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 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. C. sp. 9 animals and F1 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 F1 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 University of Maryland, College Park, in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2013

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Dedication

In memory of my father
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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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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 *fhl-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 *fhl-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$_b$ (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 $B_{3_{b,9,bh}}$ 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>
<thead>
<tr>
<th>Name</th>
<th>SNP ID</th>
<th>Sequence (5’ to 3’)</th>
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VTP-IV-8S | TCTGACTGCGAACGA
VTP-V-2F | cbv11673 AGCGCTGCTCCGGTTTCTAGATTTGGGGAACGACTTGATTTTGTATCCA
VTP-V-2R | CCATTATTCAAAACTTCCGATGCTA
VTP-V-2S | TTTGTACCTGATTTGAAA
VTP-V-6F | cbv24904 AGCGCTGCTCCGGTTTCTAGATTTGGGGAACGACTTGATTTTGTATCCA
VTP-V-6R | GCCAATTTTGACCCCCATTTTA
VTP-V-6S | TTTGTACCTGATTTGAAA
VTP-V-8F | cbv31146 GTGAGCCGTTGATCTTCATATTCC
VTP-V-8R | AGCGCTGCTCCGGTTTCTAGATTTGGGGAACGACTTGATTTTGTATCCA
VTP-V-8S | TCGATATTTTTTGTCAATT
VTP-X-9F | cbv427 GAAAAATCAGTGGTTCAGGGCTTAC
VTP-X-9R | AGCGCTGCTCCGGTTTCTAGATTTGGGGAACGACTTGATTTTGTATCCA
VTP-X-9S | GCCAATTTTGACCCCCATTTTA
VTP-UNIBIOT | Biotin-AGCGCTGCTCCGGTTTCTAGATTTGGGGAACGACTTGATTTTGTATCCA

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* sub*e* 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* sub*e* 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* sub*e* 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

<table>
<thead>
<tr>
<th>Animal</th>
<th>% Laid Embryos (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. sp. 9 F</td>
<td>93 (98)</td>
</tr>
<tr>
<td>C. sp. 9 M</td>
<td>90 (100)</td>
</tr>
<tr>
<td>F1b F</td>
<td>95 (99)</td>
</tr>
<tr>
<td>F1g F</td>
<td>83 (101)</td>
</tr>
<tr>
<td>B2b,9 F</td>
<td>24 (138)</td>
</tr>
</tbody>
</table>

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.

In contrast, they produce viable male and female progeny with C. sp. 9 males (Figure 2D, F), with about 24% of B2b,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 B2b,9 progeny than B2g,9 progeny (Figure 2D vs. F) is highly significant (chi-square $P=0.0019$, d.f. = 1). B2b,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 B2b,9 males have apparently normal gonads. While fertile B2b,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<sub>b</sub> 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<sub>b</sub> 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<sub>b</sub> (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

<table>
<thead>
<tr>
<th>Animal scored</th>
<th>C. briggsae strain(s) used</th>
<th>C. sp. 9 strain used</th>
<th>% Selfers (n)</th>
<th>% Spermatogenic (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0 n/a</td>
<td>JU1422</td>
<td>0 (n&gt;100)</td>
<td>0 (118)</td>
<td></td>
</tr>
<tr>
<td>P0 n/a</td>
<td>EG5268</td>
<td>0 (109)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F1&lt;sub&gt;b&lt;/sub&gt; AF16</td>
<td>JU1422</td>
<td>0 (230)</td>
<td>0 (92)</td>
<td></td>
</tr>
<tr>
<td>F1&lt;sub&gt;b&lt;/sub&gt; AF16</td>
<td>JU1325</td>
<td>1.2 (494)</td>
<td>0 (123)</td>
<td></td>
</tr>
<tr>
<td>F1&lt;sub&gt;b&lt;/sub&gt; AF16</td>
<td>EG5268</td>
<td>0.3 (360)</td>
<td>3.5 (114)</td>
<td></td>
</tr>
<tr>
<td>F1&lt;sub&gt;9&lt;/sub&gt; AF16</td>
<td>JU1325</td>
<td>0 (106)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F1&lt;sub&gt;b&lt;/sub&gt; VT847</td>
<td>JU1422</td>
<td>0 (34)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F1&lt;sub&gt;b&lt;/sub&gt; HK104</td>
<td>JU1422</td>
<td>0 (29)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F1&lt;sub&gt;b&lt;/sub&gt; VT847</td>
<td>JU1325</td>
<td>0 (32)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F1&lt;sub&gt;b&lt;/sub&gt; HK104</td>
<td>JU1325</td>
<td>0 (68)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F1&lt;sub&gt;b&lt;/sub&gt; VT847</td>
<td>EG5268</td>
<td>0 (26)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F1&lt;sub&gt;b&lt;/sub&gt; HK104</td>
<td>EG5268</td>
<td>0 (31)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F1&lt;sub&gt;b&lt;/sub&gt; CP116 (polyploid AF16)</td>
<td>JU1325</td>
<td>0 (137)</td>
<td>8.3 (24)</td>
<td></td>
</tr>
<tr>
<td>F1&lt;sub&gt;b&lt;/sub&gt; CP116 (polyploid AF16)</td>
<td>EG5268</td>
<td>2.3 (91)</td>
<td>15 (177)</td>
<td></td>
</tr>
<tr>
<td>B3&lt;sub&gt;b,9,bh&lt;/sub&gt; AF16</td>
<td>JU1422</td>
<td>0 (181)</td>
<td>0 (65)</td>
<td></td>
</tr>
<tr>
<td>B3&lt;sub&gt;b,9,bh&lt;/sub&gt; AF16</td>
<td>JU1325</td>
<td>0 (41)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>B3&lt;sub&gt;b,9,bh*&lt;/sub&gt; VT847 first</td>
<td>JU1325</td>
<td>0 (128)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Selfer, animal that lays embryos without mating; spermatogenic, female/hermaphrodite has sperm-like cells under DIC microscopy; -, not determined. See Figure 2 for how B3b,9,bh and B4b,9,9,b animals are constructed. *The B3b,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°C (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 F1b 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 F1b 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. 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\textsubscript{b,9} males and *C. briggsae* hermaphrodites (B3\textsubscript{b,9,bh} animals; Figure 2G). The other results from crossing B3\textsubscript{b,9,9} hybrid females with *C. briggsae* males to produce B4\textsubscript{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\textsubscript{b} female x B2\textsubscript{b,9} male) or no hermaphrodites (B2\textsubscript{b,9} female x B2\textsubscript{b,9} male).

The incidence of selfing and spermatogenesis is 0% among B3\textsubscript{b,9,bh} XX animals (Table 3). In B4\textsubscript{b,9,9,b} XX animals, the selfing incidence varies, depending upon which *C. sp.* 9 strain is used. 2.6% of XX B4\textsubscript{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\textsubscript{b,9,bh} and B4\textsubscript{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\textsubscript{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\textsubscript{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_\text{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_\text{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 \textit{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 \textit{C. briggsae} alleles completely excluded. For certain regions of \textit{C. briggsae} chromosomes II, III, IV, and X (Figure 10), it appears that either homozygosity in the B3_\text{b,9,bh} generation or heterozygosity in the previous (B2) generation adversely affects hybrid fitness.
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 ≥ 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{subscript}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.
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<sub>b,9</sub> than B2<sub>b,9</sub> 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 (Hawe 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 \textit{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 \textit{C. sp. 9} is extremely canalized in its female fate, and thus highly resistant to the action of factors that promote XX spermatogenesis. In \textit{C. elegans} and \textit{C. briggsae}, loss-of-function mutations in these factors are recessive (HODGKIN 1986; SCHEDL and KIMBLE 1988; ZHANG \textit{et al.} 1997; LI \textit{et al.} 2000; GUO \textit{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 \textit{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 \textit{C. remanei} that suggest that multiple steps are necessary for hermaphroditism to evolve (BALDI \textit{et al.} 2009).

\textit{Opportunities and limitations for the genetic investigation of hermaphroditism}

\textit{C. sp. 9} and \textit{C. briggsae} open the possibility of mapping the historically crucial variants that led to \textit{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 PAYEUR 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 \textit{C. briggsae} hermaphroditism from being rigorously evaluated. As an alternative approach, we screened 15,000 mutagenized haploid \textit{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 \textit{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 \textit{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 \textit{C. briggsae} alleles in the hybrid F1 is at least partly responsible for the recessivity of the hermaphrodite trait in this system.

\textit{Implications for the emergence of hermaphrodite lineages}

Phylogenetic studies suggest that hermaphroditism evolved multiple times in \textit{Caenorhabditis} (Cho et al. 2004; Kiontke et al. 2004). If the ability of \textit{C. sp. 9} and \textit{C. briggsae} to produce fertile hybrids (whereas all other \textit{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 \textit{C. sp. 9} today. If that ancestral female was as resistant to the effects of hermaphroditizing factors as \textit{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 lineages.

**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. briggsae* 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


Female/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).

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 germline 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).
**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
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

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

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 C9-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 C9-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.
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 hermaphroditic 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; García et al. 2007). Androdioecious males are also apparently unable to promote mating-induced behavioral changes in females that gonochoristic males are capable of (García 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 (Arness 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; Bacigalupi 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 $B_{2_{b,b}}$ 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.
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