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A Comparative Study of Feminine Reproduction in a Rhabditis Clade

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A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Biology from the College of William and Mary.

by Kathryn Rehain

Accepted for: Honors in Biology

Advisor: Dr. Diane Shakes

Dr. Jonathan Allen

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Williamsburg, Virginia

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Abstract

Here I studied the reproductive strategies of nematodes in a *Rhabditis* clade. The identified members of this clade include *Rhabditella axei* and the *Rhabditis* species *R.* sp. SB347, *R.* sp. SB372, *R.* sp. JU1782, *R.* sp. JU1783, and *R.* sp. JU1809. To better define the reproductive strategies of the hermaphrodites/females within this clade the morphology of the gonad as well as other elements of reproductive strategies were compared, with the well-studied *Caenorhabditis elegans* hermaphrodite reproductive strategy used as a standard.

It was found that the reproductive strategies of hermaphrodites/females of the *Rhabditis* clade differ from that of *C. elegans* hermaphrodites in a number of ways. In at least one, if not more, species within this clade, clear differences were documented in distal gonad morphology, the proportion of mitotically dividing cells in the distal gonad, chromosome morphology, the size of the nucleoli, spermatheca morphology, and offspring sex-ratios.

However, a number of similarities were found as well. *C. elegans, R. axei,* and SB372 gonads have comparable numbers of mitotically dividing cells. In *C. elegans, R. axei,* and SB347 meiosis resumes in the most proximal oocyte. Furthermore, *C. elegans,* SB347, and SB372 hermaphrodites all produce broods of a similar size. Lastly, the hermaphrodites/females of all species examined had didelphic gonads containing oocytes in the proximal region, and these gonads occupied a large portion of the total worm volume in all species.

In the future, these findings will be combined with molecular phylogenetic studies to examine the evolution of diverse reproductive strategies within closely related nematode species. Importantly, the reproductive features of one or more of the trioecious

species within this clade suggests that it could be an excellent model organism for the study of parasitic nematodes. Moreover, comparative studies within the *Rhabditis* clade provide an informative parallel to comparative studies within the genus *Caenorhabditis*.

Background

An Introduction to Nematodes

The phylum Nematoda is rich in history, number and diversity. Described in ancient times by the famous philosopher and early scientist Aristotle, nematodes are outnumbered only by arthropods in terms of the number of species and the number of individuals present on Earth (as reviewed in Croll and Matthews, 1977; Wharton, 1986). While many species share a morphologically similar small, thin, non-segmented "worm" body-form, nematodes as a whole utilize a surprisingly wide assortment of life-strategies (as reviewed in Malakhov and Hope, 1994). Some species are parasites of animals or plants, while others are free-living in soil, salt water, fresh water, and many other environments. Additionally, nematodes can be bacterial-feeders, fungal-feeders, algal-feeders, herbivores, omnivores, or carnivores (as reviewed in Wharton, 1986). Nematodes are in fact so numerous and diverse that Dr. Nathan Cobb, a famous nematologist, once wrote, "If all the matter in the universe except the nematodes were swept away, our world would still be dimly recognizable... we would find its mountains, hills, valleys, rivers, lakes and oceans represented by a film of nematodes," (Cobb, 1915).

One of the most studied nematode species is *Caenorhabditis elegans*, a renowned model organism for biological research. It was Dr. Sydney Brenner who voiced the need for a model organism that had three important traits. First, the organism had to be easily kept in the lab. Second, it needed to have tractable genetics. Third, the organism had to be easily observed. The nematode *C. elegans* fit the criteria, and in 1974 Dr. Brenner proposed using *C. elegans* to study genetic regulation of development (Brenner, 1974). Over time and with further study, it has become clear that *C. elegans* is an excellent

model not only to study development, but a wide range of subjects. It has since been used to study diverse topics ranging from gerontology, Parkinson's Disease, and infectious diseases to programmed cell death and the function of small RNAs (as reviewed in Ellis and Horvitz, 1986; Gami and Wolkow, 2006; Harrington et al, 2010; Abbot, 2011; Glavis-Bloom et al, 2012). Further, as the first fully sequenced multi-cellular organism, it was instrumental to the development of larger genome sequencing projects (Sulston et al, 1992).

C. elegans: A Classic Model Organism

As previously stated, *C. elegans* possesses the three traits stipulated by Dr. Brenner as necessary for a good model organism: easy to grow; simple genetics; easy to observe (Brenner, 1974). While many other nematode species are quite difficult or impractical to cultivate in a lab setting, *C. elegans* is remarkably easy to grow and maintain (as reviewed in Croll and Matthews, 1977). Naturally a free-living soil nematode, *C.elegans* can be raised in the lab either on agar plates or in liquid culture with the bacterium *Escherichia coli* as a food source. Additionally, because these worms are only about one millimeter length, large numbers can be raised in a limited space. *C. elegans* is a dioecious species, meaning it has two genders, male and self-fertile hermaphrodite (Figure 1). The presence of self-fertile hermaphrodites further increases the ease of maintaining the species in the lab (as reviewed in Blaxter, 2011).

The ability of hermaphrodites to self-propagate is also one of several features that make *C. elegans* particularly amenable for classical genetic studies. The hermaphrodites are evolutionarily designed for low inbreeding depression, indicating that a strain maintained by a self-propagating hermaphrodite will remain genetically robust (as

reviewed in Blaxter, 2011). At the same time, males can be employed to carry-out genetic crosses. In terms of genetic crosses, male sperm out-competes the hermaphrodite sperm during fertilization because it is somewhat larger (LaMunyon and Ward, 1998). The relatively short life-span of *C. elegans*, 17.7 days for males and 19.9 days for hermaphrodites, as well as the rapid development from embryos to sexually reproducing adults in about 3.5 days makes it possible to easily and quickly complete genetic experiments that require multiple generations (as reviewed in Lewis and Fleming, 1995; Hope, 1999). Moreover, advancements in preservation technology have shown that *C. elegans* can easily be cryo-preserved at -80˚C and, thus, genetic strains can be stored long-term (as reviewed in Blaxter, 2011).

Clearly *C. elegans* is easy to maintain in the lab and is useful for genetic studies. Brenner's third requirement called for an easily observable organism (Brenner, 1974). Since both the bodies and the eggshells of *C. elegans* are translucent through all life stages, it is possible to visualize both organs as well as the nuclei of individual cells without dissection of the organism (as reviewed in Blaxter, 2011). This trait allowed for the mapping of both the origin and fate of all nuclei during embryonic and post-embryonic development. These classic studies revealed that the cell lineage of *C. elegans* is invariant (Sulston and Horvitz, 1977; Sulston et al, 1983).

Beyond Brenner's original criteria, the technological advancement of laboratory tools and techniques continues to make *C. elegans* even more appealing as a model organism. *C. elegans* was the first model organism to have its entire genome (~100 Megabase) sequenced (Sulston et al, 1992). Further, a number of significant research tools, such as the use of green fluorescent protein as a transgenic marker, were pioneered

in *C. elegans* (Chalfie et al, 1994). Likewise, RNA interference, which was discovered in *C. elegans* (Fire et al, 1998), can be used to specifically knock down the function of individual genes. This technique has made it possible to conduct genome-wide reverse genetic screens and to more easily study gene function (as reviewed in Perrimon et al, 2010). Additionally, the development of online databases, such as Wormbook.org and Wormbase.org, has both accelerated and simplified the sharing of *C. elegans* research findings (as reviewed in Blaxter, 2011).

Beyond C. elegans: Other Nematodes in the Wild and in the Lab

Much of the usefulness of *C. elegans* stems from the ease of studying and generating genetic mutants of this species (Brenner, 1974). However, the study of laboratory induced genetic mutations within a single species yields little information about the natural variations produced over the course of evolutionary history. By focusing so much attention on just *C. elegans,* one convenient but likely non-typical nematode species, it is possible that the scientific community is missing out on information that the study of other diverse species could illuminate (as reviewed in Wharton, 1986). Thus, in order to study evolutionary diversity one must look beyond *C. elegans.*

C. elegans is a member of the genus *Caenorhabditis* (Figure 2). Since *C. elegans* has proven to be such a useful lab species, it is not surprising that much of the work done on nematodes other than *C. elegans* has focused on other caenorhabditids (as reviewed in Thomas, 2008). However, there is some difficulty in studying the relationships among the members of *Caenorhabditis*. This difficulty stems from the fact that while new species are often found, it is usually not possible to maintain these species in the lab

(Kiontke et al, 2011). A second issue, with studying caenorhabditids, is that there is not huge diversity within this group. As a hermaphroditic species, *C. elegans* is one of the most diverse species within this mostly gonochoristic (male/female) genus (Kiontke et al, 2004).

Caenorhabditis is part of a larger clade of more diverse nematodes known as *Eurhabditis* (as reviewed in Kiontke and Fitch, 2005). One particular small clade within in this group has drawn attention specifically for the diversity of the reproductive strategies demonstrated by its members (Figure 3). What makes studying the members of this group especially attractive is that they are sustainable in the lab. This clade includes *Rhabditella axei,* a gonochoristic species, and a number of related *Rhabditis* species, including *R.* sp. *SB347, R.* sp. *SB372, R.* sp. *JU1782, R.* sp. *JU1783,* and *R.* sp. *JU1809* (see Note 1). The *Rhabditis* species within this clade all have unusual, but mostly uncharacterized reproductive strategies (Félix, 2004; Shakes et al, 2011; Chaudhurri et al, 2011; M. Félix, W. Sudhaus, K. Kiontke, and D.A. Fitch, personal communication).

Another group of nematodes that has drawn much attention from the scientific community are the parasitic species, especially parasites of humans and live stock (Hasegawa et al, 2010; as reviewed in Molento et al, 2011). Parasites, however, are even more difficult to study in the lab than caenorhabditids, as they can be both dangerous and expensive to maintain (Croll and Matthews, 1977). Interestingly, it has been shown that the lifestyles of some parasites are similar to that of SB347, a member of the clade that can be cultivated in the lab under standard *C. elegans* growth conditions. This suggests this *Rhabditid* clade is attractive not only for the study of reproductive diversity, but also

for the study of parasitic nematodes (Runey et al, 1978; Spieler and Shierenberg, 1995; Félix, 2004; Chaudhuri et al, 2011).

Note 1: Definition of Terms Utilized from this Point Forward

- "Non-*C. elegans* species": *R. axei, SB347, SB372, JU1782, JU1783,* & *JU1809*
- "SB/JU species": *SB347, SB372, JU1782, JU1783,* & *JU1809*
- "Species of interest": *C. elegans, R. axei, SB347, SB372, JU1782, JU1783,* & *JU1809*

Rhabditis sp. SB347: An Unusual Lab Sustainable Nematode

To date, the best studied of the SB/JU species is *Rhabditis sp. SB347*. SB347 has a number of unusual reproductive characters. One of the most interesting of these characters is that the species is trioecious (Félix, 2004). This means that it has three genders: hermaphrodite, male, and female (Figure 4). This is one feature that suggests a relation to parasites. Some parasitic species also consist of three genders, however, parasites alternate between hermaphroditic and gonochoristic generations (Spieler and Schierenberg, 1995).

In *C. elegans,* hermaphrodites and males are distinguished genetically by the presence of a second X-chromosome. Hermaphrodites have two X chromosomes, while males have only one. Unlike humans, however, in which males are genetically XY, male *C. elegans* have only the single X chromosome and no Y. The same feature appears to distinguish SB347 hermaphrodites and females from SB347 males (Shakes et al, 2011). However, the feature that distinguishes SB347 hermaphrodites from SB347 females, which are somatically identical as adults, is not linked to their sex chromosomes, but rather their developmental history (Chaudhuri et al, 2011; Félix 2004).

Under normal conditions both *C. elegans* males and hermaphrodites develop from an embryo through four larval stages (Figure 5A). During this time there is continuous growth (Hope, 1999). Through the first two larval stages, L1 and L2, males and hermaphrodites are indistinguishable. During the third larval stage L3 males can be identified by a slight swelling of the tail region. The two genders diverge more obviously during the fourth larval stage L4, during which the male tail takes on its characteristic triangular shape and the hermaphrodite vulva begins to develop (as reviewed in Lewis and Fleming, 1995; Emmons, 2005; Sternberg, 2005). This pattern of development holds true for SB347 males and females (Figure 5B and 5C). SB347 hermaphrodites, however, develop slightly differently.

When a *C. elegans* population experiences a lack of food, high temperature, or a high density, juveniles will develop through an atypical developmental stage known as the dauer stage (as reviewed in Hu, 2007). The decision to form the dauer occurs late in L1, and thus worms that undergo dauering develop through an L2 pre-dauer stage and arrest in the L3 dauer (Figure 5A). The dauer stage is characterized by the formation of a thick cuticle and the sealing of the mouth. Other features of dauer larvae include a darker more slender body and a tendency to exhibit behavioral traits that promote dispersal such as nictation (Lee et al, 2011). The L3 dauer is a quiescent developmental stage, and worms in this stage will not continue development into the L4 stage until conditions become more favorable (as reviewed in Hu, 2007).

This dauer stage, which is inducible in *C. elegans*, is obligatory in SB347 hermaphrodites (Chaudhuri et al, 2011). In order for a feminine larval SB347 worm to develop into a hermaphrodite it must develop through L1, obligatory pre-dauer L2,

obligatory dauer L3 and then L4 before it can mature into a self-fertile adult (Figure 5D). Development through the obligatory dauer, under optimal conditions, delays reproductive maturity by ~24hrs compared to females. Under adverse conditions development beyond the dauer stage is delayed indefinitely. Importantly, it has been shown that developing through the dauer stage is sufficient to induce hermaphroditism in feminine worms of SB347 (Figure 5C) (Chaudhuri et al, 2011). As L1 larvae, SB347 females can be distinguished from SB347 hermaphrodites by their slightly larger gonad (Chaudhuri et al, 2011). In a study by Chaudhuri et al, this character was used as an identifier, and SB347 females and hermaphrodites were separated. When would-be-females were forced to dauer, by altering the environmental conditions, they developed into hermaphrodites. When would-be-hermaphrodites were prevented from dauering, by removing a component vital to forming the dauer hormone, the worms developed into females (Chaudhuri et al, 2011).

Why dauer? The formation of the dauer by *C. elegans* allows individuals of this species to survive despite environmentally tough conditions (as reviewed in Hu, 2007). In parasitic nematode species, dauer formation is also frequently linked to the infective stage of development (Ogawa et al, 2009). In both cases dauer stage larvae are behaviorally dispersive (Cassanda and Russell, 1975). The coupling of dauer formation and hermaphrodite development in SB347 is ideal for colonization, suggesting the species is behaviorally dispersive (Chaudhuri et al, 2011).

The feature of SB347 reproduction that originally attracted the attention of scientists is the unusual sex ratio of the offspring produced by both hermaphrodite self-fertilization and male/female outcrosses. Typically, matings between males and females

yield 50% female and 50% male offspring; whereas matings between SB347 males and females yield <5% male offspring. Conversely, when a SB347 hermaphrodite performs self-fertilization the offspring produced consist of an unusually high percentage of male offspring (~9%). In contrast, self-fertilizing XX hermaphrodites in *C. elegans* and other species produce almost exclusively feminine offspring (Hodgkin, 1987; as reviewed in Lewis and Fleming, 1995; Félix, 2004; Shakes et al, 2011).

The low percentage of male progeny produced by a male/female outcross in SB347 can be explained by an asymmetric cell division during male spermatogenesis (Shakes et al, 2011). The cellular mechanism by which self-fertilizing hermaphrodites produce unusually high percentages of male offspring remains unexplained.

Studying the Diversity of Nematodes

In order to study the diversity of nematode species, one must first be able to distinguish members of one species from another. There are a number of ways in which this can be done. A species is defined by what is known as a "species concept" (Stearns and Hoekstra, 2005). Early scientists used the *Morphospecies Concept* which defined a species based on the physical structures of its members. This concept proved unreliable and other species concepts were proposed. The *Morphospecies Concept*, however, is still used most often, but only in conjunction with other concepts (Freeman, 2008). Another well accepted and utilized species concept is the *Biological Species Concept,* which states that members of a species are able to interbreed and produce fertile offspring, and are reproductively isolated from other similar organisms. This concept, however, does not account for asexual species or inter-species hybrids. No single species concept is without

flaws, and for this reason a "species" is usually only defined as such after meeting the requirements of multiple species concepts (Stearns and Hoekstra, 2005).

Frequently, in the study of the evolutionary relationships among diverse nematode species, combinations of both molecular-genetic and morphological comparisons are utilized. The morphological features compared relate to the type of diversity being investigated. Studies on the evolution of feeding strategies typically include comparisons of mouth parts (as reviewed in Wharton, 1986). Conversely, studies of development often include comparisons of male tail and/or vulval morphology (Fitch, 2000; Félix, 2005). The focus of this work is the evolution of diverse reproductive strategies within the *Rhabditis* clade. Therefore, features of the reproductive strategy, in this case specifically those of hermaphrodites, will be investigated. As *C. elegans* is so well studied, it is the obvious standard to which the other species will be compared.

Elements of a Reproductive Strategy

In order for a species to be evolutionarily successful it must have two important qualities. It must be able to survive and it must be able to reproduce successfully (Stearns and Hoekstra, 2005). Elements that affect the overall success of a particular reproductive strategy include differences in reproductive mode, sex ratios, differences in reproductive structures, and differences in gametogenesis.

The reproductive mode of a species is defined by the sexes present and how these sexes interact to produce viable offspring. In the set of nematode species described above three different reproductive modes are exemplified. *R. axei* demonstrates the most typical reproductive mode for animals: dioecious (male/female) diploids that reproduce sexually (Karlin and Sabin, 1986). *C. elegans* utilizes a different reproductive mode:

androdioecious (male/hermaphrodite) diploids in which the hermaphrodites are self-fertile, but capable of genetically outcrossing with males (as reviewed in Lewis and Fleming, 1995). In this mode hermaphroditic self-reproduction occurs in the same generation as sexual out-crossing. Finally, SB347 and some of the other unique lab cultivatable species demonstrate a third reproductive mode, trioecious (male/hermaphrodite/female) simultaneous hermaphroditism (Charnov, 1982; Félix, 2004; Chaudhuri et al, 2011).

Another aspect of the reproductive strategy includes the sex-ratio of the offspring produced by the given reproductive mode. As previously stated, when the nematode SB347 reproduces, skewed sex-ratios are observed in the offspring (Félix et al, 2004; Shakes et al, 2011). The bias in the sex-ratios can be the result of a number of different influences such as mate competition, sex-differentiated viability, fertility forces, etc (Karlin and Lessard, 1982). In order for these unusual ratios to persist, however, they must be part of an Evolutionarily Stable Strategy (Smith and Price, 1973).

The structure of reproductive organs and the specific nature of gametogenesis also play important roles in the success of a reproductive strategy. Of the species of interest only the standard *C. elegans* gonad and gametogenesis have been studied in depth. The *C. elegans* hermaphrodite gonad consists of two U-Shaped tubular arms. While each arm has its own spermatheca (a sperm-containing pouch), the two arms are connected by a single uterus (Figure 1). Within each arm, the entire sequence of oogenesis is laid out in a linear array (Figure 6). In the distal portion of each arm, mitotically proliferating germ cells maintain the germline. As the germ cells move through this distal region, they transition from mitosis to meiosis (as reviewed in Hubbard and Greenstein, 2005).

Additionally, after the transition from mitosis to meiosis all of the cells are connected to a central core of cytoplasm known as the rachis. The cells remain attached to the rachis as they progress around the bend in the gonadal arm. During the L4 stage, the first germ-cells produced differentiate into spermatocytes. Then, as they prepare to initiate their meiotic divisions, they bud off from the rachis, completely losing their cytoplasmic connections to it. As a result of the meiotic divisions, individual spermatocytes generate four haploid spermatids. This is the only time during the hermaphrodite's life-time that sperm will be produced, and thus the number of sperm produced by self-fertilizing hermaphrodites is limited. However, their store of sperm can be replenished by mating with fertile males that inject their own sperm into the hermaphrodite (as reviewed in L'Hernoult 2006). As worms transition into adulthood they begin oogenesis.

Oogenesis in *C. elegans* differs markedly from spermatogenesis (as reviewed in Hubbard and Greenstein, 2005). Unlike in spermatogenesis, immature oocytes retain their cytoplasmic connections as they enter the proximal portion of the gonad; the rachis simply narrows. Further, the cells do not undergo meiotic divisions at this time. Instead they pause in diakinesis of meiotic prophase I. The chromosomes, at this point (diakinesis), are highly condensed, the six homologous pairs are easily visualized, and the nucleolus is still present (as reviewed in Hubbard and Greenstein, 2005). In the proximal portion of the gonad the maturing oocytes form a line with the oldest most mature oocyte nearest the spermatheca. During this time the maturing oocytes accumulate yolk lipoprotein particles that are produced in the gut. As an oocyte reaches the most proximal position early sperm cues from the sperm in the spermatheca trigger the resumption of meiosis, and both the nucleolus and the nuclear envelope break down. Further, and the

homologous pairs begin to align when the oocyte reaches the most proximal positon (as reviewed in Greenstein, 2005). Contraction of gonadal sheath cells coupled with dilation of the spermatheca forces the mature oocyte into the spermatheca. This is known as ovulation (McCarter et al, 1999). When the first mature oocyte is ovulated, it and all of the spermatids that have been produced are pushed into the spermatheca where they activate to form motile spermatozoa. The sperm will remain stored in the spermatheca for the remainder of the hermaphrodite's life (as reviewed in L'Hernoult 2006). The spermatheca also serves as the site of fertilization. The oocyte completes its meiotic divisions and forms a translucent eggshell only after fertilization. Lastly, the spermatheca-uterine valve dilates, and the new embryo enters the uterus (McCarter et al, 1999)

The Goal of This Study

The specific goal of this work is to more clearly define the reproductive strategies of the hermaphrodites, and in some cases females, of the members of a *Rhabditid* clade which includes *Rhabditella axei*. Preliminary data have already indicated diversity in spermatogenesis and thus the reproductive strategies of males within this clade (Shakes et al, 2011). Here features of hermaphrodite reproduction, within this clade, will be compared to the better studied features of hermaphrodite reproduction in *C. elegans.* In the future, hopefully, this work will help to accurately define the cohesive reproductive strategy of each species within the clade and augment models of the evolutionary relationships based on molecular phylogenetics that are currently being constructed (K. Kiontke and D.A. Fitch, personal communication).

Figure 1: *C. elegans*

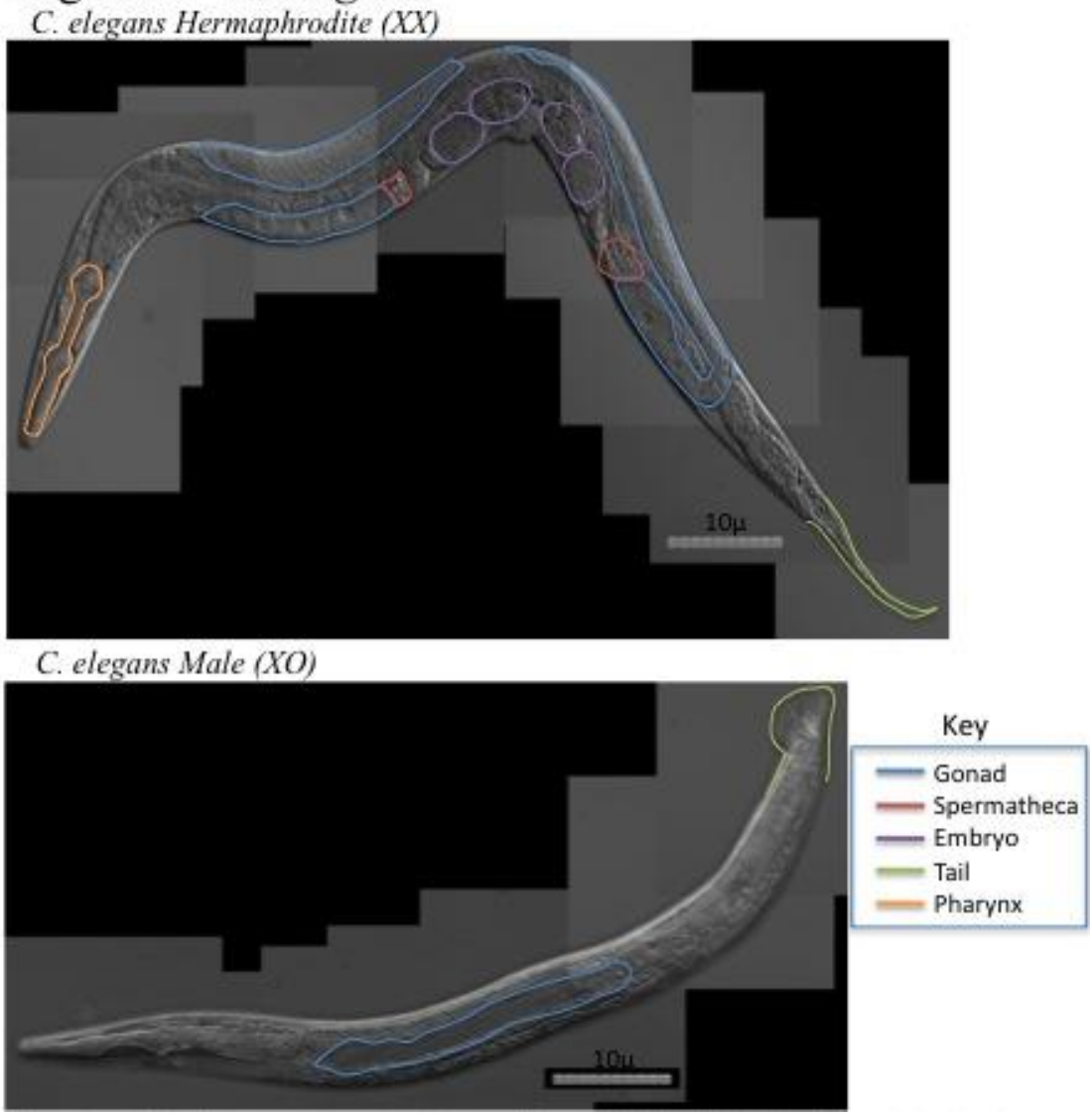


Figure 1: Both pictures are representative of the >20 observed worms. They are layered composites of Differential Interference Contrast (DIC) images of an adult *C.elegans* at 400X magnification.

The top image is of a hermaphrodite with important structures outlined according to the key. The gonad consists of two U-Shaped arms . The distal tip of each arm can be seen near the center of the body. The two arms run along the upper portion of the body before bending downwards and back toward the center.

The bottom image shows a male with important structures outlined according to the key. While the single arm of the male gonad shares the characteristic U-shape seen in the hermaphrodite, however, this character is not visible in this image because part of the gonad is at a different focal depth.

Figure 2: *Caenorhabditis Phylogeny*

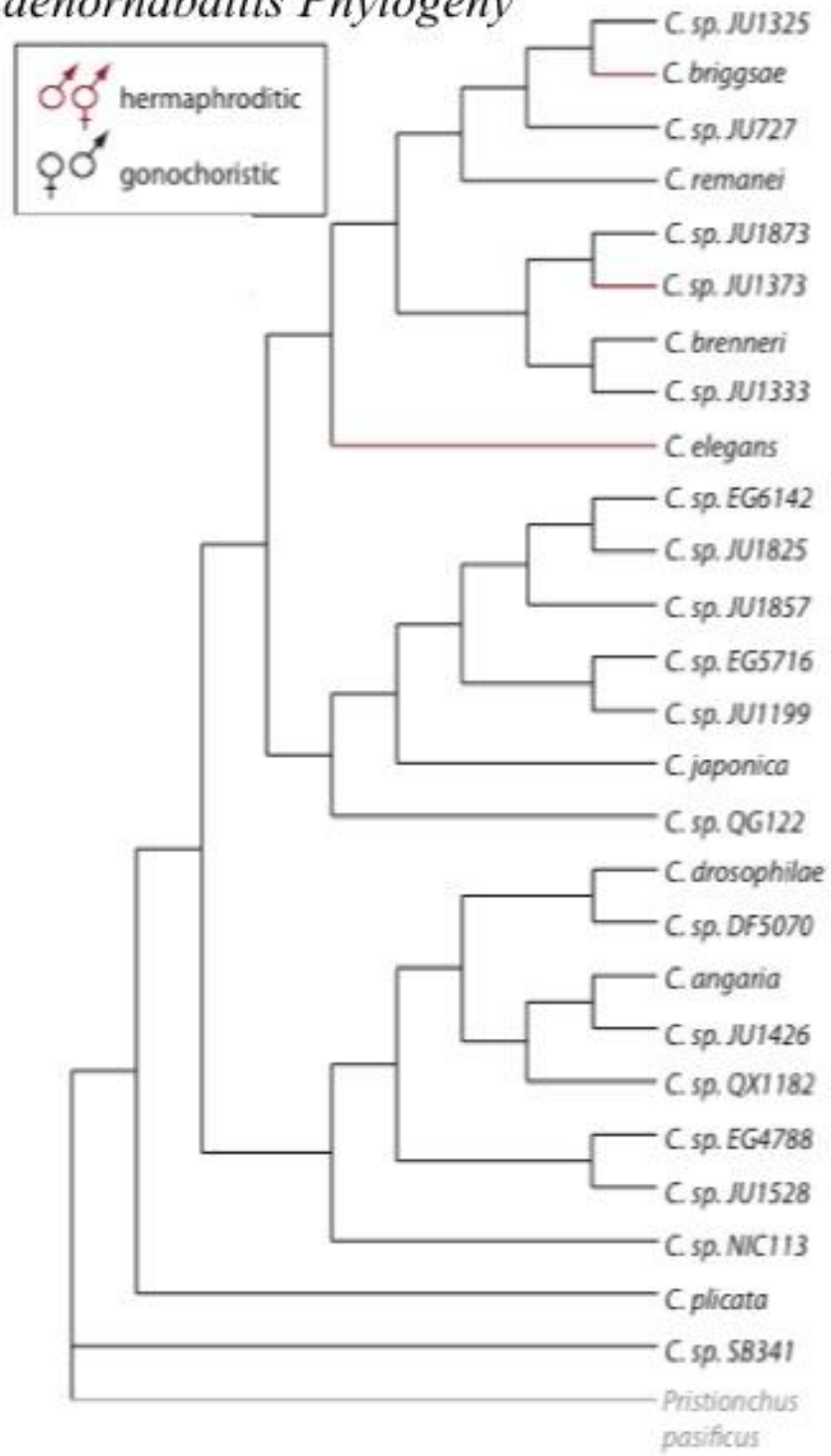


Figure 2: This figure shows the phylogenetic relationship of *Caenorhabditis* species with *Pristionchus pasificus* as an outgroup. Most species are gonochoristic with three exceptions. *C. elegans, C. briggsae, and C. sp. JU1373* which are all hermaphroditic species (adapted from Kiontke et al, 2011)

Figure 3: Phylogeny Including *C. elegans* and Members of the *Rhabditis* Clade

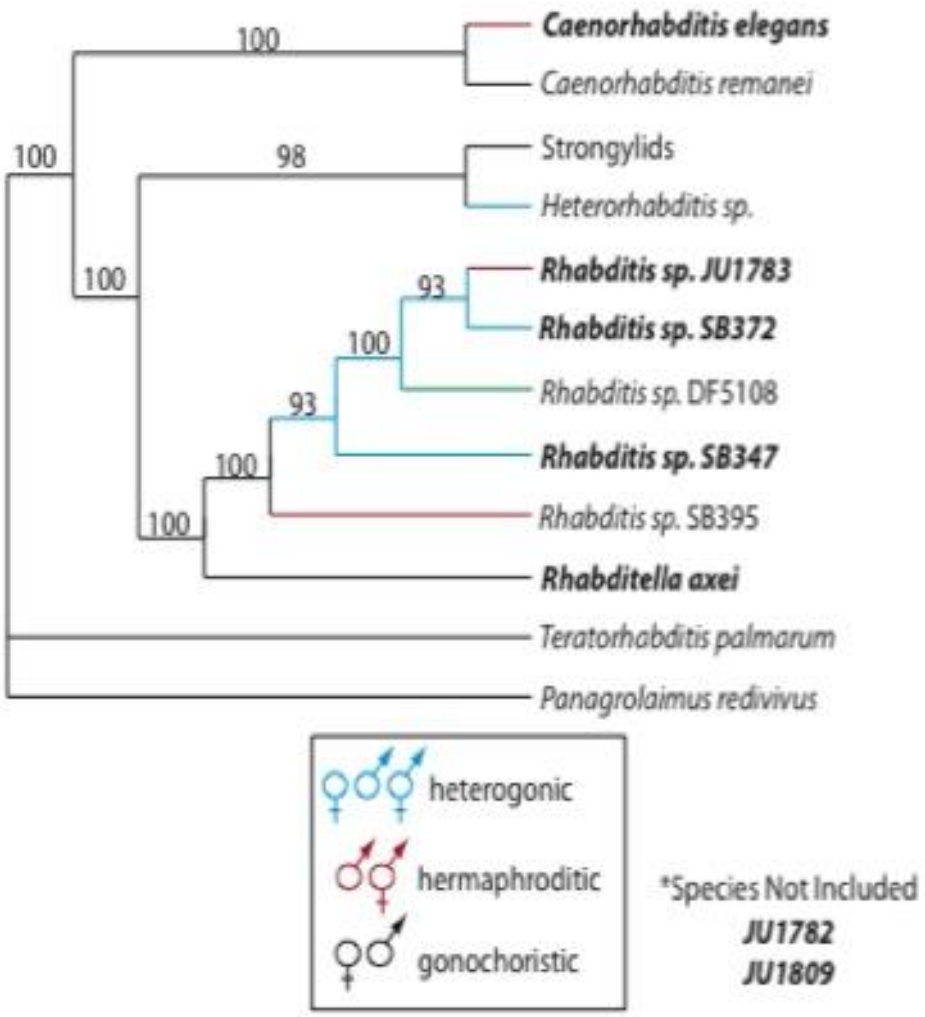


Figure 3: This figure shows the proposed relationship among some of the members of the *Rhabditis* clade. The species of interest are shown in bold face typed. *Teratorhabditis palmarum* and *Panagrolaimus redivivus* are outgroup species. The figure also shows the reproductive mode of the diverse species. Heterogonic species are shown by a blue line, hermaphroditic species by a red line, and gonochaoristic species by black lines (adapted from K. Kiontke and D. A. Fitch, personal communication).

Figure 4: *R. sp. SB347*

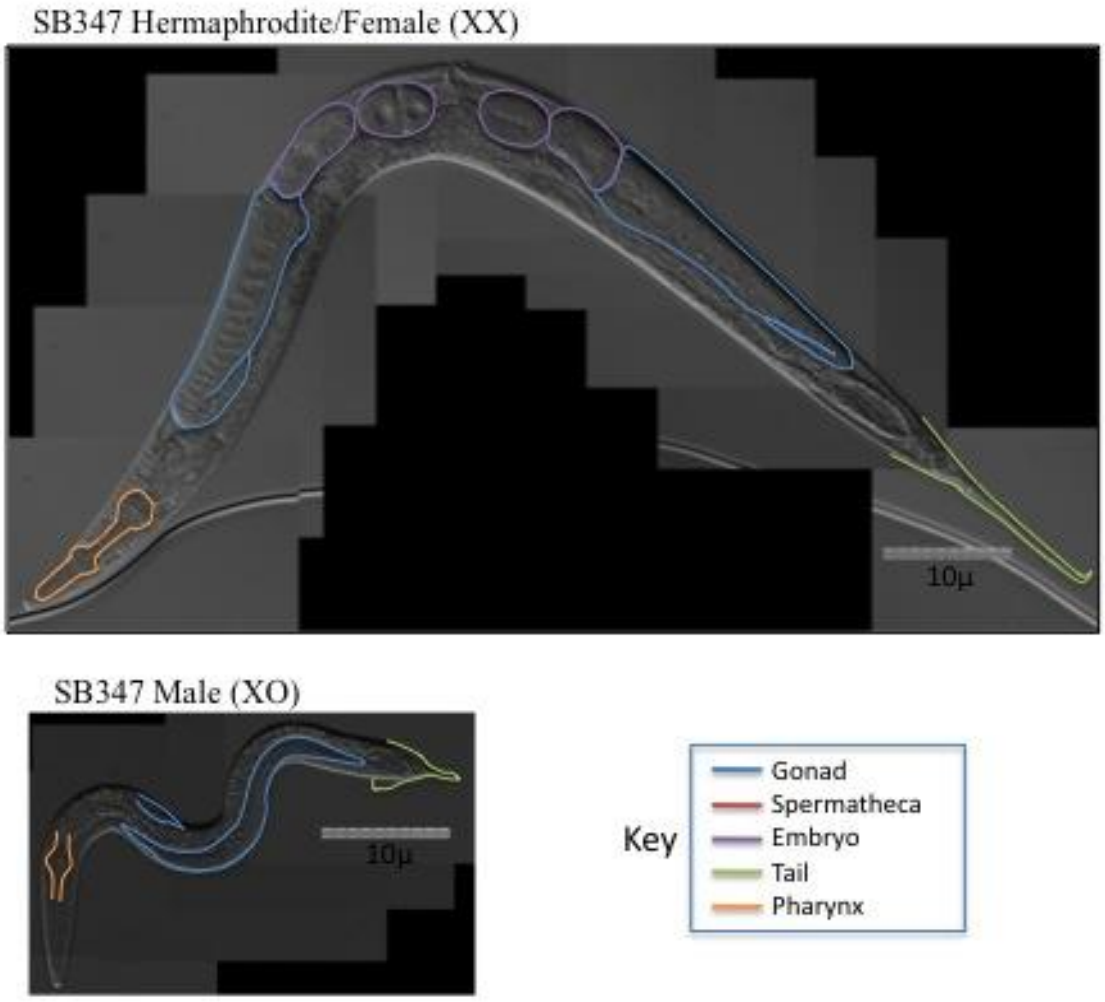


Figure 4: Both pictures are representative of the >20 observed worms.

The top image is a layered composite of Differential Interference Contrast (DIC) images of an adult *SB 347* hermaphrodite taken at 400X magnification. The bottom image, which is also a layered composite of DIC images at 400X magnification, shows an adult *SB347* male. Some of the important structures in each worm are outlined according to the key.

An image of an *SB347* female was not included as adult SB347 females are morphologically indistinguishable from adult *SB347* hermaphrodites.

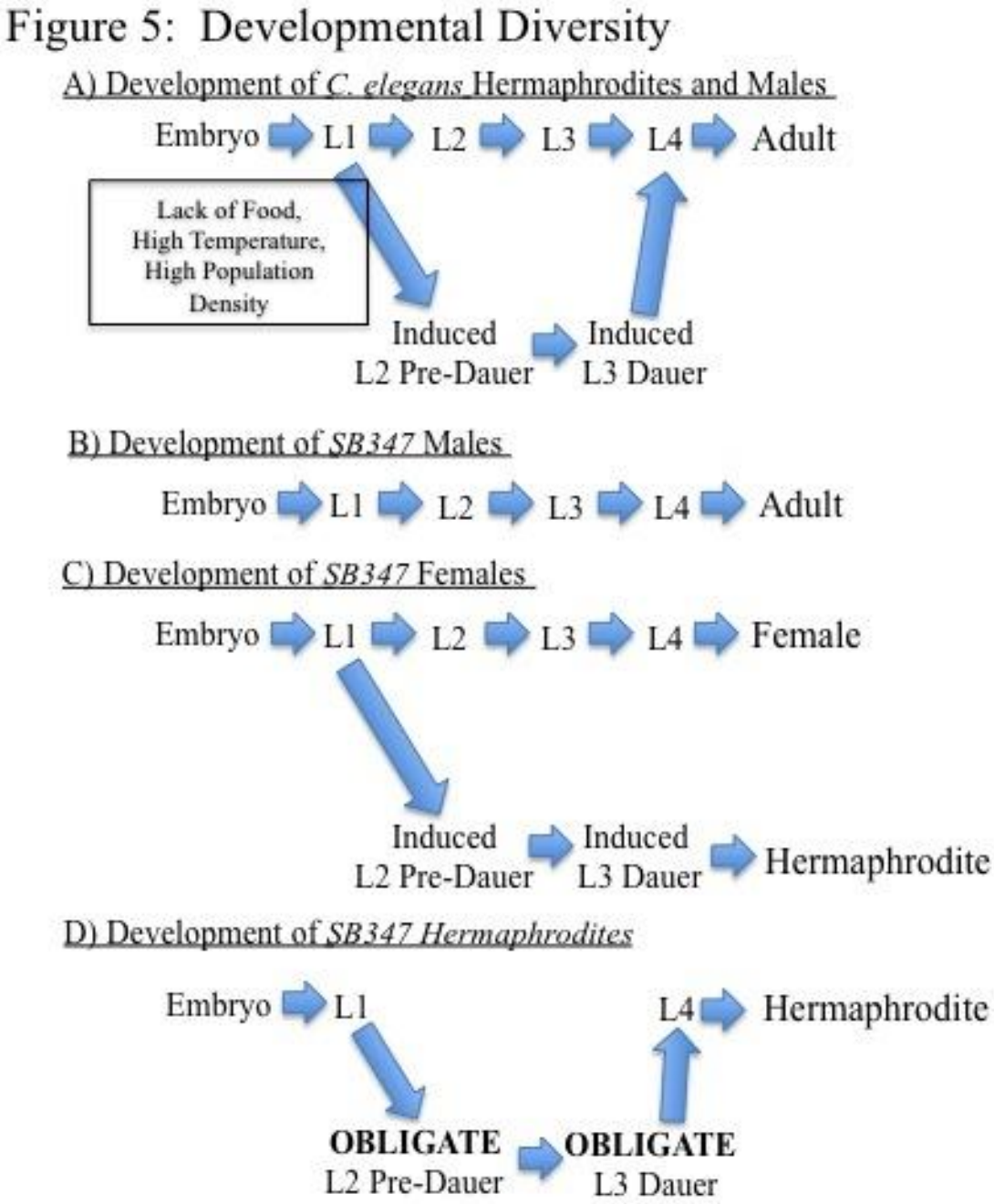


Figure 5: *C. elegans* hermaphrodites and males develop through four larval stages before reaching reproductive maturity. An optional developmental pathway called dauering can be induced by extreme environmental conditions.

SB347 hermaphrodites, males, and females develop similarly to *C. elegans*, but there are some key differences. The dauer pathway cannot be induced in males. When females are induced to dauer their gender is altered and they are hermaphrodites as adults. In SB347 hermaphrodite development the dauer stages are obligatory as opposed to inducible and last for at lest 24 hours (Chaudhuri et al, 2011).

Figure 6: Oogenesis and Oocyte Maturation in *C. elegans*

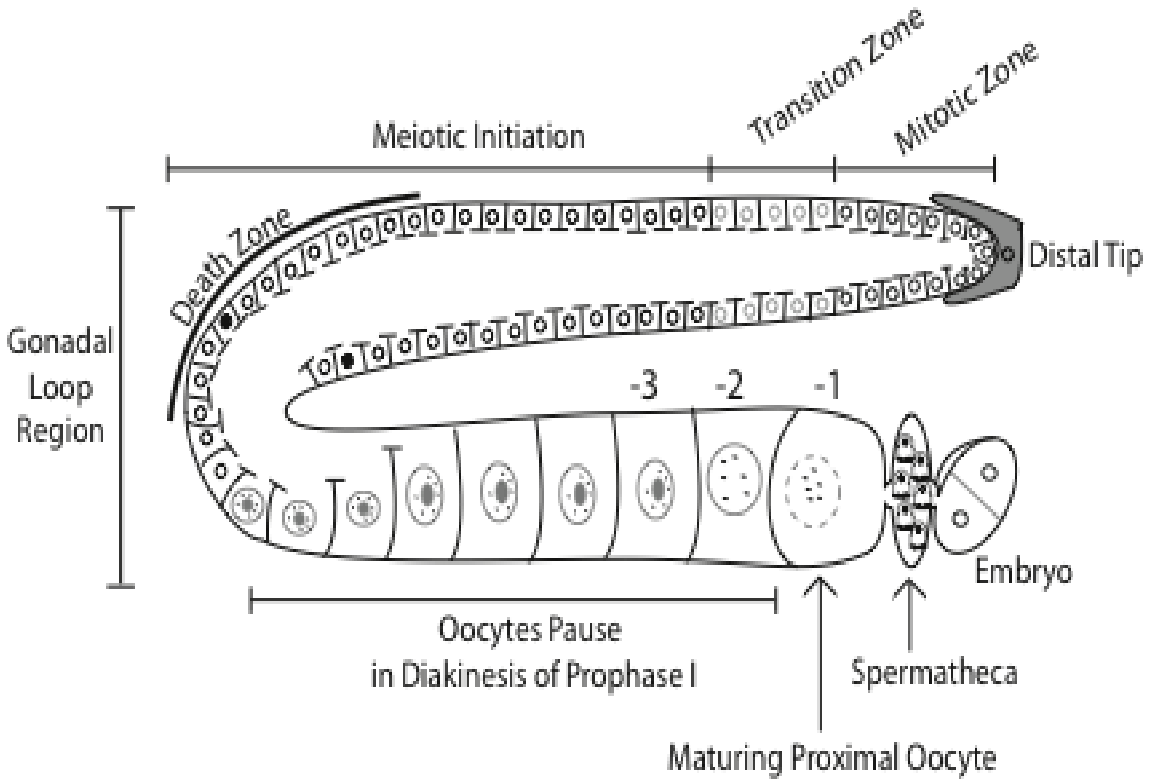


Figure 6: Above is a diagram of a single hermaphrodite gonadal arm in which oogenesis is occurring. In the distal tip germline-cells are dividing mitotically to maintain the germline. As the cells progress through the distal portion of the gonad they transition from mitosis to meiosis. In the distal portion of the gonad a central stream of cytoplasm called the rachis connects all the cells. Through the gonadal loop region some of the cells undergo apoptosis. As they enter the proximal portion of the gonad, the maturing oocytes form a single-file line with the most mature oocyte closest to the spermatheca. At this time oocytes arrest in Diakinesis of Meiotic Prophase I. The six homologous pairs of chromosomes are highly condensed and visible in each oocyte. As an oocyte approaches the most proximal portion of the gonad the nucleolus and then the nuclear envelope break down. The gonadal arm terminates in a spermatheca filled with ameboid sperm. The proximity of the sperm allows for early sperm cues to reach the proximal oocyte reinitiating meiosis. During ovulation the most proximal oocyte passes into the spermatheca and is fertilized. After fertilization the meiotic divisions are completed. Finally the newly formed embryo passes out of the spermatheca and into the uterus (Adapted from Gartner et al, 2008 and McCarter et al, 1999).

Methods and Materials

Nematode Strains and Culturing Conditions

All species were grown in culture at 20˚C on Modified Youngren's, Only Bacto-peptone (MYOB) agar plates seeded with the bacteria *E. coli* strain OP-50 (Lambie, 1994).

The strain of *C. elegans* used was *him-8(e-1489)* mutant. This mutation only affects the incidence of male production by hermaphrodites, but has no other effect on morphology of the soma or germline and thus can be used as an example of a wild-type *C. elegans* (Hodgkin et al, 1979). The *R. axei* strain (DF5006) was acquired from the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota in Minneapolis, Minnesota.

The SB/JU species were all received as gifts from Dr. Andre Pires da Silva. The species with a "JU" designation were originally isolated by the lab of M. Félix at the Institute Jacques in Paris, Fance. The "SB" species were originally isolated by the lab of W. Sudhaus at the Freie Universitaet Berlin in Berlin, Germany. More specifically, SB347 was found on ticks, *Ixodes scapularis*, in Kingston, R.I., United States by Dr. Elyes Zhioua (Félix, 2004). SB327 was discovered in a dung pile in Freiburg, Germany. JU1809 was isolated, by Dr. Marie-Anne Félix, from a rotting stem of *Symphytum officinale* in a woods near Santeuil, France. JU1782 was found in Ivry, France on the rotting stem of a *Petasites*. Finally, JU1783 was isolated by Dr. Isabelle Nuez from a star fruit in Melissa Doma, St. Denoist (A. Pires Da Silva, D. Shakes, personal communication).

Brood Count Experiments

Dauered worms were selected and placed on individual plates. Dauers were chosen to ensure that all worms would develop into self-fertile hermaphrodites. When the worms reached sexual maturity and began to lay embryos on the plate, they were moved to fresh plates. From that point forward, worms were moved to fresh plates approximately every 12 hours until they died. Data from worms that did not live at least 5 days after the first embryos appeared on the plate were excluded. This was done because worms that died early were likely unhealthy, and therefore, not reproductively typical of the species.

After a parent worm was removed from a plate, the offspring were left alone to develop. When a majority of the offspring appeared to be in the L3 stage of development an initial count of the total number of offspring on that plate was made. Once all of the offspring were either adults or in the L4 stage of development, three counts of total progeny were performed as well as three counts of just male progeny. If the results of these counts were inconsistent a fourth count was performed. Statistical analyses were then conducted on the resulting numbers to determine the average and standard deviation of the brood sizes, number of male progeny per worm, and the male percentage of the total brood.

DIC of Whole Worms

Young adult hermaphrodites were picked to 12µl of Edgar's Buffer on a Fisherfinest Premium Superfrost® slide (Edgar, 1995). Silicon grease was applied to the four corners of an 18x18mm coverslip, and the coverslip was placed over the worms. As the worms were observed through a dissecting microscope, the corners of the coverslip

were pressed down firmly enough to secure the worms to one location and limit their movement, without grossly distorting the morphology of the worms.

The live worms were then viewed immediately using Differential Interference Contrast (DIC) microscopy. An Olympus BX60 fluorescent microscope with the Wollaston prism set to give a lustrous gray image. Images were taken at 400X magnification with a Cooke cooled CCD camera for *C.elegans*, SB347, and SB372 hermaphrodites and a Qimaging ExiAqua camera for *R. axei*, JU1728, JU1783, and JU1809. At 400X magnification it is impossible to view the entire body of an adult hermaphrodite. In order to produce an image of a complete worm, overlapping images of each body section were taken and electronically stitched together using the computer program *iVision.*

DIC and Hoechst Staining of Isolated Gonads

Gonads were isolated via dissection of young adult hermaphrodites in 12µl of dissecting solution with a 27.5 gauge needle on Fisherbrand Colorfrost®/Plus slides. The dissecting solution for all species consisted of Edgar's Buffer with a 1:100 dilution of the lipid soluble DNA dye Hoechst 33428 and a species optimized dilution of the antihelminthic levamisole (Table 4). A coverslip was then added, and the gonads were viewed immediately using an Olympus BX60 fluorescent microscope with the Wollaston Prism set to give an optimal DIC image. Images were then captured using a Qimaging ExiAqua camera.

Whole Worm DAPI Staining

A droplet of a 50% M9 buffer and 50% chicken egg-white mixture was placed in the center of a circle etched on the opposite side of a Fisherfinest Premium Superfrost®

slide. Whole worms were then picked to the droplet. Then the M9/egg-white mixture was pulled away from the worms until the worms were adhered securely to the slide. The samples were then incubated in Carnoy's fixative (60% ethanol, 30% acetic acid, 10% chloroform) at room temperature for at least 1 hour.

After the incubation period the slides were rehydrated by washing them for 2 minutes each in solutions of 90% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, and finally in Phosphate Buffered Saline (PBS). After a brief dip in de-ionized water, the slides, with the exception of the area inside the etched circle, were wiped dry. Next, an 18x18mm coverslip with 5µl of Fluoro-Gel II with DAPI (4',6-diamidino-2-phenylindole), a fluorescent DNA stain, on it was placed DAPI-side down on each slide (Electron Microscopic Science). The slides were then stored overnight at 16˚C.

The next day acrylic was applied to the edges of the coverslip. Once the acrylic dried the slides were viewed with an Olympus BX60 fluorescent microscope and images were taken with a Qimaging ExiAqua camera.

Preparation for Immunofluorescence

Young adult hermaphrodites were dissected in 12µl of Edgar's Buffer and some species appropriate dilution of levamisole (Table 4). The worms were dissected with 27.5 gauge needles on a Fisherbrand Colorfrost®/Plus slide that was secondarily coated with Poly-$_{L}$-Lysine (Sigma Aldrich). The samples were fixed by "freeze cracking" followed by storage overnight in (-20˚) methanol (Miller and Shakes, 1995). Later, the slides were prepared for antibody binding by first washing them three times in PBS. Each wash lasted 10 minutes. Then the slides were washed in blocking solution (PBS +

0.5% Bovine Serum albumin + 0.1% Tween 20 and 0.04% sodium azide) for 10-30 minutes. This was done to help prevent non-specific binding of the antibody.

Labeling with Anti-Phospho-Histone-H3(ser10) Antibody

First, 27 µl of a 1:300 dilution of an anti-phospho-histone-H3(ser10) antibody in antibody buffer (blocking buffer without Tween 20) was applied to each slide. The exact anti-pHisH3(ser10) antibody used was a rabbit polyclonal antibody acquired from Upstate Biotechnology. The slides were then stored in a "humid box", a sealed container lined with a wet paper-towel intended to prevent the samples from drying-out, for 1.5 hours at 37˚C.

After this initial incubation time the slides were washed twice in PBS for two minutes each time. Then 27µl of a 1:100 dilution of an affinity purified goat anti-rabbit IgG Rhodamine (TRITC)-conjugated antibody (Jackson ImmunoResearch) in antibody buffer was applied to each slide. The slides were then incubated for 1.5-2 hours in a "humid box" at room temperature in a dark location.

Following this incubation period the slides were washed in PBS again. They were washed once for two minutes. This wash was followed by a brief dip in de-ionized water. Next excess liquid was removed from the slide was with a Kim wipe, and a coverslip, with 5µl of DAPI adhered to it, was applied DAPI-side down to each slide. The slides were then incubated a at least overnight at 16˚C in a slide folder. Acrylic was applied to the edges of the coverslip before the slides were viewed under an Olympus BX60 microscope and images were taken with a Qimaging ExiAqua camera.

Labeling with Anti-nucleolar Antibody

A mouse monoclonal antibody specific to nop-1 and fibrillarin was used to label the nucleolus of each cell. This antibody was acquired from Encore Biotechnology Inc. 27µl of a 1:200 of the anti-nucleolar antibody in antibody buffer was applied to each slide. The slides were then incubated at room temperature in a "humid box" for 2 hours. Like with the anti-pHisH3(ser10) staining, after the initial incubation the slides were washed twice in PBS for two minutes each time. Then 27µl of a 1:100 dilution of Dylight488-conjugated affinity purified goat anti-mouse IgG antibody (Jackson Immuno Research) was applied to each slide. The slides were then incubated at room temperature for 1.5-2 hours in a "humid box" stored in a dark location. After this final incubation period, the slides were washed and DAPI was applied as described in "Staining with Anti-Phospho-Histone-H3(ser10)".

Table 1: Dilution of Levamisole in Dissecting Solution

Species	Dilution of Levamisole (100X 25mM)
C. elegans	1:100
R. axei	1:100
SB372	1:200
JU1782	1:200
JU1783	1:200
JU1809	1:200

*SB347 worms were dissected without the use of levamisole.

Results

Verifying that Self-fertile SB347 and SB372 Hermaphrodites Produce an Unexpected Proportion of Male Offspring

One of the major features of the reproductive strategy of SB347 is that self-fertilizing hermaphrodites produce an atypical number of male progeny. Original reports indicated that approximately 13% of SB347 hermaphrodite progeny are male (Félix, 2004). However, later studies reported that only about 9% of hermaphrodite progeny are male (Chaudhurri et al, 2011). In order to clarify the exact percentage of male offspring that result from SB347 hermaphrodite self-fertilization the complete broods of individual hermaphrodites were analyzed. A brood will be defined as the total progeny produced over the reproductive lifetime of an individual worm. Brood count experiments were also conducted with SB372 hermaphrodites in order to determine if this element of the reproductive strategy is shared by species that are known to be close relatives of SB347 (K. Kiontke and D.A. Fitch, personal communication).

To determine the percentage of males produced by selfing hermaphrodites, complete broods of eighteen SB347 hermaphrodites and five SB372 hermaphrodites were analyzed (Tables 2-4). It was found that the brood sizes for the sample of SB347 hermaphrodites ranged from 168 to 364 progeny. The average brood size for the sample was 243 progeny. The brood sizes of the SB372 hermaphrodites sampled ranged from 152 to 321 progeny, and had an average size of 247 progeny. Both of these findings are consistent with the average number of progeny produced by selfing *C. elegans* hermpahrodites, which is 280 progeny (as reviewed in Lewis and Fleming, 1995). The average number of male progeny produced by a single worm was 21 males for SB347

hermaphrodites and 23 for SB372 hermaphrodites. Further, these males were produced consistently throughout the reproductive life of each worm.

By comparing the number of males to the total brood size, the percent males produced was calculated for each worm. The average percent male progeny for the sample of SB347 hermaphrodites was 8.7%, consistent with the results of Chaudhuri et al, 2011. The average percentage of males produced by SB372 hermaphrodites was 9.5%; similar to the percentage of males produced a by SB347 hermaphrodites. This suggests that the ability of selfing hermaphrodites to produce a higher than predicted number of male offspring is a shared characteristic of both SB347 and SB372 hermaphrodites.

Examining Intact Gonad Size Relative to Overall Body Size

In order to be evolutionarily successful, a species must be able to both survive and reproduce. However, genetic and physiological constraints make it unlikely that a single species will be both exceedingly long lived and have huge reproductive success. There are always trade-offs among traits (Stearns and Hoekstra 2005). In a naturally short lived organism, such as *C.elegans,* the ability to reproduce quickly and effectively is paramount. The significance of reproduction to an organism can manifest itself in the amount of resources allotted to it. One measure of reproductive investments is the proportion of the total body devoted to the reproductive organs (Freeman, 2008).

Like the *C. elegans* body, the bodies of these non-*C. elegans* species are transparent. This makes it is possible to view the gonad in the context of the intact worm body using DIC. In Figure 7A it can be seen that the two U-shaped arms of the *C. elegans* hermaphrodite gonad occupy approximately 2/3 of the worm volume. The gonad

of the *R. axei* female (Figure 7B) occupies a somewhat smaller portion of the worm, slightly less than 1/2 of the total worm volume. In the more recently isolated SB/JU species (Figure7C-G), the gonads occupy 1/2 to 2/3 of the worm volume. The high proportion of the worm volume occupied by the gonad in all of these species indicates a large investment in reproduction in a trait shared by *C. elegans* and the members of the *Rhabditis* clade.

Defining the Morphology of the Gonad

Beyond the size of the gonad relative to the overall body size, the DIC images in Figure 7 also facilitate comparisons of overall gonad morphology. As in *C. elegans,* the females and hermaphrodites within this clade are didelphic, that is their gonads have two arms (two ovaries or two ovatestes) (Figure 7), whereas the males are monodelphic and have a single gonadal arm (testis) (data not shown). An individual arm of the *C. elegans* hermaphrodite gonad has a long distal portion (D) with a width approximately half that of the total body width at that point (Figure 7A). The proximal portion (P) of the gonad is moderately shorter than the distal and contains a row of 3-5 maturing oocytes. Over-all, the gonad is classically characterized as U-shaped; it has a single bend, the gonadal loop region (L).

In contrast, the gonadal arms of hermaphrodites in this clade appear to differ from that of *C. elegans* in a number of ways. The distal portions (d) of the gonadal arms of these species appear to be considerably shorter than the distal portion of a *C. elegans* gonadal arm (D). In addition, these distal regions are smaller in width and contain fewer cells. As in *C. elegans,* however, the gonadal arms of the SB/JU species (Figure 7C-G) all have a single loop region (L) and the proximal portions (P) of the gonads contain a

row of maturing oocytes. However, the number of maturing oocytes in the proximal gonads of these species is higher, greater than 10 in many cases.

The gonadal arms of *R. axei* females are distinguished by the presence of an additional gonadal bend (L1,L2). This is different from both the SB/JU species and *C. elegans,* but similar arrangements occur in other nematode species including the plant parasite *Nothanguina cecidoplastes* (Whitehead, 1959). The *R. axei* gonad is also slightly longer than that of the SB/JU species. The overall patterning of the distal portion of the gonad (d) is similar to that of the SB/JU species. Maturing oocytes are present in both the medial (M) and proximal (P) portions of the *R. axei* female gonad in numbers similar to those of the SB/JU species.

Characterizing the Distal and Proximal Portions of the Gonad

In order to further define the morphology of single gonadal arms, young adult hermaphrodites were dissected and single gonadal arms were isolated for analysis under DIC optics (Figure 8). The most striking feature, which distinguishes this clade from *C. elegans,* is the markedly diminished distal region. This portion of the gonad is, not only shorter, but also houses many fewer cells (Figure 8). In an effort to confirm that there are indeed fewer cells in the distal portion of the gonadal arm, the lipid soluble DNA dye Hoechst was used to label the gonadal nuclei. This technique also allowed us to more easily examine the cellular organization with in the distal region.

In *C. elegans* the cells occupying the distal tip region of the gonad have a well-known tube-like arrangement (Figure 8A). The center of the tube is the rachis, and the outer-portion the germ-cells it connects. In order to visualize all of the nuclei in the *C. elegans* gonad arranged this way, one needs to view multiple focal planes. Figure 8A

shows the most central focal plane with both the rachis and outermost germcells of the tube structure visible. Within the *Rhabditis* clade, the cells occupying the distal region of female/hermaphrodite gonads are arranged very differently. Again, there appear to be many fewer cells. Moreover, as these cells move toward the gonadal loop region, they are arranged in a double-file line in which all nuclei can be visualized in a single focal plane. An example of this is highlighted by arrows 1 and 2 in Figure 8A. As in *C. elegans*, however, the germ-cells appear to resolve into a single file row of maturing oocytes in the proximal portions of the gonads of all species in this clade. However, in most of the SB/JU species, this row of oocytes appears to form before the bend in the gonad (Figure 8A, arrow 3), rather than after the bend as in *C. elegans* (Figure 8A, arrow 4). The row of oocytes in SB347 hermaphrodite gonads forms after the bend (Figure 8).

One feature unique to the proximal gonad of the SB/JU species is the presence of two sets of unidentified somatic cells (Figure 8B). The first set is composed of three cells and is located more distal to the uterus than the second set (Figure 8B, arrow 1). The second set seems to consist of 6-8 cells (Figure 8B, arrow 2). It is located closer to the uterus, and the cells seem to be arranged in an almost circular pattern. These somatic cells are not seen in either *C. elegans* or *R. axei*. The exact nature, structure, and function of these cells remain unknown.

Correlation of Chromosome Morphology and Nucleolus Size in Maturing Oocytes

Labeling of the DNA in the germ-cells of isolated and cold-methanol-fixed gonads with the fluorescent DNA marker 4',6-diamidino-2-phenylindole (DAPI) revealed variation in chromosome morphology in the maturing oocytes of the different species. In *C. elegans* the chromosomes in the maturing oocytes are highly condensed and

distributed throughout the nucleus; in these diakinetic oocytes, the six paired homologs can be easily distinguished (Figure 9, arrow 1). Chromosomes in the maturing oocytes of the other species, including *R. axei*, remain incompletely condensed. In *R. axei* oocytes, the chromatin is mostly uncondensed and distributed through a larger portion of the nucleus (Figure 9, arrow 2). In SB347 and SB372 oocytes the chromosomes appear more compact than in *R. axei*, however, this compaction is intermittent giving the DNA a punctate appearance (Figure 9, arrow 3). Additionally, the chromatin in SB347 and SB372 oocytes is distributed around the periphery of the nucleus near the inside of the nuclear envelop (Figure 9, arrow 4).

Chromosomes are not the only cellular components occupying the nucleus. Another major cellular component contained within the nucleus is the nucleolus, a non-membranous organelle that is the site of ribosome production (Freeman, 2008). In order to determine if the size or position of the nucleolus within the oocytes of these species might be influencing the distribution of the chromosomes isolated gonads were analyzed using an antibody against the nucleolus specific protein, Nop-1. This experiment revealed that the nucleoli in *R. axei*, SB347, and SB372 oocytes (Figure 9, arrow 6) are indeed substantially larger and occupy a larger proportion of the nucleus than those in *C. elegans* oocytes.

This suggests that the distribution of the chromatin in the oocytes of these species is affected by nucleolus size. The larger nucleolus in *R. axei*, SB347, and SB374 oocytes may prevent the chromatin from distributing more uniformly throughout the nucleus. In the *R. axei* oocytes specifically, the large nucleolus appears to occupy one half of the nucleus, while the chromatin occupies the opposite half (Figure 9). Conversely, in

SB372 the large nucleolus excludes the chromatin from the center of the nucleus and restricts the chromosomes to the periphery of the nucleus.

Mitotic Divisions of Germline Cells

In the distal zone of the *C. elegans* hermaphrodite gonad mitotically dividing cells maintain the germline, providing a constant supply of new cells for gametogenesis (as reviewed in Hubbard and Greenstein, 2005). Within this mitotic zone, the chromosomes of nuclei that are either entering or currently in M-phase label with an epigenetic mark, a post translational phosphorylation of histone H3 at amino acid serine 10 (Gurley et al, 1978). To better understand, within this clade, which nuclei within the distal gonad undergo mitotic proliferation, methanol-fixed-isolated gonads were analyzed using an anti-PhosphohistoneH3(ser10) [pHisH3(ser10)] antibody.

The distal portion of the gonads isolated from *C. elegans, R. axei*, and SB372 hermaphrodites or females were all analyzed for the distribution of pHisH3(ser10) positive nuclei. Notably, the most distal cell or cells of the gonad were never observed to be pHisH3 positive (in Figure 10 the most distal cell of each gonad is indicated by an *). This suggests that the ultimate cell or cells in each gonad are not part of the mitotic zone. If these ultimate cells prove to be like the most distal cell in the *C. elegans* gonad, they are likely non-dividing somatic cells (Hubbard and Greenstein, 2005).

In the distal portion of isolated *C. elegans* gonads, 0-4 cells were actively dividing in the mitotic zone at any one time (Figure 10, image a). In SB372 0-3 cells were observed actively dividing in the distal gonad (Figure 10, box c). While there is no dramatic difference between the number of actively dividing cells in *C. elegans* hermaphrodites and SB372 hermaphrodites; the proportion of cells dividing in the SB372

distal gonad is much greater as the distal gonad of this species contains fewer cells overall. The mitotic zones of *R. axei* female gonads contained 0-6 actively dividing cells at any one time (Figure 10, box b). The slight increase in the number of dividing cells in this species may be due to the larger size of the *R. axei* gonad.

Of the four species investigated, the only one with a truly distinctive pattern of pHisH3(ser10) labeling was SB347. In the distal portions of SB347 hermaphrodite gonads, the majority of the cells, including the most distal cells, exhibited pHisH3(ser10) labeling (Figure 10, box d). Additionally, the labeling in these cells did not always correlate with DNA labeling. This distinctive pattern was observed in multiple independent preparations, all of which included slides of non-SB347 gonads which exhibited the more typical and expected distribution of pHisH3 labeling nuclei. The labeling only disappears in SB347 after the single-file line of oocytes has formed and the oocytes have begun to mature (Figure 10, arrow 1).

While, this pattern was observed in multiple preps with the SB347 strain maintained in our lab. Other strains of SB347 were not tested. It is possible that the unique pattern of pHisH3(ser10) labeling in the distal portion of SB347 hermaphrodite gonads could be explained by either viral or bacterial infection of our lab strain (Felix et al, 2011; as reviewed in Taylor et al, 2005). The pHisH3(ser10) labeling in the non-typical cells and in areas with no correlating DNA labeling, could be the antibody labeling this epigenetic mark in either bacteria or viruses.

Re-initiation of Meiosis in the Most Proximal Oocyte

During oogenesis in *C. elegans*, meiotic progression pauses during oocyte maturation and the meiotic divisions themselves occur only after the oocyte has been

fertilized. Oocytes arrest specifically during diakinesis of meiosis I as the cell enters the proximal gonad (as reviewed in Greenstein, 2005). Meiosis is re-initiated (e.g. nuclear envelope breakdown and formation of a meiotic spindle) after the oocyte receives cues from the sperm stored in the spermatheca. The oocyte is generally in or approaching the most proximal position to the uterus when this occurs (McCarter et al, 1999). As stated previously, fluorescent staining with anti-pHisH3 antibody indicates active division (Gurley et al, 1978). Using this antibody as an indicator, oocyte position at the re-initiation of meiosis was explored.

Consistent with previous studies, chromosomes within the most proximal oocyte in a *C. elegans* hermaphrodite gonad label brightly with pHisH3(ser10) antibody, indicating that meiosis has been re-initiated and the oocytes are transitioning from meiotic prophase (G2 in the cell cycle) to M-phase (Figure 11). It appears that this pattern holds true for oocytes in both *R. axei* and SB347 (Figure 11), suggesting that the re-initiation of meiosis in the proximal oocyte is a feature of oogenesis that is shared by a many diverse nematodes.

Differences in Ovulation

In *C. elegans* hermaphrodites, ovulation is the passage of the most proximal oocyte into the spermatheca. In this species, the spermatheca functions as both a storage structure for sperm and the site of fertilization (as reviewed in Greenstein, 2005; L'Hernault, 2006). *C. elegans*, hermaphrodites ovulate once about every 23 minutes and produce about 280 progeny during their lives (as reviewed in Lewis and Fleming, 1995; McCarter et al, 1999). SB347 hermaphrodites produce a similar number of progeny (Table 2), and have similar life-spans (Félix, 2004; Chaudhuri, 2011). Given this

similarity, the frequency of ovulation was explored in SB347, with the expectation of it being the same as in *C. elegans.*

In order to determine if SB347 hermaphrodites ovulate in the same manner as *C. elegans* hermaphrodites, young adult hermaphrodites were fixed in Carnoy's solution and DAPI was used to label all of the nuclei in the body. This process made it possible to visualize the oocytes relative to the spermatheca in intact worms. If SB347 hermaphrodites ovulate in the same manner and with the same speed and frequency as *C. elegans* hermaphrodites then we anticipated an oocyte would be found within the spermatheca in a similar proportion of gonads

Using the full-body DAPI stain described, we scored for the presence or absence of oocytes within the spermatheca of individual gonads of *C. elegans* and SB347 hermaphrodites. In each case, we scored 50 spermatheca. An oocyte was determined to be physically within the spermatheca when it was clearly surrounded by sperm (Figure 12, box A) as opposed to all of the sperm being located exclusively on the uterus proximal side of the oocyte (Figure 12, B). The position of the spermatheca itself was defined by the location of clustered sperm nuclei. In the *C. elegans* hermaphrodite gonads examined, an oocyte was found within the spermatheca 11 times, 22% of the gonads. In SB347 an oocyte was observed in the spermatheca only 4 times, 8% of the gonads. This suggested that either the frequency of ovulation is lower in SB347 or, more likely, that each individual oocyte spends less time in the spermatheca before passing into the uterus in this species.

However, the low number of oocytes scored as being present in the spermatheca could reflect differences in gonad morphology. In particular, I observed that the location

of the spermatheca in the SB347 worms relative to the rest of the gonad was different than that in *C. elegans*. In *C.elegans* hermaphrodites the spermatheca is in-line with the proximal portion of the gonadal arm and ovulating oocytes are forced entirely into the spermatheca before being fertilized and moving into the uterus (Figure 12) (McCarter et al, 1999). In SB347 hermaphrodites, the spermatheca (as delineated by the location of clustered sperm) was often found either above or below the proximal portion of the gonad (Figure 13 B). Reviewing the samples from the previous experiment it was found that for 32 of the 50 SB347 hermaphrodite gonads, 64%, clusters of sperm (defining the spermatheca) were found above or below the proximal portion of the gonad. This suggested that the morphology of the spermatheca in SB347 hermaphrodites differs from the morphology of the spermatheca in *C. elegans* hermaphrodites.

Consistent with this idea, a re-examination of the SB347 samples showed that while the most proximal oocyte was rarely found within the spermatheca, as noted above, it was clearly in contact, along either its upper or lower side, with the sperm in 10 of the 50 gonads, 20% (Figure 13A). Using this revised criterion for scoring, the percent of oocytes in contact with sperm in SB347 hermaphrodites was similar to the 22% of oocytes found within the spermatheca in the *C. elegans* sample. This result suggests that in SB347 hermaphrodites, the movement of oocytes through the spermatheca occurs in a somewhat different manner and that the spermatheca may consist of a structural pocket that is more exclusively devoted to sperm storage rather than the entire structure serving the dual purpose of sperm storage and the site of fertilization.

Table 2: Brood Size

Species	N	Average	Range	Standard Deviation
SB347	18	243	168-364	+/-59
SB372	5	247	152-321	+/-71

Table 3: Number of Male Progeny per Worm

Species	N	Average	Range	Standard Deviation
SB347	18	21	8-32	+/-6.5
SB372	5	23	15-30	+/-5.4

Table 4: Percentage of Males in Total Brood

Species	N	Average	Range	Standard Deviation
SB347	18	8.7%	4.5%-12.6%	+/-2.5%
SB372	5	9.5%	7.8%-11.0%	+/-1.2%

Tables 2-4: These tables show the analysis of the brood count data collected from SB347 and SB372 hermaphrodites

Figure 7: Gonad Morphology in Intact Worm

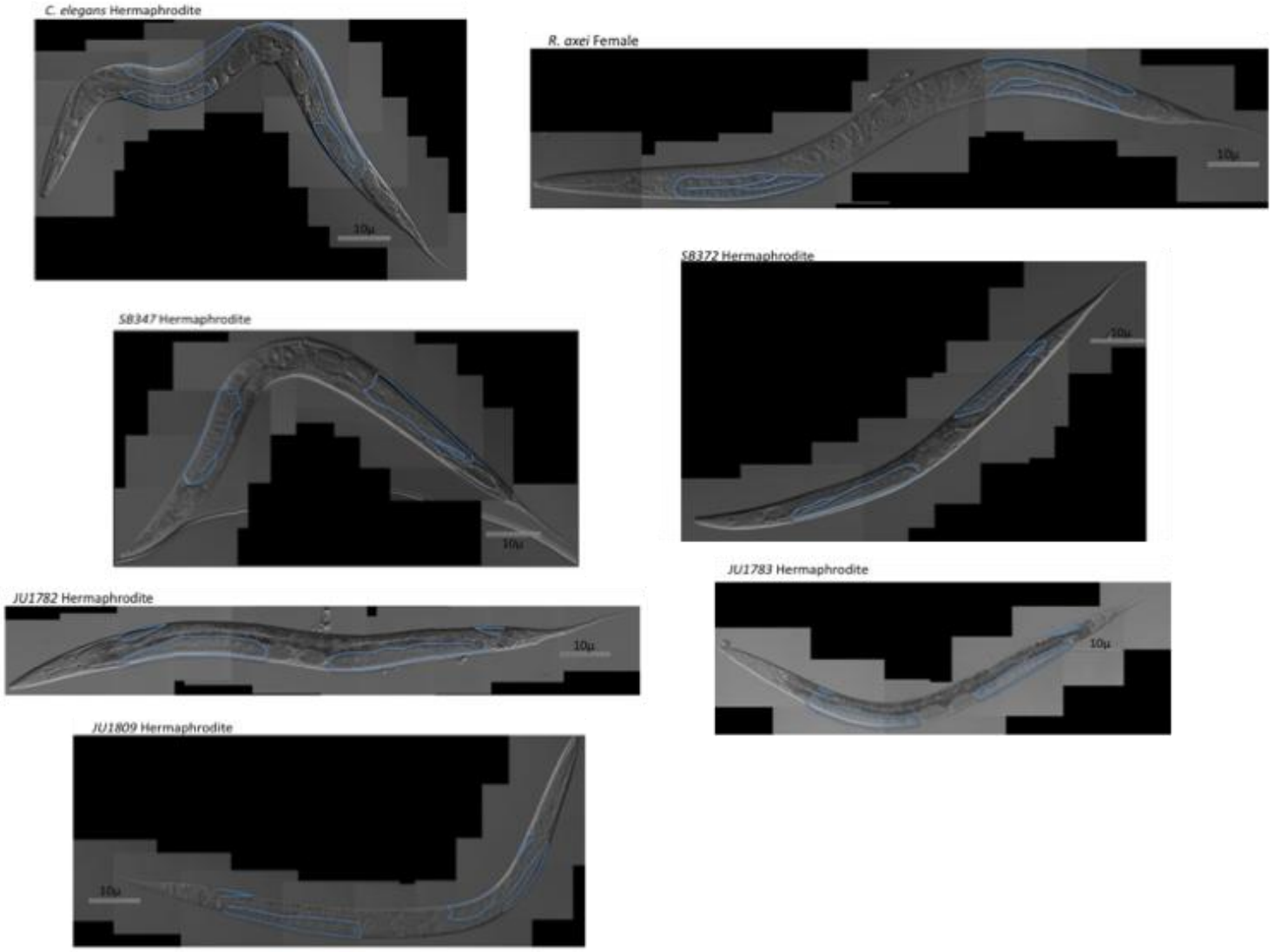


Figure 7: The images on the previous page are representative of the >20 worms observed. Each image is a DIC layered composite of a hermaphrodite worm a 400x magnification. The two gonadal arms of each worm are outlined in blue.

Image A is of a *C. elegans* hermaphrodite with (D) indicating the long distal portion of the gonad, (P) the proximal portion of the gonad, and (L) the gonadal loop region.

Image B is of a *R. axei* female. (L1) and (L2) indicate the two bends in the gonadal arms. (d), (M), and (P) mark the distal, medial, and proximal portions of the gonad respectively.

Images C-G are of the SB/JU species. (L) indicates the gonadal loop region of each gonad. The short distal portion of each gonad is indicated by (d). The row of maturing oocytes in the proximal portion of each gonad is indicated by (P).

Figure 8: Isolated Gonadal Arms

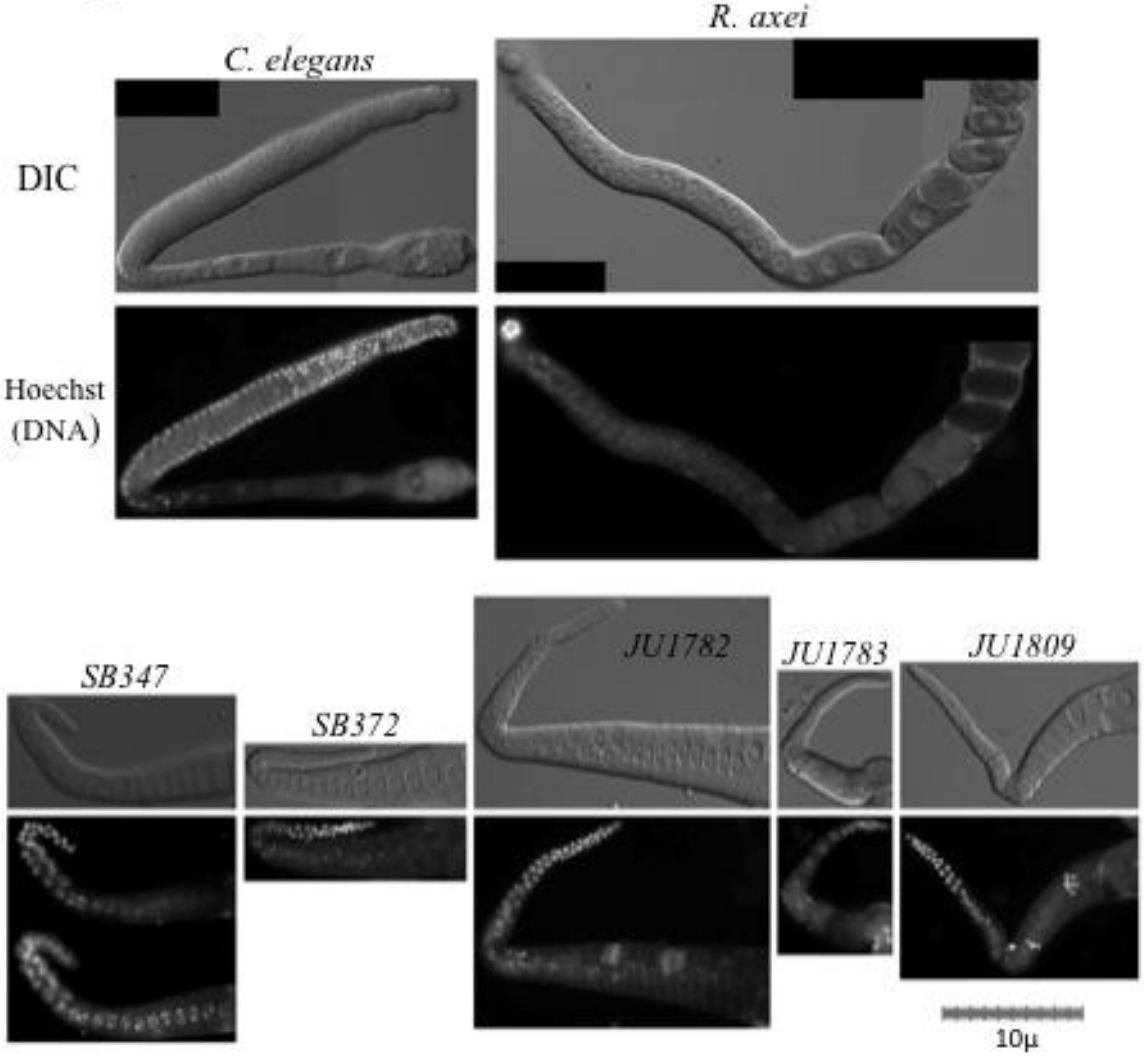


Figure 8: Above are DIC images of isolated gonads as well as the corresponding image of DNA stained with the fluorescent lipid soluble dye Hoechst. All images were taken at 400X magnification and are representative of 8-12 worms.

Figure 8A: Differences in Germ-Cell Arrangement

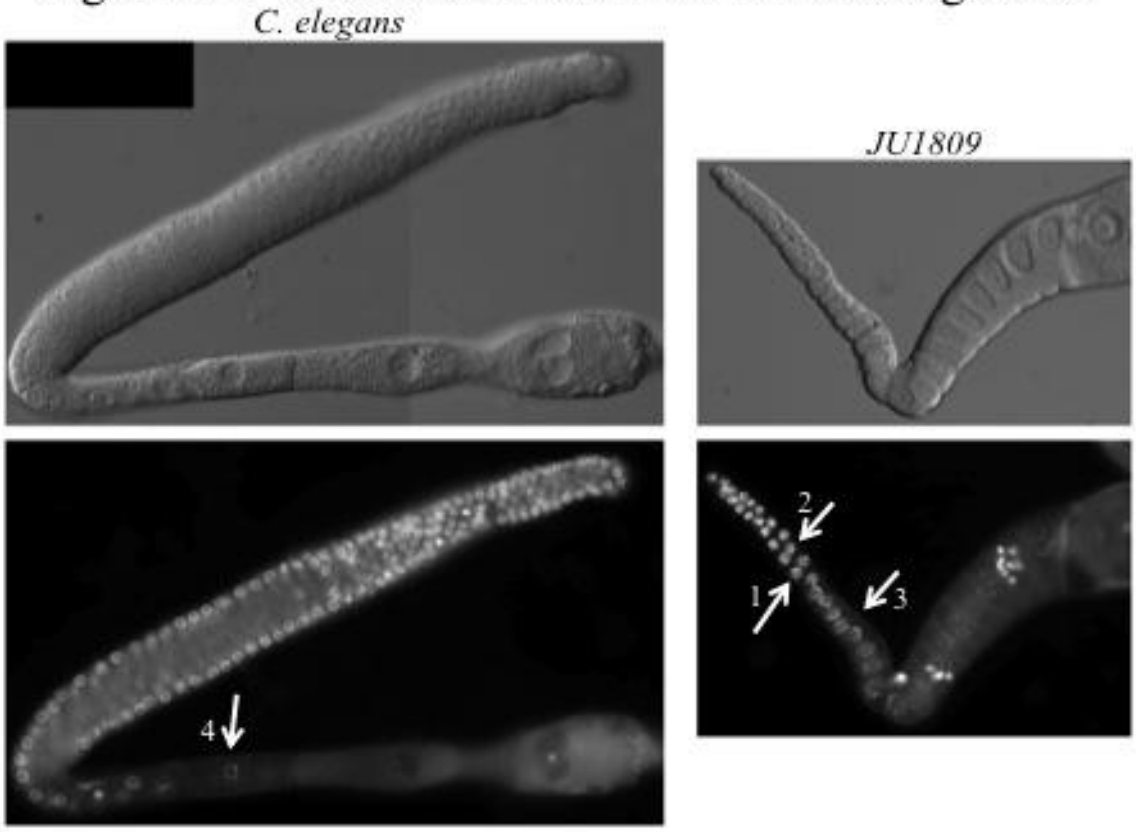


Figure 8A: Above are DIC images of isolated gonads as well as the corresponding image of DNA stained with the fluorescent lipid soluble dye Hoechst. All images are at 400X magnification and representative of the 8-12 worms observed.

Arrows 1 and 2 indicate the double-file line of cells characteristic of cell organization in the distal portion SB/JU hermaphrodite gonads. Arrow 3 indicates the single-file row of oocytes forming before the gonadal loop region in SB/JU hermaphrodite gonads. Arrow 4 indicates the formation of the single-file row of oocytes after the gonadal loop region in the *C. elegans* hermaphrodite gonad.

Figure 8B: Unidentified Somatic Cells

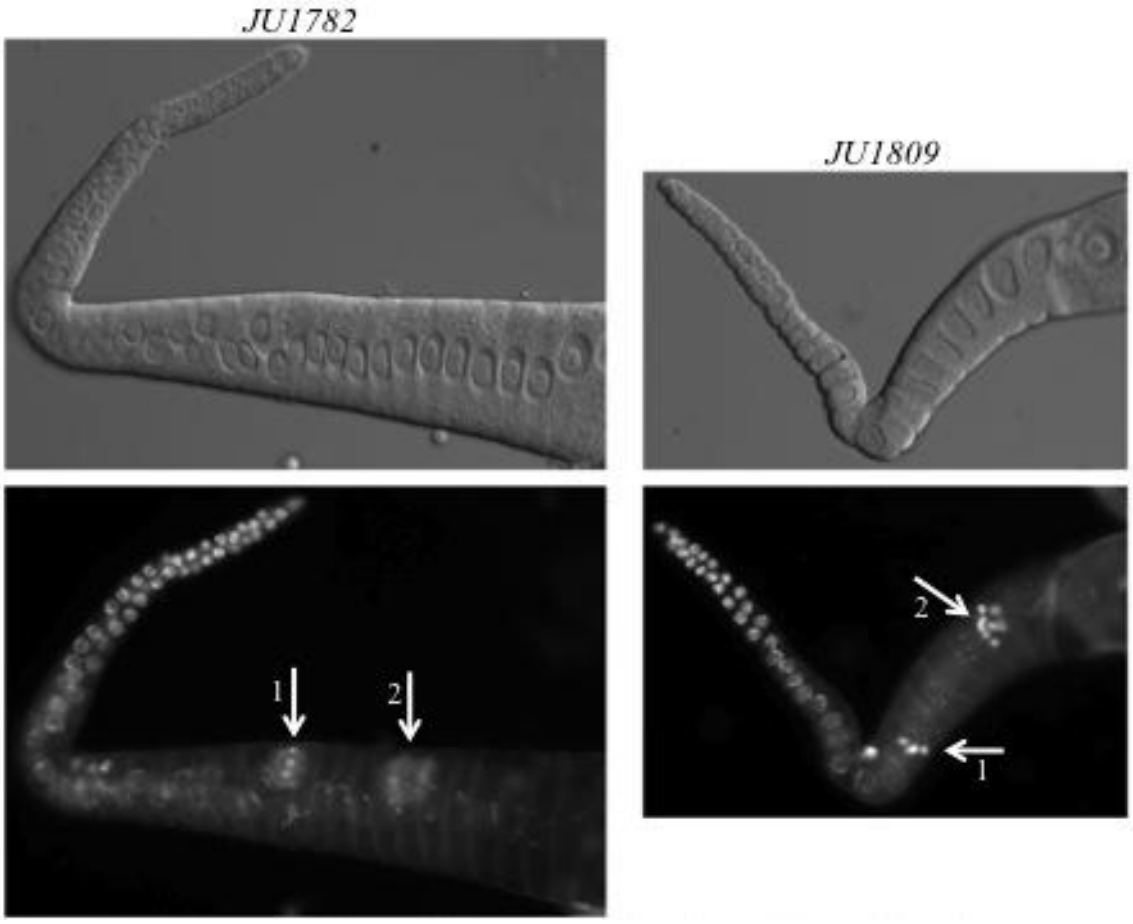


Figure 8B: Above are 400X DIC images of isolated gonads as well as the corresponding image of DNA stained with the fluorescent lipid soluble dye hoechst. Each image is representative of the 8-12 worms observed.

Arrow 1indicates the first set of three unidentified somatic cells. Arrow 2 indicates the second set of 6-8 unidentified somatic cells.

Figure 9: DNA Morphology and Nucleolus Size

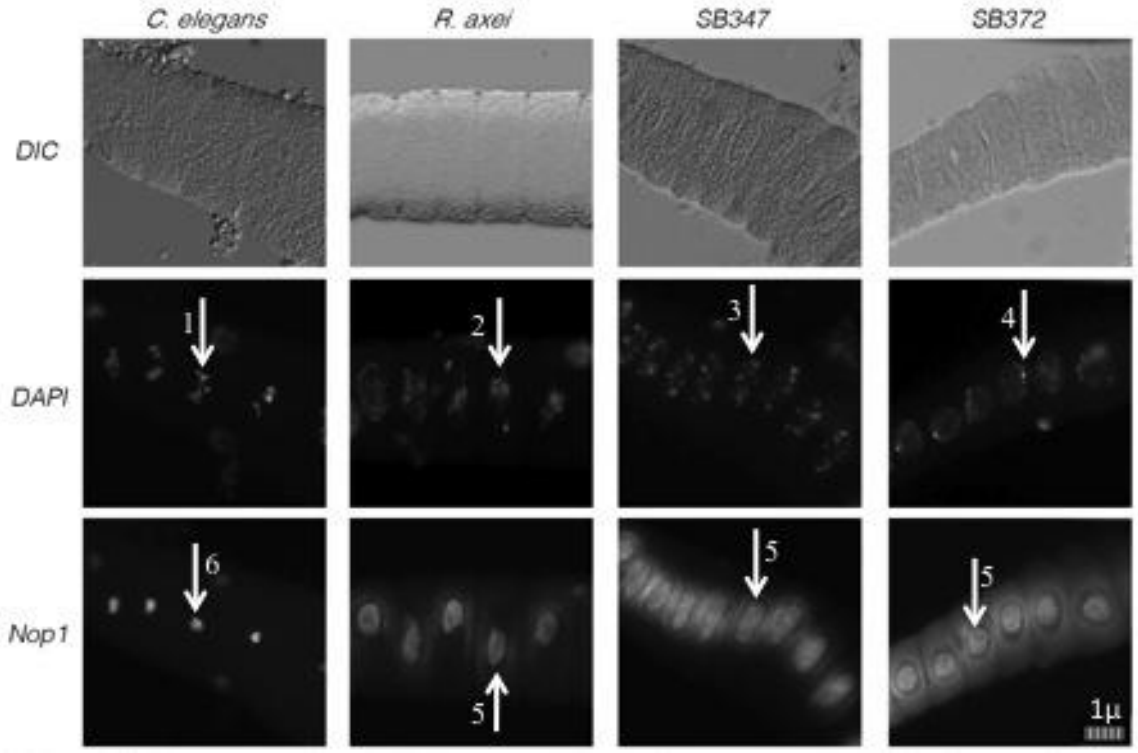


Figure 9: Above are 400X DIC images of maturing oocytes in isolated gonads as well as corresponding images of DNA stained with DAPI and of the nucleolus labeled with a fluorescent nucleolus specific antibody. Each image is representative of the 12-15 gonads observed.

Arrow 1 indicates the DNA of *C. elegans* oocytes; Arrow 2 the DNA of a *R. axei* oocyte; Arrow 3 the DNA of a SB347 oocyte; and Arrow 4 the DNA of a SB372 oocyte. The Arrows numbered 5 indicate the large nucleoli of the non-*C. elegans* oocytes. Arrow 6 indicates the nucleolus of a *C. elegans* oocyte.

Figure 10: Actively Dividing Cells of the Mitotic Zone

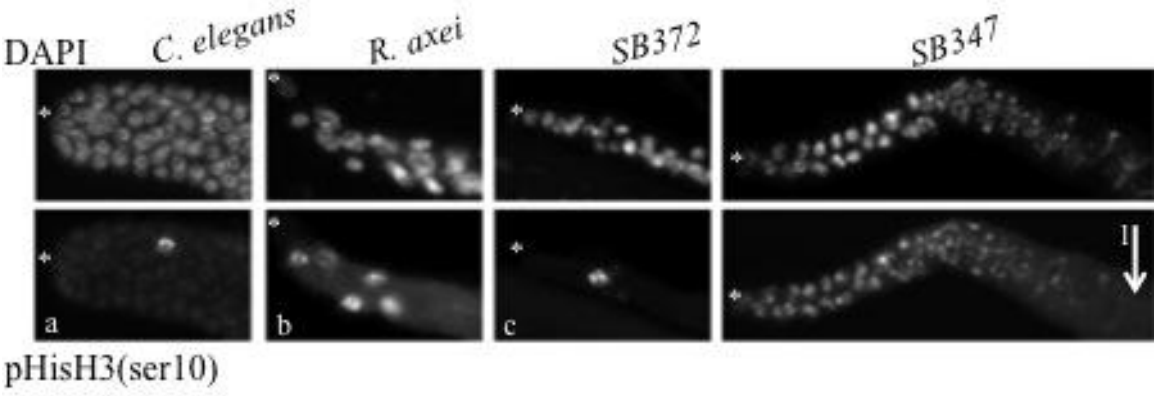


Figure 10: Above are 400 X images of DNA labeled with DAPI and corresponding immunofluorescence labeling of cells with anti-pHisH3(ser10) antibody. Each image is representative of the 8-12 gonads observed.

The most distal cell of each gonad is indicated with (*). Arrow 1 indicates the lack of pHisH3(ser10) labeling in the more mature SB347 oocytes.

Figure 11: Re-initiation of Meiosis in Proximal Oocyte

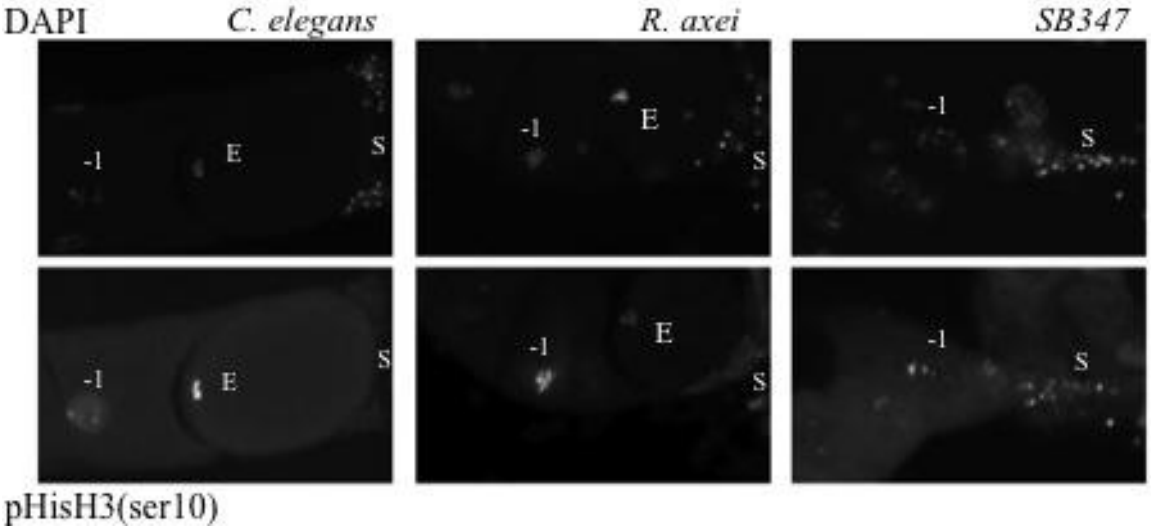
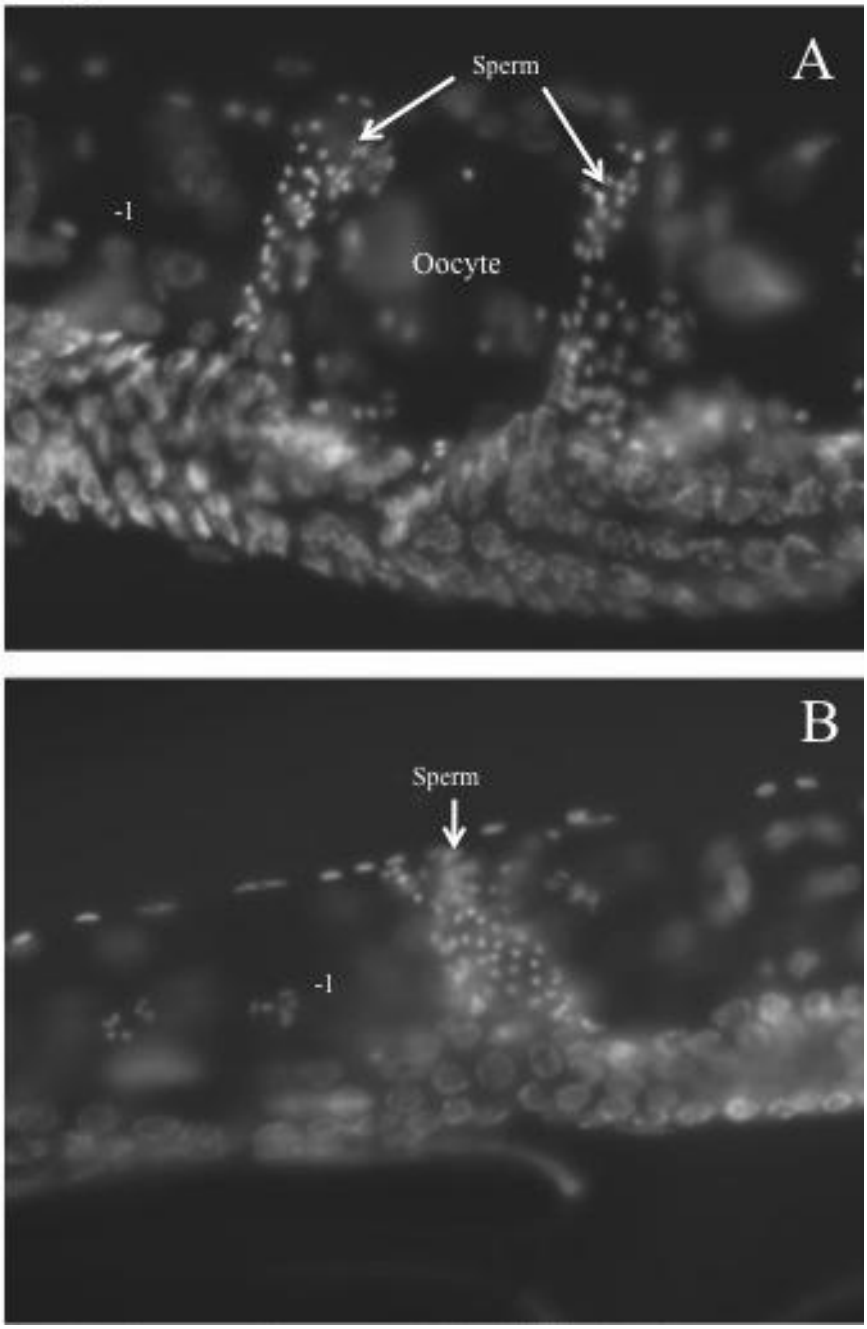


Figure 11: Above are 400X images of DNA labeled with DAPI and corresponding immunofluorescence labeling of cells with anti-pHisH3(ser10) antibody. Each image is representative of the 8-12 gonads observed.

Fertilized embryos are labeled (E). Sperm are labeled (S). The most proximal oocyte is labeled (-1).

Figure 12: *C. elegans* Ovulation



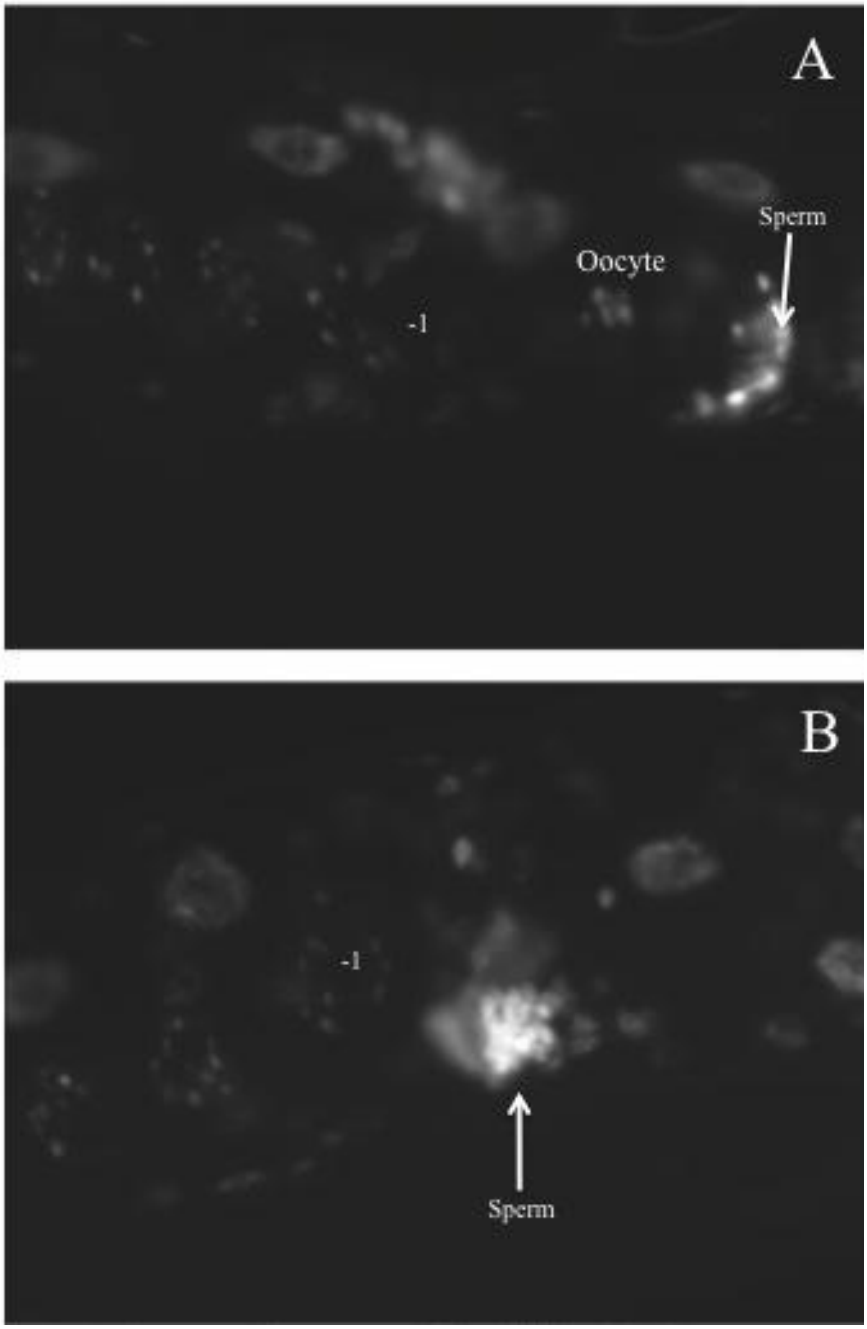
Ovulated Oocyte within the Spermatheca

No Oocyte within the Spermatheca

Figure 12: Above are 400X images of the DNA of all cells labeled with DAPI.

Box A is an image of an ovulated oocyte located within the spermatheca. Box B shows an image of the most proximal oocyte (-1) located to the left of the spermatheca, not within it.

Figure 13: *SB347* Ovulation



Ovulated Oocyte in CONTACT with Sperm

Spermatheca Located Below Proximal Oocyte

Figure 13: Above are 400X images of the DNA of all cells labeled with DAPI.

Box A is an image of an ovulated oocyte in contact with sperm. Box B shows an image of the most proximal oocyte (-1) located to the left and above the spermatheca, not within it.

Discussion

The goal of this work was to more clearly define the reproductive strategies of the hermaphrodites and, in some cases, females of a *Rhabditis* clade in reference to the well-studied reproductive strategy of *C. elegans* hermaphrodites. This study shows that both *C. elegans* hermaphrodites and the hermaphrodites/females of the *Rhabditis* clade invest greatly in reproduction. This is evidenced by the high proportion of total worm volume devoted to the gonad in all species.

One of the major features of any organism's reproductive strategy is the morphology of its gonad. Here we show that, like *C. elegans* hermaphrodites, the feminine worms of the *Rhabditis* clade all have didelphic, two-armed, gonads that contain a row of maturing oocytes in the proximal region. However, the overall shape of the gonad in this clade differs from *C. elegans*. The distal portion of the gonad in the *Rhabditis* clade is shorter, more slender, and contains fewer cells. Moreover, cells within this reduced distal region are arranged in a distinct manner. The proximal portion of the gonad in this clade normally contains many more oocytes than does the comparable portion of the *C. elegans* hermaphrodite gonad.

Within the clade, the gonad of *R. axei* appears to be the most distinct, although not all traits were characterized in all species. In contrast to all currently identified species within this clade and *C. elegans*, the gonad of *R. axei* females has two gonadal bends, whereas the other species have only one. Additionally, the *R. axei* female gonad, despite the fact that it appears to occupy a slightly smaller proportion of the worm volume compared to the other species, is longer than the gonads of the SB/JU hermaphrodites.

The gonads of SB347, SB372, JU1782, and JU1809 are distinguished from both *R. axei* and *C. elegans* by the presence of two sets of unidentified somatic cells, positioned adjacent to two regions of the proximal gonad (JU1783 has yet to be examined for the presence of these somatic cell clusters). Currently, the function of these cells remains mysterious; however, further studies may yield greater insight not only about their specific structure and function, but also about their purpose in regards to the overall reproductive strategies of these species.

Another important element of a reproductive strategy is gametogenesis. This work shows that the germlines of SB372 hermaphrodites as well as *R. axei* females are maintained by a region of mitotically dividing cells in the distal portion of the gonad, comparable to the mitotic zone found in *C. elegans* gonads. Additionally, it was shown that, like in *C. elegans*, the mitotic zones of these species do not include the most distal cells. The number of actively dividing cells in SB372 hermaphrodite and *R. axei* female gonads was found to be comparable to the number of dividing cells in the mitotic zones of *C. elegans* hermaphrodites. However, as the distal region in these species contains many fewer cells, the proportion of cells dividing is notably higher in SB372 and *R. axei.*

Our results also indicate that *C. elegans* and SB372 hermaphrodites produce similar numbers of progeny over the course of their lives. Thus, the output of embryos correlates more precisely with the number of dividing cells in the mitotic region than with either the proportion of dividing cells or the total number of nuclei in the distal gonad.

The duration of M-phase could affect the number of actively dividing cells observed at any given moment. Recent studies in *C. elegans* have been able to accurately determine the length of mitosis (Crittenden et al, 2006; Jaramillo-Lambert et al, 2007). It

is possible that the techniques utilized in these studies could be adapted to determine the length of M-phase in SB372 germcells.

It was shown that a consistent feature of oocyte maturation in *C. elegans*, SB347, and *R. axei* is that meiotically paused oocytes have resumed meiosis by the time they reach the position in the gonad most proximal to the spermatheca. In *C. elegans* hermaphrodites, a signal from the sperm, stored in the spermatheca, cues the oocytes to resume meiosis (McCarter et al, 1997). In order to see if this is the case for the members of the *Rhabditis* clade, additional experiments need to be conducted. One way to determine if sperm cues are necessary would be to deplete hermaphrodites of their sperm, by aging, and use anti-pHisH3(ser10 antibody) to test for the resumption of meiosis in the oocytes before and after insemination. For the trioecious species within this clade, comparisons between hermaphrodites and females would be even more informative, since such studies would avoid the complicating factor of aging.

Despite the similar timing of oocyte maturation, other aspects of maturing oocytes vary greatly between *C. elegans* and members of the *Rhabditis* clade. The chromatin in the proximal oocytes of *Rhabditis* clade species remains incompletely condensed, as opposed to the highly condensed chromosomes seen in *C. elegans* oocytes. Further, the nucleoli in *R. axei,* SB347, and SB372 oocytes are significantly larger and occupy a greater proportion of the nucleus than the nucleoli of *C. elegans* oocytes. This difference may explain the peripheral distribution of chromatin observed in the oocytes of *R. axei*, SB347, and SB372.

A proportionally larger nucleolus in the oocytes of *Rhabditis* clade species may reflect either a higher or different type of maternal investment in these species than in *C.*

elegans. Maternal investment is the amount of a feminine parent's resources that are devoted to its offspring. The types of resources invested vary depending on the organism (Freeman, 2008). Feminine worms in the *Rhabditis* clade may invest in their offspring by developing large nucleoli in their oocytes. The nucleolus is the site of ribosome production. Ribosomes are necessary for protein synthesis, and proteins are required for most, if not all, cellular processes (Freeman, 2008). This increased nucleolus size may allow for greater ribosome production. An increased number of ribosomes would better prepare the oocyte and the eventual embryo for the production of necessary proteins, making the embryo more self sufficient and less dependent on external resources.

In typical *C. elegans* oogenesis a number of cells in the distal portion of the gonad, near to the gonadal loop region, undergo apoptosis (as reviewed in Gartner et al, 2008). It has been hypothesized that the apoptotic cells previously served as nurse cells for the future oocytes (Gumienney et al, 1999). Perhaps, *C. elegans* hermaphrodites invest in their offspring by producing nurse cells, while *R. axei* females and SB347 and SB372 hermaphrodites invest by creating large nucleoli.

In order to examine this hypothesis it would be necessary to first confirm that apoptotic cells are not a normal aspect of oogenesis for the members of the *Rhabditis* clade. This could be accomplished by a number of methods. One of the most effective methods, especially in *C. elegans,* for identifying apoptotic cells has been labeling living cells with acridine orange (as reviewed in Gartner, 2008).

Another important feature of the reproductive strategies of these hermaphrodites is the structure of the spermatheca. In *C. elegans,* the spermatheca is a sperm containing tube into which a mature oocyte moves during ovulation. Additionally, the spermatheca

also serves as the site of fertilization in *C. elegans* (Greenstein, 2005; L'Hernault, 2006). Our studies suggest that the spermatheca structure of SB347 hermaphrodites is more of a sperm-containing pouch that allows a mature oocyte to come into contact with sperm, but never actually contains the oocyte. This type of structure has been observed in other nematodes (Bert et al, 2007). It is likely, that in SB347 hermaphrodites, the spermatheca does not serve as the site of fertilization. A future experiment, that could clarify the morphology of the SB347 spermatheca, would be to analyze the reproductive structures (gonad, spermatheca, uterus) with scanning electron microscopy as was done in Bert et al, 2007. This would reveal the three-dimensional organization of the reproductive structures.

Alternative spermatheca morphology and function could be beneficial due to the relatively small size of SB347 sperm (Shakes et al, 2011). In *C. elegans*, after an oocyte is fertilized in the spermatheca, it is forced into the uterus. When this occurs a number of sperm are forced into the uterus as well. These sperm then use their pseudopodia to crawl back into the spermatheca (L'Hernault, 2006). Previous work with SB347 shows that the sperm of this species is much smaller than *C. elegans* sperm (Shakes et al, 2011). It is possible that the small size of SB347 sperm limits either the motility or energy stores of each sperm. If SB347 spermatheca morphology and function allowed for sperm to be swept into the uterus, the smaller sperm perhaps would not be able to crawl back into the spermatheca as readily as *C. elegans* sperm; either because they do not crawl as effectively as *C. elegans* sperm or because they do not contain the requisite energy stores needed to crawl quickly or repeatedly back into the spermatheca. If sperm were unable to re-enter the spermatheca, a hermaphrodite's store of sperm would be rapidly depleted.

Additionally, other studies have suggested that SB347 males do not mate with the hermaphrodites of their species, and thus, unlike in *C. elegans,* a hermaphrodite's store of sperm cannot be augmented by mating (Chaudhuri et al, 2011). It then follows that the store of sperm in an SB347 hermaphrodite is more precious than in a *C. elegans* hermaphrodite, and thus requires greater protection. A spermatheca structure that prevents sperm from being swept into the uterus by early-embryo movement would offer the necessary protection.

One feature of specifically the SB347 hermaphrodite reproductive strategy, that originally drew much scientific attention, was the sex ratios of their offspring. Earlier studies indicated that selfing SB347 hermaphrodites produced ~9-13% male offspring (Félix, 2004; Chaudhuri et al, 2011). This differs markedly from the almost exclusively hermaphrodite progeny of selfing *C. elegans* hermaphrodites (as reviewed in Lewis and Fleming, 1995). Our studies indicate that SB347 hermaphrodites produce ~9% male progeny. Additionally, it was found that selfing SB372 hermaphrodites produce a similarly high percent of male progeny indicating that this trait may be common to hermaphrodites of the *Rhabditis* clade. More species within the clade, however, need to be examined before this can be stated definitively. It is possible that the presence of this trait in these two species is due to convergent evolution. Convergent evolution is not uncommon in nematodes; for example, hermaphroditism evolved at least three separate times within the *Caenorhabditis* genus (Kiontke et al, 2004).

Research has shown that the sex ratios of offspring as well as reproductive strategies in general are linked to the life-history-conditions under which they developed (Charnov, 1982). Very little is known about the evolutionary conditions that resulted in

these phenotypes, however, based on the observed phenotypes, it is possible to suggest characteristics of their historical environmental conditions (Eo et al, 2010). The presence of a self-fertile hermaphrodite gender in SB347, for example, suggests that the environment in which SB347 evolved favored the ability of isolated individuals to reproduce. This indicates that the environment was patchy. A patchy environment would also explain the reported link between hermaphroditism and dauering in SB347. The development of the protective dauer allows nematodes to survive, when the resources in one location are depleted, until a more plentiful environment can be reached (Cassanda and Russell, 1975).

The presence of hermaphrodites helps to maintain the species in a patchy environment. What then is the value of the other two genders? As explained previously, hermaphroditism in SB347 is coupled to dauering, which lengthens the time it takes for a juvenile SB347 hermaphrodite to reach reproductive maturity under normal conditions by ~24 hours compared to females and males (Chaudhuri et al, 2011). The presence of rapidly developing males and females in this species could allow isolated hermaphrodites to colonize a new environment more quickly and effectively.

The importance of males and females to the colonization of new environments, suggests an explanation for the high percent of male offspring produced by selfing SB347 hermaphrodites. In *C. elegans* both hermaphrodites and males are capable of dauring, and thus both genders are dispersive and capable of surviving when conditions are tough (as reviewed in Hu, 2007). Previous research with SB347 males shows that, unlike *C. elegans* males, SB347 males are incapable of dauering (Chaudhuri et al, 2011). Since SB347 males cannot dauer, they are likely non-dispersive. In order for SB347 to

maintain the valuable male gender, dispersive hermaphrodites must be able to produce a large number of males, which is why a higher percentage of selfing SB347 hermaphrodite offspring is male than would be predicted.

Moving Forward

At the start of this investigation it was known that the members of the *Rhabditis* clade had diverse reproductive strategies both relative to each other and to the well studied "model nematode" *C. elegans*; the exemplary feature being the diverse reproductive modes amongst members of the clade (Félix, 2004; K. Kiontke and D.H. Fitch, personal communication). Earlier studies of the male reproductive strategies revealed even greater diversity within the clade (Shakes et al, 2011). Differences among the reproductive strategies of the hermaphrodites were also implied by early work (Félix, 2004; Chaudhuri et al, 2011). The goal of this work was to better define the reproductive strategies of the hermaphrodites and females of this clade. Our findings show that the reproductive strategies of the hermaphrodites of the *Rhabditis* clade differ from that of *C. elegans* hermaphrodites in a number of important ways, but also have several key similarities.

In the future, this work can be coupled with molecular phylogenetic studies to establish the evolutionary histories of the traits observed. Information from phylogenies may indicate points of divergence that give insight into how diverse reproductive strategies evolve amongst closely related species (Denver et al, 2011). Moreover, the relationship of the members of this clade to known parasites can be clarified with information from molecular phylogenies. It has been hypothesized that the reproductive

mode, of SB347 in particular, is an intermediary step along the evolutionary path to parasitism (Chaudhuri et al, 2011).

A better understanding of parasitic nematodes will, potentially, help society control those species that pose a threat to human health, such as members of the genus Strongyloides (Viney, 2006). Additionally, a greater knowledge of parasitic nematodes may give us insight into species that infect crop-pest insects. Understanding these parasitic nematodes may allow us to utilize them as a means of pest control in order to protect crops (Ehlers, 2001). If a close relationship between SB347 and parasitic species of nematodes is confirmed, SB347 could be developed as a lab sustainable model for the study of parasitic nematodes.

The development of SB347 and other members of this clade as model organisms may yield a second benefit. The value of *C. elegans* to the study of many topics cannot be questioned; its value in nematology research, however, is in doubt. *C. elegans,* while a convenient species to study, is likely not typical of the phylum Nematoda (as reviewed in Wharton, 1986). More in depth comparative studies within this *Rhabditis* clade, may prove an informative parallel to comparative studies within *Caenorhabditis* that will help illuminate features of the phylum Nematoda.

However, many of the laboratory techniques utilized in research today have been optimized for use in *C. elegans* (as reviewed in Blaxter, 2011). The first step toward establishing SB/JU species as model organisms will be adapting and optimizing these techniques, such as RNAi, in the new species (as reviewed in Félix, 2008). Once this is accomplished, however, one or more new model organisms will be available that may

allow the scientific community to ask and answer questions it could not using only the previously available model systems.

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