EVOLUTION OF SEX RATIOS AND SEX DETERMINATION MECHANISMS: LESSONS FROM NEMATODES WITH THREE GENDERS

by

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Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF TEXAS AT ARLINGTON

December 2014

Acknowledgements

There are many people who have contributed to my work and supported me to get to this point and I would like to take an opportunity to thank them. First, I am deeply indebted to my advisor Dr. André Pires da Silva for his constant support and guidance during this period. His love for science, enthusiasm and patience are what kept me going for all these years, especially during difficult times. I have learnt from him not only how to perform good experiments and research, but also scientific writing, thinking critically, and presenting my research at conferences. He has always given me valuable guidance and feedback with the freedom to do whatever I wanted. I cannot imagine a better advisor. I want to thank my committee members Dr. Esther Betrán, Dr. Trey Fondon, Dr. Jeff Demuth and Dr. Ricardo Azevedo for their time and valuable input. I am very grateful to Dr. Fondon for managing our lab for all this time and to Dr. Betrán for allowing me to be her student.

I thank the Biology Department for awarding me a teaching and research assistantship and for the various travel grants. I thank the Biology department administrative staff, Linda Taylor, Gloria Burlingham, Sherry Echols, Paulette Williams, and Peggy Fisher for all their help and support in managing the admission, enrollment and graduation.

I thank all members of the Pires lab, past and present, for their inputs, experimental help and friendship. Lenore Price and Arielle Click initially introduced me to lab work and trained me. Sarah Smith, Nymisha Avadhanam and Alissa Hendricks performed many experiments with me. I am very grateful to late Dr. Chad Watkins, who was a great friend and colleague for his inputs, discussions and help both in the lab and outside. I thank Dr. Jyotiska Chaudhuri for his inputs and experimental help. Many undergraduate students have played a crucial role in an efficient functioning of the lab

and experimentation, and I want to thank Sahithi Solasa, Sandra Rodriguez, Linda Trinh, Melvin Verughese, Sueraya Naek, Christine Bateson and Kelcy Steffen for that. I also thank Dr. Sally Adams and Giusepina Zuco for all their help during my stay at Warwick, UK.

I also greatly appreciate the friendship Jacobo Reyes Velasco, David Sanchez, Claudia Marquez, Vikas Kache, Lindsey Caitlin, Bernadette Aguilar, Utpal Smart, and all other fellow graduate students and friends whose company made my time at UT Arlington enjoyable and memorable.

I cannot express enough gratitude towards the love and support of my family. My parents, Shyam Sunder Parihar and Manju Parihar, my brother Dheeraj Parihar and my loving wife Harshita Rathore have been the pillars on which this significant achievement of mine stands. It would not have been possible without their love and encouragement.

Finally, I want to thank the National Science Foundation, UT Arlington Phi Sigma Biological Honor Society, the Biology Department and UT Arlington Graduate Student Senate for providing the funding to conduct my research.

July 22, 2014

Abstract

EVOLUTION OF SEX RATIOS AND SEX DETERMINATION MECHANISMS:

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In sexually reproducing species, the decision to allocate resources towards male and female reproductive function is called sex allocation. Although theoretical and empirical work has been largely successful, the underlying mechanisms of sex allocation are not very well known. For instance, little is known about how the parents adjust the sex of the offspring in response to specific environmental conditions.

According to Mendel's law of segregation, in species that have two genders, a cross between a male and a female should give an equal proportion of male and female progeny. However, there are some species that deviate from this genetic norm and produce skewed sex ratios. For example, some parasitic nematodes (e.g., *Strongyloides*) produce exclusively XX females after crossings between XX females and XO males. Although very intriguing, the molecular mechanisms and selective pressures leading to such sex ratio distortions are difficult to study in parasites, as they cannot be cultured outside the host. Here, we are using a free-living nematode species that shows sex ratio distortion and that could be used as a model to understand mechanisms of sex ratio distortion in parasites.

For my dissertation, I have uncovered a molecular mechanism in the male germline of the free-living nematode strain SB347 that regulates the sex ratio of the cross

progeny. A large proportion of spermatids that can potentially produce males after fertilization are selectively eliminated in the male gonad. Thus, the result of crosses is mostly biased against male offspring. By understanding the mechanisms of sexual allocation and reproduction in free-living nematodes, hopefully new strategies can be created to hamper the propagation of harmful parasites.

I have also studied the evolution of sperm size, which seems to correlate with sperm competition in nematodes with three genders – males, females and hermaphrodites. I found that sperm competition is likely to be influenced by several factors, such as availability of females, proportion of hermaphrodites in a population, and production of male-attractant pheromones by females.

Unbalanced sex ratios can lead to the evolution of sex determination mechanisms that may restore the equal proportion of sexes. This is one of the reasons thought to drive the rapid evolution of sex determination mechanisms. To understand the mechanisms of sex determination, I also undertook comparative studies between *Pristionchus pacificus* and *Caenorhabditis elegans*.

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Preface

This dissertation comprises of a General Introduction chapter, three research chapters and a Conclusion chapter. The General Introduction provides the background and current knowledge of sex allocation theory, mechanisms of sex ratio distortions and the experimental model animals used in my dissertation work. In the three research chapters, I present experiments and results, followed by a final, comprehensive Conclusion chapter that includes potential direction for future research.

Chapter 1

General introduction

Darwin (1871) was fascinated to note that parents divide their reproductive resources and efforts to produce almost equal number of sons and daughters. He understood that proportions of males and females in populations were balanced by natural selection, but could not explain how sex ratios evolved. Fisher (1930) later formalized the theory for sex ratio evolution. He proposed that selection for sex ratios in a population is frequency-dependent, where an equal proportion of male and female offspring is the most evolutionarily stable state.

1.1 Theory of sex allocation

Sex allocation is defined as the allotment of parental resources between male and female offspring in sexually reproducing species (Charnov 1979; Charnov 1982). Parents are expected to apportion their resources to the offspring in a way that maximizes fitness of the parents. Since the gametes produced by males and females usually differ in size, they require differential resource investments. The allocation of sex to the offspring thus, largely depends on whether the mode of reproduction is male-female or hermaphroditic (Charnov 1982). Fisher, in his theory of equal allocation (Fisher 1930), explained that selection favors an equal genetic and resource contribution from each parent as this provides maximum fitness to an individual. This leads to selection of parents that invest resources equally in male and female offspring and a 1:1 sex ratio of the progeny in the population as a whole (Fisher 1930). An unequal investment provides advantage to the parent that can produce offspring of the sex that invests less. He established a framework for sex allocation where selection was dependent on the

frequency of the sexes, which proved to be the groundwork for all the later progresses in the field. For example, let us take a hypothetical male-female species in which production of males and females requires equal investment. If there were a higher proportion of males in the population, on an average each male would mate with less than one female and the reproductive value of females would be greater. Parents would therefore favor the production of more females. On the other hand, if there were an excess of females in the population, the reproductive value of males would be higher and parents would produce more males. Eventually, equilibrium is reached where the average reproductive value of males and females is equal. For our hypothetical example an equal number of males and females will be produced giving a sex ratio of 1:1 (Maynard Smith 1982). However, there are several examples of sex ratio bias in nature, indicating that equal sex ratios are not universally selected.

Can parents control the gender of their offspring? If so, what evolutionary forces and physiological/developmental processes are responsible for the outcome?

1.2 Biased sex ratios can be adaptive

One of the most productive areas of research on evolution of sex ratios has been on examples that violate Fisher's principle. Hamilton (1967) highlighted the impact of local mate competition, individual behavior, and selfishness of sex-linked genes within a genome as possible explanations for highly biased sex ratios. For example, if competition between males is high, Hamilton showed mathematically that in structured populations, producing relatively more females would reduce the competition among kin and subsequently increase fitness for the individuals that bias the sex ratio. Environmental conditions experienced by the parents can also influence the selection of biased sex ratios in a way that maximizes fitness of the parents (Trivers and Willard 1973). For

example, the sex ratio in great-tailed grackles is biased towards females later in the season. A decrease in the food availability during that period increases the mortality of males, which are larger than females and consume more resources (Howe 1977).

Although examples for sex allocation have been reported, relatively little is known about the mechanisms by which sex ratios can be regulated. Most of the early studies were conducted in haplodiploid hymenopteran insects (e.g., bees, wasps), in which females can control the sex of the offspring by facultatively fertilizing the oocytes. In haplodiploid insects the fertilized diploid eggs develop as a females, while the unfertilized haploid eggs develop as males (Cowan and Stahlhut 2004). Sex ratios of these insects were also used to build theoretical models for sex allocation (Hamilton 1967b; Trivers and Hare 1976; Charnov et al. 1981; Charnov 1982). However, for species with heteromorphic sex chromosomes, little is known about how sex ratios get biased (Hardy 2002; Ewen et al. 2004).

1.3 Constraint on adaptive sex ratios due to the mechanism of sex determination

The mechanisms by which organisms allocate sexes have become a subject of interest, especially in animals with other kinds of sex determination than haplo-diploidy (reviewed in West 2009, Komdeur 2012). It is predicted that manipulation of sex ratios by the parents may be more difficult in animals with chromosomal sex determination, because in these animals, a mean sex ratio of 1:1 is expected after random segregation of chromosomes at meiosis (Maynard Smith 1978; Williams 1979; Charnov 1982; Krackow 1995; Leimar 1996; Cockburn et al. 2002). However, many recent studies (mostly in birds) have shown that even under chromosomal sex determination the proportion of male and female offspring can be manipulated (Burley 1981; Madsen and

Shine 1992; Svensson and Nilsson 1996; Heinsohn et al. 1997; Komdeur et al. 1997; Sheldon 1998; Badyaev et al. 2002).

In birds, female is the heterogametic sex. Biased segregation of chromosomes during female meiosis has been proposed as the main mechanism for sex ratio adjustment (Rutkowska and Badyaev 2008). It has been hypothesized that such biased segregation could be a result of inherent differences in the sex chromosomes (size, shape, specific DNA sequences, epigenetic markers, etc.) (Solari 1993; Zwick et al. 1999; Yogev et al. 2000; Tuiskula-Haavisto et al. 2004; Bayne and Liu 2005; Gupta et al. 2006), their placement and orientation at the beginning of meiosis, interactions with microtubules (Dinkel et al. 1979; Zwick et al. 1999; Malik and Bayes 2006), and hormonal mediations. In animals in which the males are the heterogametic sex, no cellular mechanisms are known for as how the parents can control the sex ratios.

1.4 Sex chromosome segregation and meiotic drive as distorters of sex ratios in animals

It is important to note also that some examples of sex ratio bias may be due to non-adaptive mechanisms. For example, species with temperature-dependent sex determination might experience extreme temperatures in critical periods during development (Charnier 1966; Pieau 1971; Pieau 1972; Bull 1983; Lang and Andrews 1994), infection of eggs with the bacteria *Wolbachia* in arthropods can skew the sex ratio by killing males or feminizing XY individuals (Dunn and Hatcher 1997; Bouchon et al. 1998), and meiotic drive can lead to unequal transmission of sex chromosomes (Jaenike 2001).

In some populations of the fruit fly *Drosophila pseudoobscura* with meiotic drive, the mechanistic basis for sex bias is based on the failure of Y-bearing sperm to mature (Novitski and Sandler 1957; Novitski et al. 1965). The X chromosome that suppresses

the transmission of Y is called the driving X-chromosome. If the driving X-chromosome gets fixed in the population, males may become so scarce that it could potentially lead to the extinction of the species (Hamilton 1967b; Jaenike 2001).

1.5 Three-gender nematodes as experimental models

Nematodes have a broad variety of reproductive systems and thus have been excellent experimental models for studying the evolution, dynamics and population genetics of sex determination systems, and sex ratio distortion (Kiontke et al. 2004; Haag 2005; Ellis and Schedl 2007). Mating systems in nematodes include gonochoristic (male/female), hermaphroditic (including hermaphrodite/male and hermaphrodite/male/female), parthenogenetic and heterogonic (alternation between sexual reproduction and parthenogenesis). Based on phylogenetic evidence, it has been proposed that hermaphroditism has evolved several times independently from gonochoristic ancestors (Kiontke et al. 2004). However, not much is known about how these mating systems evolved.

Caenorhabditis elegans, one of the best-studied nematodes, is a hermaphrodite/male species, in which the male proportion in a population is usually very low (~ 0.1 %). Hermaphrodites are XX and produce both sperm and oocytes, allowing them to reproduce by self-fertilization or by mating with males. Males are XO and are produced as the result of a random event of chromosomal non-disjunction during hermaphrodite gametogenesis, or the result of a cross between a male and a hermaphrodite. As expected from animals with a XX/XO sex determination system, males are produced as 50% of the cross-progeny between a male and a hermaphrodite. However, in certain parasitic nematodes such as Heterorhabditis (Dix et al. 1994), the male/female cross ratio is heavily skewed towards the non-male sex. Studying the

genetics and cell biology of these deviant sexual ratios in parasites is difficult because they cannot be cultured outside the host. Only one other study has been recently reported in free-living nematodes, in which hermaphrodite-biased sex ratios occur after crossing (Shinya et al. 2014). However, the molecular basis for such bias is not known.

The free-living roundworm Rhabditidae Gen. 1 sp 1. strain SB347, henceforth referred to as SB347 has three genders (trioecious) – males, females, and hermaphrodites, and produces almost entirely non-male progeny upon crossing (Félix 2004). The three genders are also observed in the insect parasite *Heterorhabditis bacteriophora* and thus, this species could be used as a model to study the evolution of sex-ratio distortions as observed in parasitic nematodes (Dix et al. 1994). SB347 is also more closely related to parasitic species that show female biased sex ratios like *H. bacteriophora* and *Rhabditella axei* than *C. elegans* (Kiontke and Fitch, personal communication). SB347 hermaphrodites and females are very similar in appearance and the hermaphrodites are able to self-fertilize. Given the opportunity, hermaphrodites also reproduce by mating with males, but cannot fertilize other individuals (Shakes et al. 2011). SB347 females and hermaphrodites are isogenic and have identical karyotype (XX), whereas males are XO. The three genders are produced in all occasions, either by self-fertilization of hermaphrodites, or by crosses of males/females or males/hermaphrodites.

Although genetically identical, SB347 females and hermaphrodites take different paths to develop into adults, and are produced in a specific sequence by the hermaphrodite mother. The first 30 eggs of a hermaphrodite mother mostly develop into females, whereas subsequent progeny usually develops into hermaphrodites (Chaudhuri et al, 2011). The female larvae can be distinguished from hermaphrodite larvae already at the L1 stage (Fig. 1.1), by the size of the gonad primordium: larvae with large gonad

primordium develop into females and larvae with a small gonad primordium always develop through a resistant stage called dauer, to later become a hermaphrodite (Felix, 2004; Chaudhuri et al, 2011).

In *C. elegans*, the L1s become dauers only when exposed to unfavorable environmental conditions. In SB347, however, larvae with a small gonad primordium are pre-set to always become dauers, which later develop into hermaphrodites. This suggests that dauer formation and hermaphroditism are mechanistically linked. To test this, the SB347 L1 larvae with large gonads were forced to become dauers by submitting them to stressful conditions. The fact that they developed into hermaphrodites instead of females indicates that dauer formation is necessary and sufficient for hermaphrodite development (Chaudhuri et al, 2011).

Dauers can survive various environmental stresses such as lack of food, elevated temperatures, low humidity, etc. for extended periods of time (Cassada and Russell 1975). They are highly stress resistant, can stay in a non-feeding state for months and are very active. These adaptations help them survive during stress and facilitate dispersal to favorable habitats. The dauers attach themselves to insects and other animals in order to relocate to better conditions. This stage is similar to the infective juvenile stage in parasites and the genetic pathways for the two are also conserved (Ogawa et al. 2009). Since a dauer is destined to become a hermaphrodite in SB347, colonization of those favorable habitats is possible even in the absence of mates (Anderson et al. 2010).

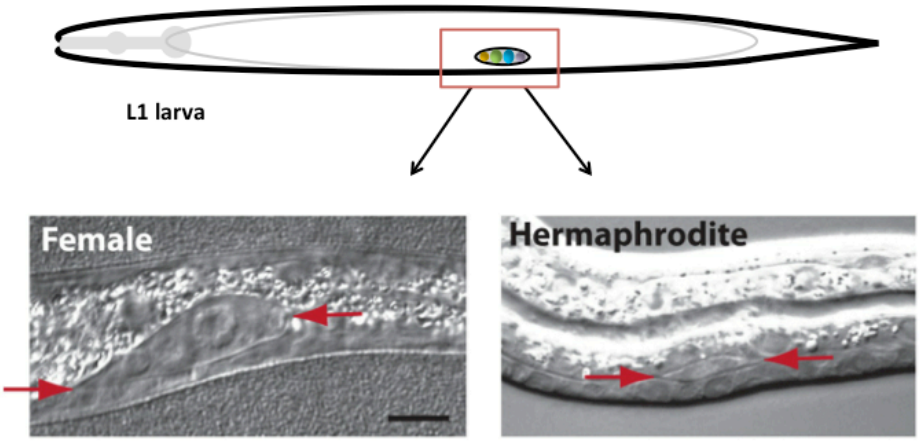

Figure 1.1 Relative size differences between the hermaphrodites and females of SB347 at the L1 stage. Both pictures were taken at equal magnification using DIC optics. The female gonad is larger than the hermaphrodite gonad. Arrows indicate the ends of the gonadal primordium. Anterior is to the left.

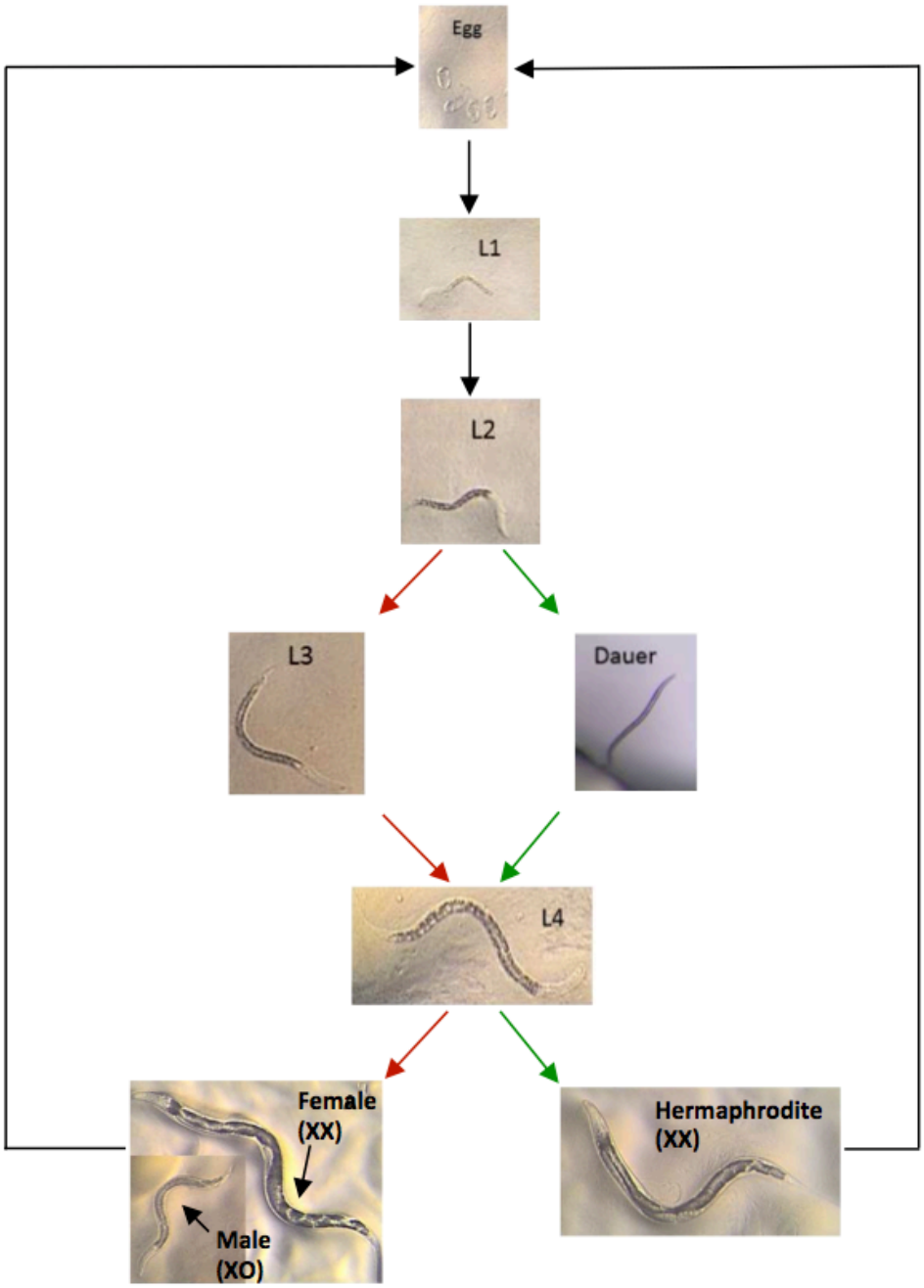

Figure 1.2 Life cycle of SB347: Larvae destined to form hermaphrodites form dauers, whereas females form L3 larvae. Males also develop from L1 to L4, but do not form dauers.

Similar to SB347, there are four other strains of free-living nematodes that are trioecious and were used as model animals for our experimentation: Rhabditidae Gen. 1. sp. 2 strain SB372, Rhabditidae Gen. 1. sp. 3 strain JU1783, Rhabditidae Gen. 1. sp. 4 strain JU1809 and Rhabditidae Gen.1. sp. 5 strain JU1782. These strains will henceforth be referred to as SB372, JU1783, JU1809, and JU1782, respectively. Reciprocal crosses were previously performed between each of these species and no viable progeny were produced. Thus, it was concluded that they are different species (Pires-daSilva, personal communication). Table 1.1 shows the locations from where each of these strains was isolated. Previous studies have shown that these trioecious species are in the same clade as SB347, and are close relatives of the facultative parasite *Rhabditella axei*, (Kiontke et al. 2004; Chaudhuri, personal communication, Fig. 1.3).

Table 1.1 Isolation information for the trioecious species used for our experiments

Strain	**Isolated from**	**Location**	**Year**
SB347	Blood-engorged deer ticks	Rhode Island, USA	2001
SB372	Horse dung pile	Freiburg, Germany	2003
JU1783	Star fruit	St. Benoist, France	2009
JU1809	Rotting stem of *Symphytum officinale*	Santeuil, France	2009

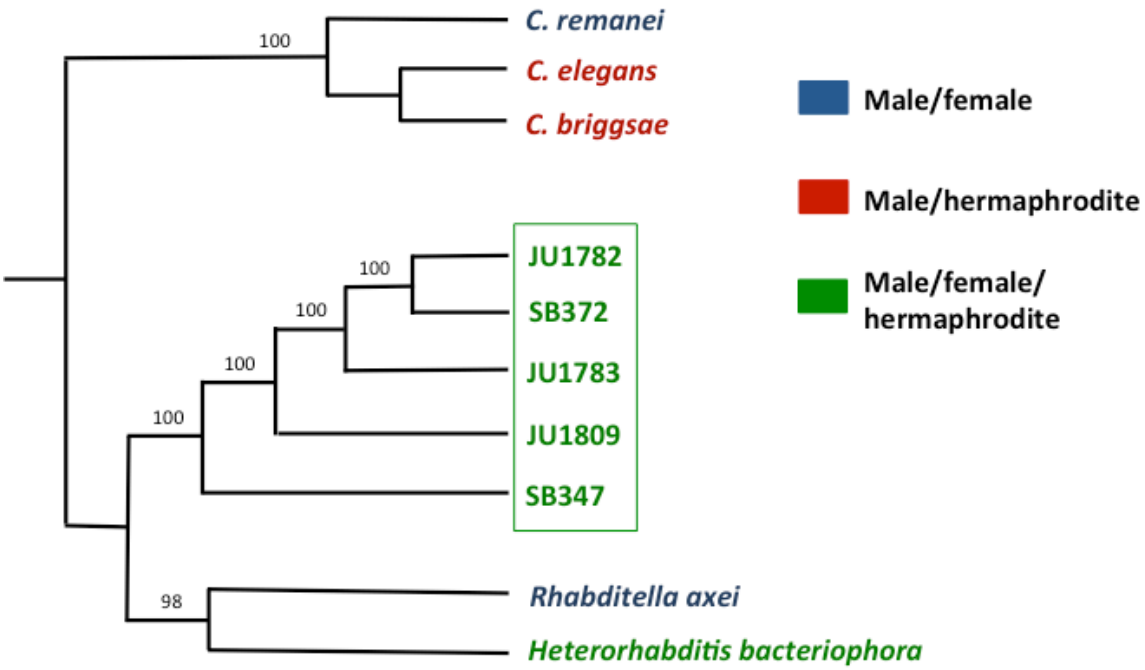

Figure 1.3 Phylogenetic tree of trioecious strains used in this work (shown in box) and their relationship with other species (Kiontke et al. 2004; Chaudhuri et al, personal communication). Phylogeny determined from weighted parsimony jackknife analysis of 28S and 18S cDNA sequences, numbers represent bootstrap values.

For my dissertation research, I employed SB347 and related trioecious nematodes to study some variables that could influence distortions in the sex ratios and drive evolution of reproductive traits. In this work, I uncovered a new molecular mechanism of sex ratio regulation in SB347, although it is unclear if selection or selfish elements explain the evolution of this trait. I have also used the knowledge available for the *C. elegans* sex-determination pathway to study the evolution of sex determination pathway in nematodes.

Chapter 2

Evolution of sperm size in trioecious nematodes

2.1 Introduction

Sexual selection, as defined by Darwin (1871) is 'the advantage that certain individuals have over others of the same sex and species in exclusive relation to reproduction. Sexual selection arises in organisms as consequence of competition for mates. It is widely accepted now that such competition is a strong selective force that results in the appearance of the most extravagant morphologies in the animal kingdom, including the giant antlers of the Irish elk and the ornamentation in males of birds-of-paradise. The evolution of these extravagant traits seems unfavorable for their survival and thus seemed to challenge the theory of evolution by natural selection. Therefore, sexual selection has generated many compelling research problems and is one of the most active and intense areas of study in evolutionary biology.

2.1.1 Origin of sexual selection

Sexual selection originates from the differential investment in the gametes by the two sexes (Trivers 1972; Andersson 1994). One sex produces a large number of small gametes and the other produces few, but large gametes (Parker et al. 1972). The evolution of two different gametes establishes a battleground for sexual conflict. The few gametes produced by female are costly, whereas the male gametes, being smaller, are comparatively cheap. It is thus predicted that males should mate with as many females as possible, so that a maximum number of their gametes are used. In doing so, males have to compete with other males that are also trying to achieve the same goal. On the other hand, since females invest a lot of resources in producing a single egg, it is of their

own best interest to pick the best mate to fertilize it. The males therefore rapidly evolve traits that either helps them fight off their competitors or make them more attractive to females.

Sexual selection refers to selection of traits solely for the purpose of increasing success at reproduction. For example, the giant antlers of elk or the bright colored feathers of peacocks do not appear to be traits evolved for survival, but have been selected only to compete with other suitors for mating. Such conspicuous sex traits are favored by sexual selection and kept in check by natural selection. However, in some cases there can be an overlap and traits may be selected by both sexual and natural selection (Andersson 1994). For example, selection for better sensory or locomotory apparatuses could evolve by means of natural (to find food and escape predators) or sexual (to find mates faster than the competitors) selection.

2.1.2 Mechanisms of sexual selection

Selection for traits specific to reproductive success mainly occurs at two levels – competition between individuals of the same sex to secure mates (intra-sexual selection), and the attractiveness of one sex to get chosen by the other (inter-sexual selection). In case of intra-sexual competition, the mechanisms of sexual selection include contests (e.g., size, strength, threat signals), early search to find mates before rivals, and endurance for remaining reproductively active for longer periods (Andersson and Iwasa 1996). Mate choice (inter-sexual selection) is decided based on phenotypic traits (ornamentation, better protection and nutrition, etc.), but the mechanisms are still a matter of debate (Andersson and Simmons 2006). A possible mechanism is that a particular phenotype indicates better fitness or correlates with some genetic component that is of benefit to the offspring (Andersson and Simmons 2006). Reproductive success

however, does not end at securing and copulating with a mate, especially in species that are not monogamous. Even after successful mating, the competition and selection continues, such as through sperm competition, selective fertilization, and killing of embryos or infants (Birkhead and Møller 1998).

2.1.3 Sperm competition

Male-male competition is common in most species, leading to selection either before or after copulation (Smith 1984; Andersson 1994). One of the major mechanisms of post-copulation sexual selection is the competition between the sperm from two or more males in order to fertilize the oocytes (Birkhead and Møller 1998). The sperm usually compete inside the female reproductive tract. This involves competition between sperm of a single male in the same ejaculate and may include sperm from other males (Parker and Begon 1993). During such a competition, a simple adaptation to improve chances of success at fertilization is to produce a great quantity of sperm to provide better odds, much like a 'lottery' (Parker 1982; Parker 1984). Such males would be at a selective advantage. However, in cases where females mate with several different males, sperm shape, size and motility may also play a role (Haig and Bergstrom 1995; LaMunyon and Ward 2002; Murray and Cutter 2011).

Theoretical modeling for evolution of sperm under competition presumes that there is a trade-off between the number and size of sperm (Parker and Partridge 1998; Murray and Cutter 2011). The higher number of sperm under competition provides an advantage by means of mere odds. However, larger sperm are able to move faster to reach the oocyte and are therefore sometimes selected over a large number of smaller sperm (Gomendio and Roldan 1991; LaMunyon and Ward 2002; Tourmente et al. 2009).

2.1.4 Sperm competition in nematodes

In nematodes, larger sperm evolves when there is stronger sperm competition (LaMunyon and Ward 1999). In self-fertilizing nematode species with males, male sperm must compete not only with sperm from other males, but also with the hermaphrodite sperm stored in their spermatheca. The predominant sperm competition however, is male-hermaphrodite because the proportion of males is extremely low and thus male sperm rarely has to compete with sperm from other males. In *C. elegans*, for example, the male sperm can outcompete the hermaphrodite sperm which is very small in size (LaMunyon and Ward 1995). The male sperm is larger and can displace the hermaphrodite sperm when inside the spermatheca.

In male-female species like *C. remanei*, males comprise about half of the population. In these species, males compete with other males, resulting in the evolution of larger sperm (LaMunyon and Ward 1999). In addition to sperm competition, the male and female must find each other and reproduce for the population to survive. Such evolutionary forces can therefore drive not only the size of the sperm, but the over all morphology and physiology of the organisms.

2.1.5 Trioecious nematodes present a unique scenario of sperm competition

Studies on nematode sperm evolution have so far been conducted mostly on male/female or hermaphrodites/male species (LaMunyon and Ward 1999; Cutter 2004; Garcia et al. 2007; Cutter 2008). Trioecious (male/female/hermaphrodite) species provide an excellent model to study evolution of traits due to reproductive selection and sex-ratio dynamics and help answer several additional questions. For example, the natural occurrence of males in *C. elegans* is very low (<1%) and therefore, the likelihood of direct sperm competition from other males is very low. It would therefore be safe to assume that

the selection pressure on the males for a larger, more efficient sperm would be lower than in species with 50% males (Garcia et al. 2007). Using species that have both females and hermaphrodites available for the males to mate will help us to directly compare and estimate the strength of selection in each case.

Female choice, facilitation in mating, and biases towards selecting a parent have been known to play a very strong role in sexual selection (Waage 1979; Thornhill and Alcock 1983). Females of some nematodes have been known to produce pheromones that attract males, driving strong competition among them (Aumann et al. 1998; Chasnov et al. 2007; Choe et al. 2012). *C. elegans* hermaphrodites however, do not produce a very strong attractant (Chasnov et al. 2007; Srinivasan et al. 2008; Srinivasan et al. 2012). This has been attributed to the fact that the hermaphrodites produce their own sperm and do not need to attract the males to fertilize their oocytes. It has been proposed that as the hermaphrodites evolved the ability to produce sperm, they lost the ability to attract males (Chasnov et al. 2007; Garcia et al. 2007). Here again, the trioecious species are a good experimental model to test if there is a differential attraction of the males towards hermaphrodites and females.

A higher attraction of males towards females presumably leads to a higher rate of mating, compared to the rate of male-hermaphrodite mating. This could have dissimilar effects on the fitness of females and hermaphrodites. For example, Gems and Riddle (1996) showed that *C. elegans* hermaphrodites, when mated with males, have a shorter lifespan than the hermaphrodites that did not mate.

It is however not a straightforward task to answer complex evolutionary questions as above, in spite of the availability of the best model systems. Especially in nematodes, closely related species may have very different mating systems (Kiontke et al. 2004). This implies that the evolutionary forces that apply to one species may not be applicable

to a closely related species. On the contrary, it could also imply that the same selective pressure results in different responses and adaptations in different species, directing them to distinct evolutionary trajectories. It therefore becomes imperative to perform a rather comprehensive comparative study in model systems that are closely related. Comparative studies on species that are closely related will provide an insight into patterns of change in sperm size due to selective forces.

For the experiments in this thesis, I use the free-living nematode species SB347, SB372, JU1782, JU1783 and JU1783. All these species are trioecious and are currently classified under the same genus (pers. commun., Karin Kiontke).

Here, I hypothesize that the sperm size is influenced and correlates with whether females produce chemical attractants. A greater incidence of attraction and mating would lead to a higher sperm competition among conspecific males, and thus the evolution of larger sperm. I also hypothesize that different amount of mating would result in the evolution of different lifespans of females and hermaphrodites.

2.2 Methods

2.2.1 Worm strains and maintenance

The following strains were used for the experiments in this chapter: SB347, SB372, JU1782, JU1783, JU1809. The strain SB347, after 50 rounds of inbreeding was renamed to APS4.

Worms were cultured and maintained at 20°C on 6 cm culture plates (unless otherwise specified) containing nematode growth medium (NGM) (Stiernagle 2006). The plates with NGM were seeded with a lawn of the streptomycin-resistant *Escherichia coli* strain OP50-1.

2.2.2 Measuring sex ratios in a population

All experiments were performed at 20°C and maintained under optimal growth conditions, with abundant food. To isolate virgin animals, L1 or L2 larvae were picked onto individual wells of 12 well plates seeded with OP50-1. Once they became adults, males were distinguished from other sexes by their copulatory apparatus in the tail. Hermaphrodites and females were differentiated by whether they laid fertile eggs or not.

2.2.3 Extraction of sperm and size measurement

To improve the adhesion of the sperm cells to the slide, slides were pre-treated with Poly-L-Lysine. A 4-5 µL drop of 100% Poly-L-Lysine was placed at the center of the charged surface of a ColorFrost Plus slide. A second slide was placed on top of it in such a way that both charged surfaces were facing each other and a thin film of liquid formed between them. Once the film was formed, slides were separated by sliding them over each other. Subsequently, slides were baked at 60°C for 1-2 hours.

To release the sperm, animals were cut using a 22 gauge needle in the presence of 5-10 µL drop of sperm buffer (90 mM NaCl, 50 mM KCl, 2 mM $MgCl_2$, 10 mM $CaCl_2$, 100 mM HEPES, and 20 mM dextrose to pH 7.8 for a 2x solution) (Shakes et al. 2011). Males were cut at the level of the *vas deferens* and hermaphrodites at the level of the spermatheca (Shakes et al. 2011). Immediately following the dissections, the slides were examined using DIC optics on a Carl Zeiss 510 confocal microscope. In case the sperm were too small to be identified by DIC optics, Hoechst 33342 (Sigma-Aldrich) was added to the sperm media at a final concentration of 100 µg/ml. Hoechst helped to visualize and identify the characteristic condensed shape of the sperm nucleus. Sperm size was measured as the cross sectional area of the spermatid for all the species using the Carl Zeiss confocal software.

2.2.4 Mating efficiency and male attraction assays

Seven virgin young adult females were allowed to mate with one virgin young adult male on a 6 cm plate that was seeded with a 1 cm diameter spot of OP50-1. The males and females were allowed to mate for one hour, after which males were removed and females were transferred onto individual wells of a 12 well NGM plate. Presence or absence of eggs was noted to determine if the females were fertilized.

Male attraction towards supernatant collected from young females and hermaphrodites was measured against a control of M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 liter, sterilized by autoclaving) (described in detail and performed by Chaudhuri 2013). The percentage of males that were attracted towards either the treatment or control, when given a choice between the two was calculated as the chemotaxis index (CI). CI is the number of males attracted to the treatment subtracted by the number of males at the control, divided by the total number of males in the experiment (Bargmann and Horvitz 1991).

2.2.5 Lifespan measurements

The lifespan assays were carried out using worms cultured on standard NGM seeded with a lawn of the bacteria *E. coli* OP50-1 at 20°C. Age was measured as number of days the worm stayed alive as adult. The worms were identified to be females or hermaphrodites at the L2 stage by the difference in the gonad morphology (Félix 2004). About 30-40 worms of the same gender were pooled onto the same 6 cm NGM plate, and a total of about 100 worms were used for each sex (3 replicates of 30-40 worms each).

For females, it takes about 2 days from L1 to adulthood. Since the hermaphrodites go through the dauer stage, which lasts for about 24 hours in case of

APS4, they take a bit longer to become adults from L2s compared to females. However, for JU1782 the duration of dauer stage is slightly variable. For JU1782, several dauers were picked and the animals that exited the dauer stage at the same time were pooled together in order to get an age-synchronized group.

For hermaphrodites, the first day of adulthood (day 1) was considered as the day they started laying eggs. To avoid self-progeny overgrowth, the hermaphrodites were transferred onto fresh plates every alternate day. To control for potential effects of transferring worms on lifespan, females were also transferred at the same rate. The time of death was defined as the point in time when worms failed to respond to a gentle poke with the pick, or when the pharynx stopped pumping. Animals that escaped the plate or died of 'non-aging' causes such as bagging (eggs hatching inside the mother) were censored.

2.3 Results

2.3.1 Proportion of males is very low in populations

Sex ratios in healthy non-crowded populations were measured by randomly picking larvae from a culture plate and allowing them to develop in isolation. For all species examined, the sex ratios were biased against males (Fig. 2.1). The proportion of males however, was very low in all the species, with the highest being in JU1783 (16.6%) and the lowest in SB372 (1.4%). Except JU1783, which had mostly females, all the species predominantly consisted of the self-fertilizing hermaphrodites (Fig. 2.1, Appendix A).

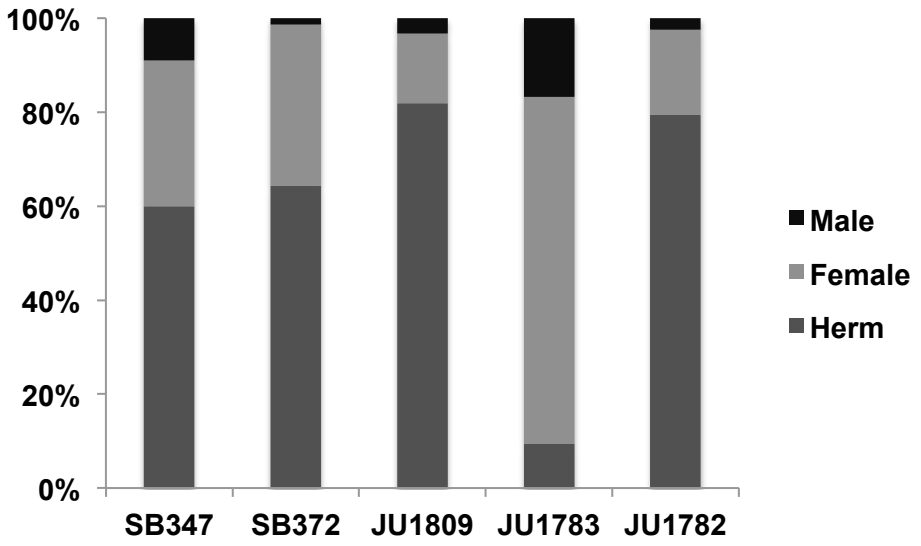

Figure 2.1. Proportion of males, females and hermaphrodites in trioecious species. All species have a female/hermaphrodite biased sex-ratio with low proportions of males. JU1783 had the highest percentage of males: 16.6%, n=192; SB347: 9.0% males, n=200; SB372: 1.3% males, n=146, JU1809: 3.2% males, n=188, JU1782: 2.4% males, n=166.

2.3.2 SB347 has the largest male and smallest hermaphrodite sperm

In nematodes, sperm competition favors the evolution of larger sperm (LaMunyon and Ward 1999). We thus hypothesize that the males of trioecious species will have larger sperm than the sperm of conspecific hermaphrodites. We measured sperm size by quantifying the cross-sectional area of these cells, both for males and the hermaphrodites. Males of SB347, SB372 and JU1809 showed significantly larger sperm in comparison to the sperm of hermaphrodites. SB347 is the species with the largest male sperm (mean area of 9.4 μm^2 ± 0.2 SE), with some sperm reaching up to 13.0 μm^2 (Fig. 2.2). SB347 also had the smallest hermaphrodite sperm, with an average area of 1.8 μm^2 ± 0.1 SE. SB347 therefore showed the largest disparity between the sizes of the male and the hermaphrodite sperm. The smallest male sperm and the largest hermaphrodite sperm was that of JU1782, with average sizes of 3.4 μm^2 (± 0.1 SE) for both sexes.

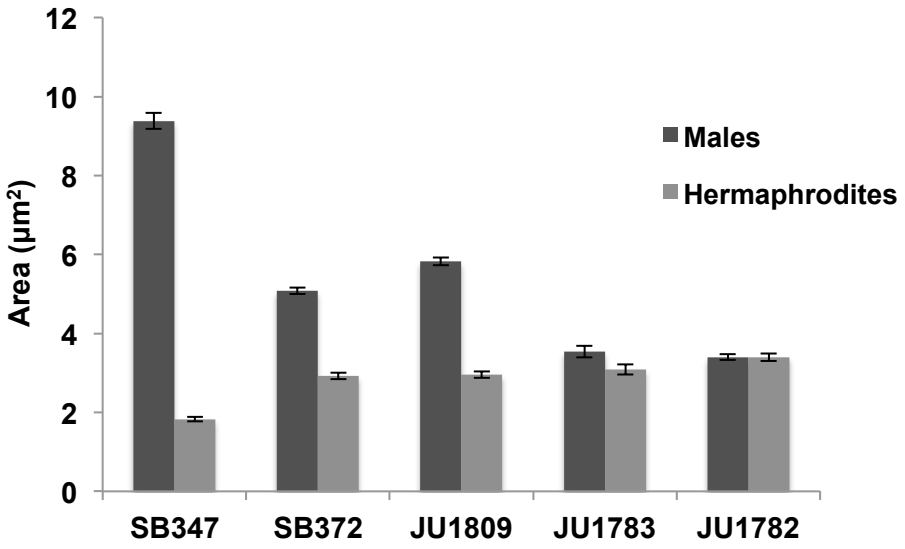

Figure 2.2. Sperm size comparison of males and hermaphrodites. Error bars represent standard error of mean. Differences between the males and hermaphrodites of SB347, SB372 and JU1809 are significant (Mann Whitney rank sum test, $p<0.01$). Spermatids were measured from at least 5 worms for each species. SB347 spermatids: n=61 for males and n=67 for hermaphrodites; SB372 spermatids: n=68 for males and n=70 for hermaphrodites; JU1809 spermatids: n=111 for males and n=50 for hermaphrodites; JU1783 spermatids: n=48 for males and n=43 for hermaphrodites; JU1782 spermatids: n=54 for males and n=47 for hermaphrodites

2.3.3 SB347 has the highest mating efficiency

In androdioecious species like *C. elegans* the male sperm must outcompete the hermaphrodite sperm to fertilize the egg. However, in trioecious species, a large fraction of the population is composed of females, which do not produce any sperm. Thus, we expect greater competition of male sperm to conspecific male sperm. It has been shown previously that males of gonochoristic species have larger sperm than the males of hermaphroditic species because of a greater sperm competition (LaMunyon and Ward 1999). Since our results showed differences between the male and the hermaphrodite sperm and magnitude of this disparity varied between species, it was of interest to investigate if the sperm size was correlated with the efficiency of the male to mate with females.

We measured mating efficiency as the number of females a single male could fertilize in one hour. Females, and not hermaphrodites were chosen for these assays because the cross progeny in hermaphrodites cannot be distinguished from their self-progeny. Also, previous experiments performed by another PhD student in the laboratory have shown little to no attraction of the males towards the hermaphrodites (Chaudhuri 2013). SB347 males had the highest mating efficiency, with each male fertilizing an average of about 4 females in one hour (Fig. 2.3). This suggests an association between the ability of the male to mate with a female and evolution of its sperm.

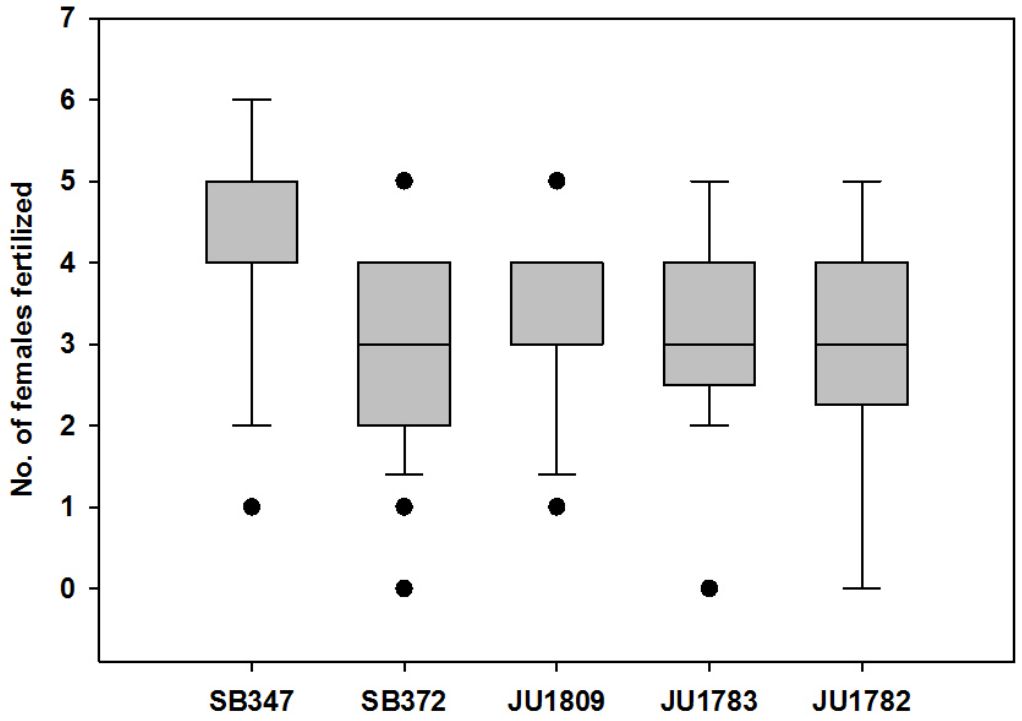

Figure 2.3. Box plot showing mating efficiency comparisons. One male was allowed to mate with 7 females for one hour and the number of females fertilized was recorded. For the number of crosses, n= 28 for SB347, n= 23 for SB372, n= 23 for JU1809, n= 21 for JU1783 and n= 20 for JU1782. Mating efficiency of SB347 is significantly higher than all the others (Mann-Whitney rank sum test, $p<0.01$ for all comparisons with SB347; U statistic for comparison with SB347 is 154.4 for JU1782, 174.5 for JU1809, 157.5 for JU1783 and 136.5 for SB372).

There are several factors that influence the ability of the male to mate with a female, and finding mates is one of the most important among these. Previous studies have shown that females produce and secrete sex pheromones that attract males for mating (Barr and Sternberg 1999; Simon and Sternberg 2002; White et al. 2007). Experiments were performed in our lab to test attractiveness of females to males (Chaudhuri 2013), where media conditioned with virgin females were tested for male attraction. The attraction was measured by using the chemotaxis index or CI (refer to Methods 2.2.4). The highest CI was observed between the SB347 males and its female conditioned media, with a mean of 0.64 (a CI of 1 means maximum chemotaxis). JU1783 and JU1782 had the lowest CI, averaging 0.02 and 0.03 respectively (Fig. 2.4). This data indicates that males of SB347 face the highest intrasexual competition in comparison to the other species. A higher attraction towards the female makes it easier for males to locate them, increasing their chances of mating.

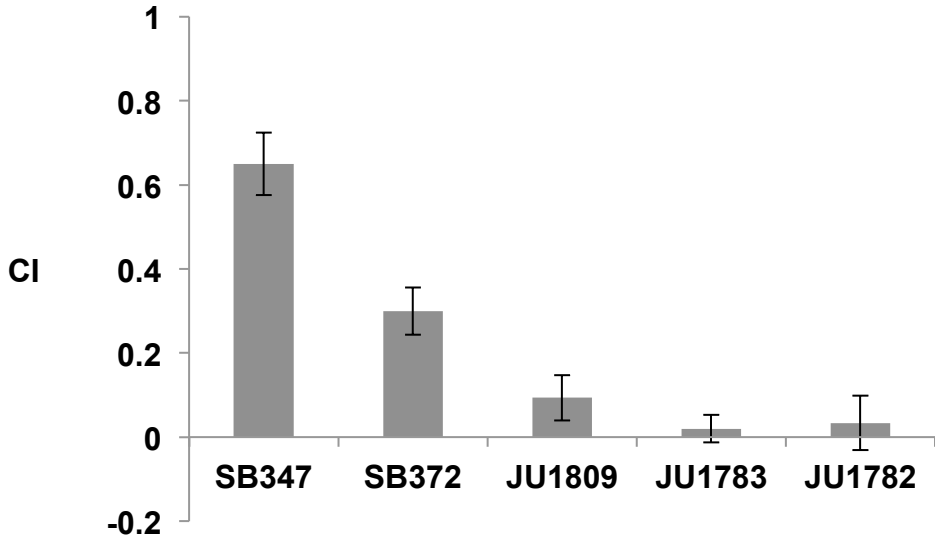

Figure 2.4. Chemotaxis indices of males of trioecious species (Pires-daSilva, personal communication). A value of 1 indicates 100% of males get attracted to the female pheromone and a negative value indicates repulsion. Error bars represent standard error.

2.3.4 Association between mating and lifespan

The evolutionary effects of sexual reproduction on an organism are not limited to the quality of gametes produced. Mating has long been postulated to increase mortality rates because of the cost associated with reproductive stress and gamete production (Williams 1957; Hamilton 1967a; Gems and Riddle 1996). Our sperm size comparisons, mating efficiency results and the CI values revealed a very striking difference between the species SB347 and JU1782.

To test a possible correlation between reproductive stress and lifespan, we also compared the lifespans of females and hermaphrodites of these two species. We hypothesized that females of SB347 would have a shorter lifespan than hermaphrodites, but the same may not be true for JU1782. This is because the SB347 females are more attractive to males and the males are very efficient at mating. The hermaphrodites do not attract males and do not face the same amount of mating stress. However, for JU1782, males are not very efficient at mating and do not show much attraction towards females. Therefore, the reproductive stress due to mating for JU1782 females and hermaphrodites should be similar and there will not be a difference in their lifespan.

The mean lifespan of SB347 hermaphrodites (n= 79) was 7.8 ± 0.2 SE days and of females was 6.7 ± 0.1 SE days (n= 112) and the difference was significant (Kaplan-Meier Log Rank Survival Analysis, $p<0.001$) (Fig. 2.5, Appendix B). However, the hermaphrodites and females of JU1782 did not show this difference in life span; hermaphrodites here had an average lifespan of 7.7 ± 0.1 SE days (n=89) and the females lived for 7.6 ± 0.1 SE days (n=72) (Kaplan-Meier Log Rank Survival Analysis) (Fig. 2.6, Appendix B).

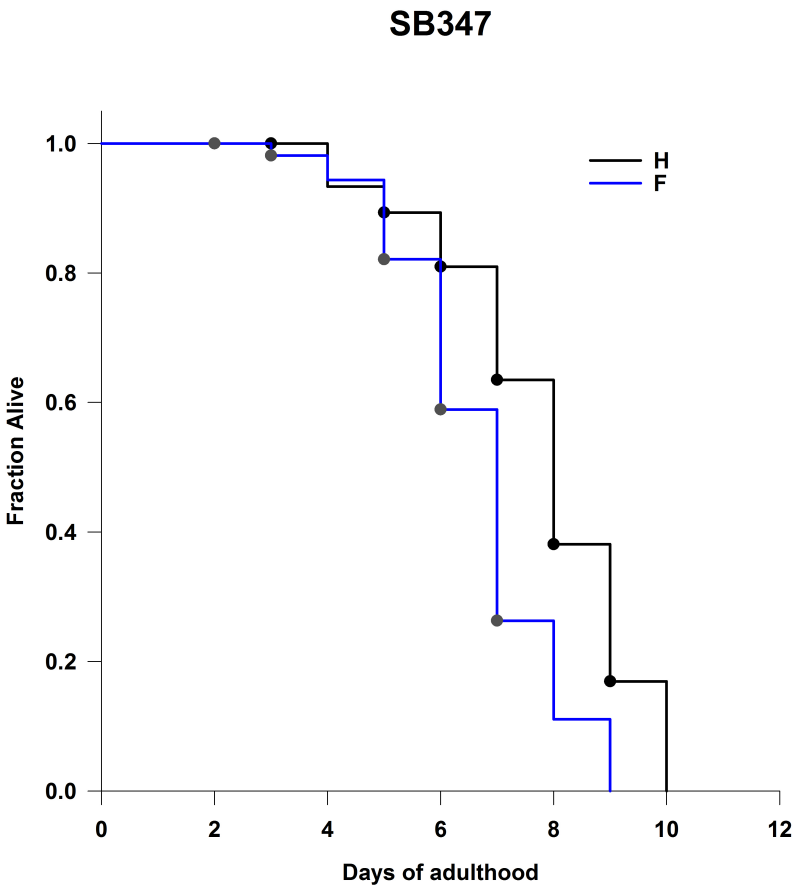

Figure 2.5 Lifespan of SB347 hermaphrodites and females. Hermaphrodites (n=79, 7.8 ± 0.2 SE) live significantly longer than females (n= 112, 6.7 days ± 0.1 SE) (Kaplan-Meier Log Rank Survival Analysis, $p<0.001$)

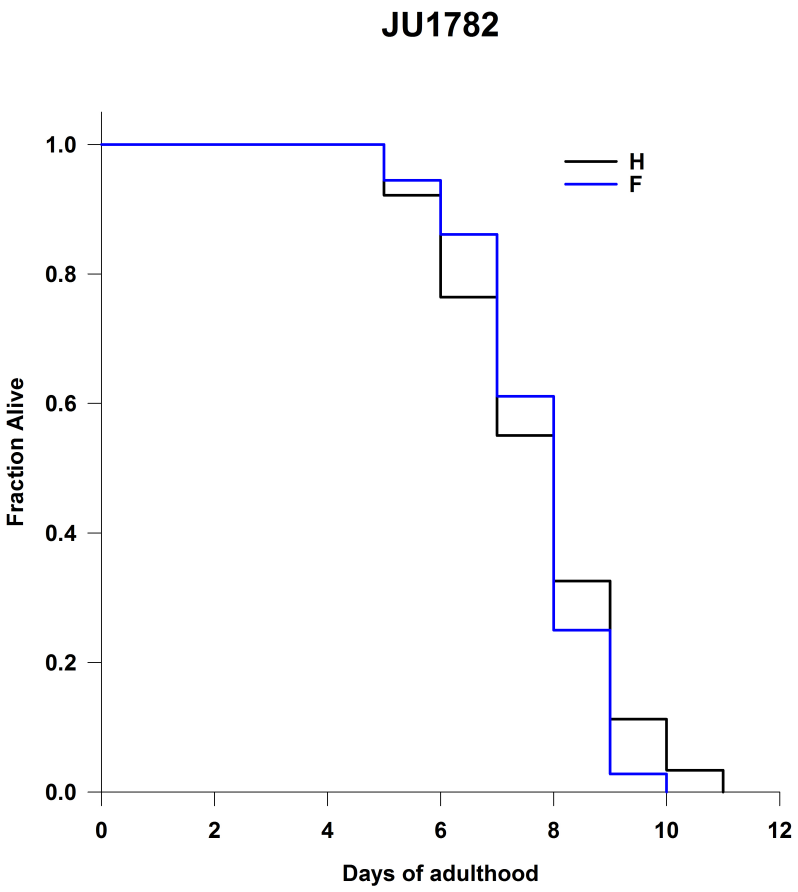

Figure 2.6 Lifespan of JU1782 hermaphrodites and females. Hermaphrodites (n=89, 7.7 ± 0.2 SE days) and females (n=72, 7.6 ± 0.1 SE days) did not show a significant difference in the lifespan (Kaplan-Meier Log Rank Survival Analysis, p = 0.429).

2.4 Discussion

Under limited resources, production of a large number of smaller sized sperm is advantageous for hermaphrodites. However, this does not prove to be a very good strategy for males for two reasons. Firstly, they predominantly mate with females because of their attraction towards female pheromones. The females in these species mate with several different males and therefore only the most competitive ones are selected. Secondly, even if a male mates with a hermaphrodite, it still needs to outcompete the hermaphrodite sperm in order to get selected.

The sex-ratios in populations for all the trioecious species examined in our experiments have a bias against males. It might appear on first glance that this being the case, males would have plenty of opportunity to mate and thus suffer little competition with sperm from other males. However, it becomes clear from the results of the attraction assays that males are attracted mostly towards females. Our data supports the hypothesis that although the increase in size of male sperm is driven by male-male competition, it is an indirect consequence of the chemical attractant produced by females. Species in which males show a higher attraction for females pose a greater male-male sperm competition compared to male-hermaphrodite species. In species where the attraction of males towards females is low (JU1783 and JU1782; Fig 2.4), the predominant sperm competition is between hermaphrodite and male sperm. It is of benefit for the hermaphrodite to produce a large number of smaller sperm rather than producing a low number of large sperm since essentially all of the hermaphrodite sperm are used to fertilize the egg. This produces a large number of progeny and facilitates rapid colonization of new habitats. In a rare instance of the hermaphrodite mating, male sperm gets preference. This again, is of benefit for the hermaphrodite as it introduces

genetic variation in the progeny and increases the chances of survival under unfavorable conditions. In either case, it makes sense for the hermaphrodites to invest more in the quantity of sperm rather than size (Murray and Cutter 2011).

Although our data shows a positive correlation between the mating efficiency, attraction of males towards females and the size of the sperm, there are other factors that could also play a role in the evolution of sperm size. Females are known to provide preferential fertilization opportunities to some sperm for their oocytes after mating with several males (Radwan 1996), and also store sperm for a long time to use it for fertilization later (Otronen et al. 2010). Such factors may be cryptic in nature and could select for traits other than a larger sperm. Furthermore, the proportion of females and hermaphrodites in a population has been reported to be very flexible depending on the environmental conditions, to the extent that early larvae destined to become females can be converted to hermaphrodites and vice-versa (Chaudhuri et al. 2011a). The role of environmental factors in more realistic conditions may explain the results that did fit the predictions. For example, we observed that in SB372 male sperm is larger than hermaphrodite sperm (Fig 2.2) and males also get attracted towards female supernatant (Fig 2.4). However, the mating efficiency of SB372 despite these two factors is low (Fig 2.3). This is a clear indication that there are other factors as mentioned above and not studied in this dissertation that can affect the evolution of the sperm size.

A larger gamete does not come without a cost and neither does sex. The males and females must spend resources and energy to find mates, and in addition to that, sexual selection may not always choose individuals that are the fittest for survival (Maynard Smith 1978). Searching for a mate, as well as the act of mating itself makes the individuals vulnerable to adversities such as predators, sexually transmitted diseases and physical wear due to loss of energy. Mating has therefore been suggested to be a reason

for shorter life span (Gems and Riddle 1996; Bakker et al. 2011; Gendron et al. 2014; Maures et al. 2014; Shi and Murphy 2014). Several factors relating to mating have been proposed to play a role in shortening the lifespan of females in mating species. In *C. elegans*, secreted compounds by the male, injury due to the copulation itself have been suggested as some of the mechanisms (Bakker et al. 2011; Gendron et al. 2014; Maures et al. 2014; Promislow and Kaeberlein 2014; Shi and Murphy 2014). However, lifespan comparisons have mostly been performed between species. Intra-specific comparisons in *C. elegans* are limited to using mutants that transform the hermaphrodite into a female (reviewed in (Gems and de la Guardia 2013; Hansen and Schedl 2013; Torgovnick et al. 2013). Experiments in which trioecious populations of *C. elegans* were generated showed that trioecy is not evolutionarily stable and such populations reverted back to androdioecy only within a few generations (Cutter 2005). SB347 has natural females that are genetically identical to the hermaphrodites and do not segregate as a Mendelian phenotype. Being able to study life-span difference in two different genders of the same species that are genetically identical but show different mating and reproductive patterns is therefore of interest to understand the mechanisms of aging.

Our preliminary experiments on lifespan with the species SB347 and JU1782 show contrasting mating efficiencies and sperm size proportions driven by sperm competition and suggest an association between mating behavior and lifespan. We do however acknowledge that so far we have made comparisons only between the two species that showed maximum contrast in mating behavior and that similar experiments on lifespan must be done on the other three species to draw stronger conclusions. Further studies are also necessary to pinpoint the exact cause of the difference between the life spans of females and hermaphrodites in these species, as this difference has previously been shown to arise due to several factors such as physical injury, secretion of

chemicals, body size, genetic factors, etc. (Ricklefs 1998; Gems 2000). In addition to that, we do not rule out the possibility that in spite of being genetically identical, the differences in lifespans of females and hermaphrodites could be inherently different due to their modes of development and other physiological differences.

Chapter 3

Apoptosis in spermatocytes of SB347 males

3.1 Introduction

3.1.1 SB347 males produce a skewed sex ratio after crossing

As expected from animals with an XO karyotype, SB347 males produce two kinds of sperm, one bearing an X chromosome and the other lacking it (nullo-X) (Shakes et al. 2011). Thus, 50% of the cross-progeny are expected to be XO males when mating with females or hermaphrodites. Surprisingly, SB347 cross progeny are comprised of less than 5% males (Félix 2004; Shakes et al. 2011). One possible hypothesis for this unexpected observation is that the majority of the nullo-X sperm is non-functional, causing an excess progeny with the XX karyotype. In this chapter, we investigate this further and uncover a novel mechanism through which SB347 males produce a lower proportion of male progeny

3.1.2 Sex allocation and adaptive sex ratios by males

In some instances it is adaptive to have a biased sex ratio, namely to minimizes competition among male siblings (Hamilton 1967a; Trivers and Willard 1973). Most early studies on sex allocation were performed on hymenopteran insects (e.g., parasitic wasps) and birds, in which female could theoretically have control over the sex of the offspring (Jones 1982; Ewen et al. 2004). In animals in which males are the heterogametic sex, however, it is more difficult to envision the mechanisms of how sex ratios could be regulated.

One of the few studies showing a role for heterogametic males in regulating the sex of the progeny is on the red deer. Fertility of males was measured in terms of the

percentage of morphologically normal sperm and more fertile males were found to produce more sons (Gomendio et al. 2006). This creates a sexual conflict between males and females and deviates from the evolutionarily stable strategy (Fisher 1930; Smith and Price 1973). The authors proposed that this could be possible either because of a different production in ratios of X and Y bearing sperm, a differential competitive edge between the two types of sperm, or because of post-mating selection of sperm by female in favor of the Y-bearing sperm.

Evidence of differential competitiveness of two types of sperm has also been reported in the androdioecious nematode *Caenorhabditis briggsae*, a close relative of *C. elegans* (LaMunyon and Ward 1997). The X-bearing male sperm outcompeted the nullo-X male sperm to fertilize the oocyte, resulting in mostly hermaphroditic (XX) progeny in the initial brood. Males are produced only after all the X-bearing male sperm is exhausted and the non-X bearing male sperm used.

3.1.3. Spermatocytes in SB347 males divide asymmetrically

Shakes et al. (2011) observed that sex ratio bias observed in cross-progeny of SB347 is due to the preferential production of functional X-bearing sperm by males. In nematodes the sperm is amoeboid and crawls by means of pseudopodia (Roberts and Ward 1982). The pseudopodia are formed by polymerization of a cytoskeleton component called major sperm protein (MSP), which is synthesized in the developing spermatocytes and then segregates to the cytoplasm of the spermatids (Roberts et al. 1986). MSP is essential to form pseudopodia, without which the spermatozoa are non-motile and unable to fertilize. In *C. elegans,* MSP segregates equally to both the X-bearing and the nullo-X spermatids, whereas non-essential contents for sperm function (e.g., histones, nuclear membrane proteins, Golgi apparatus) are discarded in to a

residual body (Roberts et al. 1986). However, SB347 does not seem to form a residual body; the non-essential contents are segregated towards the nullo-X spermatid, whereas the vital components that include MSP almost always segregates into the X-bearing spermatid (Shakes et al. 2011). The X chromosome is identified by the fact that it being unpaired, it lags behind during anaphase II of meiosis in SB347. This indicates that only the X-bearing spermatids are able to produce pseudopodia and fertilize the eggs, resulting in XX (feminine) progeny (Fig. 3.1).

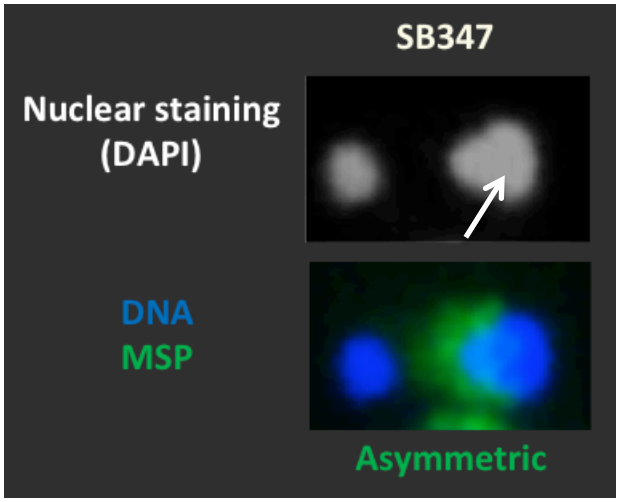

Figure 3.1. Secondary spermatocyte division SB347 (Shakes et al. 2011). SB347 shows an asymmetric division of MSP, which segregates towards the X-bearing nucleus (nucleus to the right). Arrow indicates the X chromosome that lags behind during anaphase II.

Although female biased sex ratios are common in nature, biases due to alteration in gametogenesis are not and in general, the cellular and molecular mechanisms of such distortions are poorly understood (Hardy 2002). It has been previously reported in Drosophila that males carrying a “sex ratio” X chromosome produce almost no males after crossing with females. This was because the Y chromosome degenerates during anaphase II of spermatogenesis and the Y-bearing sperm fails to mature (Policansky and Ellison 1970). Sperm bundles of different sizes have also been reported, however, without a change in the total number of sperm per bundle (Beatty and Sidhu 1969). Problems with spermatogenesis have also been reported in the ‘sex ratio’ (SR) males of stalk-eyed flies *Cyrtodiopsis dalmanni* and *C. whitei* (Presgraves et al. 1997; Wilkinson and Sanchez 2001).

For this part of my dissertation research, the specific objective is to determine the fate of the nullo-X spermatid and identify potentially conserved cellular mechanisms that could be involved in this mode of sex ratio distortion.

3.1.4. Programmed cell death (apoptosis) in germ cells

In all metazoans, one of the most common ways to maintain the quality of gametes produced is by discarding low quality gametes by programmed cell death or apoptosis. Factors such as age, DNA damage during recombination in meiosis, excess of unused gametes, or external factors such as pathogen infections could lower the quality of the gametes produced and thereby reproductive success (Aballay and Ausubel 2001; Salinas et al. 2006; Andux and Ellis 2008). Programmed cell death has been widely observed in germline and somatic development of mammals, Drosophila, nematodes and other model animal systems (Elmore 2007). This leads us to believe that one of the

possible fates for the nullo-X sperm in SB347 males could be programmed cell death (apoptosis).

About half of the *C. elegans* hermaphrodite germline undergoes apoptosis, probably to maintain the quality of gametes and as a response to DNA damage (Andux and Ellis 2008). In *C. elegans* males, apoptosis has never been observed (Gumienny et al. 1999). However, males of other species (mice, Drosophila, humans) use programmed cell death to maintain gamete quality. By taking advantage of the knowledge that apoptosis is a highly conserved pathway across metazoans (Poulin et al. 2004), in this chapter of the dissertation I will investigate whether apoptosis is the mechanism used by SB347 to eliminate the nullo-X spermatid.

3.2 Methods

3.2.1 Worm strains and maintenance

SB347 worms were cultured and maintained at 20°C as described in Chapter 2. To obtain adult virgin males, synchronous L2 larvae were isolated from a healthy culture plate onto single wells of a 12 well plate. They were used for experimentation after 24-36 hours when they became adults.

3.2.2 DIC microscopy and acridine orange staining

To identify the fate of the nullo-X sperm after the asymmetric division, we used morphological characters and acridine orange (AO) – a dye that stains apoptotic nuclei (Gartner et al. 2004).

Apoptotic cells in the germline can be identified by their distinct morphology: they are more refractile than the surrounding cells when observed under differential

interference contrast (DIC) optics. DIC (also known as Nomarski) optics is a kind of microscopy that uses the difference in the refractive indices of parts of the object to produce contrast between those parts of a specimen. DIC optics produce significantly better results in comparison to bright field or phase contrast microscopy.

Virgin one-day-old adult males were transferred onto a slide with an agar pad made of 4% agar. They were immobilized by adding 5 µL of 30 mM sodium azide in M9 buffer and covered with a coverslip. The number of apoptotic cells in the gonads was counted using DIC optics and blind scoring against a wild type control was done to avoid observational bias.

Apoptotic cells were also identified in live animals by staining with a vital dye – acridine orange and visualizing by fluorescence microscopy. AO is widely used to detect apoptosis in cells. It intercalates with DNA and produces a green fluorescence and binds electrostatically with RNA to produce red light. Apoptotic cells present a low pH which protonates acridine orange and when excited by blue light in these conditions, it emits strong green or orange fluorescence. To stain the worms with AO, 5 µL of 10 mg/mL stock solution was added to 1 mL of M9. Males to be stained were suspended in this staining solution and kept in dark for 1 hour at room temperature. After 1 hour, they were washed three times with M9 by centrifuging, re-plated on a fresh 6 cm plate with a lawn of OP50-1 and kept in dark for 1 hour. This was done so that the worms can feed on non-stained bacteria to remove the background fluorescence from the intestine. The stained and cleaned worms were then transferred onto a slide with a 4% agar pad and visualized under a fluorescent microscope.

3.2.3 Immunohistochemistry and mitochondrial staining

Sperm cells were extracted from the worms as previously described in Chapter 2. The dissected gonads were then pressed gently with a cover slip to obtain a monolayer of cells, flash frozen in liquid nitrogen, and the cover slip removed to freeze crack. Fixation of the tissue was done in 100% methanol overnight at -20°C, slides washed in PBS and blocked in 0.5% BSA. Incubations with the primary antibodies were done in a humid chamber at room temperature using the following dilutions: mouse anti-MSP 4D5 – 1:500, rabbit anti-CEP-1 – 1:200 (SantaCruz), rabbit anti-CED-3 – 1:200 (SantaCruz), and mouse anti-cyt-c 1:1000 (Upstate). For the secondary antibodies, the following dilutions were used: AlexaFluor 488 goat anti-mouse – 1:100 (Life Technologies), AlexaFluor 568 goat anti-mouse – 1:100 (Life Technologies) and AlexaFluor 488 donkey anti-rabbit – 1:200 (Life Technologies).

To observe the nuclei of the cells DAPI staining was used. Mounting medium containing DAPI (Vectashield) was used to mount the coverslips onto the slides containing the immuno-labeled tissue. The slides were then viewed on a Carl Zeiss 510 confocal microscope. Images were processed using Zeiss LSM510 Image browser and ImageJ software, and representative images are shown in 'Results'.

To stain the mitochondria in dividing spermatocytes, MitoTracker Red (Life Technologies) was used. The worms were dissected to release the sperm in sperm media and incubated with 50 nM MitoTracker in dark at room temperature for 10 mins. The sperm was subsequently fixed in 1% neutral buffered PFA for 5 mins and then mounted with mounting medium containing DAPI (Vectashield).

3.2.4 Mutagenesis

A mutagenesis screen of about 500 gametes was performed using Ethyl methane sulfonate (EMS) as described in (Chaudhuri et al. 2011a). EMS is an extremely potent mutagen that induces G:C to A:T transitions randomly throughout the genome (Anderson 1995). F3 generation males from the mutagenized parent were crossed with wild type females to determine if they produced progeny with a higher proportion of males. For the crosses, one male was allowed to mate with one virgin female. The parents were transferred to fresh plates everyday in order to avoid mixing generations. The sex of offspring from the total brood of each cross was identified by visual identification (males are morphologically different from females and hermaphrodites) and proportion of males in the brood was noted. Hermaphrodite siblings of the F3 male that produced a higher proportion of males after crossing were used to obtain a homozygous culture of the mutant.

3.2.5 Determining sex ratios of cross progeny for mutant and wild type males

Both the wild type and mutant males were crossed with wild type females to determine the proportion of male offspring produced. To isolate virgin females, L2 larvae were picked and isolated on individual wells of a 12 well plate and left to grow until adulthood. Simultaneously, L2 males were also picked from the wild type and mutant cultures and allowed to reach adulthood in isolation. Crosses between virgin adult females and males were performed in 12 well plates as mentioned above. All the progeny produced by the females was collected and the broods were scored as males or non-males (females/hermaphrodites).

3.3 Results

3.3.1 Male gonad shows cells with apoptotic morphology

Some SB347 male germ cells show morphological features of apoptotic cell death, as detected by differential interface contrast (DIC) microscopy and acridine orange (AO) staining (Fig. 3.2). Apoptotic cells were found in terminal region of the gonad, where spermatids are usually expected.

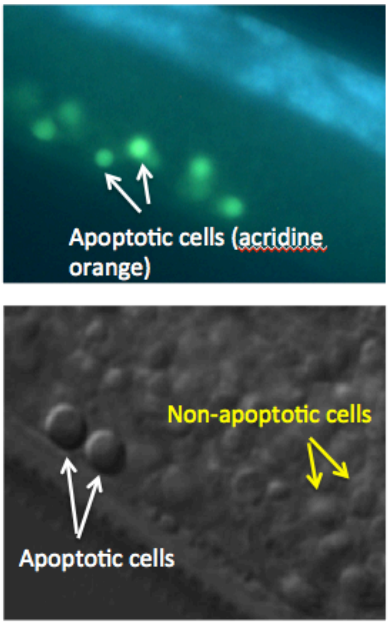

Figure 3.2. Apoptotic cells in gonad of SB347 males. The fluorescence image shows apoptotic cells stained with acridine orange (green). Under DIC optics, the apoptotic cells appear refractile and button-like. Fluorescence and DIC images shown are not from the same cells.

3.3.2 Apoptotic proteins are expressed in the nullo-X spermatid

To determine if apoptosis is occurring in nullo-X spermatids, I examined the expression of genes typically activated in apoptotic cells: CED-3, CEP-1 and cyt-c. CED-3 caspase is the most downstream gene of the core apoptotic pathway in *C. elegans* (Conradt and Horvitz 1998). It is triggered in response to many kinds of apoptotic stimuli, such as DNA damage (Derry et al. 2001; Schumacher et al. 2001; Lettre et al. 2004). The role of mitochondria and cytochrome-c (cyt-c) in apoptosis is well established in vertebrates (Li et al. 1997; Goldstein et al. 2000), but less so in *C. elegans* and Drosophila (Varkey 1999; Abdelwahid et al. 2007).

To determine which spermatid is inheriting the X chromosome, I took advantage of the observation that an unpaired chromosome usually lags behind during anaphase. The lagging X in SB347 XO males was found in anaphase II, indicating that sister chromatids of this chromosome already separate in the first meiotic division (Shakes et al. 2011). This modified meiosis results in secondary spermatocytes that will generate a nullo-X spermatid and a X-bearing spermatid. To detect the expression of CED-3, I used an antibody that recognizes the active form of this caspase. In the dividing SB347 secondary spermatocyte, CED-3 was expressed only in the nullo-X secondary spermatid (Fig. 3.3), suggesting that this gamete undergoes apoptosis.

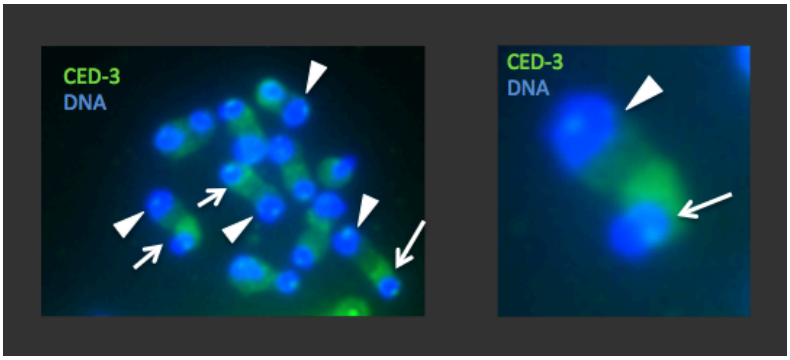

Figure 3.3. CED-3 expression in the dividing secondary spermatids of wild type SB347 males. Arrow – nullo-X nucleus; arrow head – X bearing nucleus.

Immunohistochemistry using *C. elegans* CEP-1 antibody revealed that CEP-1 is expressed only in the nullo-X spermatid of SB347 males (Fig. 3.4), suggesting that this spermatid uses a pathway similar to the *C. elegans* DNA damage pathway. Double labeling of CEP-1 and MSP showed that their expression is mutually exclusive in the daughter spermatids. CEP-1 was expressed (Fig. 3.4) in the dividing nullo-X spermatid, and MSP in the X-bearing spermatid.

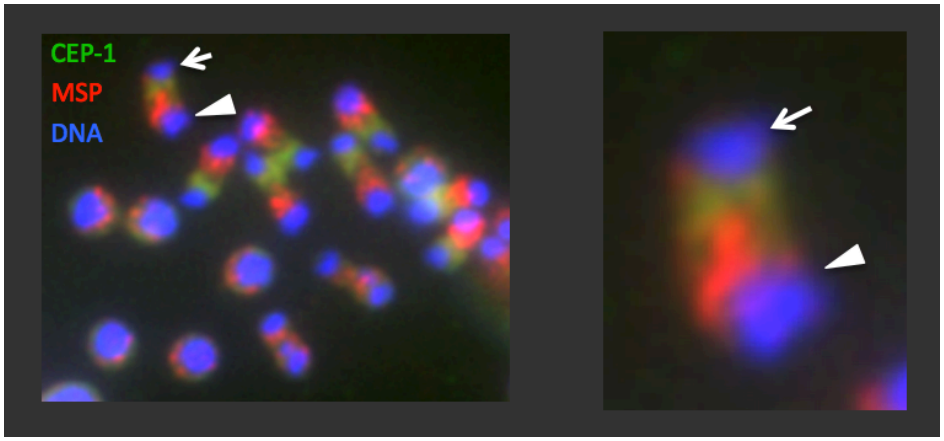

Figure 3.4. CEP-1 and MSP co-labeling in dividing secondary spermatocytes of wild type SB347 males. CEP-1 is expressed only in the nullo-X (arrow) daughter spermatid (arrow-heads: X-bearing nucleus) and MSP segregates with the X-bearing nucleus only.

To determine if the apoptosis in SB347 nullo-X spermatid is mediated by the mitochondria, as previously shown for mammals, I tested the expression of cyt-c in the dividing spermatocytes (Li et al. 1997; Goldstein et al. 2000). In mammals, cyt-c is an intermediate of apoptosis and it is released from mitochondria (Kroemer et al. 1998). Immunohistochemistry showed that cyt-c was expressed only in the nullo-X spermatid and was co-localized with CEP-1 (Fig. 3.5), but not with the mitochondria (Fig. 3.6).

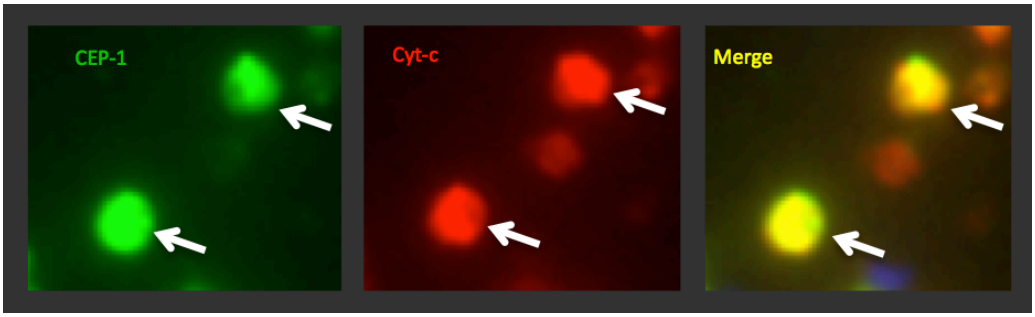

Figure 3.5. Apoptotic spermatids of SB347 males with CEP-1 and Cyt-c co-labeling.

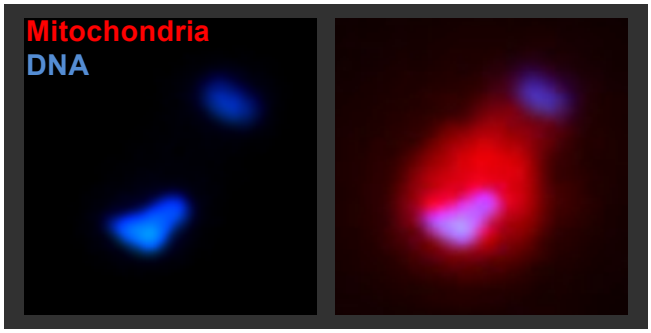

Figure 3.6 Localization of mitochondria in the dividing secondary spermatocyte. The mitochondria are partitioned asymmetrically and go to the daughter cell that has the X-chromosome. The X-bearing nucleus can be identified by the lagging-X chromosome.

3.3.3 Mutant produced more males upon crossing than the wild type and had fewer apoptotic nuclei

To determine how SB347 generate spermatids with different cytoplasmic contents, we performed EMS mutagenesis in the inbred strain APS4. We isolated a mutant named strain APS8 (*brz-1*) in which males produced a higher number of male cross progeny compared to the wild type males (Mann-Whitney U test, p= 0.003, U=66), with some producing up to 44% male offspring (Fig. 3.7). The number of refractile apoptotic cells in wild type males was higher than in the mutant (Mann-Whitney U test , p<0.001, U=277). APS8 (*brz-1*) had an average of only 8.6 apoptotic cells/gonad in contrast to 16.7 cells/gonad for the wild type males (Fig. 3.8).

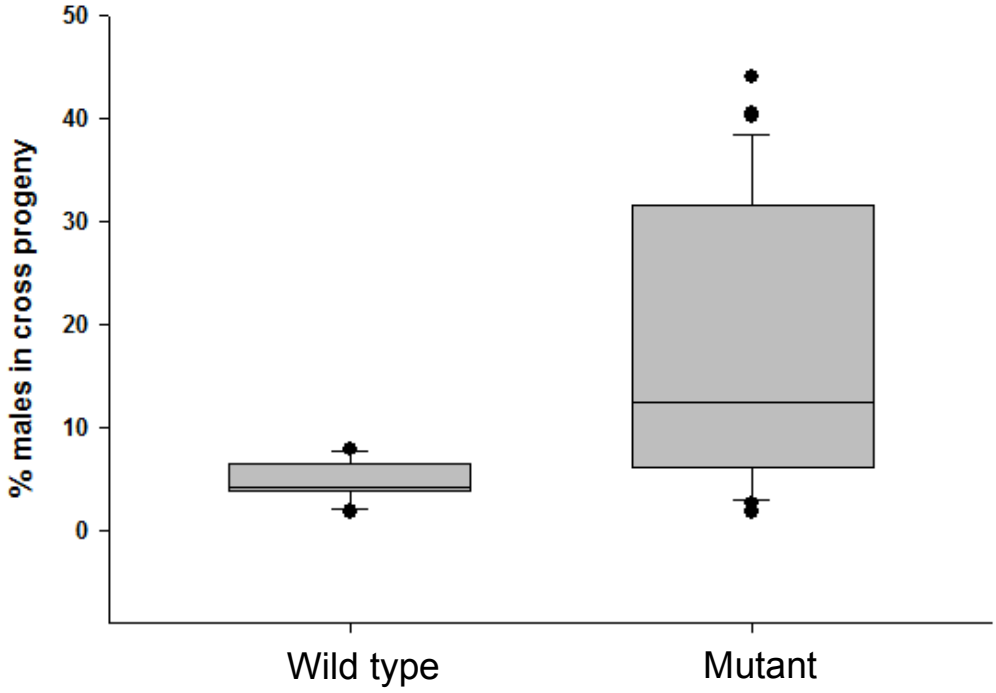

Figure 3.7. Box plot showing proportion of males obtained after crossing WT (n=10) and APS8 (n=36) (*brz-1*) males with WT females.

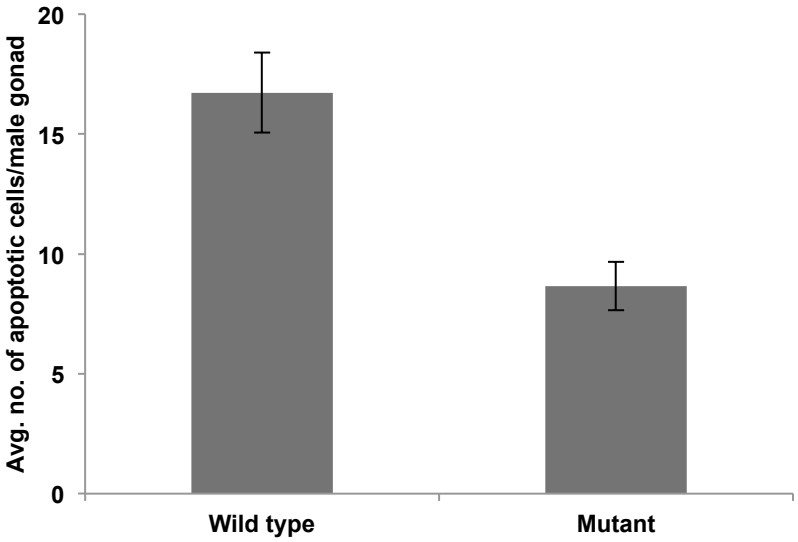

Figure 3.8. Apoptotic cells in gonad of wild type vs. mutant. Bar graph compares the number of apoptotic cells in the gonads of wild type (n=33) and mutant (n=35) SB347 males as counted under DIC microscopy. Error bars represent standard error.

3.4 Discussion

We have studied a mechanism for sex ratio regulation in the trioecious species SB347, which is XX-biased after outcrossing. Here, we have uncovered a novel mechanism of sex ratio distortion through selective apoptosis of male producing sperm in the male gonad (Fig. 3.9).

SB347 males have previously been shown to produce a functional and a non-functional gamete, where the non-functional gamete is the one that is supposed to produce males after fertilization. This gamete lacks the X-chromosome and essentially serves the function of a *C. elegans*-like residual body during spermatogenesis. This is different from *C. elegans*, in which all the non-essential cytoplasmic components are discarded in the form of a residual body. The residual body is lacks a nucleus, as is the case with the nullo-X spermatid in SB347 and does not undergo apoptosis (Huang et al. 2012). The essential cytoplasmic components, such as MSP, are segregated towards the X-bearing spermatocyte during anaphase II. When the spermatid gets activated, MSP forms the pseudopodia.

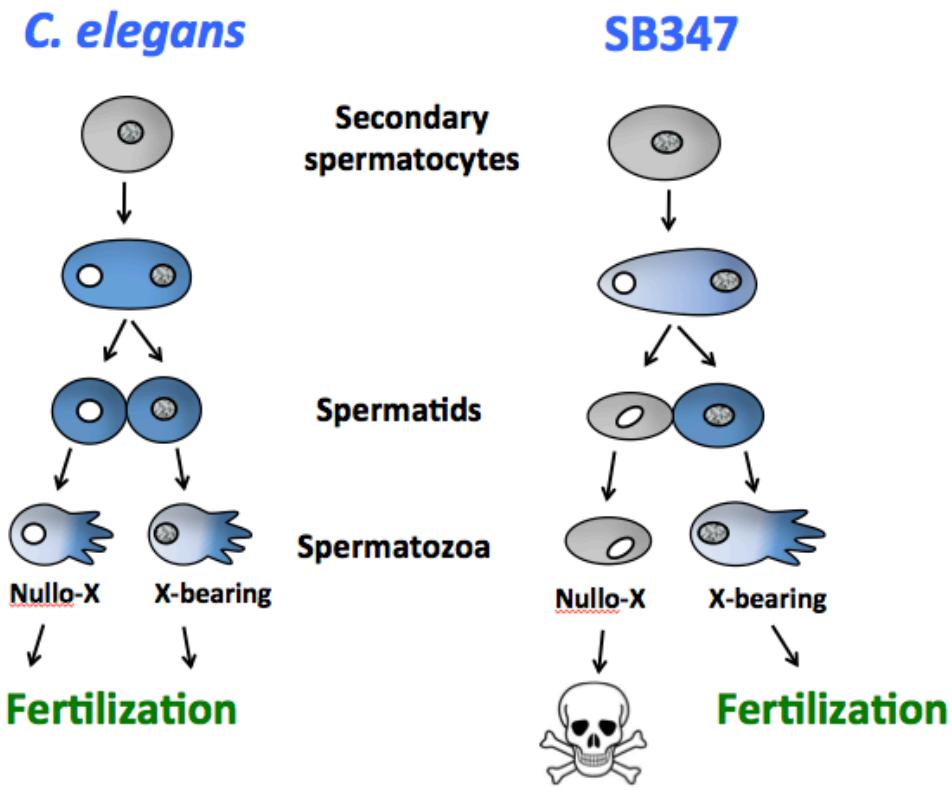

Figure 3.9. Summary of secondary spermatocyte division in *C. elegans* vs. SB347. Both X-bearing and nullo-X spermatids form spermatozoa in *C. elegans*. However, in SB347 only the X-bearing spermatid forms spermatozoa and can fertilize the egg while the nullo-X spermatid undergoes a programmed cell death. (Modified from Pires-daSilva and Parihar 2012)

Although apoptosis in *C. elegans* hermaphrodite germline is well known, there is no association between the apoptosis and the sex of the progeny. *C. elegans* germline undergoes two kinds of apoptoses – physiological, which is a normal part of oogenesis, and as a response to DNA damage or disruption of genomic integrity of the germ cell (Gumienny et al. 1999; Gartner et al. 2000).

Programmed cell death under normal conditions occurs in *C. elegans* germline. It is seen in adult hermaphrodites to maintain the quality of oocytes being produced (Andux and Ellis 2008). This is called physiological because it occurs without being triggered by any known stressors, in spite of using the same core apoptotic mechanisms described later in this section. The *C. elegans* hermaphrodite gonad is a syncytium where one oocyte matures at a time and evidence suggests that most of the oocytes die in order to provide maximum amount of cytoplasmic components for each maturing oocyte and favor quality over quantity of oocytes (Gumienny et al. 1999). It is interesting to note that there is no apoptosis involved during the spermatogenesis in either hermaphrodites or males. This kind of apoptosis shows conservation among other eukaryotes (Borum 1961; Baker 1963; Pepling 2006). In mammals, more than half of the developing oocytes undergo apoptosis and the cytoplasmic components are used by the surviving oocytes, similar to what happens in *C. elegans* (Pepling 2006).

The second kind of apoptosis observed in *C. elegans* germline is induced by DNA damage (Fig. 3.10) (Gartner et al. 2000). DNA replication is a critical step in gametogenesis and is under a very strict quality control. There are several checkpoints during DNA replication to ensure that there are no errors. These checkpoints would either repair the damage or direct the defective cell towards programmed cell death. Several factors are known to cause DNA damage, including, but not limited to oxidative damage, irradiation, replication errors and chemicals. This kind of apoptosis also utilizes the same

core pathway as physiological apoptosis, however, the expression of EGL-1 is required to trigger the downstream pathway.

In addition to the physiological and DNA damage induced apoptosis, some pathogens and environmental stressors have also been identified to bring about programmed cell death specifically in the germ cells (Aballay and Ausubel 2001; Salinas et al. 2006). These inducers do not use the physiological or the EGL-1, but have their own parallel mechanisms to trigger the core pathway (Fig. 3.10).

The core apoptotic pathway in *C. elegans* comprises of the genes CED-9 (an anti-apoptotic mammalian Bcl-2 family protein), CED-4 and CED-3 (a caspase) (Greiss et al. 2008; Bailly and Gartner 2013). All the apoptotic factors ultimately activate the caspase CED-3, which is the most downstream apoptotic regulator in *C. elegans* (Ellis and Horvitz 1986). CED-3 is synthesized as an inactive pro-enzyme and undergoes cleavage to become active in the apoptotic cell (Denault and Salvesen 2002).

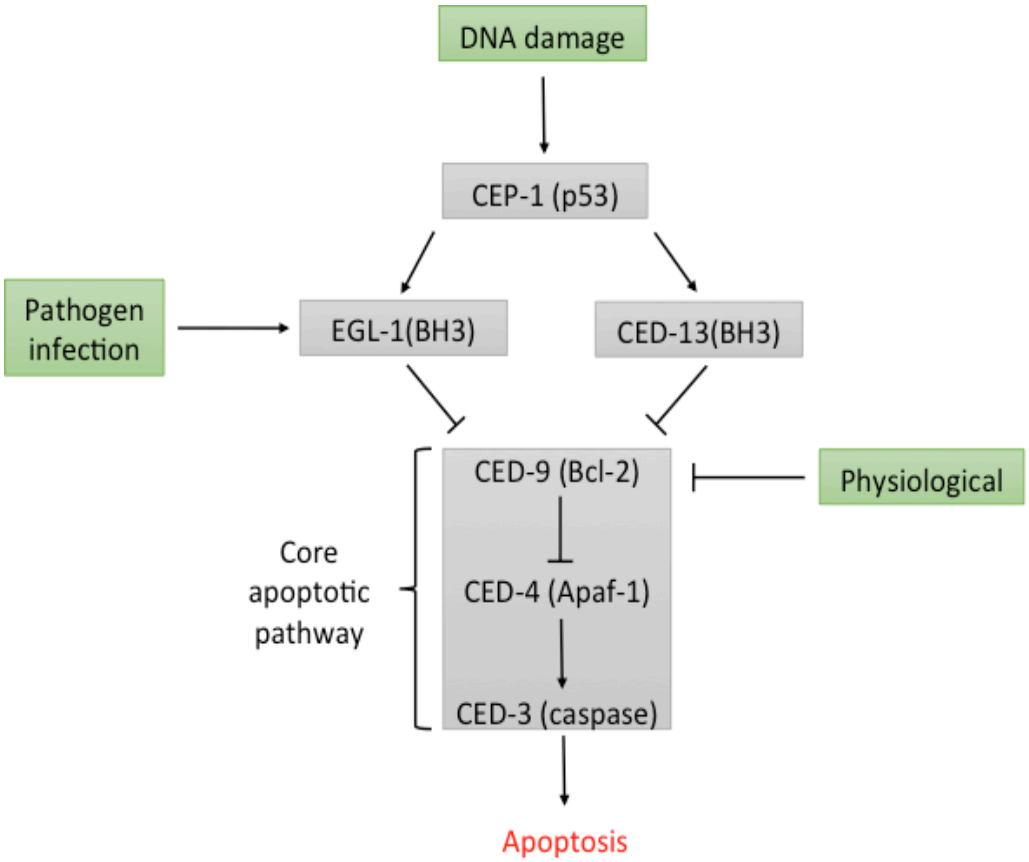

Figure 3.10. Apoptotic pathway in *C. elegans* (Figure modified from Gartner et al 2008, www.wormbook.org).

An indicator of DNA damage is CEP-1, a human p53-like gene in *C. elegans* that is expressed in cells that have DNA damage and diverts such damaged cells towards apoptosis (Derry et al. 2001; Schumacher et al. 2001; Lettre et al. 2004). p53 regulates cell cycle progression and genome stability in humans by directing cells to apoptosis in response to DNA damage or genotoxic stress. It is a tumor suppressor and mutations in it are one of the most common causes of cancer. CEP-1 in *C. elegans* is also known to have similar functions at elevated levels(Derry et al. 2001).

Although it is known that in *C. elegans* the DNA damage induced apoptosis is triggered by CEP-1, the pathway leading to the activation of CED-3 is not very well understood. This is partly due to low sequence conservation between *cep-1* and the mammalian p53. In addition to p53, *cep-1* also shows similarity with p63 and p73, making it difficult to clearly identify its role in comparison to mammalian apoptosis (Suh et al. 2006; Wang et al. 2007). In most mechanisms of apoptosis however, the mitochondria are known to play a crucial role {reviewed in (Nef et al. 2005; Rolland and Conradt 2006; Ercan et al. 2007)}. The mitochondria release several pro-apoptotic molecules (cytochrome-c, apoptosis inducing factor-AIF and endonuclease G) from the mitochondrial inter-membrane space (IMS) into the cytosol. Cyt-c is a highly conserved respiratory chain protein, released into the cytosol from IMS to induce apoptosis {reviewed in (Jiang and Wang 2004)}. It is widely believed that cyt-c is released from mitochondria to activate caspases, but more recent evidence indicates that it is also present in extra-mitochondrial locations under normal conditions (Jiang and Wang 2004). Since cyt-c and mitochondria do not co-localize in SB347 spermatids, there is the possibility the cyt-c present in the spermatid is not released by the mitochondria. Further studies would be necessary to determine the role of cyt-c in apoptosis in SB347.

Regardless, the fact that mitochondria go to the X-bearing chromosome makes sense, because these organelles are necessary for powering the sperm movement.

Microtubules have been long known to play a role in apoptosis, especially in humans and have been one of the principal targets to control cell division in cancer treatment (Mollinedo and Gajate 2006; Estève et al. 2007; Oropesa-Ávila et al. 2013). In mammals, it has also been shown that alterations in tubulin production and polymerization occur in response to DNA damage (Porter and Lee 2001). Since the only obvious difference between the functional and non-functional spermatid in SB347 males is the presence or absence of the X chromosome, we hypothesized that apoptosis of the nullo-X spermatid uses a genetic pathway similar to DNA damage induced apoptosis. This hypothesis was supported by the differential expression of CEP-1 in the two kinds of spermatids. Furthermore, the tubulin is also discarded in the nullo-X spermatid possibly playing a role in apoptosis. We find it very intriguing that and apoptotic pathway which is generally observed in mammals might be conserved in SB347.

Although in our experiments we show a differential expression of CEP-1 using antibody staining, it should be noted that CEP-1 has a constitutive expression in all cells. Our results show a clear contrast between its expression in the X-bearing and the nullo-X spermatid, which indicate that although there might be a constitutive expression of CEP-1 in both, in the nullo-X spermatid it is certainly higher. Another possible limitation of these results could be the specificity of the antibodies. The CEP-1, CED-3 and cyt-C antibodies that we used for our experiments still need to be tested in SB347 to determine if they recognize the same epitope as in *C. elegans*.

For nematodes, it is has been hypothesized that male/female (dioecious) form is the ancestral mode of reproduction and phylogenetic studies suggest that male/hermaphrodite (androdiocious) species have evolved at least 3 times independently

(Kiontke et al. 2004; Denver et al. 2011). Trioecious species like SB347 however, are considered to be evolutionary intermediates between dioecy and hermaphroditism. Ancestral females presumably evolved to produce functional sperm and become self-fertilizing hermaphrodites by mutations in only two processes (Baldi et al. 2009). One of the mutations is to lower the feminizing gene for a short period and produce sperm, and the second is to induce activation of the sperm. To explain the intermediary trioecious state of SB347, two kinds of evolutionary events could have occurred. First, mutations for production of a limited amount of sperm by some females to convert them to self-fertilizing hermaphrodites would have made SB347 trioecious. Second, genetic changes to bring about death of male producing sperm in male germline were selected. Alternatively, assuming that ancestors of SB347 were male-female, a non-adaptive female-biased sex ratio could result from a mutation that kills most of the nullo-X spermatids. This would have led to a very low proportion of males in the population and some females evolved to produce sperm as has been proposed for *C. remanei* (Baldi et al. 2009). Such a transition could convert the male-female mating system to a triocious male-female-hermaphrodite system.

In *C. elegans*, the male germ line does not undergo apoptosis and males produce a 1:1 ratio of hermaphrodites to males after crossing, however, apart from crossing males are produced at a very low proportion (~0.1%) due to a rare event of chromosomal non-disjunction. In contrast, hermaphrodite self-progeny in SB347 comprises of ~10% males. A higher degree of selection for males can be attributed here to the presence of females, which produce progeny exclusively with males. This balance of selective forces and the benefits of survival by being a hermaphrodite make SB347 a remarkable species to study transitions in mating systems. It is also important to note that although there is apoptosis in the nullo-X spermatids of SB347 males, not all of them are

killed and ~5% of them stay viable to produce male offspring. Although for my dissertation research I have not explored the survival mechanism for those ~5% nullo-X spermatids, it remains of much interest.

Chapter 4

Evolution of the sex-determination pathway

4.1 Introduction

Relatively few signaling pathways are required to regulate development and to generate the incredible diversity in animal forms (Pires-daSilva and Sommer 2003). These pathways have been postulated to evolve by several mechanisms that include gene loss, gene addition, gain of new function for an existing gene, and formation of new connections between existing genes (Wilkins 1995; Barolo and Posakony 2002). Most of the signaling pathways are highly conserved and their long history makes difficult to understand their origin and how they were built. To understand better mechanisms of evolution of pathways, the comparison of fast-evolving pathways in closely related species might give valuable insights.

One such rapidly evolving developmental pathway is the sex-determination (SD) pathway (Bull 1983; Wilkins 1995). Sex determination is the developmental switch that regulates sexual dimorphism and has been very well characterized in established experimental model systems such as *C. elegans*, the fruitfly *Drosophila melanogaster* and the mouse *Mus musculus*. Comparative studies of SD pathways in these models revealed that the mechanisms and the genes involved in SD are not conserved. It is puzzling that SD, being such a central pathway for development and reproduction, is not as conserved as other pathways such as Hedgehog, JAK/STAT, Wnt, tyrosine kinase and Notch. Only the most downstream transcription factor, with a doublesex/mab-3 (DM) domain, is conserved in Drosophila, *C. elegans* and mammals (Raymond et al. 1998). In the Caenorhabditis genus, the sex-determining genes TRA-1, TRA-2, FEM-2 and FEM-3 (de Bono and Hodgkin 1996; Kuwabara 1996; Hansen and Pilgrim 1998; Haag et al.

2002) show very little conservation at the amino acid sequence level between *C. elegans* and *C. briggsae*. The SD pathway has been extensively studied in *C. elegans* and a wealth of knowledge about the genetic mechanisms involved make it an excellent reference model for comparative studies of developmental pathways (Goodwin and Ellis 2002; Stothard and Pilgrim 2003).

4.1.1 Sexual dimorphism and SD pathway in C. elegans

C. elegans has two sexes – self-fertilizing hermaphrodites, with two X chromosomes (XX) and males with one X chromosome (XO) (Fig. 4.1). Chromosomal signals arising due to the difference in the X:A ratio control *xol-1* (XO lethal-1), the most upstream gene in the sex determination pathway (Luz et al. 2003). Activation of *xol-1* gene leads to a male phenotype, and its repression leads to the development of a hermaphrodite (Miller et al. 1988; Rhind et al. 1995). The most downstream regulator is the gene *transformer-1* (*tra-1)*, a transcription factor that activates genes specific to female fate (Hodgkin and Brenner 1977; Hodgkin 1987; Zarkower and Hodgkin 1992). *tra-1* has been shown to be conserved even in distantly related nematode species (Pires-daSilva and Sommer 2004). Essentially, *xol-1* expression levels regulate the activation of *tra-1* through a chain of inhibitory interactions involving *sdc-1-3*, *her-1*, *tra-2* and *-3*, and *fem-1* to *-3* as intermediates (Fig. 4.2).

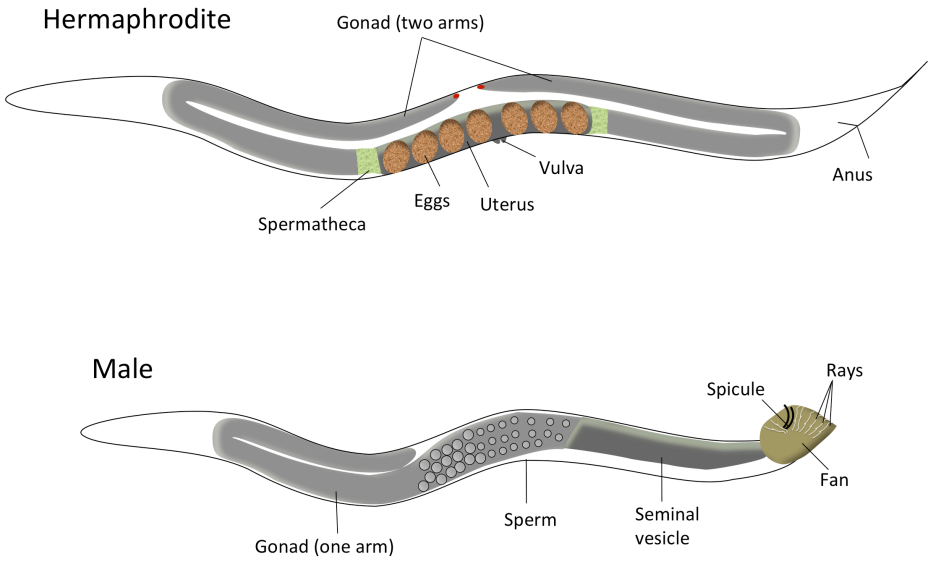

Figure 4.1: Sexual dimorphism in *C. elegans*.

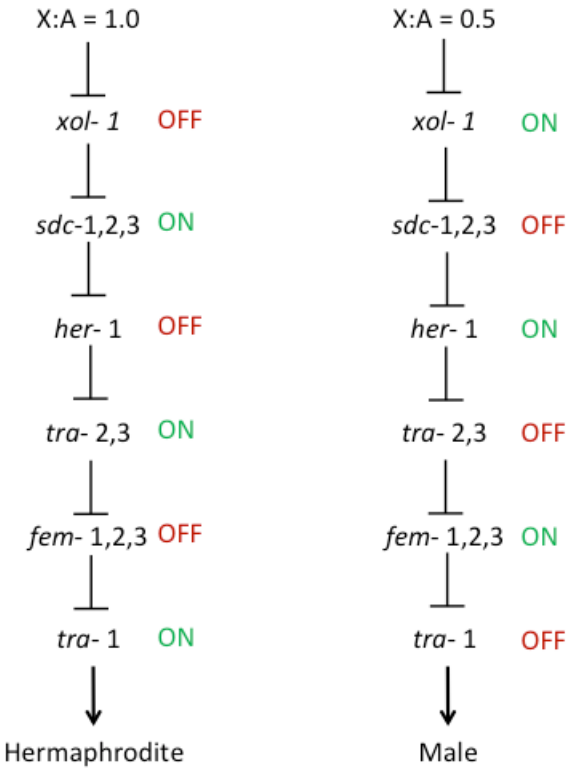

Figure 4.2: Genetic model for sex determination in *C. elegans*. The primary signal for sex determination is the ratio of X chromosomes to the set of autosomes. When the X:A ratio is 1, a cascade of negative regulations activates *tra-1* to form a hermaphrodite but for a X:A ratio of 0.5 *tra-1* is inactive (Pires-daSilva and Sommer 2004).

4.1.2 Understanding evolution of SD in nematodes by comparative studies with Pristionchus pacificus

Comparison of the somatic sex determination within the Caenorhabditis revealed that the SD pathway is conserved in this genus (Streit et al. 1999). However, very little conservation has been observed for organisms distantly related to *C. elegans*, such as *Drosophila* and mice. Therefore, a model organism that has an intermediate phylogenetic distance with *C. elegans* could be of great use for comparative studies. *Pristionchus pacificus* is a nematode that has been recently established as an excellent tool for comparative genetic studies with *C. elegans. P. pacificus* is hermaphroditic (with occasional males) and can be easily cultured and maintained in the laboratory. Its genomic sequence is available, and it is amenable to several genetic and genomic studies (Sommer et al. 1996; Dieterich et al. 2008) (Fig. 4.3).

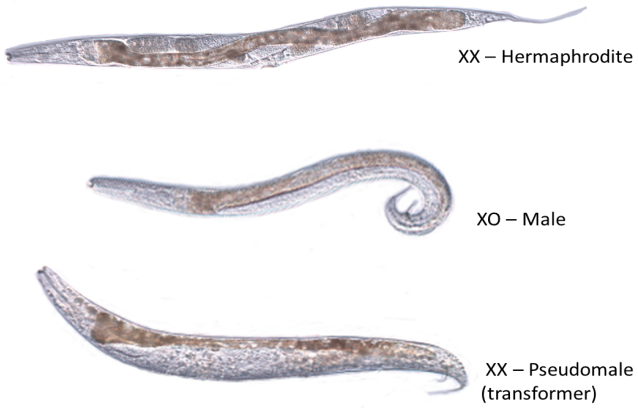

Figure 4.3. Sexual dimorphism in *P. pacificus*. Transformers are mutant animals that are genetically XX but resemble the phenotype of a male.

4.1.3 Does SD show conservation and a general pattern of evolution between C. elegans and P. pacificus?

The males and hermaphrodites of *C. elegans* have major differences in morphology and can therefore be easily identified. For example, the most striking difference between males and hermaphrodites is in the tails. The hermaphroditic tail is in the shape of a whip but the male tail is fan-like and bears the copulatory apparatus/spicule (Fig 4.1). The hermaphrodites also have a uterus containing eggs, a vulva and a spermatheca. The gonads of hermaphrodites and males are also different; males have a single lobed gonad, while the hermaphrodite gonad has two lobes. Analogous differences are also observed between males and hermaphrodites of *P. pacificus* (Fig. 4.3).

The SD pathway also has two components, the somatic SD pathway and the germline SD pathways. Mutations influencing the somatic sex determination are easier to identify compared to germline mutants because morphological phenotypes can be visually identified. Several studies comparing the somatic sex determination have been conducted in the Caenorhabditis genus and an overall conservation of the SD pathway has been observed (Streit et al. 1999; Haag and Kimble 2000; Haag et al. 2002; Stothard and Pilgrim 2003). Knowledge of the similarities or differences in the mechanism of SD between the two nematodes could be used to understand the underlying mechanisms for the evolution of the SD pathway. Previous work has revealed that the SD in *P. pacificus* is controlled by the gene *transformer-1* (*Ppa-tra-1*), a homolog of the *C. elegans, Cel-tra-1* (Pires-daSilva and Sommer 2004). This is consistent with the hypothesis that the SD pathway in *C. elegans* has evolved bottom-up with the most downstream gene (*tra-1*) being the most conserved in the genus *Caenorhabditis*. Successive genetic components to this pathway were added upstream to *tra-1* and therefore these components show

lower degree of sequence conservation. Are any other genes conserved between *C. elegans* and *P. pacificus*?

4.2 Methods

4.2.1 Mutagenesis to generate SD mutants

To identify and isolate novel SD mutants, mutagenesis was performed on the California strain of *P. pacificus*, PS312, using ethyl methane sulfonate (EMS) as described in (Chaudhuri et al. 2011b). Specifically, we screened for mutants that are genetically XX with male phenotype. This particular category of mutants was chosen because the transformers are easy to identify in the F2 generation. Mutants showing extreme transformation can be identified easily by observing the male to hermaphrodite progeny ratio (all progeny is XX, and therefore hermaphrodite). They have an abnormally developed gonad, are not as slender and agile in movement as the wild type males, and mostly do not show mating behavior (Fig. 4.3). These mutants were called the '*masculinizer*' or *mas* mutants. The proportion of males produced from a wild type hermaphrodite is about 0.1%, whereas a hermaphrodite heterozygous for the mutation produces ~25% males, most of which are XX.

F1 larvae from a mutagenized parent were isolated on 6 cm seeded NGM plates and the F2 progeny was screened for the transformer phenotype. If the F1 is heterozygous, approximately 25% of the progeny should show the transformer phenotype. The transformers cannot reproduce and therefore subsequent cultures of the mutant were maintained by isolating F1s that produced progeny with the transformer phenotype. However, in case of temperature sensitive mutants, the transformers can reproduce at the permissive temperature and were hence maintained as hermaphrodites at the permissive temperature.

To remove undesired mutations not related to the phenotype, mutants were backcrossed with wild type *P. pacificus* (California strain) at least 3 times. Backcrossing 3 times should theoretically remove 87.5% of the mutated genome. SNP mapping by crossing with the polymorphic Washington strain PS1843 revealed that the mutation *mas-27* was located on chromosome III, around the same region as *Ppa-tra-1* and was presumably allelic to it. However, *mas-25* was found to be located towards the tip of chromosome I, which was novel and was therefore chosen for further analysis.

4.2.2 Genetic mapping of the mutants

There are approximately 3% of genetic polymorphisms between the sequences of the California/Washington strains. The genetic linkage map, with over 500 markers (www.pristionchus.org), was used for positional cloning of the mutations.

Heterozygous hermaphrodites (P0) from the mutant strains were allowed to self-fertilize and lay eggs until they had depleted all of their own sperm. These sperm-depleted hermaphrodites were then crossed with males of the Washington strain PS1843. In the F1 generation, approximately 50% of the hermaphrodites should be heterozygous for the mutation (25% of total progeny). The F1 hermaphrodites were isolated and allowed to lay progeny and F2 transformers from the progeny were collected for genotyping.

In case of temperature sensitive mutants, a homozygous culture can be obtained at the permissive temperature as the mutants develop as self-fertilizing hermaphrodites. The homozygous hermaphrodites (P0) were allowed to lay eggs at the permissive temperature until sperm was depleted. They were then crossed with the wild type males of the *P. pacificus* Washington strain PS1843, The hermaphrodite cross progeny (F1), heterozygous for the mutation and the hermaphrodites from these were allowed to lay

eggs at a restrictive temperature of ~24°C. At this temperature 25% of the homozygous F2 progeny develop as transformers and these transformers were collected for genotyping.

4.2.3 Extraction of genomic DNA

F2 transformers to be genotyped were picked into single tubes containing 5 µL of lysis buffer with Proteinase K and were incubated for 1 hour incubation at 65°C. The Proteinase K was subsequently inactivated by heating sample at 95°C for 10 mins.

To isolate genomic DNA for sequencing, about 15 plates (6 cm) full of worms were washed off with M9 and the worms collected as a pellet by centrifuging. The worms were then washed 3-5 times with M9 by vortexing and centrifuging to remove the bacteria and the worm pellet was immediately frozen at -80˚C until use. The cuticle was freeze-cracked twice by flash freezing in liquid nitrogen, and genomic DNA was extracted using the reagents and protocol from Qiagen Puregene Core Kit.

4.2.4 Sequencing and physical mapping

Sequencing using Illumina platform was performed at University of Texas Southwestern Medical Center Core Genomics facility. Assembly of reads and alignment of regions was performed by Dr. Christoph Dieterich (Max Planck Institute for Developmental Biology, Tübingen, Germany). A list of candidate mutations was compiled based on whether the mutations were found in open reading frames and whether they changed the codon of aminoacid. To confirm the mutations, PCR amplified products were re-sequenced and BLASTed against the *P. pacificus* genome (www.pristionchus.org).

4.2.5 Long range PCR to amplify genomic DNA

We used the Roche Expand Long Template PCR System to amplify genomic region including ~3 kb upstream and ~1 kb downstream of the predicted gene. Nested PCR was performed on genomic DNA isolated as mentioned above and the total size of the expected amplification product was ~12 kb. Primers used for these PCR reactions are listed in Appendix C.

4.3 Results

4.3.1 masculinizer (mas) mutants

We screened approximately 3200 gametes and isolated *masculinizer* (*mas*) mutants *mas-23*, *mas-24, mas-25, mas-26, mas-27* and *mas-28*. The nomenclature for naming *Pristionchus* genes is derived from (Kenning et al. 2004). Out of these, *mas-25* was found to be a temperature sensitive mutant. In addition to the mutants we isolated, *mas-2*, a previously isolated mutant that is also temperature sensitive was available (Pires-daSilva, personal communication). The temperature sensitive mutants become transformers only at the restrictive temperature (>20°C) and can be cultured in homozygous condition as hermaphrodites at the permissive temperature of (~15°C).

4.3.2 Genetic mapping of the mas mutants

Ppa-mas-2 had been mapped to a predicted ~ 4 kb gene in the *P. pacificus* genome (Pires-daSilva, personal communication). Non-complementation in the complementation tests between *mas-2* and *mas-25* showed that they were allelic (Chaudhuri, personal communication). We tested linkage of the other mutants using two representative SSCP markers each for chromosome I and chromosome III on the F2

transformers obtained by back crossing with the Washington strain. For chromosome I, we used L-40 and L-97, and for chromosome III we used L-23 and L-70.

Two of the mutations, *mas-23* and *mas-27*, mapped to chromosome III around the same region as *Ppa-tra-1* and were presumably allelic to it. However, *mas-25* was determined to be on chromosome I, which was novel, and since it was an allele of *mas-2*, we concentrated further fine mapping efforts on *mas-25* and *mas-2*. We performed whole genome sequencing on *mas-25* and *mas-2*, and comparisons of these sequences with the wild type highlighted several mutations throughout the genome, including one for *mas-25* on chromosome I close to that predicted by SNP mapping. The SNPs were filtered based on whether they mutations were found in open reading frames and whether they changed the codon of an amino-acid. The mutation in *mas-25* was further confirmed by PCR amplification of the coding sequence around that region, followed by sequencing. BLAST alignments for the coding sequence around this locus suggest that the mutation could lie in a gene homologous to the *Cel-mcm-7* gene (Fig. 4.4). The *mcm-7* (*minichromosome maintenance protein-7*) gene in *C. elegans* is a DNA replication licensing factor that has been suggested to play important roles in embryogenesis, development of gonads, vulva formation, locomotion, etc. (Tabuse et al. 2005; Wang et al. 2007; Updike and Strome 2009). Aligning the coding sequence of the mutant with wild type indicated that *mas-25* has a point mutation changing the amino acid at position 30 from aspartic acid to tyrosine (Fig. 4.4).

Figure 4.4: Protein alignment of mas-25 and mcm-7, shows conservation of PPA-MAS-25 with CEL-MCM-7 and MCM-7 in other nematodes (Bma – *Brugia malayi*, Cbr – *Caenorhabditis briggsae*). The red arrow shows the mutation that changes the amino acid from D in wild type to Y in PPA-MAS-25.

4.4 Discussion

Studies have shown that the sex determination pathway evolves rapidly, even within the Caenorhabditis genus (Zarkower 2001; Pires-daSilva and Sommer 2004). Most of the information about evolution of sex determination is available in either from distantly related species *C. elegans* and *D. melanogaster*, where it is highly divergent, or closely related species like *C. elegans* and *C. briggsae*. Since C. elegnas and C. briggsae have the same sex determination, we chose *P. pacificus* for comparative studies. *P. pacificus* is used as a satellite nematode species for comparative studies with *C. elegans* and many genetic and genomic tools are available for such investigations. The gene *tra-1* has been previously shown to be conserved between *C. elegans* and *P. pacificus*, however, other genes involved in sex determination in *P. pacificus* remain unknown. Here, we have employed forward genetics to identify more genes involved in sex determination in *P. pacificus*.

It has been long proposed that sex determination shows a retrograde kind of evolution, where the most downstream gene is conserved between species, and newer upstream genes from other related pathways are recruited for regulation. The conservation of TRA-1 supports this hypothesis (Pires-daSilva and Sommer 2004). However, the interaction between TRA-1 and MCM-7 still remains to be determined. MCM-7 is a very important gene in *C. elegans* that has crucial developmental functions, such as in embryogenesis, formation of gonads and the vulva, etc. It is a conserved and essential protein for the initiation of replication in eukaryotic genomes acting as an unwinding enzyme and is essential for the formation of replication forks during the S phase of cell division. It has been shown previously that RNA helicases play a role in sex determination in *C. elegans* (*mog-1, mog-4* and *mog-5*) and so it can be reasonably

hypothesized that MCM-7 which has a primarily helicase function, is involved in *P. pacificus* sex-determination (Puoti and Kimble 1999). However, there is no evidence to suggest its role in sex determination in *C. elegans*. TRA-1 is a transcription factor that can be regulated by several different molecular mechanisms. In *C. elegans*, it is regulated by TRA-2 and TRA-3, however, this may or may not be the case in *P. pacificus*, and other genes or molecules, such as MCM-7, could regulate it.

Our experiments so far suggest a possible role of MCM-7 in the sex-determination of *P. pacificus* but further experimentation is required to confirm this. Transgenics have been successfully employed in *P. pacificus* for gene function knock-down and rescue (Schlager et al. 2009; Wang and Sommer 2011). For *P. pacificus*, instead of injecting the standard circular plasmids, linearized plasmids are used, along with sheared genomic DNA. These plasmids then produce extra-chromosomal arrays that are transmitted to the F1 generation. To rescue the phenotype in the mutants, genomic DNA from the wild type animals can be injected into the gonadal syncytium of the mutants along with the plasmid containing a dominant fluorescent marker and the F1 and F2 progeny can be observed for rescue. The predicted gene that corresponds to *mas-2* and *mas-25* is ~4kb in size. In order the rescue the mutants approximately 3 kb upstream of the gene and 1 kb downstream of the gene needs to be amplified, making it a total of about 8 kb. We tried to amplify this region using long range PCR but our attempts were unsuccessful, probably because the genome assembly was not correct. Therefore we could not proceed further with the rescue experiments with this approach and a different approach might be useful. However, based on the conservation of TRA-1 and its function in *C. elegans*, we can hypothesize MCM-7 is upstream of TRA-1 in *P. pacificus* too, and that it has been recruited in sex determination from interacting pathways.

Chapter 5

Conclusion and future research

Two kinds of sperm competition are possible in these trioecious species, male-male and male-hermaphrodite. Our results suggest that males that are more attracted towards females evolve larger sperm, perhaps due to greater male-male sperm competition. The hermaphrodite sperm on the other hand is much smaller than male sperm perhaps because of developmental bias. The hermaphrodites have a limited availability of developmental resource for which both the sperm and the oocytes compete (Baldi et al. 2011). The hermaphrodites therefore cannot foster the development of sperm as efficiently as males do. There is also no selective pressure on the hermaphrodite sperm since all of it is used for fertilization. Instead, the pressure on their gonad is to produce a large quantity of sperm in a very short duration before irreversibly switching to oogenesis.

5.1 Selective forces on sex ratios of trioecious nematodes

Several selective forces are possibly involved in shaping the sex ratios of trioecious species. First, male-male sperm competition imposes strong sexual selection in this species. This competition appears to be induced by a limited number of females in the population and production of attractants by them. Larger and more efficient sperm are thus favored. Second, there would also be natural selection for the production of hermaphrodites over males or females. This is because in their natural habitat, these nematodes probably face adversities such as scarcity of food, overcrowding, pathogen infections, low/high humidity and temperature fluctuations that could make the finding of mates difficult. The hermaphrodites being self-fertilizing, can colonize newer habitats on

their own. Such environmental selective forces result in the evolution of biased sex ratios as adaptations to maintain the evolutionarily stable strategy proposed by classical theories (Fisher 1930; Hamilton 1967a; West 2009).

As mentioned in the introduction, several mechanisms have been identified to affect sex ratios of offspring, including chromosomal mechanisms and selective post-fertilization mortality. In our study, we identified a new mechanism that suppresses the production of males, whereby males actively discard the male-producing spermatids. Our observations also provide a deeper understanding of how sex ratios are important in the evolution of sex determination mechanisms.

5.2 Evolution of the XX/XY and XX/XO SD systems

Several studies have documented that sex chromosomes evolved from autosomes (Muller 1914; Lahn and Page 1999; Graves 2006). Ohno (1967) proposed that in a common ancestor of all vertebrates some mutation arose that took over the function of sex determination. Therefore, presumable ancestors of the XY system evolved alleles that induced the animal to become a male (Graves 2006). In mammals, over the course of evolution, the chromosome bearing the sex-determining allele acquired more mutations and became the Y chromosome, whereas the homologous chromosome became the X. Over time, the Y chromosome lost most of its genes (and is much shorter than X), except for genes involved in male fertility (in *Drosophila melanogaster*) and sex-determination (in humans). Degeneration of the Y chromosome could occur through several mechanisms, such as fixation of deleterious mutations (Rice 1987), failure to evolve adaptively, absence of recombination (Bachtrog and Charlesworth 2002) and gene duplication (Betrán et al. 2004). The mechanisms that contribute to the Y-chromosome degeneration have been shown to vary depending on

the stage of this process (Bachtrog 2008). In Drosophila, it is the ratio of the number of X chromosomes to the number of autosomes that determines sex. Mutant *D. melanogaster* XO animals are male but sterile and the XXY animals are female, indicating that the Y chromosome is not important for sex determination but for fertility (Brosseau 1960; Charlesworth 1996). In *D. pseudoobscura*, some males carry a 'sex-ratio' polymorphism on an X-chromosome and these males produce almost exclusively female progeny. The Y chromosome in these males is degenerated during meiosis and the Y-bearing sperm fails to mature (Novitski and Sandler 1957; Novitski et al. 1965). A high frequency of males bearing the driving 'sex-ratio' X-chromosome in a population will lead to an unequal transmission of the sex chromosomes to the progeny (meiotic drive). As a result of this, the sex ratio is female-biased, and this has in fact been observed in some populations (Bryant et al. 1982; James and Jaenike 1990). A continued X chromosome drive has lead to a complete loss of the Y chromosome and evolution of new sex determination systems (XO systems, or XX systems, or other modifications) as seen in some rodent families (Arakawa et al. 2002; Marchal et al. 2003; Wilson and Makova 2009).

5.3 Selection of males and hermaphrodites in *C. elegans vs*. SB347

If a similar theory for evolution of the XX/XO system is applied towards hermaphroditic nematodes, the ancestors of hermaphroditic nematodes being gonochoristic, it can be reasonably suggested that there is a preferential transmission of the X chromosome due to the self-fertilizing hermaphrodites. The hermaphrodites produce males at a very low frequency due to spontaneous non-disjunction of the X chromosome. Since theoretically, the hermaphrodites being XX should not produce males at all, this mechanism of producing occasional males has perhaps been selected

to provide the necessary genetic diversity to prevent inbreeding depression. However, this view of male selection in *C. elegans* has been challenged by some recent studies that show that it does not suffer much from inbreeding depression (Cutter 2005; Anderson et al. 2010). These studies present evidence that natural populations of *C. elegans* that are presumably under strong selection, show a frequency of males lower than in experimental selection (Barrière and Félix 2005; Barrière and Félix 2007). Thus, a high mutation rate due to outbreeding may not necessarily be the only reason for maintenance of males. Interestingly, higher outcrossing was observed when the populations were subjected to the dauer stage, despite low initial frequencies (Morran et al. 2009). Dauer stage is induced as a response to environmental adversities. The dauer larvae resume normal development when favorable conditions return; facultative outcrossing may have been selected in this situation (Anderson et al. 2010).

SB347 hermaphrodites naturally produce a higher proportion of males (~ 15%) in comparison to androdioecious species like *C. elegans*, where males are <1% of the population. This could be because of the presence of females in SB347 that impose a strong sexual selection for males, which is absent in *C. elegans*. On the other hand, given the harsh conditions that these nematode species must survive, natural selection would favor hermaphrodites over the production of males and females. A single dauer larva can migrate to habitats and resume development when it encounters food. But, a single male or female larva cannot establish a new population on its own. Under such conditions, finding a mate for male and female within its short reproductive period may not be successful. Hermaphroditic traits thus get selected and sex ratios are skewed towards hermaphroditism. The flexibility of the genotypically XX larvae in SB347 to develop as a female or a hermaphrodite depending on the environment further supports that natural selection favors hermaphrodites in harsh conditions (Chaudhuri et al. 2011a).

The presence of males and females therefore would indicate favorable conditions for the worms to quickly multiply and colonize by producing mostly hermaphrodites. This is precisely what is observed for SB347 where a cross between male and female mostly produces hermaphrodites (this dissertation, Chaudhuri et al. 2011).

5.4 Trioecious nematodes present a new mechanism to regulate sex ratios

Through our experiments, we have unveiled a mechanism by which sex ratios get biased towards feminine progeny, by the programmed death of the male producing sperm. Although asymmetric division was observed in the spermatocytes of males of all five species studied, apoptosis of the nullo-X was studied and confirmed only in SB347 so far.

The X-bearing sperm from males has a competitive edge over the nullo-X male sperm in other nematodes too. In *C. briggsae* it is preferentially used for fertilization to produce hermaphrodites (LaMunyon and Ward 1997). *C. briggsae* males are poor maters and therefore the chance that a hermaphrodite will outcross is very low. Even though it is advantageous to produce self-fertilizing hermaphrodites, half of the sperm transferred by the *C. briggsae* male during copulation produces male progeny. In spite of being second in priority, the nullo-X sperm in *C. briggsae* gets abundant chance to fertilize the oocytes and preference over the hermaphrodite sperm. Contrary to this, SB347 males are very efficient maters, partly due to the production of attractants from females who usually mate with several males. The amount of sperm available to females of SB347 might exceed the amount of oocytes it can produce to exhaust that sperm during its reproductive period. Assuming a *C. briggsae* like selection for sperm in SB347 there is always plenty of X-bearing sperm present inside the female reproductive tract and it is quite possible that the nullo-X sperm does not get used much.

In the above scenario for SB347, if half of the male sperm (nullo-X) remain unused, it is a huge waste of resources for males. Even if a female does not mate with several males (in which case the nullo-X sperm gets used), they would result in males and not hermaphrodites. Although we do not currently have accurate record of the life history of these worms and the conditions they live in, it should be safe to assume that generally, they face harsh environments like other free-living nematodes. As discussed previously, under such conditions, hermaphrodites are preferred. Traits that would reduce the production of nullo-X sperm or enhance the production of the X-bearing sperm therefore get selected. Apoptosis of the nullo-X sperm in males of SB347 has perhaps been selected for this purpose. Further experimental evolution studies using the APS8 mutant (which produces more males after crossing than wild type) isolated in our experiments could provide more insight into the patterns of evolution.

5.5 Evolution of sex ratios and sex determination mechanisms

Sex ratio selection could lead to the evolution of new sex-determining systems and because of diverse selective pressures, it is likely that the systems will have some alterations in the underlying genetic components (Uller et al. 2007). Proper development of sexual phenotypes is one of the most important developmental processes and it would be expected that such a crucial process is highly conserved. However, the sex determination system seems to evolve faster than other developmental pathways (Wilkins 1995; Marín 1998; Pires-daSilva and Sommer 2004). There is substantial variation in the mechanisms of sex determination even in closely related species (Bull 1983; Pires-daSilva and Sommer 2004). According to the ESS, a 1:1 ratio is the stable sex ratio mainly due to frequency dependent selection, which will act against conditions or mutations that cause deviations of this sex ratio. This holds true for the primary sex

ratios of several sex determination mechanisms (Bulmer and Bull 1982). However, in some situations (such as the three-locus system in housefly) producing one sex may prove more costly than the other leading to different selective pressures (Kozielska et al. 2006). Other conditions such as sex chromosome drive, cytoplasmic sex ratio distorters can also cause alterations in the underlying sex determination system (Werren and Beukeboom 1998).

Sex determination mechanisms may also be altered in order to maintain the core signal for development of the sexual phenotype (Pomiankowski et al. 2004). If the abnormal expression of a certain gene disrupts a core signal (for e.g. *tra-1* in *C. elegans* and *P. pacificus*) of sex determination, patterns of upstream gene expression that suppress the disrupting element will be selected. In our experiments on *P. pacificus*, which is a model system developed for comparative studies with *C. elegans,* we found a putative homolog of the gene *mcm-7* that is probably required for the development of a hermaphrodite. We isolated allelic mutants of this gene in *P. pacificus*, *mas-2* and *mas-25*, which develop as males instead of hermaphrodites. *mcm-7* has a role in other developmental processes in *C. elegans* but this is the first report of it being involved in sex determination. Since the core sex determination pathway is not known in *P. pacificus* yet, further research needs to be done to identify the interactions of this gene. However, the fact that this gene has no sex determination role in species at least as distant as *Drosophila*, but is conserved for other functions suggests that sex determination recruits genes from other pathways. If there is a mutation that suppresses the development of a particular sex, a second mutation, most likely in a gene that has a suppressive effect on the first mutation, will be selected such that the evolutionarily stable strategy is maintained.

5.6 Limitations of the current results and potential future experiments

Relatively little is known about the sex determination system in trioecious species. At this moment, we cannot rule out the possibility for the presence of sex-ratio distorters on the X chromosome of SB347, leading to few males after crossing. In this work, I found that part of the mechanistic basis for the production of few males in SB347 is based on apoptosis of nullo-sperm in males. Presumably in hermaphroditic/male nematodes the fixing of a meiotic driver for the X chromosome could be more common than in male/female species, because extreme sex ratios against males do not compromise reproduction as in a male/female species. Since hermaphrodites can self-fertilize and also produce males once by non-disjunction, meiotic drive does not necessarily result in a population crash.

The precise selective forces that alter meiosis in SB347 males to make the sister chromatids segregate during meiosis I still remain to be identified. However, based on our current results we can propose the role of two predominant forces. First, selection for hermaphrodites that drives the selection of competitive X-bearing sperm. Second, selection of larger cell size for the X-bearing sperm at the expense of the nullo-X spermatid because the larger cell size provides a competitive advantage against sperm from other males and hermaphrodites. Size comparison of the X-bearing and nullo-X spermatids would reveal if in fact more cytoplasm is being transferred to the former to provide a size advantage. In the alternate scenario, if the chromatids of the X-chromosome stayed together during meiosis I this size difference would be difficult to achieve unless there was an asymmetric division in meiosis I. This scenario can be tested if mutants where the X sister chromatids stay together can be isolated.

Additional factors have been identified to influence the sex ratios and mating behaviors in at least two species of this clade, SB347 and SB372. For instance, it has

recently been found that the SB372 hermaphrodite mothers can manipulate the sex of the offspring in response to certain environmental cues (Pires-daSilva, personal communication). Under unfavorable growth conditions, the hermaphrodite mother predominantly produces hermaphroditic progeny, whereas under favorable conditions males and females are produced. Certain environmental cues, most likely in the form of chemicals, can convey specific signals to the nervous system that relays signals to the gonad produce females or hermaphrodites. It has also been shown in SB347 that the genetically XX larvae show a developmental plasticity and can be induced at an early stage to develop as females or hermaphrodites (Chaudhuri et al 2011). Furthermore, males of the trioecious species studied here show different levels of activity; SB347 males are very active and crawl on the plates at a rather fast pace compared to the other species (personal observation). Such behavioral traits have not been experimentally measured yet, but are certain to influence the mating behavior and therefore sperm size.

In our studies, we have not taken into account the factor of female choice, which in many instances might be cryptic and still drive the evolution of sperm morphology. In a study on guppies, females were artificially inseminated with equal amounts of sperm obtained from two males with contrasting characters. The results showed that females preferentially used sperm from the male that was more attractive. A possible explanation of this was that the attractive males also produce superior sperm (Evans et al. 2003).

In conclusion, we have used the comparative method to determine correlation of variables that could be affected by sexual selection in trioecious species. However, we do understand that phylogenetic comparative method using closely related species might not be fully adequate and sufficient to study adaptation (Garland and Adolph 1994; Martins 2000; Hansen 2014). Correlations observed by making comparisons between closely related species may only be due to common ancestry. Comparative studies in the future

should therefore also include more distantly related nematodes. Further experimentation also needs to be performed to verify and precisely determine the causation of the proposed adaptations. Experiments including, but not limited to establishing phylogenetic relationships, information about life history, determination of physiological and developmental differences between the species, adaptive responses to various environmental conditions and obtaining genomic information must be performed to get more complete understanding.

Appendix A

Data for sex ratios

		Hermaphrodites	Female	Male	Total
SB347	No.	120	62	18	200
	%	60	31	9	100
JU1782	No.	132	30	4	166
	%	79.5	18.0	2.4	100
JU1783	No.	18	142	32	192
	%	9.3	73.9	16.6	100
SB372	No.	94	50	2	146
	%	64.3	34.2	1.3	100
JU1809	No.	154	28	6	188
	%	81.9	14.8	3.1	100

Appendix B

Data for life span measurements

SB347 Females

Replicate #1

Date	DOA	Alive	Dead	Censored	Comments
25-May-14	1	32			
26-May-14	2	32			
27-May-14	3	29	0	3	3-M
28-May-14	4	28	0	1	1-M
29-May-14	5	26	2		
30-May-14	6	23	3		
31-May-14	7	20	1	2	2-EV
1-Jun-14	8	9	7	4	4-EV
2-Jun-14	9	5	4		
3-Jun-14	10	0	5		

Replicate #2

Date	DOA	Alive	Dead	Censored	Comments
25-May-14	1	40			
26-May-14	2	40			
27-May-14	3	40			
28-May-14	4	39	1		
29-May-14	5	39	0		
30-May-14	6	33	6		
31-May-14	7	23	7	3	1-M; 2-EV
1-Jun-14	8	8	13	2	2-EV
2-Jun-14	9	3	5		
3-Jun-14	10	0	3		

Replicate #3

Date	DOA	Alive	Dead	Censored	Comments
26-May-14	1	40			
27-May-14	2	40			
28-May-14	3	38	0	2	2-M
29-May-14	4	37	1		
30-May-14	5	35	2		
31-May-14	6	29	4	2	2-EV
1-Jun-14	7	13	16		
2-Jun-14	8	2	11		
3-Jun-14	9	0	2		

SB347 Hermaphrodites

Replicate #1

Date	DOA	Alive	Dead	Censored	Comments
14-May-14	1	41			
15-May-14	2	41			
16-May-14	3	40		1	1-M
17-May-14	4	40			
18-May-14	5	35	5		
19-May-14	6	32	3		
20-May-14	7	26	2	4	3-EV; 1-E
21-May-14	8	11	5	10	6-EV; 4-E
22-May-14	9	3	5	3	3-EV
23-May-14	10	0	3		

Replicate #2

Date	DOA	Alive	Dead	Censored	Comments
14-May-14	1	38			
15-May-14	2	38			
16-May-14	3	38			
17-May-14	4	35		3	2-M; 1-E
18-May-14	5	35			
19-May-14	6	32		3	2-EV; 1-E
20-May-14	7	25	4	3	2-EV; 1-E
21-May-14	8	14	6	5	3-EV; 2-E
22-May-14	9	6	5	3	2-EV; 1-E
23-May-14	10	1	2	3	1-EV; 2-E
24-May-14	11	0	1		

JU1782 Females

Replicate #1

Date	DOA	Alive	Dead	Censored	Comments
22-Apr-14	1	42			
23-Apr-14	2	42			
24-Apr-14	3	42			
25-Apr-14	4	42			
26-Apr-14	5	42			
27-Apr-14	6	38	4		
28-Apr-14	7	33	5		
29-Apr-14	8	23	10		
30-Apr-14	9	10	13		
1-May-14	10	2	8		
2-May-14	11	0	2		

Replicate #2

Date	DOA	Alive	Dead	Censored	Comments
22-Apr-14	1	30			
23-Apr-14	2	30			
24-Apr-14	3	30			
25-Apr-14	4	30			
26-Apr-14	5	30			
27-Apr-14	6	30			
28-Apr-14	7	29	1		
29-Apr-14	8	21	8		
30-Apr-14	9	8	13		
1-May-14	10	0	8		

JU1782 Hermaphrodites (censored worms not noted)

Replicate #1

Date	DOA	Alive	Dead	Censored	Comments
19-Apr-14	1	30			
20-Apr-14	2	30			
21-Apr-14	3	30			
22-Apr-14	4	30			
23-Apr-14	5	30			
24-Apr-14	6	27	3		
25-Apr-14	7	21	6		
26-Apr-14	8	12	9		
27-Apr-14	9	7	5		
28-Apr-14	10	3	4		
29-Apr-14	11	1	2		
30-Apr-14	12	0	1		

Replicate #2

Date	DOA	Alive	Dead	Censored	Comments
19-Apr-14	1	30			
20-Apr-14	2	30			
21-Apr-14	3	30			
22-Apr-14	4	30			
23-Apr-14	5	30			
24-Apr-14	6	26	4		
25-Apr-14	7	19	7		
26-Apr-14	8	16	3		
27-Apr-14	9	11	5		
28-Apr-14	10	4	7		
29-Apr-14	11	1	3		
30-Apr-14	12	0	1		

Replicate #3

Date	DOA	Alive	Dead	Censored	Comments
19-Apr-14	1	29			
20-Apr-14	2	29			
21-Apr-14	3	29			
22-Apr-14	4	29			
23-Apr-14	5	29			
24-Apr-14	6	29			
25-Apr-14	7	28	1		
26-Apr-14	8	21	7		
27-Apr-14	9	11	10		
28-Apr-14	10	3	8		
29-Apr-14	11	1	2		
30-Apr-14	12	0	1		

Appendix C

Primers used for nested long range PCR in *P. pacificus*

AP_1122	Forward	ACGCTCGGGCGAAATAGTCACACATCT
AP_1123	Reverse	TGTTCACAAAGTCAACCCACGCCATCT
AP_1124	Forward	TACGAAATAGGCGGTGAATGGGACGAA
AP_1125	Reverse	TTGGACGCCACCCTCGTAGATTGAGAT
AP_1172	Forward	GAGAATTCCTTAATTCCCATAAATAGC
AP_1173	Forward	TATTTCAAAAATAAAGCCCAGATCTAA
AP_1174	Forward	TATTTCCAGGATATTAGAAGAATGGTG
AP_1175	Forward	ATCAAGAGGTAAAAAGGGTTAAAATGT
AP_1176	Reverse	CAGATAGGTAATTTCAGACGGAGATAG
AP_1177	Reverse	CAGGTAAATGGAAAAGATCAAGAAATA
AP_1178	Reverse	AAAGTGTAATGAAGAAGAAGAGGTCAA
AP_1179	Reverse	AAAACAAAACACAAAATAATACCCAAA

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