ABSTRACT

Title of Document:	INVESTIGATIONS INTO THE EVOLUTION OF SELF-FERTILE HERMAPHRODITISM AND REPRODUCTIVE ISOLATION IN CAENORHABDITIS NEMATODES
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Phenotypic variation, reproductive isolation, and the consequences of selfing are important issues in biology. The nematode genus *Caenorhabditis* includes both gonochoristic and androdioecious species. However, the natural genetic variants distinguishing reproductive mode remain unknown, and nothing is known about their reproductive isolation. Here, multiple facets with respect to the hybridization of the gonochoristic *C.* sp. 9 and the androdioecious *C. briggsae* are described. A large fraction of interspecies F_1 arrest during embryogenesis, but a viable subset develops into fertile females and sterile males. Reciprocal parental crosses reveal asymmetry in male-specific viability, female fertility, and backcross viability. *C.* sp. 9 animals and F_1 hybrids are inviable at cool temperatures that *C. briggsae* can tolerate, which may reflect their geographic distribution. The segregation of the selfing trait was evaluated in multiple hybrid generations using organismal, cellular, and molecular definitions of the trait. Selfing is recessive in F_1 hybrids and was extremely rare in hybrid animals. All hybrid self-progeny are inviable. Multiple techniques were utilized to enrich the incidence of selfers in hybrid generations, and the genotyping of hybrid animals revealed segregation distortion at multiple loci. Additionally, it was found that *C. briggsae* hermaphrodites have lower numbers of self-progeny and reduced lifespan when mated with *C.* sp. 9 males. Fluorescent microscopy revealed

that *C. briggsae* hermaphrodites previously mated with *C.* sp. 9 males accumulate germ line defects, and vital staining revealed that *C.* sp. 9 sperm are capable of ectopically invading *C. briggsae* hermaphrodite tissues. *C.* sp. 9 males with feminized germ lines were incapable of promoting the mating-dependent sterilization and lifespan reduction in *C. briggsae* hermaphrodites, although they were able to mate. This suggests that the degradation of mating-related traits in hermaphroditic lineages may lead to gonochoristic male mating-induced sterility and mortality in hermaphrodites, and therefore potentially to reproductive isolation between these lineages. Presumably, this is due to relaxed sexually antagonistic selection on traits associated with sperm competition. Collectively, these investigations are among the first in a *C. briggsae*/*C.* sp. 9 system that will likely prove fruitful for future studies in reproductive isolation and the evolution of self-fertile hermaphroditism.

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

In memory of my father

Acknowledgements

In my experience as a graduate student, I have learned a great deal. One of the most lasting lessons is this: to make progress, you need the help of others.

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And finally, I must thank my family and close friends: Mom, Amy, Uncle Wayne, Aunt Anita, Uncle Curry, Aunt Kathy, Uncle Richard, Danny, Michael, Brian, Erika, Andres, and Kevin. This work would not have been possible without their love and support. I cherish these relationships, and I love them all.

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Chapter 1: Introduction

The Developmental and Genetic Basis of Animal Diversity

Diversity exists, and one of the major goals of evolutionary biology is providing an empirical explanation for this observation. However, this subject can be approached in many ways, and it has historically been framed outside of the context of developmental genetics (LAUBICHLER and MAIENSCHEIN 2007). This is unfortunate because much of animal phenotypic diversity is dependent upon diversity in embryonic and post-embryonic development because in most animals the adult individual is re-constructed every generation from a single-celled zygote (RAFF 1996). Thus, a proper understanding of animal diversity should incorporate information from developmental genetics, which is concerned with how genes and gene products influence developmental events. Such a scientific program would set out to explain how differences in genes and gene products promote differences in developmental events, which in turn would contribute to explaining the proximal causes of animal diversity.

Indeed, the maturation of comparative genomics into a reputable biological discipline has made the questions regarding the genetic basis of phenotypic diversity ever more glaring (MIKKELSEN *et al.* 2005). Clearly, there is a tremendous amount of DNA sequence difference between organisms, but for the vast majority of cases it is unknown which are developmentally relevant. Furthermore, although the genetic bases of many adaptive traits have been unearthed, for the most part the developmental biology of these traits is still unclear or inferred from studies with other systems (ABZHANOV *et al.* 2004; DOEBLEY 2004).

One potential avenue of tackling this problem is to approach it within an organismal system whose developmental genetics is already comparatively well defined. Not only would this greatly facilitate the generation of hypotheses with respect to possible underlying mechanisms of phenotypic differences, but it would also allow easier and more rigorous testing of such hypotheses. For these reasons, this is the approach that will be taken in this study. Here, the genetic basis of animal diversity will be probed within the nematode genus *Caenorhabditis*, of which the model organism *C. elegans* is a member.

C. elegans *and the Diversity of Reproductive Mode in Caenorhabditis*

C. elegans is a useful tool for investigating a wide spectrum of biological problems. Aside from the properties that lends it easily to laboratory genetics, the system has resources that include: a well-annotated genome sequence the complete cell lineage of the hermaphrodite, the complete structure of the hermaphrodite nervous system, established protocols for gene knockdown and transgenics, easily navigable online databases, and an open and helpful community of investigators (HODGKIN 2005). And in addition to this, the extreme breadth of topics successfully examined using this organism is by itself a testament to its usefulness as a subject of scientific inquiry (DE BONO and BARGMANN 1998; GRIFFITTS *et al.* 2001; RAIZEN *et al.* 2008). For these reasons this species can act as an excellent point of reference for comparative development studies (HAAG and PILGRIM 2005).

Although many examples of interspecies diversity in *Caenorhabditis* have been described (such as the number of male tail rays (BAIRD *et al.* 2005), the presence or absence of a copulatory plug (PALOPOLI *et al.* 2008), and variation in vulva specification

(FÉLIX 2007)), the arguably most consequential and obvious example of such diversity is that of reproductive mode (Figure 1). Some *Caenorhabditis* species are gonochoristic (male/female) whereas others are androdioecious (male/hermaphrodite). Hermaphrodites and females are somatically similar, and they differ mainly with respect to their germ lines. Females only make oocytes, whereas hermaphrodites briefly undergo spermatogenesis before switching to oogenesis (HAAG 2005). Thus, here is a striking interspecies difference that not only has consequences that bear on population genetics and reproductive strategies, but also one that is discrete and easily phenotypically demarcated. Furthermore, the presence of these XX sperm almost invariably leads to the presence of self-progeny. This ease of phenotyping the trait in tandem with its biological importance provides the justification for the examination of the developmental basis of its interspecies variation in this study.

Germ line sex determination in C. elegans

Since the *Caenorhabditis* genus has been chosen as the focus of this investigation due to the wealth of information provided by the *C. elegans* literature, and the hypotheses, experiments, and results of this study will be framed within the context of this information, it is prudent to briefly review the relevant background.

Because of the similar morphology and development of hermaphrodite and male sperm, as well as the presence of a male cell type in a female body, hermaphrodite spermatogenesis has long been seen as necessarily tied to the process of hermaphrodite germ line sex determination (DONIACH 1986). *C. elegans* sex is ultimately determined by the X chromosome to autosomal chromosome ratio, which influences a regulatory signal transduction pathway that specifies either the male or female fate (ZARKOWER 2006).

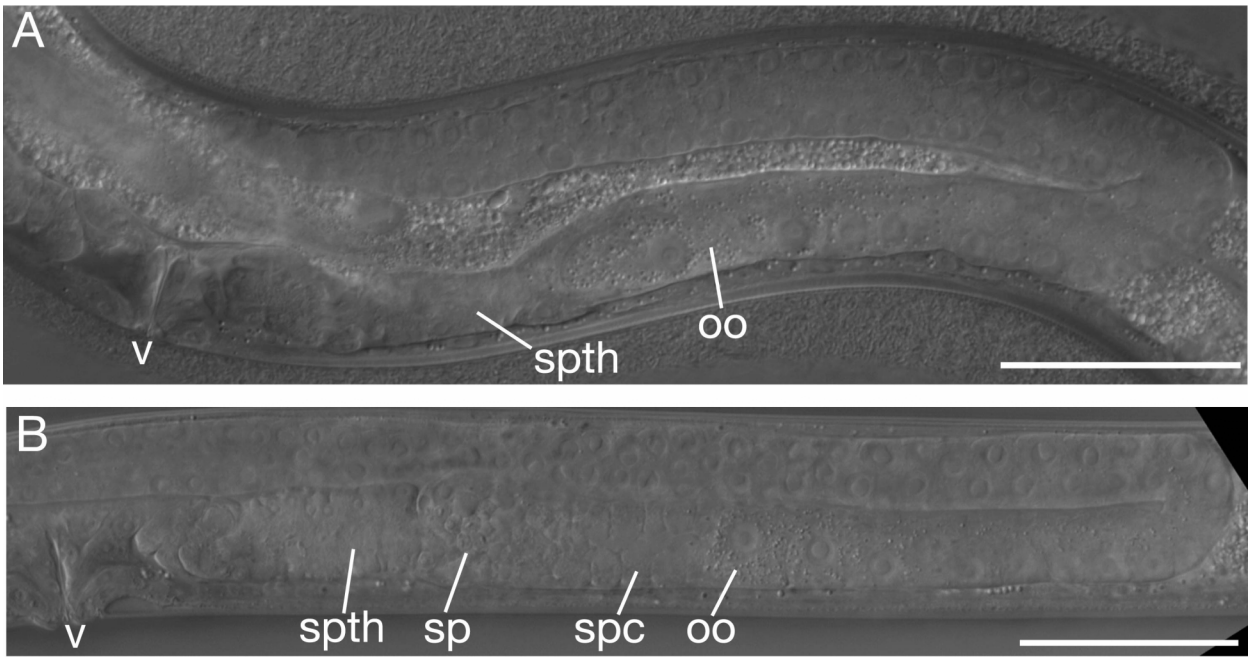


Figure 1. Variation in reproductive mode in *Caenorhabditis*. A. A young C. sp. 9 adult female. The most proximal germ cell is an oocyte (oo), and no sperm cells are present. **B.** A young *C. briggsae* adult hermaphrodite. Mature sperm (sp) are the most proximal population of germ cells, followed by developing spermatocytes (spc) and oocytes (oo). Scale bars represent 100 μm. spth, spermatheca. v, vulva. This figure is taken from work performed in Chapter 2.

Wild type males are XO, wild type hermaphrodites are XX, and they both have two copies of five autosomal chromosomes (MADL and HERMAN 1979). According to the current model, a low X:A ratio leads to the secretion of the ligand HER-1 (PERRY *et al.* 1993), which then binds the transmembrane protein TRA-2 (HAMAOKA *et al.* 2004), whose conformational change releases the FEM proteins (MEHRA *et al.* 1999) which then target the downstream female-promoting transcription factor TRA-1 for protein degradation via ubiquitination, leading to the specification of the male fate (STAROSTINA *et al.* 2007). A high X:A ratio leads to the inhibition of HER-1 expression, preventing the degradation of TRA-1, which then leads to the specification of the female fate (ZARKOWER 2006). In the hermaphrodite germ line, a set of germ line specific translational regulatory controls exist to regulate this core sex determination pathway in

order to facilitate the specification of sperm in an XX animal. Prior to spermatogenesis, the GLD-1/FOG-2 complex binds the 3' UTR of the *tra-2* transcript, inhibiting its translation (GOODWIN *et al.* 1993; CLIFFORD *et al.* 2000). This leads to a greater activity of the FEM proteins, which in turn promote male fates in the germ line (KIMBLE *et al.* 1984). Conversely, the subsequent translational inhibition of *fem-3* transcripts by the FBF complex promotes the switch from male fates to female fates (spermatogenesis to oogenesis) (ZHANG *et al.* 1997). So, genetic and molecular evidence has led to the current model that the modulation of TRA-2 and FEM-3 activity in the germ line regulates the sperm to oocyte switch in the *C. elegans* hermaphrodite (ELLIS and SCHEDL 2006).

The molecular genetics of the evolution of selfing in Caenorhabditis

There have been many advances in understanding the evolution of hermaphroditism in *Caenorhabditis* in recent years, and the synthesis of these studies can be used to generalize certain important mechanistic features of how self-fertility has evolved in this system. Phylogenetic analyses of *Caenorhabditis* suggest that self-fertile hermaphroditism evolved three times independently from gonochoristic ancestors in this lineage (Figure 2; KIONTKE *et al.* 2011). Multiple lines of evidence suggest that gene family evolution and co-option, commonly thought to be key mechanisms of evolutionary novelties (OHNO 1970; TRUE and CARROLL 2002), are important aspects of the evolution of this trait.

In *C. elegans*, the F-box protein FOG-2 is necessary for spermatogenesis in hermaphrodites but dispensable for male spermatogenesis (CLIFFORD *et al.* 2000). Using phylogenetic analysis and Southern blotting, *fog-2* was shown to be specific to *C. elegans* (NAYAK *et al.* 2005). Subsequently, the *C. briggsae* F-box gene *she-1* was found through

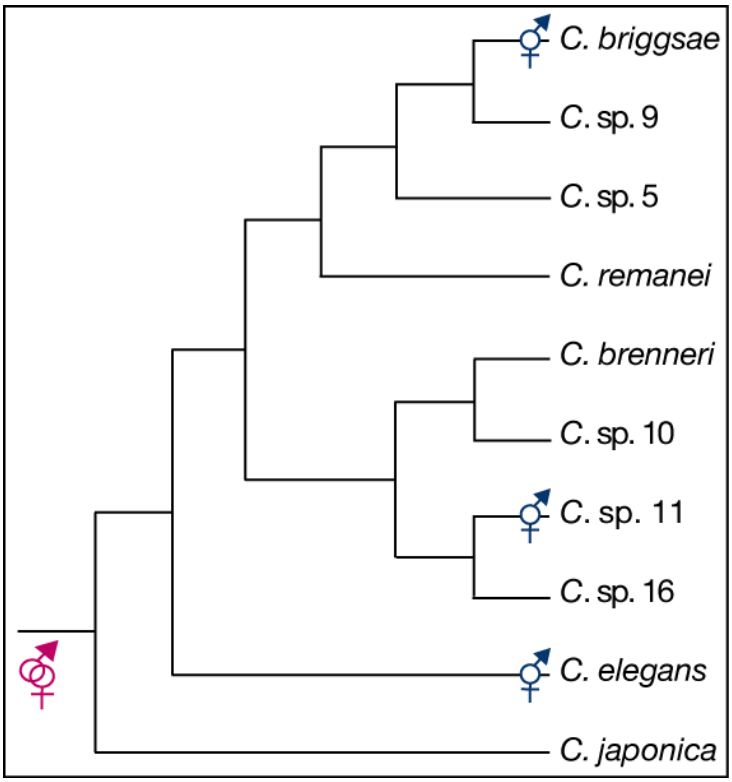


Figure 2. Self-fertile hermaphroditism evolved multiple times independently in *Caenorhabditis*. The phylogenetic relationships of the *Elegans* group of *Caenorhabditis* are displayed with *C. japonica* as an outgroup. Reproductive mode is mapped onto this phylogeny: the pink symbol represents gonochorism, whereas the blue symbols represent gains of self-fertile hermaphroditism. This phylogeny is adapted from a previous study (KIONTKE *et al.* 2011) and was inferred using eleven concatenated gene segments. This topology had high bootstrap support using three different methods of phylogenetic inference (KIONTKE *et al.* 2011).

a mutagenesis screen to be a species-specific gene necessary for hermaphrodite spermatogenesis (GUO *et al.* 2009). Therefore, surprisingly, products of different species-specific F-box gene duplication events in both *C. elegans* and *C. briggsae* were recruited independently to promote hermaphrodite spermatogenesis. Also, immunoprecipitation experiments suggest FOG-2 function is mediated through binding to GLD-1 (CLIFFORD *et al.* 2000), but yeast two-hybrid system experiments suggest that SHE-1 does not interact with *C. briggsae* GLD-1 (GUO *et al.* 2009). So, although it is remarkable that *fog-2* and *she-1* are independently derived F-box genes implicated in hermaphrodite

spermatogenesis, they probably exert their roles through different mechanisms. Regardless, these studies emphasize the importance of gene family evolution in the convergence of hermaphroditism in *Caenorhabditis*.

It is not only in the F-box family that gene duplication has been implicated in the evolution of self-fertility. The *C. elegans* RNA-binding PUF (Pumilio and FBF) family genes *fbf-1* and *fbf-2* are both redundantly required for hermaphrodite oogenesis through the transcriptional repression of the male-promoting gene *fem-3* (ZHANG *et al.* 1997). These *fbf* genes have no orthologs in *C. briggsae* (LAMONT *et al.* 2004; LIU *et al.* 2012), but the paralogous *C. briggsae* PUF family genes Cbr-*puf-2* and *Cbr-puf-1.2*, which have no orthologs in *C. elegans*, also have a role in germline sex determination (LIU *et al.* 2012). RNAi experiments that knockdown the activity of *Cbr-puf-2* together with that of *Cbr-puf-8* (or *Cbr-puf-1.2*) produce hermaphrodites that make no sperm, indicating that *puf*-2 acts redundantly with *Cbr-puf-8* and *Cbr-puf-1.2* to promote hermaphrodite spermatogenesis (BEADELL *et al.* 2011; LIU *et al.* 2012). This is in opposition to the roles of *fbf-1*, *fbf-2*, and *Ce-puf-8*, which are redundantly necessary for hermaphrodite *oogenesis* in *C. elegans* (ZHANG *et al.* 1997; BACHORIK and KIMBLE 2005). This unexpected sperm-promoting role of *puf-2* can be explained in part by its ability to bind the *Cbr-gld-1* transcript (which promotes female fates in *C. briggsae*, see below) and reduce its protein levels (LIU *et al.* 2012). In tandem with the studies on the F-box gene family, these investigations on PUF family genes reinforce the notion that gene duplication is a key factor in the evolution of self-fertility in this genus.

While gene duplications facilitate the evolution of self-fertility in multiple *Caenorhabditis* lineages, the co-option of pre-existing genes into novel roles in germline

sex determination also plays a major role. GLD-1 is a KH domain RNA-binding protein that is found throughout the *Caenorhabditis* genus (BEADELL *et al.* 2011). In *C. elegans*, *Ce-gld-1* promotes sperm-specification by regulating the sex determination gene *tra- 2* (JAN *et al.* 1999) but is also necessary for germline tumor suppression, oocyte maturation and meiotic progression (FRANCIS *et al.* 1995a; FRANCIS *et al.* 1995b). However, RNAi and loss-of-function mutations demonstrate that *Cbr-gld-1*, while retaining its roles in tumor suppression and oocyte maturation, promotes oocyte-specification in *C. briggsae* (NAYAK *et al.* 2005; BEADELL *et al.* 2011). That is, in *C. elegans*, *Ce-gld-1* loss-of-function mutations have a feminization of germline phenotype (Fog), whereas in *C. briggsae*, *Cbr-gld-1* loss-of function mutations have a masculinization of germline phenotype (Mog). RNAi knockdown of GLD-1 activity in multiple gonochoristic *Caenorhabditis* species, in tandem with probing the phenotyped animals with sex-specific germline molecular markers, suggests that the ancestral GLD-1 function was in germline tumor suppression and oocyte maintenance, and that *gld-1* has independently gained opposing sex determination roles in *C. elegans* and *C. briggsae* (BEADELL *et al.* 2011). Furthermore, another example of co-option in this system is the aforementioned *puf-8*, which in *C. elegans* acts redundantly with other PUF genes to promote female fates but in *C. briggsae* acts redundantly with other PUF genes to promote male fates. From these two examples, it is clear that the recruitment of RNA-binding proteins into novel germline sex determination roles is an important aspect of the evolution of hermaphrodite self-fertility in *Caenorhabditis*.

Indeed, the rapid evolution of RNA-binding protein function in this genus suggests that another potential general mechanism facilitating the evolution of selfing is

the evolution of the RNA targets of these proteins. It has been proposed that *cis*-regulatory changes in transcription factor binding elements are responsible for many aspects of developmental evolution (CARROLL 2008). However, the role of *cis*-regulatory changes in RNA UTR elements in such processes has been largely overlooked. The role of RNA-binding target evolution on developmental processes has been recently addressed by examining GLD-1 RNA targets in *C. briggsae* (BEADELL *et al.* 2011). In *C. elegans*, GLD-1 influences sexual fate by binding and negatively regulating the male-promoting *tra-2* 3' UTR (GOODWIN *et al.* 1993). However, comparative RNA immunoprecipitation and qRT-PCR experiments suggest that in *C. briggsae*, *Cbr-gld-1* does not associate with *Cbr-tra-2* RNA in-vivo. Furthermore, the *Cbr-tra-2* transcript has fewer GLD-1 binding elements than *ce-tra-2*, which has recently amplified them by local duplication (BEADELL *et al.* 2011). This suggests that *gld-1* has divergent functions because its ability to repress major sex determination transcripts is altered through the evolution of 3' UTR's. Determining the magnitude of the role of RNA-binding protein target evolution in the emergence of hermaphroditism in these lineages will be a promising focus of future studies.

Although the evolution of germline sex determination has long been understood as crucial in understanding the evolution of self-fertile hermaphroditism (as evidenced above), a recent study has shown that the evolution of selfing likely requires more than just changes in the sex determination pathway (BALDI *et al.* 2009). When *tra-2* activity is partially reduced in the gonochoristic *C. remanei*, females transiently produce sperm, but fail to lay self-progeny (HAAG *et al.* 2002; BALDI *et al.* 2009). However, when the sperm-activation inhibitor *swm-1* and *tra-2* are reduced in activity simultaneously, *C. remanei*

females are able to both produce sperm and lay-self progeny (BALDI *et al.* 2009). This suggests that the mechanisms promoting the evolution of self-fertility must affect more than just the sex determination pathway and must also impact crucial events downstream of the initial sexual specification of germ cells.

C. *sp. 9 and the potential for new approaches to understanding the evolution of selfing*

Although previous studies on germ line sex determination have provided insights with respect to the convergent evolution of hermaphroditism in the genus, there has been little progress in understanding the causative differences between females and hermaphrodites. Reverse genetic approaches using the gonochorist *C. remanei* have fallen short with respect to this issue (HAAG and KIMBLE 2000; HAAG *et al.* 2002), but such methods may be more effective if utilized in a gonochorist more closely related to *C. elegans* or *C. briggsae*. Furthermore, traditional forward genetics has not been successful at resolving this problem because the costly upfront work necessary for it has not been performed in any *Caenorhabditis* gonochorist. And until very recently, trait mapping approaches have proven impossible as an avenue of investigation because of the inability of any gonochoristic *Caenorhabditis* strains to produce fertile hybrid progeny with androdioecious strains.

In 2008, a new gonochoristic *Caenorhabditis* species, *C.* sp. 9, was discovered in southern India that is capable of producing fertile hybrids with the androdioecious *C. briggsae*. The existence of fertile hybrids between strains of different reproductive mode opens up the possibility of using trait mapping approaches to examine the genetic basis of hermaphroditic spermatogenesis. Furthermore, these hybrids can be utilized to

understand the barriers that isolate lineages, and these interspecies crosses can be used to investigate some of the consequences of the evolution of hermaphroditism.

C. sp. 9 *and reproductive isolation*

A longstanding problem in the biological sciences is the basis for reproductive isolation between divergent lineages. The genetic basis of postzygotic isolation has been proposed to involve the functional divergence of epistatic genetic interactions that are incompatible between lineages (CUTTER 2012). Although the genes mediating postzygotic isolation remained elusive (COYNE and ORR 2004), in recent years their molecular identity has begun to be revealed in multiple taxa (PRESGRAVES 2010). Many studies have focused on the *Drosophila* model genus (BAYES and MALIK 2009; FERREE and BARBASH 2009; PHADNIS and ORR 2009), where postzygotic isolation has been examined for decades (COYNE and ORR 2004). However, almost nothing is known about reproductive isolation in *Caenorhabditis*, although recently an intraspecific pair of epistatically incompatible factors within *C. elegans* has been characterized (SEIDEL *et al.* 2008; SEIDEL *et al.* 2011). The lack of progress, despite the otherwise favorable attributes of *Caenorhabditis*, has been largely due to the complete breakdown of hybridization in most interspecific crosses (BAIRD *et al.* 1992), which prevents genetic mapping of incompatibility factors. However, the discovery of *C.* sp. 9 (and more recently, *C.* sp. 12 (KIONTKE *et al.* 2011)) could facilitate the use of *Caenorhabditis* to address the issue of postzygotic isolation because the segregation of viable and nonviable hybrid progeny is seen. Additionally, the mechanisms of post-mating, pre-zygotic (or gametic) isolation are also poorly understood. These are reproductive barriers that occur between copulation and fertilization (COYNE and ORR 2004). And although informative studies have been

made in abalones (KRESGE *et al.* 2001) and sea urchins (PALUMBI 1998), little is known about such barriers in *Caenorhabditis*. *C.* sp. 9 and *C. briggsae* could also be used to address questions regarding gametic isolation.

Caenorhabditis *and the "selfing syndrome"*

This system is not only potentially useful for understanding the genetic basis of diversity and reproductive isolation, but also for investigating the consequences of changing reproductive mode as such. Indeed, the evolution of selfing is expected to promote profound changes in population-level genetic characteristics (GLÉMIN and GALTIER 2012). Additionally, since mating is reduced in selfing lineages, mating-related traits may also be expected to change in such populations. In flowering plants, it has long been recognized that there are marked reproductive differences between selfing and self-incompatible species. Flowers tend to be smaller, produce less pollen, produce less scent and nectar, and have less anther-stigma separation in selfing species than in outcrossing species (SICARD and LENHARD 2011). This has been referred to as the "selfing syndrome," and is generally believed to result from the lack of outcrossing and concurrent need for attracting pollinators in self-compatible species.

An analogous selfing syndrome has also been observed in *Caenorhabditis* (CUTTER 2008b). As will be further interrogated in the discussion of Chapter 3, many mating related traits have degraded in selfing *Caenorhabditis* species. Such traits that have been reduced include sperm size (LAMUNYON and WARD 1999), male maintenance (WEGEWITZ *et al.* 2008), mate discrimination (CHASNOV *et al.* 2007), and copulatory plug formation (PALOPOLI *et al.* 2008).Additionally, these traits would be expected to be maintained in obligately outcrossing species. The use then of crosses between species of

differing reproductive mode may prove useful in understanding the consequences of losing mating-related traits once selfing evolves.

The contents of these studies

Here, studies utilizing *C. briggsae* and *C.* sp. 9 are performed in order to address the issues described above. In Chapter 2, the hybrid genetics of *C. briggsae* and *C.* sp. 9 are characterized in order to provide a potential foundation for using this system to map the selfing trait. Patterns of hybrid viability and fertility are described and their potential underlying genetics are discussed. Furthermore, patterns of segregation of the selfing trait under various biological definitions are described. Additionally, multiple manipulations are used in order to potentially increase the frequency of the selfing trait in hybrid generations. And, hybrids are genotyped in order to generate hypotheses with respect to the genetic basis of selfing. These observations are then framed within the context of the genetic basis of animal diversity and reproductive isolation. This work has been published in the journal *Genetics* (WOODRUFF et al. 2010).

In Chapter 3, a curious interspecies interaction is characterized. The observation that *C.* sp. 9 males sterilize and prematurely kill *C. briggsae* hermaphrodites is described. Furthermore, it is found that these heterospecific matings cause germ line dysfunction in *C. briggsae* hermaphrodites. Additionally, it is found that *C.* sp. 9 sperm migrate ectopically in *C. briggsae* tissues, and it is experimentally determined that these cross-sperm are responsible for hermaphrodite sterilization and mortality. These findings are then discussed within the context of reproductive isolation and the consequences of the evolution of selfing. Other biological issues arising from this work (including metastasis

and sexual conflict) are also addressed. Finally, in Chapter 4, the contents of Chapters 2 and 3 are briefly summarized and future lines of investigation are discussed.

Chapter 2: Insights into species divergence and the evolution of hermaphroditism from fertile interspecies hybrids of *Caenorhabditis* nematodes

Abstract

Phenotypic variation and reproductive isolation are important problems in evolutionary genetics. The nematode genus *Caenorhabditis* includes both gonochoristic (male/female) and androdioecious (male/hermaphrodite) species. However, the natural genetic variants distinguishing reproductive mode remain unknown, and nothing is known about the genetic basis of postzygotic isolation. Here we describe the hybrid genetics of the gonochoristic *C.* sp. 9 and the androdioecious *C. briggsae*, the first *Caenorhabditis* pair capable of producing fertile hybrid progeny. A large fraction of interspecies F1 arrest during embryogenesis, but a viable subset develops into fertile females and sterile males. Reciprocal parental crosses reveal asymmetry in male-specific viability, female fertility, and backcross viability. Selfing and spermatogenesis are extremely rare in XX F1, and all hybrid self-progeny are inviable. Consistent with this, F1 females do not express male-specific molecular germline markers. We also investigated three approaches to producing hybrid hermaphrodites. A dominant mutagenesis screen for self-fertile F1 hybrids was unsuccessful. Polyploid F1 hybrids with increased *C. briggsae* genomic material did show elevated rates of selfing, but selfed progeny were mostly inviable. Finally, the use of allowed backcrosses to render the hybrid genome partial homozygous for *C. briggsae* alleles did not increase the incidence of selfing or spermatogenesis relative to the F1 generation. These hybrid animals were genotyped at 23 loci, and significant segregation distortion (biased against

C. briggsae) was detected at thirteen loci. This, combined with an absence of productive selfing, prevents formulation of simple hypotheses about the genetic architecture of hermaphroditism. In the near future, this hybrid system will likely be fruitful for understanding the genetics of reproductive isolation in *Caenorhabditis*.

Introduction

The genetic basis of phenotypic diversity is an important, albeit poorly understood phenomenon. *Caenorhabditis* nematodes provide a system that can address such an issue. *C. elegans* can act as an excellent point of reference for comparative development studies (FÉLIX 2007; LIN *et al.* 2009; SCHULZE and SCHIERENBERG 2009). *Caenorhabditis* is especially well-suited to the investigation of interspecies variation in reproductive mode, a trait of obvious organismal consequence (HAAG 2005). Some *Caenorhabditis* species are gonochoristic (male/female) whereas others are androdioecious (male/hermaphrodite; Figure 1). Hermaphrodites and females are somatically similar, but while females only make oocytes, hermaphrodites briefly undergo spermatogenesis before switching to oogenesis (ELLIS and SCHEDL 2006). This striking interspecies difference is one that is not only discrete and easily phenotypically demarcated, but is also one that has consequences that bear upon reproductive strategies and population genetics.

Many studies have addressed the evolution of germ line sex determination in *Caenorhabditis*. Phylogenetic analyses suggest that the trait has evolved convergently in this lineage multiple times (CHO *et al.* 2004; KIONTKE *et al.* 2004). Consistent with this, differences in the presence and functions of germline sex determination genes have been uncovered between the convergently evolved *C. elegans* and *C. briggsae* (NAYAK *et al.* 2005; HILL *et al.* 2006; GUO *et al.* 2009)). Similarities in germ line sex determination

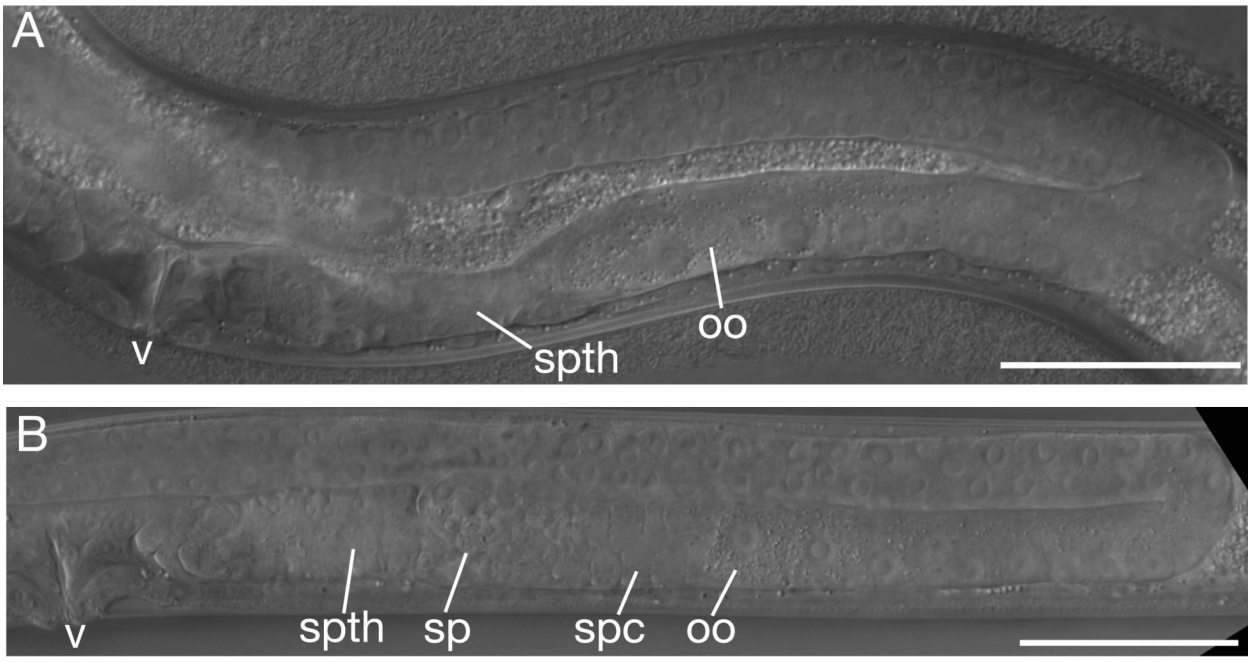


Figure 1. *C. briggsae* and *C.* sp. 9 differ in reproductive mode. A. A young *C.* sp. 9 JU1325 adult female. The most proximal germ cell is an oocyte (oo), and no sperm cells are present. **B.** A young *C. briggsae* VT847 adult hermaphrodite. Mature sperm (sp) are the most proximal population of germ cells, followed by developing spermatocytes (spc) and oocytes (oo). Scale bars represent 100 μm. "spth" = spermatheca. "v" = vulva.

between gonochoristic and androdioecious *Caenorhabditis* species have also been found (HAAG and KIMBLE 2000; CHEN *et al.* 2001; HAAG *et al.* 2002). Remarkably, reverse genetic manipulation by RNA interference can cause a *C. remanei* female to produce activated sperm and lay self-progeny (BALDI *et al.* 2009). However, despite these successes, there has been little progress in identifying the historical causative genetic differences distinguishing hermaphrodites from their female ancestors. Indeed, because the exact cause of the sperm-to-oocyte switch in *C. elegans* remains elusive (ELLIS and SCHEDL 2006) candidate-gene approaches to understanding the evolution of this trait in other *Caenorhabditis* species is problematic. The female-hermaphrodite species pairs studied thus far have been quite diverged from each other (HAAG and KIMBLE 2000; CUTTER 2008a). Here, we explore the possibility that a more closely related mixed-mode

species pair might open the door to traditional genetic trait mapping via hybrids.

In addition to the evolution of novel forms, another long-standing problem in biology is the genetic basis of postzygotic reproductive isolation. Indeed, the literature on interspecies hybrids is vast in *Drosophila* (ORR 2005) and other taxa (PRESGRAVES 2010). Recent advances in *Drosophila* (PRESGRAVES *et al.* 2003; BRIDEAU *et al.* 2006; FERREE and BARBASH 2009; PHADNIS and ORR 2009; TANG and PRESGRAVES 2009) have provided insights into the genetic bases of postzygotic reproductive isolation. Furthermore, these results are largely consistent with the notion that Dobzhansky-Muller incompatibility factors epistatically interact to promote hybrid inviability and sterility, helping to confirm a theory of how reproductive isolation can evolve (DOBZHANSKY 1937; MULLER 1942). However, the *Caenorhabditis* system has made very few contributions to this issue (BAIRD *et al.* 1992; BAIRD and YEN 2001; HILL and L'HERNAULT 2001; BAIRD 2002; SEIDEL *et al.* 2008). This is somewhat surprising considering the breadth of subjects this system has been used to examine (e.g. DE BONO and BARGMANN 1998; GRIFFITTS *et al.* 2001; RAIZEN *et al.* 2008). However, hybrid genetics has largely been impossible in this system due to the inability of any *Caenorhabditis* interspecies hybridization to successfully produce fertile hybrid progeny (BAIRD *et al.* 1992).

A new gonochoristic *Caenorhabditis* species, provisionally named *C.* sp. 9, has been discovered that is capable of producing fertile hybrids with the androdioecious *C. briggsae*. The existence of fertile hybrids between species of different reproductive mode opens up the possibility of using trait mapping approaches to examine the genetic basis of hermaphroditic spermatogenesis. Additionally, it allows the *Caenorhabditis* system to

contribute to the study of the genetics of postzygotic reproductive isolation. Here, experiments conducted in this new hybrid system that pertain to both of these issues are described.

Methods

Nomenclature

To simplify discussion of the numerous hybrid crossing schemes and hybrid generations described herein, we developed a shorthand to denote specific hybrid generations (see Figure 2). The prefixes "F" and "B" are used for intercrosses and backcrosses, respectively, and are followed by standard size numbers denoting the *n*th generation since the pure species intercross. In addition, a subscript is used to specify the species identity of each sex in the crossing scheme that produced that generation. The sex of the animal corresponding to the subscript is assumed to be male unless otherwise denoted with an "f" for female or "h" for hermaphrodite. For instance, $F1_b$ denotes the generation resulting from the *C. briggsae* male x *C.* sp. 9 female parental hybrid cross (Fig. 2A). Conversely, the generation resulting from the reciprocal parental cross would be the $F1_9$ (Fig. 2B). A generation resulting from a scheme where the P0 father was *C. briggsae* and the hybrid female progeny were subsequently backcrossed to *C.* sp. 9 males would be the $B2_{b,9}$ generation (Fig. 2D). And, when a $B2_{b,9}$ hybrid male is crossed to a *C. briggsae* hermaphrodite, the $B3_{b,9,bh}$ generation results (Fig. 2G). When the directionality of a given cross is of no consequence, the subscript will be omitted.

Maintenance and strains

Animals were maintained according to standard *C. elegans* protocols (WOOD 1988),with the exception of increased agar concentration in nematode growth medium

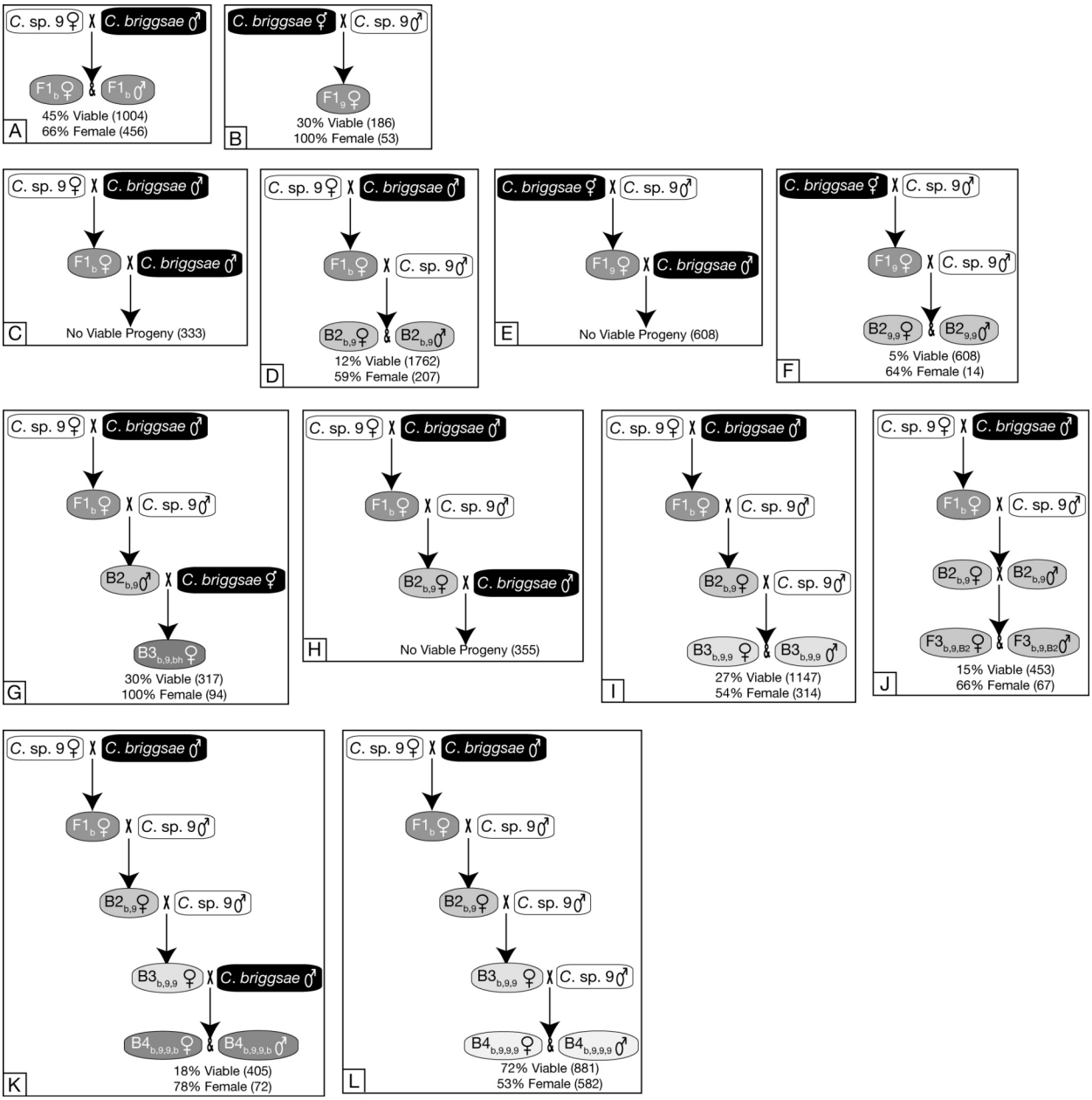


Figure 2. Summary of various hybrid crosses. Each panel represents a particular cross, with parents above and realized progeny below. Progeny are numbered via the scheme described in Methods. Numbers in parentheses represent the number of embryos and adult progeny scored to produce percentages of viable and female progeny, respectively.

(NGM) plates to 2.2%. Cultures were kept at 20°C unless otherwise indicated. Inbred lines of *C.* sp. 9 were generating through 25 generations of full-sibling inbreeding. Strains used in this study include *C. briggsae* AF16 (sequenced reference strain); *C. briggsae*

VT847 (mapping strain); *C. briggsae* HK104 (mapping strain); *C. briggsae* CP4 [*cb-unc-4* (*nm4*)]; *C. briggsae* CP99 [*cb-unc-119* (*nm67*)]; *C. briggsae* CP116 (polyploid strain, this study); *C.* sp. 9 JU1325 (wild isolate from India); *C.* sp. 9 EG5268 (wild isolate from Congo); *C.* sp. 9 JU1422 (inbred derivative of JU1325); *C.* sp. 9 JU1420 (inbred derivative of JU1325).

Determination of viability, sex ratio, and brood size

We define viability as the fraction of laid embryos that develop into adults. To measure viability, three females or hermaphrodites and five males were mated. *C.* sp. 9 females were picked at the L4 stage to ensure virginity, and all L4 *C. briggsae* hermaphrodites used for hybrid crosses were purged of all self-sperm by isolating them from males and moving them to a new plate once a day until no more embryos were seen on the plate. After mating overnight, the males were removed, the mothers were moved to a fresh plate, and the eggs on the previous plate were counted. This was repeated about every twelve hours until no more embryos were laid. The plates were scored for female and male adults six days after laying. The sex ratio has been defined as the fraction of total adults that are female. Brood sizes, defined as the number of embryos laid by a given XX animal, were determined via a similar procedure, except matings with individual mothers were used.

Fertility was measured on selected hybrid populations through single worm matings. For males, one male was placed with four wild type *C.* sp. 9 females. If embryos were present on the plate the next day, the worm was marked as fertile. For females, the test was done in a similar fashion, but with one virgin female and five *C.* sp. 9 males. The percent fertility is a measurement of the fraction of single worm matings that yield

embryos. Plates where the individual worm being assayed had fled the plate were discarded. The extent of F1 male sterility was also evaluated by determining the fraction of males with abnormal gonad morphology under differential interference contrast (DIC) microscopy.

Determination of selfing and spermatogenesis incidence in hybrids

Here, we define "selfing" as the production of embryos in the absence of mating. Because in all *Caenorhabditis* species known selfing only occurs in the presence of XX spermatogenesis, the production of self-embryos is a proxy for the production of self-sperm. To measure this in the hybrids, XX L4 animals were removed from males and left overnight at 20°C. Up to 50 L4 animals were picked to a single plate for the scoring of selfing. If embryos were observed on the plate, the plate was examined for the presence of an animal with embryos in its uterus. Typically, no more than one hybrid selfer was observed per plate. In addition, virgin young adult XX animals (produced as above) were scored for the presence of sperm-like or spermatocyte-like cells via DIC microscopy.

Immunoblotting

Protein samples were prepared by picking 100 worms into 30 µL of phosphate-buffered saline (PBS;137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4), followed by addition of 30 µL 95% Laemmli Sample Buffer (Bio-Rad, USA) + 5% beta-mercaptoethanol. SDS polyacrylamide protein gels were run according to standard methods (SAMBROOK and RUSSELL 2001). Both anti-MSP mouse monoclonal antibodies (provided by D. Greenstein(KOSINSKI *et al.* 2005) and anti-alpha-tubulin mouse antibodies (Sigma, USA) were added to the blocking solution at dilutions of 1:1000. The secondary antibody, a horseradish peroxidase-conjugated anti-mouse

conjugate (GE Healthcare), was used at a dilution of 1:1500 for 1.5 hours. Antibody-bound proteins were visualized using the SuperSignal Chemiluminescent substrate (Pierce Technology).

RT-PCR

RNA preparations were made by picking worms of the appropriate age and sex into RNA-ase free water at a concentration of 4 worms/μL, with about 200 worms per sample. TRI reagent (Molecular Research Center) was added to each preparation, and the samples were frozen at -80°C, thawed, pelleted in a microcentrifuge, and then lysed with a plastic pestle in a 1.5 mL eppendorf tube. RNA was then purified via phenol/chloroform extraction and precipitation with isopropanol. RNA was reconstituted with RNA-ase free water, using 1 μL for every four worms in the initial preparation. 5 μL of an RNA prep was used in a RT-PCR reaction using the AccessQuick kit (Promega, USA). Primers AD115 (5'-TCGACGACTTGGCTGTGCAAC-3') and AD116 (5'-TTGACGAGCTGTTTGATGCCCACC-3') were used to amplify a 245 base pair (bp) fragment of the *cb-fog-3* transcript, and primers EH37 and EH38 (HILL and HAAG 2009) were used to amplify a 250 bp fragment of all *C. briggsae* actin paralogs. Reactions were then run on a 1% agarose ethidium bromide gel to visualize the amplicons.

Mutagenesis and screening

Synchronized cultures of *C.* sp. 9 JU1422 L4 larvae were mutagenized for four hours using 50 mM ethyl methanesulfonate (EMS) according to standard methods (BRENNER 1974). Animals were washed multiple times in M9 buffer and distributed onto seeded NGM plates. Approximately 10 such mutagenized virgin *C.* sp. 9 females were then mated with roughly 20 *C. briggsae* AF16 males overnight, after which all parental

males were removed. Plates were subsequently scored for the next seven days for the presence of F2 embryos, which, given complete F1 male sterility, were likely to be due to XX self-fertility.

Construction of a polyploid C. briggsae *strain*

Polyploid *Caenorhabditis* lines have been used to determine the chromosomal basis of sex determination in this genus (NIGON 1951; MADL and HERMAN 1979). A modified version of Madl and Herman's (1979) heat shock protocol was used to generate a similar strain of *C. briggsae*. The wild-type *C. briggsae* AF16 and the dumpy *C. briggsae* CP4 (*nm4*) strains were shifted to 30°C overnight. AF16 males were crossed with CP4 hermaphrodites, and 300 F1 L4 wild-type hermaphrodite progeny were singled to separate plates. The F2 self-progeny were scored for large animals, low brood size, and a high proportion of males, all of which are indicative of polyploids (MADL and HERMAN 1979).One such animal was found, and it was confirmed to sire polyploid progeny (likely 4A:3X, see below). This animal was used to generate the CP116 strain.

DNA preparations for genotyping

For DNA preparations, worms were picked into lysis buffer (50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM MgCl2, 0.45% NP-40, 0.45% Tween 20, 0.01% gelatin) at a concentration of 2 worms/μL with about 200 worms/prep. Proteinase K was added to a concentration of 100 μg/mL, and the preparation was frozen at -80°C for at least 15 min. Samples were subsequently incubated for 1 hour at 65°C and then at 95°C for 30 min., after which they were used directly for PCR and genotyping. Control DNA samples for evaluating *C. briggsae* AF16/*C. briggsae* VT847 intrastrain segregation distortion were produced by using half AF16 worms and half VT847 worms. Samples representing the

Mendelian expectations for $B3_{b,9,bh}$ animals were produced by mixing 50% *C.* sp. 9 JU1422 (not a productive template), 40% *C. briggsae* AF16, and 10% *C. briggsae* VT847. For X-linked markers, proportions of 25% *C.* sp. 9 JU1422, 50% *C. briggsae* AF16, and 25% *C. briggsae* VT847 were used for the Mendelian expectation control DNA preparation. Here, the expectation would be different from the autosomes because the hybrid X is donated by the male (Figure 9).

Genotyping

23 polymorphic molecular markers distinguishing *C. briggsae* AF16 and VT847 were used for genotyping, and all of these markers have been mapped to a physical position on the *C. briggsae* genome sequence (KOBOLDT *et al.* 2010). 22 of these markers are single nucleotide polymorphisms (SNPs) and one insertion/deletion (indel) of 18 bp. 13 SNPs were genotyped via pyrosequencing technology and nine via restriction fragment ("snip-SNP") analysis, and the indel marker was assayed by agarose gel electrophoresis. All genotyping methods required a PCR amplification step. For the snip-SNP and indel assays, primers previously designed for interstrain *C. briggsae* mapping were used (KOBOLDT *et al.* 2010).

For pyrosequencing reactions, amplification and sequencing primers were designed around the SNPs of interest using software provided by the manufacturer (Qiagen, USA formerly Biotage; Table 1). For the PCR step, 0.5 μL of a DNA preparation (as described above) was used with in a 30 μL mixture containing: 0.5 μM of an untailed primer, 0.1 μM of a tailed primer, 0.4 μM of a universal biotinylated primer, 0.25 mM of each dNTP, 1 X ThermoPol PCR Buffer (New England Biolabs), 1.5 mM Mg^{2+}, and 1 unit of *Taq* DNA polymerase per 10 μL. A single biotinylated primer was

used for all pyrosequencing PCR reactions (AYDIN *et al.* 2005). The cycling conditions were as follows: 95°C denature (2 min.), [95°C denature (30 sec.), 60°C annealing (30 sec.), 72°C extension (30 sec.)], 72°C extension (5 min.) with the bracketed subroutine repeated for 40 total cycles. The same conditions were used for each assay. Assays that only amplified *C. briggsae* DNA, and failed to amplify *C.* sp. 9 DNA were used for genotyping. 5 μL of all PCR reactions were visualized on an agarose gel to confirm amplification. Single-stranded PCR amplicons were purified with streptavidin sepharose beads (GE Healthcare, USA) according to the manufacturer's instructions. Pyrosequencing reactions were performed using the PyroMarkTM Q96 ID machine (Qiagen, USA) according to the manufacturer's instructions. Resulting data were analyzed using Allele Quantification software provided by the manufacturer. This estimates allele frequencies of the polymorphic alleles through integration of the pyrogram peaks (LAVEBRATT *et al.* 2004).

For snip-SNP assays, the PCR conditions are as above with the exceptions that only untailed, non-biotinylated primers were used (0.5 μM each), and that the reaction volumes were 25 μL. Additionally, the extension time was increased to 1 min. Here, primers designed by Koboldt et al. (2010) were used. After amplification, 1.2 μL of the appropriate restriction endonuclease (NEB) was added to reaction and incubated at the appropriate temperature for 2 hours. In order to quantify the allele frequencies of the polymorphisms, a standard curve for every assay was generated by performing the assay on DNA preps of known AF16/VT847 allele frequencies of 0.5/0.5, 0.6/0.4, 0.7/0.3, 0.8/0.2, and 0.9/0.1. AF16, VT847, *C.* sp. 9 JU1422, and AF16/VT847 F2 controls were also run. All control and test DNA were run out on the same 1% ethidium bromide

agarose gel to visualize the polymorphic bands. Band intensities were quantified using the ImageJ software (ABRAMOFF *et al.* 2004). The ratio of the VT847 and AF16 diagnostic band intensities were found for the control reactions. These values were plotted against their known allele frequencies and a best-fit regression line was then used to estimate the allele frequencies of the test reactions. This same general process was used for the indel marker, but here no restriction digest step was necessary, and the bands were run out on a 2% agarose gel to resolve the bands differing in size by 18 base pairs. To facilitate the comparison between the Mendelian control and hybrid $B3_{b,9,bh}$ results, the raw data were normalized by forcing the average allele frequency of each Mendelian expectation control to its known value, and the raw data for the hybrid $B3_{b,9,bh}$ results were normalized using the same metric.

Table 1. Pyrosequencing Primers.

Name	SNP ID	Sequence (5' to 3')
VTP-I-9F	cbv26002	AAATTCTGGGCCGTCTGGAT
VTP-I-9R		AGCGCTGCTCCGGTTCATAGATTGGTTTTAGCTGGTCCCGAGTTATT
VTP-I-9S		ACTGATCCTAATTGGTTAT
VTP-II-6F	cbv3168	AGCGCTGCTCCGGTTCATAGATTTCTCGGAAAATTTGAAATTGGA
VTP-II-6R		CCCTCACACTGCCAAAGTATTTA
VTP-II-6S		TACTTTTCACTTTTGAAAAT
VTP-II-9F	cbv27609	AAGTGGACAGTGTGGGGAACC
VTP-II-9R		AGCGCTGCTCCGGTTCATAGATTGACAAACAGATTGGGGCCACTAT
VTP-II-9S		CCATCCATTGGAAATT
VTP-III-2F	cbv14644	GGGCCATCCTCTTTTGTTAGCT
VTP-III-2R		AGCGCTGCTCCGGTTCATAGATTAGAGCCTACGATGCCTGGTATG
VTP-III-2S		TCTTTTGTTAGCTTCATTG
VTP-III-6F	cbv26660	AGCGCTGCTCCGGTTCATAGATTGGTTTTGAAAGAAGTTGCAGTGA
VTP-III-6R		GCTCGAAAAACATGACATTTTAA
VTP-III-6S		CTAAATTGCCTAAAATTGAT
VTP-III-7F	cbv19012	AGCGCTGCTCCGGTTCATAGATTGCTGCCGATGAGCAGAGAAA
VTP-III-7R		AGTAGCACCCCGGCCAAATT
VTP-III-7S		CCCTTACCTTATTGGTTG
VTP-IV-1F	cbv557	AGCGCTGCTCCGGTTCATAGATTAAATGGATGGGAATGCACTAATGA
VTP-IV-1R		TCTTTTTTGTTCCCATGAAGTCG
VTP-IV-1S		TTTACTTTGCTGGAAAAC
VTP-IV-2F	cbv17008	GGAGCCAAGATAATAAACCTCAAA
VTP-IV-2R		AGCGCTGCTCCGGTTCATAGATTCCCTTTAAAAAGAGATGCAGTGA
VTP-IV-2S		TTACGTTTTAAAAAGATGAG
VTP-IV-8F	cbv5985	TGCCCGAAAGTAGTCCTCCATA

VTP-IV-8R		AGCGCTGCTCCGGTTCATAGATTGGGAACGACTTGATTTTGTATCCA
VTP-IV-8S		TCTGACTGCGAACGA
VTP-V-2F	cbv11673	AGCGCTGCTCCGGTTCATAGATTTCCGATGTGTTCGTTTAGAAAGA
VTP-V-2R		CCATTATTCAAACTTCCGATGCTA
VTP-V-2S		TTTGTACCTGATTGAAA
VTP-V-6F	cbv24904	AGCGCTGCTCCGGTTCATAGATTGAATGTGGTCGGAAAAAAATTTA
VTP-V-6R		GCACTTTTGACCCCCATTTTA
VTP-V-6S		CCAAAACAAAAACCATG
VTP-V-8F	cbv31146	GTGAGCCGTTGATCTTCATATTCC
VTP-V-8R		AGCGCTGCTCCGGTTCATAGATTTCGGTCTTTGCACTGAAAAGTTT
VTP-V-8S		TCGATATTTTTTGTTCAATT
VTP-X-9F	cbv427	GAAAAATCAGTGTTCGAGGCTTAC
VTP-X-9R		AGCGCTGCTCCGGTTCATAGATTAAAGGTTTTCGGCTTCTGAGCT
VTP-X-9S		GCACTAGAATAAGTGAAAAG
VTP-UNIBIOT		Biotin-AGCGCTGCTCCGGTTCATAGATT

At the end of the primer name: "F" denotes it as a forward PCR primer, "R" denotes it as a reverse PCR primer, and "S" denotes it as the sequencing primer for the Pyrosequencing reaction. "VTP-UNIBIOT" is the biotinylated universal PCR primer (AYDIN *et al.* 2005).

Results

C. briggsae *and* C. *sp. 9 produce fertile hybrids*

A summary of the various hybrid crosses and backcrosses we have examined and the naming scheme used to describe them is presented in Figure 2. Prior to this study, the highest reported *Caenorhabditis* interspecies hybrid viability is 6% for crosses of *C. remanei* females to *C. briggsae* males (BAIRD *et al.* 1992). In contrast, one third to one half of hybrid F1 progeny from reciprocal crosses of *C. briggsae* and *C.* sp. 9 were viable, with the remainder arresting during development. This viability is dependent on

the direction of the parental cross. When *C.* sp. 9 is the mother the F1 have a viability of 45%, whereas when *C. briggsae* is the mother the viability is 30% (Figure 2A,B). This difference between these reciprocal crosses can be accounted for entirely by an extreme difference in male-specific viability. When *C.* sp. 9 is the mother 34% of the progeny are male, whereas when *C. briggsae* is the mother no viable F1 male progeny were observed. The average brood size (70 embryos laid, n=5, s.e.=11), sex ratio, and viability of the $F1_b$ are all significantly different (t-test $P<0.01$) from the conspecific *C.* sp. 9 cross. Here the average brood size is 259 (n=3, s.e.=32); the sex ratio is 53% female (n = 1890); and the viability is 82% (n =2312).

$F1_b$ males exhibit delayed development and most are atypically small. No cross ever performed with F1 males was successful, and all males examined under Nomarski microscopy (n=94) had gonadal defects (Figure 3). All lacked obvious spermatocytes or sperm, and 37% had no gonad at all. In contrast, using single-pair mating tests (see Methods), the vast majority of F1 females successfully produce embryos when crossed with *C.* sp. 9 males (Table 2). The resulting $B2_9$ progeny are less viable than the F1 (Figure 2D, F), but surviving females are comparable in fertility to F1 females (data not shown), and viable males, roughly one quarter of which are fertile, are also produced (Table 2 and Figure 2G).

Multiple asymmetries in postzygotic isolation exist between C. briggsae *and* C. *sp. 9*

In addition to the F1 sex ratio and viability asymmetries described above, other instances of asymmetry were observed in later hybrid generations. F1 females in both reciprocal crosses are completely unable to produce viable progeny with *C. briggsae* males (Figure 2C, E), with all progeny arresting during embryonic development. In

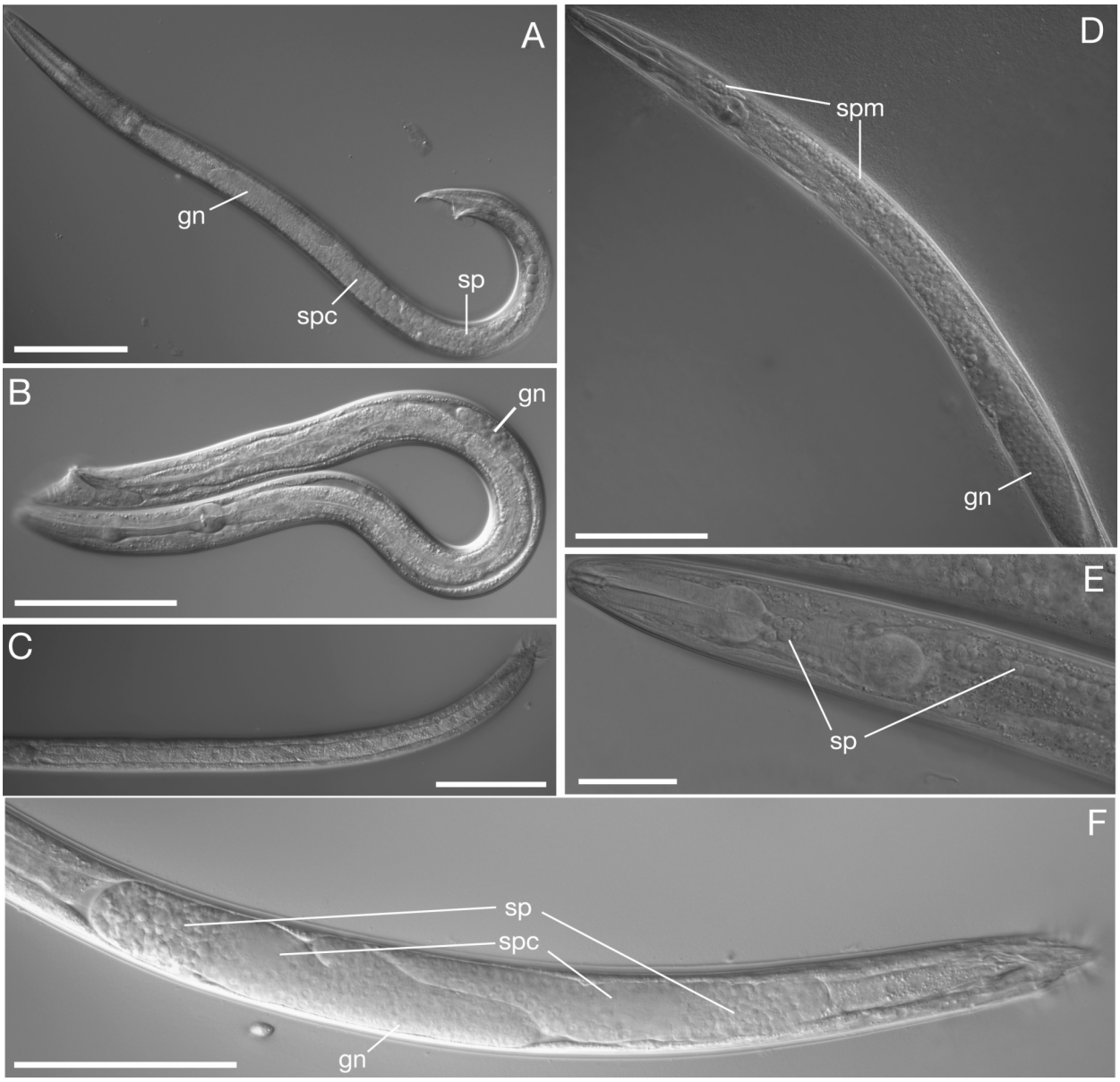


Figure 3. Hybrid males have abnormal gonads. A. A wild-type *C.* sp. 9 JU1422 adult male, with a wild-type gonad (gn), spermatocytes (spc) and sperm (sp). **B.** A hybrid $F1_9$ male with an underdeveloped gonad (gn) and no mature germ cells. **C.** A hybrid $F1_9$ male with no discernible gonad. **D.** A hybrid backcross ($B2_{b,9}$) male with sperm (sp) located abnormally in the anterior. **E.** A higher magnification image of the head of a hybrid backcross male with mislocalized sperm. **F.** $B2_{b,9}$ male with developing spermatocytes (spc) and sperm (sp) oriented to both the anterior and posterior of the animal. Scale bars represent 100 µm for all panels except for E, where it represents 50 µm. Hybrids shown in panels B-C were generated with the lines *C.* sp. 9 JU1325 and *C. briggsae* AF16, and the hybrids shown in panels C-F were generated with the lines *C.* sp. 9 1422 and *C. briggsae* AF16.

Table 2. Incidence of Fertility in Hybrid Animals

Animal	*% Laid Embryos (n)*
C. sp. 9 F	93 (98)
C. sp. 9 M	90 (100)
$F1_b$ F	95 (99)
$F1_9$ F	83 (101)
$B2_{b,9}$ F	24 (138)

The incidence of fertility was determined through single worm mating tests. Animals were mated with *C.* sp. 9 males or females. All hybrids were derived using the *C. briggsae* strain AF16 and the *C.* sp. 9 strain JU1422.

contrast, they produce viable male and female progeny with *C.* sp. 9 males (Figure 2D, F), with about 24% of $B2_{b,9}$ males being fertile (Table 2). Furthermore, the viability of B2 progeny depends on the identity of the P0 mother; the roughly two-fold greater viability of $B2_{b,9}$ progeny than $B2_{9,9}$ progeny (Figure 2D vs. F) is highly significant (chi-square P=0.0019, d.f. = 1). $B2_{b,9}$ males exhibit a wider range of male germline phenotypes than F1 males (Figure 3D-F). This included gonads with sperm oriented towards the anterior (Figure 3D), animals with sperm apparently localized outside of the gonad (Figure 3E), and gonads with female-like dual polarity (Figure 3F). 34% (N=167) of $B2_{b,9}$ males have apparently normal gonads. While fertile $B2_{b,9}$ males can successfully mate with *C. briggsae* hermaphrodites to produce hybrid progeny (Figure 2G), their sisters can never produce viable hybrid progeny with *C. briggsae* males (Figure 2H). Hybrid females can only be backcrossed to *C. briggsae* males to produce viable hybrid progeny after being backcrossed with *C.* sp. 9 for two generations (Figure 2K).

In addition to the above asymmetries, pure species *C.* sp. 9 males greatly reduce

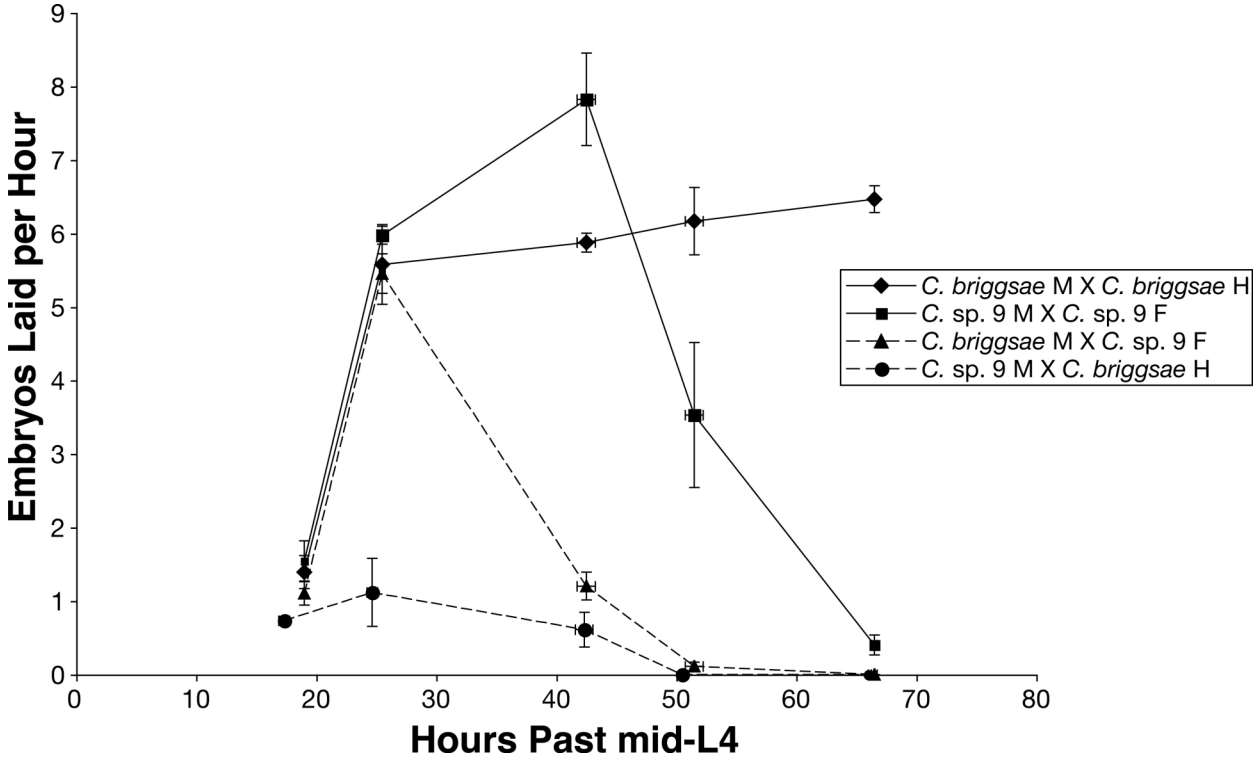


Figure 4. *C.* sp. 9 males reduce the brood size of *C. briggsae* hermaphrodites. The rate of embryo-laying for conspecifics and hybrids over time. Conspecific crosses are solid lines, and hybrid crosses are dashed lines. Individual females or hermaphrodites were mated with four males overnight, after which the females were moved without the males to a new laying window twice a day. At least three replicates were performed for every cross. The error bars represent one standard error. The lines *C.* sp. 9 JU1325 and *C. briggsae* AF16 were used for all observations.

the brood size of *C. briggsae* hermaphrodites and prevent them from laying self-progeny (Fig. 4). About fifty hours after mating with a conspecific male, *C. briggsae* hermaphrodites are laying an average of 6 embryos per hour. However, after mating with *C.* sp. 9 males, *C. briggsae* hermaphrodites stop laying altogether by this time, despite the presence of both sperm and oocytes. *C.* sp. 9 females stop laying embryos about 67 hours after mating (with either conspecific or *C. briggsae* males). Examination of such post-reproductive *C.* sp. 9 under DIC optics indicates revealed that they had consistently run out of sperm.

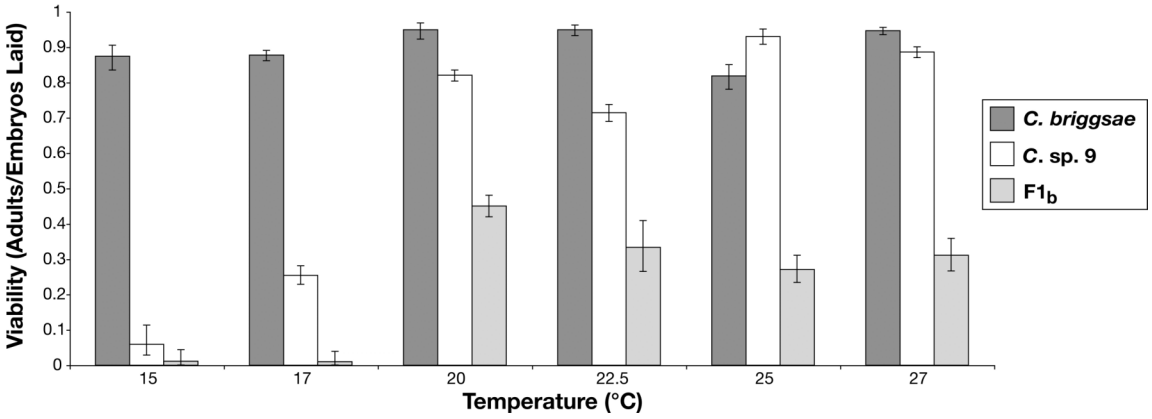


Figure 5. Viabilities of *C. briggsae*, *C.* sp. 9, and $F1_9$ hybrids at different temperatures. Viability was measured as the number of adults resulting from the total number of embryos laid (N ≥ 136 for all groups). All hybrid $F1_9$ were generated from crossing *C. briggsae* males to *C.* sp. 9 females. Error bars represent 95% confidence intervals. The lines *C.* sp. 9 JU1325 and *C. briggsae* AF16 were used for all observations. Sample sizes at temperatures 15°C, 17°C. 20°C, 22.5°C, 25°C, and 27°C, respectively: *C. briggsae* AF16 (342, 1883, 377, 867, 472, 1800); *C.* sp. 9 JU1325 (136, 1078, 2312, 1396, 534, 1616); Hybrid $F1_b$ (174, 195, 1004, 162, 510, 383).

C. *sp. 9 has a low viability at temperatures below 20°C*

It was noticed that *C.* sp. 9 strains grow poorly at 15°C, so the viability of *C.* sp. 9 and hybrid F1 animals were examined at a range of temperatures (Fig. 5). At 27°C, the viability of *C. briggsae* is 95% and the viability of *C.* sp. 9 is 89%. However, at lower temperatures (i.e. below 20°C), *C. briggsae* has a much higher viability than does *C.* sp. 9 (87% vs. 6% at 15°C, respectively). Hybrid F1 animals always displayed viability much lower than either *C.* sp. 9 or *C. briggsae* at all temperatures observed, but are similar to *C.* sp. 9 in performing particularly poorly below 20°C.

Hermaphroditism is rare in hybrid F1 XX animals

Because *C.* sp. 9 and *C. briggsae* differ in reproductive mode, the germ line sex of hybrid XX animals is of considerable interest. Three phenotypes were used to assess the presence of hermaphroditism among hybrid animals: the incidence of selfing (i.e. the fraction of apparent XX animals that laid embryos with eggshells in the absence of

males); the incidence of spermatogenesis (the fraction of XX adults that appeared to have sperm-like cells under DIC

microscopy); and the presence of sperm-specific molecular markers in XX hybrids.

Both selfing and spermatogenesis are rare in the F1 (Table 3). Overall, the incidence of any detectable selfing in the F1 is very low, and all selfed embryos observed died prior to hatching. However, the incidence of selfing varies with respect to the strain of *C.* sp. 9 that is used. When the wild isolates JU1325 and EG5862 are crossed with *C. briggsae*, selfing is observed in 1.2% and 0.3% of hybrid F1, respectively. If the inbred line JU1422 is used, then selfing is never seen. The use of other strains of *C. briggsae,* such as HK104 and VT847, do not reveal any significant increase in the incidence of selfing (Table 3). Progeny of the reciprocal parental crosses differ in their incidence of selfing, although not significantly so.

Because selfing requires more than just the generation of sperm and oocytes in a female soma (BALDI *et al.* 2009), XX hybrids were also observed under DIC to investigate the possibility that many hybrids made sperm (and were in fact hermaphrodite) but were unable to produce self-progeny. The incidence of spermatogenesis in young adult XX hybrid F1 animals is higher than that of the incidence of selfing itself, but never exceeds a few percent (Table 3). Also, in most F1 females germ line development is delayed with respect to *C.* sp. 9 females. In *C.* sp. 9 females, typically at least one mature oocyte is fully developed by young adulthood (Figure 1A). However, in hybrid F1 animals, oftentimes no differentiated germ cells or incomplete oocytes ("ooids") are seen in the proximal germ line of young adult XX animals (Figure 6A,B).

Table 3. Hermaphrodites in Hybrid Generations

Animal scored	C. briggsae *strain(s) used*	*C. sp. 9 strain used*	*% Selfers (n)*	*% Spermatogenic (n)*
P0	*n/a*	JU1422	0 (n>100)	0 (118)
P0	*n/a*	EG5268	0 (109)	-
$F1_b$	AF16	JU1422	0 (230)	0 (92)
$F1_b$	AF16	JU1325	1.2 (494)	0 (123)
$F1_b$	AF16	EG5268	0.3 (360)	3.5 (114)
$F1_9$	AF16	JU1325	0 (106)	-
$F1_b$	VT847	JU1422	0 (34)	-
$F1_b$	HK104	JU1422	0 (29)	-
$F1_b$	VT847	JU1325	0 (32)	-
$F1_b$	HK104	JU1325	0 (68)	-
$F1_b$	VT847	EG5268	0 (26)	-
$F1_b$	HK104	EG5268	0 (31)	-
$F1_b$	CP116 (polyploid AF16)	JU1325	0 (137)	8.3 (24)
$F1_b$	CP116 (polyploid AF16)	EG5268	2.3 (91)	15 (177)
$B3_{b,9,bh}$	AF16	JU1422	0 (181)	0 (65)
$B3_{b,9,bh}$	AF16	JU1325	0 (41)	-
$B3_{b,9,bh}$*	VT847 first AF16 second	JU1325	0 (128)	-

$B4_{b,9,9,b}$	AF16	JU1422	0 (202)	-
$B4_{b,9,9,b}$	AF16	JU1420	0 (396)	-
$B4_{b,9,9,b}$	AF16	JU1325	2.6 (117)	-

Selfer, animal that lays embryos without mating; spermatogenic, female/hermaphrodite has sperm-like cells under DIC microscopy; -, not determined. See Figure 2 for how $B3_{b,9,bh}$ and $B4_{b,9,9,b}$ animals are constructed. *The $B3_{b,9,bh}$ generation used for the genotyping shown in Figure 10 was constructed with three *Caenorhabditis* strains as in Figure 9.

However, the stacking oocyte phenotype characteristic of normal unmated females is seen in most older hybrid F1 animals (Figure 6C). This, in tandem with the result that most hybrid F1 females are fertile (above, Table 2), suggests that germ line development is delayed but otherwise normal in F1 females. Indeed, young adult F1 females observed to have no differentiated germ cells under DIC microscopy were rescued, and all displayed the stacking oocyte phenotype after about 12 hours at 20℃ (n=10). Aside from rare hermaphrodites with clear populations of spermatocytes (Figure 6D), no hybrid F1 XX animals displayed sexually ambiguous populations of germ cells proximal to the oocytes.

The rarity of spermatogenesis and selfing in hybrid F1 XX animals does not entirely exclude the possibility that the hermaphroditism trait is codominant in this system. Despite the lack of morphologically sperm-like cells in the vast majority of F1 XX animals, their germ-lines could still possess cryptic male characteristics. Indeed, sex-specific germ line molecular markers have been used in *C. elegans* to reveal the sexual

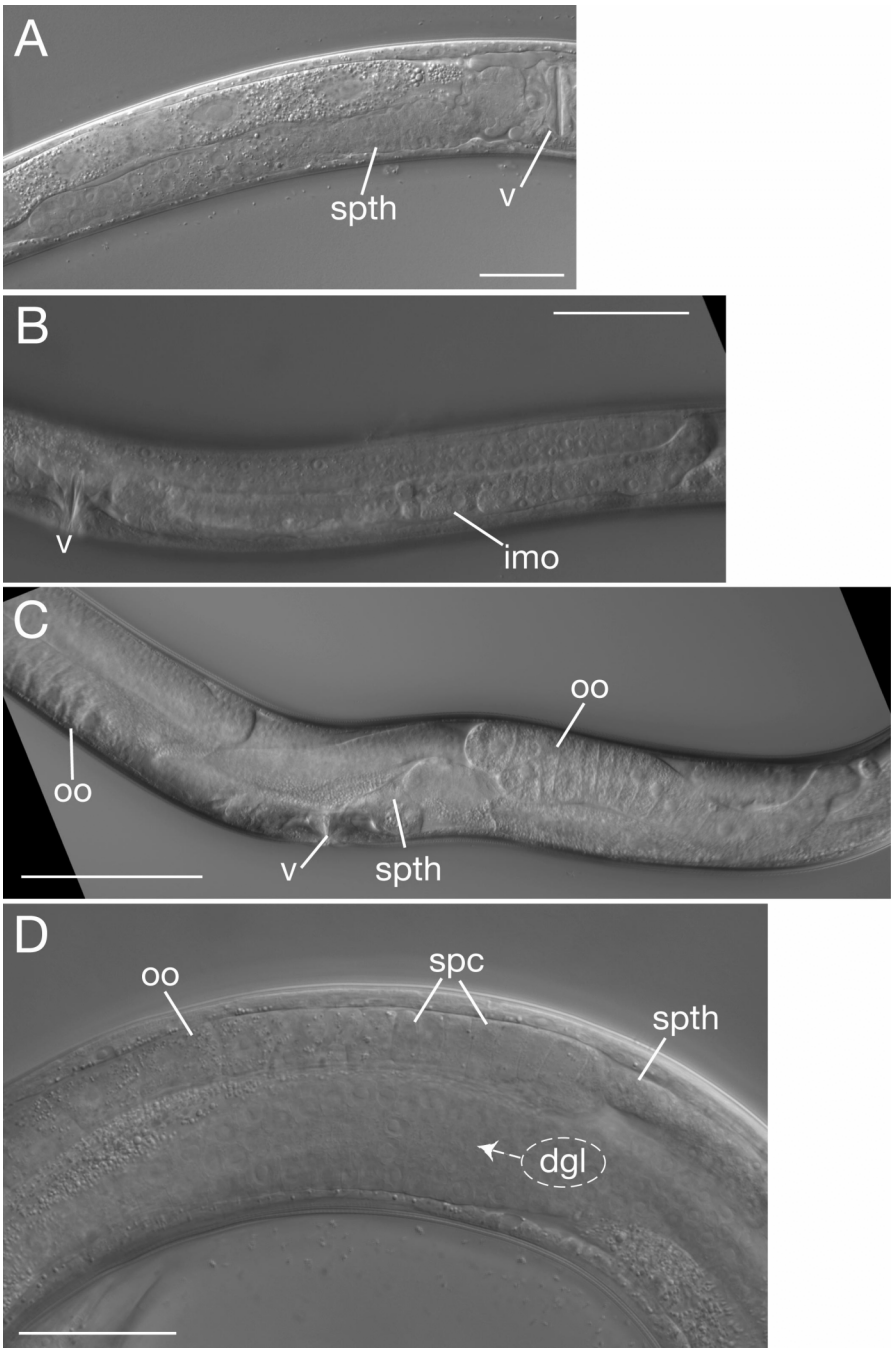


Figure 6. Hybrid F1 female germ lines. A. A young hybrid F1 adult female displaying delayed germ-line development with no discernible differentiated germ cells despite having an adult vulva (v). The empty spermatheca (spth) is noted. **B.** A young hybrid F1 adult female with small, proximal immature oocytes (imo). **C.** A hybrid F1 adult female with stacking oocytes (oo) and an empty spermatheca (spth). **D.** A rare hybrid F1 adult hermaphrodite with clear proximal spermatocytes (spc) and oocytes (oo). This animal was recovered and failed to lay any embryos. "dgl" denotes the distal germ line. Scale bar represents 50 μm for panels A, B, and D; 100 μm for panel C. The lines *C*. sp. 9 JU1325 and *C*. *briggsae* AF16 were used for all panels, with the exception of panel D, wherein

the *C.* sp. 9 line EG5468 was used.

identity of germ cells in the absence of morphological characteristics (JONES *et al.* 1996). Furthermore, the observed delayed oogenesis in young adult F1 XX animals is suggestive of codominant hermaphroditism. To investigate this possibility, the possible expression of male-specific molecular markers was examined in the $F1_b$ generation. Major Sperm Protein (MSP) is a crucial sperm cytoskeletal protein that is also implicated in oocyte maturation (SMITH 2006). *fog-3* is a TOB-domain protein that is necessary for spermatogenesis in *C. elegans*, *C. briggsae*, and *C. remanei* (CHEN *et al.* 2001). Neither MSP nor *fog-3* transcripts are detectably expressed in XX $F1_b$ L4 and young adult animals, though both are detectable in hermaphrodites and males (Figure 7A-B). These results suggest that most hybrid F1 XX animals do not harbor germ cells with cryptic male character, and that with only rare exceptions the female germ line state is dominant in XX hybrid F1.

Attempts to produce hybrid hermaphrodites via mutagenesis

The genetic mechanism underlying female dominance may be due to a small number of hyperactive female-promoting genes in XX *C.* sp. 9 germ cells. If this were the case, then mutation of one of these genes could permit hermaphrodite-like levels of spermatogenesis in hybrid F1 XX animals, and perhaps thereby provide enough viable F2 progeny to allow establishment of hybrid hermaphrodite lines. To test this possibility, mutagenized *C.* sp. 9 females were mated with *C. briggsae* males, and their F1 progeny screened for the ability to produce viable self-progeny. No such F1 were uncovered after screening ~15,000 mutagenized haploid genomes. In *C. elegans*, this treatment would

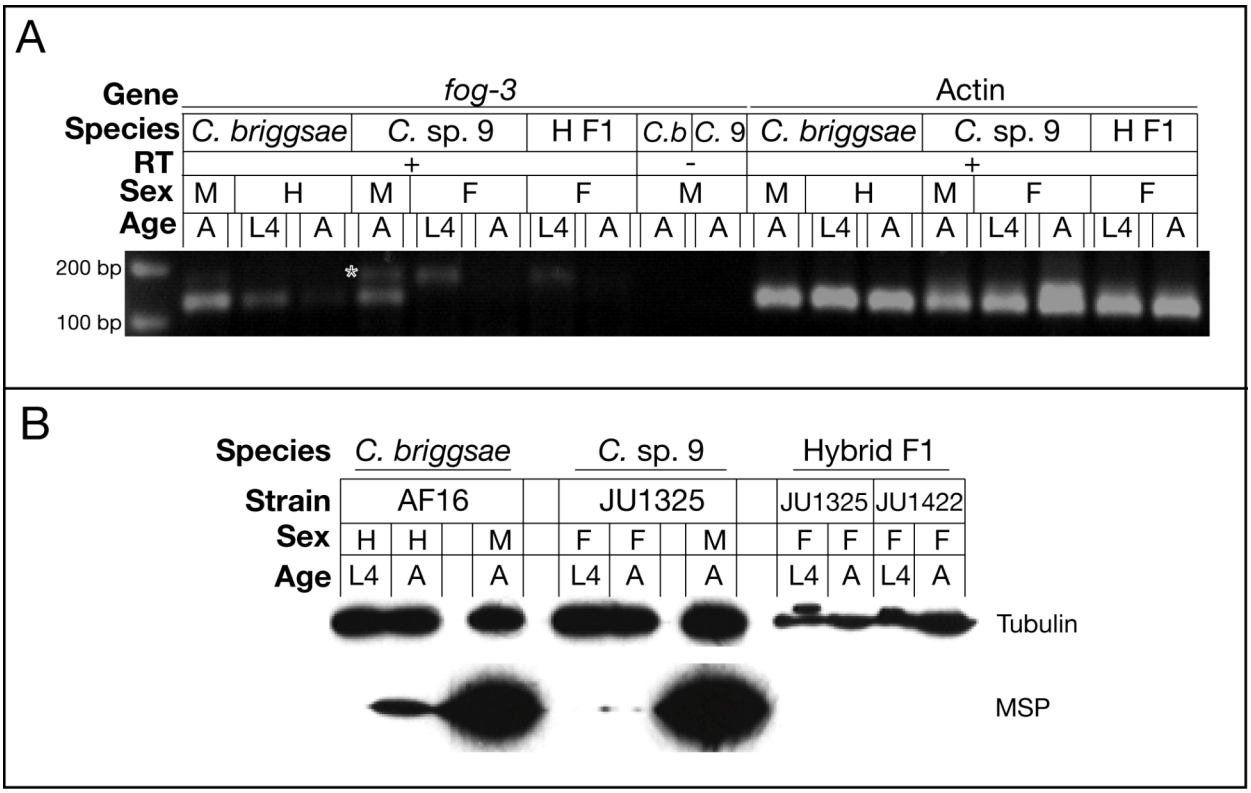


Figure 7. Sperm-specific molecular markers in hybrid F1 females. A. RT-PCR for the sperm-specific transcript *fog-3*. Primers specific for an actin transcript were used as a positive control. All corresponding actin and *fog-3* reactions used RNA from the same preparations in equal quantities. The asterisk denotes a non-specific *C*. sp. 9 amplicon. "H" = hermaphrodite. "A" = adult. "H F1" = hybrid $F1_9$. **B.** Western blot for the sperm-specific protein MSP (Major Sperm Protein). An anti-tubulin antibody was used as a positive control. Asterisks denote nonspecific proteins. All protein preparations were made with 100 worms. Blot was exposed overnight to ensure greatest possibility of protein detection in the hybrids.

result in an average of 7.5 null mutations per gene (BRENNER 1974).

Extent of F1 XX spermatogenesis is sensitive to the dosage of C. briggsae *genetic material*

As an alternative to the oligogenic canalization hypothesized above, the genetic mechanism underlying the recessivity of hermaphroditism could be due to haploinsufficiency, perhaps at many loci. That is, the hermaphroditism trait may be rarely expressed in the F1 because there is only one copy of the *C. briggsae* genome in the F1

instead of the two copies that exist in the parent. To test this possibility, a tetraploid strain of *C. briggsae*, CP116, was created. Two lines of evidence suggest that hermaphrodites in this strain are 4A:3X tetraploids. First, there is a high incidence of male progeny arising from virgin CP116 hermaphrodites (39.7%, N adult progeny=229). In addition, when CP116 diakinesis-stage oocytes were examined under fluorescent microscopy using Hoechst staining, a majority of them contained 11 or 12 chromosomes (70%, n animals=40; Figure 8B). No such oocyte observed had less than 9 chromosomes. This is in contrast to wild type *C. briggsae* oocytes, which all contain 6 chromosomes (Figure 8A).

Tetraploid (likely 4A: 2X) *C. briggsae* CP116 males were crossed with diploid *C.* sp. 9 females to generate a triploid hybrid F1 with a 2:1 ratio of *C. briggsae* to *C.* sp. 9 genetic material. When triploid hybrid F1 are produced using wild isolates of *C.* sp. 9, the incidence of selfing increases to 2.3%, and the incidence of spermatogenesis increases significantly to 14% when compared to diploid hybrid F1 (chi-square p-value=0.006, d.f.=1; Table 3). Surprisingly, a small proportion of *C. briggsae* CP116/*C.* sp. 9 EG5268 self-progeny progress through embryonic development. Most undergo larval arrest, but one adult F2 was observed. It did not lay any embryos, but was observed to have both sperm and oocytes. No viable triploid hybrid F1 are produced when *C. briggsae* CP116 is crossed to the inbred *C.* sp. 9 strain JU1422. The great increase in both overt selfing and spermatogenesis in triploids with excess *C. briggsae* gene content suggests that hybrid F1 XX germ line fate is at least somewhat sensitive to the dosage of "hermaphroditizing genes" and that haploinsufficiency can partly account for the dominance of the female germ line state in the hybrid F1.

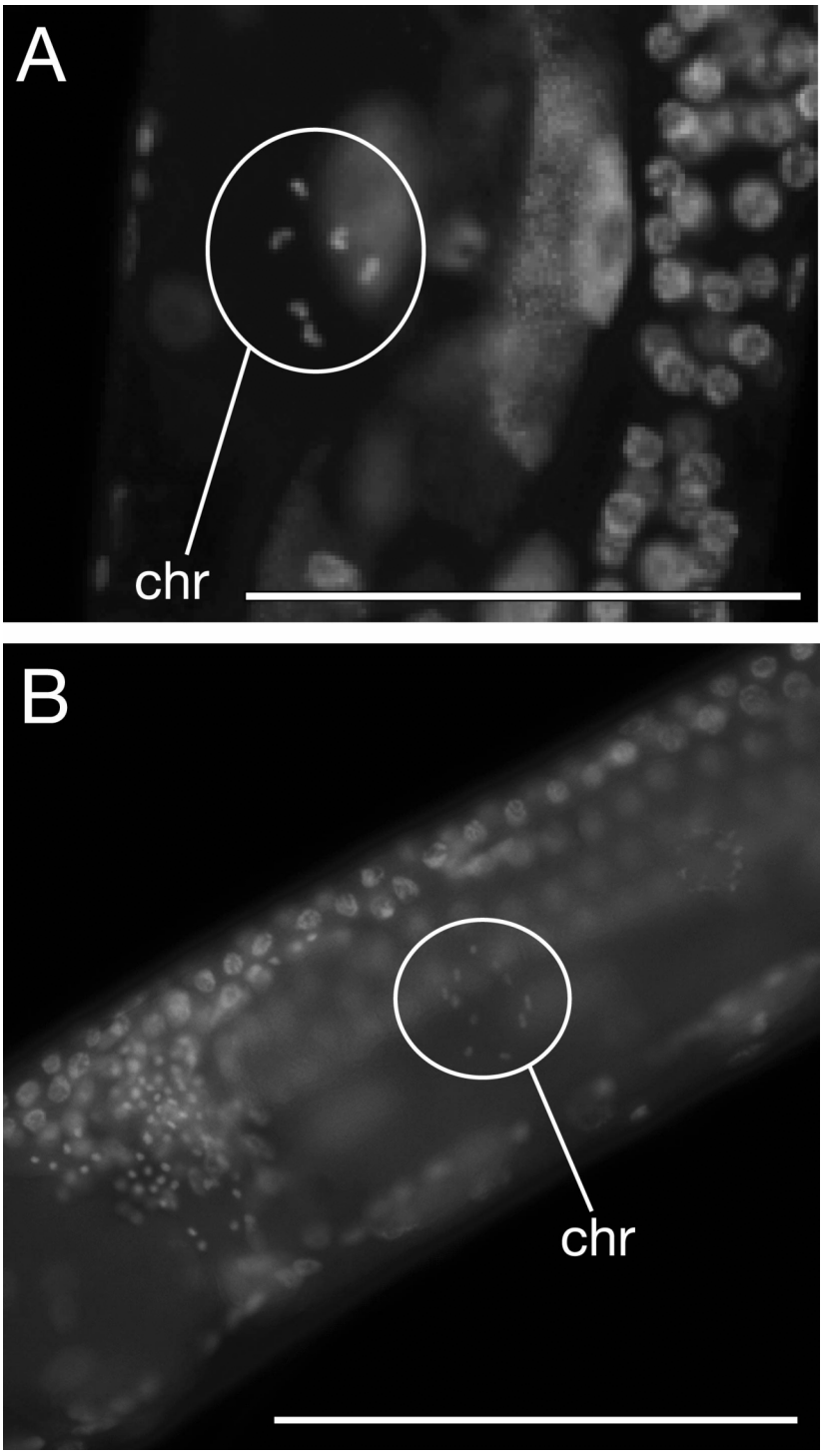


Figure 8. A polyploid strain of *C. briggsae*. A. A wild-type *C. briggsae* AF16 hermaphrodite with 6 chromosomes. Scale bar represents 50 µm. **B.** A polyploid *C. briggsae* CP116 hermaphrodite with 11 chromosomes. Scale bar represents 100 µm. "chr" denotes the chromosomes in both panels. Both of these images are focused upon oocytes arrested in diakinesis stage of meiotic prophase I, with DNA stained with Hoechst 33258.

Partial homozygosity of C. briggsae *loci in the hybrids does not reveal hermaphroditism*

To further investigate the potential of this hybrid system for understanding the genetic basis of hermaphroditism, possible segregation of the hermaphroditism trait was examined in recombinant hybrid generations. Segregation of the trait in the traditional F2 intercross and *C. briggsae* backcross populations cannot be examined due to the developmental and reproductive incompatibilities of the hybrid system and to the recessivity of the hermaphroditism (see above). For these reasons, more unconventional crossing designs were used to produce animals with substantial homozygosity for *C. briggsae* alleles, which allows potential segregation of the recessive hermaphroditism trait if it has a simple genetic architecture and key alleles are not linked to hybrid lethality and sterility factors.

Two generations were investigated for the segregation of the hermaphroditism trait. One is the progeny of $B2_{b,9}$ males and *C. briggsae* hermaphrodites ($B3_{b,9,bh}$ animals; Figure 2G). The other results from crossing $B3_{b,9,9}$ hybrid females with *C. briggsae* males to produce $B4_{b,9,9,b}$ animals (Figure 2K). These generations should be homozygous for *C. briggsae* alleles at a non-zero fraction of their genomes if they undergo a Mendelian pattern of segregation. Other crosses potentially yielding hybrids with homozygous *C. briggsae* regions were examined but either yielded no viable progeny ($F1_b$ female x $B2_{b,9}$ male) or no hermaphrodites ($B2_{b,9}$ female x $B2_{b,9}$ male).

The incidence of selfing and spermatogenesis is 0% among $B3_{b,9,bh}$ XX animals (Table 3). In $B4_{b,9,9,b}$ XX animals, the selfing incidence varies, depending upon which *C.* sp. 9 strain is used. 2.6% of XX $B4_{b,9,9,b}$ animals were selfers when the wild isolate strain of *C.* sp. 9, JU1325 was used, but none were seen with JU1420 and JU1422, the

inbred strains derived from it (Table 3). This figure is not significantly different from that observed in hybrid F1 produced with JU1325 (1.2%; chi-square p=0.5075, d.f.=1). These results suggest that partial homozygosity for *C. briggsae* genes in hybrids does little or nothing to allow reemergence of the hermaphroditism trait.

Hybrid animals show segregation distortion

It is possible that hermaphrodites are absent in the $B3_{b,9,bh}$ and $B4_{b,9,9,b}$ generations because multiple *C. briggsae* alleles must be homozygous for the trait to be observed. However, the patterns of hybrid viability suggest that there may be certain genotypes that promote hybrid lethality. If such hybrid lethal loci were linked to loci essential for hermaphrodite development, key genotypes would become inaccessible. To investigate this possibilities, $B3_{b,9,bh}$ animals were genotyped at multiple loci to determine the extent of segregation distortion in this hybrid generation.

The crossing scheme in Figure 9 was used in order to allow the use of previously generated *C. briggsae* genetic markers (KOBOLDT *et al.* 2010). One strain of *C. briggsae* (VT847) was used as the P0 *C. briggsae* parent, and another strain of *C. briggsae* (the AF16-derived *cb-unc-119* (*nm69*) CP99) was used for the final backcross (Figure 9). This creates $B3_{b,9,bh}$ hybrids that have one entire copy of the *C. briggsae* CP99 genome, and one hybrid genome copy expected to contain roughly 25% *C. briggsae* VT847 DNA. Such hybrids would thus be expected to be homozygous for *C. briggsae* at a given locus 25% of the time. Only assays that amplified *C. briggsae* DNA and failed to amplify *C.* sp. 9 DNA were utilized. Thus, homozygosity at a given *C. briggsae* locus would be revealed by presence of both polymorphic variants for the two *C. briggsae* strains (AF16/VT847; Figure 9). In contrast, heterozygosity at the species level

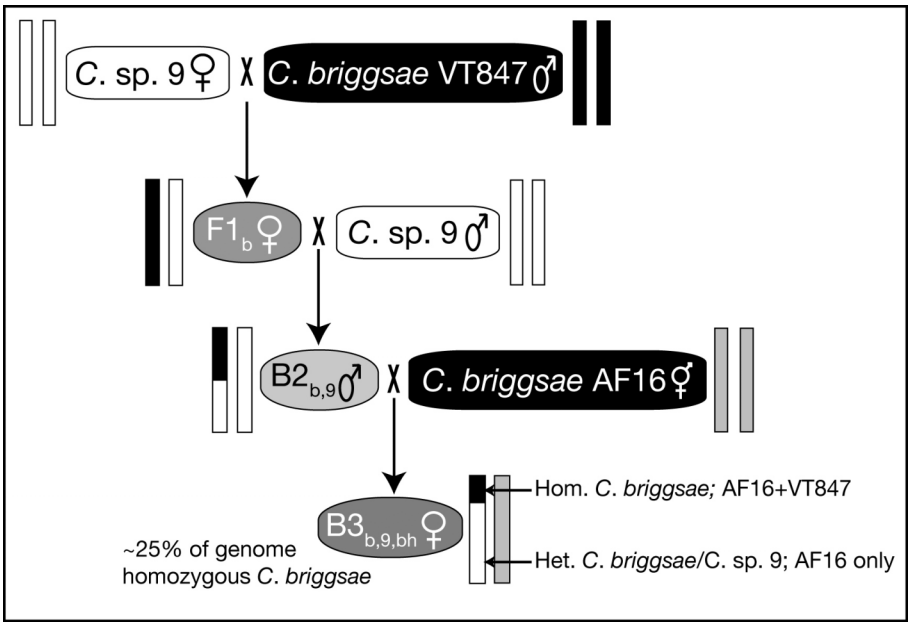


Figure 9. Scheme for genotyping hybrid animals with partially homozygosity for *C. briggsae* alleles. The genotyping $B3_{b,9,bh}$ generation. Existing markers for mapping *C. briggsae* mutations (KOBOLDT *et al.* 2010) were used to genotype hybrid animals. The *C. briggsae* strain VT847 was used for the parental cross, whereas a different *C. briggsae* strain, the AF16-derived *Cb-unc-119(nm68)* strain CP99, was used for the final cross. The mapping generation was then heterozygous for AF16 and VT847 when homozygous for *C. briggsae* and was homozygous AF16 when heterozygous *C. briggsae*/*C.* sp. 9. Following a Mendelian pattern of segregation, a given locus was expected to be heterozygous AF16/VT847 (or homozygous *C. briggsae*) 25% of the time.

(*C. briggsae*/*C.* sp. 9) would be revealed through hemizygosity for the *C. briggsae* strain AF16 (Figure 9).

23 markers were used with an average distance of 3.98 MB (~12.4 cM) (HILLIER *et al.* 2007) between markers (Figure 10). Since the average size of the *C. briggsae* block of the recombinant hybrid chromosome was expected to be 12.5 cM, the assays provide reasonable power to detect segregation distortion if it were present. All 23 markers were confirmed to be dimorphic in *C. briggsae* AF16 and VT847, and no inter-strain segregation distortion was seen (Figure 11; (ROSS *et al.* 2011). Control DNA preparation was made with proportions of *C. briggsae* AF16, *C. briggsae* VT847, and *C.* sp. 9

JU1422 animals equal to the proportions that would be expected in the hybrid $B3_{b,9,bh}$ generation if all of the genetic loci behaved in a Mendelian fashion. This facilitated the recognition of segregation distortion when compared with the hybrid $B3_{b,9,bh}$ generation genotypes.

Among the 23 markers genotyped, thirteen displayed significant deviation (Mann-Whitney U test p-value < 0.05) from the Mendelian expectation (Figure 10). Three markers showed no difference from expectation, and seven markers displayed a non-statistically significant deviation from the Mendelian expectation, all in the same direction. Strikingly, all 20 observed instances of substantial segregation distortion were under-representations of *C. briggsae* alleles, which is itself a highly significant deviation from random error model (binomial sign test, $P<0.0001$). However, at no marker locus were *C. briggsae* alleles completely excluded. For certain regions of *C. briggsae* chromosomes II, III, IV, and X (Figure 10), it appears that either homozygosity in the $B3_{b,9,bh}$ generation or heterozygosity in the previous (B2) generation adversely affects hybrid fitness.

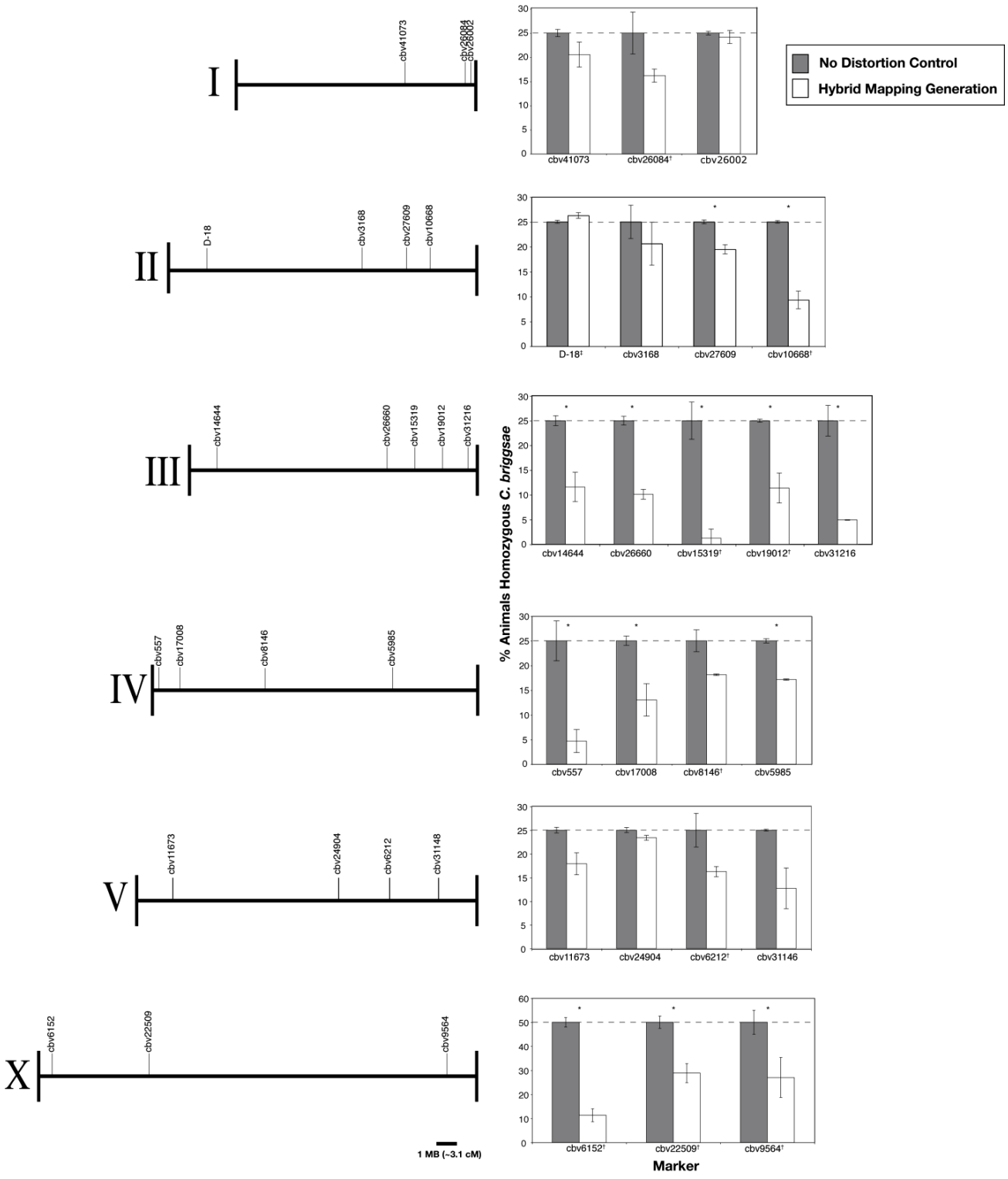

I
cbv41073
cbv26084
cbv26002
No Distortion Control
Hybrid Mapping Generation
II
D-18
cbv3168
cbv27609
cbv10668
III
cbv14644
cbv26660
cbv15319
cbv19012
cbv31216
IV
cbv557
cbv17008
cbv8146
cbv5985
V
cbv11673
cbv24904
cbv6212
cbv31148
X
cbv6152
cbv22509
cbv9564
1 MB (~3.1 cM)
% Animals Homozygous C. briggsae
Marker

Figure 10. Patterns of segregation of loci in hybrids with homozygosity for some *C. briggsae* alleles. Animals in the hybrid $B3_{b,9,bh}$ generation (Figure 8A), which represents the backcross with the highest potential to produce *C. briggsae* homozygosity, were used for genotyping. On the left are the physical positions of the markers used for the genotyping. On the right is displayed the percentage of $B3_{b,9,bh}$ homozygosity for *C. briggsae* at each locus. Pyrosequencing, RFLP, or indel analysis was used to estimate the frequencies of *C. briggsae* AF16 and *C. briggsae* VT847 alleles for each marker. The control (gray bars) was a mixture of *C. briggsae* AF16, *C. briggsae* VT847, and *C.* sp. 9 JU1422 worms in the same proportions expected for hybrid alleles under Mendelian segregation. Note that *C.* sp. 9 DNA was included to mimic the hybrid genome composition, but does not support PCR amplification of polymorphic *C. briggsae* sites. The percent *C. briggsae* homozygosity measured for the Mendelian controls were normalized to 25% to allow direct comparisons of the measurements of the hybrid markers. All DNA preparations used have at least 200 worms each. All tests had $N \geq 3$. The error bars represent one standard error of the mean. * = $P < 0.05$ for Mann-Whitney U test. † = this marker genotyped using RFLP analysis. ‡ = this marker is an indel of 18 base pairs. All other markers were SNP's genotyped through Pyrosequencing.

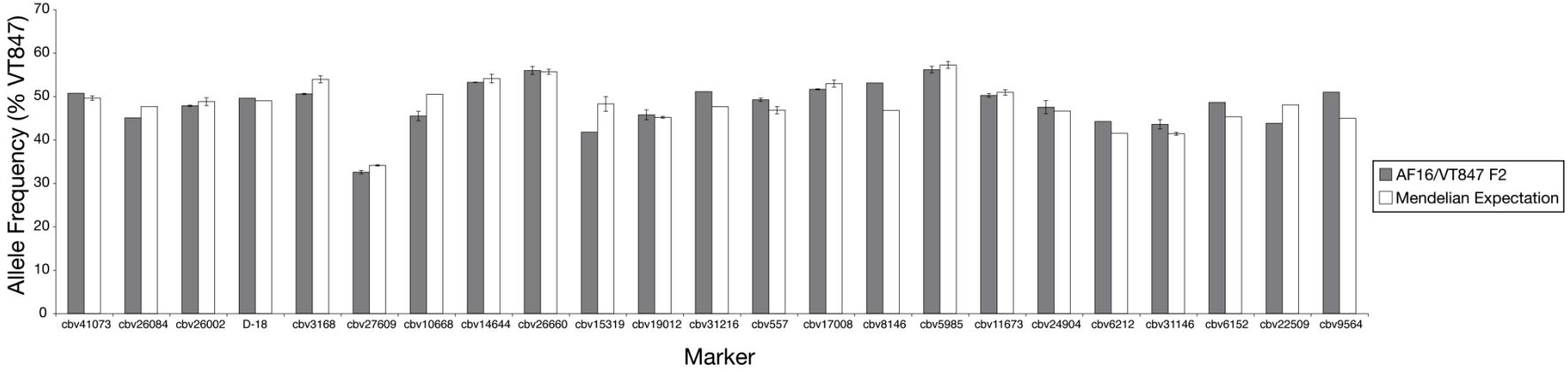


Figure 11. Markers display no between-strain segregation distortion. The estimates of VT847 allele frequencies of an AF16/VT847 F2 generation. Gray bars represent the data for the AF16/VT847 F2 generation. The Mendelian expectation control (white bars) was a mixture of *C. briggsae* AF16 and *C. briggsae* VT847 worms in the proportions expected of these alleles under Mendelian segregation (50:50). Markers were genotyped using Pyrosequencing, RFLP, or indel analysis. All DNA preparations had at least 200 worms each. Error bars represent one standard error of the mean. Points without error bars represent one observation

Discussion

Reproductive isolation in Caenorhabditis

The genetic basis of postzygotic isolation has been an intensely studied problem (COYNE and ORR 2004), and recent advances in fruit flies (FERREE and BARBASH 2009), mice (MIHOLA *et al.* 2009), fish (KITANO *et al.* 2009), and plants (BIKARD *et al.* 2009) have provided insights into the mechanisms that promote reproductive barriers. Recent work has also revealed genetic factors responsible for intraspecies postzygotic isolation in *C. elegans* (SEIDEL *et al.* 2008). However, little is known about the genetics of interspecies postzygotic isolation in *Caenorhabditis* due to the previous absence of fertile hybrids between *Caenorhabditis* species (BAIRD *et al.* 1992). The recently discovered *C.* sp. 9 promises to allow application of the genetic and genomic tools of the *Caenorhabditis* model genus to the problem.

The types of asymmetric patterns observed in the *C.* sp. 9/*C. briggsae* hybrid generations are not uncommon in hybrid systems. In many such systems, the heterogametic sex (XY/XO males or ZW/ZO females) is the disadvantaged sex with respect to hybrid viability and fertility (PRESGRAVES 2008). This common phenomenon is referred to as Haldane's Rule, and clearly applies to the *C.* sp. 9/*C. briggsae* hybrids: F1 males are either dead or sterile, depending upon cross direction, despite the presence of fertile F1 females in both directions. Many theories have been put forth to explain Haldane's Rule, including the dominance theory, the "faster X" theory, and the "faster male" theory (COYNE and ORR 2004). Whichever applies, the leading candidate mechanism for incompatibility is the interaction of Dobzhansky-Muller incompatibility factors (BURKE and ARNOLD 2001; COYNE and ORR 2004), and for the *Caenorhabditis*

system several hypotheses can be framed with such factors in mind. The difference in the F1 sex ratio between reciprocal crosses (Figure 2A, B) can be accounted for entirely by a difference in male-specific viability. This asymmetry in male viability could be explained by hemizygous X-linked *C. briggsae* factors that promote male inviability in the presence of *C.* sp. 9 autosomal factors. This explanation for this male-specific lethality is consistent with the dominance and faster-X theories of Haldane's rule.

In *C. briggsae*/*C. remanei* hybrid F1, an unusual Haldane's Rule phenomenon is seen, in which XO male hybrids are transformed into females in a strain-dependent manner (BAIRD 2002). Although the possibility of male-to-female transformation was not specifically addressed here, the observation of hybrid males with bipolar gonads (Figure 3F) is suggestive that partial sexual transformation may occur in hybrids. Additionally, the segregation of both fertile and infertile males in the $B2_{b,9}$ generation suggests that this system can be utilized to determine the genetic basis of hybrid male infertility. Indeed, the number of fertile males in this hybrid generation (24%; Table 2) suggests that as few as two loci may be needed to restore fertility in this generation (but see discussion of genotyping results below). This hybrid system will likely prove useful in teasing apart the basis of Haldane's rule in *Caenorhabditis*.

In addition to Haldane's Rule, another common pattern of asymmetry is the strong dependence of hybrid F1 viability and fertility upon the directionality of the parental cross. This phenomenon has recently been dubbed "Darwin's corollary to Haldane's Rule" (TURELLI and MOYLE 2007), and it is in effect here. If *C.* sp. 9 is the mother in the parental cross, viable male progeny are produced (Figure 2A), a larger percentage of F1 females are fertile (Table 2), and more viable B2 progeny are produced (Figure 2D vs.

2F). F1 progeny exhibit lower fitness with respect to all of these categories when *C. briggsae* is the P0 mother. The older age of the *C. briggsae* mothers used in these experiments (see Methods) may be partly responsible for the poor performance of their progeny. However, although this is a possible explanation for the lowered viability and fertility of the F1 females, it is unlikely that moderate aging of the *C. briggsae* P0 mother would sex-specifically reduce F1 male viability to zero. Indeed, crosses using L4 *C. briggsae* AF16 hermaphrodites and *C.* sp. 9 JU1325 males also failed to produce males (data not shown). It is also unlikely it could explain the lower viability of $B2_{9,9}$ than $B2_{b,9}$ embryos. This hybrid system then also has the potential to reveal potential causes of Darwin's corollary.

As an alternative to interactions between zygotic factors, there could be parental factors in the *C. briggsae* hermaphrodite or *C.* sp. 9 male gametes that adversely affect hybrid F1 fitness. The mutation rate of the *C. briggsae* mitochondrial genome has been reported to be much faster than that of other *Caenorhabditis* species (HOWE and DENVER 2008), and this difference could facilitate a nuclear-mitochondrial genome incompatibility between *C. briggsae* mitochondria and *C.* sp. 9 nuclear genes that is not reciprocal (BOLNICK *et al.* 2008). This could provide a potential explanation for differences in F1 female fertility and backcross viability in reciprocal crosses. Alternatively, *C. briggsae* paternal effect factors may explain why hybrid progeny are inviable when F1 and backcross females are crossed to *C. briggsae* males (Fig. 2C, E, and H). Since F1 and backcross females are capable of producing viable hybrid progeny with *C.* sp. 9 males (Fig. 2D, F, and I), and because backcross males can produce viable progeny with *C. briggsae* hermaphrodites (Fig. 2G), there may be a *C. briggsae* paternal

effect factor that is incompatible with a hybrid background. As *C. briggsae* males can produce viable F1 hybrids, however, the hybrid's zygotic genotype may also have to have substantial *C. briggsae* homozygosity in order for this particular incompatibility to arise. The ability of *C. briggsae* males to produce viable progeny with $B3_{b,9,9}$ females (Figure 2K and Table 3) is consistent with this.

The $B3_{b,9,bh}$ genotyping results also provide some insights into the genetic basis of hybrid male sterility in this system. That 24% of $B2_{b,9}$ males are fertile is consistent with the existence of two unlinked *C.* sp. 9 loci that must be homozygous to allow male fertility. However, all loci genotyped were detectably homozygous for the *C. briggsae* allele in the $B3_{b,9,bh}$ generation, implying that no region of the *C. briggsae* genome was absolutely excluded from fertile $B2_{b,9}$ males. Although clearly some genomic regions affect hybrid fitness more than others (particularly regions of chromosomes II, III, IV, and X; Figure 10), this suggests that there are multiple epistatic interactions between the *C. briggsae* and *C.* sp. 9 genomes that contribute to lowered hybrid fitness. Such polyfactorial interspecies incompatibility has been reported for both plants (e.g. RIESEBERG *et al.* 1999; JIANG *et al.* 2000; TAYLOR *et al.* 2009) and other animals (e.g. ROGERS and BERNATCHEZ 2006; GOOD *et al.* 2008). In contrast, individual genes have been shown to play major roles in hybrid incompatibilities in *Drosophila* (PRESGRAVES 2010), *C. elegans* (SEIDEL *et al.* 2008), and mice (MIHOLA *et al.* 2009).

Evolutionary implications for recessivity of the hermaphrodite germline

The discovery of productive hybridization between the gonochoristic *C.* sp. 9 and the androdioecious *C. briggsae* suggested that these species could help reveal the genetic architecture of hermaphrodite development. That selfing is almost completely recessive

is itself an important and surprising insight. In particular, this implies that no *C. briggsae* gene (or set of genes) in a single copy is sufficient for robust hermaphroditism in a gonochoristic background. This observation suggests that the female germ-line state in *C.* sp. 9 is extremely canalized in its female fate, and thus highly resistant to the action of factors that promote XX spermatogenesis. In *C. elegans* and *C. briggsae*, loss-of-function mutations in these factors are recessive (HODGKIN 1986; SCHEDL and KIMBLE 1988; ZHANG *et al.* 1997; LI *et al.* 2000; GUO *et al.* 2009) A. Doty unpublished results), indicating they are not individually dose-sensitive in present-day hermaphrodites. Therefore, perhaps an important part of the evolution of selfing *C. briggsae* was the weakening of female germline sexual canalization, so that the "sexual oscillator" that has been proposed to effect limited spermatogenesis (HAAG 2009) can function.

We also note that the defining attributes of hermaphrodite germline development are not always congruent in their expression in hybrid generations. In a few cases, the incidence of spermatogenesis is higher than the incidence of overt selfing within a given hybrid generation. This suggests that in the hybrids selfing can become defective at multiple stages in the process. It would also suggest that it is more difficult to complete the self-fertilization and laying of embryos than it is to simply produce sperm. This is consistent with recent studies in *C. remanei* that suggest that multiple steps are necessary for hermaphroditism to evolve (BALDI *et al.* 2009).

Opportunities and limitations for the genetic investigation of hermaphroditism

C. sp. 9 and *C. briggsae* open the possibility of mapping the historically crucial variants that led to *C. briggsae* hermaphroditism. However, the attempts to do this described here have so far been thwarted. One obvious problem is the extreme

postzygotic isolation between these two species. Due to the inability to make self-fertile hybrid progeny, typical QTL designs based on recombinant inbred selfing lines are impossible (DOROSZUK *et al.* 2009; MOYLE and PAYSEUR 2009). This necessitates the use of unconventional designs to tackle this problem in a genetic mapping context (Fig. 9).

Another, larger problem is the low segregation of hermaphroditism in hybrid generations (Table 3), which falls to zero when inbred lines of *C.* sp. 9 are used. Further, in generations where hybrid hermaphrodites are observed, the incidence of hermaphroditism is no different from that seen in the F1 generation, indicating it is not due to rare combinations of homozygous *C. briggsae* alleles. The inability of *C.* sp. 9 inbred lines to yield any hybrid hermaphrodites suggests that either XX spermatogenesis in hybrids is especially sensitive to parental inbreeding depression, or that there is standing genetic variation in *C.* sp. 9 for factors that specifically facilitate XX hybrid spermatogenesis. If the latter is true, then there should be heterogeneity among *C.* sp. 9 inbred lines with their ability to create hybrid hermaphrodites. This has not been observed, but only a small number of inbred lines have been investigated. However, even if such a polymorphic *C.* sp. 9 factor existed, its identity would not explain how *C. briggsae* became hermaphroditic. This would require the mapping of a *C. briggsae* hermaphroditizing factor that is not polymorphic.

Though it has proven difficult to use this system to map specific *C. briggsae* hermaphroditic factors, mechanisms patterning germline sex in this hybrid system can still be explored. One possibility is that this recessive trait has a complex (i.e. polygenic) genetic architecture. However, the presence of widespread segregation distortion biased against *C. briggsae* alleles in hybrids (Fig. 10) prevents any such hypotheses about the

nature of the genetic architecture of *C. briggsae* hermaphroditism from being rigorously evaluated. As an alternative approach, we screened 15,000 mutagenized haploid *C.* sp. 9 JU1422 genomes for a mutant that would facilitate the generation of hybrid F1 selfers. No such animal was observed. Given that loss-of-function alleles are generated for an average size gene about once in about every 2,000 haploid mutagenized genomes (ANDERSON 1995), it is unlikely that there is any one *C.* sp. 9 feminizing factor that is responsible for the dominance of the female state in the hybrid germ-line. However, an increased dosage of *C. briggsae* factors to the hybrid background through the use of polyploids does elevate the incidence of hermaphroditism, and in certain cases can almost triple it in the F1 (Table 3). This suggests that the haploinsufficiency of hermaphrodite-promoting *C. briggsae* alleles in the hybrid F1 is at least partly responsible for the recessivity of the hermaphrodite trait in this system.

Implications for the emergence of hermaphrodite lineages

Phylogenetic studies suggest that hermaphroditism evolved multiple times in *Caenorhabditis* (CHO *et al.* 2004; KIONTKE *et al.* 2004). If the ability of *C.* sp. 9 and *C. briggsae* to produce fertile hybrids (whereas all other *Caenorhabditis* pairwise crosses cannot) can be used to infer that these two species are recently diverged sister taxa (a inference further supported by CUTTER *et al.* 2010; RABOIN *et al.* 2010), then the most parsimonious scenario is one in which the last common ancestor of these two species had a female germ-line, similar to *C.* sp. 9 today. If that ancestral female was as resistant to the effects of hermaphroditizing factors as *C.* sp. 9, then mating with gonochoristic relatives would have completely destroyed the nascent trait. How, then, was a hermaphrodite lineage successfully established?

One simple scenario posits a single dominant factor sufficient for hermaphroditism arising in a gonochoristic population, which then rapidly fixes due to the reproductive fitness benefits of selfing (SMITH 1978). Alternatively, multiple hermaphrodite-promoting factors (with one or more being recessive) arise and accumulate within a gonochoristic population, and are fixed in the population by physical or ecological isolation and resulting inbreeding. That our results support the latter, more convoluted process is unexpected because of the empirical evidence (SCHEDL and KIMBLE 1988; BALDI *et al.* 2009) as well as the multiple, independent gains of selfing within the *Caenorhabditis* lineage, which may suggest that the process that leads to the evolution of hermaphroditism would be one that is relatively simple. However, it is important to note that this is predicated upon the notion that the germ-line state of *C*. sp. 9 is a good proxy for the common ancestor of *C*. sp. 9 and *C. briggsae*. Given the degree of reproductive isolation demonstrated to exist between these two species, it is entirely possible that such an assumption is unsound. It is even possible that the strongly canalized *C*. sp. 9 female germline state has evolved since the origin of *C. briggsae* to prevent loss of gonochorism through either new mutations or hybridization with *C. briggsae* (which is cosmopolitan and has been so for a long period of time) (DOLGIN *et al.* 2008). Indeed, it is thought that these selfing *Caenorhabditis* lineages are short-lived and that pure selfing could quickly lead to extinction due to the accumulation of deleterious alleles (LOEWE and CUTTER 2008).

Other differences between C. *sp. 9 and* C. briggsae

In addition to their reproductive barriers, other differences between *C. briggsae* and *C*. sp. 9 have been observed. One peculiar difference is that mated *C*. sp. 9 females

run out of sperm long before *C. briggsae* hermaphrodites do (Fig. 4), even though the assays for the former initially placed multiple male mates with each female. The average *C.* sp. 9 total brood size of 259 is striking given that *C. elegans* hermaphrodites can lay over 1000 embryos after a single mating assay (HODGKIN 1986). In the similarly gonochoristic *C. remanei*, outbred crosses of only 6 hours duration with single males (DOLGIN *et al.* 2007) produce fecundities similar to those we report here for *C.* sp. 9 mated with multiple males for at least twice as long. This suggests that *C.* sp. 9 females cannot take full advantage of abundant sperm. This may be a general feature of the species, for example as a consequence of female spermatheca size, barriers to remating, male effects on female fecundity (DIAZ *et al.* 2010), or as a consequence of unintended inbreeding in our laboratory stocks. Additionally, the reduction of *C. briggsae* hermaphrodite brood size by *C.* sp. 9 males is at least in part due to a decrease in hermaphrodite survivorship after such matings (A. Chang pers. comm.; Chapter 3).

A more notable difference, however, is that *C.* sp. 9 animals have strikingly lower viabilities at low temperatures whereas *C. briggsae* animals have relatively high viabilities at similar temperatures (Fig. 5). Interestingly, this apparent pattern of *C.* sp. 9 as a high temperature specialist and *C. briggsae* as a temperature generalist correlates with these species' geographic distributions. *C. briggsae* has a relatively wide geographic range across at least five continents, and over 100 isolates have been found in regions as disparate in temperature as Iceland and India (CUTTER *et al.* 2010). Thus far *C.* sp. 9 has only been found in tropical nations such as India and Congo, but this may change with more sampling. Despite the small sample size, the locations of its isolation and its low fitness at low temperatures are consistent with this species having a geographic range

restricted to high temperature environments. If this is the case, then *C.* sp. 9 and *C. briggsae* provide a new system within which to potentially understand the evolution of generalist and specialist modes of ecological adaptation. In tandem with the reproductive isolation between these species, these differences will likely prove the comparative *C.* sp. 9/*C. briggsae* system to be fruitful for future studies.

Future prospects

We have described some attributes of the first hybrid genetic system in *Caenorhabditis*, formed between the gonochoristic species *C.* sp. 9 and the androdioecious species *C. briggsae*. Though mapping factors that distinguish hermaphrodites from true females has thus far been thwarted, a range of studies could be envisioned that would exploit this system's combination of speed and experimental resources to further studies of species formation, the evolution of mating systems, and any other phenotypic differences that may be discovered between the two species. Such experiments will be greatly aided by genomic tools for *C.* sp. 9. Indeed, a set of male- and female -specific transcriptome profiles (R. Jovelin and A. Cutter pers. comm.) and a genome sequence (F. Piano pers. comm.) of *C.* sp. 9 are currently being constructed. There is clearly great potential for this system that will begin to be realized in the near future.

Chapter 3: *C.* sp. 9 sperm sterilize, decrease lifespan, and ectopically migrate in *C. briggsae* hermaphrodites

Abstract

In *Caenorhabditis*, self-fertile hermaphroditism has evolved multiple times independently from gonochoristic ancestors. In these lineages, the incidence of outcrossing has largely decreased, which has also corresponded with a degradation of mating-related traits in these lines. Additionally, it has been observed that there is a lifespan cost to mating in *C. elegans* hermaphrodites. Here, it is observed that *C. briggsae* hermaphrodites have lower numbers of self-progeny and reduced lifespan when mated with males of the gonochoristic species *C.* sp. 9. Fluorescent microscopy reveals that *C. briggsae* hermaphrodites previously mated with *C.* sp. 9 males accumulate endomitotic oocytes, suggesting that *C.* sp. 9 males promote ovulation and oocyte maturation defects in *C. briggsae* hermaphrodites. This is somewhat surprising because this heterospecific cross produces viable progeny, showing hybrid fertilization can be successful. Additionally, microscopy also reveals the mislocalization of sperm outside of the uterus and spermatheca in these mated hermaphrodites. Ectopic sperm were found near the bend of the gonad, the distal germ line, and outside of the gonad including near the pharynx. To further investigate the role of *C.* sp. 9 cross-sperm in *C. briggsae* hermaphrodite mortality and fecundity, *C.* sp. 9 males with feminized germ lines were produced using *fog-3* RNAi. These spermless males were incapable of promoting the mating-dependent sterilization and lifespan reduction in *C. briggsae* hermaphrodites, although they were able to perform the mating behavior and deposit copulatory plugs. Additionally, SEM shows that conspecific mating promotes cuticle damage and bacterial accumulation in *C.*

elegans hermaphrodites, as previously suggested. Ectopic sperm localization was also observed in conspecific crosses of the gonochoristic species *C. remanei* and *C.* sp. 9, and *C.* sp. 9 sperm were found to more frequently mislocalized in *C. remanei* females than in conspecific crosses. Collectively, this suggests that alternative patterns of sexually antagonistic coevolution in divergent lineages, in tandem with the degradation of mating-related traits in hermaphroditic lineages, may lead to gonochoristic male mating-induced sterility and mortality in hermaphrodites and therefore potentially to reproductive isolation between these lineages. Presumably, this is due to relaxed sexually antagonistic selection on traits associated with sperm competition in hermaphroditic linages.

Introduction

When traits that are beneficial in one sex harm the other, sexual conflicts emerge (ARNQVIST and ROWE 2005). Although first recognized relatively recently (PARKER 1979), such conflicts are now understood to be widespread and have been found in a wide range of taxa (ARNQVIST and ROWE 2005). Additionally, it has been proposed that sexually antagonistic coevolution may involve arms races that can promote divergence and reproductive isolation (RICE 1998). Both experimental (RICE 1996; MARTIN and HOSKEN 2003) and theoretical (GAVRILETS 2000) work are consistent with this notion, although other studies have failed to support the idea (WIGBY and CHAPMAN 2006; BACIGALUPE *et al.* 2007).

Furthermore, the evolution of selfing is predicted to have consequences on the dynamics of these conflicts. Particularly, sexual conflicts are expected to be reduced in selfing lineages due to the absence of mating (GLÉMIN and GALTIER 2012). This prediction is consistent with findings in *Arabidopsis* (SPILLANE *et al.* 2007; FOXE and

WRIGHT 2009) and *Caenorhabditis* (PALOPOLI *et al.* 2008).Additionally, it has also been proposed that changes in reproductive mode may hasten sexual conflict-driven divergence (BRANDVAIN and HAIG 2005).However, there are few experimental studies that have addressed these concepts at the intersection of sexual conflict, reproductive isolation, and the evolution of reproductive mode.

Self-fertile hermaphroditism has evolved from gonochoristic ancestors multiple times independently in the *Caenorhabditis* lineage (KIONTKE *et al.* 2011). Sexual conflicts are inferred to exist in *Caenorhabditis* due to asymmetries in lifespan costs to copulation (GEMS and RIDDLE 1996) and the presence of copulation plugs (PALOPOLI *et al.* 2008). And, as lines differing in reproductive mode are also reproductively isolated from one another (Chapter 2; BAIRD *et al.* 1992), the *Caenorhabditis* system can provide a unique opportunity to address questions that converge on these issues. Here, a cryptic sexual conflict in *Caenorhabditis* is revealed between interspecific matings of *C*. sp. 9 and *C. briggsae*, and *C*. sp. 9 male sperm are found to sterilize and prematurely kill *C. briggsae* hermaphrodites. This could be interpreted as a conflict-driven phenomenon made more acute due to the evolution of hermaphroditism.

Methods

Maintenance and strains.

Animals were maintained according to standard *C. elegans* procedures (WOOD 1988), with the exception of increased agar concentration in NGM plates to 2.2% in order to discourage animals from burrowing underneath the surface of the plate. Cultures were raised at 20°C unless otherwise indicated. Strains used in this study include: *C. briggsae* AF16, *C*. sp. 9 JU1325, *C. remanei* EM464 and *C. elegans him-5* (*e1490*) DR461.

Fecundity measurements.

One L4 female or hermaphrodite was placed with four heterospecific or conspecific males per plate and left overnight. In the case of assaying for self-brood size alone, hermaphrodites were left alone on a plate without males. The next day, males were removed altogether and XX animals were transferred to a new plate. All resultant embryos and larvae were then counted. This process was repeated every day until the day where <10 embryos/larvae were found on the plate. The total number of embryos and larvae laid for a given individual mother accounted for a single brood size measurement.

Lifespan measurements.

Seven L4 females/hermaphrodites were placed with 10 heterospecific or conspecific males per plate and left overnight. The next day, XX animals were assayed for mortality by being touched on the head with an eyebrow hair glued to a toothpick. If the animal performed a backwards locomotive response to the touch, it was scored as alive. If it did not, it was scored as dead. This was performed every day for at least seven days. Every two days, XX animals and males were transferred to new plates in order to prevent the confusing of progeny with parents. Additionally, in these assays, XX animals were kept under continuous mating conditions: when males died or crawled off the plate, they were replaced with new males. XX animals that crawled off the plate were excluded from the lifespan measurements. Lifespan statistics were performed with the OASIS online application (YANG *et al.* 2011).

Hoechst staining and vital staining of sperm.

The nuclei of animals were visualized using Hoechst 33258 staining. 7 XX animals were mated with 10 heterospecific or conspecific males per plate for 1-3 days,

and then XX animals were fixed in 100% methanol overnight at 4°C. Then, the animals were washed three times in M9 buffer and then incubated in 1 μg/ml Hoechst in M9 buffer for 5 minutes. The animals were then mounted for fluorescent microscopy and imaging.

Male sperm were fluorescently labeled *in vivo* with MitoTracker® Red CMXRos (Invitrogen)(KUBAGAWA *et al.* 2006). Males were incubated in 1 mM dye for 2-3 hours, and then left on a plate to recover overnight. Subsequently, these males were mated with virgin young adult XX animals for 1-4 hours (matings with *C. elegans* males were allowed to run overnight). Virginity was assured by isolating XX L4 animals from males before reaching adulthood. Mated XX animals were then mounted on 10% agarose pads (FANG-YEN *et al.* 2009) for DIC and fluorescence imaging. Automated time-lapse photography was performed with the Open Lab software package and a Zeiss Axioskop 2 equipped with DIC and fluorescence microscopy.

Electron microscopy.

7 *C. elegans him-5* hermaphrodites were either left alone or mated with 10 conspecific males per plate for 5 days. Worms were moved every two days to ensure they were not confused with their progeny. Animals were then fixed in 2% glutaraldehyde overnight. Animals were washed and post-fixed in osmium tetroxide for 30 minutes, and then dehydrated in an ethanol series. Animals were then critical point dried from carbon dioxide, mounted, and coated with gold. Animals were then imaged using a scanning electron microscope.

RNAi and scoring germ line feminization phenotypes.

A 929 base pair fragment including coding sequence homologous to *fog-3* was

PCR amplified from *C*. sp. 9 genomic DNA using primers flanked with 5' T7 promoters. The reaction was gel purified using the QIAquick kit (Qiagen), and the resultant template was then utilized for in vitro transcription using the MAXIscript kit (Ambion) to make dsRNA. The dsRNA was recovered using phenol-chloroform extraction and isopropanol precipitation, and the dsRNA was then introduced into the animals via maternal microinjection. The male progeny of injected animals were scored for the feminization of germline (Fog) phenotype using DIC microscopy via standard methods (WOOD 1988). The worms were mounted on 2% agarose pads and immobilized with 50 mM sodium azide. Only males with clearly defined oocytes and no observable sperm were used for sterilization and lethality experiments. Fog males were placed in a drop of M9 buffer on a plate and allowed to recover for 30 minutes. These males were then utilized for experiments as described above. Control wild-type males were mounted, drugged, and allowed to recover for the same amount of time in order to remove these as confounding factors.

Results

C. sp. 9 *males promote sterilization and mortality in* C. briggsae *hermaphrodites*

It was previously observed that *C. briggsa*e hermaphrodites cease laying self-progeny soon after mating with *C.* sp. 9 males (Chapter 2, Figure 4). Indeed, exposure to *C.* sp. 9 males promotes an extreme reduction in *C. briggsae* hermaphrodite fecundity in comparison to unmated hermaphrodites (Figure 1). *C. briggsae* hermaphrodites lay an average of 275 embryos (n=4) in the absence of males. When paired with heterospecific *C.* sp. 9 males, these hermaphrodites maintain a significantly lower average brood of 33

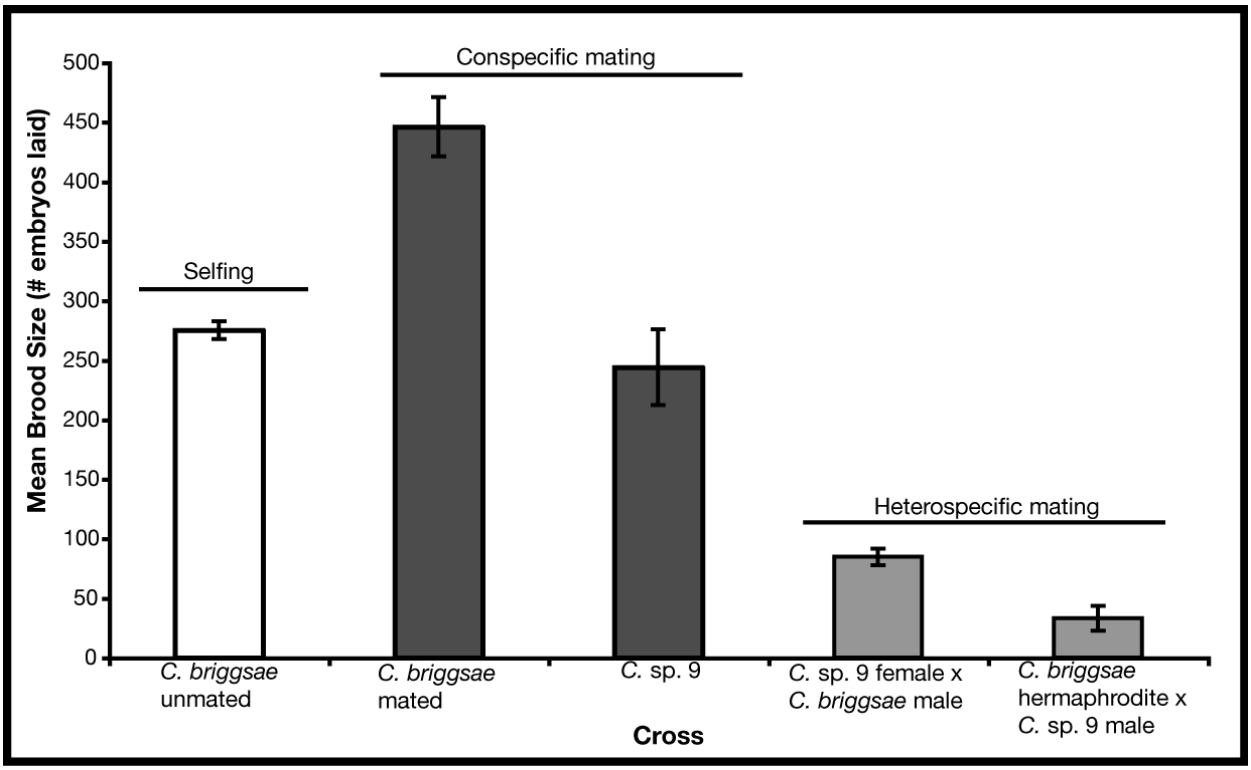
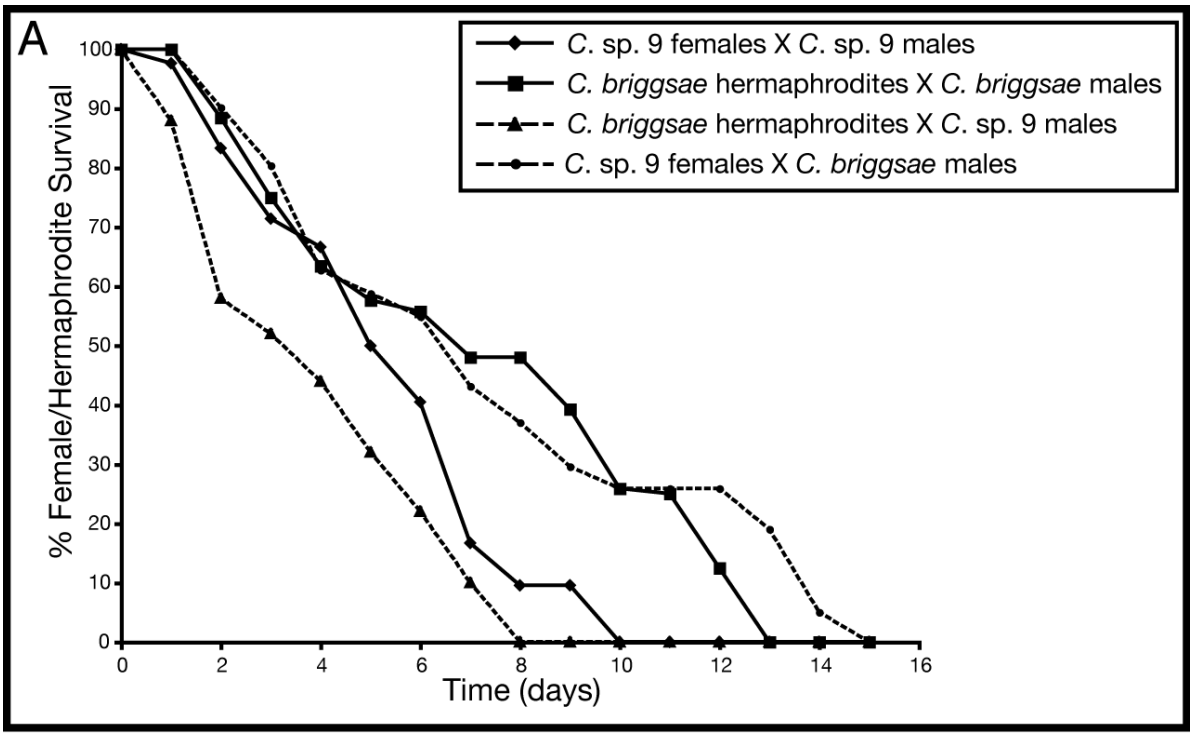


Figure 1. Gonochoristic males inhibit selfing in hermaphrodites. Mean female/hermaphrodite brood sizes of conspecific and heterospecific *C*. sp. 9 and *C. briggsae* crosses. Error bars represent one standard deviation of the mean. Sample sizes ranged from 4-6. The fecundity difference between conspecifically mated and heterospecifically mated *C. briggsae* hermaphrodites is statistically significant (Mann-Whitney U p-value=0.014).

(n=4). Mating with conspecific males does not promote this sterilization but rather is associated with a higher fecundity than selfing by itself (Figure 1).

Because there is a lifespan cost to mating in *C. elegans* hermaphrodites, the loss of fecundity imposed by *C*. sp. 9 males on *C. briggsae* hermaphrodites may be related to premature mortality. Consistent with this, under continuous mating conditions (see Methods). *C. briggsae* hermaphrodites have a shorter lifespan when mated with heterospecific *C*. sp. 9 males than when mated with conspecific males (Figure 2). When mated with conspecific males under continuous mating conditions, *C. briggsae*


A
C. sp. 9 females X C. sp. 9 males
C. briggsae hermaphrodites X C. briggsae males
C. briggsae hermaphrodites X C. sp. 9 males
C. sp. 9 females X C. briggsae males
% Female/Hermaphrodite Survival
Time (days)

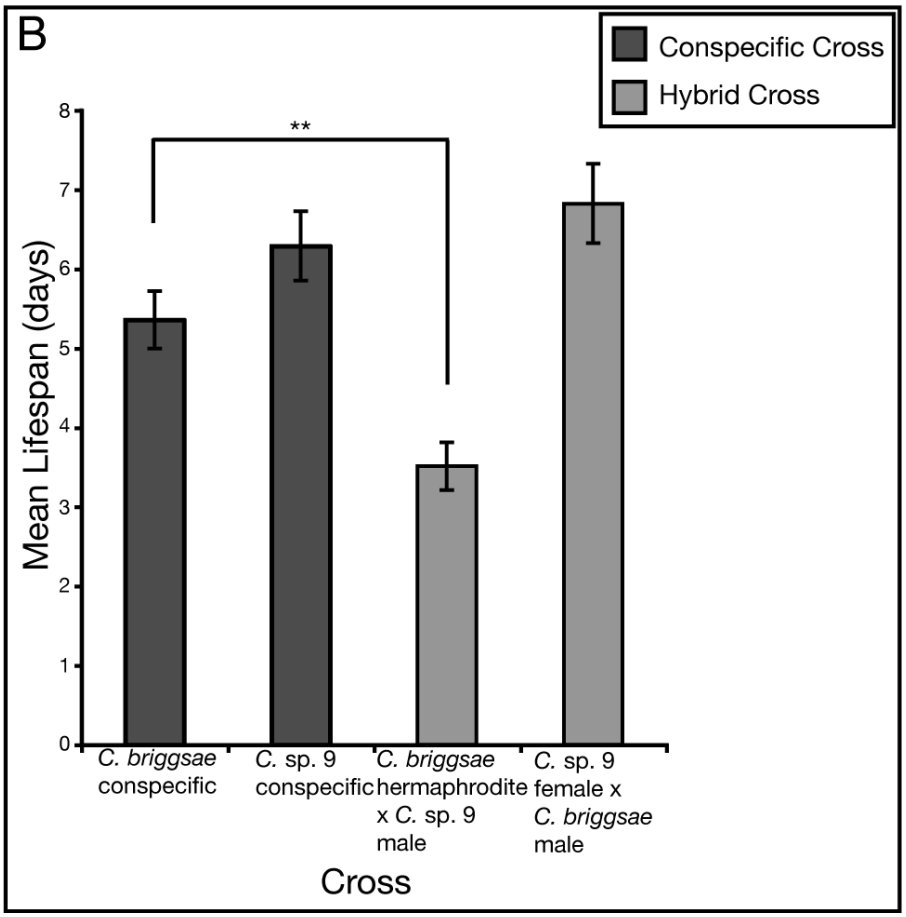

B
Conspecific Cross
Hybrid Cross
**
Mean Lifespan (days)
C. briggsae conspecific
C. sp. 9 conspecific
C. briggsae hermaphrodite x C. sp. 9 male
C. sp. 9 female x C. briggsae male
Cross

Figure 2. Gonochoristic males reduce lifespan in hermaphrodites. (A) Lifespan curves of *Caenorhabditis* females/hermaphrodites in conspecific (solid lines) and heterospecific (dashed lines) crosses during continuous mating conditions. Sample sizes ranged from 42-52. (B) Mean maternal lifespans in conspecific and heterospecific *C.* sp. 9 and *C. briggsae* crosses. Error bars represent one standard error of the mean. The lifespan difference between conspecifically mated and heterospecifically mated *C. briggsae* hermaphrodites is statistically significant (Fisher's Exact Test p-value at 50% mortality=0.008).

hermaphrodites had a lifespan at 50% mortality of 5 days (n=42). When mated with *C.* sp. 9 males, their lifespan at 50% mortality drops significantly to 3 days (n=50). This species-specific asymmetry in mating-induced mortality is not observed when hermaphrodites are not under continuous mating conditions. However, since most *C. briggsae* self-progeny are laid in the first two days (PRASAD *et al.* 2011), and that only one heterospecific mating event is sufficient to promote *C. briggsae* sterilization (J. Ting and A. Cutter, pers. comm.), this premature mortality cannot explain the loss of fecundity in *C. briggsae* alone.

Mating promotes cuticle damage and bacterial accumulation in C. elegans

Previous investigations reported that the most likely cause of mating-induced mortality in *C. elegans* hermaphrodites is the physical act of mating itself, and the lifespan costs persist even in the absence of fertilization (GEMS and RIDDLE 1996). It was also suggested that copulation induces damage and/or bacterial accumulation on the cuticle surface of hermaphrodites (GEMS and RIDDLE 1996). To investigate this as a potential mechanism of the lifespan cost to mating in *Caenorhabditis* XX animals, scanning electron microscopy (SEM) was performed on mated and unmated *C. elegans* hermaphrodites (Figure 3). SEM revealed a higher incidence of both cuticle damage and bacterial accumulation in mated hermaphrodites when compared to unmated hermaphrodites. Whereas none of the observed unmated hermaphrodites (n=8) showed

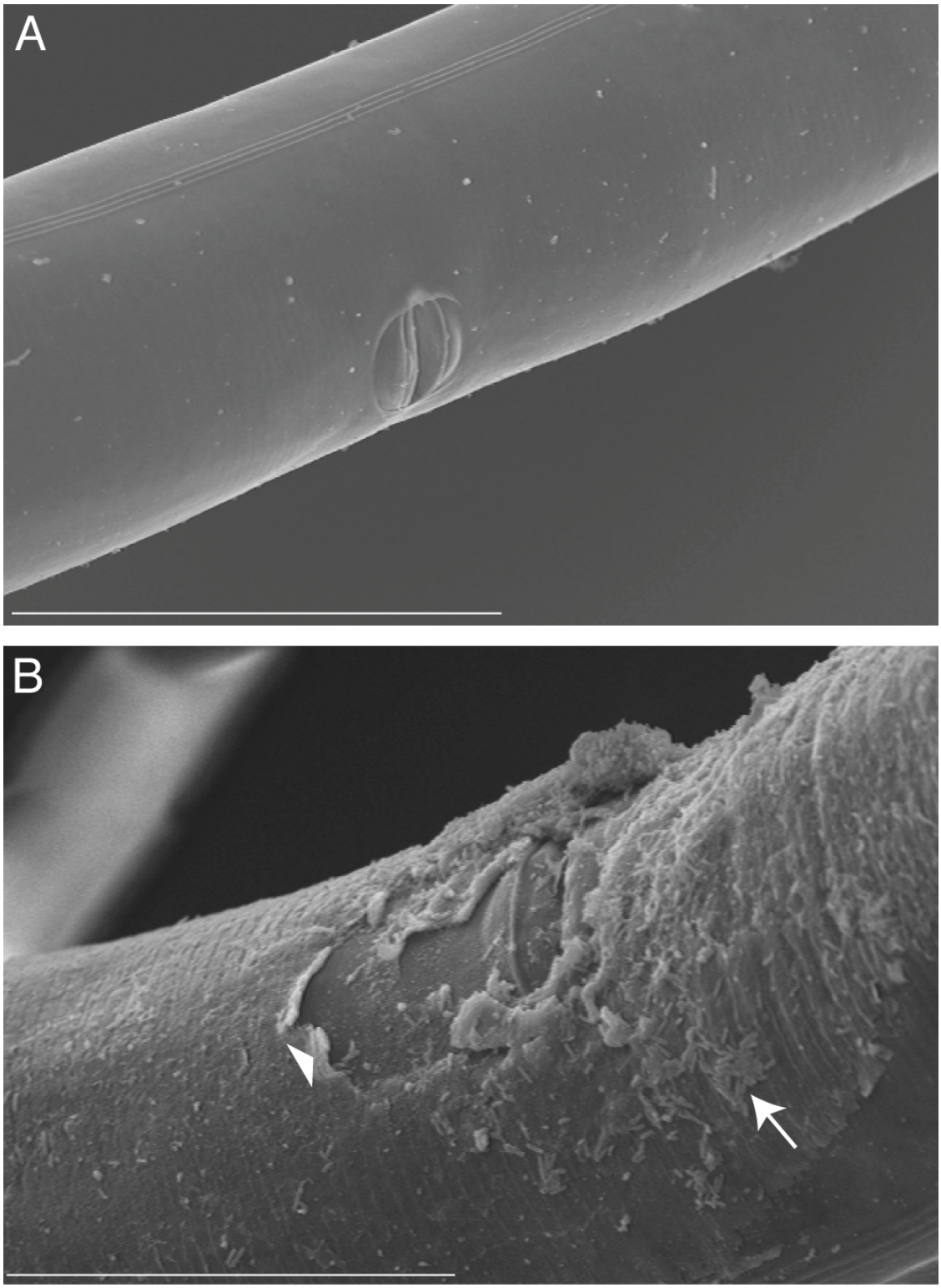


Figure 3. Conspecific mating promotes cuticle damage and bacterial accumulation in *C. elegans*. (A) SEM of an unmated *C. elegans* hermaphrodite vulva. Scale bar, 100 microns. (B) SEM of a mated *C. elegans* hermaphrodite vulva. Here, bacterial accumulation (arrow) and tearing (arrowhead) on the cuticle surface around the vulva are apparent. Scale bar, 50 microns.

any evidence of cuticle damage near the vulva, a fraction (4 out of 9) of the mated hermaphrodites displayed tearing of the cuticle surface localized near the vulva. Such injury may be expected in this region in mated hermaphrodites because males probe the area around the vulva with their spicules during copulation (GARCIA *et al.* 2001). Additionally, mated hermaphrodites had a higher incidence (5 out of 9) of bacterial accumulation, whereas unmated hermaphrodites had no such growths. Both of these observations are consistent with previous predictions (GEMS and RIDDLE 1996). And since mating behavior within the Elegans group of *Caenorhabditis* is highly conserved (KIONTKE *et al.* 2011; THOMAS *et al.* 2012), it is likely that similar patterns of cuticle damage and bacterial accumulation occur in other species of this clade, including *C.* sp. 9 and *C. briggsae*. However, *C.* sp. 9 and *C. briggsae* males deposit mucin-rich copulatory plugs upon the vulva of their mates at the end of mating (HODGKIN and DONIACH 1997; PALOPOLI *et al.* 2008). Since these plugs are likely to obscure cuticle features around the vulva under SEM, mated XX animals from these species were not examined under SEM.

C. sp. 9 *males promote oocyte maturation defects in* C. briggsae *hermaphrodites*

To further investigate the sterilization of *C. briggsae* by *C.* sp. 9 males, *C. briggsae* hermaphrodites mated to *C.* sp. 9 males were examined with DIC microscopy. After three days of mating, *C. briggsae* hermaphrodites mated to conspecific males displayed wild-type phenotypes (Figure 4A). In contrast, hermaphrodites mated to *C.* sp. 9 males had striking germ line abnormalities (Figure 4B), such as disorganized proximal germ line material and ectopic, distally localized oocytes. In addition, egg-laying had ceased due to sterilization. Also, in the absence of egg-laying, copulatory plugs tended to

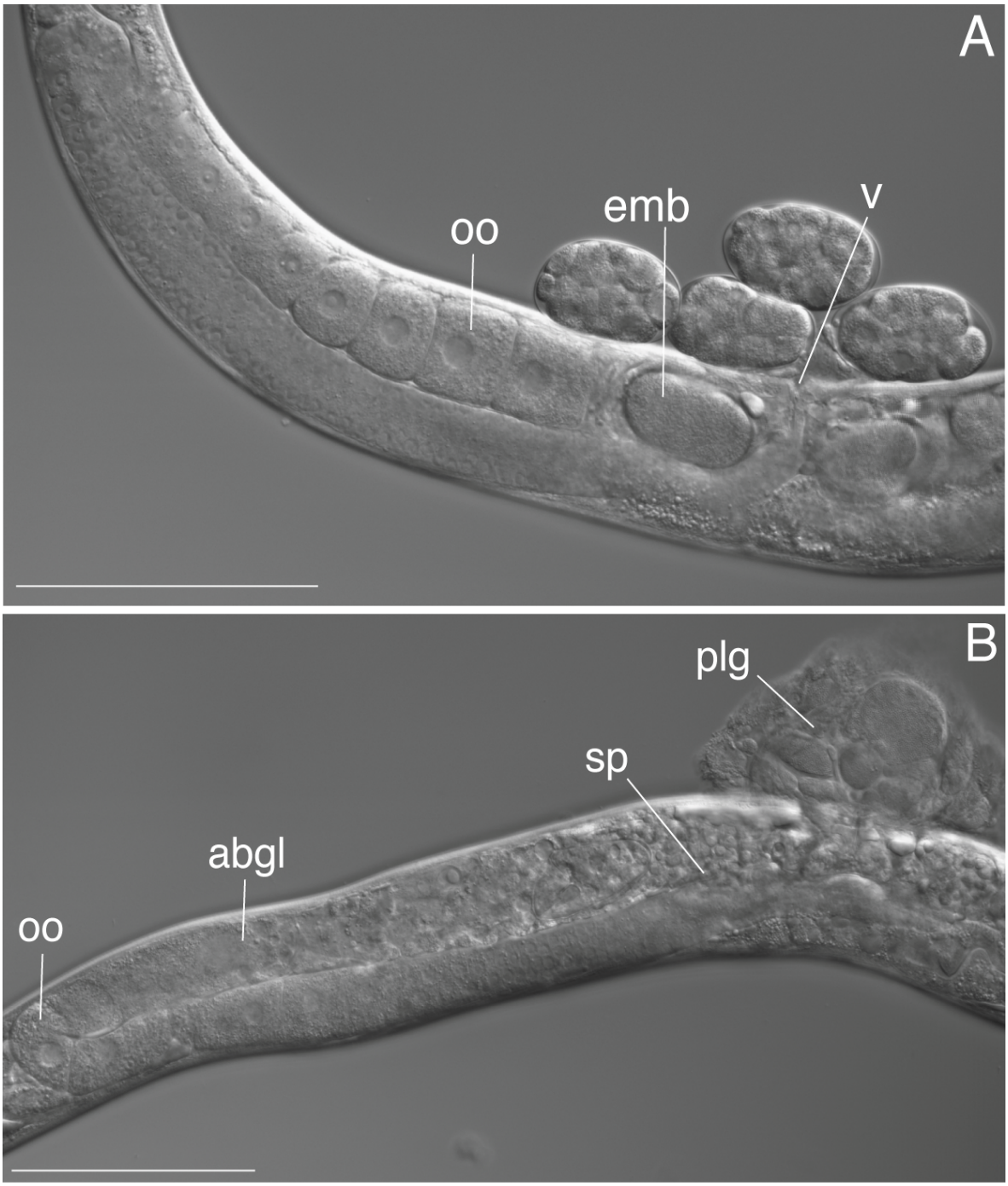


Figure 4. *C*. sp. 9 males promote germ line defects in *C. briggsae* hermaphrodites. (A) A wild-type *C. briggsae* hermaphrodite germline after mating three days with *C. briggsae* males. Oocytes (oo), embryos (emb), and the vulva (v) are marked. (B) A wild-type *C .briggsae* hermaphrodite germ line after mating three days with *C*. sp. 9 males. Here, there is the abnormal localization of the most clearly defined proximal oocyte at the bend of the gonad arm, the presence of disorganized, abnormal germ line material (abgl) throughout the proximal gonad, and the absence of embryos in the uterus. Scale bars represent 100 microns in both panels.

accumulate on the vulva and promote bacterial growth. Such plug accumulation was not observed in hermaphrodites mated to conspecific males.

The proximal mass of disorganized germ line tissue seen in heterospecifically-mated hermaphrodites was reminiscent of the endomitotic oocyte phenotypes of *tra-1* mutants (HILL and HAAG 2009). The accumulation of endomitotic oocytes in the proximal gonad is emblematic of ovulation defects in *C. elegans* (WARD and CARREL 1979; IWASAKI *et al.* 1996; KIM *et al.* 2013) and are easily identifiable with DNA staining due their polyploidy.

To examine if *C.* sp. 9 males induce oocyte maturation defects in *C. briggsae* hermaphrodites, mated hermaphrodites were fixed and stained with Hoechst 33258 to visualize DNA under fluorescent microscopy. After one day of mating with heterospecific males, *C. briggsae* hermaphrodites displayed a high frequency of endomitotic oocyte accumulation (54%, n=78; Figure 5B). This fraction increased (91%, n=70) after two days of mating with heterospecific males. No endomitotic oocytes were observed among *C. briggsae* hermaphrodites mated with conspecific males (n=51, Figure 5A). This

evidence suggests that *C.* sp. 9 males promote oocyte maturation defects in *C. briggsae* hermaphrodites.

C. sp. 9 *male sperm are mislocalized in* C. briggsae *hermaphrodites*

In addition to endomitotic oocytes, Hoechst staining also uncovered the presence of ectopically localized sperm in heterospecifically mated *C. briggsae* hermaphrodites. In virgin or conspecifically mated *C. elegans* hermaphrodites, sperm actively localize to the spermatheca. Upon ovulation, fertilization occurs, and the newly formed zygote pushes

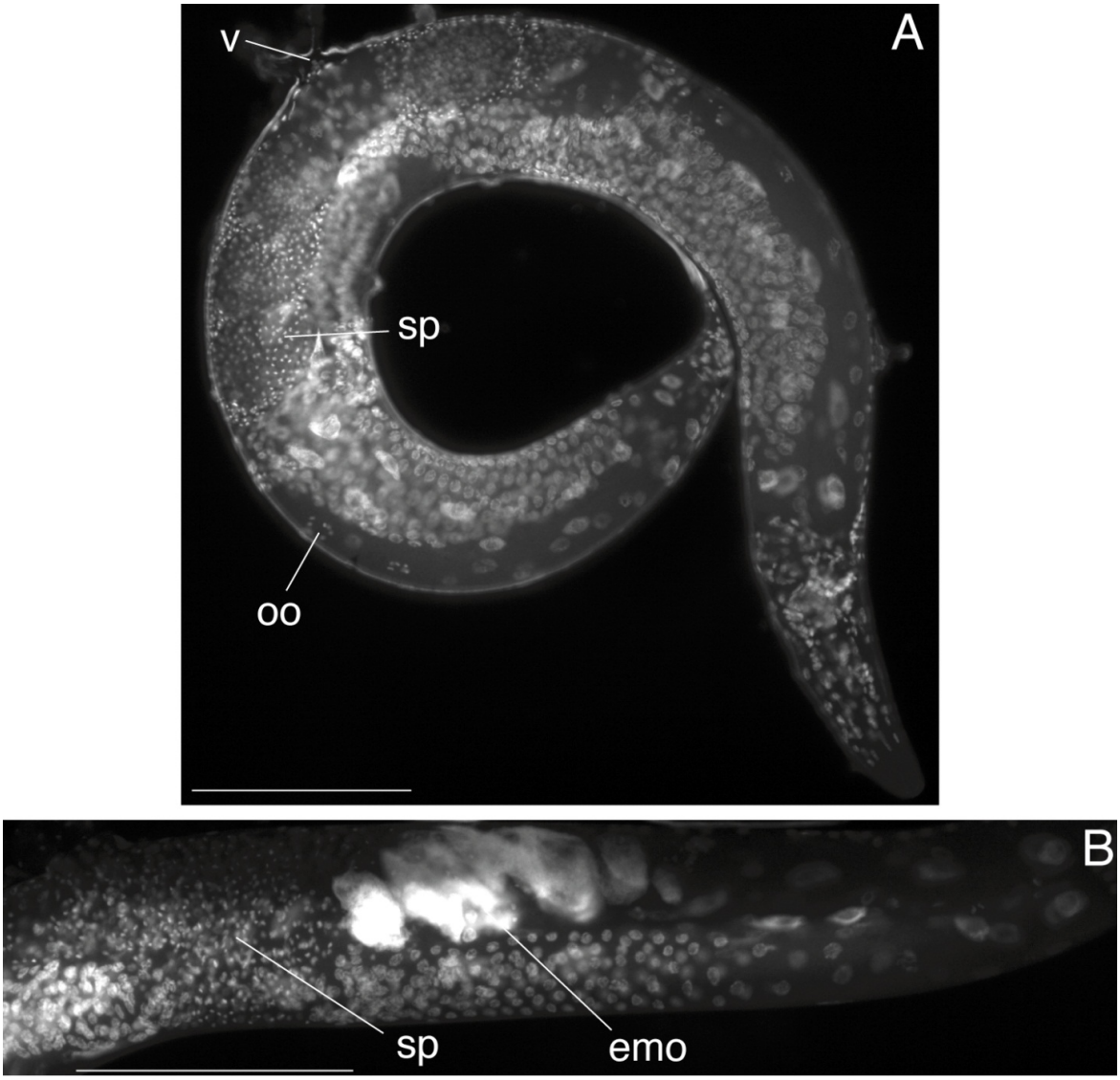


Figure 5. *C.* sp. 9 males promote oocyte maturation defects in *C. briggsae* hermaphrodites. (A) A wild-type *C. briggsae* hermaphrodite stained with the DNA-labeling Hoechst stain one day after mating with *C. briggsae* males. Sperm (sp) are noted. (B) A wild-type *C. briggsae* hermaphrodite with Hoechst staining one day after mating with *C*. sp. 9 males. The presence of endomitotic oocytes (emo) in the proximal germ line is noted. Scale bars represent 100 microns in both panels.

most of the sperm into the uterus and the sperm then crawl back into the spermatheca to fertilize the next oocyte (L'HERNAULT 2006). Similarly, in *C. briggsae* hermaphrodites mated with *C. briggsae* males, all sperm were observed in either the spermatheca or the uterus under Hoechst staining (n=51). However, when *C. briggsae* hermaphrodites were mated with *C*. sp. 9 males overnight, a substantial fraction displayed ectopic sperm (40%,

n=78; Figures 6-7). This fraction increased (81%, n=70) after two days of mating. Most ectopic sperm were localized to the proximal gonad, although sperm were oftentimes observed in the distal gonad as well as outside of the gonad, including in the head of the animal (Figure 6G-H).

To further investigate this phenomenon, and to confirm that the ectopic sperm were not self-sperm (that is, sperm produced by the *C. briggsae* hermaphrodite), a vital dye was used to stain *C.* sp. 9 sperm. Within six hours of mating to *C. briggsae* hermaphrodites, *C. briggsae* male sperm were properly localized (and restricted to) the spermatheca (Figure 6A-B) and uterus in all animals (n=52). On the other hand, when mated with *C. briggsae* hermaphrodites, *C.* sp. 9 male sperm were seen to be mislocalized in the vast majority of cases within six hours of mating (90%, n=188; Figure 6G-H; Figure 7). Additionally, within six hours, invasion of cross-sperm into non-gonadal tissues was also observed in some hermaphrodites (7%, n=159; Figure 6G-H). *C. briggsae* male sperm were never observed in ectopic locations when mated to *C.* sp. 9 females (n=70; Figure 6E-F).

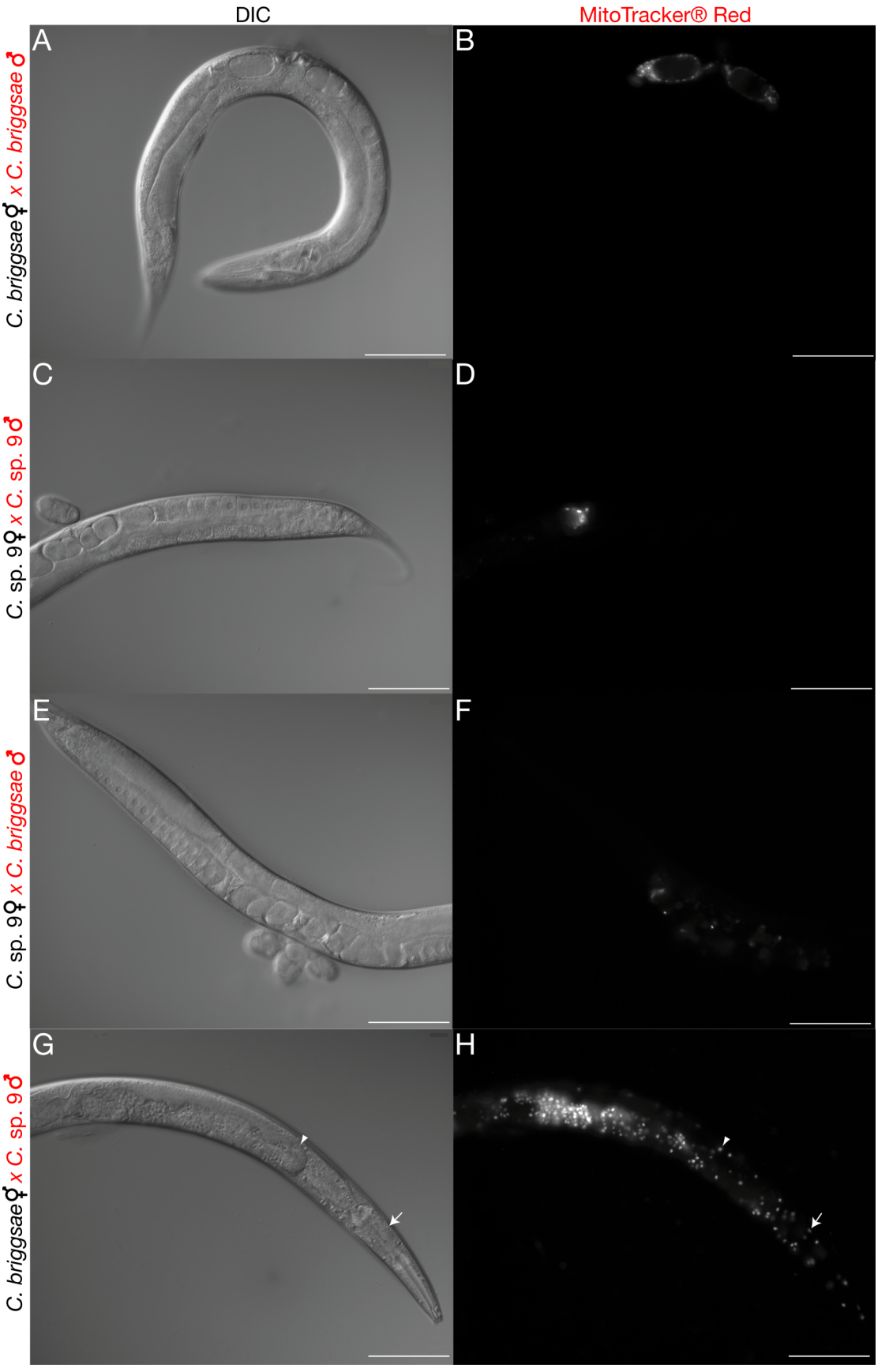

DIC
MitoTracker® Red
A
B
C
D
E
F
G
H
C. briggsae ♀ x C. briggsae ♂
C. sp. 9 ♀ x C. sp. 9 ♂
C. sp. 9 ♀ x C. briggsae ♂
C. briggsae ♀ x C. sp. 9 ♂

Figure 6. *C.* sp. 9 male sperm are mislocalized in *C. briggsae* hermaphrodites. Females/hermaphrodites were mated with males vitally labeled with MitoTracker® Red and imaged under DIC (left panels) and fluorescence microscopy (right panels) to visualize transferred sperm. (A-B) A *C. briggsae* hermaphrodite mated with labeled conspecific males. (C-D). A *C.* sp. 9 female mated with labeled conspecific males. (E-F) A *C.* sp. 9 female mated with labeled *C. briggsae* males. (G-H) A *C. briggsae* hermaphrodite mated with labeled *C.* sp. 9 males. Here, sperm are localized outside of the spermatheca and vulva. Ectopic sperm in the developing germ line (arrowhead) and in the head (arrow) are noted. All scale bars represent 100 microns.

Sperm are mislocalized in conspecific and heterospecific crosses of gonochoristic species

To investigate the generality of this sperm mislocalization phenomenon, labeling experiments were performed in various *Caenorhabditis* species (Table 1). Notably, sperm were seen to be mislocalized in a fraction of gonochoristic, conspecifically mated *C.* sp. 9 and *C. remanei* females (Table 1, Figure 8). Additionally, an asymmetry was observed in interspecies crosses of *C. remanei* and *C.* sp. 9: *C.* sp. 9 male sperm were far more effective in invading *C. remanei* female ectopic tissues than *C. remanei* sperm (Table 1).The ability of *C. remanei* sperm to mislocalize in *C.* sp. 9 females was comparable to *C.* sp. 9 sperm (Table 1). And, *C.* sp. 9 sperm were found to ectopically localize in more than half of observed *C. elegans* hermaphrodites (Table 1). Thus, hermaphrodites appear to be particularly susceptible to *C.* sp. 9 sperm mislocalization, although *C. briggsae* hermaphrodites are most affected.

Because there was an asymmetry in sperm mislocalization between *C.* sp. 9 and *C. remanei*, it was hypothesized that there may also be an asymmetry of mating-dependent female mortality. Indeed, the median lifespan of *C. remanei* significantly decreases from 7 to 5.5 over eight days when continuously mated with *C.* sp. 9 males instead of conspecific males (Figure 9). This lifespan reduction is not as severe as that observed between *C. briggsae* hermaphrodites and *C.* sp. 9 males (Figure 2), and this is

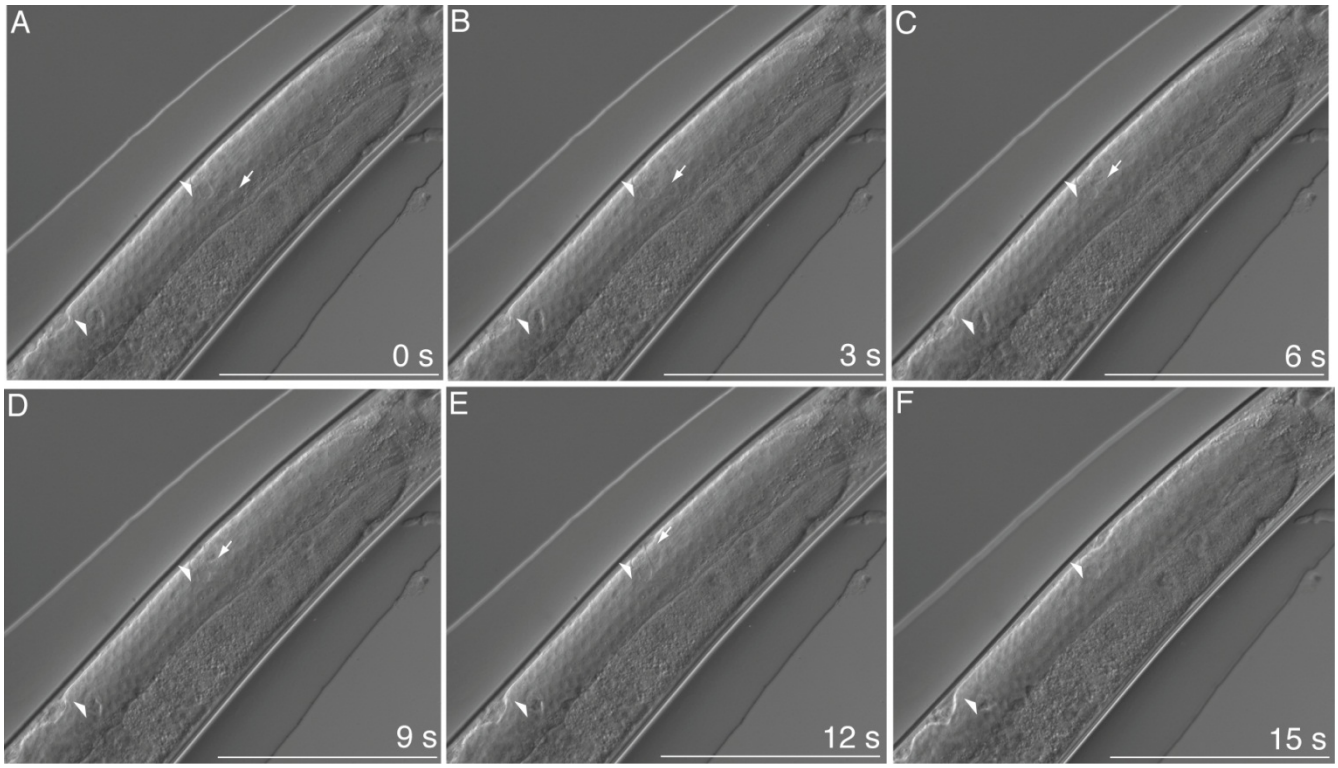


Figure 7. Ectopically migrating *C.* sp. 9 sperm in a *C. briggsae* hermaphrodite gonad. (A-F) A time-lapse series of images demonstrating the rapid movement of a *C.* sp. 9 sperm (arrow) in the distal gonad of a *C. briggsae* hermaphrodite. Images were taken in 3 second intervals. By the last frame (F), the migrating sperm has crawled out of the focal plane. Stationary ectopic sperm (arrowheads) are also noted. Scale bars represent 100 microns.

correlated with severity of ectopic sperm localization (Table 1).

Fog-3 *promotes sperm fate in* C. sp. 9 *males*

To determine whether *C.* sp. 9 sperm are responsible for *C. briggsae* hermaphrodite sterilization and premature death, *C.* sp. 9 males lacking sperm had to be generated. This was achieved by injecting dsRNA homologous to *C9-fog-3* into gravid *C.* sp 9 females and scoring their male progeny for the feminization of germline (Fog) phenotype. More specifically, the Fog phenotype is defined by a germ line filled with cells of the female fate (oocytes) and not male fate (sperm). In *C. elegans*, *fog-3* encodes

Table 1. Mislocalization of Sperm in Various Crosses

Cross	Heterospecific cross	Reproductive mode difference	% Ectopic sperm (n)	% Sperm outside of gonad (n)
C. briggsae males x *C. briggsae* hermaphrodites	-	-	0 (52)	0 (52)
C. sp 9 males x *C.* sp. 9 females	-	-	5 (119)	0 (119)
C. remanei males x *C. remanei* females	-	-	8 (104)	0 (104)
C. elegans males x *C. elegans* hermaphrodites	-	-	0 (9)	0 (9)
C. briggsae males x *C.* sp. 9 females	+	+	0 (70)	0 (70)
C. sp 9 males x *C. briggsae* hermaphrodites	+	+	90 (188)	8 (119)
C. sp 9 males x *C. elegans* hermaphrodites	+	+	51 (41)	0 (41)
C. sp 9 males x *C. remanei* females	+	-	31 (165)	6 (165)
C. remanei males x *C.* sp. 9 females	+	-	5 (100)	2 (100)
C. briggsae males x *C. elegans* hermaphrodites	+	-	0 (13)	0 (13)

The results of observations utilizing vitally dyed male sperm in various crosses. The "Heterospecific cross" column displays whether or not the cross is between species: -, conspecific cross, +, heterospecific cross. The "Reproductive mode difference" column notes whether or not the species differ in reproductive mode: -, same reproductive mode, +, differing reproductive mode. The "% ectopic sperm" column denotes the percentage of observed females/hermaphrodites displaying labeled sperm outside of the spermatheca and uterus. The "% sperm outside of the gonad" column shows the percentage of observed females/hermaphrodites with labeled sperm outside of the gonad entirely.

a Tob-related protein that feminizes the germline in both males and females and does not affect somatic sex-determination (ELLIS and KIMBLE 1995; CHEN *et al.* 2000). Additionally, this gene has been shown to be functionally conserved in multiple *Caenorhabditis* species (CHEN *et al.* 2001), so it was potentially likely to also be functionally conserved in *C.* sp. 9. Indeed, among the male progeny of dsRNA-injected mothers, 42% (n=264) exhibited the Fog phenotype (Figure 10), and 17% exhibited germ lines of ambiguous sexual fates or mixtures of male and female fates. Thus, wild-type

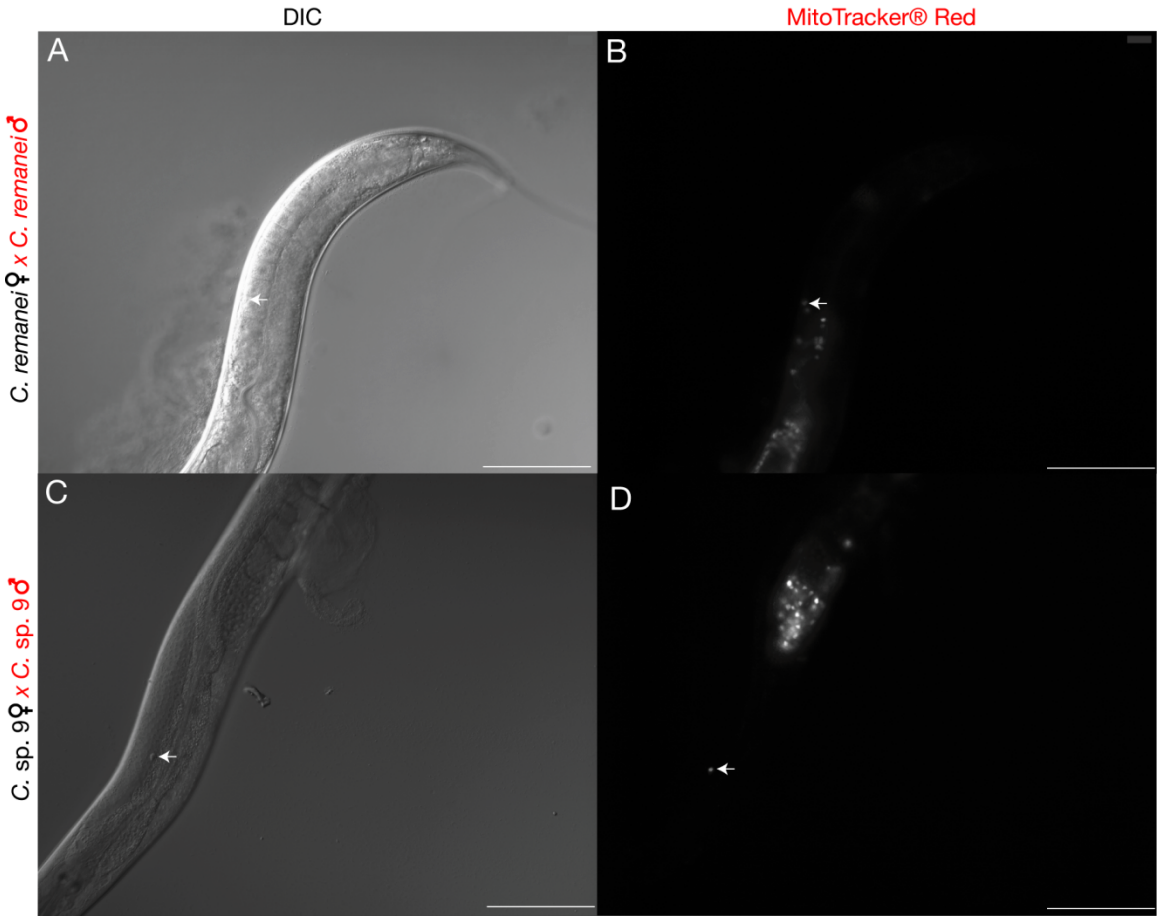


Figure 8. Sperm can migrate outside of the spermatheca in conspecific crosses of gonochoristic species. Females were mated with males vitally labeled with MitoTracker® Red and imaged under DIC (left panels) and fluorescence microscopy (right panels) to visualize transferred sperm. (A-B) A *C. remanei* female mated with labeled conspecific males. (C-D) A *C.* sp. 9 female mated with labeled conspecific males. Arrows denote sperm outside of the uterus or spermatheca. Scale bars at the lower right of panels represent 100 microns.

levels of *C*9-*fog-3* activity are necessary to prevent the feminization of the germ line in *C.* sp. 9 males. These germline feminized males were observed performing the mating behavior and were capable of depositing copulatory plugs (and therefore potentially other seminal fluids). These males would then be suitable for determining whether sperm are responsible for *C. briggsae* hermaphrodite sterilization and premature death.

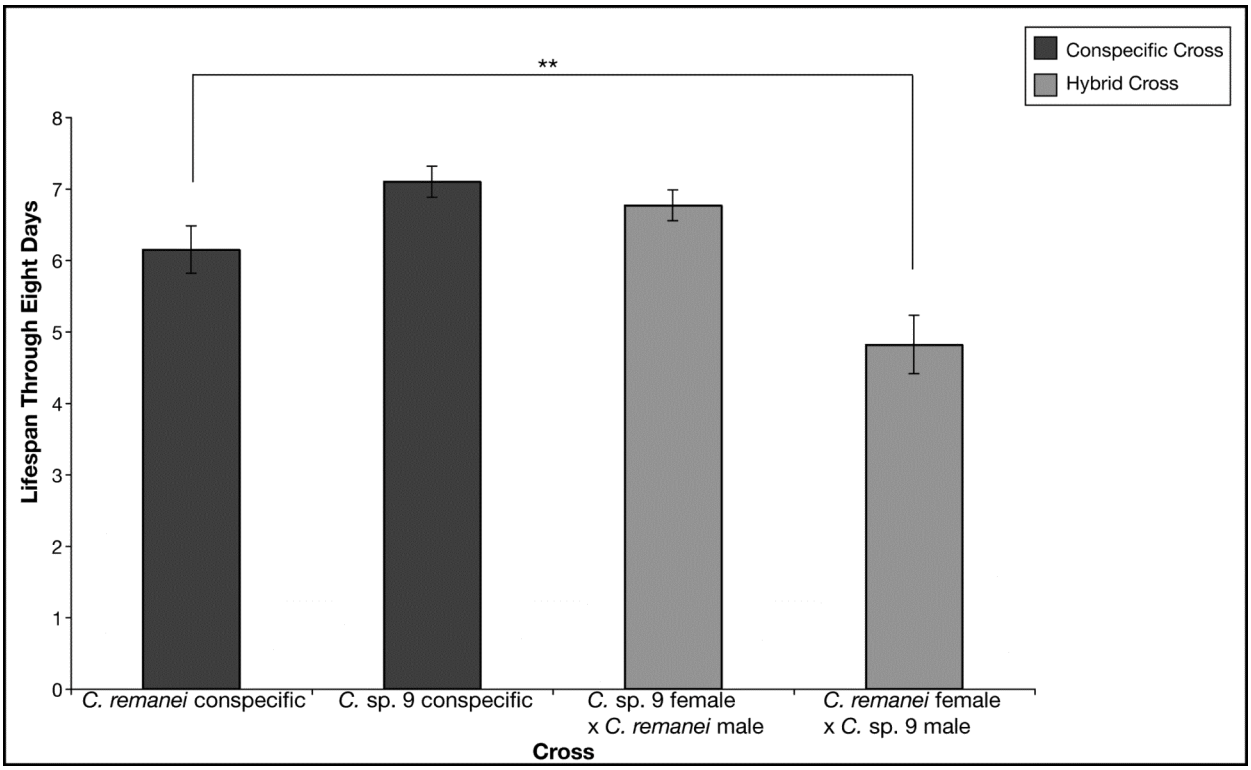


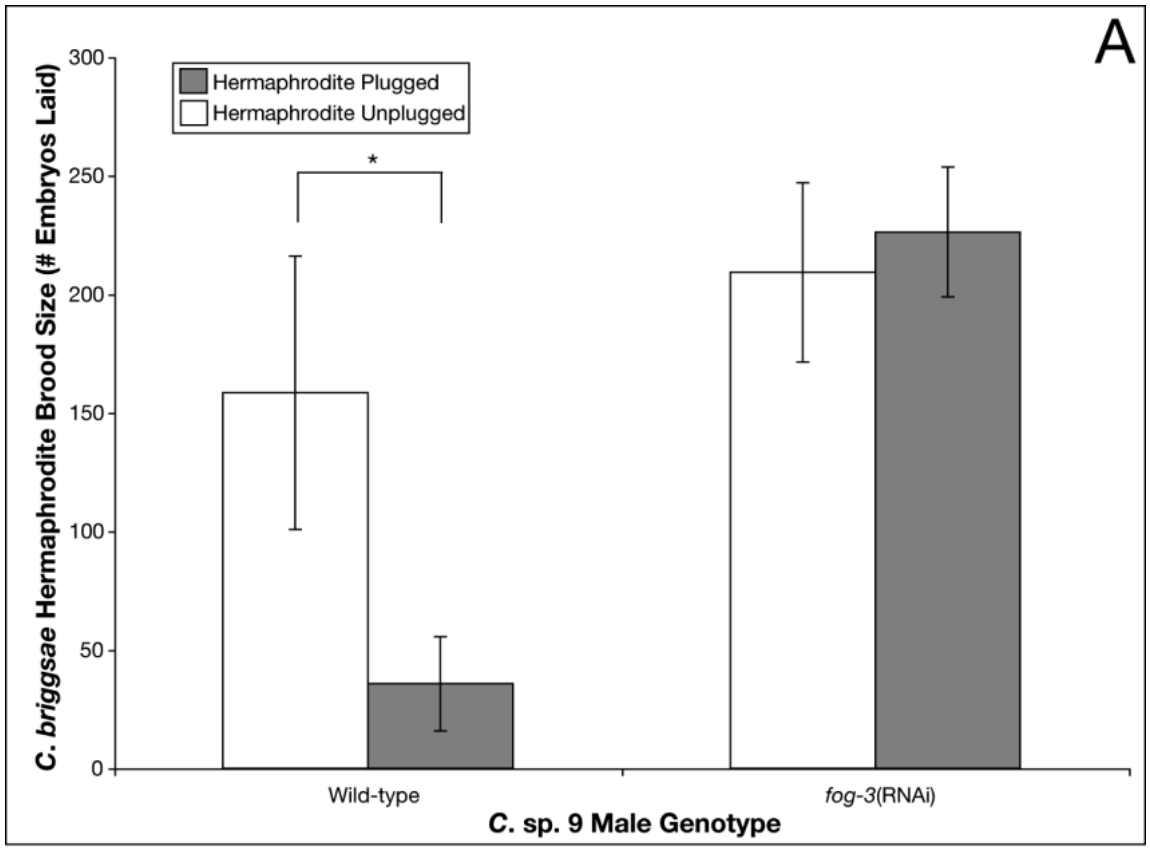
Figure 9. *C.* sp. 9 males reduce the lifespan of gonochoristic *C. remanei* females. Mean maternal lifespans through eight days of continuous mating in conspecific and heterospecific *C.* sp. 9 and *C. remanei* crosses. Error bars represent one standard error of the mean. The lifespan difference between conspecifically mated and heterospecifically mated *C. remanei* females is statistically significant (Mann-Whitney U p-value=0.005). Sample sizes ranged from 38-42.

C. sp. 9 *male sperm are necessary for mating-dependent sterilization and mortality of C. briggsae hermaphrodites*

To determine if *C.* sp. 9 sperm are responsible for the sterilization and premature death of *C. briggsae* hermaphrodites, *C. briggsae* hermaphrodites were mated with *C.* sp. 9 *C*9*-fog-3(RNAi)* males, and their subsequent fecundity and lifespan were measured. Indeed, the *C.* sp. 9 mating-dependent brood size reduction observed in *C. briggsae* hermaphrodites disappears when germline feminized males are utilized (Figure 11A). The average fecundity of successfully mated *C. briggsae* rises significantly from 36 (n=16) to 226 embryos laid (n=13) when wild-type *C.* sp. 9 males are replaced with

Figure 10. *fog-3* is required for spermatogenesis in *C*. sp. 9 males. (A) A wild-type *C*. sp. 9 male, with spermatocytes (spc) and sperm (sp). (B) A *fog-3*(RNAi) *C*. sp. 9 male produces oocytes (oo) and no sperm. Scale bars are 100 microns in both panels.

males unable to properly produce sperm. Additionally, *C. briggsae* hermaphrodite lifespan is rescued when wild-type *C*. sp. 9 males are replaced with sperm-deficient males (Figure 11B). This manipulation causes the mean lifespan over seven days to rise from 4.4 (n=25) to 6.6 days (n=26). Thus, it is likely that heterospecific *C*. sp. 9 sperm are responsible for premature death and sterilization in *C. briggsae* hermaphrodites.


A
Hermaphrodite Plugged
Hermaphrodite Unplugged
*
C. briggsae Hermaphrodite Brood Size (# Embryos Laid)
300
250
200
150
100
50
0
Wild-type
fog-3(RNAi)
C. sp. 9 Male Genotype

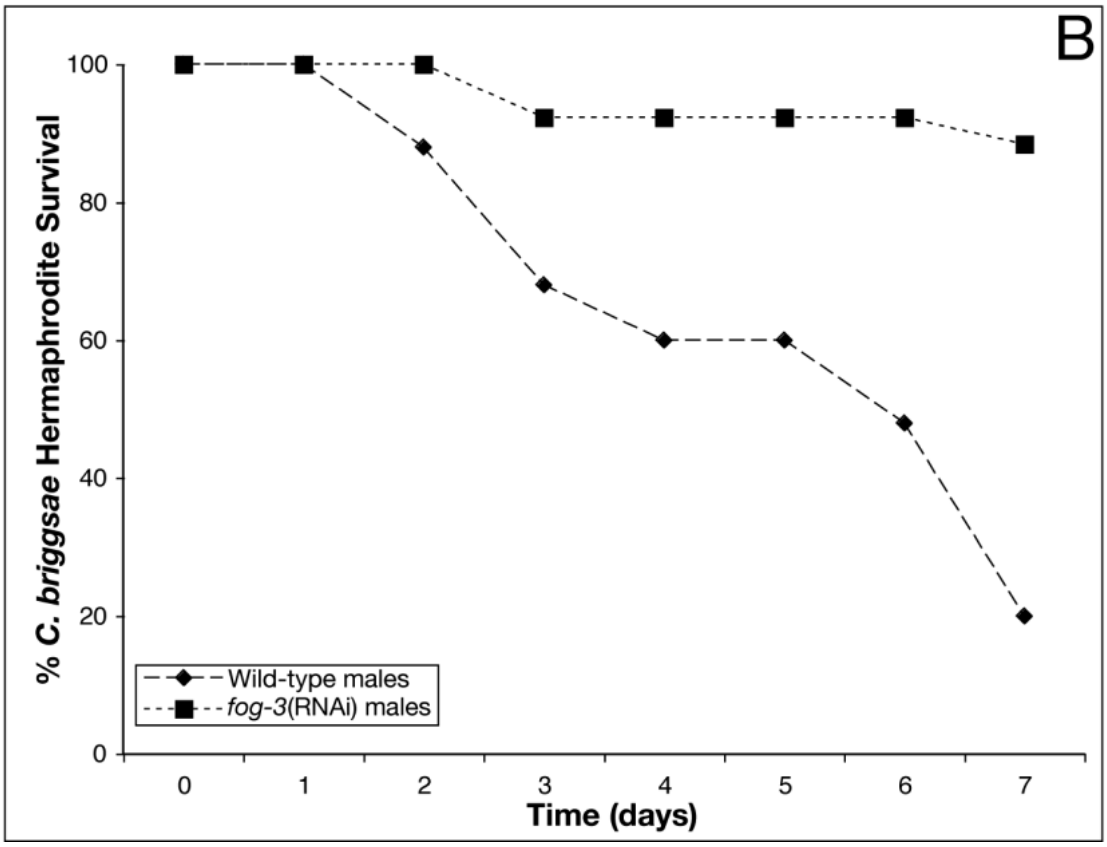

B
% C. briggsae Hermaphrodite Survival
100
80
60
40
20
0
Wild-type males
fog-3(RNAi) males
0
1
2
3
4
5
6
7
Time (days)

Figure 11. *C.* sp. 9 sperm are necessary for the mating-dependent brood size and lifespan reduction in *C. briggsae* hermaphrodites. (A) Mean brood sizes of *C. briggsae* hermaphrodites when mated with wild-type or *fog-3*(RNAi) *C.* sp. 9 males. After one day of mating, the presence or absence of copulatory plugs on hermaphrodite vulvae was noted, and it was used as a marker for successful copulation. Mating by wild-type *C.* sp. 9 males resulted in a decrease in *C. briggsae* hermaphrodite fecundity (Mann-Whitney U p-value=0.02), whereas *fog*-3(RNAi) *C.* sp. 9 males that successfully mated with *C. briggsae* hermaphrodites displayed no such brood size reduction (Mann-Whitney U p-value=0.4). The difference between wild-type and *fog-3*(RNAi) successfully-mated hermaphrodites was highly significant (Mann-Whitney U p-value=0.0006). Error bars represent one standard deviation of the mean. Sample sizes ranged from 8-16. (B) Lifespan curves of *C. briggsae* hermaphrodites when continuously mated with similarly treated wild-type (n=25) and *fog-3*(RNAi) (n=26) *C.* sp. 9 males. *fog-3*(RNAi) males were unable to promote a reduction in *C. briggsae* hermaphrodite lifespan comparable to wild-type males. This lifespan reduction is statistically significant (Mann-Whitney U p-value=0.00002).

Discussion

Sperm competition in Caenorhabditis

Sperm competition occurs when sperm from multiple males vie for the fertilization of oocytes within a single female (PARKER 1970). While initially observed in insects (PARKER 1970), sperm competition has subsequently been observed across multiple plant and animal taxa and is likely a major and widespread phenomenon (BIRKHEAD and MØLLER 1998). Indeed, the asymmetric pattern of sterilization and mortality in heterospecific crosses observed in this study is likely to be in part a consequence of sperm competition.

There is much evidence for the existence of sperm competition in *Caenorhabditis* nematodes. In the androdioecious *C. elegans*, male cross-sperm are able to outcompete the self-sperm of hermaphrodites and consequently fertilize the majority of that animal's oocytes (WARD and CARREL 1979). In *C. elegans*, male sperm are larger than hermaphrodite sperm, and larger sperm crawl faster than smaller sperm (LAMUNYON and WARD 1998). Additionally, there is intraspecific variation in male sperm size in *C. elegans*, and males with larger sperm produce more progeny in multiply mated hermaphrodites than do males with smaller sperm (LAMUNYON and WARD 1998; MURRAY *et al.* 2011). Furthermore, the males of obligately outcrossing gonochoristic species have larger sperm than those of facultatively outcrossing androdioecious species (LAMUNYON and WARD 1999; BALDI *et al.* 2011), suggesting that sexual selection is maintaining large sperm size in gonochoristic lineages. Indeed, experimental evolution studies in *C. elegans* revealed the evolution of larger sperm size in obligately outcrossing lines compared to hermaphroditic lines after 60 generations (LAMUNYON and WARD

2002). And, theory predicts the evolution of male defensive adaptations that inhibit female remating in addition to offensive adaptations that increase the ability to outperform competitor sperm (PARKER 1970). In line with this, copulatory plugs are present in *Caenorhabditis* (BARKER 1994). These plugs are capable of inhibiting the fertilization success of secondarily mating males (BARKER 1994; PALOPOLI *et al.* 2008), and the existence of these plugs are polymorphic in androdioecious species, whereas they are fixed in gonochoristic species (HODGKIN and DONIACH 1997; PALOPOLI *et al.* 2008), suggesting they are maintained by sexual selection. There is then likely to be sperm competition in *Caenorhabditis*, and such competition is expected to be more intense in obligately outcrossing gonochoristic species.

Here, males from a gonochoristic species with intense sperm competition were paired with hermaphrodites from an androdioecious species where sperm competition is likely to be relatively more relaxed. The consequences are dramatic: these heterospecific sperm ectopically invade hermaphrodite tissues, sterilizing and killing them in the process. The directionality of this asymmetry (that is, that the gonochoristic sperm hurt the hermaphrodite and not the androdioecious sperm hurting the gonochoristic female) is likely due to the maintenance of sperm competition in the gonochoristic lineage. *C.* sp. 9 sperm are larger (BALDI *et al.* 2011) and likely faster than *C. briggsae* sperm. *C.* sp. 9 sperm are clearly more aggressive than *C. briggsae* sperm, given that they are able to ectopically invade the tissues of not only hermaphrodites but conspecific females as well, albeit at a lower frequency. It is possible that this aggressiveness confers a competitive advantage to *C.* sp. 9 sperm, which would suggest that sexually antagonistic coevolution might maintain female resistance (ARNQVIST and ROWE 2005) to *C.* sp. 9 sperm

aggressiveness in order to avoid the costs associated with ectopic sperm invasion.

However, there remains an alternative to the interpretation that this trait was driven by sperm competition. As sperm competition refers to post-copulatory male-male competition, cryptic female choice refers to post-copulatory female choice (EBERHARD 1996). The existence of cryptic female choice in the form of ejaculate ejection has been suggested in *C. elegans* (KLEEMANN and BASOLO 2007). And indeed, it is possible that in *C.* sp. 9, females preferentially allow more aggressive sperm to fertilize their oocytes. This could be achieved through differences in the structure or composition of the female reproductive tract, such as a physically stronger oviduct or a spermatheca extracellular matrix that sperm have more difficulty crawling through. Such roles of female gonad structure in cryptic female choice have been demonstrated in spiders (HUBER 1993). However, it is oftentimes difficult to discriminate between sperm competition and cryptic female choice, and the emphasis on sperm competition here reflects that of the *C. elegans* literature. Regardless, it is important to note the possible role of cryptic female choice in the evolution of this phenomenon.

It is unclear whether the aggressive *C.* sp.9 sperm predate the divergence of *C.* sp. 9 and *C. briggsae*. If so, then relaxation of the competition that maintains this sperm aggression may have led *C. briggsae* hermaphrodites to evolve weaker defenses, leading to their sterilization and death when exposed to them. However, as appealing as this interpretation is, it also is possible that aggressive sperm represent a bout of sexually antagonistic coevolution after the two lineages diverged. Indeed, divergent lineages could evolve such differences in sperm aggression and female/hermaphrodite resistance independent of differences in reproductive mode, and there is evidence for such

dynamics, including asymmetries in ectopic sperm localization in heterospecific crosses of species with shared reproductive mode (Table 1; see section "Sexually antagonistic coevolution" below).

Gametic isolation and fertilization in Caenorhabditis

It is clear from the observations above that *C.* sp. 9 sperm are responsible for the sterilization of *C. briggsae* hermaphrodites in heterospecific matings. What is not so clear, however, is the mechanism by which these sperm induce this sterility. The germ line defects seen in heterospecifically mated *C. briggsae* hermaphrodites suggest that multiple factors such as inefficient fertilization resulting from intrinsic gametic isolation, ovulation inhibition, and the out-competition of self-sperm by heterospecific sperm may be involved.

Fertilization in *Caenorhabditis* nematodes requires sperm activation, oocyte maturation, and ovulation in order to proceed successfully. *Caenorhabditis* sperm are notable in that they do not migrate with the aid of a typical eukaryotic flagellum, but rather with a pseudopod (SMITH 2006). Sperm activation is the final stage of spermatozoan development wherein motility is gained, and in *Caenorhabditis*, this is promoted through an extracellular regulatory pathway that induces the release of sperm secretory factors into the extracellular space and the growth of the pseudopod (CHU and SHAKES 2013). Activated sperm crawl to the spermatheca and wait for ovulating oocytes in order to fertilize them, although sperm are oftentimes pushed into the uterus by new zygotes and then crawl back to the spermatheca again (L'HERNAULT 2006).

In *C. elegans*, oocyte maturation and ovulation are induced by the presence of activated sperm in the spermatheca, and these events occur prior to, and independent of,

fertilization (McCarter *et al.* 1999). Oocyte maturation is the set of events that prepare the oocyte for embryogenesis including the completion of meiosis, DNA synthesis, and protein synthesis; ovulation is the contraction of the gonadal sheath cell that pushes the maturing oocyte from proximal gonad into the spermatheca so it can be fertilized (Kim *et al.* 2013). Ovulation and oocyte maturation are both induced by Major Sperm Protein (MSP; Miller *et al.* 2001), a sperm-specific cytoskeletal component that also generates nematode sperm motility (King *et al.* 1994). The MSP signal induces oocyte maturation and ovulation through interacting with the gonadal sheath cell (Miller *et al.* 2003). The gonadal sheath cell forms gap junctions with oocytes (Hall *et al.* 1999), and these are used to transduce the MSP signal from the sheath cell to the oocyte (Govindan *et al.* 2006). Thus, sperm induce oocyte maturation and ovulation before fertilization occurs. Additionally, heterospecific matings between multiple *Caenorhabditis* species has revealed that the mechanisms of the induction of ovulation and oocyte maturation are largely conserved despite the inviability of the embryos these matings produce (Hill and L'Hernault 2001).

With the above in mind, the sterilization and germ line phenotypes observed in heterospecifically mated *C. briggsae* hermaphrodites can be addressed. These animals are observed to be sterilized (Figure 1), have the accumulation of endomitotic oocytes (Figure 5B), and display the presence of ectopic cross-sperm distal to the spermatheca (Figure 6G-H, Figure 7). This is likely due to a combination of sperm competition, intrinsic gametic isolation and the inhibition of proper ovulation, which results in the accumulation of prematurely maturing oocytes.

Since reciprocal heterospecific crosses of *C. briggsae* and *C.* sp. 9 produce much

smaller broods than those of conspecific crosses, it is possible that there is a degree of intrinsic gametic isolation between these two species. Gametic isolation is a post-mating pre-zygotic form of reproductive isolation wherein fertilization is deficient after successful copulation (COYNE and ORR 2004). Intrinsic gametic isolation occurs when fertilization fails upon sperm-oocyte contact, and this has been observed between species of externally fertilizing taxa such as abalones (KRESGE *et al.* 2001) and sea urchins (PALUMBI 1998). This likely occurs due to the incompatibility of sperm-oocyte cell surface recognition proteins between divergent lineages (VACQUIER and SWANSON 2011). Surprisingly, the *C. elegans* sperm-oocyte recognition proteins are not known (although there are candidates; MARCELLO *et al.* 2013), so hypotheses about the molecular evolution of gamete recognition in *C. briggsae* and *C.* sp. 9 cannot be directly addressed. And although gametic isolation is known to exist between certain *Caenorhabditis* species (BAIRD *et al.* 1992), complete intrinsic gametic isolation between *C.* sp. 9 sperm and *C. briggsae* oocytes does not completely explain the sterilization of *C. briggsae* hermaphrodites. *C.* sp. 9 males are capable of producing viable hybrid progeny when crossed with *C. briggsae* hermaphrodites (Chapter 2). Additionally, even if complete intrinsic gametic isolation existed, this alone would not explain the sterilization, because presumably, the *C. briggsae* self-sperm should still be available to fertilize their self-oocytes. *C.* sp. 9 male sperm are somehow interfering with this process.

Even though *C.* sp. 9 sperm are capable of fertilizing *C. briggsae* oocytes, it is possible that only a small fraction is capable of doing so. If this is true, then sterilization might be a consequence. In *C. elegans*, there are certain classes of fertilization-defective mutants that do not affect sperm motility (L'HERNAULT *et al.* 1988). When males bearing

these sort of mutations are mated to conspecific wild-type hermaphrodites, they have lower self-fecundity than unmated hermaphrodites (SINGSON *et al.* 1999). Thus, male sperm continue to outcompete and displace self-sperm even though they are fertilization-defective. In the case of *C. briggsae* hermaphrodites and *C.* sp. 9 males, a similar phenomenon may be going on but to a greater degree because *C.* sp. 9 sperm are larger than *C. briggsae* sperm (BALDI *et al.* 2011). Thus, one factor contributing to sterilization could be the ability of *C.* sp. 9 sperm to exclude *C. briggsae* self-sperm from their own oocytes, while, at the same time, not being at all efficient in fertilizing those oocytes.

Additionally, it is likely that *C.* sp. 9 sperm inhibit proper ovulation in *C. briggsae* hermaphrodites. The vast majority of *C. briggsae* hermaphrodites mated to *C.* sp. 9 males displayed the accumulation of endomitotic oocytes in the gonad two days after mating (Fig. 4B). In *C. elegans*, mutations that cause defective ovulation promote just this phenotype (IWASAKI *et al.* 1996). In fertilization-defective mutants of *C. elegans* that do not affect ovulation, endomitotic oocytes are formed, but they are ovulated and laid as an unfertilized oocyte (WARD and CARREL 1979). Since in these *C. briggsae* animals, these endomitotic oocytes begin to accumulate and degrade in the proximal gonad, they are not being properly ovulated.

The mechanism by which C. sp. 9 sperm inhibit *C. briggsae* ovulation is unclear. Since the regulatory mechanism controlling oocyte maturation and ovulation (MSP) is the same in *C. elegans* (KIM *et al.* 2013), MSP is a highly conserved protein across nematodes (SCOTT *et al.* 1989), and multiple reciprocal heterospecific *Caenorhabditis* crosses can induce ovulation (HILL and L'HERNAULT 2001), it is unlikely that this defect in ovulation is due to any significant divergence between sperm-ovulation signaling

pathways as such. Rather, a somewhat more plausible explanation is the physical blockage of ovulation by the sperm themselves. *C*. sp. 9 sperm are larger, faster, and more aggressive than *C. briggsae* male sperm. It is possible that any of these factors alone or in combination simply impede the proper contraction of the gonadal sheath cell and oocyte expulsion. Indeed, sperm are observed to be crawling through the oviduct into the proximal germ line (Figure 6H). In conspecific crosses, ovulation is able to push the oocyte and surrounding sperm away from the proximal gonad into the uterus. If the sperm are aggressive enough to violate the boundaries that they are pushed away from in the conspecific condition, it is possible that, *en masse*, they are able to prevent the proper contraction of the *C*. *briggsae* gonadal sheath cell and subsequent oocyte expulsion.

However, it is also possible that premature oocyte maturation resulting from ectopic sperm in the proximal gonad is itself the cause of ovulation problems. Since in *C. elegans*, mutations in the oocyte maturation pathway that promote premature maturation do not have ovulation defects (BURROWS *et al.* 2006), this may not be the best explanation alone. Another possibility is that since *Caenorhabditis* sperm release a suite of secretory factors upon activation (CHU and SHAKES 2013), *C*. sp. 9 sperm secrete factors that inhibit *C*. *briggsae* ovulation but do not affect *C* sp. 9 ovulation.

The evidence above then suggests that a combination of sperm competition, inefficient fertilization resulting from intrinsic gametic isolation, and inhibited ovulation result when *C*. *briggsae* hermaphrodites encounter *C*. sp. 9 males. This results in sterilization. Although sterilization upon heterospecific mating appears to be a unique observation, it is not unprecedented. In heterospecific matings of certain *Drosophila* species, an insemination reaction occurs wherein a hard mass forms in the vagina after

copulation, which can result in obstructed fertilization and sterilization (PATTERSON 1946; KNOWLES and MARKOW 2001).

Lifespan costs to mating

Costs to reproduction are expected to be asymmetrical in males and females due to the sex-specific differential investment in gamete size or anisogamy (ANDERSSON 1994). These costs include increased exposure to predation (SIH *et al.* 1990), disease (NORRIS and EVANS 2000), costs to foraging time (ALCOCK *et al.* 1977), physical damage (CRUDGINGTON and SIVA-JOTHY 2000), and the inhibition of proper immune function (ROLFF and SIVA-JOTHY 2002), among others. Specifically, lifespan costs to reproduction have been observed in many taxa (MCKINNEY *et al.* 1983; FOWLER and PARTRIDGE 1989; WESTENDORP and KIRKWOOD 1998). In *Drosophila melanogaster*, it has been shown that seminal fluid proteins are capable of modulating the mating-reduced physiology of females in addition to increasing their mortality (CHAPMAN *et al.* 1995). In *C. elegans*, a hermaphrodite cost to mating was observed which was found to be independent of sperm and fertilization (GEMS and RIDDLE 1996). Additionally, this investigation found there was cost to *Caenorhabditis* males if left together in large numbers in the absence of female or hermaphrodite mates (GEMS and RIDDLE 2000).

It was predicted that the lifespan cost to mating in *C. elegans* hermaphrodites was due to physical damage and bacterial accumulation as a result of the physical act of copulation, and not due to progeny production or the receipt of sperm (GEMS and RIDDLE 1996). Using SEM on conspecifically mated and unmated *C. elegans* hermaphrodites, such cuticle damage and bacterial accumulation is just what was observed in this study (Figure 3). However, among a heterospecific cross in a divergent *Caenorhabditis* lineage,

it was found that sperm were largely responsible for the cost of mating (Figure 11B), in contrast to what was seen in *C. elegans*. Thus, despite their highly conserved behavioral and anatomical reproductive biology, the lifespan costs to mating can evolve rapidly in this genus. Furthermore, this appears to be a seldom recognized mechanism of mating-related mortality in females, as most copulation-related lifespan costs observed in other taxa appear to derive from the seminal fluid or physical damage (ARNQVIST and ROWE 2005).

However, some important caveats must be made when interpreting that *C.* sp. 9 sperm are the cause of lifespan costs (as well as sterilization) in heterospecific matings to *C. briggsae*. The deduction that sperm causes mortality was derived from the use of *fog-3*(RNAi) males (Figures 10-11). These males were observed to lay copulatory plugs and were able to perform the mating behavior with *C. briggsae* hermaphrodites. However, it is possible that their biology differs from wild-type *C.* sp. 9 males aside from their inability to generate sperm and inhibit oocyte production, and there have been no in-depth characterization of *fog-3* mutant phenotypes in *C. elegans* aside from those related to sex determination (ELLIS and KIMBLE 1995). For instance, it is possible that their behavior is also modulated, and perhaps their mating rate or spicule insertion rate is decreased. Indeed, in certain sex-determination mutants of *Drosophila melanogaster*, male-mating behavior is abnormal (MANOLI *et al.* 2005). Also, it is possible that sperm production influences the components of the seminal fluid in wild-type males. In *C. remanei*, it was observed that a connection between the germ line and seminal vesicle is necessary for certain mating-related, male-induced modulations of female behavior (GARCIA *et al.* 2007). However, these *fog-3*(RNAi) males must be able to transfer some

of the seminal fluid because they are able to deposit copulatory plugs, the components of which are produced in the vas deferens (PALOPOLI *et al.* 2008). If *fog-3* is indeed pleiotropic in these ways, then the interpretation of sperm being the cause of mortality (and sterilization) would be unsound. However, given that *C*. sp. 9 sperm are also capable of ectopic tissue invasion (Fig. 5-6), it is the most likely explanation for this mortality.

Additionally, this is a lifespan cost to mating that is exaggerated in a heterospecific cross. Since *Caenorhabditis* hermaphrodites are capable of laying cross-progeny beyond ten days of adulthood (WU *et al.* 2012), it is possible that this asymmetrical lifespan cost to mating in *C. briggsae* hermaphrodites could be a form of reproductive isolation. That is, the death a heterospecific mate before it has finished siring progeny would reduce hybrid fitness. However, the maternal death occurs after most of its embryos have otherwise been laid (WU *et al.* 2012), and the sterilization of *C. briggsae* hermaphrodites is independent of maternal death (J. Ting and A. Cutter, pers. comm.). It is then possible that the premature death is not a strong isolating factor in this case. Indeed, although rarely described, reproductive isolation by maternal death is not unprecedented. In a horrific form of mechanical isolation, heterospecific crosses of certain Japanese carabid beetles can result in the male copulatory piece breaking off and tearing the female's reproductive organs, sometimes resulting in her death (SOTA and KUBOTA 1998).

With respect to the genetics of aging in C. elegans

In *Caenorhabditis* studies where mortality is measured, it is appropriate to address the expansive literature on the genetics of aging in *C. elegans* (KENYON 2010). A multitude of long-lived mutants of *C. elegans* have been discovered, and many molecular

pathways have been implicated in the modulation of lifespan, including insulin signaling (KIMURA *et al.* 1997), TOR signaling (JIA *et al.* 2004), and AMP kinase signaling (APFELD *et al.* 2004). Many of these aging genes also regulate metabolism, and the modulation of metabolism has been linked to prolonged lifespan in multiple taxa including fruit flies and mice (KENYON 2010). Additionally, many of the genes that prolong lifespan in *C. elegans* are also implicated in the regulation of the dauer larva, a polyphenic developmental trajectory that is induced in stress conditions (HU 2007). Germ cells have also been shown to be important in modulating lifespan in *C. elegans*. Lifespan is increased in animals where the germ line has been removed (HSIN and KENYON 1999). Additionally, in some long-lived mutants of *C. elegans*, important germ line determinants are misexpressed in somatic tissues (CURRAN *et al.* 2009). Considering there are multiple molecular pathways known to affect lifespan, as well as the importance of the germ line in the modulation of lifespan, there is the possibility that the causes of mortality seen in this study may intersect with those discovered by the investigators of aging in *C. elegans*. However, although possible, it seems likely that the phenomenon observed here is independent of the metabolic and dauer regulatory pathways responsible for modulating lifespan. Aging studies rarely measure lifespan in the presence of mating, so these pathways are not necessarily implicated in mating-related lifespan reduction. And more importantly, these studies do not address heterospecific sperm that can invade ectopic tissues. A more parsimonious explanation for increased mortality is simply the violation of somatic tissues by these migrating cells, as opposed to their potential ability to regulate certain pathways.

The invasion of sperm into ectopic tissues

In this study, the migration of *C*. sp. 9 sperm into ectopic tissues of *C*. *briggsae* hermaphrodites was observed. Although many *C. elegans* mutants that disrupt germ cell fate specification and function have been characterized (GREENSTEIN 2005; L'HERNAULT 2006; KIMBLE and CRITTENDEN 2007), none have been observed that promote such behavior in mature spermatozoa (to the best of my knowledge). This then appears to be a novel *Caenorhabditis* phenomenon. However, mislocalized sperm were observed in backcross *C*. sp. 9/*C*. *briggsae* hybrid males (Chapter 2, Fig. 3E). At the time, this was presumed to be due to the hybrid breakdown of proper anterior-posterior specification in the adult gonad, and indeed, there are reasons to suppose that this may be a different sort of phenomenon. Mislocalized sperm in hybrid males are derived from themselves, and not from outcrossing. Additionally, ectopic sperm in hybrid males appeared to be not activated and not migratory. But, alternatively, it is possible that the hybrid genome of the somatic cells expresses a factor that attracts sperm, causing them to be ectopically localized. This is an intriguing possibility given that it addresses the important question of why *C*. sp. 9 sperm are capable of escaping the gonadal tissues in *C*. *briggsae* hermaphrodites. That is, one possible reason the sperm do this is that they are attracted to some somatically-expressed *C*. *briggsae* factor. Another explanation is that since *Caenorhabditis* sperm release proteases upon activation (Shakes 2011), perhaps there is a cross-species incompatibility when *C*. sp. 9 proteases encounter the reproductive system of the *C*. *briggsae* hermaphrodite. That is, *C*. sp. 9 male proteases may breakdown the tissues of *C*. *briggsae* hermaphrodites, causing sperm to become mislocalized. Using electron microscopy to examine the structural integrity of *C*. *briggsae* tissues after heterospecific mating could provide some insight into this possibility.

The novel phenomenon of ectopic sperm bears a striking resemblance to cell migration and metastasis in cancer (HANAHAN and WEINBERG 2011). *C. elegans* has long been used as a model for understanding the biology of cancer (KIRIENKO *et al.* 2010). The development of the uterine-vulval connection (SHERWOOD *et al.* 2005) as well as the migration of the distal tip cell (BLELLOCH and KIMBLE 1999) have both been proposed as models for cell invasion and metastasis. Although these comparisons to metastasis and invasion are sound, they are regardless utilizing the development of wild-type, endogenous tissues as a proxy for the abnormal violation of adult tissues. Here, that is precisely what is seen. And given that *C.* sp. 9 sperm are able to invade the proximal gonad of *C. elegans* hermaphrodites (Table 1), the sophisticated genetic tools of that system can be used to address the molecular basis of tissue integrity and resistance to metastasis.

The degradation of mating-related traits in androdioecious lineages

In androdioecious lineages, males are dispensable for propagation. In laboratory *C. elegans* populations, the frequency of males is quite low (~0.2%) and approaches the frequency of non-disjunction of the X chromosome in hermaphroditic germ cells (HODGKIN 1983). Estimated male frequencies of natural *C. elegans* populations are also low (~1%), although notably higher than laboratory populations (BARRIÈRE and FÉLIX 2005). Because mating is dispensable and so infrequent in androdioecious lineages, mating-related traits should be under weaker selection in these lineages. Indeed, a large number of mating-related traits are seen to degrade in these species (THOMAS *et al.* 2012).

As mentioned above, sperm are smaller and less competitive in males of

androdioecious species (LAMUNYON and WARD 1999). Additionally, males of androdioecious species are less efficient at mating successfully than gonochoristic males (CHASNOV and CHOW 2002). That is, whereas obligately outcrossing strains are able to maintain high male frequencies indefinitely, in many androdioecious strains, artificially enhanced male frequencies drop precipitously after only a few generations (WEGEWITZ *et al.* 2008). Also, androdioecious males appear to be impaired in species-specific mate recognition; androdioecious males mate more with gonochoristic females than conspecific hermaphrodites (CHASNOV *et al.* 2007; GARCIA *et al.* 2007). Androdioecious males are also apparently unable to promote mating-induced behavioral changes in females that gonochoristic males are capable of (GARCIA *et al.* 2007). Gonochoristic males also spend more time mating than androdioecious males (CHASNOV and CHOW 2002). And as mentioned above, the presence of copulatory plugs is polymorphic in androdioecious lineages, whereas it is fixed in gonochoristic species (PALOPOLI *et al.* 2008). Mutations that severely impair male development have also been seen to segregate in natural populations of androdioecious species (HODGKIN and DONIACH 1997). Clearly, multiple mating-related traits are degrading in hermaphroditic lineages.

Here, the case could be made that a novel *Caenorhabditis* mating-related trait has been observed and that it also has been weakened in an androdioecious species. Namely, this would be the ability of somatically female animals to resist hyperaggressive cross-sperm. *C. briggsae* and *C. elegans* hermaphrodites are clearly less able than *C.* sp. 9 females to resist ectopic migration of *C.* sp. 9 sperm, and it is possible that this is a consequence of the evolution of hermaphroditism and the relaxed maintenance of mating-related traits. Indeed, a meta-analysis of interspecies crosses in flowering plants with

divergent reproductive modes has suggested that selfing species are particularly susceptible to hybridization dysfunction resulting from sexual conflicts in largely outcrossing species (BRANDVAIN and HAIG 2005).

Also, many studies on the consequences of the evolution of hermaphroditism have focused on male traits, and here is an example of a more rarely studied female-related mating trait (KLEEMANN and BASOLO 2007). Additionally, this would be a most dramatic potential consequence of the evolution of hermaphroditism: sterilization and death by sperm when encountering a heterospecific male. However, although the connection between decreased resistance and the evolution of hermaphroditism is alluring, there remains the possibility that the evolution of the resistance to hyperaggressive sperm has little to do with reproductive mode as such, but rather with alternative patterns of sexually antagonistic coevolution in divergent lineages.

Sexually antagonistic coevolution

Sexual conflicts arise when certain traits beneficial to one sex are detrimental to the other (ARNQVIST and ROWE 2005). Here, a cryptic sexual conflict defined by sperm aggressiveness and female resistance has been revealed in a heterospecific cross. Although it is possible that this asymmetry resulted from the evolution of hermaphroditism and the concurrent degradation of hermaphrodite resistance to aggressive sperm, another possibility is that sexually antagonistic coevolution can account for it alone. Indeed, the ability of *C.* sp. 9 sperm to invade the tissues of a substantial fraction of gonochoristic *C. remanei* females (Table 1) is consistent with this.

Sexual conflict has been proposed to be a potential engine of reproductive isolation (RICE 1998). In *Drosophila*, the accessory seminal gland proteins (Acps)

implicated in a sexual conflict have been shown to evolve rapidly (BEGUN *et al.* 2000), as do reproductive proteins in general (SWANSON and VACQUIER 2002). Experimental evolution studies have shown that both male and female traits associated with this Acps-related sexual conflict in *Drosophila* can evolve substantially within a number of generations (RICE 1996; HOLLAND and RICE 1999). Additionally, an experimental evolution study in dung flies suggested that higher degrees of sexual conflict can promote greater divergence (MARTIN and HOSKEN 2003). However, work in other systems provides no evidence for a link between sexual conflict and reproductive isolation (WIGBY and CHAPMAN 2006; BACIGALUPE *et al.* 2007) So although some theoretical work suggests sexual conflict can be a potent driver of reproductive isolation (GAVRILETS 2000), the idea remains controversial.

If divergent trajectories of sexually antagonistic, coevolutionary arms races have resulted in this asymmetric difference in hermaphrodite resistance and male sperm aggressiveness, then the question of what promoted such aggressiveness in the *C.* sp. 9 lineage (or a lack thereof in other lineages) remains. Very little is known about the species-specific ecology of *Caenorhabditis* animals (FÉLIX and BRAENDLE 2010). *C.* sp. 9 is known to be a high-temperature specialist, whereas *C. briggsae* is a cosmopolitan generalist (Chapter 2). Furthermore, temporal stratification of *Caenorhabditis* species populating the same geographic area has also been described (FÉLIX and DUVEAU 2012). *C. remanei* has also been observed to be restricted to temperate regions (SUDHAUS and KIONTKE 2007), so it is possible that temperature-driven or other habitat-driven evolutionary trajectories have somehow led to differences in the evolution of this sexual conflict. Additionally, it is known that *Caenorhabditis* worms form phoretic relationships

with insects and snails in order to find new nutrient sources (KIONTKE and SUDHAUS 2006; FÉLIX and BRAENDLE 2010). Furthermore, in some cases, these associations are likely to be critical for the maintenance of proper life history (TANAKA *et al.* 2012). It is then possible that differences in phoretic associations may be responsible for differences in the evolution of a sexual conflict. Additionally, *Caenorhabditis* worms are now known to proliferate on rotting fruit (KIONTKE *et al.* 2011), but it is unknown to what degree of fruit preference there exists between species. Any of these ecological differences may be accountable, and particularly, if any of these differences are capable of increasing the population size of *C.* sp. 9 relative to other lineages, then one would potentially expect a more rapid evolution of a sexual conflict and subsequent reproductive barriers in this lineage (GAVRILETS 2000).

Conclusion

Here, the mating-induced death and sterilization of *C. briggsae* hermaphrodites by *C.* sp. 9 males was investigated. It was found that heterospecific matings induce the inhibition of ovulation and the promotion of germ line defects in hermaphrodites. Furthermore, it was observed that *C.* sp. 9 cross-sperm are able to migrate into ectopic tissues and that these sperm are likely to be the cause of hermaphrodite sterilization and death. These phenomena are likely due to an incompatibility between heterospecific reproductive systems, which could result from differing trajectories of sexually antagonistic coevolution. Such differences could also be influenced by the evolution of self-fertile hermaphroditism, wherein sexual selection is relaxed and mating-related traits degrade.

Since *C.* sp. 9 and *C. briggsae* are able to make fertile hybrid progeny, there

exists the opportunity of using this system to understand the genetic basis of this sexual conflict. However, although mapping the aggressive sperm trait will be difficult due to the high frequency of sterile males in hybrid generations (Chapter 2), this does not preclude the possibility of examining the genetic basis of female resistance to such sperm. Furthermore, these phenomena are potentially relevant to a wide range of fields of biological interest including sperm competition, reproductive costs, cell-cell signaling, the germ line, sexual conflict, reproductive isolation, and cell invasion and metastasis. Additionally, since *C.* sp. 9 sperm can ectopically migrate in *C. elegans* tissues, the sophisticated tools of that system can potentially also be used to address such problems.

Chapter 4: Future directions and conclusion

This work is among the first to study the biology of *C. briggsae* and *C.* sp. 9 hybridization. This system can provide insights into the evolution of phenotypic diversity, the genetic basis of reproductive isolation, and, because these species differ in reproductive mode, the consequences of the evolution of selfing.

Chapter 2 described the general hybrid genetics of *C. briggsae* and *C.* sp. 9 within the context of studying the genetic basis of hermaphroditism. Various measures of fitness and the selfing trait were taken in multiple different hybrid generations. Attempts to increase the hybrid selfing rate were made, and hybrids were genotyped to reveal segregation distortion in such generations. Inferences on the genetic basis of postzygotic isolation of these lineages were made from the patterns of inviability and sterility observed in hybrid generations. Additionally, the recessivity of the selfing trait suggested that perhaps inbreeding and subsequent isolation from gonochoristic ancestors may be necessary for hermaphroditism to evolve.

Chapter 3 described an asymmetric pattern of sterilization and mortality resulting from interspecific mating. *C.* sp. 9 sperm were found to sterilize and prematurely kill *C. briggsae* hermaphrodites. Additionally, these sperm were found to promote germ line abnormalities in *C. briggsae* hermaphrodites, and they were seen to migrate in ectopic tissues of these hermaphrodites. This phenomenon was interpreted as a result of a sexual conflict derived from male-male sperm competition in gonochorists that has been relaxed in a hermaphroditic lineage where mating is rare.

One of the major aims of this work was to utilize two lines divergent in reproductive mode in order to understand the genetic basis of that difference (and

potentially phenotypic diversity in general) using a mapping approach. In as much as this work fell short of linking specific molecular genetic variants to XX spermatogenesis, it could be argued that this system is inappropriate for understanding the genetic basis of phenotypic diversity. Indeed, the high degree of reproductive isolation and lack of hybrid selfers precludes traditional mapping studies for understanding the genetic basis of hermaphroditism. However, this does not mean this system is altogether unfit for such investigations. For instance, it is still possible that additional manipulations may yield hybrid selfers. Recent work suggests that certain wild isolates can produce viable $B2_{b,b}$ progeny (KOZLOWSKA *et al.* 2012). If this is true, then mapping selfing could still be possible. Additionally, further mutagenesis screens or RNAi feeding screens could be utilized to reveal hybrid selfers.

Even if mapping approaches do turn out to be altogether unfeasible, this system can still be used to address questions regarding the genetic basis of selfing. For instance, hybrid F_1 females were utilized as a sensitized female background in order to examine the consequences of *gld-1* knockdown (BEADELL *et al.* 2011). This system could also potentially be used to address the genetic basis of diversity in phenotypes aside from reproductive mode. For example, *C.* sp. 9 and *C. briggsae* differ in temperature-dependent viability (Chapter 2, Figure 5), and there are multiple conceivable approaches for addressing the genetics of this trait. Considering their presumed differing geographic distributions, there may be many other ecologically relevant traits in which they diverge that could be interrogated genetically. This suggests that there remains much potential for this system to be utilized for understanding the genetic basis of phenotypic diversity.

The existence of *C*. sp. 9 also opens up the *Caenorhabditis* model genus to the study of the genetics of interspecies postzygotic isolation for the first time. This was long impossible due to the lethal and sterile phenotypes of other interspecies crosses (BAIRD and SEIBERT 2013). Although identifying genes involved in hybrid dysfunction is a formidable task, it is most certainly possible. Mutagenesis in parental lines combined with scoring F_1 for the restoration of viability or fertility could reveal genes responsible for such phenotypes. Additionally, sampling multiple polymorphic parental lines could also potentially reveal the restoration of hybrid dysfunction. This was shown to be the case in crosses *Drosophila melanogaster* and *D. simulans*, where a wild isolate of *D. melanogaster* was found to be unique in restoring hybrid F_1 male fertility (HUTTER and ASHBURNER 1987). This line of work led to the cloning of the incompatibility gene *Hybrid male rescue* (BARBASH *et al.* 2003), and a similar natural polymorphism revealed the molecular identity of the *D. melanogaster*/*D. simulans* incompatibility gene *Lethal hybrid rescue* (WATANABE 1979; BRIDEAU *et al.* 2006). Indeed, it has been found that polymorphisms in *C*. sp. 9 and *C. briggsae* can affect hybrid fitness (KOZLOWSKA *et al.* 2012), so this proposed line of investigation may be sound. Additionally, since hybrid male fertility segregates in a *C*. sp 9/*C. briggsae* backcross population (Chapter 2, Table 2), it is possible that this trait could be mapped in order to find *C. briggsae* alleles responsible for hybrid male sterility. Additionally, introgression lines could potentially be used to map *C*. sp. 9/*C. briggsae* incompatibility factors, and many have already been constructed (YAN *et al.* 2012). Hopefully, much of the initial descriptions of *C*. sp.9/*C. briggsae* hybrid fitness discussed in this work will be utilized in understanding the genetic basis of reproductive isolation in the future.

Overall, this work provides a foundation for future studies in the genetics of postzygotic isolation. Additionally, it has described a novel phenotype and consequence of the evolution of selfing in the increased susceptibility to sperm metastasis. Although negative consequences of selfing such as the accumulation of deleterious alleles has been largely discussed (LOEWE and CUTTER 2008), this work describes (to the best of my knowledge) the first case of a potential consequence of selfing that involves sterilization and premature death upon encountering the wrong mate.

References

ABRAMOFF, M. D., P. J. MAGELHAES and S. J. RAM, 2004 Image Processing with ImageJ. *Biophotonics International* **11:** 36-42.

ABZHANOV, A., M. PROTAS, B. R. GRANT, P. R. GRANT and C. J. TABIN, 2004 Bmp4 and morphological variation of beaks in Darwin's finches. Science **305:** 1462-1465.

ALCOCK, J., C. E. JONES and S. L. BUCHMANN, 1977 Male mating strategies in the bee *Centris pallida* Fox (Anthophoridae: Hymenoptera). American Naturalist**:** 145-155.

ANDERSON, P., 1995 Mutagenesis, pp. 31-58 in *Caenorhabditis elegans: Modern Biological Analysis of an Organism*, edited by H. F. A. S. EPSTEIN, DIANE C. Academic Press, San Diego, CA.

ANDERSSON, M. B., 1994 *Sexual selection*. Princeton University Press.

APFELD, J., G. O'CONNOR, T. MCDONAGH, P. S. DISTEFANO and R. CURTIS, 2004 The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in *C. elegans*. Genes & development **18:** 3004-3009.

ARNQVIST, G., and L. ROWE, 2005 *Sexual Conflict*. Princeton University Press.

AYDIN, A., M. R. TOLLAT, S. BAHRING, C. BECKER and P. NURNBERG, 2005 New universal primers facilitate Pyrosequencing. *Electrophoresis* **27:** 394-397.

BACHORIK, J. L., and J. KIMBLE, 2005 Redundant control of the Caenorhabditis elegans sperm/oocyte switch by PUF-8 and FBF-1, two distinct PUF RNA-binding proteins. Proceedings of the National Academy of Sciences of the United States of America **102:** 10893-10897.

BACIGALUPE, L., H. CRUDGINGTON, F. HUNTER, A. MOORE and R. SNOOK, 2007 Sexual conflict does not drive reproductive isolation in experimental populations of *Drosophila pseudoobscura*. Journal of evolutionary biology **20:** 1763-1771.

BAIRD, S., C. DAVIDSON and J. BOHRER, 2005 The genetics of ray pattern variation in Caenorhabditis briggsae. BMC evolutionary biology **5:** 3.

BAIRD, S. E., 2002 Haldane's rule by sexual transformation in *Caenorhabditis*. *Genetics* **161:** 1349-1353.

BAIRD, S. E., and S. R. SEIBERT, 2013 Reproductive isolation in the Elegans-Group of Caenorhabditis. Natural Science **5:** 18-25.

BAIRD, S. E., M. E. SUTHERLIN and S. W. EMMONS, 1992 Reproductive Isolation in

Rhabditidae (Nematoda: Secernentea); Mechanisms That Isolate Six Species of Three Genera. *Evolution* **46:** 585-594.

BAIRD, S. E., and W.-C. YEN, 2001 Reproductive isolation in *Caenorhabditis*: terminal phenotypes of hybrid embryos. *Evolution & Development* **2:** 9-15.

BALDI, C., S. CHO and R. E. ELLIS, 2009 Mutations in Two Independent Pathways Are Sufficient to Create Hermaphroditic Nematodes. Science **326:** 1002-1005.

BALDI, C., J. VIVIANO and R. E. ELLIS, 2011 A bias caused by ectopic development produces sexually dimorphic sperm in nematodes. Current Biology **21:** 1416-1420.

BARBASH, D. A., D. F. SIINO, A. M. TARONE and J. ROOTE, 2003 A rapidly evolving MYB-related protein causes species isolation in Drosophila. Proceedings of the National Academy of Sciences **100:** 5302-5307.

BARKER, D. M., 1994 Copulatory plugs and paternity assurance in the nematode *Caenorhabditis elegans*. Animal Behaviour **48:** 147-156.

BARRIÈRE, A., and M.-A. FÉLIX, 2005 High Local Genetic Diversity and Low Outcrossing Rate in *Caenorhabditis elegans* Natural Populations. Current Biology **15:** 1176-1184.

BAYES, J. J., and H. S. MALIK, 2009 Altered heterochromatin binding by a hybrid sterility protein in Drosophila sibling species. Science **326:** 1538-1541.

BEADELL, A. V., Q. LIU, D. M. JOHNSON and E. S. HAAG, 2011 Independent recruitments of a translational regulator in the evolution of self-fertile nematodes. Proceedings of the National Academy of Sciences **108:** 19672-19677.

BEGUN, D. J., P. WHITLEY, B. L. TODD, H. M. WALDRIP-DAIL and A. G. CLARK, 2000 Molecular population genetics of male accessory gland proteins in Drosophila. Genetics **156:** 1879-1888.

BIKARD, D., D. PATEL, C. LE METTE, V. GIORGI, C. CAMILLERI *et al.*, 2009 Divergent Evolution of Duplicate Genes Leads to Genetic Incompatibilities Within *A. thaliana*. Science **323:** 623-626.

BIRKHEAD, T. R., and A. P. MØLLER, 1998 *Sperm competition and sexual selection*. Academic Press.

BLELLOCH, R., and J. KIMBLE, 1999 Control of organ shape by a secreted metalloprotease in the nematode *Caenorhabditis elegans*. Nature **399:** 586-590.

BOLNICK, D. I., M. TURELLI, H. LOPEZ-FERNANDEZ, P. C. WAINWRIGHT and T. J. NEAR,

2008 Accelerated Mitochondrial Evolution and "Darwin's Corollary": Asymmetric Viability of Reciprocal F1 Hybrids in Centrarchid Fishes. Genetics **178:** 1037-1048.

BRANDVAIN, Y., and D. HAIG, 2005 Divergent mating systems and parental conflict as a barrier to hybridization in flowering plants. The American Naturalist **166:** 330-338.

BRENNER, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics **77:** 71-94.

BRIDEAU, N. J., H. A. FLORES, J. WANG, S. MAHESHWARI, X. WANG *et al.*, 2006 Two Dobzhansky-Muller Genes Interact to Cause Hybrid Lethality in *Drosophila*. Science **314:** 1292-1295.

BURKE, J. M., and M. L. ARNOLD, 2001 Genetics and the fitness of hybrids. Annual Review of Genetics **35:** 31-52.

BURROWS, A. E., B. K. SCEURMAN, M. E. KOSINSKI, C. T. RICHIE, P. L. SADLER *et al.*, 2006 The C. elegans Myt1 ortholog is required for the proper timing of oocyte maturation. Development **133:** 697-709.

CARROLL, S. B., 2008 Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. Cell **134:** 25-36.

CHAPMAN, T., L. F. LIDDLE, J. M. KALB, M. F. WOLFNER and L. PARTRIDGE, 1995 Cost of mating in Drosophila melanogaster females is mediated by male accessory gland products. Nature **373:** 241-244.

CHASNOV, J., and K. L. CHOW, 2002 Why are there males in the hermaphroditic species *Caenorhabditis elegans*? Genetics **160:** 983-994.

CHASNOV, J., W. SO, C. CHAN and K. CHOW, 2007 The species, sex, and stage specificity of a *Caenorhabditis* sex pheromone. Proceedings of the National Academy of Sciences **104:** 6730-6735.

CHEN, P.-J., S. CHO, S.-W. JIN and R. E. ELLIS, 2001 Specification of Germ Cell Fates by FOG-3 Has Been Conserved During Nematode Evolution Genetics **158:** 1513-1525.

CHEN, P.-J., A. SINGAL, J. KIMBLE and R. E. ELLIS, 2000 A Novel Member of the Tob Family of Proteins Controls Sexual Fate in *Caenorhabditis elegans* Germ Cells. Developmental biology **217:** 77-90.

CHO, S., S.-W. JIN, A. COHEN and R. E. ELLIS, 2004 A phylogeny of *Caenorhabditis* reveals frequent loss of introns during nematode evolution. Genome Research **14:** 1207-1220.

CHU, D. S., and D. C. SHAKES, 2013 Spermatogenesis, pp. 171-203 in *Germ Cell Development in C. elegans*. Springer.

CLIFFORD, R., M.-H. LEE, S. NAYAK, M. OHMACHI, F. GIORGINI *et al.*, 2000 FOG-2, a novel F-box containing protein, associates with the GLD-1 RNA binding protein and directs male sex determination in the C. elegans hermaphrodite germline. Development **127:** 5265-5276.

COYNE, J. A., and H. A. ORR, 2004 *Speciation*. Sinauer, Sunderland, MA.

CRUDGINGTON, H. S., and M. T. SIVA-JOTHY, 2000 Genital damage, kicking and early death. Nature **407:** 855-856.

CURRAN, S. P., X. WU, C. G. RIEDEL and G. RUVKUN, 2009 A soma-to-germline transformation in long-lived *Caenorhabditis elegans* mutants. Nature **459:** 1079-1084.

CUTTER, A. D., 2008a Divergence times in Caenorhabditis and Drosophila inferred from direct estimates of the neutral mutation rate. Mol Biol Evol **25:** 778-786.

CUTTER, A. D., 2008b Reproductive evolution: symptom of a selfing syndrome. Current Biology **18:** R1056-R1058.

CUTTER, A. D., 2012 The polymorphic prelude to Bateson–Dobzhansky–Muller incompatibilities. Trends in ecology & evolution **27:** 209-218.

CUTTER, A. D., W. YAN, N. TSVETKOV, S. SUNIL and M.-A. FELIX, 2010 Molecular population genetics and phenotypic sensitivity to ethanol for a globally diverse sample of the nematode *Caenorhabditis briggsae*. Molecular Ecology **19:** 798-809.

DE BONO, M., and C. I. BARGMANN, 1998 Natural Variation in a Neuropeptide Y Receptor Homolog Modifies Social Behavior and Food Response in *C. elegans*. Cell **94:** 679-689.

DIAZ, S. A., D. T. HAYDON and J. LINDSTROM, 2010 Sperm-limited fecundity and polyandry-induced mortality in female nematodes *Caenorhabditis remanei*. Biol J Linn Soc **99:** 362-369.

DOBZHANSKY, T., 1937 *Genetics and the Origin of Species*. Columbia University Press, New York.

DOEBLEY, J., 2004 The genetics of maize evolution. Annu Rev Genet **38:** 37-59.

DOLGIN, E. S., B. CHARLESWORTH, S. E. BAIRD and A. D. CUTTER, 2007 Inbreeding and outbreeding depression in Caenorhabditis nematodes. *Evolution* **61:** 1339-1352.

DOLGIN, E. S., M. A. FELIX and A. D. CUTTER, 2008 Hakuna Nematoda: genetic and phenotypic diversity in African isolates of *Caenorhabditis elegans* and *C. briggsae*. Heredity **100:** 304-315.

DONIACH, T., 1986 Activity of the sex-determining gene tra-2 is modulated to allow spermatogenesis in the C. elegans hermaphrodite. Genetics **114:** 53-76.

DOROSZUK, A., L. B. SNOEK, E. FRADIN, J. RIKSEN and J. KAMMENGA, 2009 A genome-wide library of CB4856/N2 introgression lines of *Caenorhabditis elegans*. Nucleic Acids Res **37:** e110.

EBERHARD, W., 1996 *Female control: sexual selection by cryptic female choice*. Princeton University Press.

ELLIS, R., and T. SCHEDL, 2006 Sex determination in the germ line, pp. in *WormBook*, edited by T. *C. E.* R. COMMUNITY. WormBook.

ELLIS, R. E., and J. KIMBLE, 1995 The fog-3 gene and regulation of cell fate in the germ line of Caenorhabditis elegans. Genetics **139:** 561-577.

FANG-YEN, C., S. WASSERMAN, P. SENGUPTA and A. D. SAMUEL, 2009 Agarose immobilization of *C. elegans*. Worm Breeder's Gazette **18:** 32.

FÉLIX, M.-A., and C. BRAENDLE, 2010 The natural history of *Caenorhabditis elegans*. Current Biology **20:** R965-R969.

FÉLIX, M.-A., and F. DUVEAU, 2012 Population dynamics and habitat sharing of natural populations of Caenorhabditis elegans and C. briggsae. BMC biology **10:** 59.

FÉLIX, M. A., 2007 Cryptic quantitative evolution of the vulva intercellular signaling network in Caenorhabditis. Curr Biol **17:** 103-114.

FERREE, P. M., and D. A. BARBASH, 2009 Species-specific heterochromatin prevents mitotic chromosome segregation to cause hybrid lethality in Drosophila. PLoS Biol **7:** e1000234.

FOWLER, K., and L. PARTRIDGE, 1989 A cost of mating in female fruitflies. Nature **338:** 760-761.

FOXE, J. P., and S. I. WRIGHT, 2009 Signature of diversifying selection on members of the pentatricopeptide repeat protein family in *Arabidopsis lyrata*. Genetics **183:** 663-672.

FRANCIS, R., M. K. BARTON, J. KIMBLE and T. SCHEDL, 1995a gld-1, a tumor suppressor gene required for oocyte development in Caenorhabditis elegans. Genetics **139:** 579.

FRANCIS, R., E. MAINE and T. SCHEDL, 1995b Analysis of the multiple roles of gld-1 in germline development: interactions with the sex determination cascade and the glp-1 signaling pathway. Genetics **139:** 607.

GARCIA, L. R., B. LEBOEUF and P. KOO, 2007 Diversity in mating behavior of hermaphroditic and male–female Caenorhabditis nematodes. Genetics **175:** 1761-1771.

GARCIA, L. R., P. MEHTA and P. W. STERNBERG, 2001 Regulation of Distinct Muscle Behaviors Controls the *C. elegans* Male's Copulatory Spicules during Mating. Cell **107:** 777-788.

GAVRILETS, S., 2000 Rapid evolution of reproductive barriers driven by sexual conflict. Nature **403:** 886-889.

GEMS, D., and D. L. RIDDLE, 1996 Longevity in *Caenorhabditis elegans* reduced by mating but not gamete production. Nature **379:** 723-725.

GEMS, D., and D. L. RIDDLE, 2000 Genetic, behavioral and environmental determinants of male longevity in *Caenorhabditis elegans*. Genetics **154:** 1597-1610.

GLÉMIN, S., and N. GALTIER, 2012 Genome evolution in outcrossing versus selfing versus asexual species, pp. 311-335 in *Evolutionary Genomics*. Springer.

GOOD, J. M., M. D. DEAN and M. W. NACHMAN, 2008 A complex genetic basis to X-linked hybrid male sterility between two species of house mice. *Genetics* **179:** 2213-2228.

GOODWIN, E., P. OKKEMA, T. EVANS and J. KIMBLE, 1993 Translational regulation of< i> tra</i>-2 by its 3′ untranslated region controls sexual identity in C. elegans. Cell **75:** 329-339.

GOVINDAN, J. A., H. CHENG, J. E. HARRIS and D. GREENSTEIN, 2006 $G\alpha_{o/i}$ and $G\alpha_s$ Signaling Function in Parallel with the MSP/Eph Receptor to Control Meiotic Diapause in *C. elegans*. Current biology **16:** 1257-1268.

GREENSTEIN, D., 2005 Control of oocyte meiotic maturation and fertilization, pp. in *WormBook*, edited by T. *C. E.* R. COMMUNITY. WormBook.

GRIFFITTS, J. S., J. L. WHITACRE, D. E. STEVENS and R. V. AROIAN, 2001 Bt toxin resistance from loss of a putative carbohydrate-modifying enzyme. Science **293:** 860-864.

GUO, Y., S. LANG and R. E. ELLIS, 2009 Independent recruitment of F box genes to regulate hermaphrodite development during nematode evolution. Curr Biol **19:** 1853-1860.

HAAG, E., and D. PILGRIM, 2005 Harnessing Caenorhabditis genomics for evolutionary developmental biology. Current Genomics **6:** 579-588.

HAAG, E. S., 2005 The evolution of nematode sex determination: *C. elegans* as a reference point for comparative biology, pp. in *WormBook*, edited by T. *C. E.* R. COMMUNITY. WormBook.

HAAG, E. S., 2009 Convergent evolution: regulatory lightning strikes twice. Curr Biol **19:** R977-979.

HAAG, E. S., and J. KIMBLE, 2000 Regulatory elements required for development of caenorhabditis elegans hermaphrodites are conserved in the *tra-2* homologue of *C. remanei*, a male/female sister species. Genetics **155:** 105-116.

HAAG, E. S., S. WANG and J. KIMBLE, 2002 Rapid coevolution of the nematode sex-determining genes *fem-3* and *tra-2*. Curr Biol **12:** 2035-2041.

HALL, D. H., V. P. WINFREY, G. BLAEUER, L. H. HOFFMAN, T. FURUTA *et al.*, 1999 Ultrastructural Features of the Adult Hermaphrodite Gonad of *Caenorhabditis elegans*: Relations between the Germ Line and Soma. Developmental biology **212:** 101-123.

HAMAOKA, B. Y., C. E. DANN III, B. V. GEISBRECHT and D. J. LEAHY, 2004 Crystal structure of Caenorhabditis elegans HER-1 and characterization of the interaction between HER-1 and TRA-2A. Proceedings of the National Academy of Sciences of the United States of America **101:** 11673-11678.

HANAHAN, D., and R. A. WEINBERG, 2011 Hallmarks of cancer: the next generation. Cell **144:** 646-674.

HILL, K. L., and S. W. L'HERNAULT, 2001 Analyses of reproductive interactions that occur after heterospecific matings within the genus Caenorhabditis. Dev Biol **232:** 105-114.

HILL, R. C., C. E. DE CARVALHO, J. SALOGIANNIS, B. SCHLAGER, D. PILGRIM *et al.*, 2006 Genetic flexibility in the convergent evolution of hermaphroditism in Caenorhabditis nematodes. Dev Cell **10:** 531-538.

HILL, R. C., and E. S. HAAG, 2009 A sensitized genetic background reveals evolution near the terminus of the Caenorhabditis germline sex determination pathway. Evol Dev **11:** 333-342.

HILLIER, L. W., R. D. MILLER, S. E. BAIRD, A. CHINWALLA, L. A. FULTON *et al.*, 2007 Comparison of *C. elegans* and *C. briggsae* genome sequences reveals extensive conservation of chromosome organization and synteny. PLoS Biol **5:** e167.

HODGKIN, J., 1983 Male phenotypes and mating efficiency in *Caenorhabditis elegans*. Genetics **103:** 43-64.

HODGKIN, J., 1986 Sex determination in the nematode *C. elegans*: analysis of *tra-3* suppressors and characterization of fem genes. Genetics **114:** 15-52.

HODGKIN, J., 2005 Introduction to genetics and genomics, pp. in *WormBook*, edited by T. C. E. R. COMMUNITY. WormBook.

HODGKIN, J., and T. DONIACH, 1997 Natural variation and copulatory plug formation in *Caenorhabditis elegans*. Genetics **146:** 149-164.

HOLLAND, B., and W. R. RICE, 1999 Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. Proceedings of the National Academy of Sciences **96:** 5083-5088.

HOWE, D. K., and D. R. DENVER, 2008 Muller's Ratchet and compensatory mutation in *Caenorhabditis briggsae* mitochondrial genome evolution. BMC Evol Biol **8:** 62.

HSIN, H., and C. KENYON, 1999 Signals from the reproductive system regulate the lifespan of *C. elegans*. Nature **399:** 362-366.

HU, P. J., 2007 Dauer, pp. in *WormBook*, edited by T. *C. E.* R. COMMUNITY. WormBook.

HUBER, B., 1993 Female choice and spider genitalia. Bolletino de la Accademia Gioenia de Scienza Naturale **26:** 209-214.

HUTTER, P., and M. ASHBURNER, 1987 Genetic rescue of inviable hybrids between Drosophila melanogaster and its sibling species. Nature **328:** 331-333.

IWASAKI, K., J. MCCARTER, R. FRANCIS and T. SCHEDL, 1996 *emo-1*, a *Caenorhabditis elegans* Sec61p gamma homologue, is required for oocyte development and ovulation. The Journal of cell biology **134:** 699-714.

JAN, E., C. K. MOTZNY, L. E. GRAVES and E. B. GOODWIN, 1999 The STAR protein, GLD-1, is a translational regulator of sexual identity in Caenorhabditis elegans. The EMBO journal **18:** 258-269.

JIA, K., D. CHEN and D. L. RIDDLE, 2004 The TOR pathway interacts with the insulin signaling pathway to regulate *C. elegans* larval development, metabolism and life span. Development **131:** 3897-3906.

JIANG, C., P. CHEE, X. DRAYE, P. MORRELL, C. SMITH *et al.*, 2000 Multilocus interactions restrict gene introgression in interspecific populations of polyploid Gossypium (cotton). *Evolution* **54:** 798-814.

JONES, A. R., R. FRANCIS and T. SCHEDL, 1996 GLD-1, a cytoplasmic protein essential for oocyte differentiation, shows stage- and sex-specific expression during *Caenorhabditis elegans* germline development. Dev Biol **180:** 165-183.

KENYON, C. J., 2010 The genetics of ageing. Nature **464:** 504-512.

KIM, S., C. SPIKE and D. GREENSTEIN, 2013 Control of oocyte growth and meiotic maturation in *Caenorhabditis elegans*, pp. 277-320 in *Germ Cell Development in C. elegans*. Springer.

KIMBLE, J., and S. L. CRITTENDEN, 2007 Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in *Caenorhabditis elegans*. Annu. Rev. Cell Dev. Biol. **23:** 405-433.

KIMBLE, J., L. EDGAR and D. HIRSH, 1984 Specification of male development in< i> Caenorhabditis elegans:</i> The< i> fem</i> genes. Developmental biology **105:** 234-239.

KIMURA, K. D., H. A. TISSENBAUM, Y. LIU and G. RUVKUN, 1997 *daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. Science **277:** 942-946.

KING, K. L., M. STEWART and T. M. ROBERTS, 1994 Supramolecular assemblies of the *Ascaris suum* major sperm protein (MSP) associated with amoeboid cell motility. Journal of cell science **107:** 2941-2949.

KIONTKE, K., N. P. GAVIN, Y. RAYNES, C. ROEHRIG, F. PIANO *et al.*, 2004 Caenorhabditis phylogeny predicts convergence of hermaphroditism and extensive intron loss. Proc Natl Acad Sci U S A **101:** 9003-9008.

KIONTKE, K., and W. SUDHAUS, 2006 Ecology of Caenorhabditis species, pp. in *WormBook*, edited by T. *C. E.* R. COMMUNITY. WormBook.

KIONTKE, K. C., M.-A. FÉLIX, M. AILION, M. V. ROCKMAN, C. BRAENDLE *et al.*, 2011 A phylogeny and molecular barcodes for Caenorhabditis, with numerous new species from rotting fruits. BMC evolutionary biology **11:** 339.

KIRIENKO, N. V., K. MANI and D. S. FAY, 2010 Cancer models in *Caenorhabditis elegans*. Developmental Dynamics **239:** 1413-1448.

KITANO, J., J. A. ROSS, S. MORI, M. KUME, F. C. JONES *et al.*, 2009 A role for a neo-sex chromosome in stickleback speciation. Nature **461:** 1079-1083.

KLEEMANN, G. A., and A. L. BASOLO, 2007 Facultative decrease in mating resistance in hermaphroditic *Caenorhabditis elegans* with self-sperm depletion. Animal Behaviour **74:** 1339-1347.

KNOWLES, L. L., and T. A. MARKOW, 2001 Sexually antagonistic coevolution of a postmating-prezygotic reproductive character in desert Drosophila. Proceedings of the National Academy of Sciences **98:** 8692-8696.

KOBOLDT, D. C., J. STAISCH, B. THILLAINATHAN, K. HAINES, S. E. BAIRD *et al.*, 2010 A toolkit for rapid gene mapping in the nematode *Caenorhabditis briggsae*. BMC Genomics **11:** 236.

KOSINSKI, M., K. MCDONALD, J. SCHWARTZ, I. YAMAMOTO and D. GREENSTEIN, 2005 *C. elegans* sperm bud vesicles to deliver a meiotic maturation signal to distant oocytes. Development **132:** 3357-3369.

KOZLOWSKA, J. L., A. R. AHMAD, E. JAHESH and A. D. CUTTER, 2012 Genetic variation for postzygotic reproductive isolation between Caenorhabditis briggsae and Caenorhabditis sp. 9. Evolution **66:** 1180-1195.

KRESGE, N., V. D. VACQUIER and C. D. STOUT, 2001 Abalone lysin: the dissolving and evolving sperm protein. Bioessays **23:** 95-103.

KUBAGAWA, H. M., J. L. WATTS, C. CORRIGAN, J. W. EDMONDS and E. SZTUL, 2006 Oocyte signals derived from polyunsaturated fatty acids control sperm recruitment in vivo. Nature cell biology **8:** 1143-1148.

L'HERNAULT, S., 2006 Spermatogenesis, pp. in *WormBook*, edited by T. *C. E.* R. COMMUNITY. WormBook.

L'HERNAULT, S. W., D. C. SHAKES and S. WARD, 1988 Developmental genetics of chromosome I spermatogenesis-defective mutants in the nematode *Caenorhabditis elegans*. Genetics **120:** 435-452.

LAMONT, L. B., S. L. CRITTENDEN, D. BERNSTEIN, M. WICKENS and J. KIMBLE, 2004 FBF-1 and FBF-2 Regulate the Size of the Mitotic Region in the< i> C. elegans</i> Germline. Developmental cell **7:** 697-707.

LAMUNYON, C. W., and S. WARD, 1998 Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*. Proceedings of the Royal Society of London. Series B: Biological Sciences **265:** 1997-2002.

LAMUNYON, C. W., and S. WARD, 1999 Evolution of sperm size in nematodes: sperm competition favours larger sperm. Proceedings of the Royal Society of London. Series B: Biological Sciences **266:** 263-267.

LAMUNYON, C. W., and S. WARD, 2002 Evolution of larger sperm in response to experimentally increased sperm competition in *Caenorhabditis elegans*. Proceedings of the Royal Society of London. Series B: Biological Sciences **269:** 1125-1128.

LAUBICHLER, M. D., and J. MAIENSCHEIN, 2007 *From embryology to evo-devo: a history of developmental evolution*. MIT Press Cambridge, MA.

LAVEBRATT, C., S. SENGUL, M. JANSSON and M. SCHALLING, 2004 Pyrosequencing-based SNP allele frequency estimation in DNA pools. Hum Mutat **23:** 92-97.

LI, W., R. BOSWELL and W. B. WOOD, 2000 *mag-1*, a homolog of Drosophila mago nashi, regulates hermaphrodite germ-line sex determination in *Caenorhabditis elegans*. Dev Biol **218:** 172-182.

LIN, K. T., G. BROITMAN-MADURO, W. W. HUNG, S. CERVANTES and M. F. MADURO, 2009 Knockdown of SKN-1 and the Wnt effector TCF/POP-1 reveals differences in endomesoderm specification in *C. briggsae* as compared with *C. elegans*. Dev Biol **325:** 296-306.

LIU, Q., C. STUMPF, C. THOMAS, M. WICKENS and E. S. HAAG, 2012 Context-dependent function of a conserved translational regulatory module. Development **139:** 1509-1521.

LOEWE, L., and A. D. CUTTER, 2008 On the potential for extinction by Muller's ratchet in *Caenorhabditis elegans*. BMC Evol Biol **8:** 125.

MADL, J. E., and R. K. HERMAN, 1979 Polyploids and sex determination in *Caenorhabditis elegans*. Genetics **93:** 393-402.

MANOLI, D. S., M. FOSS, A. VILLELLA, B. J. TAYLOR, J. C. HALL *et al.*, 2005 Male-specific fruitless specifies the neural substrates of Drosophila courtship behaviour. Nature **436:** 395-400.

MARCELLO, M. R., G. SINGARAVELU and A. SINGSON, 2013 Fertilization, pp. 321-350 in *Germ Cell Development in C. elegans*. Springer.

MARTIN, O. Y., and D. J. HOSKEN, 2003 The evolution of reproductive isolation through sexual conflict. Nature **423:** 979-982.

MCCARTER, J., B. BARTLETT, T. DANG and T. SCHEDL, 1999 On the Control of Oocyte Meiotic Maturation and Ovulation in *Caenorhabditis elegans*. Developmental biology **205:** 111-128.

MCKINNEY, F., S. R. DERRICKSON and P. MINEAU, 1983 Forced copulation in waterfowl. Behaviour**:** 250-294.

MEHRA, A., J. GAUDET, L. HECK, P. E. KUWABARA and A. M. SPENCE, 1999 Negative regulation of male development in Caenorhabditis elegans by a protein–protein interaction between TRA-2A and FEM-3. Genes & development **13:** 1453-1463.

MIHOLA, O., Z. TRACHTULEC, C. VLCEK, J. C. SCHIMENTI and J. FOREJT, 2009 A mouse speciation gene encodes a meiotic histone H3 methyltransferase. Science **323:** 373-375.

MIKKELSEN, T. S., L. W. HILLIER, E. E. EICHLER, M. C. ZODY, D. B. JAFFE *et al.*, 2005 Initial sequence of the chimpanzee genome and comparison with the human genome. Nature.

MILLER, M. A., V. Q. NGUYEN, M.-H. LEE, M. KOSINSKI, T. SCHEDL *et al.*, 2001 A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation. Science Signaling **291:** 2144.

MILLER, M. A., P. J. RUEST, M. KOSINSKI, S. K. HANKS and D. GREENSTEIN, 2003 An Eph receptor sperm-sensing control mechanism for oocyte meiotic maturation in *Caenorhabditis elegans*. Genes & development **17:** 187-200.

MOYLE, L. C., and B. A. PAYSEUR, 2009 Reproductive isolation grows on trees. Trends Ecol Evol **24:** 591-598.

MULLER, H., 1942 Isolating Mechanisms, Evolution, and Temperature. Biol. Symp. **6:** 71-125.

MURRAY, R. L., J. L. KOZLOWSKA and A. D. CUTTER, 2011 Heritable determinants of male fertilization success in the nematode *Caenorhabditis elegans*. BMC Evolutionary Biology **11:** 99.

NAYAK, S., J. GOREE and T. SCHEDL, 2005 *fog-2* and the evolution of self-fertile hermaphroditism in Caenorhabditis. PLoS Biol **3:** e6.

NIGON, V., 1951 Polyploidie experimentale chez un Nematode libre, *Rhabditis elegans*. Biol. Fr. Belg. **85**.

NORRIS, K., and M. R. EVANS, 2000 Ecological immunology: life history trade-offs and immune defense in birds. Behavioral Ecology **11:** 19-26.

OHNO, S., 1970 *Evolution by gene duplication*. London: George Alien & Unwin Ltd. Berlin, Heidelberg and New York: Springer-Verlag.

ORR, H. A., 2005 The genetic basis of reproductive isolation: insights from Drosophila. Proc Natl Acad Sci U S A **102 Suppl 1:** 6522-6526.

PALOPOLI, M. F., M. V. ROCKMAN, A. TINMAUNG, C. RAMSAY, S. CURWEN *et al.*, 2008 Molecular basis of the copulatory plug polymorphism in *Caenorhabditis elegans*. Nature **454:** 1019-1022.

PALUMBI, S. R., 1998 Species formation and the evolution of gamete recognition loci.

Endless forms: species and speciation. Oxford Univ. Press, Oxford, UK**:** 271-278.

PARKER, G. A., 1970 Sperm competition and its evolutionary consequences in the insects. Biological Reviews **45:** 525-567.

PARKER, G. A., 1979 Sexual selection and sexual conflict. Sexual selection and reproductive competition in insects **123:** 166.

PATTERSON, J., 1946 A new type of isolating mechanism in Drosophila. Proceedings of the National Academy of Sciences of the United States of America **32:** 202.

PERRY, M., W. LI, C. TRENT, B. ROBERTSON, A. FIRE *et al.*, 1993 Molecular characterization of the her-1 gene suggests a direct role in cell signaling during Caenorhabditis elegans sex determination. Genes & development **7:** 216-228.

PHADNIS, N., and H. A. ORR, 2009 A single gene causes both male sterility and segregation distortion in Drosophila hybrids. Science **323:** 376-379.

PRASAD, A., M. J. CROYDON-SUGARMAN, R. L. MURRAY and A. D. CUTTER, 2011 Temperature-dependent Fecundity Associates with Lattitude in Caenorhabditis briggsae. Evolution **65:** 52-63.

PRESGRAVES, D. C., 2008 Sex chromosomes and speciation in Drosophila. Trends Genet **24:** 336-343.

PRESGRAVES, D. C., 2010 The molecular evolutionary basis of species formation. Nat Rev Genet **11:** 175-180.

PRESGRAVES, D. C., L. BALAGOPALAN, S. M. ABMAYR and H. A. ORR, 2003 Adaptive evolution drives divergence of a hybrid inviability gene between two species of Drosophila. Nature **423:** 715-719.

RABOIN, M., A. TIMKO, D. HOWE, M. A. FÉLIX and D. DENVER, 2010 Evolution of Caenorhabditis mitochondrial genome pseudogenes and *Caenorhabditis briggsae* natural isolates. Mol. Biol. Evol. **27:** 1087-1096.

RAFF, R. A., 1996 *The shape of life*. University of Chicago Press, Chicago, IL.

RAIZEN, D. M., J. E. ZIMMERMAN, M. H. MAYCOCK, U. D. TA, Y. J. YOU *et al.*, 2008 Lethargus is a *Caenorhabditis elegans* sleep-like state. Nature **451:** 569-572.

RICE, W. R., 1996 Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. Nature **381:** 232-234.

RICE, W. R., 1998 Intergenomic conflict, interlocus antagonistic coevolution, and the evolution of reproductive isolation, pp. 261-270 in *Endless forms: species and speciation*, edited by D. J. HOWARD and S. H. BERLOCHER.

RIESEBERG, L. H., J. WHITTON and K. GARDNER, 1999 Hybrid zones and the genetic architecture of a barrier to gene flow between two sunflower species. *Genetics* **152:** 713-727.

ROGERS, S. M., and L. BERNATCHEZ, 2006 The genetic basis of intrinsic and extrinsic post-zygotic reproductive isolation jointly promoting speciation in the lake whitefish species complex *(Coregonus clupeaformis)*. *J Evol Biol* **19:** 1979-1994.

ROLFF, J., and M. T. SIVA-JOTHY, 2002 Copulation corrupts immunity: a mechanism for a cost of mating in insects. Proceedings of the National Academy of Sciences **99:** 9916-9918.

ROSS, J. A., D. C. KOBOLDT, J. E. STAISCH, H. M. CHAMBERLIN, B. P. GUPTA *et al.*, 2011 Caenorhabditis briggsae recombinant inbred line genotypes reveal inter-strain incompatibility and the evolution of recombination. PLoS genetics **7:** e1002174.

SAMBROOK, J., and D. W. RUSSELL, 2001 *Molecular cloning : a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

SCHEDL, T., and J. KIMBLE, 1988 *fog-2*, a germ-line-specific sex determination gene required for hermaphrodite spermatogenesis in *Caenorhabditis elegans*. Genetics **119:** 43-61.

SCHULZE, J., and E. SCHIERENBERG, 2009 Embryogenesis of *Romanomermis culicivorax*: an alternative way to construct a nematode. Dev Biol **334:** 10-21.

SCOTT, A., J. DINMAN, D. SUSSMAN and S. WARD, 1989 Major sperm protein and actin genes in free-living and parasitic nematodes. Parasitology **98:** 471-478.

SEIDEL, H. S., M. AILION, J. LI, A. VAN OUDENAARDEN, M. V. ROCKMAN *et al.*, 2011 A novel sperm-delivered toxin causes late-stage embryo lethality and transmission ratio distortion in C. elegans. PLoS biology **9:** e1001115.

SEIDEL, H. S., M. V. ROCKMAN and L. KRUGLYAK, 2008 Widespread genetic incompatibility in *C. elegans* maintained by balancing selection. Science **319:** 589-594.

SHERWOOD, D. R., J. A. BUTLER, J. M. KRAMER and P. W. STERNBERG, 2005 FOS-1 promotes basement-membrane removal during anchor-cell invasion in *C. elegans*. Cell **121:** 951-962.

SICARD, A., and M. LENHARD, 2011 The selfing syndrome: a model for studying the genetic and evolutionary basis of morphological adaptation in plants. Annals of Botany **107:** 1433-1443.

SIH, A., J. KRUPA and S. TRAVERS, 1990 An experimental study on the effects of

predation risk and feeding regime on the mating behavior of the water strider. The American Naturalist **135:** 284-290.

SINGSON, A., K. L. HILL and S. W. L'HERNAULT, 1999 Sperm competition in the absence of fertilization in *Caenorhabditis elegans*. Genetics **152:** 201-208.

SMITH, H., 2006 Sperm motility and MSP, pp. in *WormBook*, edited by T. C. E. R. COMMUNITY. WormBook.

SMITH, J. M., 1978 *The Evolution of Sex*. Cambridge University Press, Oxford, UK.

SOTA, T., and K. KUBOTA, 1998 Genital lock-and-key as a selective agent against hybridization. Evolution**:** 1507-1513.

SPILLANE, C., K. J. SCHMID, S. LAOUEILLÉ-DUPRAT, S. PIEN, J.-M. ESCOBAR-RESTREPO *et al.*, 2007 Positive darwinian selection at the imprinted MEDEA locus in plants. Nature **448:** 349-352.

STAROSTINA, N. G., J.-M. LIM, M. SCHVARZSTEIN, L. WELLS, A. M. SPENCE *et al.*, 2007 A CUL-2 Ubiquitin Ligase Containing Three FEM Proteins Degrades TRA-1 to Regulate< i> C. elegans</i> Sex Determination. Developmental cell **13:** 127-139.

SUDHAUS, W., and K. KIONTKE, 2007 Comparison of the cryptic nematode species Caenorhabditis brenneri sp. n. and C. remanei (Nematoda: Rhabditidae) with the stem species pattern of the Caenorhabditis Elegans group. Zootaxa **1456:** 45-62.

SWANSON, W. J., and V. D. VACQUIER, 2002 The rapid evolution of reproductive proteins. Nature Reviews Genetics **3:** 137-144.

TANAKA, R., E. OKUMURA, N. KANZAKI and T. YOSHIGA, 2012 Low survivorship of dauer larva in the nematode *Caenorhabditis japonica*, a potential comparative system for a model organism, *C. elegans*. Experimental Gerontology.

TANG, S., and D. C. PRESGRAVES, 2009 Evolution of the Drosophila nuclear pore complex results in multiple hybrid incompatibilities. Science **323:** 779-782.

TAYLOR, S. J., M. ARNOLD and N. H. MARTIN, 2009 The genetic architecture of reproductive isolation in Louisiana irises: hybrid fitness in nature. *Evolution* **63:** 2581-2594.

THOMAS, C. G., G. C. WOODRUFF and E. S. HAAG, 2012 Causes and consequences of the evolution of reproductive mode in *Caenorhabditis* nematodes. Trends in Genetics.

TRUE, J. R., and S. B. CARROLL, 2002 Gene co-option in physiological and morphological evolution. Annual review of cell and developmental biology **18:** 53-80.

TURELLI, M., and L. C. MOYLE, 2007 Asymmetric postmating isolation: Darwin's corollary to Haldane's rule. Genetics **176:** 1059-1088.

VACQUIER, V. D., and W. J. SWANSON, 2011 Selection in the rapid evolution of gamete recognition proteins in marine invertebrates. Cold Spring Harbor Perspectives in Biology **3**.

WARD, S., and J. S. CARREL, 1979 Fertilization and sperm competition in the nematode *Caenorhabditis elegans*. Developmental biology **73:** 304-321.

WATANABE, T., 1979 A gene that rescues the lethal hybrids between Drosophila melanogaster and D. simulans. Japanese Journal of Genetics **54**.

WEGEWITZ, V., H. SCHULENBURG and A. STREIT, 2008 Experimental insight into the proximate causes of male persistence variation among two strains of the androdioecious *Caenorhabditis elegans* (Nematoda). BMC ecology **8:** 12.

WESTENDORP, R. G., and T. B. KIRKWOOD, 1998 Human longevity at the cost of reproductive success. Nature **396:** 743-746.

WIGBY, S., and T. CHAPMAN, 2006 No evidence that experimental manipulation of sexual conflict drives premating reproductive isolation in *Drosophila melanogaster*. Journal of evolutionary biology **19:** 1033-1039.

WOOD, W. B. (Editor), 1988 *The Nematode Caenorhabditis elegans*. CSHL Press, Cold Spring Harbor, NY.

WOODRUFF, G. C., O. EKE, S. E. BAIRD, M.-A. FÉLIX and E. S. HAAG, 2010 Insights into species divergence and the evolution of hermaphroditism from fertile interspecies hybrids of Caenorhabditis nematodes. Genetics **186:** 997-1012.

WU, D., P. M. TEDESCO, P. C. PHILLIPS and T. E. JOHNSON, 2012 Fertility/longevity trade offs under limiting-male conditions in mating populations of *Caenorhabditis elegans*. Experimental Gerontology.

YAN, C., Y. BI, D. YIN and Z. ZHAO, 2012 A Method for Rapid and Simultaneous Mapping of Genetic Loci and Introgression Sizes in Nematode Species. PloS one **7:** e43770.

YANG, J.-S., H.-J. NAM, M. SEO, S. K. HAN, Y. CHOI *et al.*, 2011 OASIS: online application for the survival analysis of lifespan assays performed in aging research. PloS one **6:** e23525.

ZARKOWER, D., 2006 Somatic sex determination. WormBook.

ZHANG, B., M. GALLEGOS, A. PUOTI, E. DURKIN, S. FIELDS *et al.*, 1997 A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. Nature **390:** 477-484.