cells within clusters are dividing as the clusters shift from distal to proximal locations . It remains unknown how these cells are being maintained. The character of these cells most closely matches the description of spermatogonia found in fruit flies and humans. In *C. elegans* adults, a somatic distal tip cell is known to maintain mitotically-

dividing germ cells via a Notch based cell signaling pathway (Kimble and White 1981). However, as some of these clusters with the gonads of *R.* sp. SB347 hermaphrodites are positioned far from the distal tip cell, they may require an alternate mechanism of maintenance.

In other systems, spermatogonia divide mitotically to yield a predictable number of spermatocytes, and then each these spermatocytes divide meiotically to yield four spermatids that mature into functional spermatozoa. Evidence suggests that meiotically dividing *R.* sp SB347 spermatocytes in both males (Shakes et al., 2011) and hermaphrodites (18B, blue arrow) do not produce residual bodies characteristic of *C. elegans*, but instead deposit tubulin (and likely other nonessential cellular components) into MSP negative spermatids after anaphase II. During male spermatogenesis, nonessential cellular components are specifically deposited into nullo-X spermatids (Figure 14, Shakes et. al). However this new discovery of a similar asymmetric spermatocyte division in XX hermaphrodites suggests suggests that the asymmetric segregation of MSP and other cellular components can occur in both XO and XX spermatocytes and does not require an asymmetric cue of an unpaired X chromosome. Although the two events may be coupled in male spermatocytes, there must be an independent driver of this asymmetric partitioning event in XX spermatocytes.

In light of these findings, I suggest that the MSP negative clusters of dividing cells found along *R.* sp. SB347 hermaphrodite germlines are

mitotically-dividing spermatogonia that generate daughter cells which may become spermatocytes. This study presents a novel finding in the context of nematode spermatogenesis as spermatogonia-like cells outside of the traditional mitotic zone have not been reported in either hermaphrodites or males.

Future Directions

Another unanswered question regarding these spermatogonia is how are they are physically associated with the rest of the gonad, and how are they acquiring nutrients? They may be directly attached to the oocytes or the central rachis, or they may be able to move independently along oocytes by unknown mechanisms. They may also acquire nutrients directly from the intestine; for example, yolk proteins in *C. elegans* are produced in the intestinal cells and secondarily transported to the developing oocytes (Hall et al. 1999; Kimble and Sharrock 1983). As a model for studying spermatogenesis, the *R.* sp. SB347 hermaphrodite germline may provide a more extreme example than *C. elegans* of developing spermatocytes lacking supporting somatic cells such as Sertoli cells.

Spermatogenesis in *C. elegans* hermaphrodites occurs during the L4 stage before switching to oocyte production (Kimble and White 1981). As young adults, hermaphrodites have a large pool of ~300 sperm in the spermatheca to fertilize ovulating oocytes. These sperm compete to fertilize oocytes, with the largest sperm gaining preferential access (LaMunyon and Ward 1997). This feature of spermatogenesis is markedly different from *R.* sp. SB347 hermaphrodite spermatogenesis, in which there does not seem to be a large pool of spermatozoa waiting for oocytes, but rather the production of a few spermatozoa followed by the

ongoing production of both spermatozoa and ova (Shakes Lab, unpublished).

The population studies discussed in Ch. 1 showed that reproductive lifespan of *R.* sp. SB347 hermaphrodites is much longer than it is in *C. elegans* hermaphrodites. Considering that both *R.* sp. SB347 and *C. elegans* wild type strains have similar brood sizes, this data has implications regarding ovulation rates. Within the *C. elegans* hermaphrodite gonad, all sperm are produced during the L4 stage and stored in the spermatheca at the proximal end of each gonad arm until fertilization. Oocytes are produced during oogenesis. Oocytes mature in response to a hormonal (MSP) signal from spermatozoa (McCarter et al. 1999; Miller et al. 2001). Presuming that oocyte maturation in *R. sp.* SB347 hermaphrodites is also simulated by MSP, *R.* sp. SB347 oocytes may be stimulated at different rates based on their proximity to sperm clusters. In my own studies, I did not observe major fluxes in ovulation rates based on progeny counts in 12 hour brood intervals in *R.* sp. SB347 (Ch 1), but future studies on the ovulation timing of individual oocytes in sequence might reveal a correlation between ovulation timing and proximity to spermatocyte clusters.

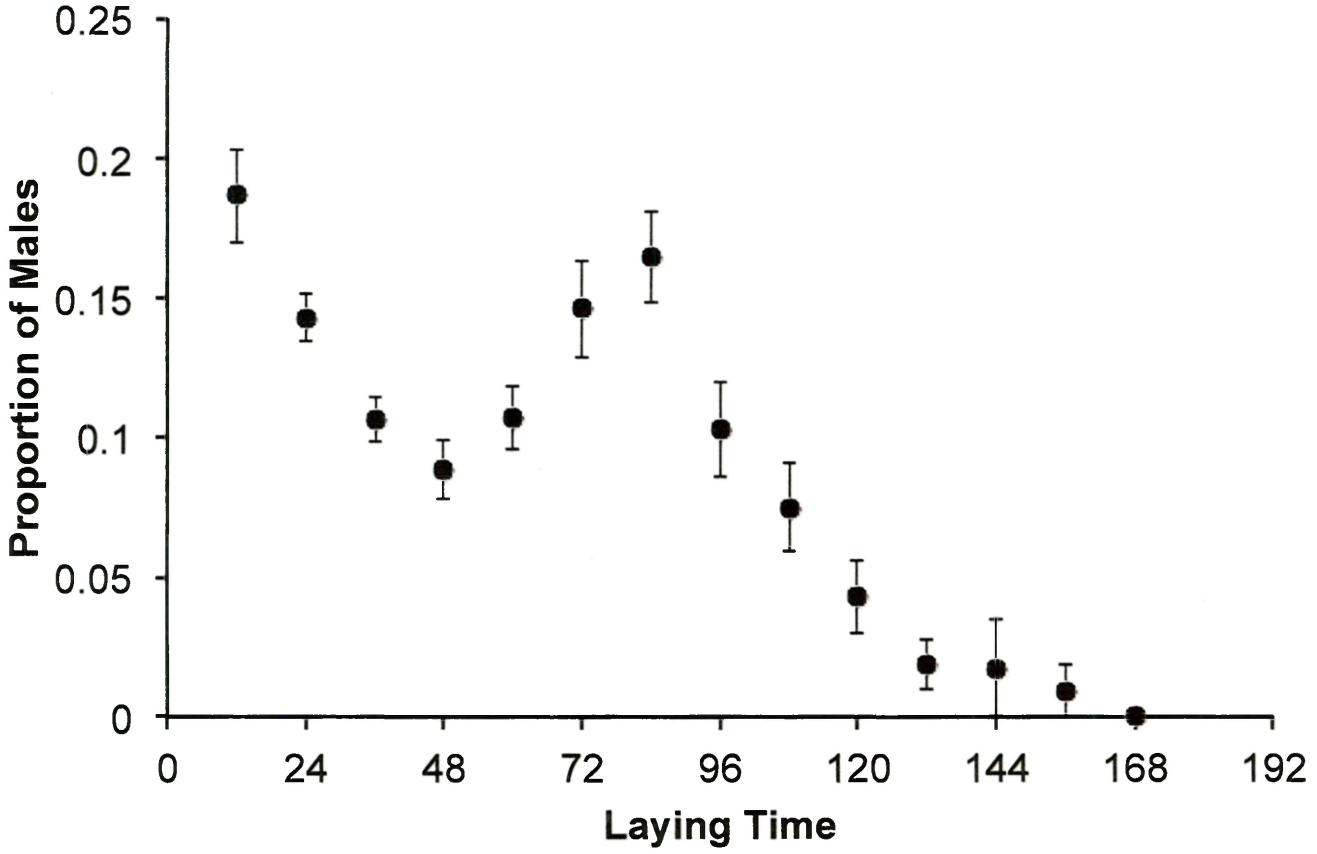
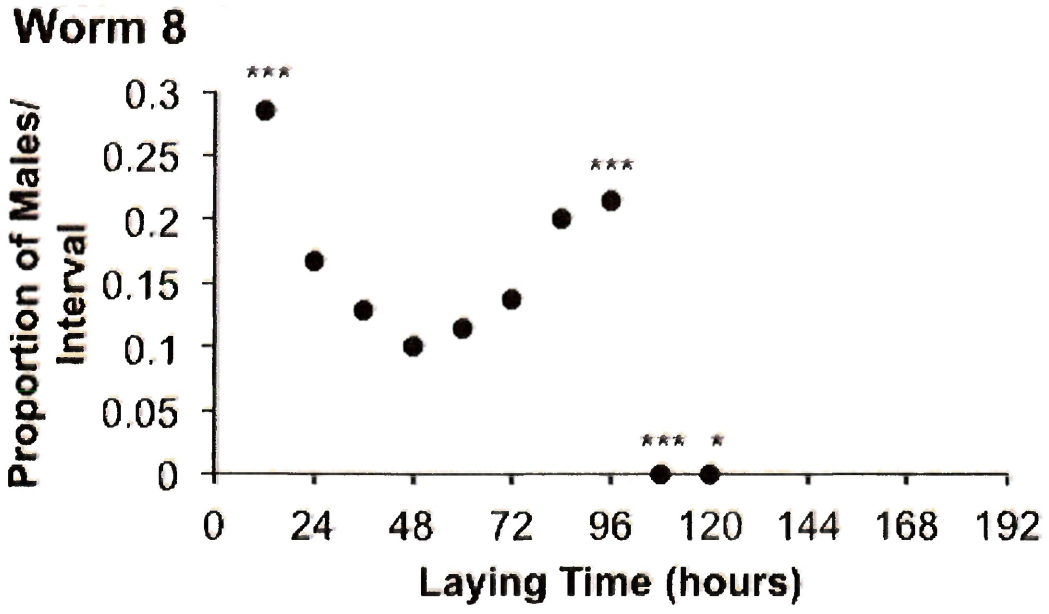
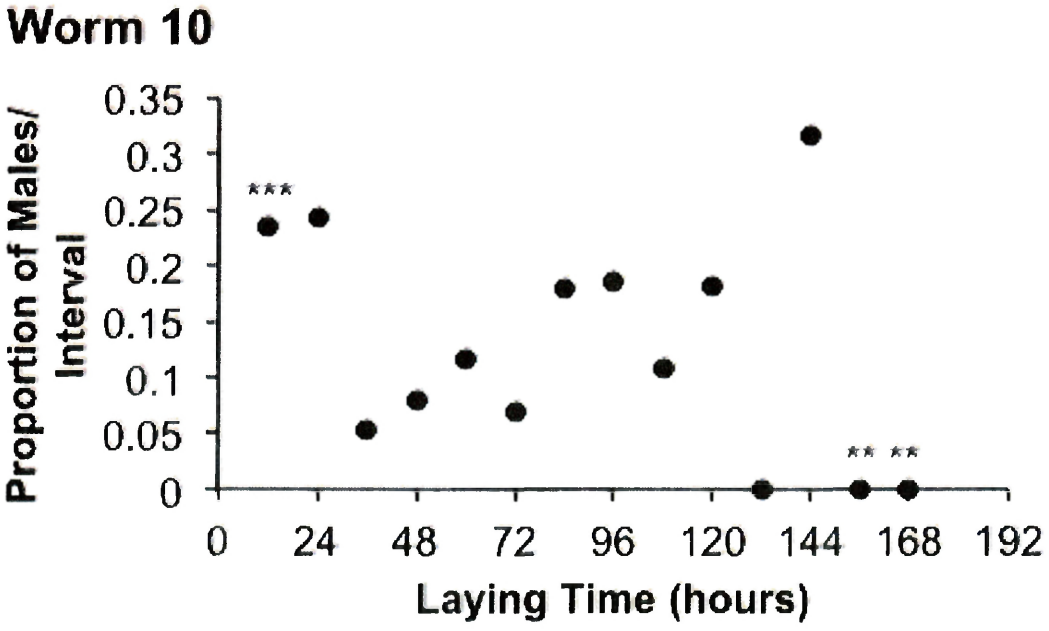


Figure 1: Proportion of XO male progeny in *Rhabditis* sp. SB347 self-broods varies over time. Average male proportions were calculated by dividing the number of males by the total number of progeny for each 12-hour interval (Average ± S.E.; n=36 complete broods).


Worm 8
Proportion of Males/ Interval
0.3
0.25
0.2
0.15
0.1
0.05
0
***
***
***
*
0
24
48
72
96
120
144
168
192
Laying Time (hours)


Worm 10
Proportion of Males/ Interval
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
***
** **
0
24
48
72
96
120
144
168
192
Laying Time (hours)

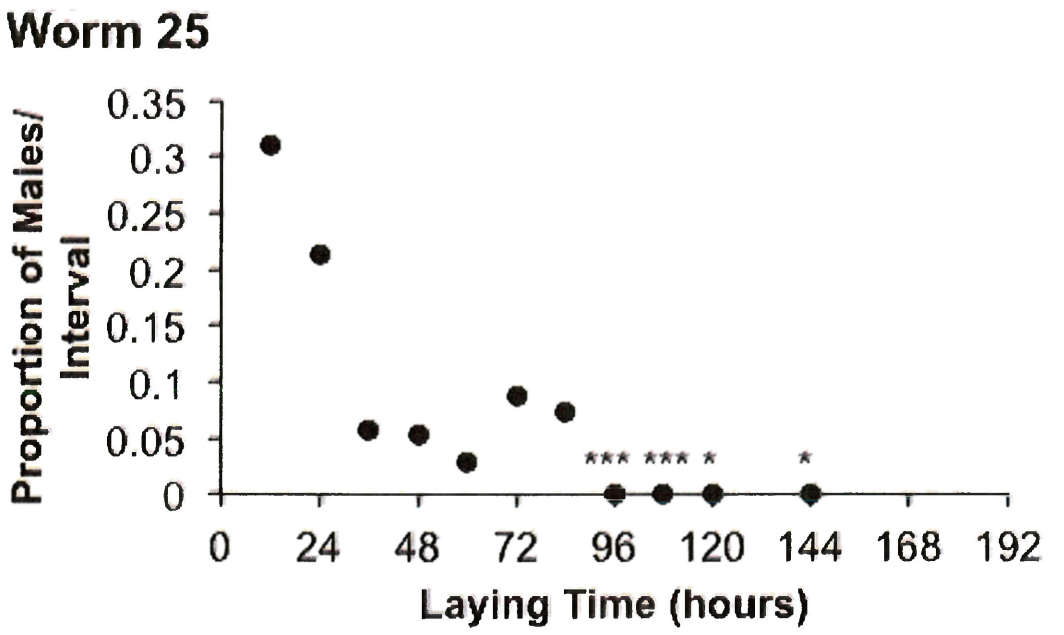


Figure 2: Individual *Rhabditis* sp. SB347 hermaphrodites produce males in different patterns throughout self-broods. Worm 8 demonstrates two peaks of male production (>10.5%). Worm 10 shows >2 peaks in male production. Worm 25 shows an initial peak of male production. The male proportions were calculated by dividing the number of males by the total number of progeny for each 12-hour interval. Three asterisks indicate total progeny count/interval between 10 and 20. Two asterisks indicate total progeny count/interval between 5 and 10. One asterisk indicates total progeny count/interval less than 5. (Male production is from worm 8, 10, and 25 in Supplementary Tables 1 and 2).

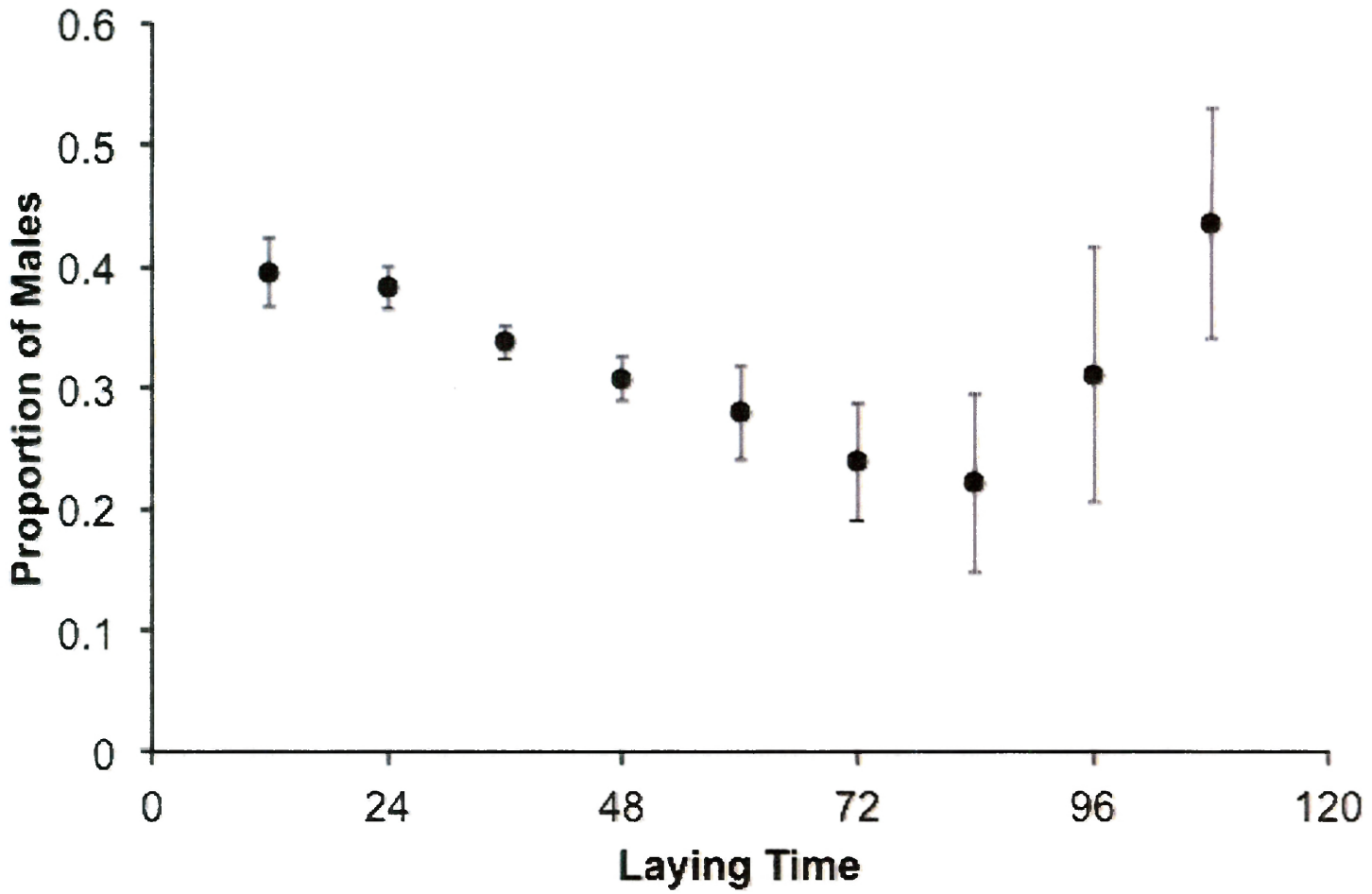


Figure 3: Proportion of XO male progeny in *him-8* self-broods remains relatively constant over time of high reproduction. Average number of progeny decreased markedly after 48 hours, resulting in large standard error bars beyond that time point. Male proportions were calculated by dividing the number of males by the total number of progeny for each 12-hour interval (n=20 complete broods).

	Number of complete broods counted	Average brood size	Percent males
Caenorhabditis elegans wild type (N2)	15	227 ± 13	ND
Caenorhabdtis elegans him-8	20	162 ± 6	34.6 ± 1.1
Rhabditis sp. SB347	112	249 ± 8	10.5 ± 0.4

Table 1: *Rhabditis* sp. SB347 hermaphrodites have 200-300 progeny in self-broods. Brood sizes and percentages are quoted as mean ± standard error. ND indicates not done, literature reports 0.02% male progeny in *him*-8 self-broods (Hodgkin et al. 1979).

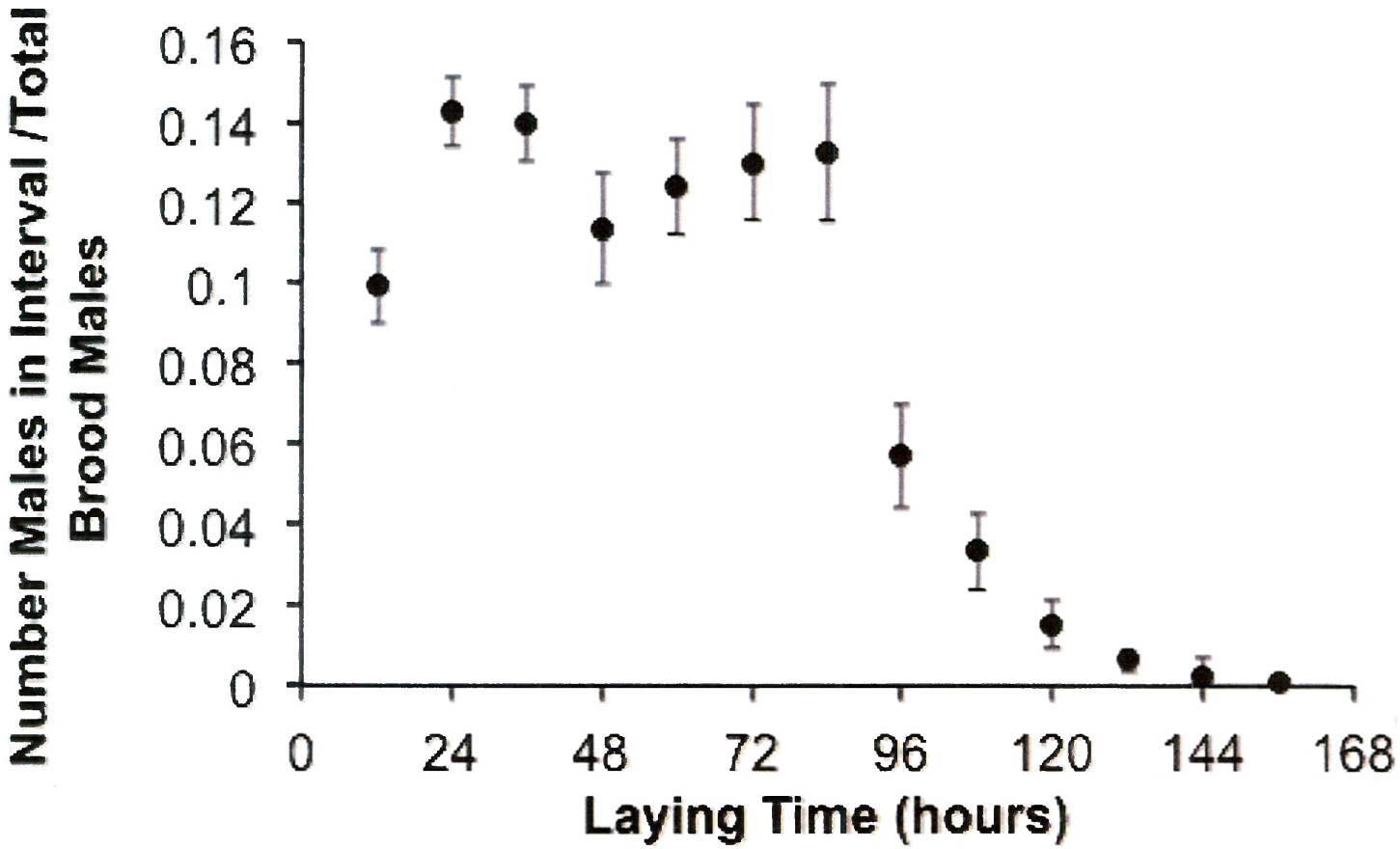


Figure 4: *Rhabditis* sp. SB347 hermaphrodites produced constant numbers of males during time of high reproduction. During the first 84 hours of reproduction, hermaphrodites produced 83% of their total broods. Results show averages of proportion of males produced within an interval out of total males produced within that specific self-brood (n=36 complete broods).

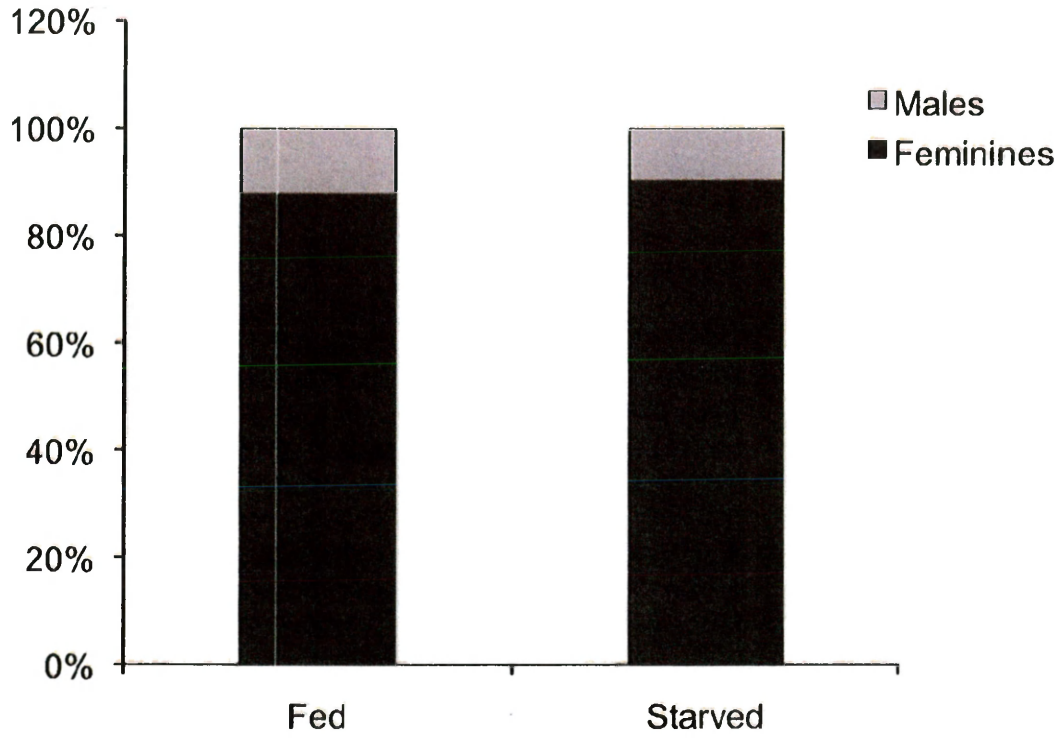


Figure 5: Nutritional deprivation of pre-dauer *Rhabditis* sp. SB347 hermaphrodites is associated with smaller proportion of male progeny. Results show average proportion males in broods from two treatment groups of celibate, adult hermaphrodites. Fed group (12.2%, n=6 983 progeny, n=853 males in 33 complete broods). Starved group (9.8%, n=5 291 progeny, n=521 males in 24 complete broods). Differences in total broods were insignificant (p=0.663) while differences in proportion males were significant (p=0.036).

Rhabditis sp. SB347 Treatment	Number of complete broods counted	Average brood size	Percent males
Fed	33	212 ± 13	9.7 + 0.9
Starved	24	220 ± 15	12.2 ± 0.7

Table 2: *Rhabditis* sp. SB347 hermaphrodite self-brood structures under varying pre-dauer conditions. Brood sizes and percentages are quoted as mean ± standard error.

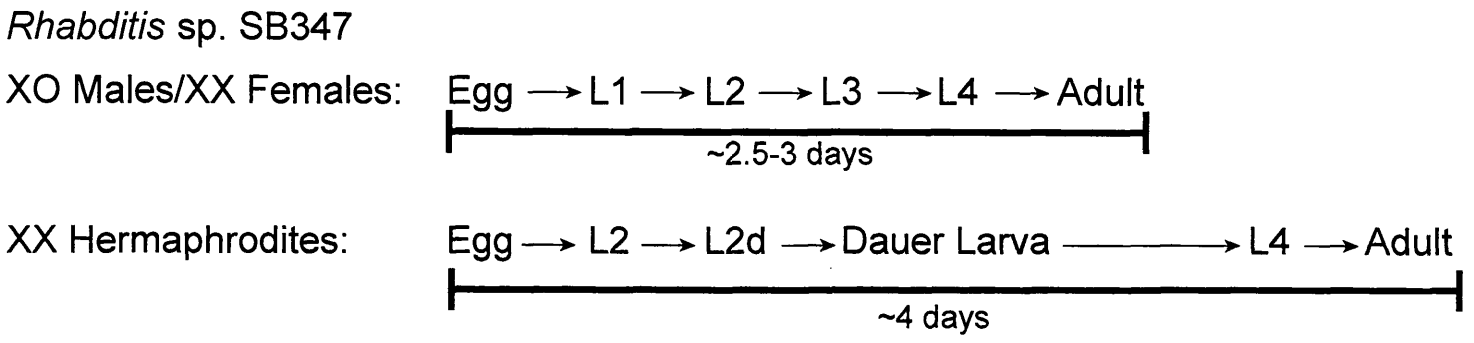
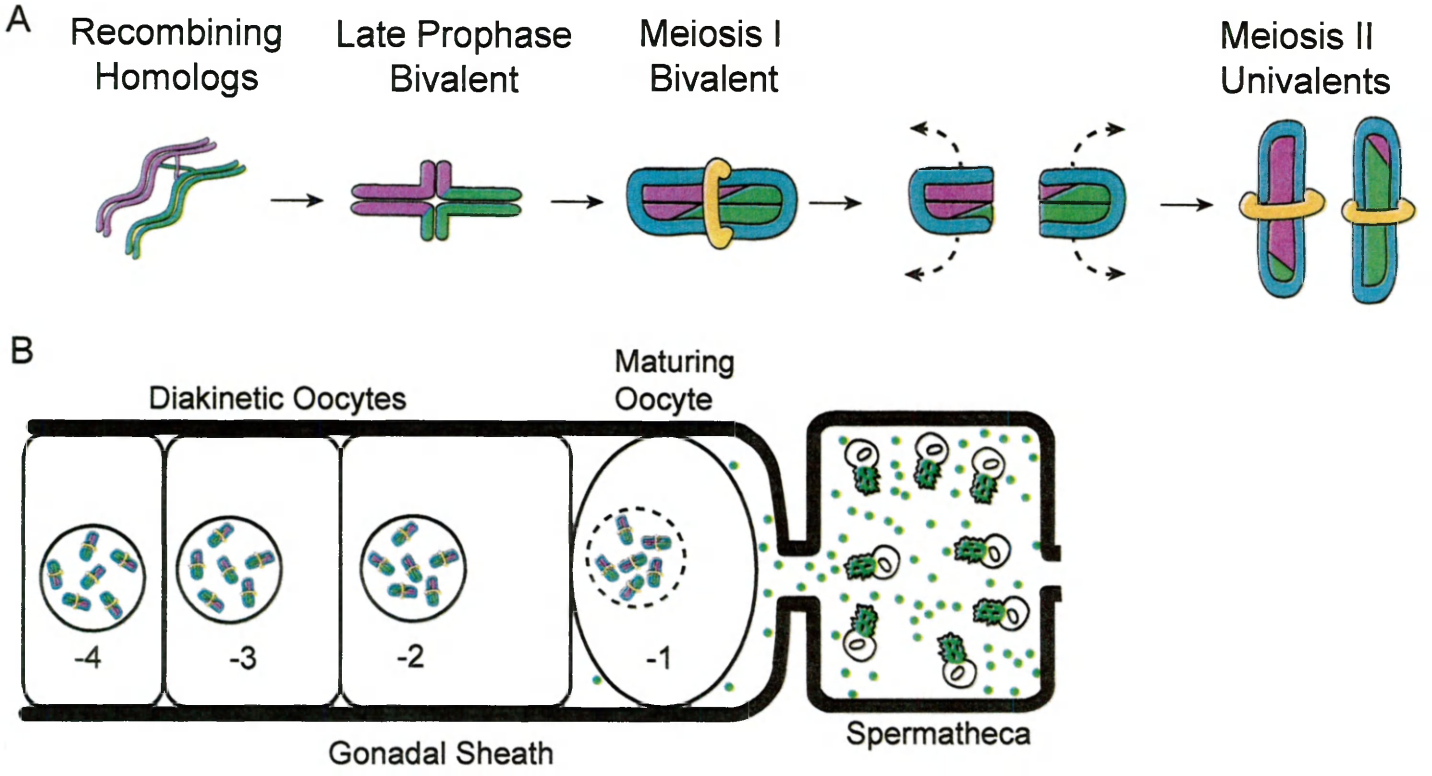


Figure 6: Life cycle and developmental stages of *Rhabditis* sp. SB347 animals. Males and females develop through four larval stages. They become sexual capable adults after 2.5-3 days. Hermaphrodites develop through an obligatory nonfeeding dauer stage. If left on food, hermaphrodites can exit dauer diapause in as little as 24 hours. In total, hermaphrodites develop into sexually capable adults in as little as 4 days, but can remain dauer larvae for months. Larval stages are indicated by "L". (Felix 2004; Adapted from Chaudhuri et al. 2011).


A
Recombining Homologs
Late Prophase Bivalent
Meiosis I Bivalent
Meiosis II Univalents
B
Diakinetic Oocytes
Maturing Oocyte
-4
-3
-2
-1
Gonadal Sheath
Spermatheca
C
Meta I
End View
Side View
Ana I
Meta II
End View
Side View
Ana II
Oocyte

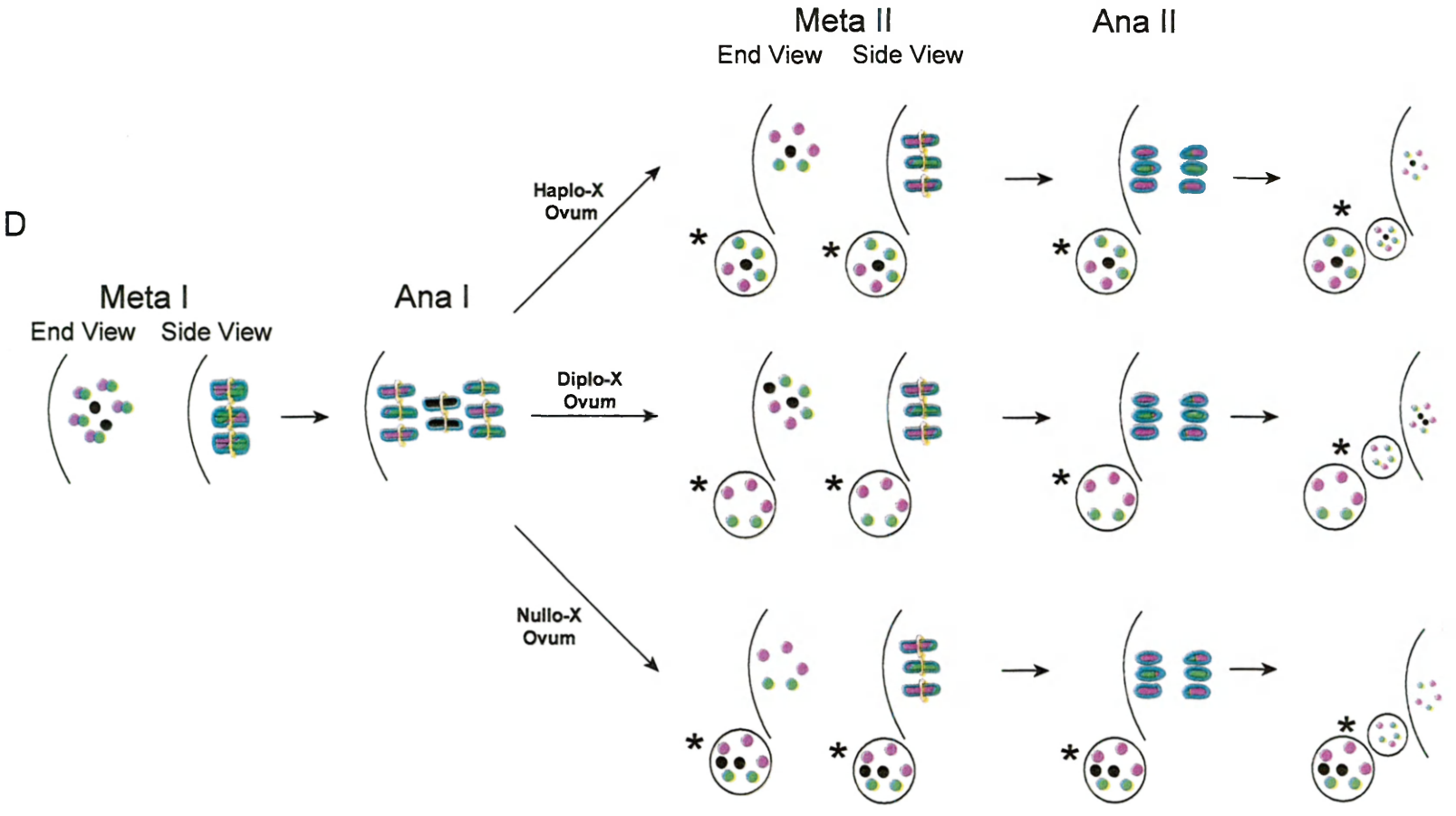


Figure 7: Schematic of oocyte meiosis in *Caenorhabditis elegans* hermaphrodites. (A) After homologs pair, nonsister chromatids recombine during pachytene. The chromosomes condense into bivalents during diakinesis in late prophase. Homologous chromosomes segregate during meiosis I to yield two univalent. The sister chromatids within the univalent will segregate during meiosis II. (B) While arrested in diakinesis, the oocytes wait for an MSP-mediated hormonal signal from the sperm to stimulate meiotic maturation (Yan

et al. 2003; Albertson and Thomson, 1993; Miller et al. 2001; Kim et al. 2013). Meiotic maturation includes nuclear envelope breakdown (dotted circle in -1 oocyte) and acentriolar spindle formation, which are critical steps in preparation for the meiotic divisions (Yan et al. 2003; Albertson and Thomson 1993; Miller et al. 2001; Kim et al. 2013). (C) In wild type *C. elegans* hermaphrodites, homologs are paired at the metaphase plate and associated with a meiotic spindle (not shown). The spindle elongates, forms a barrel shape, shortens, and rotates (not shown, Albertson and Thomson, 1993). During anaphase I, a midbody of microtubules forms between homologous chromosomes (not shown), which segregate equally to opposite spindle poles; and, in a highly asymmetric division, half of the genetic material is extruded into a small polar body, (indicated by an asterisk). Meiosis II begins as chromosomes align at the metaphase plate to prepare for segregation of sister chromatids. The barrel-shaped spindle forms around the chromosomes, shortens, and rotates (not shown, Albertson and Thomson, 1993). The sister chromatids segregate away for each other as one set of chromosomes is extruded into a second polar body and its haploid complement is retained in the final product of oogenesis, the ovum (Zickler and Kleckner 2015; Lui and Colaiacovo 2013). (D) In *him-8* hermaphrodites, the homologs of the X chromosome are unpaired during meiosis I, and either segregate to opposite spindle poles (Haplo-X ovum), remain within the oocyte (Diplo-X ovum), or are extruded into the polar body (Nullo-X ovum). Sister chromatids segregate in anaphase II. Autosomal chromosomes are shown in green and pink. X chromosomes are shown in black. Kinetochores are shown in light blue. Chromosome-specific motoproteins are shown in yellow. Metaphase and anaphase figures are depicted next to the oocyte cortex, represented by a curved line. (Adapted from Kosinski et al. 2005; Kuwabara 2003; and Wignall and Villeneuve 2009).

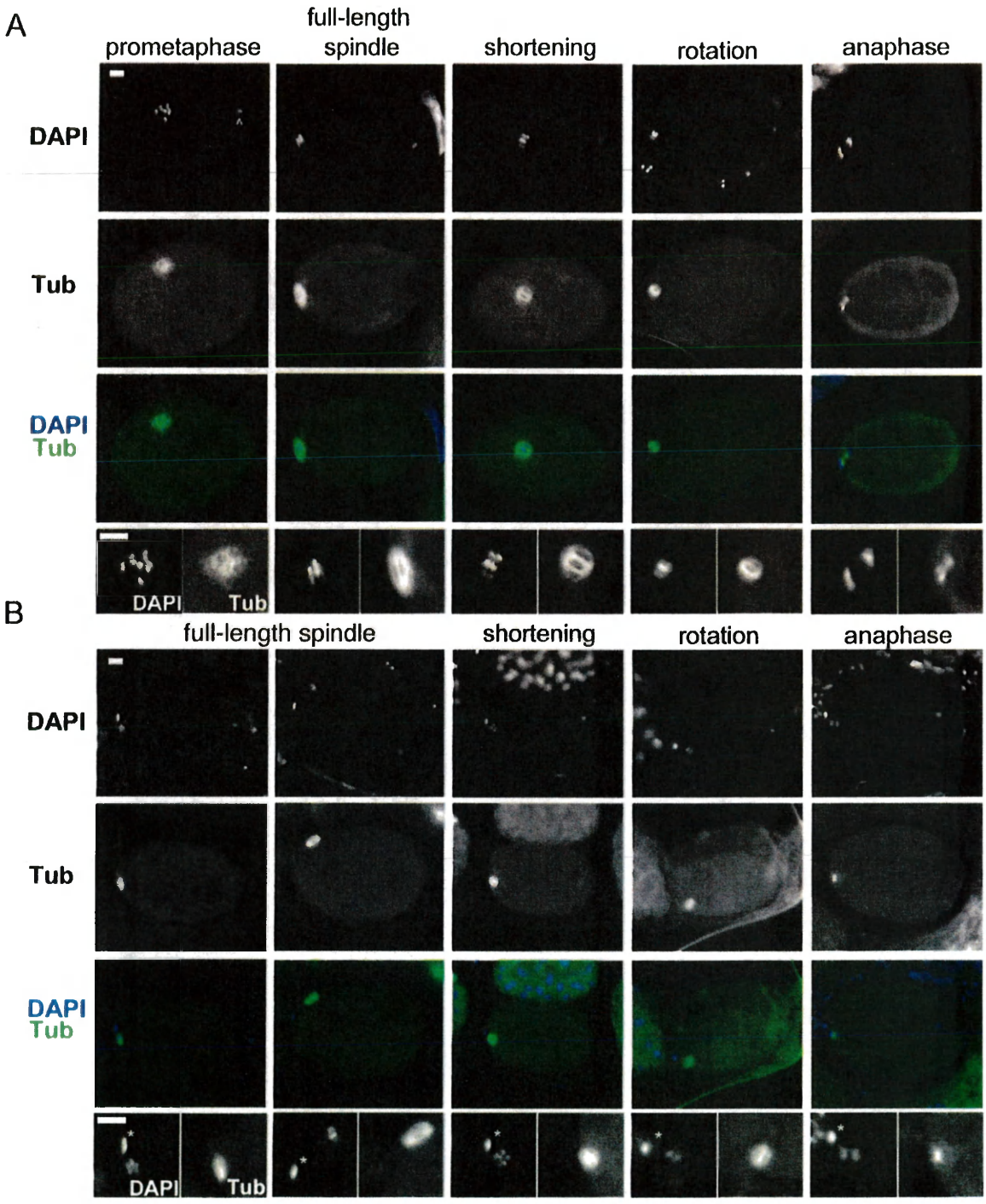

A
prometaphase
full-length spindle
shortening
rotation
anaphase
DAPI
Tub
DAPI
Tub
DAPI
Tub
B
full-length spindle
shortening
rotation
anaphase
DAPI
Tub
DAPI
Tub
DAPI
Tub

Figure 8: Stages of oocyte meiosis in *Caenorhabditis elegans*. Images show fixed preparations of meiotic one-cell embryos from dissected hermaphrodite germlines in meiosis I (A) and meiosis II (B). Prometaphase I spindle is shown at the embryo cortex oriented down the pole-to-pole spindle axis (far left, A) and in a perpendicular plane (adjacent image in the series). The spindle shortens, rotates toward the embryo cortex, and then the microtubules rearrange to form a midzone between the chromosomes during anaphase. The midzone lengthens to form a midbody and the first polar body is extruded. This process is repeated during meiosis II (B). For each embryo, a 2X magnification of the chromosomes (left) and meiotic spindle (right) are included at the bottom of the image. Images were acquired by epifluorescence microscopy. Asterisks (*) indicate polar bodies. Sperm chromatin mass is marked in the upper left image by ^. Scale bar, 5 μm.

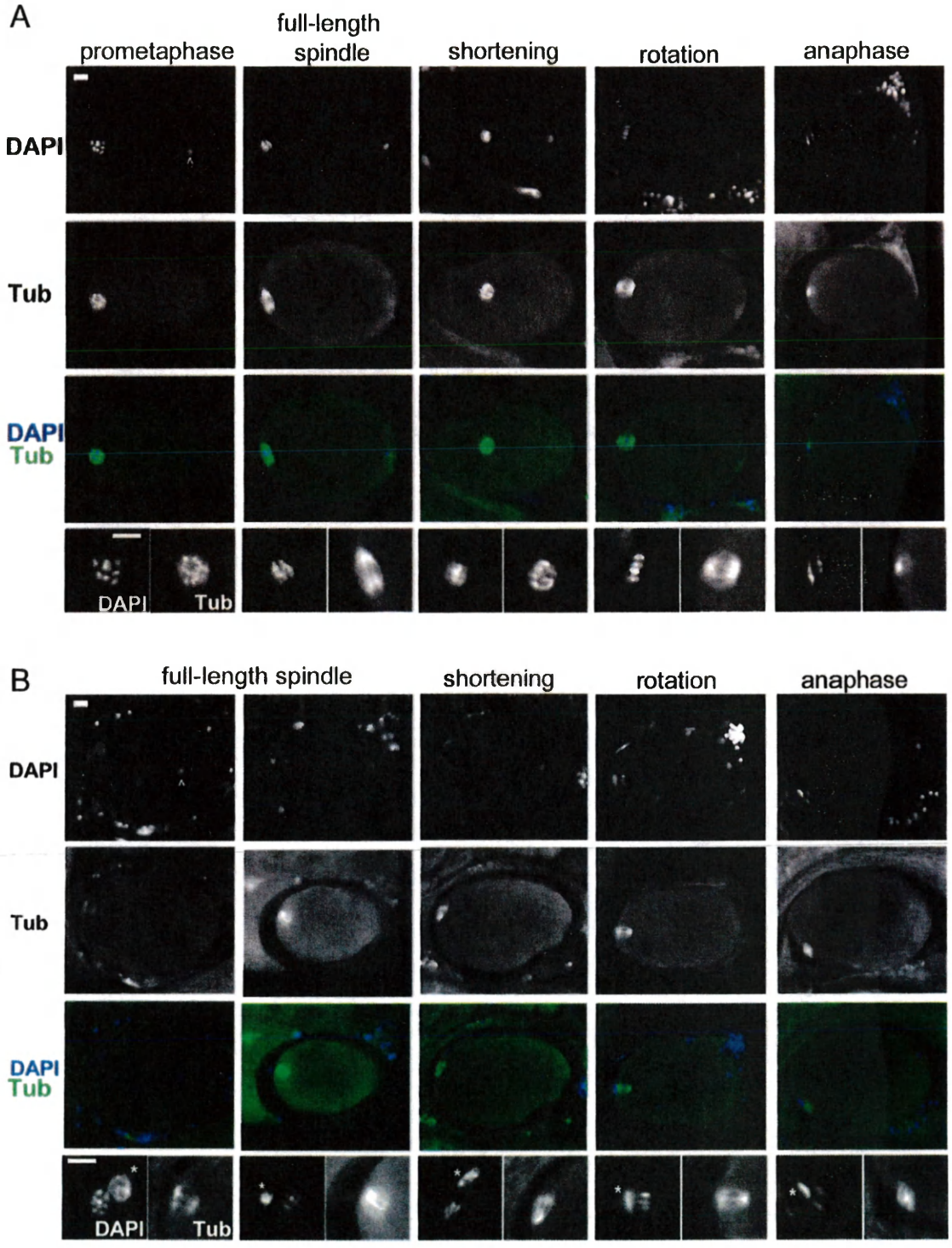

A
prometaphase
full-length spindle
shortening
rotation
anaphase
DAPI
Tub
DAPI
Tub
DAPI
Tub
B
full-length spindle
shortening
rotation
anaphase
DAPI
Tub
DAPI
Tub
DAPI
Tub

Figure 9: Stages of oocyte meiosis in *Rhabditis* sp. SB347. Images show fixed preparations of meiotic one-cell embryos from dissected hermaphrodite germlines in meiosis I (A) and meiosis II (B). Prometaphase I spindle is shown at the embryo cortex oriented down the pole-to-pole spindle axis (far left, A) and in a perpendicular plane (adjacent image in the series). The spindle shortens, rotates toward the embryo cortex, and then the microtubules rearrange to form a midzone between the chromosomes during anaphase. The midzone lengthens to form a midbody (anaphase) and the first polar body is extruded (far left, B). This process is repeated during meiosis II (B). For each embryo, a 2X magnification of the chromosomes (left) and meiotic spindle (right) are included at the bottom of the image. Images were acquired by epifluorescence microscopy. Asterisks (*) indicate polar bodies. Sperm chromatin mass is marked in the upper left image by ^. Scale bar, 5 µm.

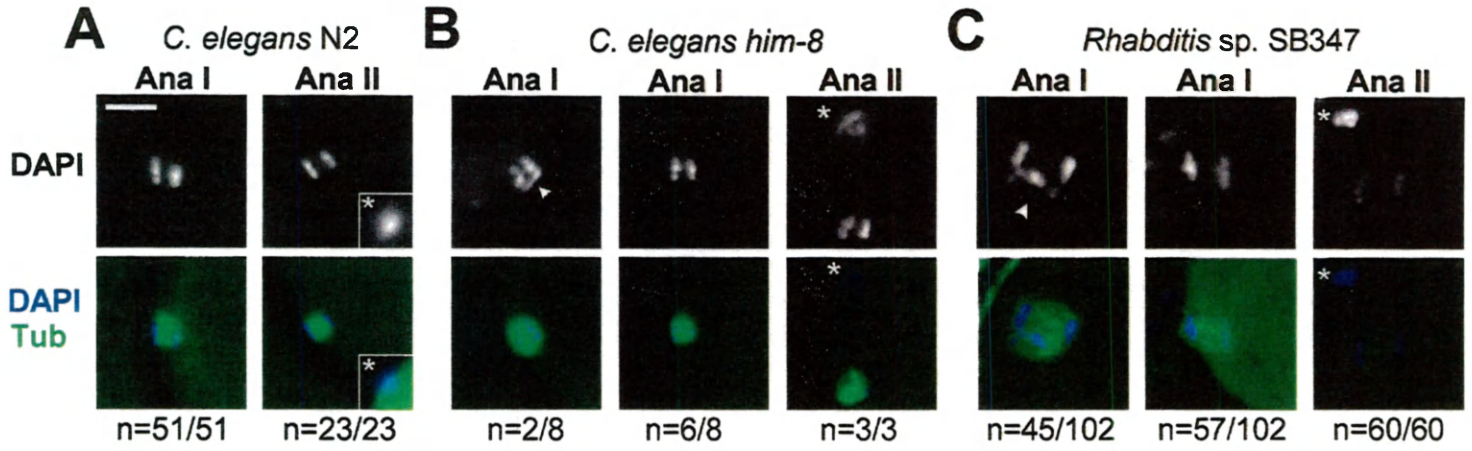


Figure 10: Chromosomes lag during anaphase I in meiotic one-cell embryos of selfing hermaphrodites from *Caenorhabditis elegans him-8* mutants and *Rhabditis* sp. SB347. Images of anaphase figures from fixed meiotically-dividing one-cell embryos. (A) *C. elegans* N2 (B) *C. elegans him-8*. (C) *R.* sp. SB347. Fixed embryos were stained with DAPI and anti-α-tubulin antibody. Examples of lagging chromosomes are shown in the left-most images for him-8 and *R.* sp. SB347. Arrowheads indicate lagging chromosomes Quantification of the results are noted below each representative image. Asterisks (*) indicate polar bodies. Insets in (A) indicate polar bodies that did not fit within the frame. Scale bar, 5 μm.

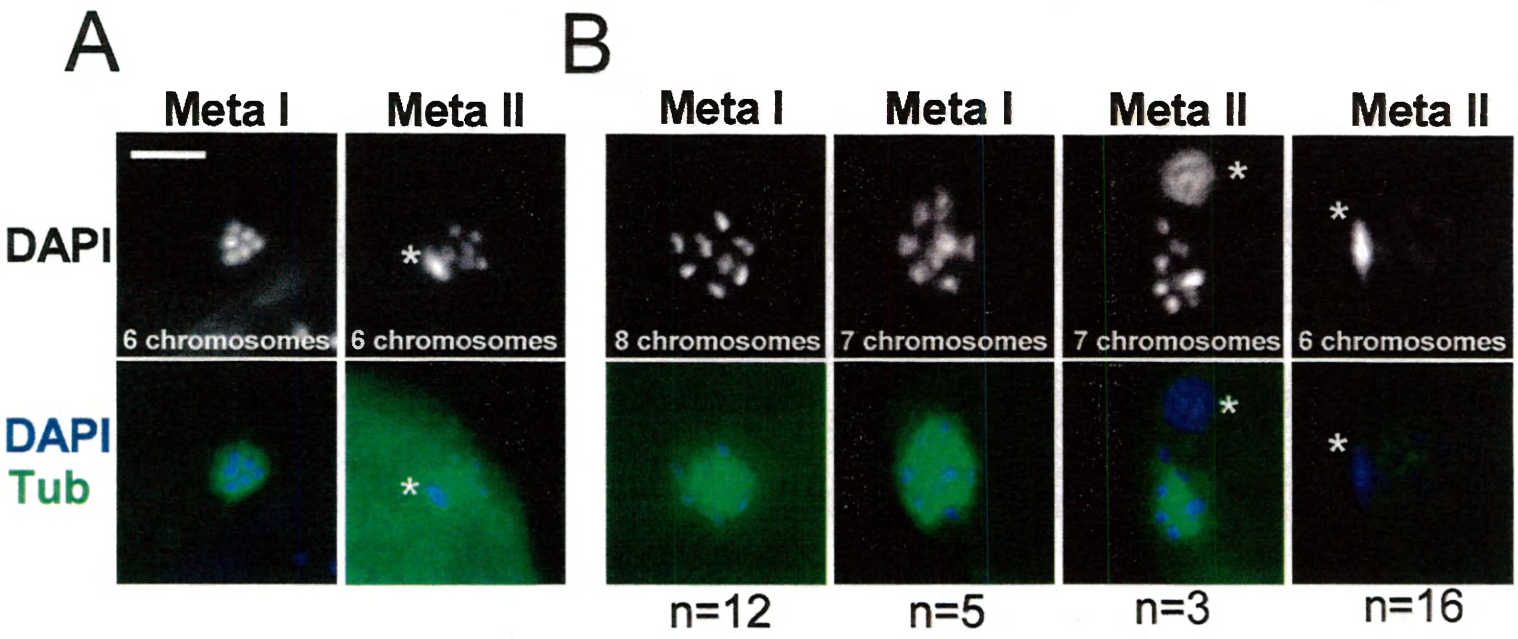


Figure 11: DAPI-staining bodies are lost between metaphase I and metaphase II in *Rhabditis* sp. SB347 hermaphrodite self-embryos. Images of metaphase plates from fixed meiotic one-cell embryos viewed down the pole-to-pole spindle axis. (A) *Caenorhabditis elegans* N2 oocyte meiosis from self-embryos . (B) *R.* sp. SB347 oocyte meiosis from self-embryos. Fixed embryos were stained with DAPI and anti-α-tubulin antibody. Asterisks (*) indicate polar bodies. The number of times of each spindle classification was observed in *R.* sp. SB347 oocyte meiosis is indicated below the panel. Scale bar, 5 μm.

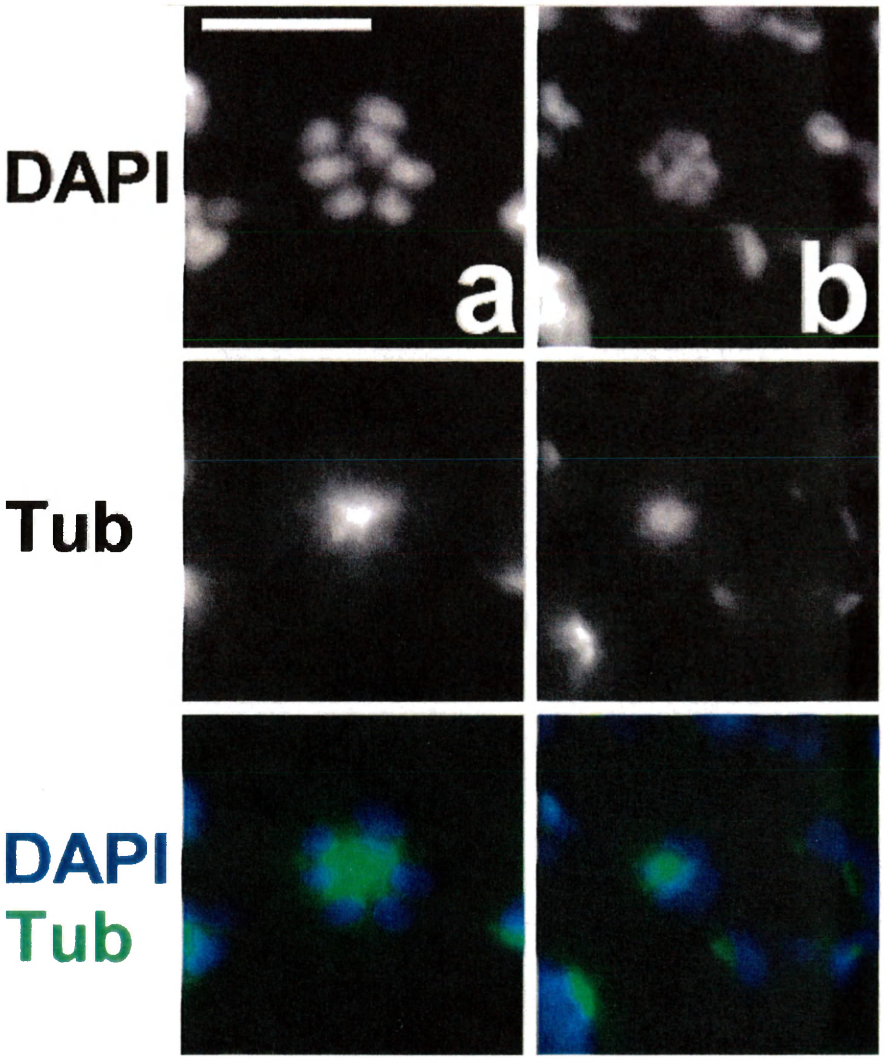


Figure 12: Primary and secondary spermatocytes of *Rhabditis* sp. SB347 XO males contain 7 DAPI-staining bodies. Fixed spermatocytes were stained with DAPI and anti-α-tubulin antibody. Dividing primary spermatocytes are shown in (a) (n=33), and dividing secondary spermatocytes are shown in (b) (n=56). Scale bar, 5 μm.

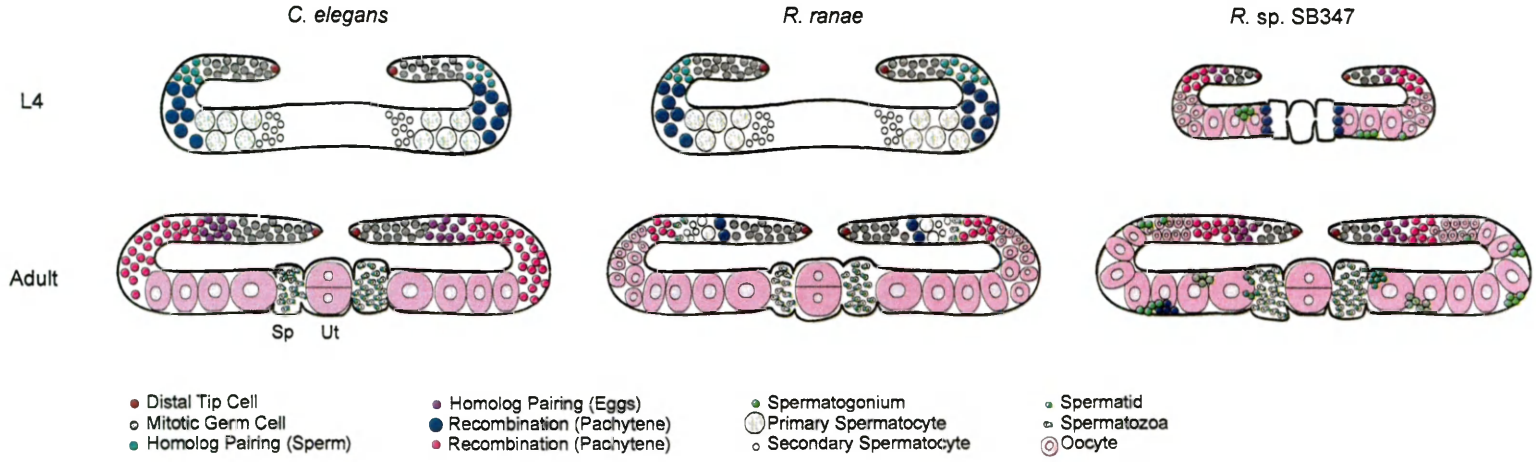


Figure 13: Different methods of germline development in nematodes. Schematics of germlines within developing (L4) and adult hermaphrodites of *Caenorhabditis elegans*, *Rhabdias ranae,* and *Rhabditis* sp. SB347. Spermatogenesis in *C. elegans* hermaphrodites is confined to the final larval stage (L4), before a permanent switch to oogenesis. Spermatogenesis in *R. ranae* occurs in larval animals but alternates with oogenesis in adults. Spermatogenesis in *R.* sp. SB347 occurs slightly before oogenesis in L4 animals, but simultaneously with oogenesis in adults. Sp indicates spermatheca and Ut indicates uterus.

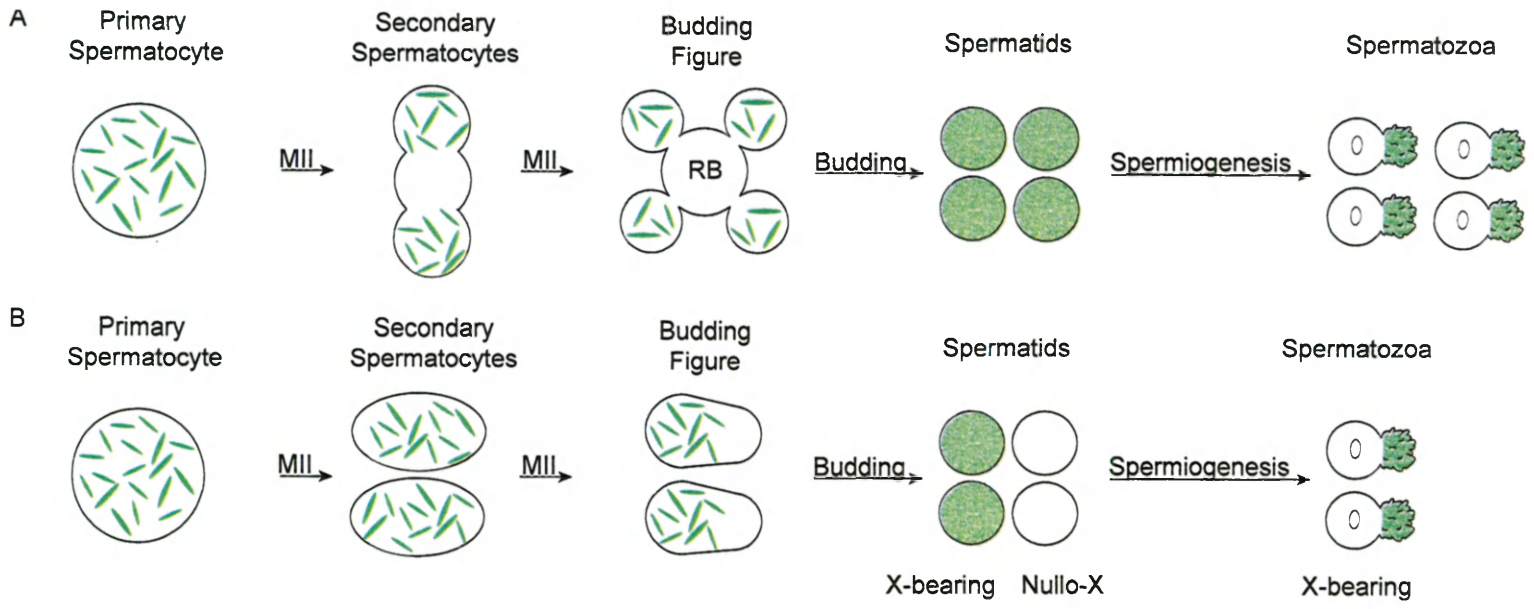


Figure 14: Schematic of spermatogenesis in *Caenorhabditis elegans* and *Rhabditis* sp. SB347 XO males. (A) Schematic diagram of spermatogenesis in a *C. elegans* XO male. Spermatogenesis involves the replication of DNA followed by two rounds of spermatocyte divisions to yield spermatids that mature into spermatozoa capable of fertilization. The unpaired X homolog segregates to one of two secondary spermatocytes during meiosis I (not shown). Sister chromatids segregate during meiosis II (not shown). Primary and secondary spermatocytes have Major Sperm Protein (MSP) organized into Fibrous Bodies (FBs). Spermatids bud off of a central residual body (RB), leaving behind ribosomes, tubulin, and other cellular components no longer necessary for fertilization. Spermatids activate through spermiogenesis to yield spermatozoa. Within spermatozoa, MSP localizes to the pseudopod. (B) Schematic diagram of spermatogenesis in a *R.* sp. SB347 XO male. During meiosis I, sister chromatids of the unpaired X segregate to each secondary spermatocyte. During meiosis II, each X chromosome segregates to a spermatid. MSP, an essential motility protein, is segregated to the X-bearing spermatid, while nonessential cellular components segregate to the nullo-X spermatid. No residual body is formed. MSP localizes to the pseudopods of the X-bearing spermatids, forming viable spermatozoa. MSP (green) is packaged within rod-

like structures in spermatocytes, but localizes to the pseudopod in mature spermatozoa. (Adapted from Smith 2005; L'Hernault 2006; Shakes et al. 2011).

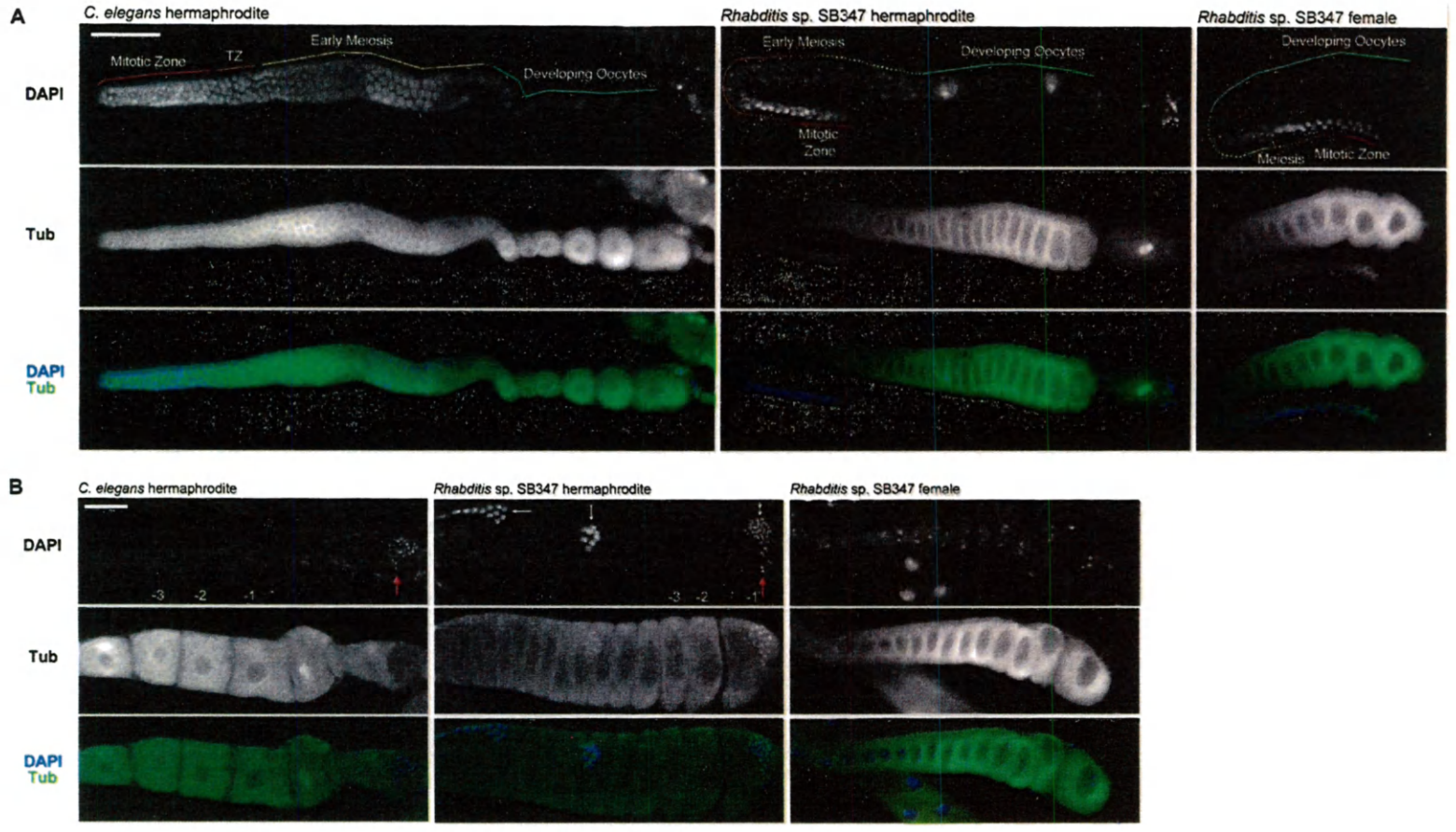


Figure 15: Germline organization in XX animals of *Caenorhabditis elegans* and *Rhabditis sp.* SB347. (A) The overall structure of the germline showing the large number of mitotically dividing nuclei in *C. elegans* hermaphrodites compared to both *R.* sp. SB347 hermaphrodites and females. Scale bar, 40 μm (B) Images of the proximal regions on hermaphrodite germlines show clusters of unusual cells (white arrows) of

unknown function in the germlines of *R.* sp. SB347 hermaphrodites, but not in *C. elegans* hermaphrodites or *R. sp.* SB347 females (B) White arrows in DAPI image of *R.* sp. SB347 hermaphrodites indicates cells that are present only in these hermaphrodites. Double white arrow indicates cells that are associated with tubulin spindles. Red arrow indicates the tubulin-negative, hypercondensed chromatin mass of spermatozoa in both *C. elegans* and *R.* sp. SB347 hermaphrodites. Images are 400 X magnification. Scale bar, 25 μm.

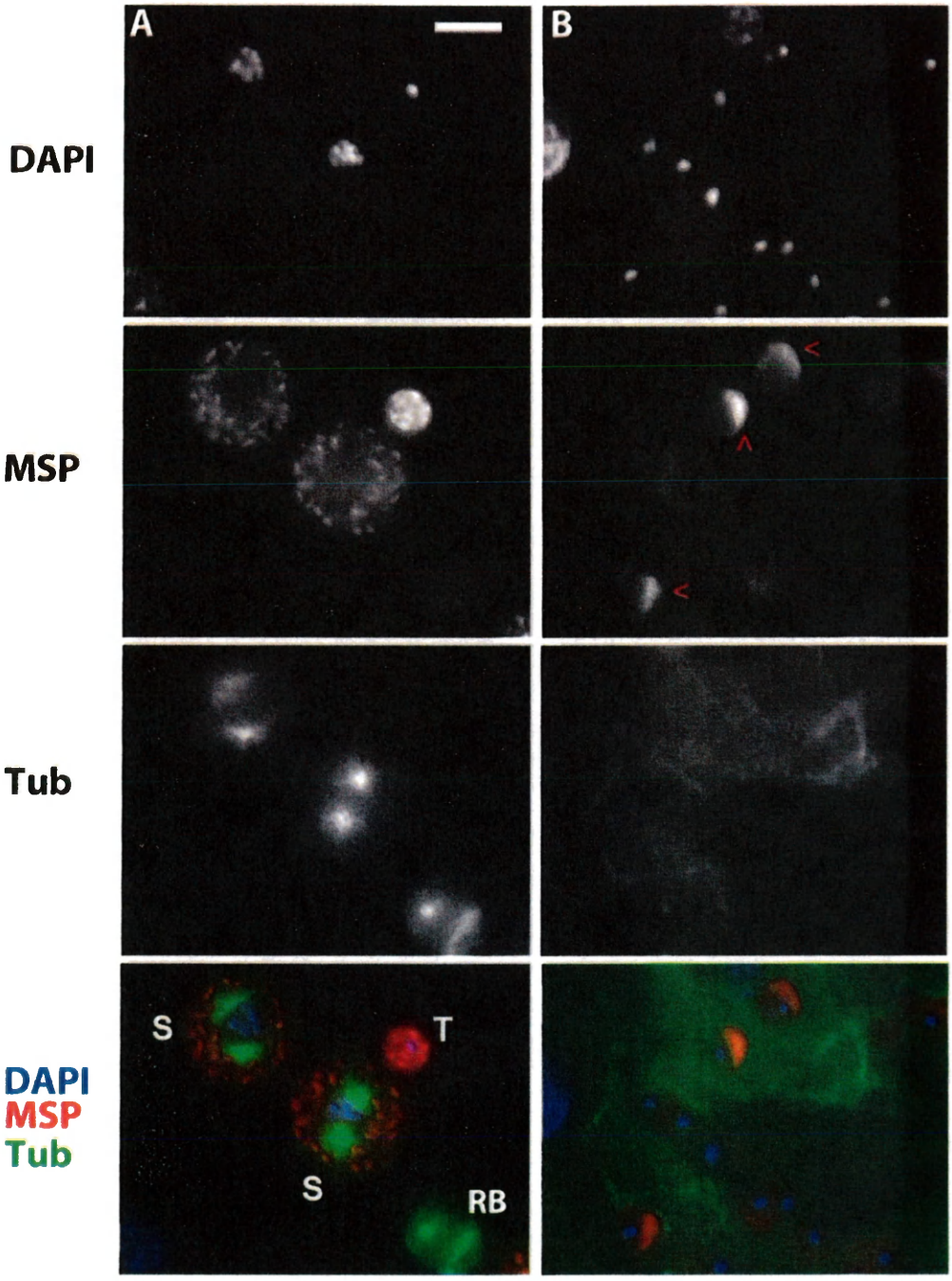


Figure 16: Stage-specific MSP patterns during *Caenorhabditis elegans* (*him-8*) spermatogenesis. (A) Dividing primary spermatocytes (S) in (A) are MSP positive with the MSP packaged into distinct fibrous bodies. These meiotically dividing spermatocytes have prominent tubulin spindles in *him-8* males. Haploid spermatids (T) lack tubulin and have cytosolic MSP. RB indicates residual body. (B) In crawling spermatozoa MSP localizes to the pseudopods (red carets, ^). These cells are tubulin

negative. Images are 2X enlargements of images taken at 600X magnification. Scale bar, 5 μm.

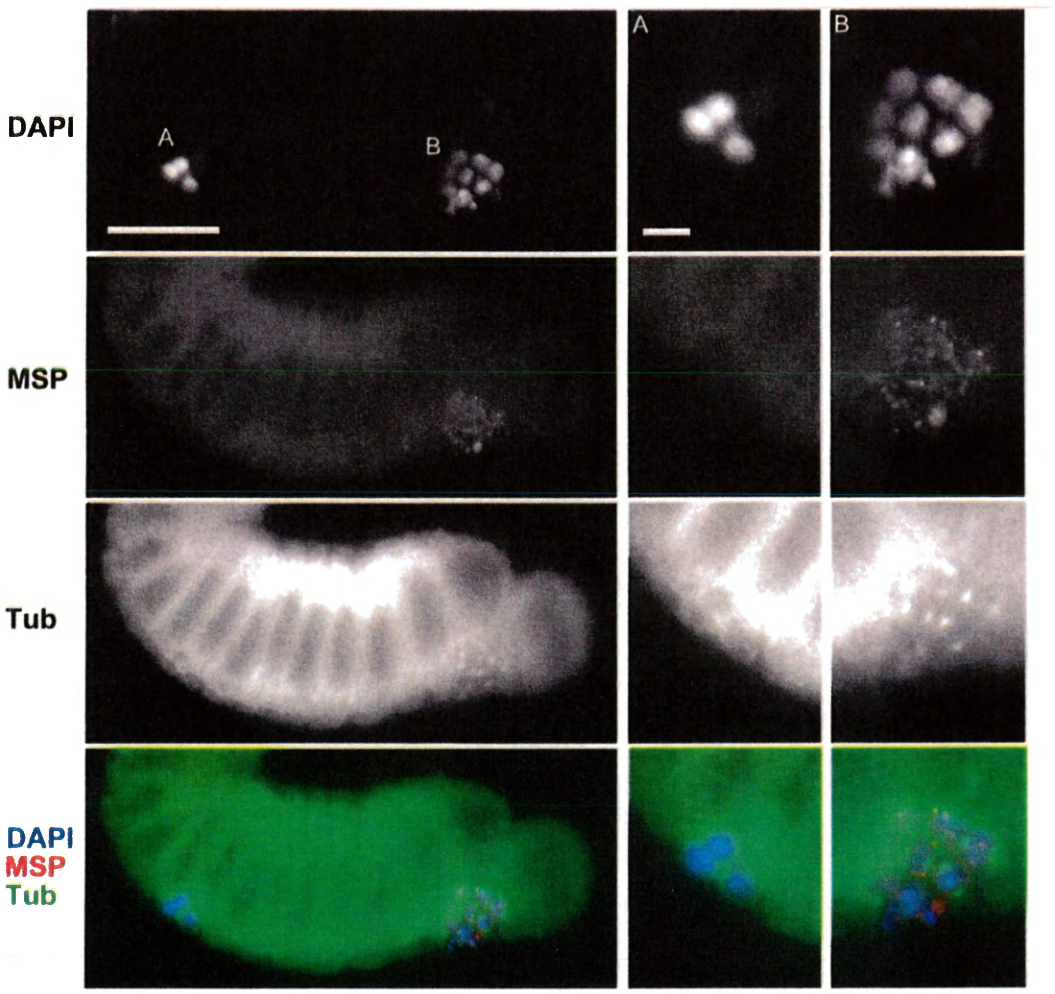


Figure 17: *Rhabditis* sp. SB347 hermaphrodite germlines contain clusters of MSP positive and MSP negative cells along their germlines. In (b), cells are dividing and MSP is packaged into fibrous bodies. Leftmost image is 400 X magnification. Scale bar, 25 µm. (a) and (b) show 2X enlargements of 600 X magnification. Scale bar, 5 µm.

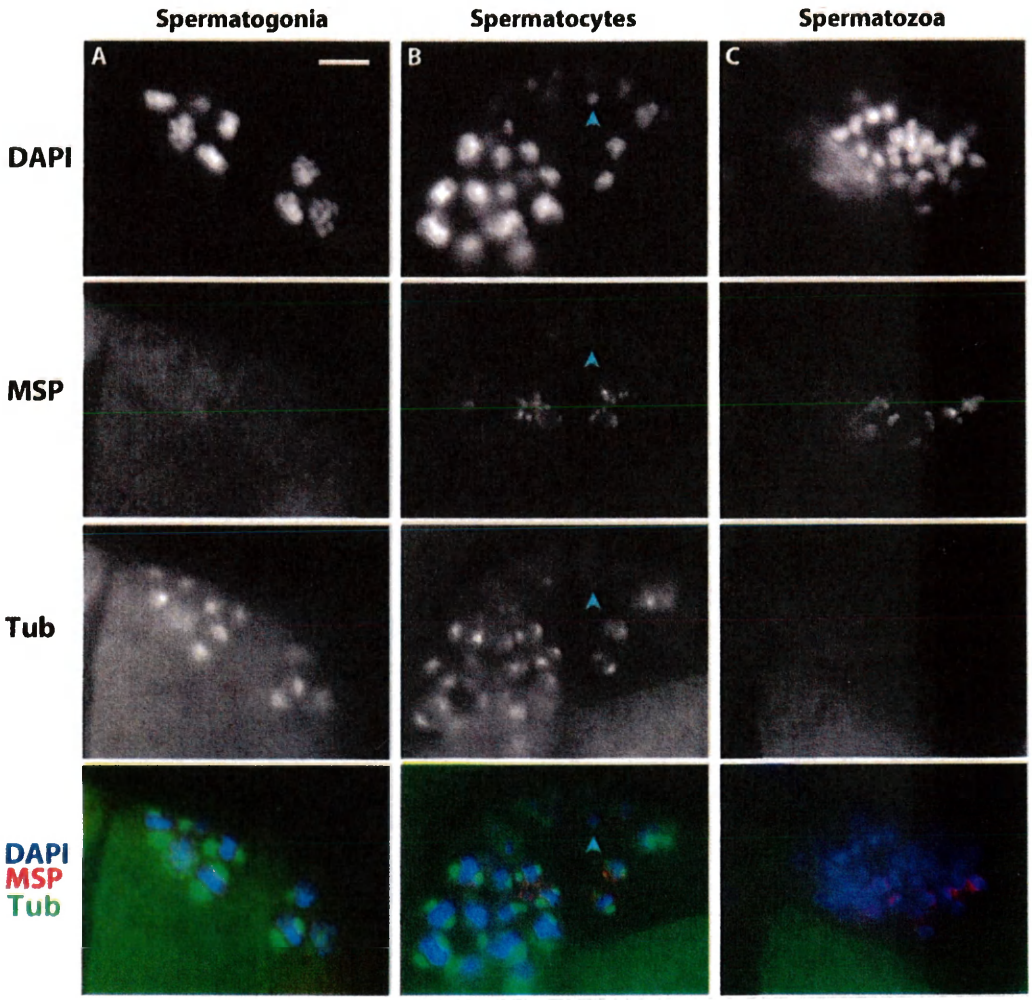


Figure 18: Spermatogenesis in *Rhabditis sp.* SB347 hermaphrodites. (A) Synchronously dividing metaphase cells associated with a –1oocyte. (B) MSP positive mitotically-dividing cells associated with a –1 oocyte as well as MSP negative spermatids associated with tubulin (blue arrowhead). (C) Activated MSP positive spermatozoa within the spermatheca. Spermatozoa are tubulin negative and MSP is localized to pseudopods. Images are 2X enlargements taken at 600 X magnification. Scale bar, 5 μm.

	Laying Time (hours)													
Worm	12	24	36	48	60	72	84	96	108	120	132	144	156	168
1	18	41	56	63	69	60	46	24	6	8	8	2	0	
2	15	36	54	46	57	26	37	21	15	6	3			
3	15	36	51	49	55	36	39	26	29	19	30	16	7	
4	13	31	44	46	50	49	43	24	35	15	2	6	3	
5	9	30	33	26	26	13	18	9	12	6	2	1	1	
6	12	33	46	47	42	45	47	35	31	11	18	12	5	
7	18	33	51	51	45	27	25	14	7	1	2			
8	14	36	31	30	35	29	30	14	17	2	0			
9	16	38	60	66	51	28	25	15	13	5	3	3	2	
10	17	33	56	63	69	57	50	43	37	22	25	19	9	8
11	13	38	47	24	33	6	2	2						
12	14	28	33	38	51	48	39	22	20	21	17	11	3	
13	17	36	47	50	61	49	41	26	21	13	5	5	2	
14	12	33	43	38	61	55	46	28	25	20	10	3	3	
15	15	34	35	29	45	42	49	31						
16	12	27	43	36	49	51	47	19	0	5	4	1	0	
17	18	40	47	15	38	43	46	29	32	17	19	9	2	
18	23	38	57	47	32	21	14	3	2	0				
19	26	43	39	41	31	28	20	9	6	5	2			
20	26	41	90	81	82	73	61	37	5	3	1	0		
21	22	39	52	57	40	38	30	32	20	5	2	1	1	
22	25	42	60	66	51	57	35	20	10	5	0			
23	19	34	49	60	58	35	20	8	3					
24	26	36	40	46	39	38	33	27	20	8	3	0		
25	29	47	35	38	34	34	27	19	13	2	0	1		
26	19	32	51	55	48	49	43	33	2					
27	16	30	50	56	65	53	36	28	26	20	10	2	2	1
28	18	28	34	23	35	27	11	5	1	0				
29	13	36	47	37	37	30	37	29	21	21	11	0	1	0
30	17	37	49	52	22	23	16	7	1	2				
31	17	43	58	51	52	46	38	20	7	3	1	1	0	
32	16	37	48	61	43	27	20	8	3	2				
33	12	33	43	52	43	31	23	12	15	13	8	4	1	1
34	15	25	37	47	25	20	8	7	3	2	2			
35	14	15	19	18	33	32	23	21	19	21	20	11	7	2
36	13	31	42	57	44	20	13	12	8	3	1	0		

Supplementary Table 1: Total progeny counts in self-broods of *Rhabditis* sp. SB347 hermaphrodites over time. The number zero indicates that no progeny were produced during this time interval, but that

the worm had not expired. Empty boxes indicate that the worm had expired.

	Laying Time (hours)													
Worm	12	24	36	48	60	72	84	96	108	120	132	144	156	168
1	3	6	4	6	5	3	5	5	1	0	0	0	0	
2	3	2	5	1	4	0	3	0	1	0	0			
3	4	5	7	6	4	4	10	0	5	1	2	0	1	
4	2	5	4	2	1	3	7	1	3	1	0	0	0	
5	2	6	4	6	8	4	5	0	1	0	0	0	0	
6	2	7	6	10	8	4	10	2	8	0	0	0	0	
7	2	3	3	9	10	8	5	1	1	0	0			
8	4	6	4	3	4	4	6	3	0	0				
9	3	4	5	6	11	2	8	3	0	0	0	0	0	
10	4	8	3	5	8	4	9	8	4	4	0	6	0	0
11	2	8	9	0	4	2	0	0						
12	1	3	5	2	7	7	4	3	4	5	3	0	0	
13	3	6	6	9	7	2	7	2	2	1	0	0	0	
14	4	5	3	1	5	5	4	3	6	2	0	0	0	
15	3	5	3	1	2	3	5	10						
16	3	4	8	2	4	7	12	2	0	0	0	0	0	
17	4	6	4	0	6	7	1	0	1	1	1	0	0	
18	5	5	6	4	4	6	2	0	0	0				
19	8	3	4	2	1	1	1	0	0	0	0			
20	7	3	4	6	11	14	20	11	0	0	0	0		
21	5	2	6	4	1	11	2	1	0	0	0	0	0	
22	4	3	0	8	4	13	5	2	1	0	0			
23	1	4	7	3	2	5	2	2	0					
24	4	6	10	2	5	4	9	6	5	1	0	0		
25	9	10	2	2	1	3	2	0	0	0	0	0		
26	2	2	3	1	6	8	5	7	0					
27	1	5	6	11	6	4	4	6	6	5	0	0	0	0
28	2	4	3	2	5	2	3	0	0	0				
29	5	4	5	2	2	7	8	4	1	2	0	0	0	0
30	0	7	7	7	5	7	2	2	0	0				
31	6	6	6	7	4	2	4	1	0	0	0	0	0	
32	2	4	3	5	1	3	3	0	0	0				
33	0	8	3	8	2	3	6	0	3	0	1	0	0	0
34	2	5	4	9	5	9	1	1	0	0	0			
35	5	2	2	0	3	1	2	1	0	1	1	0	0	0
36	0	5	8	4	2	3	6	0	0	0	0	0		

Supplementary Table 2: Total male counts in self-broods of *Rhabditis* sp. SB347 hermaphrodites over time. The number zero indicates that no progeny were produced during this time interval, but that

the worm had not expired. Empty boxes indicate that the worm had expired.

	Laying Time (hours)								
Worm ID	12	24	36	48	60	72	84	96	108
1	22	45	63	48	14	0	1	0	0
2	8	45	53	38	20	0	1	0	0
3	15	35	46	44	25	4	0	0	4
4	14	47	67	52	21	1	0	0	0
5	23	30	31	26	19	11	3	4	4
6	6	36	52	42	22	9	3	1	0
7	3	30	45	29	31	15	1	0	0
8	11	42	59	48	21	16	3	0	0
9	14	36	41	34	7	0	0	0	0
10	10	35	54	45	0	0	0	1	0
11	10	51	30	43	30	4	0	0	0
12	17	48	38	46	25	9	3	1	0
13	11	56	47	48	10	1	0	0	0
14	10	38	16	21	1	0	0	0	0
15	15	58	49	28	1	1	1	0	0
16	21	42	44	54	23	3	2	0	0
17	9	46	43	34	20	0	0	0	1
18	12	38	48	46	10	1	0	0	0
19	7	39	40	42	1	0	0	0	1
20	9	31	61	44	14	2	0	0	0

Supplementary Table 3: Total progeny counts in self-broods of *him-8* hermaphrodites over time. The number zero indicates that no progeny were produced during this time interval, but that the worm had not expired.

	Laying Time (hours)								
Worm ID	12	24	36	48	60	72	84	96	108
1	6	11	28	16	2	0	0	0	0
2	2	18	22	8	5	0	1	1	0
3	6	15	16	6	11	2	0	0	2
4	6	19	24	18	11	0	0	0	0
5	8	10	7	9	9	6	1	1	1
6	3	17	20	12	10	4	1	1	0
7	1	13	13	8	11	0	0	0	0
8	4	18	24	15	11	5	1	1	0
9	6	16	16	10	1	0	0	0	0
10	4	12	16	16	0	0	0	0	0
11	8	22	11	14	8	1	0	0	0
12	5	17	13	16	6	2	0	0	0
13	4	15	14	15	4	0	0	0	0
14	4	18	6	11	0	0	0	0	0
15	4	31	15	10	0	0	0	0	0
16	6	13	11	12	7	1	0	0	0
17	5	17	17	12	3	0	0	0	0
18	6	9	17	12	3	0	0	0	0
19	2	15	9	7	0	0	0	0	1
20	4	12	18	18	5	1	0	0	0

Supplementary Table 4: Total male counts in self-broods of *him-8* hermaphrodites over time. The number zero indicates that no progeny were produced during this time interval, but that the worm had not expired.

Methods

Maintenance of Nematode Strains

All nematode strains were maintained at 20°C on MYOB agar plates freshly seeded with *Escherichia coli* slow-growing mutant strain, OP-50 (Brenner 1974; Church et al. 1995).

Rhabditis sp. SB347 was provided by Dr. Andre Pires da-Silva (University of Warwick) and was originally isolated by Walter Sudhaus from a tick, *Ixodes scapularis*, in Kingston, Rhode Island (Felix 2004). The *Caenorhabditis elegans* strains include wildtype N2 (Bristol) from the *Caenorhabditis* Genetics Center and the *him-8* (*e1489)* mutant (Brenner 1974). Bristol N2 was used as a negative control for cytological studies and as a reference for germline structure compared to *R.* sp. SB347. The *him-8* strain was used as a positive control because *him-8* hermaphrodites produce a large proportion of male progeny due to missegregation of X chromosomes during oocyte meiosis (Hodgkin et al. 1979; Cortes et al. 2015).

Brood Studies

To specifically study hermaphrodites, *R.* sp. SB347 dauers were picked to fresh culture plates (Felix 2004; Chaudhuri et al. 2011). Because *R.* sp. SB347 dauers mature exclusively into hermaphrodites, selecting dauers ensures both celibacy and complete broods (Felix 2004).

In the case of *him-8* hermaphrodites, worms were picked as fourth stage larvae (L4s). Animals were allowed to develop for 12-16 hours. From the pools of *R.* sp. SB347 and *him-8* hermaphrodites, animals in their final L4 to adult molt were picked to fresh culture plates to ensure precise developmental synchrony. Parental hermaphrodites were subsequently transferred to fresh culture plates at twelve-hour intervals until they expired. All brood studies were conducted at 20°C. Offspring were counted and scored as feminine or male 36-48 hours after hatching.

Dissection and Fixation

R. sp. SB347 hermaphrodite worms were mass picked from MYOB culture plates as dauer larvae and allowed to develop for 12-16 hours. From these populations, synchronized, celibate hermaphrodites were selected during their final molt (time=0). Animals were dissected at various stages of adult development until 72 hours after the final molt. Beyond this time point, isolated gonads did not withstand dissecting conditions and were not usable.

Animals were placed on poly-L-Lysine-subbed (Sigma Aldrich) ColorFrost/Plus glass slides (Fisher Scientific) in 7 µL of Edgar's Buffer (Edgar, 1995) containing 0.25 mM levamisole and 1.25% glucose. Levamisole was added to anesthetize the worms and glucose was added to establish isotonicity with *R.* sp. SB347 embryos, which require higher osmolarity than their *C. elegans* counterparts. Animals

were dissected with a 30-gauge syringe needle by cutting them at the vulva to release both gonad arms and embryos. Glass coverslips were placed over the sample to adhere to the slide with four dots of silica grease. Light pressure was carefully applied to coverslips until a slight depression in cell morphology was observed when looking through the dissecting microscope. Slides were flash frozen in liquid nitrogen, cover slips were removed by freeze-cracking to crack egg shells, and tissue samples were fixed with -20°C methanol for at least 24 hours (Miller and Shakes 1995).

Immunostaining

Following fixation, tissue samples were washed three times for five minutes each wash with 1X Phosphate Buffered Saline (PBS). Washing samples with PBS rehydrates them in preparation for antibody binding. Then slides were placed in blocking buffer (PBS containing 0.5% Bovine Serum Albumin, 0.04% sodium azide, and 0.1% Tween-20) for 25 minutes to prevent antibodies from binding non-specifically.

Staining Microtubules with Anti-α-Tubulin Antibody

Fluorescein isothiocyanate (FITC) conjugated anti-α-tubulin monoclonal antibody DM1A (Sigma) was diluted to a concentration of 1:100 with antibody buffer (PBS containing 0.5% BSA and 0.04% sodium azide). The dilution was kept on ice. A hydrophobic barrier was smeared

around the dissected tissue on the slide, and 25 µL diluted antibody was applied to each sample. Slides were incubated in a dark, humid chamber for 1.5-2 hours at room temperature. After incubation, slides were dip-washed in 1XPBS, followed by deionized water. Excess liquid was wicked off the sample with a Kimwipe. Finally, glass coverslips with 7 µL of Fluoro Gel II mounting media with DAPI (4',6-diamidino-2-phenylindole, Electron Microscopy Services) were placed over the sample. Slides were kept in a protective slide book at 4°C and viewed within 48 hours.

Staining Modified Histones with Anti-Phospho-Histone-H3(ser 10) Antibody

Anti-phospho-histone-H3(ser 10) rabbit polyclonal antibody (Upstate Biotechnology) was diluted to a concentration of 1:300 with antibody buffer (PBS containing 0.5% BSA and 0.04% sodium azide). The dilution was kept on ice. A hydrophobic barrier was smeared around the dissected tissue on the slide, and 25 µL diluted antibody was applied to each sample. Slides were incubated in a dark, humid chamber for 1.5-2 hours at room temperature. After incubation with primary antibody, slides were washed twice for two minutes each wash in 1XPBS.

Then affinity purified goat anti-rabbit IgG Rhodamine (TRITC)-conjugated antibody (Jackson ImmunoResearch) was diluted to 1:100 concentration in antibody buffer (PBS containing 0.5% BSA and 0.04% sodium azide). The dilution was kept on ice, and 25 µL of diluted

secondary antibody was applied to each sample. Slides were incubated in a dark, humid chamber for 1.5-2 hours at room temperature.

After the incubation with secondary antibody, slides were washed in 1XPBS for two minutes, followed by a dip-wash in deionized water. Excess liquid was wicked off the sample with a Kimwipe. Finally, glass coverslips with 7 µL of Fluoro Gel II mounting media with DAPI (Electron Microscopy Services) were placed over the sample. Slides were kept in a protective slide book at 4°C and viewed within 48 hours.

Staining Major Sperm Protein with Anti-MSP Antibody

Anti-MSP rabbit polyclonal antibody in glycerol (Kosinski et al. 2005), provided by David Greenstein, was diluted to a concentration of 1:20 with antibody buffer (PBS containing 0.5% BSA and 0.04% sodium azide) and stored as a substock at 4°C. The substock was diluted to a concentration of 1:900 with antibody buffer. The dilution was kept on ice. A hydrophobic barrier was smeared around the dissected tissue on the slide, and 25 µL diluted antibody was applied to each sample. Slides were incubated in a dark, humid chamber for 1.5-2 hours at room temperature. After incubation with primary antibody, slides were washed twice for two minutes each wash in 1XPBS.

Then affinity purified goat anti-rabbit IgG Rhodamine (TRITC)-conjugated antibody (Jackson ImmunoResearch) was diluted to 1:100 concentration in antibody buffer (PBS containing 0.5% BSA and 0.04%

sodium azide). The dilution was kept on ice, and 25 µL of diluted secondary antibody was applied to each sample. Slides were incubated in a dark, humid chamber for 1.5-2 hours at room temperature.

After the incubation with secondary antibody, slides were washed in 1XPBS for two minutes, followed by a dip-wash in deionized water. Excess liquid was wicked off the sample with a Kimwipe. Finally, glass coverslips with 7 µL of Fluoro Gel II mounting media with DAPI (Electron Microscopy Services) were placed over the sample. Slides were kept in a protective slide book at 4°C and viewed within 48 hours.

Epifluorescence Microscopy

All images were acquired under epifluorescence using an Olympus BX60 microscope equipped with a QImaging EXi Aqua cooled CCD camera with an Olympus PlanApo 40x or 60x objective lens and IPLab software. Nomarksi optics were used for differential interference contrast imaging. Images were minimally processed to enhance contrast with IPLab software or Adobe Photoshop.

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