

NEMATODES WITH THREE GENDERS: MODELS FOR THE EVOLUTION OF
MATING SYSTEMS, PARASITISM AND AGING

by

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To
my parents for all their moral support, love and inspiration
throughout my life.

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Abstract

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SYSTEMS, PARASITISM AND AGING

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All living organisms reproduce and age. These are the two basic facets of life, but in many aspects poorly understood.

The majority of the species are male/female. The proportion of genders in population can vary significantly among animals and plants, but the mechanisms and evolutionary reasons for such variation are poorly understood. In some species of nematode (roundworm) parasites, for example, only female progeny is generated after a cross between a male and female. To understand how gender-ratio distortion happens and how it evolved, we are using a non-parasitic nematode that can be cultured in the laboratory, but that is similar in many aspects to parasitic nematode. For my thesis, I studied the evolution, maintenance and the underlying cellular mechanisms that govern extreme skewed sex ratios in free-living and parasitic nematode species. This knowledge could be used to screen for drugs that further suppress the production of males in the future, helping to eradicate important parasites that cause debilitating diseases worldwide in livestock and humans.

Aging rates can vary dramatically in various species, but it is not understood why and how this happens. I found that our model nematode produce two genders that age at significantly different rates and that respond differently to cellular stress, although they

are genetically identical. Here, I clarified the mechanism behind this gender difference in lifespan. This could help design potential targets for drugs to address age-related diseases to improve our health-span in future.

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Chapter 1

Evolution of Mating Systems

1.1 Introduction

Reproduction is the most fundamental feature that defines life and therefore it is important to examine what kind of selective forces play a significant role in the stability and evolution of mating systems. Natural selection has favored the evolution of diverse modes of reproduction: organisms reproduce sexually or asexually, self-fertilize or outcross, and exist as either separate sexes or hermaphrodites. One of the biggest challenges in evolutionary biology (Futuyma, 2005) is to determine how different modes of reproduction evolved (Charlesworth, 2006).

In the animal kingdom, nematodes exhibit one of the most diverse modes of reproduction. According to phylogenetic studies, male/female (dioecy) is the ancestral mode of reproduction in nematodes (Kiontke & Fitch, 2005). Most nematode species are dioecious but hermaphroditism by self-fertilization evolved at least ten times independently in nematodes (Kiontke & Fitch, 2005). Other modes of reproduction include heterogony (alternation of generation between outcrossing and selfing) and parthenogenesis (egg develops without fertilization) (Kiontke & Fitch, 2005). Thus, nematodes serve as an excellent model for studying evolution of mating systems.

Mating systems can evolve in various different ways (Figure 1-1). In the process of transition from outcrossers to selfers or vice-versa, there are intermediate steps of mixed breeding systems (Figure 1-1). An example of a mixed breeding system is the one present in the model nematode *Caenorhabditis elegans* (*C. elegans*). *C. elegans* is a sexually dimorphic species with hermaphrodites and males (androdioecious). It can reproduce either by self-fertilization or by mating with males.

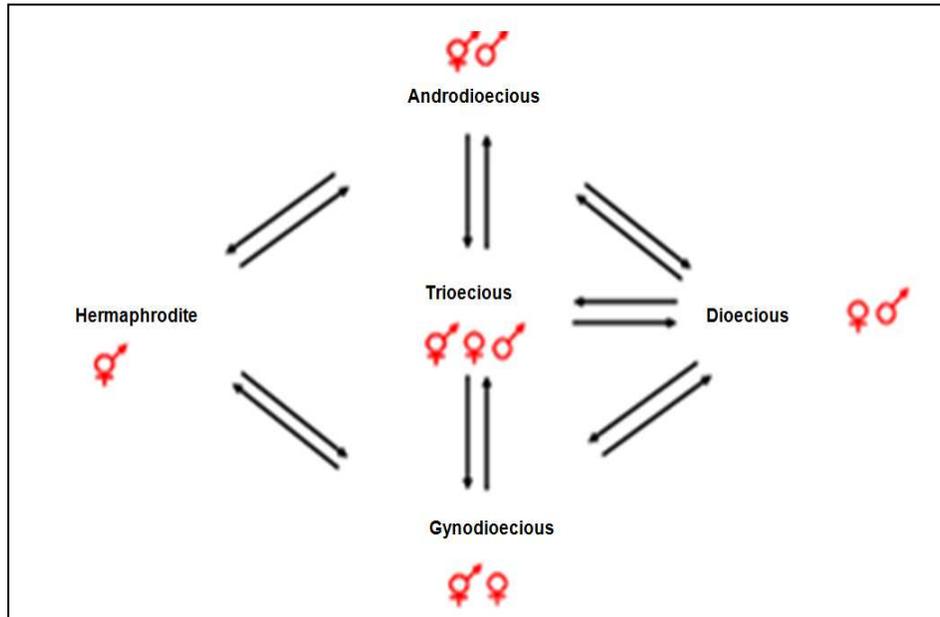


Figure 1-1 Possible mating type transitions between hermaphroditism and dioecy. Androdioecy (hermaphrodites & males) is the most common intermediate stage of transition and trioecy (hermaphrodites, males & females) is the least common. Modified from (Weeks, 2006).

Androdioecy evolved independently from dioecy in a few nematode lineages (Kiontke, 2004). This type of mating system combines the advantages of selfing (e.g. reproductive assurance) with the advantages of outcrossing. However, mixed breeding systems (e.g. androdioecy, trioecy, gynodioecy) are thought to be evolutionarily unstable (Gregorius *et al.* 1983; Weeks *et al.* 2006). Among the mixed breeders trioecy is hypothesized to be the most unstable of all mating systems (Lloyd 1975; Charlesworth & Charlesworth, 1978; Charlesworth, 1984; Pannell, 2002; Wolf & Takebayashi, 2004, Weeks *et al.* 2006, Williams, 2006). Empirical studies show that an engineered trioecious population of the model nematode *C. elegans* becomes androdioecious within few generations (Kephart *et al.* 1999; Stewart, 2002; Cutter, 2005).

In this thesis we will use the free-living and lab-culturable nematode Rhabditidae Gen. 1. sp. 1 strain SB347 (henceforth referred as SB347) to study how new mating systems evolve and how trioecy is maintained.

1.1.1 A Strategy towards Evolution of a New Mating System by Skewing Sex Ratios

This chapter will focus on a comparative study to understand the phylogenetic relationship between SB347 and its close trioecious relatives and variations in the proportion of males. This comparative study of the sex ratios will eventually help determine how species with predominantly selfing hermaphrodites maintain males.

According to the evolutionary theory, the evolutionarily stable strategy (ESS) is to produce equal number of females and males or a sex ratio of 1:1 in a population (Fisher, 1930; Hamilton, 1967; Smith & Price, 1973). There will be an equal investment of resources (Smith & Price, 1973) and parental care for both the sexes to be in equilibrium (Fisher, 1930; Hamilton, 1967). However, there can be different ecological, genetic and environmental factors (e.g. temperature, sex chromosome mediated meiotic drive, pheromones, bacterial infection) that can distort sex ratios from a usual 1:1 male/female ratio (West, 2009; Charnov, 1982; Hamilton, 1967). The consequence of such distortion is a counterbalancing selection to increase frequency of the less common sex (Fisher, 1930; Jaenike, 2001). In presence of mating competition between brothers and close relatives, female-biased sex ratios become more common (West, 2009; Charnov, 1982).

Examples for animals with sex ratio distortions are abundant. In a wide range of host arthropod species, for example, sex ratios can be distorted by the bacteria of the genus *Wolbachia* (Werren, 1995). The bacterium is inherited through cytoplasm and can manipulate host sex ratio by feminizing genetically male hosts or killing them during host embryogenesis (Werren *et al.* 1995; Hurst *et al.* 1999).

In insects with haplo-diploid sex determination, in which an unfertilized egg develops into a male and a fertilized egg develops into a female (Cowan & Stahlhut, 2004), many examples are known for extreme sex ratios (Hamilton, 1967). A female mother can control the sex ratio of the progeny by varying the number of unfertilized eggs.

SB347 can reproduce either by self-fertilization or outcrossing similarly to the well-established model nematode *C. elegans*. *C. elegans* has an XX/XO sex determination system, in which outcrossing generates 50% of each sex (hermaphrodites and males). In the nematode SB347, however, outcrosses generate less than 5% male in the progeny (Félix, 2004; Shakes *et al.* 2011).

In this chapter, we will examine the type of sex determination in SB347, and survey closely related species to determine if there is conservation of rates of production of males. This would reflect the potential dynamics of self-fertilization and outcrossing in these species. We predict that a balance between selfing and outcrossing rates influenced by the proportion of males may be responsible for the stability of the mixed breeders.

1.2 Methods

1.2.1 Strains

In this study we used the following four trioecious strains: Rhabditidae Gen. 1. sp. 1 strain SB347, Rhabditidae Gen. 1. sp. 2 strain SB372 (henceforth SB372), Rhabditidae Gen. 1. sp. 3 strain JU1783 (henceforth JU1783), Rhabditidae Gen. 1. sp. 4 strain JU1809 (henceforth JU1809). The strains were maintained at 20°C and cultured using standard worm culture methods (Stiernagle, 2006). We used the *Escherichia coli*

strain OP50-1 (with ampicillin and streptomycin resistance), instead of OP50 that has only ampicillin resistance.

1.2.2 PCR Amplifications and Sequencing For Phylogenetic Analysis

Total RNA was extracted from mixed stage of worms for individual species following a tissue extraction protocol using Trizol reagent (Invitrogen). RT-PCR was performed by first-strand cDNA synthesis (Invitrogen Superscript III kit). This was followed by PCR amplification of cDNA for partial segments of the following genes: genes for SSU, LSU rRNA and also the largest segment of the RNA polymerase II. Primers used and PCR cycle was followed as described in (Kiontke *et al.* 2004). Please see appendix for the list of primers of the overlapping sequences and PCR conditions used. PCR products were sequenced using ABI Big Dye Terminator version 3.0. in an ABI 3130 DNA Sequencer (Applied Biosystems). Sequences were assembled at NYU and phylogenetic tree was constructed using weighted parsimony analysis.

1.2.3 Obtaining Sex Ratios

Sex ratios of SB347 and its close relative were obtained by picking worms individually into single plates in their fourth larval stage. They were allowed to develop to adulthood and lay progeny. Hermaphrodites were distinguished from female progeny by their ability to lay fertilized eggs in individual wells of twelve or twenty four well plates. To perform crosses hermaphrodites were allowed to lay eggs till they are out of self-sperm (approximately 3-4 days into adulthood). At the conclusion of crosses between hermaphrodites / males and females/males, both females and hermaphrodites were singled in individual plates. Broods for both self- and cross- fertilization experiments were scored till the animals stop laying anymore eggs. Our sex ratios only include data where loss of animals was less than 10% between embryo and adult stages. The sex ratios assays were performed as described in Shakes *et al.* 2011 and Chaudhuri *et al.* 2011.

1.3 Results

1.3.1 SB347 is Closely Related To Other Trioecious Free-living and Parasitic Heterogonic Species

Various trioecious nematodes that included SB347 were sent to us by Marie-Anne Félix (IBENS, France), who has been doing a comprehensive collection of free-living nematodes from the wild. Previous work established that the trioecious SB347 is a close relative of the dioecious *Rhabditella axei* (*R. axei*) (Kiontke & Fitch, 2005). In previous studies, *Heterorhabditis* (Dix *et al.* 1994) and other three species from *Rhabditis* (Maupas, 1900) were reported to be trioecious. The species identified by Maupas were not available to include in our studies. To determine the relationship between SB347 and other recently isolated trioecious nematodes, I sequenced SSU rRNA, LSU rRNA and RNA Polymerase II (RNAP2) genes. The resultant phylogenetic tree was generated in collaboration with the group of Karin Kiontke and David Fitch (New York University) (Figure1-2).

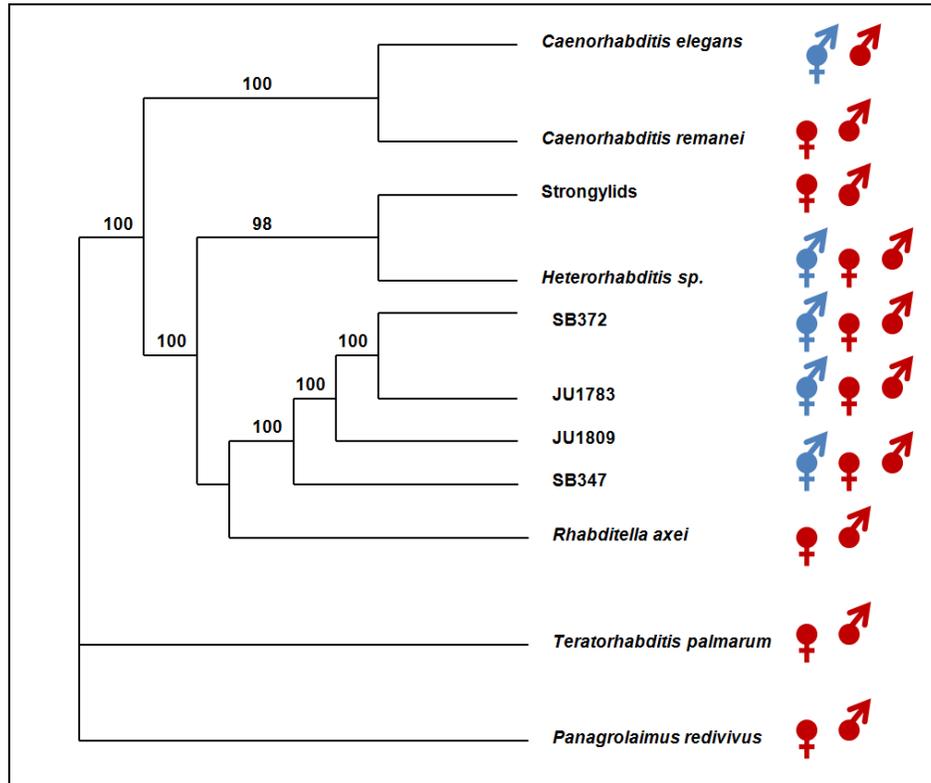


Figure 1-2 Phylogenetic relationship between SB347 and its close relatives. Weighted maximum parsimony analysis (a transversion weighted twice a transition) of 18S, 28S and RNAPII (RNA Polymerase II) complementary DNA (cDNA) sequences for a selection of rhabditid nematodes and the *Panagrelus redivivus* as outgroup representative. Numbers on branches denote support values based on bootstrap analysis with 100 repeats (10 rounds of tree-search for each repeat) using a heuristic search algorithm. Symbols on the right denote genders in each species. (Tree was constructed by Karin Kiontke, NYU, based on sequencing data generated at Genomics Core Facility, UTA).

1.3.2 SB347 and Its Free-living Close Trioecious Relatives Exhibit Female Biased Sex Ratios

Three recently isolated free-living trioecious species closely related to SB347 are Rhabditidae Gen. 1. sp. 2 SB372, Rhabditidae Gen. 1. sp. 3. JU1783, Rhabditidae Gen. 1. sp. 4 JU1809. We performed test crosses in both directions among all the four strains in all combinations to confirm that they are individual species. No cross-species hybrids were observed, indicating that they are different species. To determine the rate of production of males, about five outcrosses and five selfing lines were used for each species. Similar to SB347, ratios in the three other species result in female-biased progeny from both self - and outcross (Fig. 1.3). JU1783 had the highest proportion of males (30%, $n = 154$) after crossing, whereas SB347 had the lowest (3%, $n = 714$). In progeny resulting from self-fertilization, male proportion ranges from nearly 8% ($n = 2110$) in SB372 to nearly 20% ($n = 2181$) in JU1783. The proportion of males never reaches 50% of the total brood under any crossing condition for any of the four species studied. One possibility for skewed sex ratios is the embryonic death of one of the sexes. However, no dying embryos or dead larvae were observed in self-fertilization and outcross experiments in any of the four species ($n > 80$ broods).

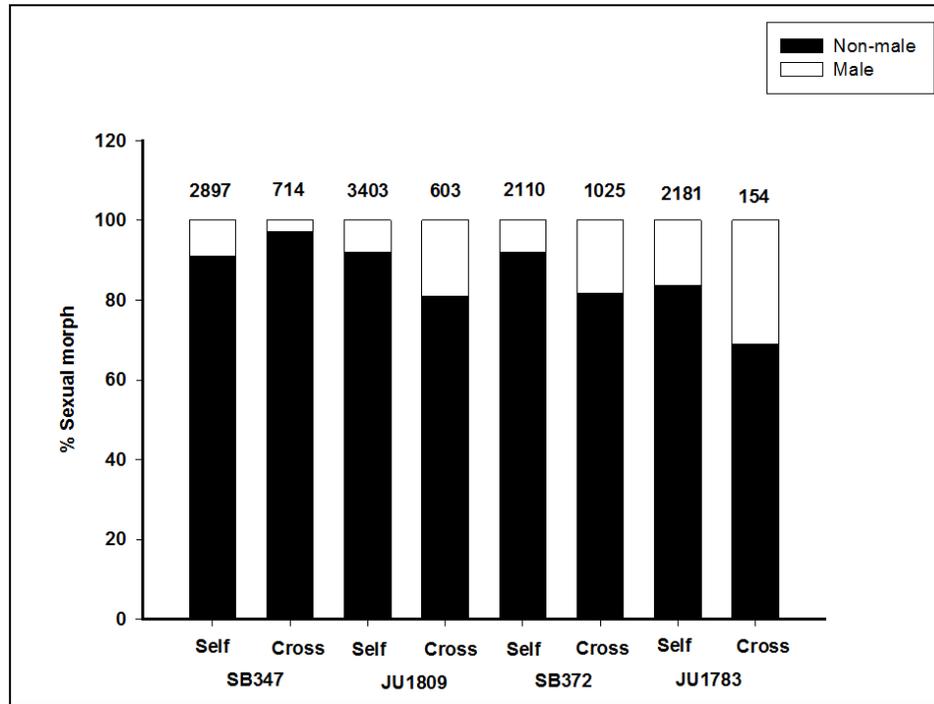


Figure 1-3 Sex ratios of SB347 and its close relatives. Non-male vs. male sex ratio data generated after selfing and outcross in SB347 and its close trioecious relatives. Numbers above the bar denote the total number of animals scored from self-fertilization and outcross experiments for each species.

1.4 Discussion

Given that hermaphrodites reproduce more efficiently than males/females and that they avoid the two-fold cost of sex (Smith, 1978), it is predicted that a trioecious mating system will be unstable. For a species with an XX/XO sex determination system it is likely that females will rapidly disappear from the population and males will be generated from rare non-disjunction event. The maintenance of males may be important for outcrossing and therefore for rapid adaptation (Ohta & Cockerahm, 1974; Glémin, 2003, Morran *et al.* 2009). Although the “right” proportion of males is difficult to predict, it has to be high enough to prevent inbreeding depression and lack of genetic diversity (Charlesworth, 2006). Therefore, a stable survival strategy for an intermediate mixed mating system could be to maintain a balance between selfing and occasional outcrossing.

Previously, the stability studies for mixed breeders were carried out in genetically polymorphic *C. elegans* populations, where male percentages are 0.1% in self-progeny and are produced from rare chromosomal non-disjunction event in hermaphrodites (Hodgkin *et al.* 1979). To compare the trioecious species with *C. elegans*, we first generated a phylogenetic tree of these species. We found that they form a clade of closely related species, where dioecy seems to be the ancestral mating type (Figure 1-2). Males in these species were found to have higher proportions of males than *C. elegans*, although crosses resulted in very few male cross-progeny. Contrary to *C. elegans*, most of the males were generated from self-fertilization (Figure 1-3). We hypothesize that by keeping the male frequency relatively low, the two-fold cost of sex (Smith, 1978) is minimized.

We suggest that SB347, which has an XX/XO sex determination mechanism (Shakes *et al.* 2011) similar to *C. elegans*, may have evolved a higher rate of

chromosomal non-disjunction that results in comparatively higher proportion of males from self-fertilization (Figure 1-3). This compensates for the low proportion of males resulting from outcrossing (Figure 1-3).

We conclude that SB347 and its close free living trioecious relatives can serve as excellent models for studying regulatory mechanisms that can control and distort their optimal sex ratios to evolve into a new mating system.

Chapter 2

Regulation of Sexual Plasticity in a Nematode that Produces Males, Females and Hermaphrodites

2.1 Introduction

In the previous chapter we discussed the proportion of males and the mechanisms that control the masculine fate in this species. In the present chapter, we will characterize the female/hermaphrodite sex determination mechanism in SB347. From a previous study on this species it was reported that the hermaphrodites obligatorily go through a non-feeding stage, the 'dauer' larvae ('enduring' in German) (Félix, 2004). In *C. elegans* and most terrestrial nematodes, larvae typically arrest as dauers under unfavorable growth conditions (Lee, 2002). They resume larval development towards adulthood once conditions are optimal for growth. In SB347, dauer formation is different from other nematodes because it is not facultative, but obligatory for the development of hermaphrodites (Félix, 2004; Chaudhuri *et al.* 2011) (Figure 2-1). SB347 females, however, do not go through dauer under standard optimal culture conditions.

In the dauer stage, free-living nematodes seek for animals (arthropods, mammals) to attach and migrate to a different location for better growth conditions. This behavior known as phoresy has been considered as an intermediate step to how parasites evolved from their free-living ancestors (Anderson, 1984; Poulin, 2007). The genetic pathway controlling the development of dauers in free living nematodes and infective juveniles in parasites is highly conserved. For example, the infective juvenile (IJ) stage of mammalian parasites belonging to genus *Strongyloides* and dauer stage of free-living *C. elegans* and *Pristionchus pacificus* has been reported to be regulated by a conserved endocrine mechanism (Ogawa *et al.* 2009).

The link between dauer formation and hermaphroditism is also found in more distantly related species relative to SB347, such as the entomopathogenic nematode *Heterorhabditis bacteriophora* (Dix *et al.* 1992). Given that *H. bacteriophora* also produces males, female and hermaphrodites, we suggest that SB347 is an excellent model for studying evolution of complex mating system in parasitic nematodes. Our study in this chapter aims to test if dauer formation and sex determination are mechanistically linked in SB347.

2.2 Methods

2.2.1 Identification of Genders and Determining Sex Ratios

SB347 was cultured in standard NGM plates seeded with *Escherichia coli* OP50 strain (Wood, 1988; Stiernagle, 2006). To determine the gender, animals were placed individually on single wells of twelve - or twenty-four-well plates and allowed to develop until adulthood. Individuals that laid eggs in absence of a mating partner were classified as hermaphrodites; otherwise they were classified as females or males. Females were further confirmed by presence of dead oocytes in the media. Male animals could be easily distinguished from hermaphrodites/females by the presence of distinct male copulatory organ or spicule in their tail.

Sex ratios in the F1 generation were determined by placing the selfing parent in an individual well of a twelve-or twenty-four-well plate. The parent was transferred to a new well each day of adulthood. This was to avoid mixing the original parents with its progeny. Larvae hatched from eggs laid by the parent was monitored and counted. There was occasional discrepancy between embryo and adult counts. We are uncertain about the reason behind such discrepancy but it could be due to embryonic lethality or dauer migration. However, brood counts were discarded where discrepancy was more than 10%.

Temporal sequence of gender was determined by allowing a selfing parent to lay eggs for 15 hours in isolation at 20°C in presence of cholesterol and abundant *E. coli* OP50. We assayed ten broods that accounted for a total of 376 non-male animals. In the other set of experiment, eggs laid within first 15 hours by a selfing parent was allowed to develop in NGM plates under dauer inducing conditions in absence of cholesterol kanamycin-killed *E. coli* OP50. Twenty broods totaling 351 non-male F1 offsprings were cultured under these conditions.

2.2.2 Gonad Primordium Measurements

Under normal culture conditions, female and hermaphrodite larvae of SB347 can be distinguished by the mid-L1 stage due to different rates of gonad development (Félix, 2004). To examine this size difference, gonad primordium between females and hermaphrodites was measured using a stereoscope (80X magnification) or Nomarski microscope (1000X magnification). In some cases the gonad sizes overlapped. To avoid any discrepancy, we considered length above 14µm as 'large' and anything below that as 'small'. However, mis-identification of gender resulted in about 10% of the cases due to the overlap in size.

2.2.3 Dafachronic Acid Assays

Δ7- dafachronic acid (dissolved in ethanol; synthesized as described previously [Motola et al. 2006; Giroux & Corey, 2007]) mixed with concentrated *E. coli* OP50 was used to spot each well of a twelve-well plate that holds 2 ml of media. Concentrated *E. coli* OP50 was prepared by re-suspending an overnight culture in 1/5 volume of 0.9% NaCl. Pre-selected mid-L1 animals with small gonads were individually placed in the bacterial spot. For each concentration of dafachronic acid, about 10 replicates were performed.

2.2.4 Statistical Analysis

Statistical analysis of the data was performed using SigmaPlot version 11.0. Statistical significance among proportions was determined using the non-parametric Mann-Whitney test. For more than two groups, a Kruskal-Wallis test was performed followed by the post hoc Mann-Whitney test.

2.3 Results

In SB347, reproduction by selfing or outcrossing results in males, females and hermaphrodites. SB347 self-fertilizing hermaphrodites are protandrous, meaning that they first produce sperm and then oocytes. Hermaphrodites produce about 300 sperm that are stored in the spermatheca, which are later utilized to fertilize their own oocytes. Therefore, they undergo recombination during gametogenesis. Males can be clearly distinguished from non-males by their distinctive copulatory structures. The soma of females and hermaphrodites is identical, but their germline is distinguished by the production of sperm by the hermaphrodites (Figure 2-1 A). In collaboration with another group, we found that the male versus non-male sex determination is mediated by an XX/XO system (Shakes *et al.* 2011). However, the female/hermaphrodite sex determination mechanism was still not clear.

Previous studies showed that hermaphrodites and females differ in post-embryonic development (Félix, 2004). Females (and males) pass through four distinct larval stages marked L1 through L4. Hermaphrodites, however, enter an obligatory non-feeding dauer stage after larval stage L1. They stay as dauer for 24 hours, and then resume normal development to reproductive adulthood (Félix, 2004) (Figure 2-1 B). Dauers can withstand long periods of stressful conditions that include lack of food, elevated temperatures and hazardous chemical exposure (Cassada & Russell, 1975). In

most nematodes, dauer formation is a facultative stage induced when L1s encounter unfavorable growth conditions.

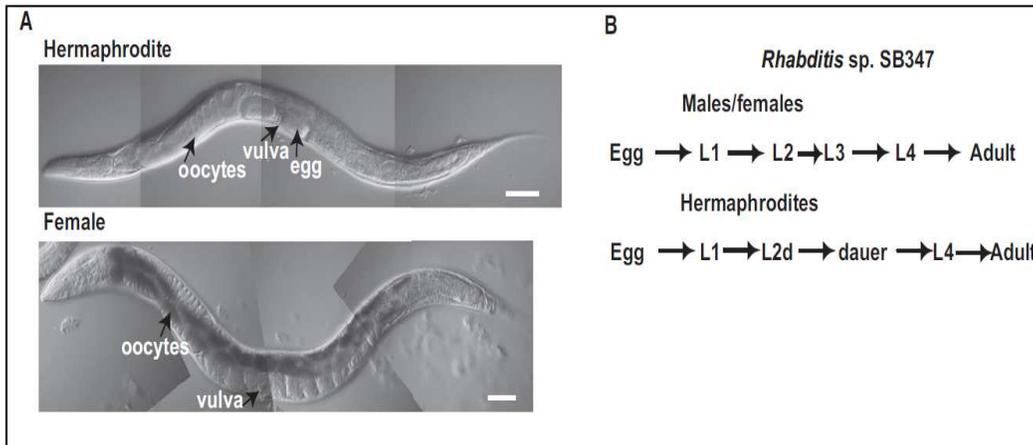


Figure 2-1 Alternative genders in SB347. A) Hermaphrodite germline produces egg by self-fertilization (top panel). Female and hermaphrodite possess morphologically identical soma. Scale bars represent 65µm. B) Males and females do not go through dauer stage.

Hermaphrodites go through an obligatory nonfeeding dauer pathway.

2.3.1 Dauers Obligatorily Develop into Hermaphrodites

Following dauer recovery, *C. elegans* has the ability to develop into either hermaphrodites or males. To confirm if dauers develop into all three genders in SB347, we tracked dauers to adulthood to confirm their gender. To isolate dauers we took advantage that they are resistant to 1% SDS, a detergent. The remaining animals after SDS treatment were confirmed to be dauers by their fast movement and long, slender morphology. After reproductive maturity under normal growth conditions, all the dauers (n = 1,015) developed into hermaphrodites. We also tracked the other set of animals that bypassed dauer pathway under normal growth conditions. These animals that did not pass through dauer formation developed into female adults (n = 69).

2.3.2 Culture Conditions Can Induce Hermaphrodite Development

To test if the passage through dauer changes the germline sex determination, we isolated young larvae that usually become females. These larvae could be identified because SB347 hermaphrodite mothers produce mostly female F1 within the first 15 hours of adulthood (Figure 2-2). To induce dauer formation of females, we removed cholesterol from the media, which is a precursor for some endocrine hormones named dafachronic acids (Matyash *et al.* 2004; Ogawa *et al.* 2009; Wang *et al.* 2009). When nematodes lack these hormones, they arrest as dauers (Gerisch *et al.* 2001; Fielenbach and Antebi, 2008). When SB347 female-fated larvae were induced to go through dauer, most developed into hermaphrodites ($P < 0.001$, post hoc Mann-Whitney U test) (Figure 2-3).

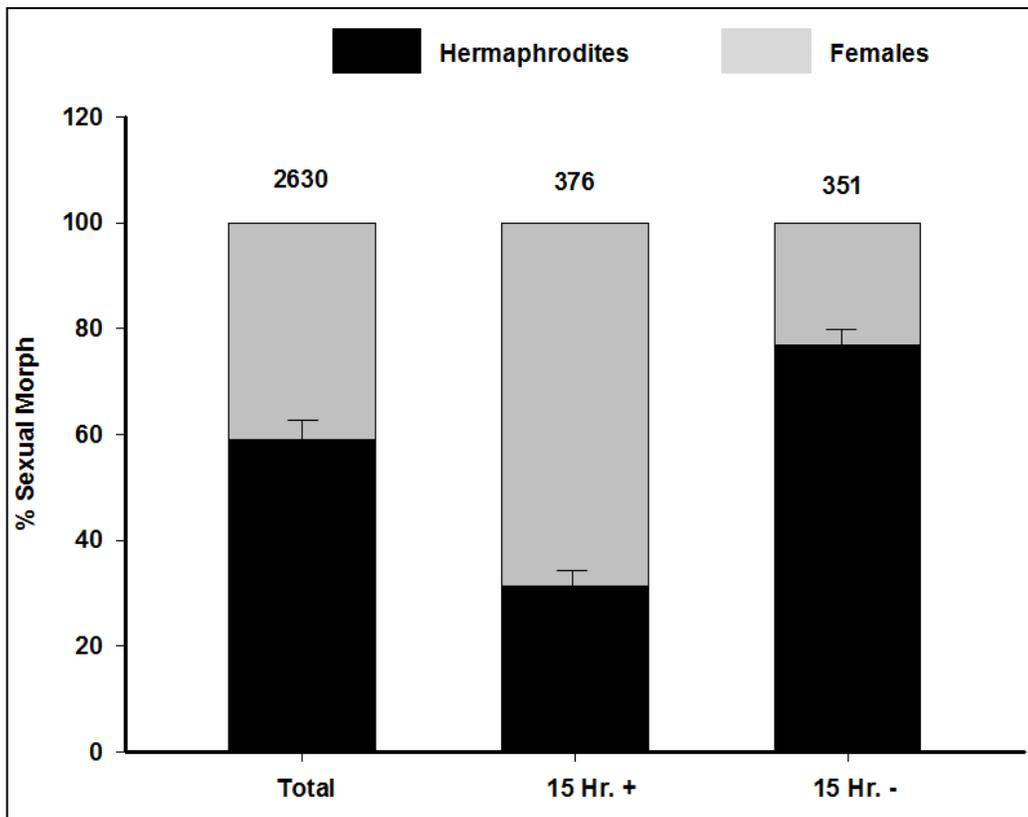


Figure 2-2 Dauer inducing conditions result in hermaphrodite development in SB347. In a total brood from hermaphrodite parents there are more hermaphrodite animals than females (Total). However, larvae hatched from eggs laid in the first 15 hours from a selfing parent develop as females under standard culture conditions in presence of cholesterol (15 Hr. +). In absence of cholesterol and low food, a higher proportion of the female larvae hatched from eggs laid in the first 15 hours from a selfing parent develop as hermaphrodites (15 Hr.-) ($P < 0.001$, post hoc Mann-Whitney U test). Number of animals assayed under each condition is mentioned on top of each column. Data are represented as mean \pm standard error of the mean of ten replicates.

2.3.3 Gonad Primordia Differ in Size Between Female and Hermaphrodite Larvae of

SB347

It was previously noted that the size of the gonad primordium is different between hermaphrodites and females at the L1 stage (Félix, 2004). This could be used as additional criteria to identify females and hermaphrodites at early stages, so that their sex determination could be manipulated. To quantify the difference in size in gonad primordia between females and hermaphrodites, we measured gonad primordia of L1 stage females and hermaphrodites. The size of the gonad primordium of a mid-L1 female larva is nearly twice the length of a mid-L1 hermaphrodite larva (Figure 2-3).

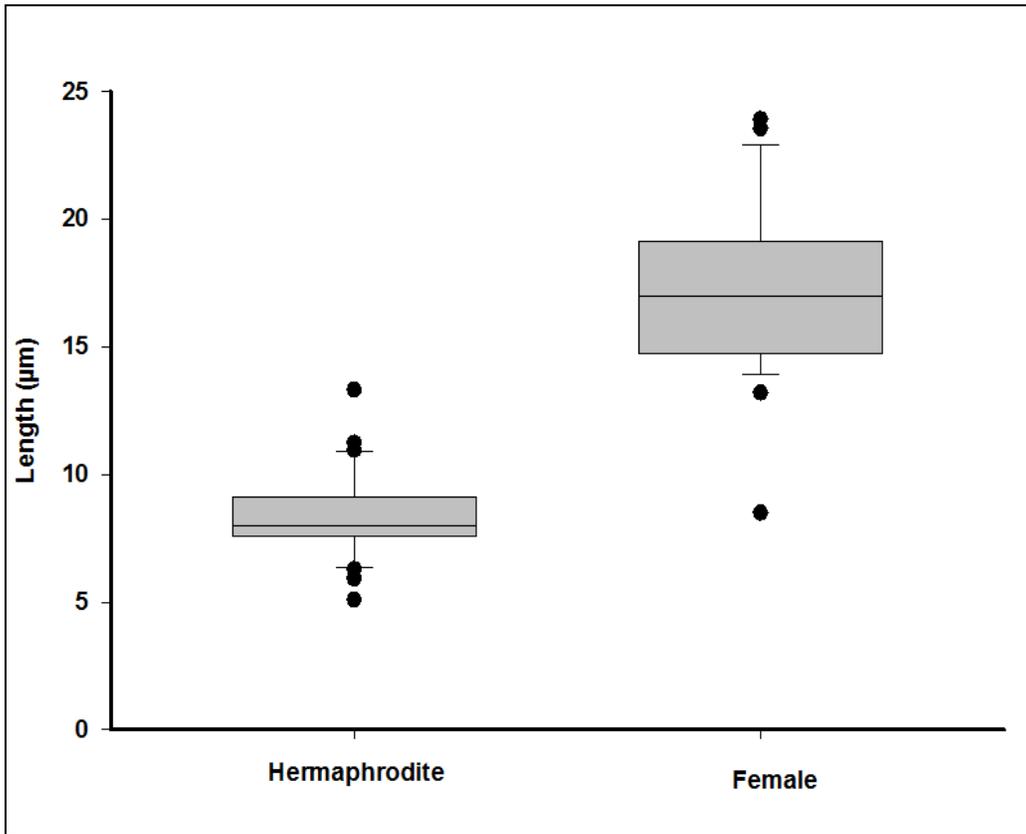


Figure 2-3 Gonad primordium differs between females and hermaphrodites in mid-L1 stage of development. Sizes of the gonad primordium for females and hermaphrodites are shown in the figure. The horizontal line in the box represents the median for the gonad length. Sample sizes for the measurements were 126 and 97 for hermaphrodites and females respectively.

2.3.4 Larvae with Large Gonads Can Be Induced to Become Hermaphrodites by Redirecting Development Through the Dauer Pathway

To confirm if we can redirect development in female-fated animals to become hermaphrodites, we pre-selected larvae based on size of gonad primordium using an 80X magnification of a dissecting microscope (as previously described). Under standard culture conditions, 84% of the large gonad (LG) larvae (n = 291) developed into females (Figure 2-3). To test if we can redirect large gonad female-fated larvae to become dauers, we subjected them to dauer-inducing conditions. In absence of cholesterol and low food, 74% of the LG larvae (n = 61) developed into dauers. LG larvae with the potential to become females eventually developed as reproductively mature hermaphrodites under dauer inducing conditions (Figure 2-3). We observed that sometimes there is a discrepancy in the number of eggs and the adults. We interpreted this discrepancy due to death of an animal or migration of dauers out of the plates. No embryonic death was noticed. However, dauers are a highly dispersive stage of development. Therefore, in our assays, we considered brood that had less than 10% discrepancy between number of eggs and number of adults due to escape of dauers from the culture plates .

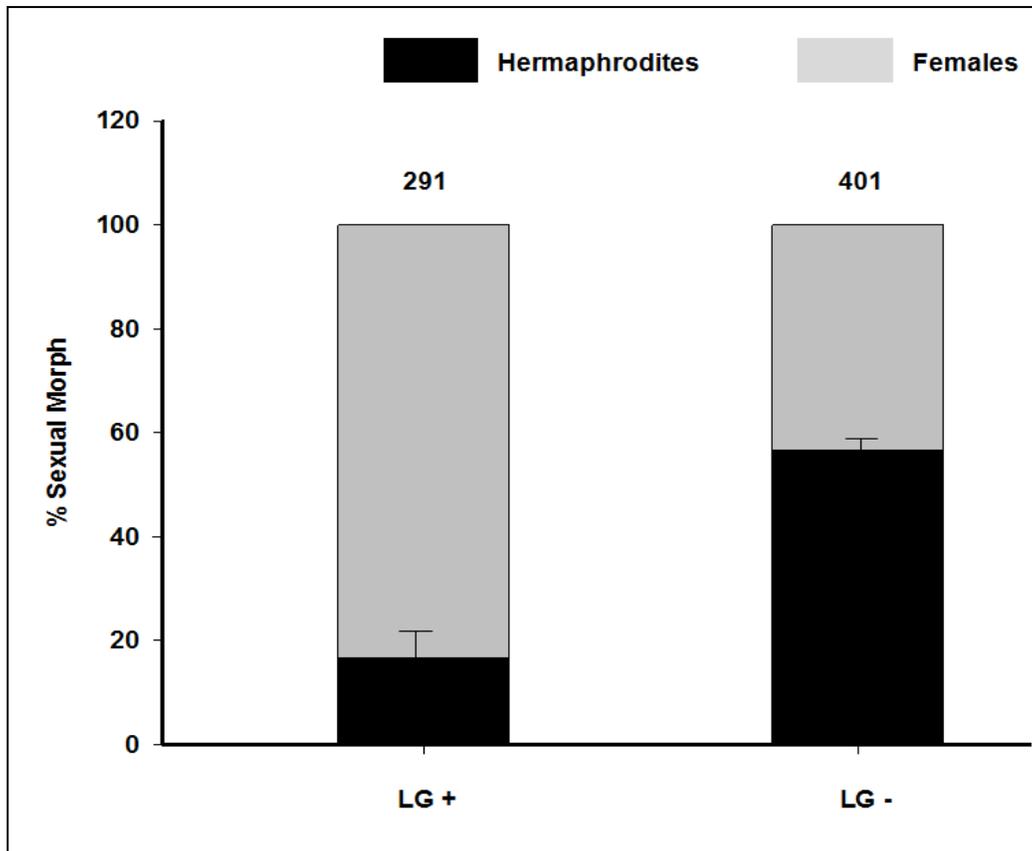


Figure 2-4 Large gonad animals develop as hermaphrodites under dauer inducing conditions. Larvae with large gonads (LG) potentially develop into females under standard culture conditions (LG+). However, under lack of cholesterol and low food, mid-L1 stage LG animals can be redirected to develop into adult hermaphrodites (LG -). The proportion of animals developing as hermaphrodites under dauer inducing conditions is significantly high compared to normal culture conditions ($P < 0.001$, post hoc Mann-Whitney U test). Sample sizes are mentioned on top of each column. Data are represented as mean \pm standard error of the mean of ten replicates.

2.3.5 Larvae with Small Gonads Specified for Hermaphrodite Development Can Be Redirected to Become Females by Blocking the Dauer Pathway

In the reverse study, we wanted to check if we can redirect development of hermaphrodite-fated larvae in adult females. Accordingly, we incubated mid-L1 stage SB347 larvae in $\Delta 7$ - dafachronic acid (DA) to test the necessity of the dauer pathway for hermaphrodite development in this species. Endocrine steroid hormone dafachronic acid (DA) is a ligand for the transcription factor and nuclear hormone receptor DAF-12 and a potent dauer inhibitor. This ligand-receptor interaction is highly conserved in a wide variety of nematode species (Motola *et al.* 2006; Ogawa *et al.* 2009; Wang *et al.* 2009). Incubation of animals with 100 μ M $\Delta 7$ -DA resulted in a high percentage of conversion from hermaphrodite to female (97%, n = 86) compared to the control (25%, n = 146). We observed a dose-dependent response to DA and higher proportions of females were obtained on larval exposure to higher concentration of the hormone (Figure 2-5). We also confirmed that DA is specifically blocking the dauer pathway. The majority of the small gonad L1 animals (86%, n = 120) did not become dauer on incubation with 200 nM $\Delta 7$ -DA. Therefore, DA is redirecting hermaphrodite-specific mid-L1 larvae to become females by blocking the dauer pathway.

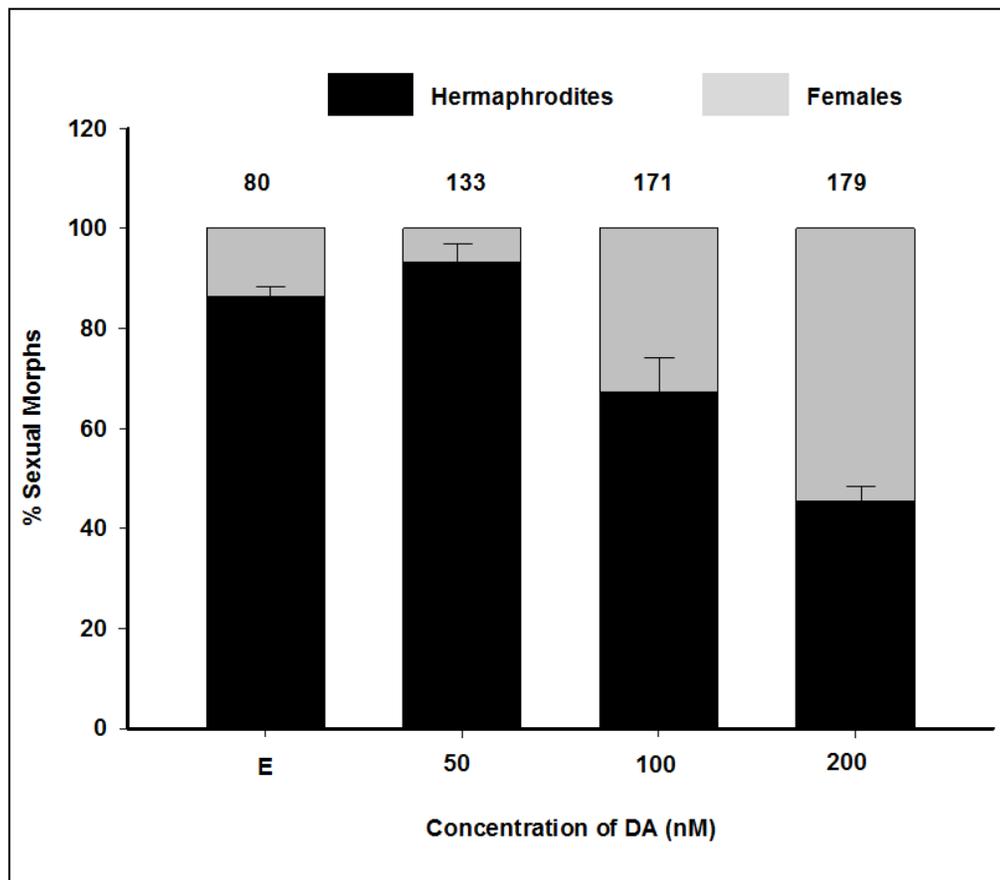


Fig 2-5 Dafachronic acid (DA) redirects hermaphrodite to female development. Mid-L1 stage larvae with small gonads specified to become hermaphrodites can be redirected to become females in a dose-dependent manner. Larvae with small gonads were incubated in Ethanol (E, control) and different concentrations of DA. Data are represented as mean \pm standard error of the mean of seven to ten replicates. Sample sizes are mentioned above each column. Differential mortality or dauer migrations were not considered in the calculations.

2.3.6 Males of SB347 Did Not Form Dauers

Previous studies have shown that SB347 hermaphrodites produce 13% male offspring in F1 generation (Félix, 2004). Our studies confirmed that result. Total broods from self-fertilization resulted in similar percentage (9%, n = 10 total broods) of males in the F1 generation (Shakes *et al.* 2011; this study). Outcrosses resulted in less than 2% male progeny which was accounted for due to the preferential fertilization of X-bearing sperm over nullo-X sperm (Shakes *et al.* 2011).

Dauers harvested from overcrowded, starved plates of SB347 always developed into hermaphrodites (n = 1,015). However, direct observation into the starved plates indicated presence of males. We hypothesized, that males cannot form dauers. We pre-selected L1 larvae from standard culture plates based on the large B cell on their tail that is typical of male larvae. They were incubated on cholesterol-free media. None of the male larvae molted into dauer stage (n = 100).

2.4 Discussion

SB347 exhibits three genders and is an example of a mixed breeding system. Mixed breeders are considered an intermediate evolutionary step between obligate outcrossers (gonochrostatic male/female species) and obligate selfers (self-fertilizing hermaphrodites) (Gregorius *et al.* 1983; Weeks *et al.* 2006). However, theoretical models predict that this kind of intermediate mating system is evolutionary unstable (Gregorius *et al.* 1983). In support of this theory, *C. elegans* mutant populations mimicking trioecious species (consisting of males, females and hermaphrodites) revert to androdioecy (hermaphrodites/males) within a few generations (Stewart & Phillips, 2002; Cutter, 2005). SB347 seems to have a stable trioecious system. However, it is difficult to compare between *C. elegans* and SB347 due to the difference in developmental programming between the two species. Unlike *C. elegans*, gender development in SB347 depends on

the timing of egg laying by the hermaphrodite, the type of cross that produces the offspring and also the availability of natural precursors in the environment for producing endogenous endocrine hormones. The SB347 hermaphrodite, inbred for over ten generations, produces an average of 46% hermaphrodites, 44% females and 10% males (n = 10 total broods, 2,897 scored F1s).

In SB347, sexual fate of female/hermaphrodite is specified in early embryogenesis (Félix, 2004). The gonad primordium of an L1 female larva is larger compared to a hermaphrodite larva (Figure 2-3). Under stressful culture conditions, in absence of cholesterol and low food, large gonad (LG) female larvae can be induced to pass through the dauer pathway and develop as hermaphrodite adult (Figure 2-4). Thus, the dauer pathway is linked to hermaphrodite development in this species. In the reverse experiment, passage through dauer pathway was inhibited in small gonad hermaphrodite-fated larva using a potent dauer inhibitor, $\Delta 7$ -dafachronic acid (DA), these larvae developed into female adults (Figure 2-5). Therefore, in SB347, the dauer pathway is acting as a regulatory switch that is controlling the germline sex determination in females/hermaphrodites. However, the detailed mechanism behind this regulation is still not clear. Regulation of reproductive gene expression by epigenetic mechanisms has been reported to be co-related with the dauer pathway in *C. elegans* (Hall *et al.* 2010). It has also been found that post-dauer hermaphrodites produce more sperm and hence more offspring compared to hermaphrodites that bypass the dauer pathway. The higher reproductive output is due to the epigenetic changes in post-dauer animals (Hall *et al.* 2010). It is possible that in SB347 the link between dauer and hermaphrodite gender results in epigenetic changes that render an XX animal to produce active sperm and self-fertilize. Further studies are required to confirm this hypothesis.

The connection between the dauer pathway and germline sex determination in hermaphrodites of SB347 may be the result of the evolution of an adaptive strategy. Dauers are morphologically, physiologically and behaviorally adapted for dispersal (Cassada & Russell, 1975). In a complex ecological environment under stressful conditions and in absence of a mating partner, coupling the dauer pathway with hermaphrodite development may be ideal for rapid colonization of a new territory by self-fertilization. However, more ecological data related to each gender may be required to confirm if this coupling has evolved to enable hermaphrodites to explore new food sources.

SB347 shares many distinct features with parasitic nematodes that follow complex life-cycles and mating systems. Very similar to SB347, the hermaphrodites (but not females) of the genus *Rhabdias* obligatorily pass through a diapause stage. In addition to this, the majority of the *Rhabdias* males are produced from selfing rather than outcrossing (Runey *et al.* 1978). It is possible that nematode parasites that alternate between hermaphroditic and gonochoristic generations evolved from their sexually polymorphic free-living ancestors (Spieler & Schierenberg, 1995). This makes SB347 an excellent model for understanding evolution of complex mating systems in parasites.

Chapter 3

Killing of Spermatids During *Heterorhabditis bacteriophora* Spermatogenesis

3.1 Introduction

The evolutionary stable strategy (ESS) followed by most organisms in a population is to produce a sex ratio of 1:1 or to have equal number of males and females (Edwards, 1884; Fisher, 1930; Hamilton, 1967; Smith & Price, 1973). According to Fisher's principle, selection would favor parental expenditure of resources for both sexes in equal amounts. An unequal resource allocation for male or female is predicted to be subject of counter balancing selection until the expenditure becomes equal for both the sexes (Fisher, 1930, Hamilton, 1967). Also, according to classical genetics, Mendel's first law predicts an equal proportion of males and females from a cross between an XX female and an XY (or XO) male. However, as discussed earlier, extraordinary sex ratios are not uncommon in nature (Hamilton, 1967).

Metazoans are well known for exhibiting distorted sex ratios by undergoing unequal sex chromosome segregation (Pires-daSilva, 2012; Denver *et al.* 2011). Particularly, in nematodes, these kind of skewed sex ratios are predicted to be due to local competition among males for mating partners (Hamilton, 1967; LaMunyon & Ward, 1999). Furthermore, by producing more female/hermaphrodite progeny they can colonize the territory much faster by reducing the cost of sex (Smith, 1978) in the presence of their competitors.

Nematodes have evolved different mechanisms to skew sex ratios. In the free living nematode *Caenorhabditis briggsae* the initial progeny comprises of feminine sex because of the fertilization advantage of the larger size of the X-bearing sperm compared to the nullo-X sperm (LaMunyon & Ward, 1999). SB347 which produces only 5% of male progeny from an outcross uses a different mechanism. In this species, only

the X-bearing sperm is capable to fertilize oocytes because the nullo-X sperm is non-functional (Shakes *et al.* 2011). Interestingly, the close parasitic relatives of SB347 also exhibit sex ratio distortion by producing more females from an outcross. For example, both the insect parasite *Heterorhabditis bacteriophora* (*H. bacteriophora*) (Dix *et al.* 1994) and the rat parasite *Strongyloides ratti* (Harvey & Viney, 2001) exhibit female biased sex ratios resulting from an outcross. However, the cellular basis of such distortion is still unknown in *H. bacteriophora*, the parasitic relative of SB347. *H. bacteriophora* comprises of three genders: males, females and hermaphrodites. The infective juveniles develop into hermaphrodites when inside the host. Unlike most free living nematodes, SB347 dauers also obligatorily develop as hermaphrodites (Figure 3-1). The infective stage in a parasite is supposed to have evolved from free living dauer larvae (Anderson, 1984) and more importantly vertebrate parasites are suggested to have evolved from parasites of arthropods (Anderson, 1992, Blaxter *et al.* 1998). Furthermore, similar to SB347 (Félix, 2004; Shakes *et al.* 2011), the non-male percentage in insect parasite *Heterorhabditis bacteriophora* is less than 5% under any mating condition (Dix *et al.* 1994).

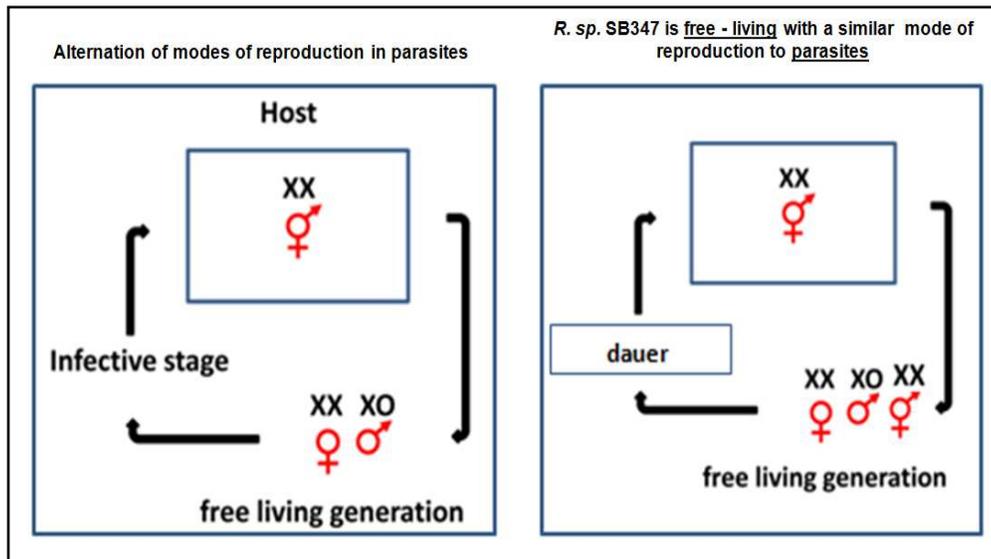


Figure 3-1 Similarity in life-history characteristics between SB347 and the parasite *H. bacteriophora*. SB347 hermaphrodites obligatorily pass through the dauer larval stage and produces males, females and hermaphrodites in the next generation. In the insect parasite *H. bacteriophora*, passage through infective juvenile stage is obligatory for XX animals that eventually develop as hermaphrodites inside the host. Hermaphrodites lay eggs that develop as males and females under free living condition in the next generation. Outcross between males and females results in infective juveniles that are highly dispersive and infect a new host.

Sex ratios can be distorted due to many factors that include temperature (Kamel & Mrosovsky, 2006) or even post-zygotic embryonic lethality (Haldane, 1922; Orr, 1997). However, in this chapter we will focus only on understanding events during spermatogenesis in males that has the ability to skew sex ratios resulting from an outcross in *H. bacteriophora*. From our previous study in SB347, we already know that in an XX/XO sex determination system sex ratios from an outcross can be distorted due to

the production of non-functional and non-motile male-producing spermatid that is unable to fertilize (Shakes *et al.* 2011). The phenomenon of skewed sex ratios is well conserved in nematodes but not well understood. In contrast to many studies on this topic, our approach will focus neither on expanding current theories of sex allocation (West, 2009) nor on identifying new transcriptional and translational regulator of sex determination (Hill *et al.* 2006; Guo *et al.* 2009). Instead, we will study potentially conserved, cellular level modifications in the production of functional gametes that control skewed sex ratios in the heterogonic parasitic nematode *H. bacteriophora*. We hypothesize, that the parasite *H. bacteriophora* has an XX/XO sex determination system and sex ratios are skewed due to a conserved mechanism of production of non-functional spermatid during male spermatogenesis.

We also hypothesize SB347 and *H. bacteriophora* may have evolved this similar mechanism of distorting sex ratios to produce more feminine sex among its progeny because this may be an adaptive strategy for fast colonization in the absence of conspecific males.

3.2 Methods

3.2.1 Immunohistochemistry and Microscopic Studies

Studies on *H. bacteriophora* spermatogenesis were carried out using dissected male gonads. Gonads were dissected in 7 μ l sperm buffer on ColorFrost Plus slides (Fisher Scientific). Slides were extra charged using a coating of Poly-L-Lysine (Sigma). Dissected tissues were covered with a cover slip that had dots of silicone grease at all the four corners. A monolayer of spermatocytes and spermatids was obtained by application of gentle pressure diagonally on the cover slip. Tissues were subjected to quick freeze cracking in liquid nitrogen (-80°C) followed by fixation in 100% methanol overnight in -20°C. After subsequent washes in PBS, tissue was blocked with PBS plus

0.5% BSA. This was followed by incubations with required primary and secondary antibodies. Both washing and antibody incubations were carried out at room temperatures. The following primary antibodies were used at their specific dilutions as mentioned: mouse anti-MSP 4D5 at 1:800, FITC-conjugated anti- α -tubulin monoclonal DM14 (Sigma) at 1:100, rabbit polyclonal cep-1 IgG at 1:400 and rabbit polyclonal ced-3 IgG at 1:300 dilutions. The following secondary antibodies were used as per requirement at the specified dilutions: goat anti-mouse IgG at 1:100 or 1:50 (Alexa Fluor 488, Life Technologies, Invitrogen), goat anti-rabbit IgG (Alexa Fluor 488, Life Technologies, Invitrogen) at 1:100 or 1:50 dilutions and goat anti-mouse IgG (Alexa Fluor 568). Double antibody labeling was performed by incubating tissue in desired combination of diluted primary antibodies mixed at 1:1. This was followed by incubation in a mixture of respective secondary antibodies at 1:1. Slides were mounted in mounting media with DAPI (Vectashield). DAPI/DIC and immuno-stained images of methanol fixed sperm spreads were viewed using Zeiss 510 Meta confocal/fluorescent microscope equipped with a AmScope Sony 5.0 MP cooled CCD color microscope camera. DIC images for live cells were viewed in Leica DM LB2 microscope equipped with Nomarski optics and coupled with an Olympus CM2 camera. Images were analyzed and processed using ImageJ software. The representative image for each of the figures was based on observation of sperm spreads from > 50 individual animals. Only uniform or conserved cytology within a single male gonad and across animals were considered for the representative image. Protocol was based as described in Shakes *et al.* 2011.

3.3 Results

3.3.1 During Male Spermatogenesis *H. bacteriophora* Secondary Spermatocytes Undergo Asymmetric division Similar to SB347

SB347 uses XX/XO sex determination system to determine male versus non-male fate (Shakes *et al.* 2011). Outcrosses result in very few males because of modifications in spermatogenesis of the male. In SB347 males, there is asymmetric partitioning of essential cellular components to produce non-functional nullo-X spermatids in meiosis II (Shakes *et al.* 2011) (Figure 3-2A). Similar to free-living SB347, insect parasite *H. bacteriophora* also exhibits distorted sex ratios from an outcross (Dix *et al.* 1994). We hypothesized that a similar underlying mechanism during male spermatogenesis skews sex ratios between SB347 and *H. bacteriophora*, given their relative position in the phylogenetic tree (Figure 1-2).

In *C. elegans*, during meiosis II, the spermatids bud from a secondary spermatocyte empty all their non-essential cytoplasmic components in an anucleate central residual body (Ward & Klass, 1982; Ward, 1986; L'Hernault, 2006) (Figure 3-2B). No residual body formation similar to *C. elegans* spermatogenesis was observed in either SB347 or *H. bacteriophora* (Figure 3-2C).

In *H. bacteriophora*, secondary spermatocytes divide both symmetrically and asymmetrically. The percentage of secondary spermatocytes undergoing asymmetric division in *H. bacteriophora* was 90% (n = 50, n = number of secondary spermatocytes). Similar to SB347, asymmetric division results in morphologically two different types of spermatids (Figure 3-2C). The stage of meiosis in *H. bacteriophora* was decided based on the relative sizes of the spermatocytes (Figure3-2C).

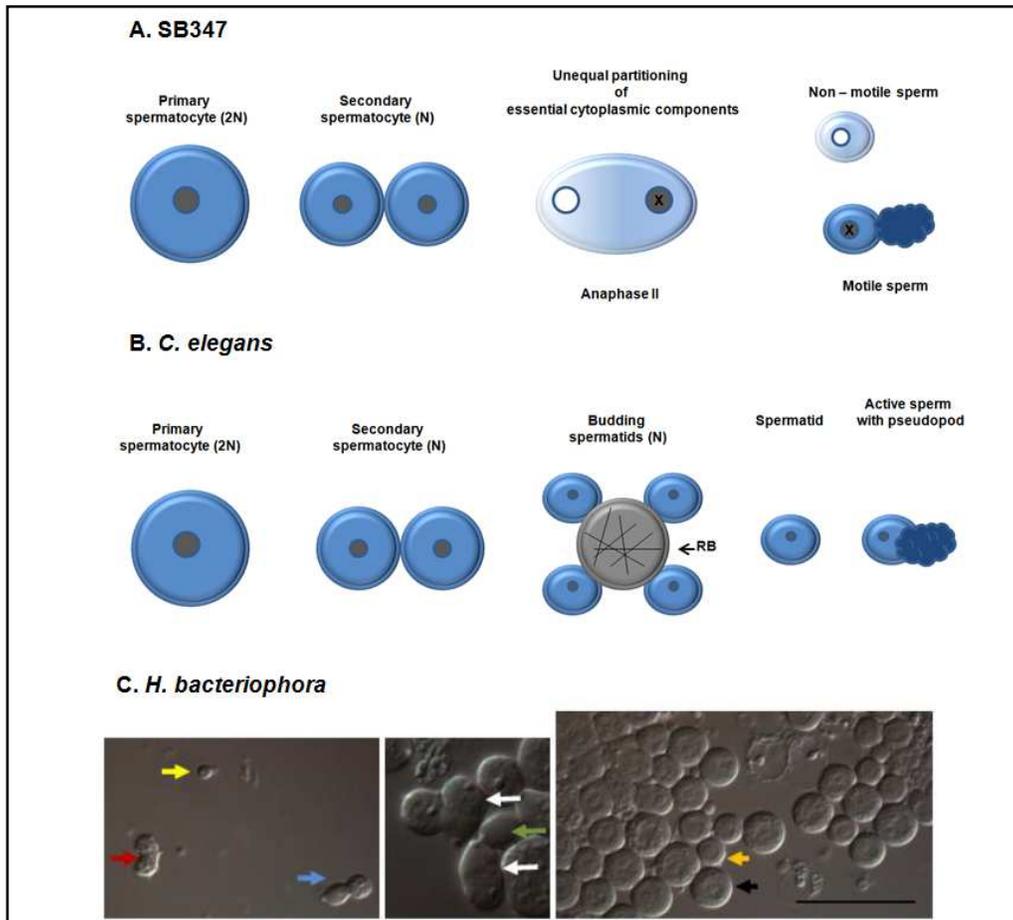


Figure 3-2 Comparison of male spermatogenesis between SB347, *C. elegans* and *H. bacteriophora*. A. Asymmetric partitioning of cytoplasmic components in anaphase II resulting in functional and non-functional sperm in SB347 (Shakes *et al.* 2011). B. Spermatogenesis in *C. elegans* results in the formation of a central residual body (RB) in meiosis II. When spermatids bud from the secondary spermatocytes they discard non-essential cytoplasmic components (e.g. tubulin, actin etcetera) in the anucleate residual body. N denotes the ploidy of the cell. C. Symmetric (red) and asymmetric (blue) partitioning of cytoplasmic components in *H. bacteriophora*. Unlike asymmetric division, symmetric division in anaphase II distributes cytoplasmic components equally to both the

spermatids. Motile sperm is shown in yellow arrow. Second panel: asymmetrically dividing secondary spermatocytes results in two morphologically different spermatids. A 'smooth' spermatid (green arrow) and 'rough' spermatids resulting from secondary spermatocytes (white arrow). Third panel: relative sizes of primary (black) and secondary (orange) spermatocytes. Figures in first and third panels are in scale; scale bar is 30 μm .

3.3.2 Lagging-X Was Not Observed during Meiosis in H. bacteriophora

A diploid cell in *H. bacteriophora* consists of 7 pairs of chromosomes (Figure 3-3A). Progression of spermatocytes through meiosis I and meiosis II did not reveal any lagging-X. However, in anaphase II secondary spermatocyte partitions cytoplasmic components both symmetrically and asymmetrically (Figure 3-3B). Asymmetric division gives rise to two spermatids with different physical appearance and surface features. Symmetric division gives rise to identical spermatids.

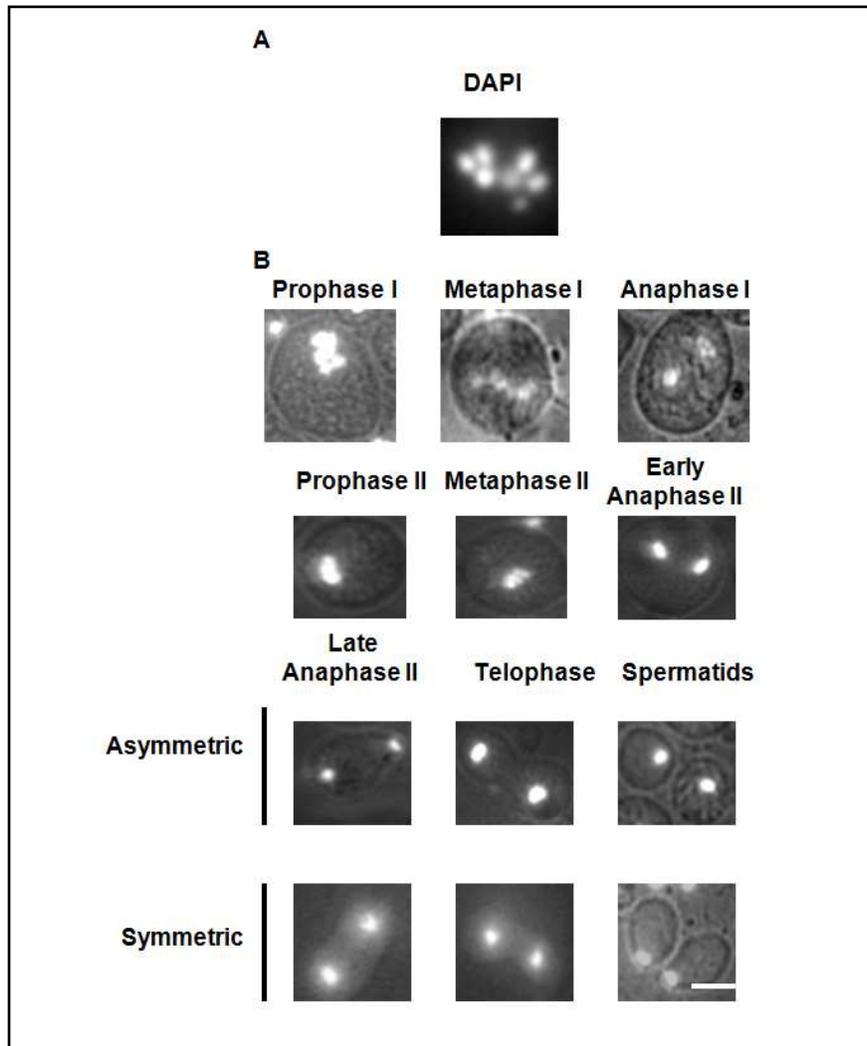


Figure 3-3 Different stages of meiosis in spermatogenesis of *H. bacteriophora*. A. A diploid cell has 7 pairs of chromosomes. B. Primary spermatocyte progresses through meiosis giving rise to four daughter cells at the end of meiosis II. Both asymmetric and symmetric divisions were observed in anaphase II. Asymmetric division gives rise to spermatids of different physical appearance and surface features. Spermatids look alike after a symmetric division. Scale bar is 5 μm . All figures in panel B are in the same scale.

3.3.3 In *H. bacteriophora* Major Sperm Protein (MSP) Segregates Asymmetrically in Meiosis II in Majority of the Secondary Spermatocytes

Major sperm protein (MSP) is an essential cytoplasmic component required for sperm motility. It is initially dispersed throughout the cytoplasm in a developing spermatid and is eventually restricted to the pseudopod in an active motile sperm (Ward & Klass, 1982; L'Hernault, 2006). In the absence of MSP the spermatid fails to produce pseudopod and is unable to fertilize. In *H. bacteriophora*, 'pear' shaped asymmetrically dividing secondary spermatocytes that partition MSP unequally are observed at high percentage (90%, n = 50, n = number of secondary spermatocytes). These asymmetrically dividing spermatocytes accumulate essential cytoplasmic component MSP only on one side (Figure 3-4A). This event is similar to the spermatogenesis in free living species SB347 (Shakes *et al.* 2011) (Figure 3-4B). In *C. elegans* MSP partitions equally and both the X-bearing and nullo-X spermatids develop into functionally active sperm (Figure 3-4B).

In *H. bacteriophora* during anaphase II, two kinds of division occur in secondary spermatocytes. Symmetric division results in the partitioning of the essential cytoplasmic component MSP equally to the resulting spermatids. However, asymmetric division results in spermatids, only half of which has the ability to develop into active sperm due to the presence of MSP (Figure 3-4C). In summary, unlike *C. elegans* spermatogenesis (Ward & Klass, 1982; Ward, 1986; L'Hernault, 2006), and similar to SB347 (Shakes *et al.* 2011), spermatogenesis in *H. bacteriophora* is associated with asymmetric partitioning of the essential cytoplasmic component MSP required to produce active sperm.

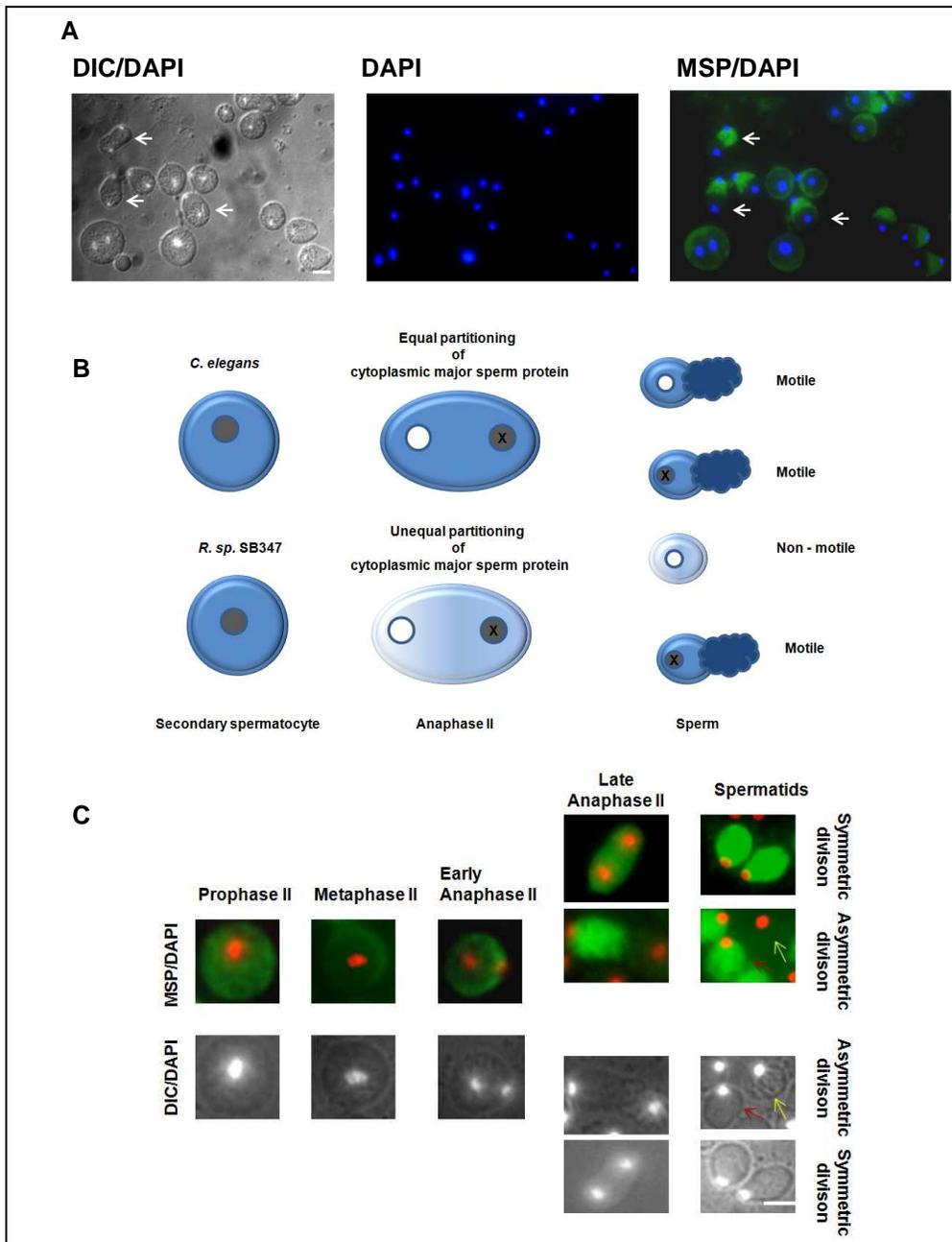


Figure 3-4 Symmetric and asymmetric partitioning of major sperm protein in meiosis II during spermatogenesis in *H. bacteriophora*. A. Majority of the secondary spermatocytes undergoes asymmetric division partitioning unequally an essential cytoplasmic

component MSP (Major Sperm Protein) required for sperm motility (white arrow). Scale bar is 5 μm . B. In *C. elegans* MSP segregates equally producing all motile sperm. Unlike *C. elegans*, during anaphase II, MSP segregates unequally in SB347. The X- bearing spermatid (marked by the letter X) gets all the MSP producing motile sperm. However, the male producing nullo-X spermatid without MSP is non-motile and cannot fertilize. C. Secondary spermatocytes in *H. bacteriophora* undergo both symmetric and asymmetric division in anaphase II. Symmetric division shows equal partitioning of major sperm protein (MSP) resulting in two functional spermatids with MSP. Asymmetric division results in one functional spermatid with MSP (red arrow) and one non-functional spermatid without MSP (yellow arrow). Scale bar is 5 μm .

3.3.4 Abnormal Random Budding Events and Anucleated Cells Were Identified During *H. bacteriophora* Spermatogenesis

In *C. elegans*, two haploid spermatids (both having MSP) are produced as a result of a budding event from a diploid secondary spermatocyte as described earlier in Figure 3-2C. Anucleate residual body is formed simultaneously as the secondary spermatocyte divides. Non-essential cytoplasmic components (e.g. tubulin, actin) are emptied in the residual bodies by the budding spermatids during *C. elegans* spermatogenesis (Ward & Klass, 1982; Ward, 1986; L'Hernault, 2006). Random budding events were identified at a low percentage (4%, n= 50, n= number of secondary spermatocytes) in *H. bacteriophora* (Figure 3-5A). Similar to SB347, and unlike *C. elegans*, residual body formation was not observed during anaphase II. However, anucleate cells with similar features to a *C. elegans* residual body were present (Figure 3-5B). These random budding events and occasional residual bodies were too inconsistent to be considered as part of a main spermatogenesis event.

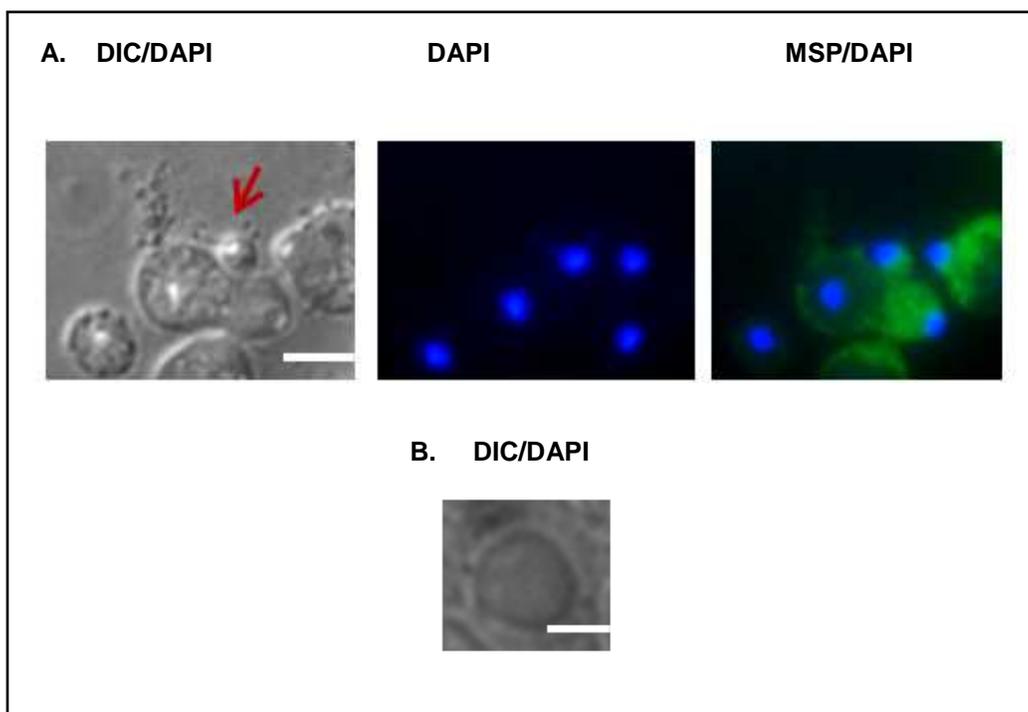


Figure 3-5 Random budding event and presence of anucleated cell during *H. bacteriophora* spermatogenesis. A. Budding events from asymmetrically dividing secondary spermatocytes were rarely observed (red arrow). DAPI/DIC and MSP staining revealed the presence of both DNA and MSP in the budding cells. Scale bar is 5 μm. B. DAPI/DIC staining showed presence of cells that do not contain any DNA but looked similar to a *C. elegans* residual body. Scale bar is 5 μm.

3.3.5 Cytoplasmic Component Tubulin Partitions Asymmetrically towards One Pole of an Asymmetrically Dividing Spermatocyte in Anaphase II

In *H. bacteriophora*, the tubulin dynamics during an asymmetric division is similar to the MSP partitioning event. Microtubules are symmetric during metaphase II but towards the end of anaphase II they become localized towards one cell and eventually

get discarded by the cell. Therefore, motile sperm do not have any tubulin (Figure 3-6). Similar microtubule dynamics was observed in SB347 where the X-bearing sperm initially gets majority of the tubulin. However, during the elongation phase following anaphase II, tubulin shifts towards the nullo-X sperm and is eventually discarded by the cell (Shakes *et al.* 2011). In *C. elegans*, tubulin is discarded into the central residual bodies during the budding of spermatids described earlier in Figure 3-2C.

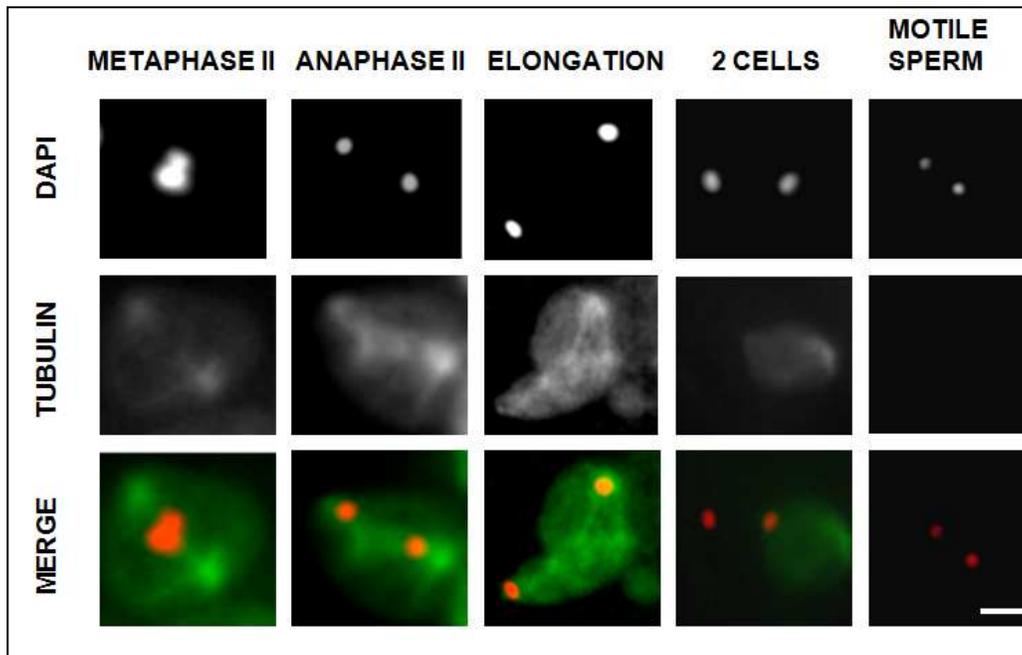


Figure 3-6 Tubulin is initially partitioned asymmetrically in anaphase II and eventually discarded by the cell that receives it. During an asymmetric division in anaphase II, microtubules are localized more towards one pole and are eventually discarded by the cell. Motile sperm do not contain tubulin. Scale bar is 5 μ m. All the figures are in same scale.

3.3.6 During an Asymmetric Partitioning of MSP, Expression of the Apoptosis Gene *cep-1* Is Associated to the Cell That Does Not Receive MSP

We hypothesized that the non-functional spermatid resulting from the asymmetric division is essentially undergoing a programmed cell death. To confirm this, we performed antibody staining against the apoptotic marker CEP-1. In *C. elegans*, apoptosis induced by DNA damage is regulated by the gene *cep-1* which is an ortholog of the human tumor suppressor gene p53 (McGee *et al.* 2012). We observed that in the majority of the dividing secondary spermatocytes *cep-1* segregates to one side of cell (Figure 3-7A). However, the functional spermatid that receives MSP during an asymmetric division does not express *cep-1*. Therefore, the non-functional spermatid lacking MSP is undergoing a *cep-1* mediated apoptosis. Presence of CEP-1 is not evident in the rare symmetrically dividing spermatocytes that receives MSP on both sides and hence produce two functional spermatids (Figure 3-7B).

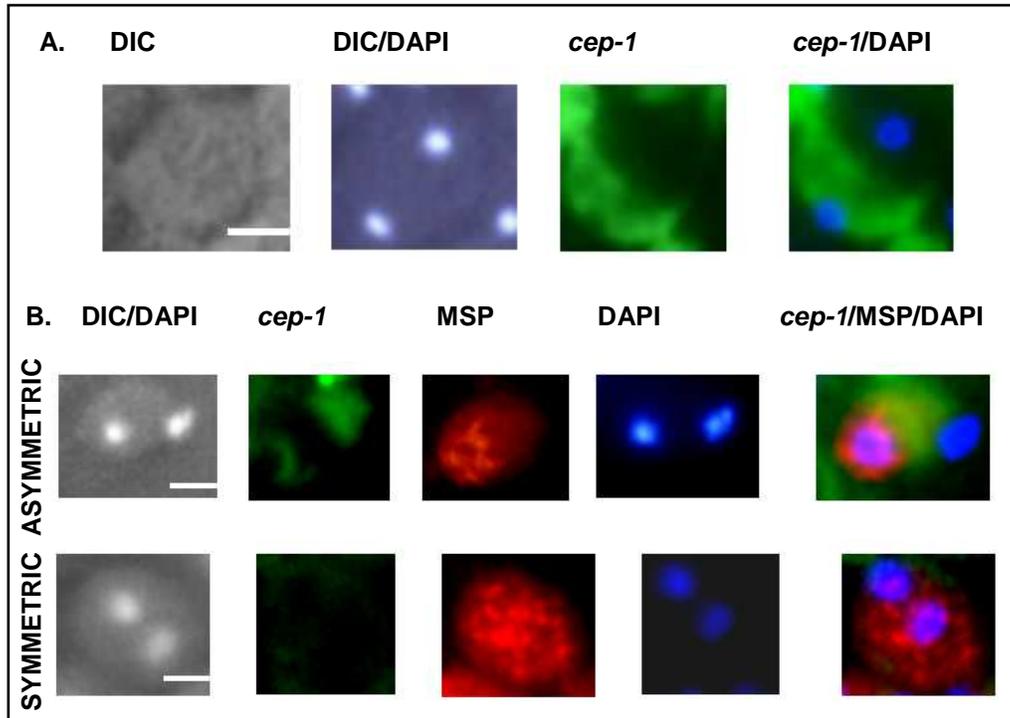


Figure 3-7 In an asymmetrically dividing secondary spermatocyte *cep-1* is expressed towards one pole and opposite to that of MSP. Scale bar is 5 μ m. A. *cep-1* is expressed towards one pole and not the other in a spermatocyte during anaphase II. B. The pole expressing *cep-1* is opposite to the pole expressing MSP during asymmetric division.

Symmetrically dividing secondary spermatocytes do not express *cep-1*.

Scale bars are 5 μ m.

3.3.7 Expression of *ced-3* is Biased Towards One Pole During an Asymmetric Division in Meiosis II

To further confirm an event of apoptosis in the non-functional spermatid we performed antibody staining against another marker CED-3. In *C. elegans*, the protein CED-3 has been reported to trigger an apoptotic death of the cell. It is an example of a cysteine protease that has a conserved role of programmed cell death in mammals (Yuan *et al.* 1993). To further confirm that an event of apoptotic death occurs in one of the cells in *H. bacteriophora* during meiosis II, we used antibody against the core apoptotic marker CED-3. We found, clear expression of *ced-3* towards one pole during anaphase II (Figure 3-8).

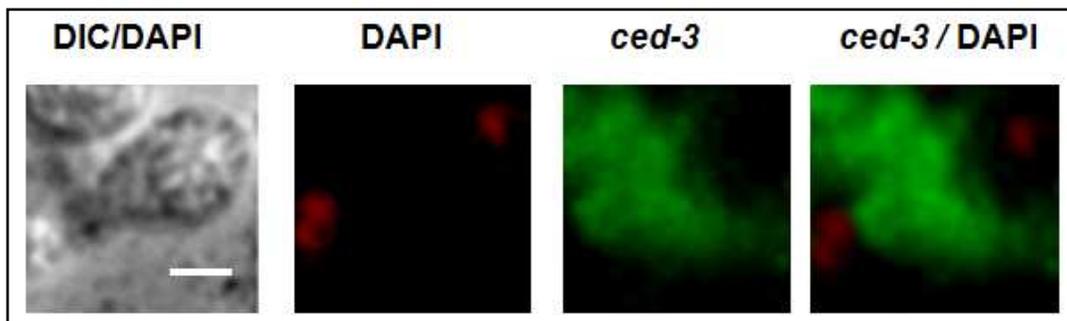


Figure 3-8 Expression of *ced-3* is biased towards one cell during asymmetric division of secondary spermatocyte. In anaphase II expression of the core apoptotic marker *ced-3* is asymmetric. Scale bar is 5 μ m.

3.4 Discussion

Heterogony, which is the alternation between a hermaphroditic or parthenogenetic generation with an amphimictic generation, is present in some vertebrate and invertebrate parasitic nematodes. Because of the similarities in the mode of reproduction between the parasite *Heterorhabditis bacteriophora* and the free-living SB347, we decided to investigate if the mechanisms underlying sex ratio distortion were also similar. As discussed earlier, both SB347 (Shakes *et al.* 2011) and *H. bacteriophora* (Dix *et al.* 1994) produce less than 5% males in cross progeny. Distortion in sex ratios can occur mainly by the following reasons: a) embryonic lethality (Haldane, 1922; Orr, 1997) or b) post-meiotic gametic dysfunction due to differential expression of genes mediated by sex chromosome (Cazemajor *et al.* 2000) or c) even segregation of sex chromosomes in a nonrandom manner during meiosis (Kubai *et al.* 1982). In SB347, biased sex ratio towards females is an outcome of an X-chromosome mediated meiotic drive that results in functional and non-functional gametes during male spermatogenesis. We predicted that similar to SB347, the insect parasite *H. bacteriophora* may have evolved a similar mechanism to produce functional and non-functional gametes. From our spermatogenesis studies in *H. bacteriophora*, we do not find any evidence for sex chromosomes. Interestingly, we found that the non-functional gametes in *H. bacteriophora* undergo an apoptosis-mediated cellular death. We hypothesize that this intriguing phenomenon of killing of gametes during a male spermatogenesis event has the potential to play a key role in regulating sex ratios in *H. bacteriophora*.

Our data suggests *H. bacteriophora* undergoes both symmetric and asymmetric partitioning of cytoplasmic components resulting in two morphologically different types of spermatids. The high frequency of asymmetric division is evident from the monolayer sperm spread under DIC optics (Figure 3-2). Unlike SB347, meiotic studies of

spermatogenesis in *H. bacteriophora* did not show any evidence of lagging-X either in meiosis I or in meiosis II (Figure 3-3). In SB347, unpaired lagging-X was present in anaphase II. This was due to the premature separation of sister chromatids in meiosis I. In addition to this, essential cellular components required to make a functional sperm segregate asymmetrically to the X-bearing spermatid in SB347 (Figure 3-2A) (Shakes *et al.* 2011). The outcome of asymmetric division is an unequal distribution of the major sperm protein (MSP) that is essential for the formation of pseudopod required for sperm motility (Figure 3-4). Although we are dubious about the presence of X-chromosome, *H. bacteriophora* asymmetrically partitions essential cellular components to one of the developing spermatids during anaphase II (Figure 3-4). The essential major sperm protein (MSP) required for sperm motility is unequally partitioned in majority of the secondary spermatocytes during anaphase II. However, rare symmetric partitioning also occurs. During meiosis II in *H. bacteriophora*, the majority of the secondary spermatocytes undergo asymmetric division producing two spermatids of very distinct morphological features. The remaining secondary spermatocytes divide symmetrically resulting in two identical spermatids.

In *C. elegans* primary spermatocytes initiate assembly of MSP into fibrous bodies until anaphase II. Eventually MSP gets localized into the developing spermatids and non-essential cytoplasmic components (e.g. tubulin, actin) end up in the residual bodies that act as a 'garbage disposal' for the dividing secondary spermatocytes. Fibrous bodies depolymerize in the spermatids resulting in MSP getting distributed throughout the cytoplasm. Following activation of spermatid, MSP gets localized in the pseudopod of the active sperm (Roberts *et al.* 1986; Shakes *et al.* 2011). The formation of a residual body characteristic of *C. elegans* spermatogenesis was not observed in SB347, although rare budding events and occasional anucleate residual bodies were located in *H.*

bacteriophora (Figure 3-5). The partitioning process of cellular components is mediated by physical forces generated by the microtubules and actin filaments, both of which end up in the residual body following the partitioning event (Shakes *et al.* 2011). As mentioned earlier, residual bodies were occasionally observed in *H. bacteriophora* spermatogenesis (this study, Poinar & Hess, 1985). Sometimes they contained DNA and resulted from a budding event from an asymmetrically dividing secondary spermatocyte (Figure 3-5).

The asymmetric partitioning of cellular components in *H. bacteriophora* such as tubulin was found to be localized more towards one pole during the elongation stage immediately following anaphase II. Staining against tubulin eventually disappeared and there was no evidence of tubulin in the resulting spermatids (Figure 3-6). This event is very similar to SB347 where more tubulin was initially deposited towards the putative X-bearing spermatid (Shakes *et al.* 2011). Therefore, in *H. bacteriophora* the essential cytoplasmic components required for making a functional sperm are asymmetrically distributed by forces radiating from the microtubules.

We wanted to investigate the fate of the non-MSP bearing spermatid resulting from the asymmetric division. Our hypothesis was that lack of MSP rendering the spermatid to be non-functional may eventually lead to its death. Interestingly, the non-functional spermatid lacking MSP does undergo an apoptosis-mediated cellular death. This was confirmed by *cep-1* antibody staining (Figure 3-7), a marker for apoptosis. The gene *cep-1* triggered DNA damage-induced apoptosis in germ cells and is an ortholog of the human tumor suppressor gene p53 (Rutkowski *et al.* 2011; McGee *et al.* 2012). Rare symmetric divisions did not show any evidence of expression of *cep-1* (Figure 3-7). Asymmetric cell death event was further confirmed by examining the expression of the core apoptotic marker *ced-3* (Figure 3-8), a highly conserved caspase that mediates

apoptosis in several organisms including *C. elegans* (Yuan *et al.* 1993). Both these markers were expressed constitutively in one of the developing spermatids (Figs. 3-7, 3-8).

Our data suggests a potential mechanism for the biased sex ratios observed in *H. bacteriophora*. First, we demonstrate a conserved asymmetric partitioning event of essential cytoplasmic components between a free-living nematode SB347 and its close parasitic relative *H. bacteriophora*. Second, we show that the asymmetric partitioning event is coupled with a programmed cellular death of the non-functional spermatids in the parasite. Together, these events may serve as a potential mechanism for skewing sex ratios in *H. bacteriophora* that was earlier documented to produce very few males from an outcross (Dix *et al.* 1994). Although we were unable to locate any putative X-chromosome in this species, we cannot completely rule out the possibility of its existence. Further studies would be required to confirm this. However, in this study, we illustrate a very intriguing event where apoptosis mediated cellular death is linked to male spermatogenesis that has the potential for skewing sex ratios in a parasitic population.

From an evolutionary perspective, insect parasite *H. bacteriophora* may have evolved a similar mechanism of producing non-functional spermatids from its free living ancestor SB347 to skew sex ratios in a population. This is selectively advantageous under conditions when population sizes are relatively large and sibling matings are very frequent. Under these conditions, possibilities of random mating are highly questionable, giving rise to ratios that are skewed from the expected 1:1 resulting from an outcross (Fisher, 1930; Hamilton, 1967). Therefore this kind of selective death of the non-functional spermatids with the potential to produce more female offspring may be an adaptive trait for rapid colonization.

Chapter 4

Sex Pheromone Production Correlates with Lifespan

4.1 Introduction

According to the evolutionary theory of aging, animals with a longer period of fecundity following reproductive maturity will senesce at a slower rate compared to animals that do not reproduce that long (Williams, 1957; Hamilton, 1966; Charlesworth, 1994). Consequently, Rhabditid nematodes like *C. elegans* that reproduce for only a few days after sexual maturity undergo rapid senescence. In addition to this, mortality rates are also increased by extrinsic factors like mating (Gems and Riddle, 1996), despite its beneficial roles under conditions of changing environment and high mutation rates (Morran *et al.* 2009). Therefore, we would predict that lineages with the ability to self-fertilize would avoid the costs of physical injury associated with mating and would eventually evolve a longer life span. In contrary, outcrossing lineages with male and female animals must mate in order to reproduce and would evolve to live shorter. However, comparative studies show contradictory results (McCulloch and Gems, 2003; Gardner *et al.* 2006). In the genus *Caenorhabditis*, hermaphrodites of *C. briggsae* and *C. elegans* live shorter compared to the females of *C. remanei* (McCulloch and Gems, 2003). In contrast, the genus *Oscheius* displays an opposite pattern: the hermaphrodites of species *O. myriophila* live longer than the females of *O. dolichura* and *O. dolichuroides*. These contradicting results may be due to confounding factors that may influence life-span including internal hatching of larvae (endotokia matricide) observed in *Caenorhabditis* self-fertilizing species (McCulloch and Gems, 2003). Among other factors, that may influence life-span are differences in genotypes among strains of a species that may reach a two-fold difference in median life-span (McCulloch and Gems,

2003). Body size also plays a role with size differences among genetically identical individuals influencing life-span (Gardner *et al.* 2006).

In order to bypass confounding variables affecting life-span, comparative studies among females and hermaphrodites belonging to the same species may help understand factors that may influence life-span. Here, we study a species of nematode comprising of males, females and hermaphrodites in which the females display differential mating pattern by attracting males more efficiently compared to the hermaphrodites. We hypothesize, as an evolutionary consequence of the antagonistic pleiotropy theory of aging (Williams, 1957), production of pheromones in females is potentially energy consuming and may be affecting the life-span in females causing them to senesce significantly faster compared to the hermaphrodites. We also hypothesize that hermaphrodites evolved to self-fertilize can bypass pheromone production eventually living longer.

4.2 Methods

4.2.1 Lifespan Assays

To assure genetic homogeneity, SB347 was inbred for 50 generations by selfing single individuals and was renamed as strain APS3. APS3 was cultured on standard NGM plates seeded with the *Escherichia coli* OP50-1 strain, a streptomycin-resistant derivative of *Escherichia coli* OP50 (Wood, 1988; Stiernagle, 2006). Age refers to days following adulthood and worm lifespan assays were performed at 20°C in the absence of 5-fluoro-2'-deoxyuridine (FUdR). Hermaphrodites were transferred every other day to new plates to eliminate confounding progeny and were marked as dead or alive. To control for transfer of worms as a variable that affects lifespan, females were also transferred to a new plate in the same day as hermaphrodites. The time of death was

determined by the lack of response to the touching with a platinum pick. The total number of animals from at least three replicates is denoted by 'n' in the legend of the figures. All life-span experiments were performed in 6 cm NGM plates with a bacterial spot of ~ 5 mm *Escherichia coli* strain OP50-1 (OP-50-1 is resistant to both ampicillin and streptomycin unlike OP-50 that is resistant to ampicillin) . To test the effect of mating on mortality of hermaphrodites, 44 sets of crosses were made. In each set, six males were cultured with two hermaphrodites for 18 hours. Worms that underwent 'matricide' or that crawled off the plate were censored. Statistical analysis of lifespan was performed on Kaplan-Meier survival curves in SigmaPlot (v11.0; Systat Software, Inc., San Jose, CA, USA) by logrank (Mantel-Cox) tests.

4.2.2 Male Responsiveness Assays

Assay plates were prepared one day before the experiment by dropping 13 μ l of OP50-1 *E. coli* culture on a fresh NGM plate to make a lawn of ~ 5 mm diameter. 5 males were placed in the center of the bacteria lawn and their response to 5 hermaphrodites or 5 females were observed for 4 minutes in each assay. If males attempted to mate with the opposite sex by using their tails to scan the body to reach the vulva, their response was scored as positive. To determine if males respond to mated females, each female was mated with 4-5 males for 5-6 hrs. To determine if males respond to converted females or converted hermaphrodites, 5 females was mated with 15 males for 5-6 hrs. All experiments were performed with 5 replicates.

4.2.3 Male Attraction Assays

The supernatant was collected from five young adults incubated in 100 μ l of M9 buffer for 12 hours at room temperature. The assay was performed on a 1.5% agar pad placed on top of a Petri dish lid. 3 μ l of supernatant was added in one side of the slide and 3 cm apart from the control drop that just contained M9. 15 young-adult males were placed on

the midline between the spots. After 30 min, male worms were scored on the basis of their location. The remaining worms were scored as “neither spot”. The same procedure was performed for hermaphrodites. At least ten replicates were performed for each gender, totaling 150 males for each treatment. We also computed male attraction by taking the three numbers (test, control, and neither) to calculate the chemotaxis index (CI) (Bargmann and Horvitz, 1991). CI is defined by the number of males attracted to the test spot subtracted by the number of males in the control spot, divided by the total number of males assayed. A CI of one indicates that all worms are attracted to the chemical in the test spot, a CI close to zero indicates no attraction, whereas a negative CI indicates repulsion.

For the competition experiments of males between hermaphrodites and females, 3 μ l of supernatant of hermaphrodites was added in one side of the slide and 3 cm apart from the supernatant from females. 15 males were placed in the midline, as described below. Ten replicates totaling 150 males were performed for this experiment. To determine at which stages females start secreting male attractants, female larvae were pre-selected based on their gonad size.

4.2.4 Sex Conversion Assays

APS3 hermaphrodite fated larvae were converted to adult female animals following the protocol as described in methods section of chapter 2. The protocol for conversion of female fated larvae to adult hermaphrodites is also described in the methods section of the same chapter.

4.2.4 Environmental Stress Assays

Four different types of environmental stress experiments were performed. For the oxidative stress assays 2 days adult animals (L4 was treated as day 1 of adulthood) were subjected to 200 mM Paraquat (Methyl Viologen Dichloride Hydrate, 98%,

Sigma-Aldrich) for 2 hours. To track the reactive oxygen species (ROS) we used a probe called 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA). ROS was then estimated using a Bio-Tek Synergy 2 Multi-detection microplate reader and data was analysed in Gen-5 Software package (Bio-Tek Instruments, Inc. Winooksi, VT USA). Assay plates were manufactured by Corning, Inc. (Lowell, MA, USA).

For the heat-shock assay, 2 days adult animals were incubated at 35°C for 2 hours. They were then rescued to regular NGM plates and life-span was assayed.

For the osmotic-stress assays animals were subjected to hypertonic NGM plates containing 200mM NaCl. For acute stress, they were incubated in the hypertonic plates for 15 minutes and for chronic stress they were incubated for 2 hours. Animals were rescued to regular plates after the acute and chronic incubation periods and life-span was assayed.

For the hypoxic stress, animals were incubated in a vinyl hypoxic chamber that is fitted with an oxygen sensor and automated controller set for 2% or 20% O₂ concentration (Coy Laboratory Products, Glass Lake, MI, USA). We performed our hypoxic assay in presence of 2% oxygen. They were rescued to regular NGM plates after the incubation for 12 hours and measured for life-span.

4.2.5 Exo-metabolome Sample Preparation

Chemical analysis of the supernatant (lyophilized and worm water) was carried out by our collaborators in the Schroeder lab in Cornell University, Ithaca, NY.

2 L of a three-week mixed stage culture of APS3 in S-complete media with 20% w/v OP50 at 20°C was centrifuged at 13000 g followed by filtration to remove bacteria. The supernatant collected was frozen over a dry ice-acetone bath, lyophilized to a fine powder, and extracted with 100 mL of a 95:5 mixture of ethanol and water for 16 h. The resulting exo-metabolome sample was concentrated *in vacuo*, resuspended in methanol,

filtered, and used for HPLC-MS/MS without further processing. Single-sex exo-metabolome samples were obtained from incubating 800 virgin females, males, and hermaphrodites in 800 μ l M9 buffer. After centrifugation, the culture supernatants were lyophilized as described above and extracted with 1 ml of methanol each. The extracts were concentrated *in vacuo*, resuspended in methanol, filtered, and used for selective ion monitoring (SIM)-HPLC-MS analysis.

4.2.6 HPLC-MS/MS, and SIM-HPLC-MS Protocols

HPLC-MS/MS and selective ion monitoring (SIM)-HPLC-MS were performed using an Agilent 1100 Series HPLC system equipped with an Agilent Eclipse XDB-C18 column (9.4 x 250 mm, 5 μ m particle diameter) connected to a Quattro II spectrometer (Micromass/Waters) using a 10:1 split. For HPLC, a 0.1% acetic acid-acetonitrile solvent gradient was used at a flow rate of 3.6 mL/min, starting with an acetonitrile content of 5% for 5 min which was increased to 100% over a period of 40 min. Exo-metabolome samples were analyzed by HPLC-ESI-MS in negative ion mode using a capillary voltage of 4.0 kV and a cone voltage of -40 V. HPLC-MS/MS screening for precursor ions of $m/z = 73.0$ performed using argon as collision gas at 2.1 mtorr and 40 eV. For the analysis of exo-metabolome samples of mixed-stage APS3 liquid cultures, the mass spectrometer was operated in scanning mode for a mass range of m/z 200-700. For exo-metabolome samples of single-sex worm cohorts, the spectrometer was operated in selective ion monitoring (SIM) mode and the following ions were selectively observed: $m/z = 247$ (ascr#9) and $m/z = 275$ (ascr#1). Quantification of ascr#9 and ascr#1 in these samples was based on integration of the SIM-HPLC-MS signals from the corresponding ion-traces. Concentrations were calculated using response factors determined with synthetic standards.

4.2.7 Quantification of Ascarosides

Quantification of ascr#1 and ascr#9 in APS3 single-sex worm cohort samples were based on integration of the SIM-HPLC-MS signals from the corresponding ion-traces. Absolute amounts of ascarosides in each sample were calculated using response factors determined with synthetic standards of ascr#1 and ascr#9. The amounts calculated were then normalized to the number of worms used for exo-metabolome preparation (n = 800) to reflect the amount of ascarosides produced by a single worm. Finally, physiological concentrations of ascr#1 and ascr#9 in samples used for male attraction assays were calculated from the ascaroside amounts per worm, taking into account the number of worms used for the assays (n = 5) and the volume of M9 (100 μ l) used for incubating the single sex worm cohorts.

4.2.8 Productivity of Hermaphrodites After Sperm-depletion

Hermaphrodites were allowed to self until they exhausted their supply of self-sperm. Ten sets of crosses with one sperm-depleted hermaphrodite and one male were cultured together until the death of the hermaphrodite. Cross-progeny was counted for each day.

4.3 Results

4.3.1 In SB347 Virgin Hermaphrodites Live Longer Compared to Virgin Females

To examine for any influence of body size on life-span in genetically identical individuals (Gardner *et al.* 2006), we measured body volumes in APS3 adult females and hermaphrodites. In APS3 there were no significant differences in mean body volume over 3 days of adulthood between females and hermaphrodites ($n = 10$ for each gender, $P = 0.843$ using the Student's t test). This strain therefore allows direct comparisons of female and hermaphrodites while controlling for genetic variation, phylogeny and body size.

Under normal culture conditions, APS3 virgin adult hermaphrodites live significantly longer compared to virgin adult females (Log-Rank test, $P < 0.001$) (Figure 4-1).

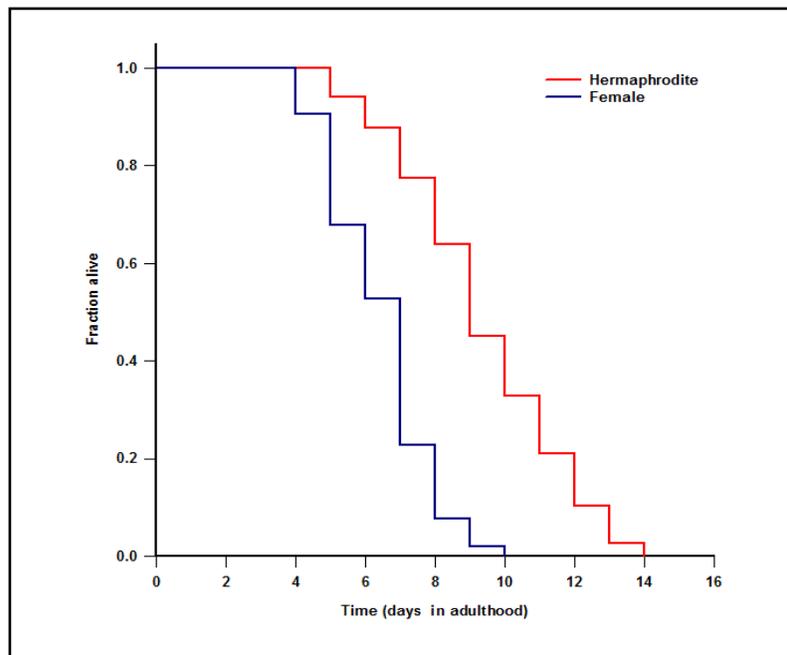


Figure 4-1 Hermaphrodites in APS3 have a longer life-span compared to females. The mean life-span of virgin adult hermaphrodites (9.0 ± 0.2 SEM days, $n = 186$) is significantly higher than the mean adult life-span of virgin females (6.2 ± 0.2 SEM days, $n = 53$) (Log-Rank test, $P < 0.001$).

4.3.2 Mating affects Life-span in Both Hermaphrodites and Females

Since mating is generally associated with costs that affect lifespan (Daly 1978; Gems and Riddle 1996), we first tested whether APS3 hermaphrodite and female lifespan are affected by copulating with males. We observed that mated APS3 hermaphrodites and females have a shorter adult lifespan than unmated counterparts (Figure 4-2A, 4-2B) indicating that mating is also a factor for extrinsic mortality in this species.

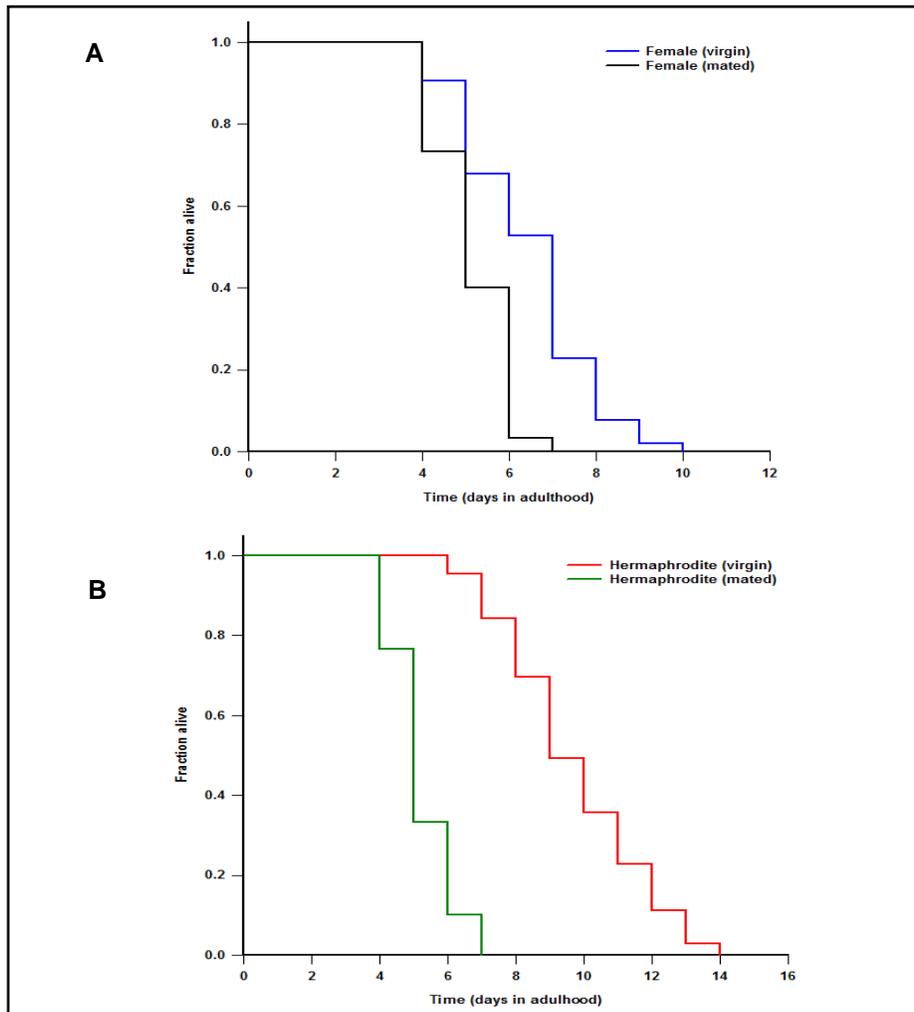


Figure 4-2 Mating shortens life-span in both females and hermaphrodites. A. The mean adult lifespan of virgin females is longer than of mated females (Log-Rank, $P < 0.001$, virgin females: 6.2 ± 0.2 SEM days, $n = 53$; mated females: 5.2 ± 0.2 SEM days, $n = 30$).

B. The mean lifespan of virgin adult hermaphrodites is longer than of mated hermaphrodites (Log-Rank, $P < 0.001$, virgin hermaphrodites: 9.0 ± 0.2 SEM days, $n = 183$; mated hermaphrodites: 5.2 ± 0.2 SEM days, $n = 30$).

4.3.3 Females Are More Attractive to Males Than Are Hermaphrodites with Self-sperm

The ancestral mating system in nematodes is gonochorism, in which females only make oocytes and must mate with males to produce progeny (Kiontke and Fitch, 2005). To assure reproduction, females secrete potent sex pheromones to attract males for mating (Bone and Shorey, 1978; Chasnov *et al.* 2007; Izrayelit *et al.* 2012). Because mating is required for reproduction of females, we hypothesized that APS3 females retained the ancestral character of secreting male-attractant chemicals. Given the fitness cost associated with mating, we also hypothesized that hermaphrodites with self-sperm, capable of self-fertilization, would be less attractive to males. To test these hypotheses, conditioned media obtained from virgin females or hermaphrodites were assayed for male attraction. In competition experiments, males were significantly more attracted to the female supernatant than the hermaphrodite supernatant (Figure 4-3). These male attraction assays suggest that APS3 females secrete a male attractant to the medium that is reduced or absent in hermaphrodites with self-sperm.

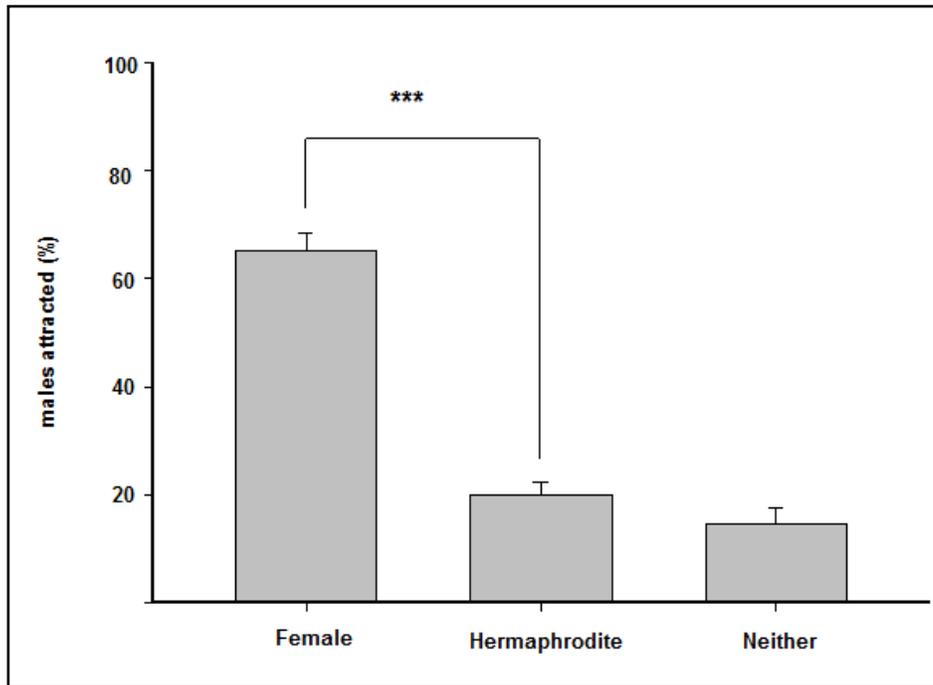


Figure 4-3 Adult virgin females attract significantly higher number of males compared to hermaphrodites with sperm. A higher proportion of males are attracted to conditioned supernatant of females than of hermaphrodites with sperm (one-way ANOVA, $P < 0.001$). The graph represents the mean \pm SEM of ten independent experiments with 15 males in each. $P < 0.001$ is denoted by ***.

4.3.4 Hermaphrodites Delay Mating with Males

Since mating is a major factor for extrinsic mortality, we further hypothesized that APS3 hermaphrodites have evolved to postpone their attractiveness to males, thereby extending their reproductive life and increased fecundity. To test this, we measured the percentage of response of males to the opposite sex using an assay developed previously for *C. elegans* (Morsci *et al.* 2011). As expected, we observed that a higher proportion of males attempt to mate with females than with hermaphrodites with self-

sperm (Figure 4-4). However, male response to hermaphrodites increases once they become sperm-depleted (Figure 4-4). These results suggest that the delay of hermaphrodites to attract males is an adaptation to increase reproductive output by postponing mating-related fitness costs.

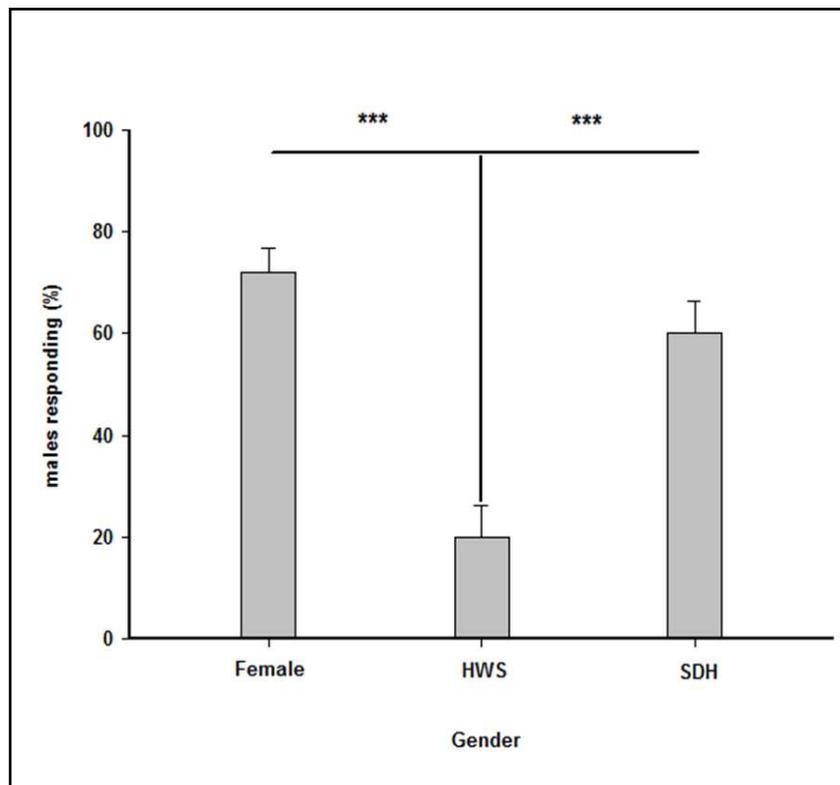


Figure 4-4 Males are attracted to sperm-depleted hermaphrodites. Males of APS3 respond more to females and sperm-depleted hermaphrodites (SDH) animals than to hermaphrodites with sperm (HWS) (one-way ANOVA, $P < 0.001$). $P < 0.001$ is denoted by ***.

4.3.5 Secretions from Females and Sperm-depleted Hermaphrodites Are Sex Pheromone in Nature

The male response to females and sperm-depleted hermaphrodites could be due to a signal that is subject to natural selection (e.g., pheromone) or a cue that is not (e.g., a waste product) (Viney and Franks 2004). To distinguish between these alternatives, we tested whether the female response to males and the secretion of the chemical are dependent on life stage and mating status (Chasnov *et al.* 2007). We found that males are not attracted to females in the first hour after mating and that females recover their attractiveness after about 24 hours (Figure 4-5A). We further showed that the putative sex pheromone is secreted during adulthood but not at larval stages (Figure 4-5B). By laser ablation studies, we also found that the secretions are from the female somatic gonad and uses the vulva as an exit (Figure 4-5 C). These findings indicate that the secretion of the male attractant is regulated, and therefore consistent with the characteristics of a sex-pheromone that is subject to natural selection.

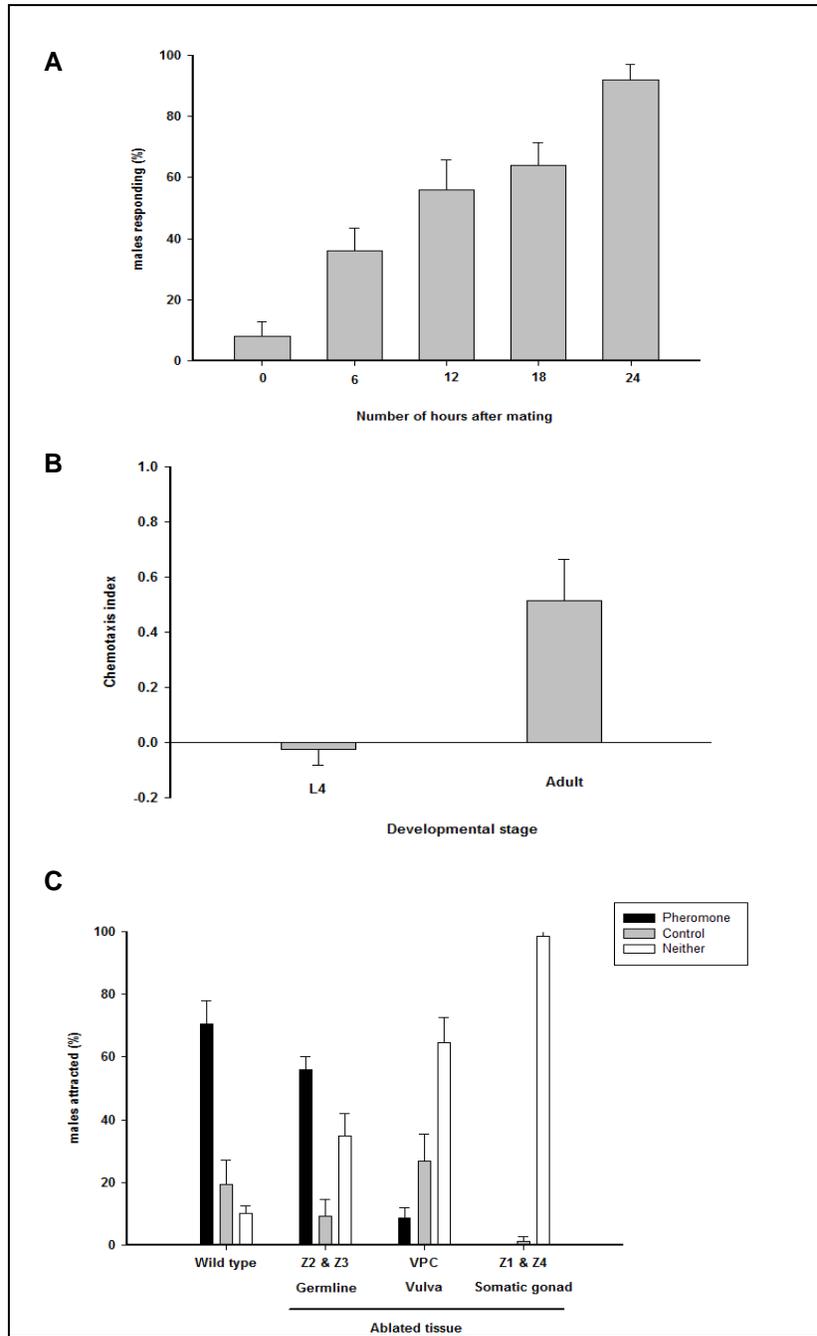


Figure 4-5 Male attractant from females is sex-pheromone in nature. A. Male responsiveness to females over time after mating. B. Chemotaxis index (CI) of males to

females in the fourth larval stage (L4) or as adults (see Materials and Methods for how to calculate CI). C. Somatic gonad secretes the pheromone that exits the vulva to the exterior. The graph in (A), represent the \pm SEM of 5 independent experiments. The graphs in (B) and (C) represent the mean \pm SEM of ten independent experiments with 15 males in each

4.3.6 The Sex Pheromone Has Ascaroside Properties

To elucidate the chemical structure(s) of the sex pheromone, we analyzed exo-metabolome samples of APS3 females and hermaphrodites for the presence of any of the ~170 ascarosides recently identified from *C. elegans* and other nematodes (Bose *et al.* 2012; Choe *et al.* 2012; von Reuss *et al.* 2012), using a combination of HPLC-MS/MS and highly sensitive selective ion monitoring (SIM)-HPLC-MS. Ascarosides are nematode-specific small molecules that serve a wide variety of signaling functions that include the regulation of mating and larval development (Srinivasan *et al.* 2008; Choe *et al.* 2012; von Reuss *et al.* 2012). We detected two ascarosides, ascr#1 and ascr#9 (Choe *et al.* 2012), in the exo-metabolome samples of females and sperm-depleted hermaphrodites (Figure 4-6 A), whereas no significant amounts of ascarosides were detected in exo-metabolome samples of hermaphrodites with self-sperm (Figure 4-6 B). By testing synthetic ascr#1 and ascr#9 in the male attraction assay, we found that males are attracted to both compounds at femtomolar to picomolar concentrations (Figure 4-6 C). At high nanomolar concentrations, however, they are repulsive to males (Figure 4-6C). To determine if the concentration of ascarosides found to be attractive to males is physiological, we measured the amount of ascarosides ascr#1 and ascr#9 produced per worm. As shown in Fig. 4-6 D, amounts of ascaroside ascr#1 and ascr#9 produced by females and sperm-depleted hermaphrodites are in the range expected to be attractive to males.

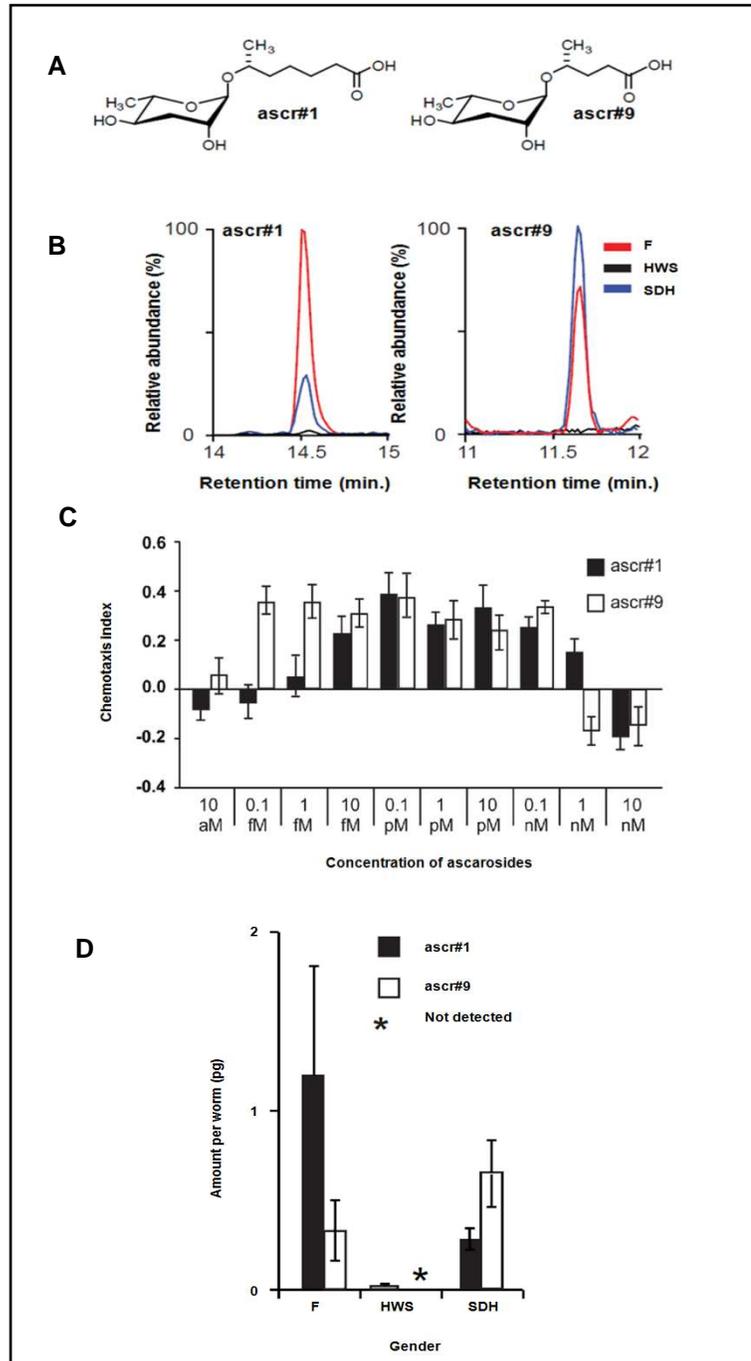


Figure 4-6 Chemical identity of the sex pheromone. A. Structures of ascr#1 and ascr#9. B. SIM-HPLC-MS analyses showing molecular ion traces for ascr#1 (left) and ascr#9

(right) in exo-metabolome samples of females (F), hermaphrodites with sperm (HWS) and sperm-depleted hermaphrodites (SDH). C. Chemotaxis index of males towards synthetic ascr#1 and ascr#9. This graph represents the mean \pm SEM of ten independent experiments with 15 males in each. D. Amount of ascarosides produced per worm of each gender.

4.3.7 Hermaphrodites Redirected from Female Sexual Fate Attract Males and Live Shorter

From our previous study we know that the dauer pathway is linked to germline sex determination in APS3. Hermaphrodites in APS3 obligatorily pass through dauer stage. By inhibiting dauer we were able to convert 'would be hermaphrodites' to females. In the reverse assay, we were able to redirect hermaphrodite development from 'would be females' by inducing dauer formation at an early larval stage of development (Chaudhuri *et al.* 2011). One of the major pathways regulating the dauer pathway is the insulin/IGF signaling pathway (Hu, 2007) which also regulates life-span in worms, flies and mice (Piper *et al.* 2007). In this study, we hypothesized that dauer pathway may be linked to life-span and pheromone production. Therefore we induced dauer formation in 'would be female' larvae and redirected them to become hermaphrodites. We also tested if the converted hermaphrodites attract males. Interestingly, the converted hermaphrodites do not live as long in comparison to the wild-type hermaphrodites (Figure 4-7A). However, they elicit significantly higher male response that was absent in wild-type hermaphrodites (Figure 4-7B).

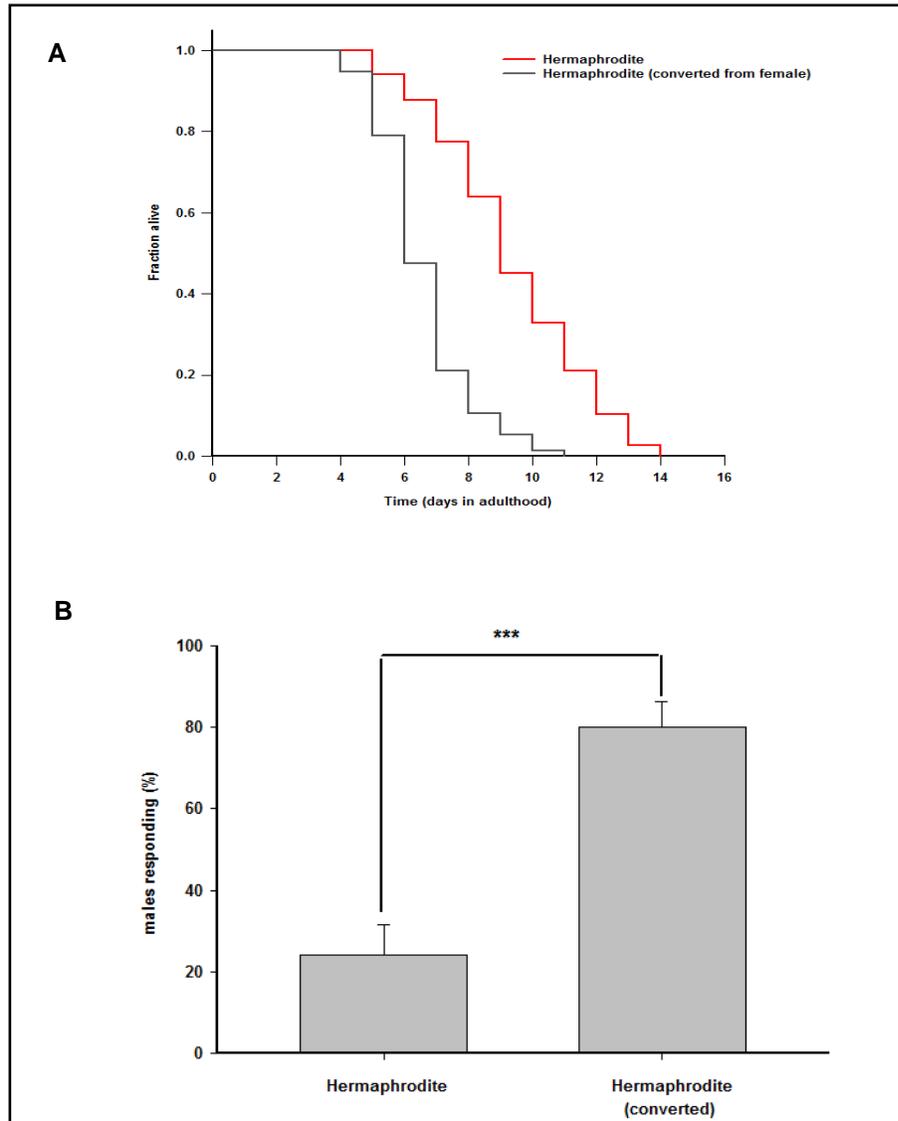


Figure 4-7 Hermaphrodites converted from ‘would be females’ live shorter but attract males. A. The mean adult lifespan of virgin hermaphrodites is longer than that of hermaphrodites converted from females (Log-Rank, $P < 0.001$, virgin hermaphrodites: 9.0 ± 0.2 SEM days, $n = 183$; converted hermaphrodites: 7.5 ± 0.3 SEM days, $n = 30$). B. Male responsiveness to converted hermaphrodites is significantly higher compared to wild-type hermaphrodites (Student's t test, $P < 0.001$). $P < 0.001$ is denoted by ***.

4.3.8 Females Redirected from Hermaphrodite Sexual Fate Live Longer and Do Not Attract Males

In the reverse study we inhibited dauer formation in 'would be hermaphrodites' and converted them into females. The converted female animals were found to live significantly longer than the wild-type females (Figure 4-8A). Unlike wild-type females, they fail to attract males (Figure 4-8B).

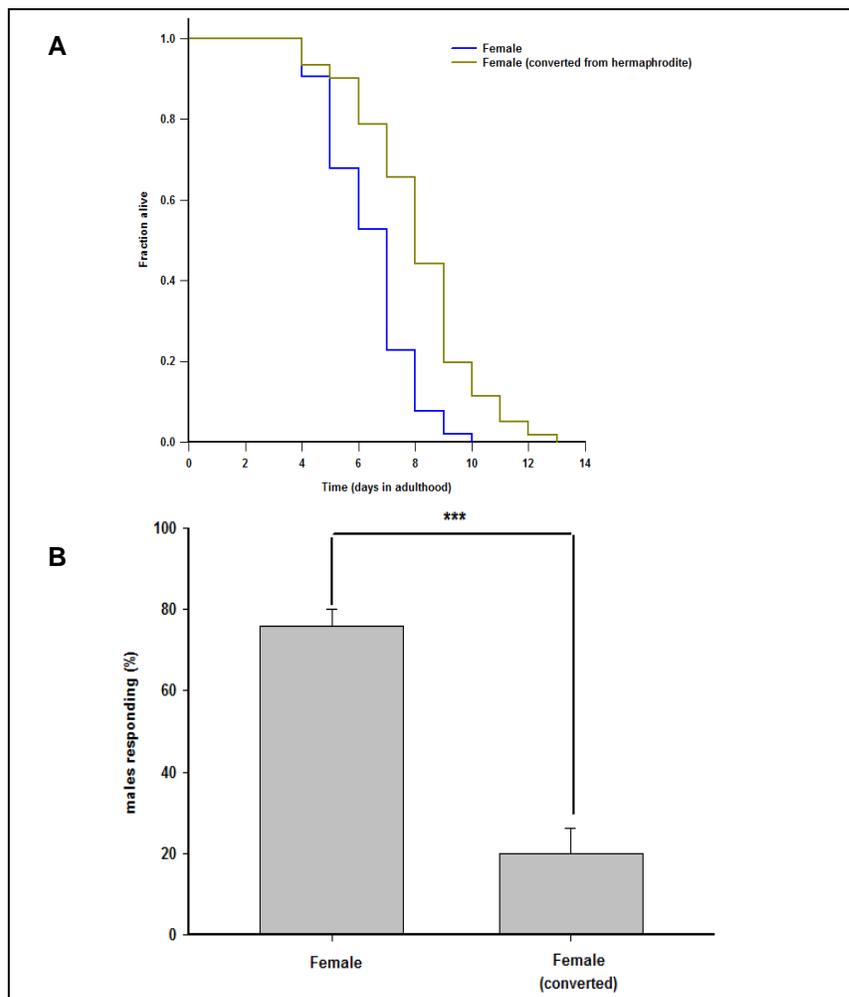


Figure 4-8 Females converted from 'would be hermaphrodites' live longer but do not attract males. A. The mean adult lifespan of virgin females is shorter than that of females

converted from hermaphrodites (Log-Rank, $P < 0.001$, virgin females: 6.2 ± 0.2 SEM days, $n = 53$; converted females: 8.5 ± 0.4 SEM days, $n = 61$).B. Male responsiveness to wild-type females is significantly higher compared to converted females (Student's t test, $P < 0.001$). $P < 0.001$ is denoted by ***.

4.3.9 The Ability to Withstand Oxidative Stress and Heat-shock is Better in Hermaphrodites Compared to the Females

Environmental stress is a key regulator of multiple human diseases and it has been shown there is substantial homology among regulators of stress that is shared among organisms (Rodriguez *et al.* 2013). We hypothesized that the long-lived hermaphrodites in APS3 may have evolved the ability to withstand stress better compared to the females. We found that hermaphrodites are capable of handling stress in a more efficient way than the females. Here, we show that females exhibit significantly higher accumulation of reactive oxygen species (ROS) under stress with an external oxidative stress inducer, Paraquat at a concentration of 200mM (Figure 4.9A). Cellular reactive oxygen ROS is produced as a by-product of the electron transport pathways inside the cell. They keep accumulating with age and causes oxidative damage to the proteins, lipids and DNA inside the cell (Gems & Doonan, 2009). This happens when the ROS levels are higher and the cellular redox balance is perturbed. We also show that hermaphrodites withstand heat-shock better compared to the females. Although, it suffers a significant reduction in life-span, they still live longer than the females (Figure 4.9B).

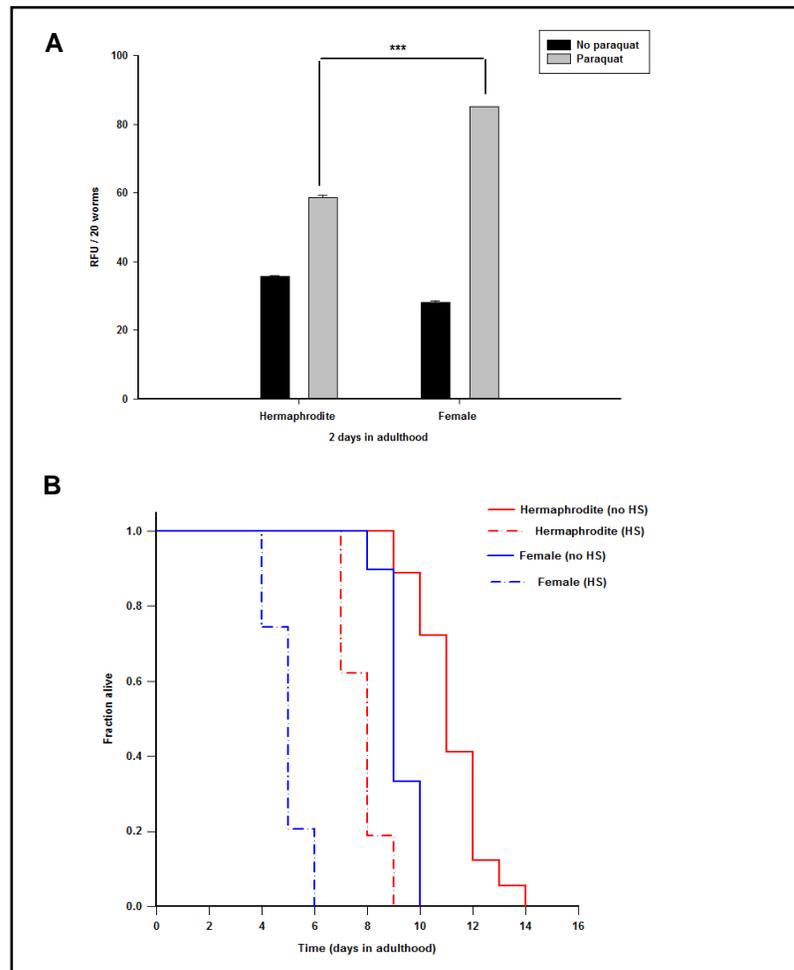


Figure 4-9 Hermaphrodites withstand oxidative stress and heat-shock better compared to the females. A. The accumulation of reactive oxygen species (ROS) due to oxidative stress inducer Paraquat is significantly higher in 2 days adult females compared to 2 days adult hermaphrodites (Student's t test, $P < 0.001$). 20 worms were assayed in three independent trials under each condition for two genders. B. Under heat-shock hermaphrodites live significantly longer compared to females (Log-rank, $P < 0.001$, hermaphrodite: 6.9 ± 0.1 SEM days, $n = 88$; female: 4.2 ± 0.1 SEM days, $n = 90$). $P < 0.001$ is denoted by ***.

4.4.0 Both Under Chronic and Acute Osmotic Stress Hermaphrodites Live Longer Than Females

To understand the effect of osmotic stress on hermaphrodite and female animals, they were independently subjected to chronic stress for 2 hrs. and acute stress for 15 mins. in NGM plates containing 200mM NaCl. Under both conditions, survival performance was better in hermaphrodites compared to the female animals (Figure 5.0A & 5.0B). However, 12 hours incubation was lethal to both the genders.

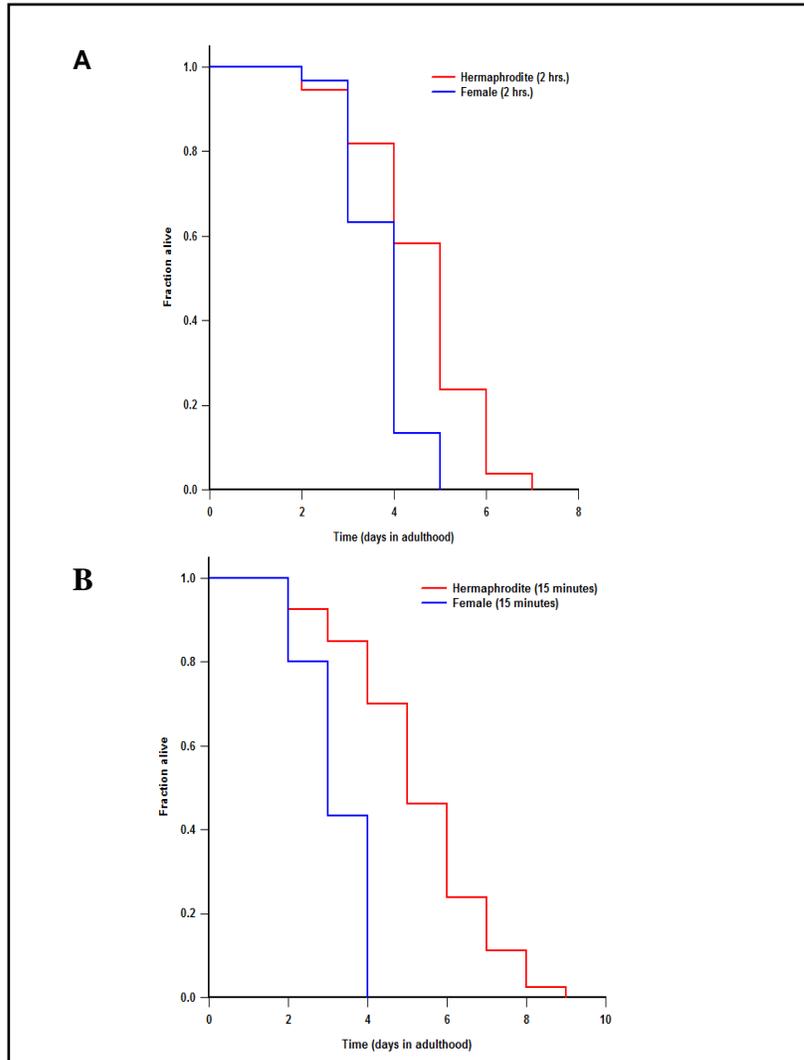


Figure 4-10 Hermaphrodites exhibit longer life-span than females both under chronic and acute osmotic stress. A. Under chronic osmotic stress for 2 hrs. hermaphrodites live significantly longer compared to females (Log- rank, $P < 0.001$, hermaphrodite: 4.6 ± 0.2 SEM days, $n = 55$; female: 3.7 ± 0.1 SEM days, $n = 30$). B. Under acute osmotic stress for 15 minutes, hermaphrodite life-span is longer compared to the females (Log- rank, $P < 0.001$, hermaphrodite: 5.3 ± 0.2 SEM days, $n = 80$; female: 3.2 ± 0.1 SEM days, $n = 30$).

4.4.1 Hermaphrodites Resist Hypoxic Stress More Efficiently Compared to Females

Environmental stress like hypoxia plays key role in modulating longevity in the nematode *C. elegans*. However, the regulation has been found to be complex (Leiser *et al.* 2013). Therefore, to estimate how efficiently APS3 hermaphrodites and females handle hypoxic stress, we subjected them to 2% oxygen for 12 hours. Similar to handling any other environmental stress, under hypoxic conditions hermaphrodites survived significantly longer compared to the females (Figure 5-1).

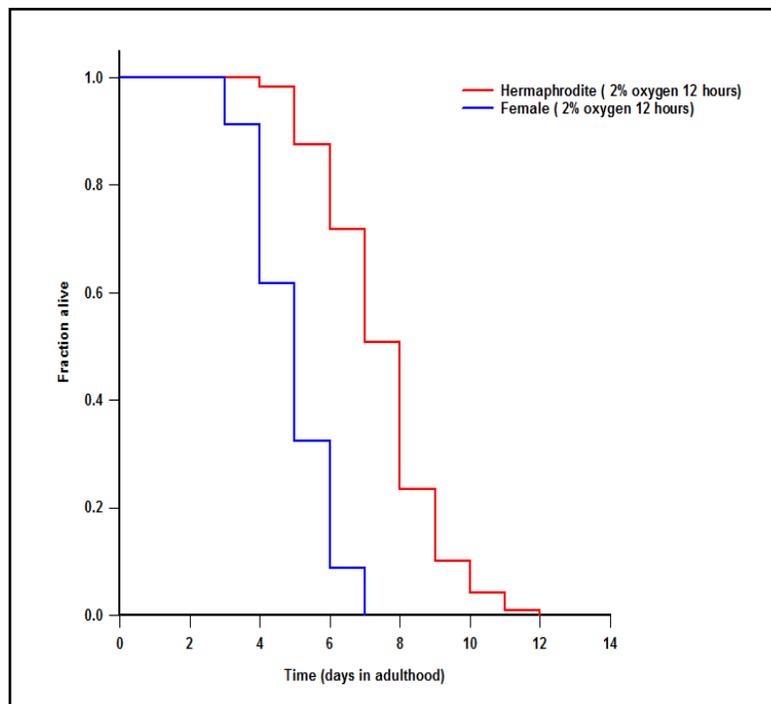


Figure 4-11 Hypoxic condition adversely affects female animals compared to the hermaphrodites. Under hypoxia, the life-span of hermaphrodites is significantly higher compared to the females (Log-rank, $P < 0.001$, hermaphrodite: 7.5 ± 0.1 SEM days, $n = 120$; female: 4.9 ± 0.1 SEM days, $n = 34$).

4.4 Discussion

The lifespan of nematodes can range from a few days to more than a decade (Gems, 2000; Gardner *et al.* 2004). Consistent with the evolutionary theory of aging (Williams, 1957; Hamilton, 1966), typically the longest-lived nematodes have lower extrinsic mortality. When parasitic nematodes are inside the host, for example, they are presumably protected from non-physiological mortality caused by environmental hazards, predators, pathogens and variations in food supply. However, lifespan is also influenced by body size and genetic factors (Ricklefs, 1998). It is possible that lifespan of the parasitic generation is longer because of their usually larger size than the genetically identical free-living generation (Gems 2000; Gardner *et al.* 2006). APS3, which shares features with some parasitic nematodes such as the production of three genders (Runey *et al.* 1978), have hermaphrodites and females of identical sizes and genomes but that differ in their lifespan. In the present study, some of the factors influencing the lifespan of APS3 hermaphrodites and females were identified.

4.4.1 Early Senescence in Females is a Trade-off Due to Production of Sex-pheromones Early in Life

APS3 females and hermaphrodites are produced in every generation, either from a selfing hermaphrodite parent or as the result of males crossing with hermaphrodites or females (Félix, 2004; Shakes *et al.* 2011). Herein we report a significant difference in lifespan between hermaphrodites and females when cultured in the absence of males (Figure 4-1). In addition to this, we provide some evidence that these differences in lifespan may have evolved due to a higher chance of early mortality in females compared to hermaphrodites. Mating with males is a strong predictor for early mortality and affects the lifespan of both genders equally (Figs. 4-2A and 4-2B). However, when given the choice, males prefer to mate with females rather than to hermaphrodites with self-sperm

(Figure 4-3). Early mortality experienced by females is due to production of a signal characteristic of a sex-pheromone produced from their somatic gonad (Figure 4-5). APS3 hermaphrodites do not require mating with males for reproduction within their first week of adult lives because they can use their self-sperm. They do not secrete any pheromone early in their adulthood. We found that hermaphrodites delay the attraction of males until late in their adult lives (Figs. 4-4 and 4-6), thereby postponing mating and allowing them to live longer than females, which are early producers of sex pheromones. Therefore, APS3 hermaphrodites reduce the chances of extrinsic mortality early in life by delaying pheromone production and produce more progeny initially by selfing and then by mating. They also minimize the energy requirement to produce the sex-pheromones early in life. However, females must produce the sex pheromones in order to mate and in doing so, it uses a lot of energy. From a physiological perspective, this may have a potential to decrease life-span in females. There is no doubt that the male-attracting sex-pheromone has its benefits early in the life of a female when they need a mate to reproduce. However, this costs them later in life due to an excessive metabolic energy usage. Interestingly, in both females and sperm-depleted hermaphrodites the ascarosides *ascr#1* and *ascr#9* have been identified as potent male attractants at physiological concentrations (Figure 4-6).

In summary, the two phases of reproduction in hermaphrodites (selfing followed by crossing) maximizes the reproductive output and extend their reproductive lives. Selection is therefore predicted to delay senescence on these animals, which explains the differences in lifespan between virgin hermaphrodites and females. In addition to this, there also seems to be a trade-off and an antagonistic effect of the pheromone production in females later in their life that could be explained by the antagonistic pleiotropy theory of aging (Williams, 1957). This was further confirmed when we found

that the females converted into hermaphrodites live shorter than the wild-type hermaphrodites and secrete sex-pheromones during their self-reproductive period. In the reverse experiment, hermaphrodites converted into females lived longer than wild-type females and failed to trigger any male response (Figs. 4-7 & 4-8). This clearly explains there could be a possible mechanistic link between the pathways regulating pheromone production and controlling life-span in APS3 hermaphrodite and female animals.

Our results also show that as the hermaphrodites evolved to live longer by postponing the production of pheromone, they also evolved an intrinsic ability to withstand environmental stress. Under all four (oxidative, osmotic, heat-shock and hypoxic) stress hermaphrodites exhibited a longer life-span compared to the females (Figs. 4-9, 5-0, 5-1). This may be an adaptive trait that the hermaphrodites acquired as they evolved to live longer and reproduce more for rapid colonization of new and changing habitats.

4.4.2 A Model for the Evolution of Lifespan Plasticity

Although the classic evolutionary theory of aging is derived from a population genetics model in which the selection and combination of specific alleles determine lifespan (Rose, 1991), dramatic differences in lifespan can evolve in genetically identical animals. In *Strongyloides ratti*, for example, differences in lifespan and the rate of aging between the parasitic and free-living form can be as large as 80-fold (Gardner *et al.* 2006). In the current study, APS3 was inbred for fifty generations, assuring that females and hermaphrodites derived from the same selfing parent are genetically identical. Differences in lifespan between genders, therefore, are most likely to be the reflection of differences in gene expression. The study of APS3 and other nematodes with lifespan plasticity might provide insights into mechanisms for the evolution of lifespan that include changes in gene expression (Thompson *et al.* 2009). Because APS3 is a free-living

species, it has many of the advantages that made *C. elegans* as powerful model system for laboratory studies.

Differences between APS3 hermaphrodites and females are not restricted to adult lifespan, but also include distinct developmental paths. In contrast to *C. elegans*, which develops into a dauer when exposed to detrimental environmental conditions, APS3 hermaphrodites obligatorily pass through the dauer stage (Félix 2004; Chaudhuri *et al.* 2011). Dauers are larvae that can endure hazardous chemicals, lack of food and extreme variations in temperature. Intriguingly, APS3 hermaphrodites develop into dauers even when culture conditions are optimal, remaining in this stage for about 24 hours. APS3 females, however, bypass the dauer stage when cultured in the same conditions, developing directly into reproducing adults (Félix, 2004). There is a possibility that the enhanced ability to withstand environmental stress is due to this obligatory link with dauer pathway. Differences in gene expression in hermaphrodites after dauer development have been previously reported in *C. elegans* (Hall *et al.* 2010). It is possible that passage through the dauer stage alters the epigenetic status of hermaphrodites, reflecting in gene expression alterations that influence lifespan. This scenario would be compatible with the antagonistic pleiotropy hypothesis for senescence as mentioned earlier, which assumes that genes beneficial early in life might have adverse consequences in later stages (Williams, 1957). Future research on the epigenetic and physiological basis for differences between females and hermaphrodites should provide further insights into detailed mechanisms involved in the evolution of lifespan.

Appendix A

List of primer sequences and PCR conditions

Primers for Amplifying Small Subunit (SSU) rRNA (Two Overlapping Segments)

(based on Kiontke *et al.* 2004)

First segment:

DF18S-Aa, 5'-TACCTGATTGATTCTGTCAG (forward)

DF18A-16, 5'-CACCTACTCGYACCTTGTTACGACT (reverse)

Second segment:

G18S4a, 5'-GCTCAAGTAAAAGATTAAGCCATGC (forward)

DF18S-B, 5'-GGCGATGATCCAGCTGCAGGTTC (reverse)

Primers for Amplifying Large Subunit (LSU) rRNA (Three Overlapping Segments)

First segment:

KK28S-1, 5'-AAGGATTCCCTTAGTAACGGCGAGTG (forward)

KK28S-4, 5'-GCGGTATTTGCTACTACCAYYAMGATCTGC (reverse)

Second segment:

KK28S-3, 5'-AACGACCCGAAAGATGGTGA ACTATGC (forward)

KK28S-6, 5'-AGTTACTCCCGCCGTTTACCCGC (reverse)

Third segment:

KK28S-5, 5'-AGGTAAGGGAAGTCGGCAAAGTAGATCC (forward)

KK28S-16, 5'-TCAATCAAAGGATAGTCTCAACAGATCGC (reverse)

Primers for Amplifying RNA polymerase II (RNAP2) (Three Overlapping Segments)

First segment:

KKRNAP-1P, 5'-AACGTCGAAGGAAAGCGTATTCCDTTYGG (forward)

KKRNAP-4P, 5'-CGCCAGTGCTCCGACCATYTCWCCNGGYTG (reverse)

For RT-PCR:

KKRNAP-6P, 5'-CGCCAAGTGTTACATTCTTNGCHGANACWCC (reverse)

Second segment:

KKRNAP-7P, 5'-GGAATCTCGAAACAGGCTCARTAYAAYGC (forward)

KKRNAP-10P, 5'-GAGACATCTCAAGAACGCRTCRTCYTCCAT (reverse)

Third segment:

KKRNAP-9P, 5'-CAAGCGATTGCTCAGCCNGGWGARATGG (forward)

KKRNAP-12P, 5'-TGAACAACGCATGAGAGCWCCVACYTCYTG (reverse)

PCR conditions (based on Kiontke *et al.* 2004)

1x	95°C	7 min
15x	94°C	15sec
	60°C-0.3°C each cycle (30 sec)	
	72°C	3 min
25x	94°C	15 sec
	52°C	30 sec
	72°C	3 min
1x	72°C	7 min
	4°C	hold

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Biographical Information

Jyotiska Chaudhuri graduated from college in 2005 and he received a Bachelor of Technology degree in Biotechnology from West Bengal University of Technology in India. After finishing his undergraduate studies, he engaged himself in voluntary research at the Indian Association for the Cultivation of Science, Kolkata, India. Later in 2006, he moved to Scotland, UK, to pursue his Masters degree. He carried out his Master's research under the guidance of Dr. Vivian Blok at the Scottish Crop Research Institute on characterizing pathogenic genes in a plant parasitic nematode. In 2007, he earned his Master of Science (MSc) degree from the University of Abertay Dundee, UK. On completion of his MSc, he moved to United States to pursue his doctoral studies at The University of Texas, Arlington, under the supervision of Dr. André Pires da Silva. His research focused on understanding why and how new reproductive modes evolve in free-living and parasitic roundworms. He also clarified a mechanism underlying gender difference in life-span using his experimental free-living roundworm species. In August, 2013, he received his PhD in Quantitative Biology from UT Arlington. Both reproduction and aging are essential components of every living organism. His PhD work has the potential to answer key questions regarding designing drugs to treat parasite borne diseases and also improve health-span of aging individuals in the future. Besides pursuing his PhD, he was a Graduate Teaching Assistant in the Biology Department for five years. While pursuing graduate studies at UTA, he was awarded the William L. and Martha Hughes Fellowship for the study of Biology and also the Graduate Dean's Dissertation Fellowship. He aspires to continue post-doctoral research in the field of aging with a vision to engage in a career in academia in the future.