

## ABSTRACT

Title of Dissertation

CHARACTERIZATION OF THE ROLE OF  
THE FEM GENES IN THE SEX  
DETERMINATION PATHWAY OF  
CAENORHABDITIS BRIGGSÆ

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In the genus *Caenorhabditis*, self-fertile hermaphrodites in *C.elegans* and *C.briggsæ* evolved from females by developing the ability to generate a limited number of self sperm. The *fem* genes are crucial for spermatogenesis and the sperm-to-oocyte switch in *C.elegans* hermaphrodites. RNAi results of the *fem* genes in *C.briggsæ* hermaphrodites differed from results in *C.elegans*, suggesting regulation of germ line sex determination pathway differs between the two species. To more definitively address this possibility, and to further investigate the role of the *fem* genes in the sex determination pathway of *C.briggsæ*, deletion mutants of *Cbr-fem-2* and *Cbr-fem-3* were generated and characterized. Double *Cbr-tra-1;Cbr-fem-2* and

*Cbr-tra-1;Cbr-fem-3* were also generated to further characterize the role of the *fem* genes and their relationship to *tra-1*.

Our results show that while the somatic role of the *fem* genes have been conserved in both species, their germline role differs. Males of both species require the *fem* genes for somatic development and to suppress oocyte production. However, *C.briggsae* hermaphrodites do not require the *fem* genes for spermatogenesis or the sperm-to-oocyte switch. The double mutant analysis results suggest that, unlike *C.elegans*, *Cbr-tra-1* remains epistatic to the *Cbr-fem* genes in the germline sex determination system in *C.briggsae*. While there is overall similarity in phenotypic categories between the double mutants, the percentages within each category differs. The double *tra-1;fem3* mutant phenotype differs significantly from the single *tra-1* mutant, suggesting a role for *Cbr-fem-3* in regulating *Cbr-tra-1* activity. A previously undescribed Emo phenotype was also discovered in both single and double mutants in *C.briggsae* and in *C.elegans tra-1* mutant alleles e1099 and e1781.

The overall results of this study are consistent with the convergent evolution of hermaphroditism within the genus *Caenorhabditis* and suggest considerable genetic flexibility in this developmental pathway.

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## **Chapter One: Introduction**

### 1.1. Challenges of Evolutionary Studies

Scientific research addresses a broad range of questions. Sometimes the goal is to understand a process, such as DNA translation; a structure, such as actin filaments; or a function, such as the role of phosphorylation in gene regulation. These studies can involve work within single species or comparative work between species. Sometimes the research seeks to address larger questions of evolutionary processes and relationships, often through the comparative study of process, structure or function.

Historically, evolutionary studies have been challenging, and remain so today, due largely to the retrospective nature of the research. Evolution is often difficult to directly observe, so research begins with the results of evolution and infers past processes and relationships based on current observations. This requires the development of methodologies to indirectly investigate the origins and diversity of life, the relationships between species and the processes responsible for the diversity and relationships. These methodologies have generated considerable conflict within the research community (Amundson 1998; McOuat 2001; Maienschein 1991).

Conflicts relating to issues of homology, methodologies and interpretation in the field of evolutionary comparative embryology during the late 19<sup>th</sup> and early 20<sup>th</sup> centuries resulted in a rejection of that method in favor of population genetics as an evolutionary mechanism during the development of the Modern Synthesis (Amundson 2001; Gilbert 2003.) Interest in evolution continued within the

experimental and physiological embryology community, however. Advances in techniques during the 20<sup>th</sup> century and continuing today have allowed a greater understanding of the genetic and molecular underpinning of developmental processes, and the crucial role these processes play in evolution. There is a growing consensus among evolutionary researchers that to understand the evolution of form it is necessary to understand the evolution of the developmental mechanisms underlying form (Gilbert 1996; Goldschmidt 1982; Gould 1980; Robert 2001). This has resulted in a reevaluation of the Modern Synthesis as well as the reemergence of evolutionary developmental biology as a discipline.

## 1.2. The History of Evo-Devo

Modern Biology has its roots in the field of natural history established in the 18<sup>th</sup> century (Allen 1975; Appel 1987; McOuat 2001). Natural history was primarily a descriptive endeavor. As the biblical account of Creation was taken as fact, naturalists believed there was no need to identify laws or mechanisms operating within nature. There were none; God was the answer. If each species is uniquely created, there is no need for comparative analysis. Therefore naturalists sought to describe, classify and collect the natural world and glorify God in the process (Rehbock 1983; p7). As exploration of the known world expanded, and discoveries of new species (both extant and extinct) increased, it became more difficult to accept the simplistic view of the constancy of uniquely created species. Geological studies showed the Earth had a history of change over time, and fossil discoveries showed species were not immutable and eternal. If species are mutable, comparative studies become a valid assessment of species identification and species change. If change is

a common aspect of life, then understanding the laws governing that change is a legitimate avenue of research. The field of *philosophical* natural history, which sought to identify patterns in nature and the laws governing those patterns, emerged.

By 1800, naturalists in Germany and France were using the word *biology* to describe the “theoretical as well as descriptive science of living things” (Rehbock 1983; p6). Early philosophical naturalists struggled to define methodologies and interpretation for this emerging field of biology. Two views emerged, the primacy of function in defining patterns, as exemplified by work of Georges Cuvier and the importance of form in guiding patterns, as exemplified by the work of Etienne Geoffroy Saint-Hilaire.

Georges Cuvier (1769-1832) espoused a teleological and functional view of the natural world. Animal form was the result of the coordination of all parts to produce an integrated, functional whole. The form was the manifestation of the functional needs of the animal, based on the “conditions of existence” for each animal. In the *Regne animal* (English translation 1834) Cuvier proposed an animal classification system based on four distinct classes, or embranchments, distinguished by distinct nervous system plans. Any commonality between embranchments was simply a matter of common functional need. “Let us then conclude that, if there are any resemblances between the organs of the fish and those of other classes, it is only insofar as there are resemblances between their functions” (quoted in Coleman 1964; p156). The embranchments were distinct; therefore no transitional forms between the embranchments could exist. This system would be the basis of zoological classification throughout most of the 19<sup>th</sup> century. As noted in Appel (1975; p90),

Cuvier advocated analytical and empirical analysis, but believed you could not analyze the properties of animals via experimentation. He believed a natural method of classification based on comparative anatomy was comparable to analytical methods used in other sciences.

Geoffroy Saint-Hilaire (1772-1844) proposed a transcendental approach to comparative anatomy that he proclaimed would replace classification and description of species. It was based on the belief that all life was built on a single general body plan devised by the Creator. Diversity was simply the result of modification of the plan, or archetype.

Thus the forms in each class of animal, however they may vary, all result at bottom from organs common to all; nature refuses to make use of new ones. Thus all the most essential differences that affect each family belonging to the same class come solely from another arrangement, from a complication – in a word, from a modification – of these same organs. (quoted in Le Guyander 2004)

Geoffroy also believed the common body plan restricted the size of subsequent modifications. “A normal or pathological organ never flourishes to an extraordinary degree, with its being the case that another organ of its system or of its relations suffers to the same degree” (Saint-Hilaire 1822).

Nature was the result of dynamic interactions of universal laws. An animal’s place in life was determined by its anatomical organization, not its ultimate function. Determination of analogous (what we now call homologous) structures in animals was crucial to this view, as Geoffroy viewed homologous structures as evidence of the archetype, an ideal form imposed on nature by the Creator. He began a research program based on morphological analysis centered on the identification of

homologous structures (Saint-Hilaire 1822). He also noted that the study of embryological form often provided a more reliable guide in determining homology, as development processes could obscure homologous structures (Appel 1987; p85).

Although major differences between the two views are obvious, both emphasized the need to seek biological laws. Simple teleological explanations were no longer sufficient to explain animal form and diversity. Both believed in a common body plan (Cuvier's four embranchments and Geoffroy's archetype), and the ability to determine relationships between species through commonly shared features (homologies). Both men contributed to the development of modern evolutionary thought. Cuvier's emphasis on the adaptation of form to function can be found in the basic tenets of population biology. Adaptation is considered a strong force in the development of the genetic structure of populations (see Orr 2005 for brief history of genetic adaptation). Saint-Hilaire's belief in a single body plan is reminiscent of the concept of constraint in evolutionary developmental theories (see Maynard Smith 1985 for discussion of developmental constraint and evolution). Through the work of both men and their supporters, the groundwork for the acceptance of evolution as force in nature was laid, and important investigative tools were developed.

Evolutionary developmental biology can trace its roots to the work of German comparative embryologists beginning in the 1820s. As discussed in Gould (1980), the concept of recapitulation, i.e. the stages of development in higher animals corresponded to the adult forms of lower animals, became a central tenet of the German school of *Naturphilosophie*. Embryologists such as Lorenz Oken, J.F. Meckel and Etienne Serres shared a belief that nature is governed by a single set of

laws, and that the study of embryological development was the best mechanism for deducing those laws. Although the underlying theory was flawed, the importance of comparative studies in understanding relationships among organisms was reinforced.

Much early embryonic research focused on the actual process itself. Christian Pander discovered the three germ layers; Heinrich Rathke studied a diverse group of organisms, describing for the first time the pharyngeal arches, and Karl Ernst von Baer discovered the notochord (Gilbert 2006). As noted by Gilbert (2006), their work transformed embryology into a specialized branch of science.

von Baer was a harsh critic of Recapitulation. He noted that during early development all vertebrate embryos appear similar. As development progresses, the special features of each distinct group and species emerge. From these observations he formulated what is now called “von Baer’s laws”, which in summary state: development proceeds from general characters (i.e. phylum level) to more specific characters (i.e. species level), and rather than passing through the adult stages of lower animals, higher animals emerge from the lower animal embryonic stages over the course of development.

The field of comparative embryology morphed into evolutionary embryology as the result of the convergence of a number of ideas and discoveries. Saint-Hilaire’s concept of unity of type implied a common body plan uniting all of life. His work identifying this common type required the identification of homologies, and it was believed those homologies were most easily identified in embryos. The work of the earlier embryologists in developing detailed descriptions of the developmental process made the use of embryos for identification of homology the ‘gold standard’.

The similarity of the early embryonic stages was noted by Darwin and offered as proof of common descent (Darwin 1902). This connected embryology to evolution, and so after the publication of Darwin's *Origins*, homologous larval structures were believed to be evidence of shared ancestry and relationships.

Ernst Haeckel is perhaps the best-known evolutionary embryologist, although I suggest he was more interested in phylogeny than embryology. His Biogenetic Law "Ontogeny recapitulates phylogeny" was a form of parallelism – the stages of development of a species parallel the phylogeny of that species. A strong proponent of Recapitulation, Haeckel's goal was to use development to determine the origin of major animal groups, and ultimately to identify the first metazoan (which he called *Gastraea*). He often created hypothetical groups to "fill in the blanks" when structures in embryonic development did not have a similar extant group with the embryonic structure. Thus developmental stages were proxies for the stages in a species evolution. Haeckel actively sought publicity for his theory, with impressive results. His books were translated into 25 languages, and some had as many as 12 editions (Sander and Schmidt-Ott 2004). Despite strong criticism and lack of support among many of his fellow embryologists, his theory became part of the "scientific psyche". It found its way into textbooks and general acceptance despite lack of scientific validation, and has caused numerous headaches for future evolutionary scientists.

Not all evolutionary embryologists were fixated on a strict phylogenetic approach to embryology. Francis Balfour, while accepting recapitulation, focused on understanding and identifying phylogenetically significant homologies, as well as



detailing the actual processes of development. As opposed to Haeckel, Balfour believed natural selection operated on the larva and embryo in the same manner as it did in adult forms, and determining these “secondary changes” from primary ancestral patterns was a crucial goal of evolutionary embryology. As stated in his *Treatise*:

The satisfactory application of embryological data to morphology Depends upon a knowledge of the extent to which the record of ancestral history has been preserved in development. Unless secondary changes intervened this record would be complete; it becomes therefore of the first importance to the embryologist to study the nature and extent of the secondary changes likely to occur in the foetal or the larval stage. (1885; p360)

Other embryologists, including Gavin de Beer and Walter Garstang rejected recapitulation completely, focusing instead on changes in the timing of developmental events and larval adaptations as key contributors to species evolution (Hall 2000). They were interested in using embryological analysis to understand processes, such as heterochrony, plasticity and constraint that modified embryonic development and led to species diversification. As Garstang argued, “ontogeny does not recapitulate phylogeny: it creates it”(qtd. in Hall 2000)

Following the untimely death of Francis Balfour, many of his students left the field of embryology. William Bateson was perhaps the most vocal and vehement in his critique of the discipline. “The Embryological Method then has failed not for want of knowledge of the visible facts of development but through ignorance of the principles of Evolution” (Bateson 1894:p9). A true believer in the approach initially, he increasingly questioned the validity of using hypothetical groups (such as

Protognathostomata) to determine phylogenetic relationships, beginning a paper published in 1886 as follows:

Of late the attempts to arrange genealogical trees involving hypothetical groups has come to be the subject of some ridicule, perhaps deserved. But since this is what modern morphological criticism in great measure aims at doing, it cannot be altogether profitless to follow this method to its logical conclusion. That the results of such criticisms must be highly speculative, and often liable to grave error, is evident. (Bateson 1886)

In his studies of the hemichordate *Balanoglossus*, he noted such a wide range of larval forms that not only made determining ancestor from descendent difficult, but identifying the different species based on their larval forms was problematic (Hall 2005). His research led him to believe the study of variation was the key to understanding evolution. “Variation, in fact, is Evolution....the readiest way then of solving the problem of Evolution is to study the facts of Variation” (Bateson 1894: p6). Bateson left the field of embryology for the emerging field of genetics and the study of variation.

The late 19<sup>th</sup> century saw an expansion of embryological techniques. Improved microtomes allowed for more detailed analysis of embryological stages. Methods to actively manipulate live specimens were also being developed. The era of experimental embryology had begun. Major players in the development of this field include Wilhelm His, Wilhelm Roux and August Weismann. His, a strong critic of Haeckel, believed embryology should be a study of developmental processes, not an attempt to determine phylogeny. Weismann studied developing cells and suggested the nucleus contained the hereditary units (Winther 2001). Wilhelm Roux actively manipulated developing embryos, perhaps most famously in his 1888 experiments

with two-celled embryos (Roux 1888). He destroyed one of the cells with a hot needle, with the goal of testing Weismann's germ plasm model. Although his experimental design was flawed (he did not remove the punctured blastomere from its partner) the importance of this work was the belief that experimentation could provide the answer. Roux founded a new journal, Archiv für Entwicklungsmechanik, and experimental approach, *Entwicklungsmechanik* (developmental mechanics), in 1895. He believed experimentation was the only legitimate method in science, and due to the difficulty of observing embryonic development, manipulative experimentation provided the only way of obtaining information. He totally rejected the evolutionary embryology approach as exemplified by Haeckel.

The rediscovery of Mendel's work in 1900 opened new experimental opportunities, as well as conflicts and competitions. The experimental, or physiological, embryologists of the early 20<sup>th</sup> century were focusing on how the fertilized egg becomes an adult, and which compartment, the nucleus or the cytoplasm, controls the process. Mendel's work suggested some kind of "element" (now called gene) that controlled the inheritance of traits and by extension must be involved in development. E.B. Wilson, along with Theodor Boveri and Nettie Stevens, conducted research that supported the nucleus, and more specifically the chromosomes, as the controller of inheritance. Thomas Hunt Morgan believed the cytoplasm-controlled inheritance, but his work ultimately showed nuclear chromosomes were responsible for the development of inherited characters.

Historical accounts of this time show the fields of embryology and genetics diverging by the 1930s. There is much discussion of the hostilities between the two

disciplines (Gilbert, et al 1996; Gilbert 2003; Hall 2005). Geneticists saw the embryologists as out of touch with modern scientific methods and the embryologists viewed geneticists as uninformed regarding the actual development of organisms. A main player in the ultimate “banishment” of embryology from evolutionary theory was T.H.Morgan. He clearly separated the two fields by describing genetics as the study of the transmission of inherited traits while embryology was the study of the expression of inherited traits. He ridiculed a major concept within embryology in the early 20<sup>th</sup> century, that of morphogenetic fields, “areas of embryological information that created webs of interactions such that any cell was defined by its position within its respective field” (Gilbert et al 1996). It has been suggested his hostility was due to fear of competition. Neither morphogenetic field nor gene had been seen, so the field provided an alternative to the gene as a developmental force (Gilbert *et al* 1996). By 1937 one of Morgan’s students, Theodosius Dobzhansky had redefined evolution as changes in gene frequency and by 1951 he declared, “Evolution is a change in the genetic composition of populations. The study of mechanisms of evolution falls within the province of population genetics” (qtd. in Gilbert *et al* 1996). Population genetics at the time did have advantages. It focused on microevolution, which can be observed over short periods of time and defined in mathematical terms. It provided a mechanism – change in allele frequency. It provided financial support. The Atomic Energy Commission funded research in population genetics at a time when evolutionary studies were struggling for funding. Some historians argue that in an attempt to make embryology more scientific, embryologists distanced themselves

from evolution, and genetics filled the void in the development of the Modern Synthesis (Allen G.E. 1978; Maienschein L. 1991).

A review of developmental research during the first part of the 20<sup>th</sup> century argues against an abandonment of evolution. Ivan Schmalhausen (1884-1963), a Russian developmental biologist, developed the concept of morphogenetic correlations. He believed organisms must be studied holistically and that evolution must be viewed as a coordinated process of the entire organism. “Since the organism is an interconnected whole, it must keep its property of wholeness also in the course of evolution” (qtd. in Levit *et al* 2006)). C.H. Waddington isolated homeotic mutants in the late 1930s, analyzing these mutants in terms of the influence of genes on early embryonic processes. He believed evolutionary studies needed to focus on the processes that translate the information in the genotype into the phenotype of an organism. R. B. Goldschmidt criticized the Modern Synthesis, arguing small genetic changes could not cause large scale change. Both researchers viewed all evolutionarily important changes as alterations in development. Raff and Love note the work of N. J. Berrill in the comparative embryology of ascidians as well as D.T. Anderson’s work in the ontogeny and phylogeny of annelids and arthropods as “characterizing a school of highly expert anatomists and comparative embryologists who carried on vigorous programs rightly construed as Evo-devo during the middle half of the 20<sup>th</sup> century” (Raff and Love 2004).

Alfred Kuhn developed a research program beginning in 1924 aimed at bringing together genetics, development and physiology. His work during the middle of the 20<sup>th</sup> century led him to conclude that regulatory changes and new combinations

of developmental processes are primarily responsible for phenotypic change. Divergent phenotypes share common developmental mechanisms that vary in regulation between the phenotypes. As noted by Laubrichler and Rheinberger (2004), these views include many of the central tenets of present day evolutionary developmental biology. In addition, they note Kuhn connects the late 19<sup>th</sup> century program of developmental evolution with modern molecular developmental genetics, including the discovery of the Homeobox. Kuhn was a student of August Weismann and continued the study of problems central to developmental evolution in his own work. Beginning in the early 1950s, Kuhn collaborated with Ernst Hadorn on a number of projects. By the 1960s, Kuhn no longer taught graduate students, but among Hadorn's students were Walther Gehring and Rolf Nothiger. They continued the work of their mentor on developmental problems such as imaginal disks and homeotic mutations. While at Yale, Gerhing met Eric Wieschaus, who followed Gerhing to Switzerland. Chistiane Nusslein-Volhart, influenced by Kuhn's developmental physiology lectures, was looking for a place to do developmental genetic work, and joined the lab in Switzerland. The rest, as they say, is history. This group laid the foundations of molecular developmental genetics, generating methods that expanded the field of evolutionary developmental studies.

Gilbert suggests evo-devo was conceived in 1977 and born in 2000. (Gilbert 2003). I suggest it was conceived more than one hundred years ago, with the establishment of embryology as a discipline. It has gone through a very long developmental process, through many stages, to emerge, as Wilhelm Roux predicted in 1895, as the second type of developmental mechanics (Gilbert et al 1996).

### 1.3. Evolutionary developmental biology as a research program

This research project is grounded in the field of evolutionary developmental biology, a field that seeks to identify the changes in developmental processes that result in the evolution of phenotypic traits. “Evo-devo opens the black box between genotype and phenotype” (Hall 2003). Advances in genetic and molecular techniques during the last half of the 20<sup>th</sup> century resulted in studies that uncovered striking similarities in the genes and pathways that determine early embryonic patterning for the majority of metazoan phyla. Homologous genes and pathways were found to control the development of diverse morphologies. The discovery of Hox gene clusters throughout the bilaterian metazoan and their spatial colinearity was truly mind-blowing (Barolo and Posakony 2002; Wilkins 2002; Gehring 1985). These findings lead some to question if the corollary could be true – could different genes and pathways control the development of similar morphologies?

Comparative studies of multiple species provide a good system to answer these questions. Optimal studies share a number of characteristics. They have interesting, tractable variation among closely related species. The species have diverged enough to provide variation to study, while retaining enough similarity to establish phylogenetic relationships. If species are too closely related, not enough variation may exist between them to enable study. If they are too distantly related, the amount of evolutionary change between them may erase the evidence of how the divergence occurred. Optimal model systems also have a large amount of genetic developmental data, including sequenced genomes for the closely related species.

They also have molecular tools, such as transgenes, molecular markers and forward and reverse genetic techniques.

#### 1.4. *Caenorhabditis* as a Comparative Developmental System

The nematode *Caenorhabditis* genus fits all of the above criteria (Fig.1).

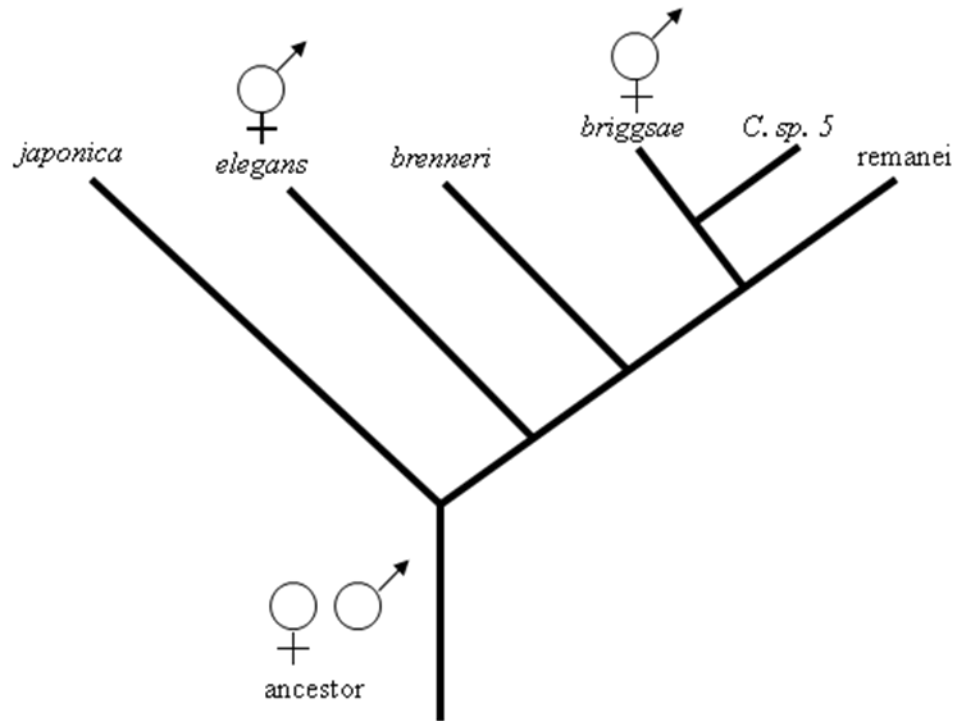


Figure 1. Phylogenetic relationships within the Elegans group of the genus *Caenorhabditis*. The tree follows Dolgin et al (2008) and Kiontke and Fitch (2005), and is based on sequence analysis from SSU and LSU rRNA genes, and coding regions from genes for RNAP2, *par-6*, and *pkc-3*.

*Caenorhabditis* are small, free-living, soil-dwelling worms, easily maintained on agar medium and a diet of bacteria. Brood sizes are large and the life cycle, comprised of four larval stages prior to adulthood, is short; approximately 3 days at optimal temperature of 20C. Since being chosen by Sydney Brenner in the late 1960s as a model organism to study developmental processes (Brenner 1974), a large amount of genetic and developmental data has been amassed for a number of developmental



forward and reverse genetics, RNA interference (RNAi), transgenes, and numerous molecular and phenotypic markers. The genomes of both *C. elegans* and another species within the Elegans clade, *C. briggsae*, have been completely sequenced, and the sequencing of three additional species within the Elegans group is in progress.

### 1.5. Sex Determination Systems Diversity Within the Metazoa

The discovery of the general conservation in both structure and expression of the *Hox* genes across the metazoans highlights both the underlying unity of animal life as well as the importance of “getting your body plan right” (Gehring 1985; Manzanares 2000). Further investigation of the Hox genes revealed diversity in both the details of their genetic organization and their roles in the developmental process. They appear to have a general role in specifying segment identity along the anterior-posterior axis during embryonic development, but their upstream regulators and downstream effectors vary widely among the Metazoa (Wilkins 2002). A pattern of both conservation of key regulatory gene families and diversity of their regulators and effectors is common among the Metazoans. Examples include *tinman*, involved in heart development in both flies and mice, and Pax-6, involved in eye development in organisms as diverse as human, mice, flies and mollusks.

Equally important to the success and survival of a species is the production of the next generation. In sexually reproducing organisms, the production of haploid male and female germ cells is a common mechanism, although there is variation in whether the cells occur in a single animal (hermaphroditic) or separate animals (gonochoristic). The sex determining mechanisms that control the production of the germ cells vary widely (Morrish and Sinclair 2002), and appear to lack conserved key

regulatory genes. Given the general conservation of the *Hox* genes despite the diversity of body plans, the lack of conservation of sex determination mechanisms throughout the animal kingdom is surprising. In fact, sex determination systems display a pattern opposite to that of many other developmental systems. A wide variety of seemingly unrelated developmental mechanisms generate a common outcome – male and female.

Much is known regarding the diversity of sex determining mechanisms (Bull 1983). As noted by Wilkins (2002), the initial observations of chromosomal differences between males and females occurred in studies of insects at the start of the 20<sup>th</sup> century. The discovery of what came to be known as sex chromosomes led to a search for them in additional animal and plant species, and over the course of the 20<sup>th</sup> century a wide range of systems were uncovered (Bull 1983; Marin and Baker 1998; Morrish and Sinclair 2002).

Sex determination mechanisms fall into two broad categories; environmental mechanisms and genetic mechanisms. Recent work indicates both mechanisms can operate within a single species (Radder, et al. 2008; Quinn, et al. 2007).

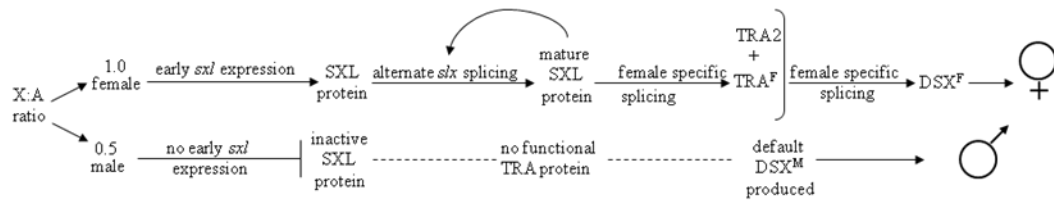
In environmental sex determination (ESD), the initiating cue is a variable factor in the environment. Temperature is a common environmental cue for many reptile species. For many species of turtles, lower temperatures produce males while higher temperatures produced females. For many lizard and crocodylian species the opposite is true. A mixture of these patterns is also observed. For example, in the Australian lizard *Amphibolurus muricatus*, eggs incubated at either low (23-26C) or high (30-33C) produce female offspring. Intermediate temperatures (27-30C) produce

both sexes. (Crews, et al. 1995; Warner and Shine 2008). The mechanism for this process has been extensively studied in turtles, and it is thought to involve temperature-sensitive promoters for genes encoding steroidogenic enzymes, as well as genes encoding steroid hormone receptors (Crews, et al 1994). Local sex ratios are an environmental sex determinant in some Perciform hermaphroditic fish species. In some fish species, social groupings are comprised of a group of small individuals of one sex and a few to one dominant individual(s) of the opposite sex. If the larger individual leaves the group, the next largest individual of the opposite sex may switch sex and assume the dominant role (Devlin, et al 2002). Population density influences the sex ratio of the parasitic mermithid nematodes. In a lightly infected host, only females are produced. Conversely, highly infected hosts produce only males. Mixed populations are found in intermediate parasite loads (Christie 1929; Harlos, et al 1980).

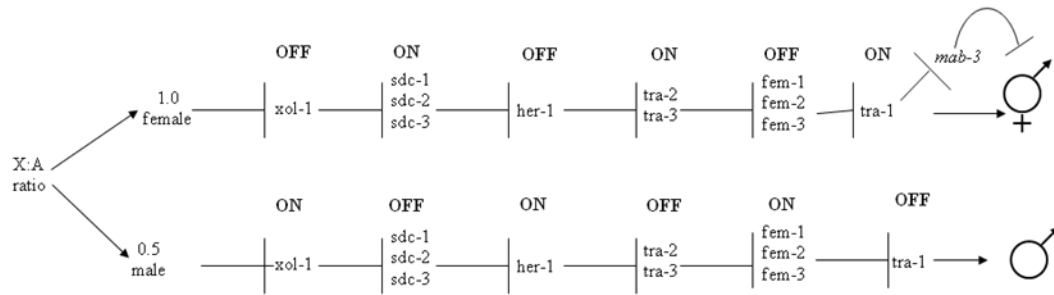
In genetic sex determination (GSD), the initiating cue is through some genetic factor(s). Genetic sex determination systems vary widely. Sex chromosomes systems can be either XX/XY, with males the heterogametic sex, or ZW/ZZ, with females as the heterogametic sex. These systems are probably the most common sex determining mechanisms in animals (Bull 1983). Mammals employ the XX/XY system. The *Sry* gene located on the Y chromosome determines male fate. *Sry* is both necessary and sufficient for the initiation of testis determination and the development of male sexual characteristics in most mammals (Wilhelm et al 2007). Avian sex is determined via the ZW/ZZ system, but the mechanism of determination is unclear (Clinton 1998).

Other genetic systems employ the ratio of the number sex chromosomes to sets of autosomes. This system is in both *C. elegans* and *Drosophila melanogaster*, although the details of the systems vary (Fig.2).

*D. melanogaster* has sex chromosomes of the XX/XY type, but the Y does not contain a male determining factor. It does, however, contain genes required for male fertility (Charlesworth 2001). A 1:1 ratio of X chromosomes to autosomes ( $X:A=1.0$ ) activates the feminizing switch gene, *sex-lethal* (*Sxl*). Active *Sxl* controls the processing of *transformer* (*tra*) gene mRNA, which in turn controls the female-specific splicing of the *doublesex* (*dsx*) mRNA (with the assistance of *tra-2*). This female Doublesex protein ( $Dsx^F$ ) combines with the product of the *intersex* gene to form a transcription factor that controls female-specific traits by repressing genes responsible for male differentiation. Female differentiation genes are not repressed and are therefore active. In XY animals, *Sxl* is not active; therefore no functional Tra protein is made. *tra* pre-mRNA is made in both sexes, but without active *Sxl* protein, a non-functional Tra protein is made in males. *Dsx* is active in both males and females. The male Doublesex protein ( $Dsx^M$ ) acts directly as a transcription factor to direct male-specific traits by the opposite mechanism responsible for female fate. Female differentiation genes are repressed; male differentiation genes are not repressed and therefore active. (Baker and Wolfner 1988; Belote et al 1989; Nothiger et al 1987).



A.



B.

Figure 2. Sex determination pathway in *Drosophila melanogaster* and *Caenorhabditis elegans*. (A.) In *Drosophila melanogaster*, the X:A ratio activates sex-specific mRNA splicing that results in either male or female development. (B.) In the *Caenorhabditis elegans* somatic sex determination pathway, a 1:1 ratio of X chromosomes to autosomes impacts a signaling pathway composed of a secreted ligand (encoded by *her-1*) and its membrane receptor (encoded by *tra-2*), which ultimately regulates the most downstream regulator, the female-promoting *tra-1*. (Figure based on Manolalou et al 2006).

In *C. elegans*, a hermaphroditic species, hermaphrodites are XX and males have just one X chromosome. A detailed description of their sex determination system is found in the following section, but it is essentially a negative regulatory cascade that controls the activity of the most downstream regulator *transformer-1* (*tra-1*). Active *tra-1* promotes female development (Zarkower 2006).

Additional genetic mechanisms used by some organisms include dominant male determiners, dominant female determiners and multiple genes with additive effects are also used as genetic sex determinant mechanisms. (Wilkins, 2002; Haag and Doty, 2005).

The wide variety of sex determination mechanisms suggests sex determination systems may have evolved independently numerous times over the course of evolution. Nothinger and Steinmann-Zwicky (1985) proposed a general mechanism for the evolution of sex determination pathways. After surveying a group of 14 insect genera, they observed a core portion of the pathway, from *Sxl* to *dsx*, was conserved across genera. The differences in pathways were limited to the switch mechanisms for the most upstream regulator, *Sxl*. This mechanism, the “retained core pathway”, involves retention of a core pathway with differences in upstream regulations that result in different activity levels of the pathway or genetic location of the switch mechanism. Study of the sex determination system of *C. elegans* led Wilkins to propose a different mechanism, the “retrograde addition model” (Wilkins 2002). Noting the complexity of the pathway when its ultimate function is rather simple (control of *tra-1* activity), and the observations of Jacob as evolution as a “tinkerer” (Jacob 1983), he suggests that pathways evolve in a piecemeal pattern, beginning from the most downstream element and adding regulatory elements upstream.

Molecular studies lend support to both models of pathway evolution. Although sex determination is chromosomal in mammals and birds and temperature-dependent in reptiles, studies show a possible conserved gonadal-development pathway composed of DAX1, WT1 and SF1 (Western et al 2000). Studies also provide support for an ancestral, rapidly evolving sex determination pathway. Raymond et al (1998) identified a region of sequence relatedness between the *mab3* gene in *C. elegans* and *dsx* in *Drosophila* in a region encoding the DNA-binding domain. Both of these genes are involved in male specific developmental processes.

They named this domain “DM” and identified a DM gene in humans, DMRT1, which may be involved in testis specification (Raymond et al 1998). Since this discovery, many DM genes have been identified in a number of vertebrates and studies suggest a role in male development in a number of the species (Raymond 2000). Recently a DM family gene possibly involved in sex determination was discovered in cnidarians as well (Miller 2003). The DM genes may represent a conserved, ancestral downstream regulator.

Rapid evolution of sex determination systems is observed between closely related species. The *Sxl* gene of *Drosophila* has been isolated from many dipteran species, and despite being well conserved does not appear to be involved in sex determination. Between *C. elegans* and *C. briggsae*, many genes involved in sex determination are poorly conserved, yet appear to maintain their role and interactions the pathway (deBono and Hodgkin 1996; Haag et al 2002; Stothard and Pilgrim 2003; Wang and Kimble 2001), suggesting selection is operating at the level of phenotype.

The observations of deep conservation and rapid evolution reinforces the use of closely related species to study the evolution of sex determination systems

#### 1.6. Sex determination System in *Caenorhabditis*

The sex determination system of *C. elegans* has been extensively studied (Hodgkin 1986; Kuwabara and Kimble 1992; Kuwabara and Perry 2001; Goodwin and Ellis 2002; Hodgkin 2002), and provides a well defined reference point for comparative studies within the genus. Comparative studies require variation between species, and this is found within the *Caenorhabditis* genus. *C. elegans* and *C. briggsae*, as well as two other species within the larger *Caenorhabditis* genus, produce

males and hermaphrodites, while the remaining species produce males and true females (larger genus phylogenetic tree not shown) (Fig.1). Phylogenetic studies suggest hermaphroditism has evolved from male/female species numerous times in rhabditid nematodes (Sudhaus, et al. 1996; Kiontke, et al. 2004;), and recent work has supported this scenario for the *Elegans* group of *Caenorhabditis* species (Cho, et al. 2004; Kiontke, et al. 2004).

Hermaphrodites are anatomically female, with a double-armed gonad leading to a central vulva, whip-like tail, and a generally broader body than males (Fig.3.). Each gonad arm contains a single spermatheca, to which male and self-derived sperm are attracted and stored. Hermaphrodites produce a limited amount of amoeboid sperm (200-300) during the third (*C. elegans*) or fourth (*C. briggsae*) larval stage of development before switching to oocyte production for their remaining life span.

Within the hermaphroditic species, males are produced at a low frequency (~0.2%) by X-chromosome non-disjunction. Males have a single armed gonad and a thinner body compared to hermaphrodites/females (Fig.3). The male tail, composed of fans, rays and spicules, is specialized for mating. These structures form during the fourth larval stage (Emmons 2005). Males produce amoeboid sperm that are 50% larger than hermaphrodite sperm. Male sperm have a competitive advantage over self-sperm (Ward and Carrel 1979; LaMunyon and Ward 1994) Hermaphrodites are capable of mating with males (but not with each other). The resulting cross-progeny are approximately 50% male.





Figure 3. DIC images of *C. briggsae* AF16 wild type animals. XX hermaphrodite (left) have a two-armed gonad (both arms can be seen, extending both distal and proximal from the centrally located vulva). XO males (right) have a single armed gonad, and a tail specifically modified for mating. Labels: e, embryo; v, vulva; o, oocyte; s, sperm; sp, spermatheca; mt, male tail.

Sexual fate in *C.elegans* is controlled by a regulatory pathway (Fig. 4). Whose activity differs between the sexes. It is called a core or global pathway as it impacts the sexual fate of all cells and tissues. The core pathway is regulated in the germline of hermaphrodites to allow a brief period of spermatogenesis.

The primary determinant of sex in *C. elegans* is the X:A ratio (See Table 1 for gene name and function information). A high ratio (XX) represses *xol-1* transcription, resulting in hermaphrodite development. A low ratio (XO) allows high *xol-1* expression, resulting in male development (Cline and Meyer 1996). *xol-1* activity controls sexual fate via a negative regulatory pathway, ultimately resulting in the activation (in hermaphrodites) or repression (in males) of the terminal transcriptional regulator *Ce-tra-1*. At the center of this pathway is a signal transduction cascade consisting of *Ce-her-1*, *Ce-tra-2* and *Ce-tra-3*, as well as the *Ce-fem* genes (*fem-1*, 2 and 3) (Kuwabara and Perry 2001; Goodwin and Ellis 2002; Wilkins 2002). The *Ce-tra*

genes promote female fate; *her-1* and the *Ce-fem* genes promote male fate. The same core pathway is used for both somatic and germline sex determination. Additional genes are responsible for the modulation of activity of core pathway genes within the hermaphrodite germline. These modulations are required to produce both sperm and oocytes.

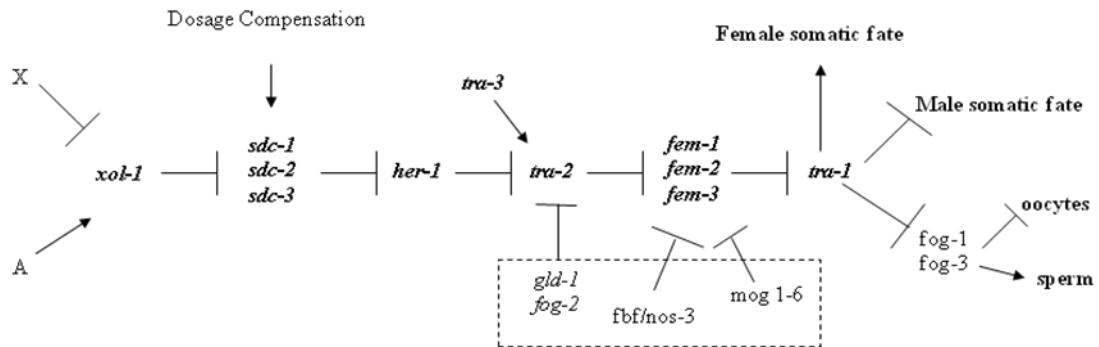


Figure 4. Core sex determination pathway in *C. elegans*. The core pathway comprises the genes in bold type. Positive interactions are shown by arrows. Negative interactions are indicated by bars. Above the core pathway are genes and processes involved in somatic cell fate. Below the core pathway and within the dashed box are germline modifications to the core pathway which allow a period of spermatogenesis in hermaphrodites. *fog-1* and *fog-3* are specific to the germline. Figure adapted from Ellis and Schedl 2007 and Zarkower 2006.

**Table.1. Gene naming in *C. elegans***

The gene naming system is based on the mutant phenotype of the gene, in abbreviated form. Gene names are italicized and lower case; proteins are not italicized and are capitalized

<b>Gene</b>	<b>Main mutant phenotype</b>	<b>Normal function</b>
<i>fbf</i>	<i>fem-3</i> mRNA binding factor produces excess sperm and no oocytes	helps maintain germline stem cell pop. involved in sperm to oocyte switch
<i>fem</i>	<b>feminizing</b> soma and germline males transformed into female hermaphrodites transformed into females	promotes male development
<i>fog</i>	<b>feminization of germline</b> only oocytes produced	promotes spermatogenesis
<i>gld</i>	<b>defective in germline</b> development	promotes oogenesis
<i>her</i>	male → <b>hermaphrodite</b>	promotes male development
<i>mog</i>	<b>masculinization of germline</b> only sperm produced	promotes oogenesis
<i>nos</i>	<b>Nanos</b> related lethal and developmental problems (homolog of <i>Drosophila</i> Nanos)	germline development promotion of switch
<i>sdc</i>	sex and <b>dosage</b> compensation defective, XX animals inviable and masculinized	involved in dosage compensation by hypoactivation of hermaphrodite X chromosome
<i>tra</i>	<b>transforms</b> hermaphrodites → male	promotes female development
<i>xol</i>	<b>XO</b> lethal – males die	promotes male fate

The *Ce-sdc* genes regulate *her-1* expression as well as decrease the expression of both X chromosomes in XX animals (Meyer 2005). HER-1, a small extracellular protein, promotes male function, by binding to the transmembrane protein TRA-2 (Hunter and Wood 1992; Hamaoka et al 2004; Kuwabara 1996a). Epistasis analysis indicates *Ce-tra-2* promotes hermaphrodite development by inhibiting the activity of the *Ce-fem* genes (Hodgkin 1986). *tra-2* has weak similarity to *Patched*, (Kuwabara et al 1992). As all three *fem* genes are expressed at high levels in both sexes (Ahringer et al 1992; Pilgrim et al 1995; Gaudet et al 1996), it is believed they are inhibited by TRA-2 via protein-protein interactions (Goodwin and Ellis 2002). *Ce-fem-1* encodes a protein that contains ankyrin-like repeats (Spence et al 1990). Studies found that FEM-1 was able to bind GST-FEM-2 (Tan et al 2001). *Ce-fem-2* encodes a serine/threonine

type 2C phosphatase that interacts directly with FEM-3 (Pilgrim et al 1995; Chin-Sang and Spence 1996). *fem-3* encodes a novel protein (Ahringer et al 1992) that in addition to interacting with FEM-2, also interacts directly with the intracellular portion of TRA-2 (Mehra et al 1999). Although the exact mechanism is unclear, it is believed an interaction between TRA-2 and FEM-3 inactivates the *fem* genes, allowing hermaphrodite development to occur. In XO males, HER-1 is believed to bind to the extracellular portion of TRA-2, which then prevents TRA-2 from binding to FEM-3 and inhibiting male development. The FEM proteins are then able to inhibit TRA-1A, which allows male development to occur.

Until recently, the exact mechanism of TRA-1A inhibition via the FEM proteins was unknown. Starostina et al (2007) have determined that TRA-1A is regulated by degradation via a CUL-2-based ubiquitin ligase complex. FEM-1 is the substrate-recognition subunit, and FEM-2 and FEM-3 as cofactors. TRA-1 is a Gli/Ci family transcription factor (Zarkower and Hodgkin 1992). *C. elegans* does not have a canonical *Hedgehog* (*Hh*) signalling pathway, but the similarity of *tra-1* to *Cubitus interruptus* and *tra-2* to *Patched* suggests the sex-determination pathway of *C. elegans* may be a highly diverged *Hedgehog* pathway (Kuwabara et al 1992). *Ce-tra-1* codes for two differentially processed transcripts, producing the protein TRA-1A (1109 amino acids) and TRA-1B (287 amino acids). TRA-1A contains five C2H2 Zinc fingers and binds DNA. TRA-1B has only the first two zinc fingers, does not bind DNA and has no known function (Zarkower and Hodgkin 1992; Zarkower and Hodgkin 1993). TRA-1A is known to negatively regulate three male-specific genes, *Ce-egl-1*, *Ce-mab-3* and *Ce-fog-3* (Chen and Ellis 2000; Conradt and Horvitz 1999, Yi

et al 2000), suggesting negative regulation is crucial to the control of sex determination.

The feature that distinguishes *Caenorhabditis* hermaphrodites (*C. elegans* and *C. briggsae*) from *Caenorhabditis* females (*C. remanei*, *C. brenneri*, *C. japonica* and *Sp. 5*) is the ability to generate sperm. This is accomplished by modulation of the core sex determination pathway (Fig.4). The *Ce-fem* genes are necessary for spermatogenesis in both males and hermaphrodites. In *C. elegans*, *Ce-tra-2* is negatively regulated via translational repression by *Ce-gld-1* and *Ce-fog-2* (Barton et al 1987; Schedl and Kimble 1988; Goodwin et al 1997; Puoti et al 2001). This allows the *fem* genes to be active, and spermatogenesis to occur. *Ce-fem-3* must then be negatively regulated to allow oogenesis to occur. This is accomplished post-transcriptionally by *Ce-fbf-1,2* and *Ce-nos 3*, as well as *Ce-mog 1-6* (Ahringer and Kimble 1991; Kuwabara and Perry 2001).

### 1.7. Research Project Rationale

Homology is a concept first used in reference to morphological traits (Owen 1843). It traditionally has been defined as similarity between organisms due to common ancestry, identified by shared characters. While a seemingly straightforward definition, the identification of “shared characters” has been problematic (Hall 1994; Butler and Sidel 2000; Dickinson 1995).

As the molecular and developmental underpinnings of many structures were identified, an understanding emerged that homology may be defined and investigated on multiple levels (Sanetra et al 2005; Wagner 2007). As noted by Sommer (2008), homology at the organ level does not always mean homology of the underlying

developmental process. It is possible for homologous organs to be formed through non-homologous genes or developmental pathways.

As more of the molecular underpinnings of the sex determination system within *Caenorhabditis* have been identified, in particular, the sperm to oocyte switch, the general applicability of the mechanisms involved in the switch has come into question. Is this the only way to evolve hermaphroditism in *Caenorhabditis*? As *C. briggsae* is also a hermaphroditic species, and closely related to *C. elegans*, do they share the same genetic mechanisms regulating the sperm to oocyte switch? Did they evolve hermaphroditism convergently, or is it an example of a homologous developmental pathway producing a homologous trait? Previous studies in other systems (Hoekstra and Nachman 2003) suggest it is possible that non-homologous pathways have come to specify a homologous outcome within *Caenorhabditis*. It is also possible that different modifications of a common developmental genetic mechanism produced the similar phenotypic outcome of hermaphroditism (True and Haag 2001). If the developmental pathway utilized in the two species is the same, then a common origin of hermaphroditism is possible, although the phylogeny would then suggest *C. remanei* and *Sp 5*. reversed to gonochorism, which, based on parsimony, seems unlikely.

Three lines of evidence suggest that hermaphroditism evolved convergently, via distinct modifications at the germline level of the core sex-determination pathway.

First, recent phylogenetic studies suggest *C. elegans* and *C. briggsae* are not as closely related as once thought. *C. briggsae* is more closely related to *C. remanei*, and the newly discovered *Sp. 5*, both male-female species (Cho et al 2004; Dolgin et al 2008; Kiontke et al. 2004). It has also been suggested that *C. elegans* evolved

hermaphroditism earlier than *C. briggsae* (Cutter et al 2008). Cutter noted little difference in either the overall patterns of codon usage bias or replacement site substitutions among the species of the *Caenorhabditis* genus, and no significant difference in codon bias in *C. briggsae* relative to its gonochoristic sister species, *C.sp.5*. Self-fertility results in a drastic reduction of effective population size, leading to the accumulation of slightly deleterious mutations via genetic drift. Over time the accumulation of these slightly deleterious mutations should result in a decline in codon usage bias and an elevated rate of replacement-site substitutions in coding sequences. The lack of significant difference in codon usage bias between *C. briggsae* and *C.Sp.5* suggests that the common ancestor of *C. briggsae*, *C. remanei*, and *Sp. 5* may have diverged from the *C. elegans* lineage prior to the evolution of hermaphroditism in *C. briggsae*. If this is the case, the initial environmental conditions at the time of the evolution of hermaphroditism could have widely differed. Additionally, if *C. elegans* and *C. briggsae* began to diverge prior to the evolution of hermaphroditism, then the developmental pathways from which hermaphroditism evolved could also have differed, and unique modifications of that pathway would not be surprising.

Second, although most of the *C. elegans* sex determination genes have orthologs in *C. briggsae*, there is an exception. *C. briggsae* lacks a homolog for *fog-2*, a gene whose function is crucial for the repression of *tra-2* (and therefore sperm production in hermaphrodites). *fog-2* is the result of a recent, tandem duplication, differing from its most closely related paralog (FTR-1) primarily at the C-terminus. This region is necessary and sufficient for GLD-1 binding (Clifford et al 2000; Nayak et al 2005). Additionally, the *Cbr-gld-1* mutant phenotype is Mog (masculinization of

germline), opposite that of *Ce-gld-*, suggestive of a different wild-type function (Alana Doty, unpublished results; Clifford et al 2000; Nayak et al 2005). As stated earlier, *Ce-fog-2* and *Ce-gld-1* are required to negatively regulate *Ce-tra-2* translation to allow spermatogenesis. Yet, *Cbr-tra-2* mutants display a phenotype similar to *Ce-tra-2* mutants (Kelleher et al 2008). This data suggests that there is negative regulation of *tra-2* in *C. briggsae*, and the mechanism of repression differs from that of *C. elegans*.

Third, experiments using RNA interference (RNAi) against the *fem* genes within a hermaphroditic sister species, *C. briggsae* result in the same somatic phenotype as *C. elegans*, but a different germline phenotype (Table 2) (Stothard et al 2001; Haag et al 2002). Regulation of the *Ce-fem* genes, in particular post-transcriptional negative regulation of *Ce-fem-3*, is required for the sperm to oocyte switch within that species (Puoti et al 2001; Ahringer and Kimble 1991; Ahringer et al 1992). If the RNAi results are valid, a crucial aspect of hermaphroditic regulation differs between the two species, suggesting the regulation of the sperm to oocyte switch occurs at another level of the core sex determination pathway.

There is an important caveat to the RNAi results. While RNAi is a robust technique in *C. elegans*, it is less effective in both *C. briggsae* and *C. remanei* ( Haag and Kimble 2000; Haag et al 2002; Kuwabara, 1996b). Additionally, RNAi produces “knock-downs” not “knock-outs”, so true mutations are needed to confirm the validity of the RNAi results.

The specific goal of this research project was to elucidate the role of the *Cb-fem* genes (*Cbr-fem-1*, Wormbase sequence name CBG19924; *Cbr-fem-2*, Wormbase sequence name CBG15267; *Cbr-fem-3*, Wormbase sequence name CBG21774) in the



sex determination system of *Caenorhabditis briggsae*, either confirming or refuting the RNAi results. To accomplish this, reverse genetics screens, based on screens successfully conducted in *C. elegans* (Liu et al 1999) were used to generate deletion mutants in *C. briggsae fem-2* and *fem-3*. Homozygous mutant strains were then generated and characterized. If the RNAi results are accurate, it is expected that hermaphrodites would have no discernable mutant phenotype. The RNAi results in XO males are more ambiguous, making a strong statement of expected results problematic. However, based on the phenotypes of *C.elegans* loss-of-function mutants, some degree of feminization is a likely outcome.

Table 2. Comparison of *Ce-fem* and *Cbr-fem* mutant phenotypes

	<i>C.elegans</i> ( <i>lf</i> mutants & RNAi)	<i>C.briggsae</i> (RNAi)
<i>fem-1</i>	XX Fem	XX <b>no phenotype</b>
	XO Fem	XO <b>partially Fem</b>
<i>fem-2</i>	XX Fem	XX <b>no phenotype</b>
	XO Fem (@25°)	XO <b>partially Fem</b>
<i>fem-3</i>	XX Fem	XX <b>no phenotype</b>
	XO Fem	XO <b>soma partially Fem, germline male</b>

Haag, Wang, & Kimble (2002); Stothard & Pilgrim(2002); A. Spence, (pers. comm.)

## Chapter Two: Deletion Screen Methods

### 2.1. Introduction

There are currently no methods of targeted mutagenesis available for *Caenorhabditis*. Therefore, to generate deletion mutations in particular genes, random mutagenesis is performed and the desired mutation is identified by PCR (Liu et al 1999).

A reverse deletion screen was employed in this project. In this method a deletion mutation is generated in a gene of interest, and the resulting phenotype is characterized (Jansen et al 1997; Liu et al 1999). There are risks associated with this method. A large number of genomes must be screened, and as the mutagenesis process is random there is no guarantee of obtaining a deletion in the desired gene. Additionally, a deletion may be obtained, but it may not result in an identifiable phenotype.

The method chosen was based on a highly successful method developed by the *C. elegans* Knockout Consortium (<http://celeganskoconsortium.omrf.org>). The Consortium recovers deletions in roughly fifty percent of targeted loci from a million genome library (<http://www.mutantfactory.ouhsc.edu/protocols.asp>). Modifications to this protocol have been developed to increase the detection of small deletions (Edgley et al 2002) and to extend the time a mutagenized library can be screened by freezing the mutagenized population (Ahringer 2006). The modifications were not employed in this protocol. Small deletions may not result in a null mutation and the goal of this screen was to develop a method that would detect primarily large, null

(hopefully!) mutations. Frozen libraries required more of an investment of time to optimize that was warranted for only three genes. Additionally, post freeze recovery of the deleted mutant has been shown to be inconsistent (*C. elegans* Knockout Consortium). The possible loss of a deleted population seemed more of a negative than the time involved in generating multiple libraries. Modifications made were primarily in the timing of mutagenesis and DNA preparation, due to developmental differences between the two species. Oogenesis is initiated later in *C. briggsae* relative to *C. elegans*. and *C. briggsae* holds fewer fertilized oocytes relative to *C. elegans*.

Many reagents were available for mutagenesis, including EMS (ethyl methane sulfonate), DEB (1,2:3,4-diepoxybutane), ultraviolet light combined with 4,5',8-trimethylpsoralen (UV/TMP), and ENU (N-ethyl-N-nitrosourea). UV/TMP has been shown to have a higher deletion rate, but a lower forward mutation rate as compared with EMS (Jansen et al 1997). Gengyo-Ando and Mitani (2000) showed that increasing both the UV intensity and TMP concentrations increased the forward mutation rate, but there was also an increase in F1 sterility and decrease in progeny. ENU has been shown to generate a wide range of non-null mutations, but it exhibits high toxicity to the nematodes (DeStasio and Dorman 2001). EMS was chosen based primarily on availability and ease of use. In addition, Liu et al reported an average deletion size of ~ 1320 bp using EMS (Liu et al 1999). As the average size of the *Cbr-fem* genes is 3.9 kb, a 1.3kb deletion would most likely result in significant loss of function.

Reverse genetic screens rely on high-throughput PCR methods of mutation analysis. These methods present numerous challenges in obtaining consistent, reliable results. Often the balance between optimal method and high volume is difficult to achieve. Primer design and optimization of amplification conditions are crucial factors in successful screens.

Many factors negatively impact reliable PCR results. Contaminants in DNA preps interfere with PCR amplification. Given the crude nature of the DNA preps used in this method, consistent amplification is often difficult to achieve. The goal of deletion screens is to generate null mutants. While null mutants can be achieved by small, even point mutations, the protocol employed in the screen cannot detect either category, so reasonably large deletions are needed. However, large PCR products are often difficult to amplify consistently. Although the genes in question are not large (~2.7 to 6 Kb unspliced + UTR), consistent amplification of PCR products larger than ~ 2 Kb is often problematic. The deleted product might ultimately be small, but the wild type control and heterozygotes may not consistently amplify, making confirmation of deletion difficult.

The primers are designed flanking the gene(s) of interest, and the nested nature of the protocol requires the each flanking set to be located in close proximity. This limits the ability to obtain optimal GC content and can lead to problems in consistent results. Many factors are considered when choosing primer location. Regions both 5' and 3' of genes impact expression, so when possible, roughly 50-100 bases up and downstream of each gene is targeted for deletion in addition to the actual gene itself. To ensure the primers were capable of consistent and accurate

amplification, they were first tested on high quality phenol/chloroform extracted DNA. Initial PCR were performed at the melting temperature ( $T_m$ ) calculated by the company that conducted the primer synthesis (IDT). Elongation time was determined by length of fragment (roughly 1min per 1Kb). Initial total rounds of amplification were set at 35 cycles. Based on results, the conditions were modified until consistent results were obtained. A gradient program was used in some cases to obtain optimal annealing temperature. Once optimal PCR conditions were determined for use with high quality DNA, each primer set was tested on DNA prepared using the deletion screen method (see Table 1, Appendix II, for PCR programs used in the screen).

To increase the chance of a deletion occurring in each gene, the gene sequence was divided into two or more regions (depending on total gene size), and nested primer sets were designed for each region (see Table 2, Appendix II, for list of Primers used in the screen). This increased the chances of detecting deletions occurring in any region of the gene.

One disadvantage of random mutagenesis is the generation of background extraneous mutations in addition to the desired deletion. Therefore, prior to characterization of the mutant phenotype, the mutant strains must be outcrossed multiple times. The protocol employed for outcrossing was based on a protocol defined in Fay (2006) with some modification. Nested PCR, rather than a phenotypic marker, is used to identify the genotype of the deleted worms and recover deletion populations. The basic scheme can be found in Figure 5.

## 2.2. Strain Maintenance

Deletion mutagenesis was performed on the *C. briggsae* wild isolate AF16. Strains were maintained using standard *C. elegans* methods (Wood 1988), using 2.2% agar to discourage burrowing. Unless otherwise stated, incubations during the screen were conducted at 20°C. The mutagenized population was stored on agar plates at 15°C.

## 2.3. Deletion Protocol

A detailed deletion protocol can be found in the appendix. An overview of the process is shown in Figure 5.

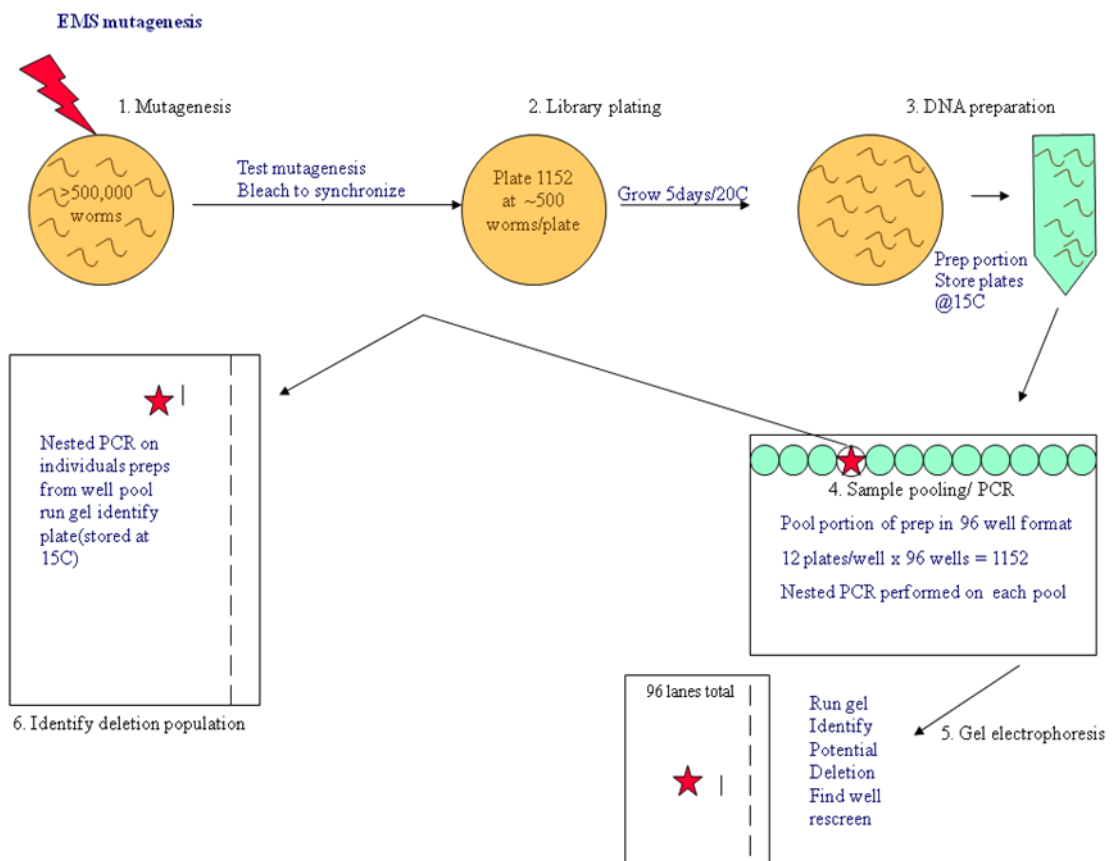


Figure 5a. Deletion screen. Generating mutagenized population and identifying the potential deletion population.

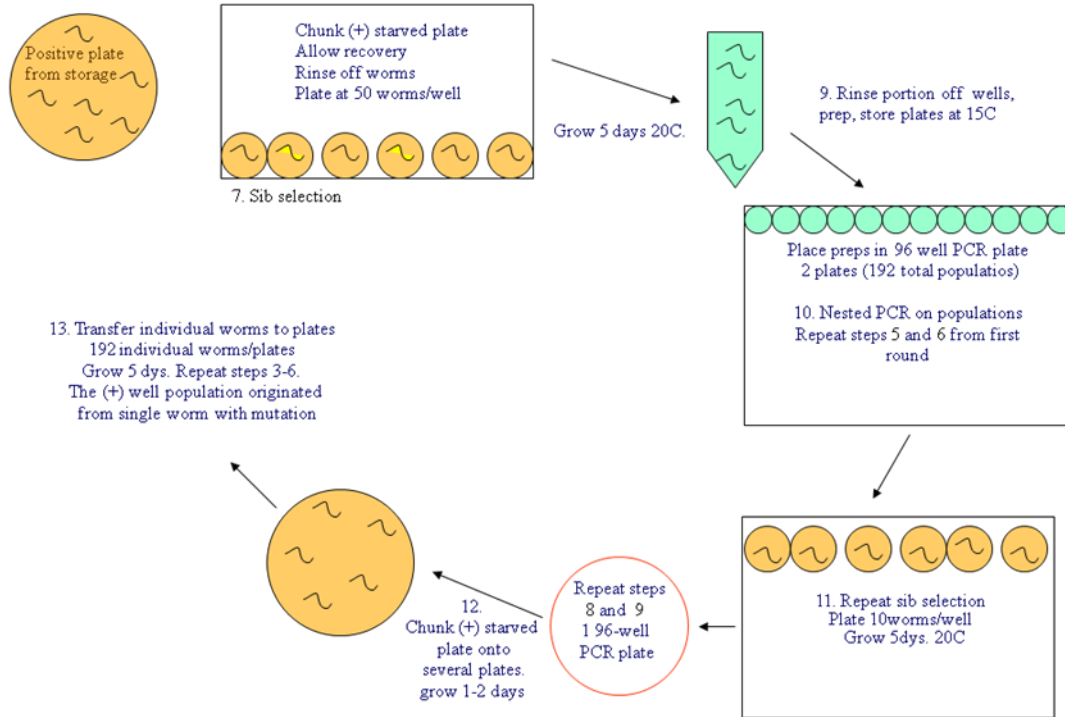


Figure 5b. Isolation of mutant population founded by a single mutant worm

### 2.3.1. Synchronization of population for mutagenesis

Worms were grown on 6 small (60x15mm) nematode growth medium (NGM; wood 1988) plates spotted with *E.coli* of the uracil auxotroph strain (OP50) to generate a large starting population. The worms were washed off with M9 buffer and treated with a bleach solution (40% NaOH; 60% bleach) to collect eggs. Egg count was estimated and if sufficient to generate ~ 500,000 worms for mutagenesis, the eggs were plated onto 15 large (95x15mm) NGM plates spotted with OP50, then incubated for approximately 52 hours. The plates were checked after 24 hours and the worms washed off and replated onto fresh plates if the original plates were starved out. Plates were monitored at one-hour intervals to determine when the majority of the worms were at the late L4 stage. When the majority reached the late L4 stage, the

worms were washed off the plates with M9 salts (Wood 1988) and collected in a final volume of 12 ml of M9 in a 50 ml Nalgene conical tube.

### 2.3.2. EMS mutagenesis

EMS was added to 4 ml of M9 to a final concentration of 0.2M EMS. This solution was then added to the worm suspension for a final EMS concentration of 50 mM. The tube was sealed with parafilm and rocked for 4 hours on a nutator placed in a fume hood at room temperature. Following mutagenesis the worms were split into 4 50 ml conical tubes and washed 5x with M9 buffer. After each wash the worms were collected by centrifugation at 1000rpm, 4° C for 5 minutes. The worms were plated onto 15-20 large (95x15mm) NGM plates spotted with OP50 and allowed to grow for 24 hours. The worms were washed off with M9 and treated with a bleach solution to collect eggs. The eggs were then split into 4 50 ml conical tubes containing 40 ml eggs suspended in M9 solution. The tubes were sealed with parafilm and rocked on a nutator at 4° C overnight. Roughly 500 embryos were plated onto 3 large (95x15mm) seeded (OP50) plates and incubated overnight at 25° C. The following day these plates were examined for 5 to 10% dead embryos and obvious defective worms (dumpy, rollers, etc.). If the correct level of mutagenesis was achieved, the mutagenized population was spun down, rinsed twice to remove any dauer pheromone present, and plated onto 1152 small (60x15mm) NGM plates seeded with OP50 at a concentration of 500 worms per plate. The plates were incubated for 5 days.



### 2.3.3. First round of DNA preps and initial PCR reactions

A portion of the worm populations was washed off the seeded NGM plates with water (containing 60 mg/ml streptomycin; 5 mg/ml nystatin) as follows: the plates were grouped and labeled to correspond to the 96 wells of a deep 96-well plate (A1 – H12), for a total of 12 deep well plates (12x96=1152 plates). Approximately 200 µl of the worm and water solution from the seeded NGM plates was placed in the corresponding wells of a deep 96-well plate. An equal volume of lysis solution containing ProteinaseK (recipe in appendix) was added to each well. The plates were covered, mixed thoroughly, and then placed at  $-80^{\circ}$  C for 2-3 hours. Following freezing, the deep well plates were incubated in a hybrid oven overnight at  $65^{\circ}$  C. The following morning the plates were briefly mixed and centrifuged at 2000 rpm for 1 minute. Twelve 96-well PCR plates were labeled to correspond to the twelve 96-well plates and then 150 µl of crude lysis DNA prep was transferred to the corresponding wells of each PCR plate. An additional deep 96-well plate was labeled and 50ul of worms suspended in lysis solution was added to the appropriate wells. This deep well plate contained the pooled populations that will be tested in the first round of PCR reactions. The wells were mixed, and using a multichannel pipettor, 200 µl of pooled DNA preparation was added to the corresponding wells of three 96-well PCR plates. All PCR plates were sealed and proteinase K inactivated. The PCR plates containing individual samples were also proteinase K inactivated, then stored at  $-20^{\circ}$  C. One of the pooled PCR plates was used for PCR reactions; the remaining two plates were backups in case of contamination or loss of the primary plate.

Nested PCR reactions were performed (see the appendix for specific conditions) on the pooled reactions. Inner reactions were run on 1% agarose gels containing ethidium bromide (0.5  $\mu\text{g/ml}$ ) for visualization. Potential deleted populations had a band smaller than wild-type product and were selected for further testing (for example see Figure 1, Appendix II). These lanes were identified and PCR reactions (12 per positive hit) were set up using the individual samples corresponding to the lane location. The false positive rate is fairly high for this procedure, but if the hit is real 1 of the 12 lanes will have the deleted product.

#### 2.3.4. Sib selection; populations founded by 50 worms per plate

Once a hit was identified, the plate corresponding to the location was removed from storage at 15°C. The plate were usually starved out, so it was chunked onto large (95x15mm) NGM plates seeded with OP50 and the population was allowed to recover for 1-2 days. The worms were then washed off with M9 and plated onto NGM agar seeded with OP50 in 32 x 6-well flat bottom tissue culture plates at a concentration of 50 worms per plate (9600 total worms). The plates were incubated for 5 days and the PCR prep repeated using 50ul worms (in water with streptomycin and nystatin) and an equal volume of lysis solution with ProteinaseK. Nested PCR was performed, and positive lanes (plates) identified. Often at this stage more than one plate contained mutagenized worms. Each plate was identified, then chunked onto large plates and allowed to recover. Two populations were chosen to continue the screening process. The remaining populations were frozen as insurance if one of the deletion populations was lost.

#### 2.3.5. Sib selection; populations founded by 10 worms per plate

Once the deletion populations recovered, two were chosen to continue the screen. The worms on the plates were washed off with M9 and plated onto 16 x 6-well flat bottomed tissue culture plates containing NGM and seeded with OP50 at a concentration of 10 worms per plate (960 worms per population). The plates were incubated for 5 days, and the DNA prep (50  $\mu$ l worms and water and equal volume of lysis solution containing ProteinaseK) and nested PCR reactions were performed. Positive plates were identified, chunked and allowed to recover. Two populations were chosen to continue the screening process. The remaining populations were again frozen as insurance if one of the deletion populations was lost.

#### 2.3.6. Sib selection; populations founded by single worms

Positive lanes were again identified and corresponding plates located. The plates were chunked onto large NGM plates and allowed to recover for 1 to 2 days. Individual worms were picked onto small NGM plates. The goal was to screen 96 individual worms for each positive plate. This was not always possible, as there were not always enough worms on the recovered plates. It was sometimes necessary to move forward with 3 or more populations at this point to ensure enough single plates. The plates were incubated for 5 days, and the DNA prep (100  $\mu$ l worms suspended in water and an equal volume of lysis solution containing ProteinaseK) and nested PCR reactions were performed. Positive plates were identified. A single worm carrying the deletion (most likely a heterozygote) founded these plates.

### 2.3.7. Outcrossing of strains

The final deletion mutation populations were outcrossed to AF16 (wild-type) a minimum of four times to eliminate spurious deletions (Figure 6). The deleted PCR product was gel purified, and sequenced to determine the exact location of the lesion. Primers were designed within the deleted region and designated wild-type (WT) only. These primers were used to genotype worms to isolate homozygous strains. Once homozygous strains were generated, allele and strain numbers were assigned and frozen stocks generated.

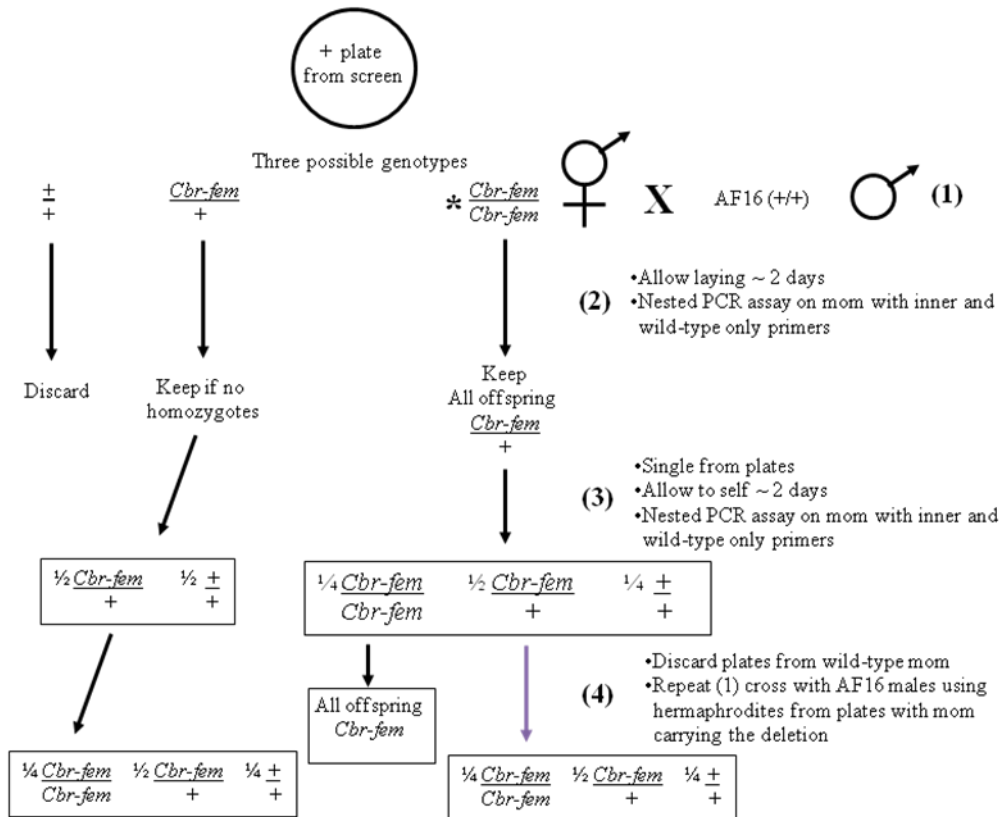


Figure 6. Outcrossing deletion strain scheme. \* *Cbr-fem-2* and *Cbr-fem-3* homozygotes were isolated from the (+) plates from the screen, so this schema was used for all outcrossing of strains

## 2.4. Results

After screening approximately 4 million haploid genomes, deletions were identified in all three *Cbr-fem* genes. (see Table 3, Appendix II, for sizes of wild-type; deletion and wild-type only fragments) :

### 2.4.1. *Cbr-fem-1* (Figures 7 and 8)

A 2 kbp deletion was generated using the “B” primer set, resulting in the deletion of greater than 40% of the coding sequence. During the process of outcrossing and generating a homozygous strain the PCR reactions became very inconsistent. Additional primers were designed and PCR conditions modified in an attempt to solve the problem, but nothing worked consistently with crudely prepared genomic DNA samples.

A homozygous strain was never obtained, and further progress was eventually blocked when frozen stocks of the initial population containing the deletion proved inviable when thawed. Thus, despite numerous attempts to salvage the deletion allele from the mutagenesis, it was ultimately lost.

### 2.4.2. *Cbr-fem-2* (*nm27*): (Figure 7 and 9)

A 1.6 kbp deletion was generated using the “B” primer set, resulting in the deletion of the entire phosphatase region as well as part of the 3' UTR. The original right primer sites were included in the 3' UTR deleted region. Fortunately, the sites were part of a tandem repeat, and the second primer sites were preserved. This allowed for recovery of the deletion. Outcrossing was performed and a self-fertile homozygous strain was generated. The allele designation for *Cbr-fem-2* is *nm27*; the strain designation is CP36.

### 2.4.3. *Cbr-fem-3* (nm63) (Figure 7 and 10)

A 1.1 kbp deletion was generated using the “A” primer set, resulting in the deletion of approximately 38% of the coding region. The deletion removed residues conserved between *C. elegans* and *C. briggsae* as well as residues known to be essential for function in *C. elegans*. The addition of three adenines during the deletion repair process maintained the correct reading frame for the remaining exons (as long as splicing was unaffected). Outcrossing was performed and a self-fertile homozygous strain was generated. The allele designation for *Cbr-fem-3* is nm63; the strain designation is CP48.

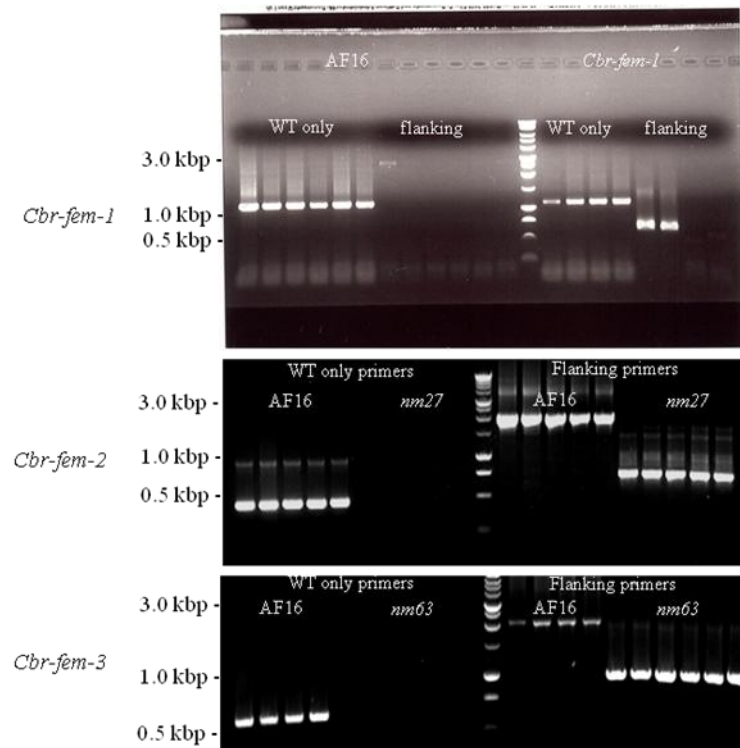


Figure 7. Identification of deletion strains by single-worm PCR with nested flanking and WT-only primers. AF16 wild-type animals display both full length flanking and WT-only product. In *Cbr-fem-1* animals only heterozygotes with deleted flanking product and WT-only product were produced prior to loss of the deletion strain. *Cbr-fem-2*(nm27) and *Cbr-fem-3*(nm63) homozygotes produce deleted flanking product and no WT-only product. Replicates are to confirm homozygous populations and ensure assay reproducibility.

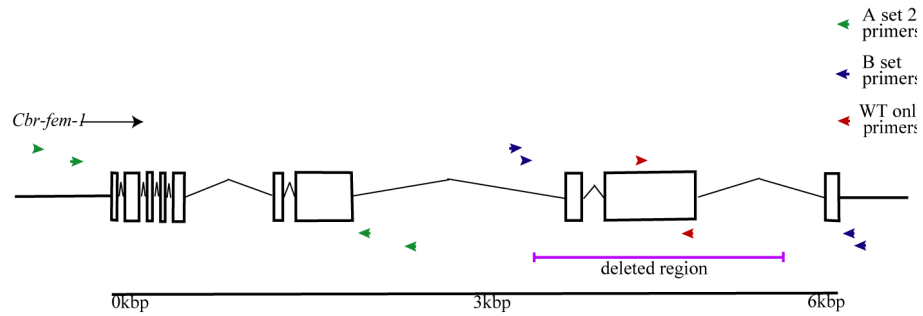


Figure 8. *Cbr-fem-1* deleted region and primer location. (List of primers used in the deletion screen as well as PCR product size and PCR conditions used to generate the products can be found in Appendix II).

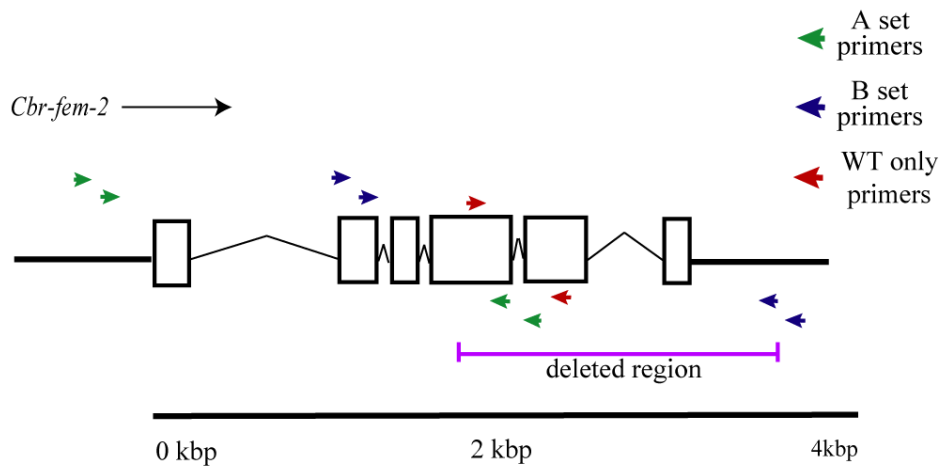


Figure 9. *Cbr-fem-2* deleted region and primer location. (List of primers used in the deletion screen as well as PCR product size and PCR conditions used to generate the products can be found in Appendix II).

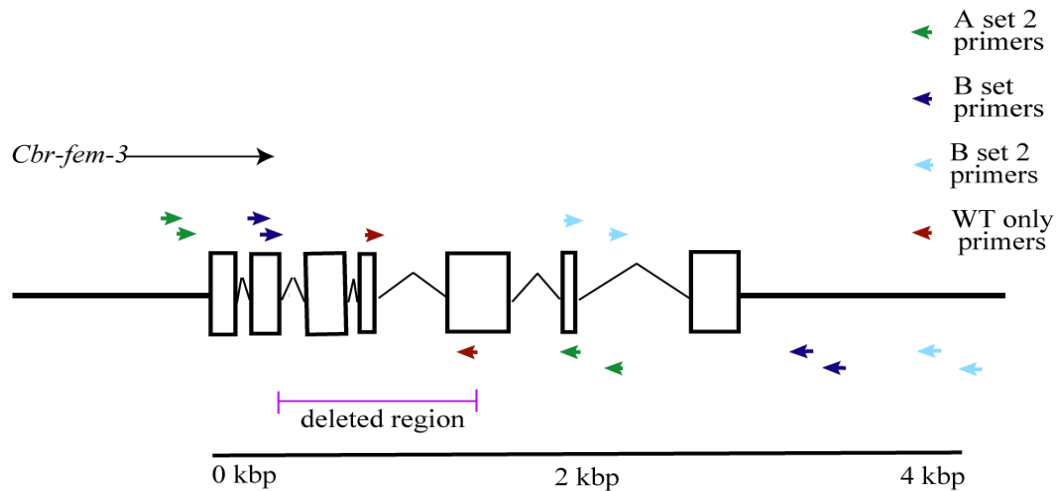


Figure 10. *Cbr-fem3* deleted region and primer location. (List of primers used in the deletion screen as well as PCR product size and PCR conditions used to generate the products can be found in Appendix II).

## 2.5. Conclusions

This project was the first use of the *C. elegans* reverse genetic screen method in *C. briggsae*, and the results prove it is a viable method for generating deletion mutants. Approximately one million haploid genomes were screened in each of four screens, with one deletion mutant identified in each screen (the first screen isolated a *fem-3* deletion, but subsequent isolation of the strain was unsuccessful). The *C. elegans* Knockout Consortium estimates approximately  $4 \times 10^6$  mutagenized genomes must be screened to identify a deletion in a particular gene (<http://www.mutantfactory.ouhsc.edu/protocols.asp>). The results of this project are in line with that estimation; four million haploid genomes were screened to obtain the mutations in the *Cbr-fem* genes.

Future optimization of this protocol should focus on developing more robust crude lysis preps. A major problem with each of the screens was inconsistent PCR



reactions. This resulted in the need to repeat numerous PCR reactions, increasing the length and cost of the screen. Should this method be used to isolate numerous genes, then investing the time in generating a frozen mutagenized library is probably a viable option.

## Chapter Three: Characterization of Deletion Mutants

### 3.1. Introduction

Null mutations in the *C. elegans fem* genes result in complete feminization of both XX and XO animals, but display maternal rescue and temperature-dependent effects (Hodgkin 1986). *Ce-fem-2*  $m^{+}z^{-}$  XX animals are hermaphrodites, and *Ce-fem-2*  $m^{+}z^{-}$  XO animals are somatically male at all temperatures. At 20° C the maternally rescued males sire noticeably fewer offspring compared to N2 (wild-type); at 25° C the males were sterile, despite containing sperm. *Ce-fem-3* XX animals are completely feminized whether the mother is homozygous or heterozygous. The offspring are as fertile as normal XX hermaphrodites. However, two doses of the wild-type *fem-3* gene appear to be required for complete self-fertility in hermaphrodites. *Ce-fem-3/+* hermaphrodites have brood sizes roughly 80% of wild type. *Ce-fem-3*  $m^{+}z^{-}$  XO animals have an intersexual phenotype. Some are self-fertile, however, they produce very few offspring.

The RNAi results in *C. briggsae* discussed in the Introduction suggested the germline phenotype differs between the two species, and the underlying mechanism controlling the phenotype might be different as well. These results indicate that the mutant phenotype generated by actual mutants would differ as well. However, given the fact that RNAi is less effective in *C. briggsae* (Haag and Kimble 2000; Haag et al 2002; Kuwabara, 1996b) and it does not completely eliminate gene function, true mutations were generated in *Cbr-fem-2* and *Cbr-fem-3* to both confirm the mutant phenotype and investigate the role of the *fem* genes in the sex determination system of *C. briggsae* (see Chapter 2).

### 3.1.1. Assessing *Cbr-fem* XX phenotype

If the RNAi results were accurate, then the XX phenotype would be evident in the process of outcrossing, as the homozygous XX animals would be hermaphroditic.

Once self-fertility was established (see Chapter 2), it was then important to check for maternal effects, brood size (less/more than wild-type), and any effects of the allele in the heterozygous state. These extensive crosses were only done for the first deletion mutant, *Cbr-fem-2(nm27)*. As the *Cbr-fem-3(nm63)* phenotype displayed the same initial phenotype, it was decided to just focus on brood size and maternal effect to characterize the XX null mutant phenotype.

Phosphatase activity has been shown to be necessary for the sex-determination function of *Ce-fem-2* (Chin-Sang and Spence 1996). The *Cbr-fem-2* deletion removes the entire phosphatase region (refer to Figure 11) and is likely a null mutation. Although the *Cbr-fem-3* deletion removes amino acids known to be essential for *Ce-fem-3* function, it is an in-frame deletion and therefore a truncated protein with some function might be generated. Additionally, the interaction between FEM-2 and FEM-3 is conserved in *C. briggsae* (Stothard and Pilgrim 2006) so it is possible that only one of the proteins is necessary for hermaphrodite development. A double XX *Cbr-fem-2(nm27);Cbr-fem-3(nm63)* was generated to address this possibility.

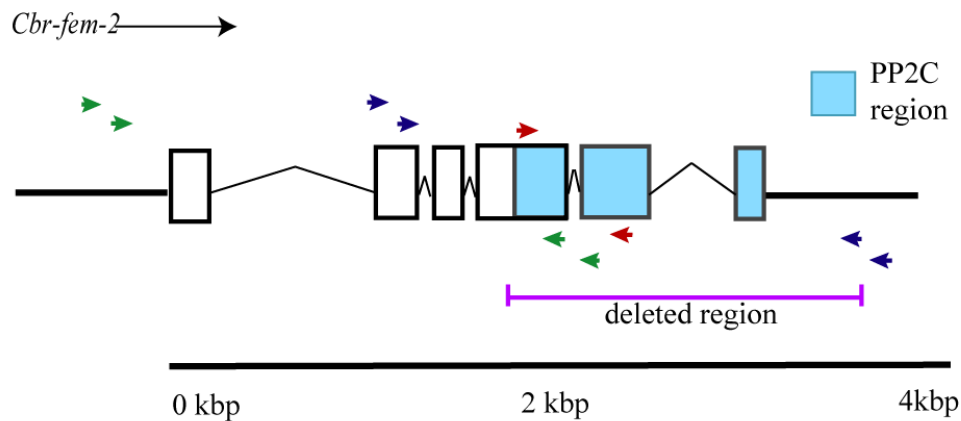


Figure 11. PP2C phosphatase region and deleted region of *Cbr-fem-2*

### 3.1.2. Assessing *Cbr-fem* XO phenotype

The initial XO phenotypic assay employed was crude sex ratios. The self-fertility of the *Cbr-fem-2* and *Cbr-fem-3*, and the PCR assay used to assess the state of the *Cbr-fem* mutation, enabled genetic crosses to be quickly performed to determine if sexual transformation of *Cbr-fem* XO animals might have taken place. If sexual transformation occurs, the percentage of males generated in the cross will be less than produced in a wild-type cross.

Two additional assays were developed to more definitively assess possible XO feminization. The first involved suppression of male development in a high incidence of males (Him) strain. Again, if sexual transformation occurs the percentage of males in this strain should decrease.

The second, and most definitive, involved the use of genetic markers for outcrossing and karyotype, which allowed the identification of individual XO feminized animals.

### 3.1.3. Assessing germline transcription of *Cbr-fem* genes

To address the possibility that a difference in phenotype between *C. elegans* and *C. briggsae* was due to differences in germline transcription, in situ hybridizations were also performed.

### 3.2. Methods used to Characterize Deletion Mutants

Strains were maintained using standard *C. elegans* methods (Wood 1988), using 2.2 % agar to discourage burrowing. All mutant strains were generated from AF16, a wild type isolate of *Caenorhabditis briggsae*. Reagents were prepared as per the protocols found in the Appendix.

Mutations used in this study include *Cbr-dpy(nm4) II*, *Cbr-fem-2(nm27) III*, *Cbr-fem-3(nm63) IV*, and *syIs802[myo-2::GFP] X*.

#### 3.2.1. Method to assess *Cbr-fem* XX hermaphrodite fertility

Once outcrossing was complete, single unmated hermaphrodites were placed on large (95x15mm) NGM plates spotted with OP50 bacteria. Large plates were employed as it was observed single hermaphrodites tend to leave the agar surface, crawling onto the side of the plate and desiccating. They were allowed to lay their progeny, and then removed to be genotyped by PCR assay (see Appendix I for details of PCR assay). The offspring were observed to see if they hatched and developed to adulthood, and to see if the offspring were also self-fertile. Another group of single, unmated hermaphrodites were placed on large (95x15mm) NGM plates, and then moved to new plates every 8 – 10 hours to make counting offspring easier and to decrease the possibility of the hermaphrodite leaving the plate. Brood sizes were

determined for hermaphrodites who laid embryos for at least two days. This was the only method of characterization performed for *Cbr-fem-3(nm63)*.

Additional crosses were performed for the first deletion mutant isolated, *Cbr-fem-2(nm27)*, to determine the effects of the allele in the heterozygous state as well the presence of any maternal effect. The same method as above was employed. The complete list of crosses performed can be found in Table 2. Crosses were repeated a minimum of 5 times, then averaged to obtain the mean brood size.

Table 3. Sex ratios and *Cbr-fem* genotypes

Mother	Father	Mean brood size* (SEM)	% male (SEM or upper bound <sup>††</sup> )	No. of Crosses	Progeny counted
AF16	self	200 (8.9)	0.0 (0.2)	6	1203
AF16	AF16	288 (42.2)	39.7 (3.26)	5	1439
AF16	<i>Cbr-fem 2(nm27/+)</i>	273 (61.7)	34.6 (2.08)	5	1364
<i>Cbr-fem 2(m27)</i>	Self	124 (6.3)	0.0 (0.1)	17	2110
<i>Cbr-fem 2(m27)</i>	AF16	209 (23.6)	39.6 (1.87)	10	2093
<i>Cbr-fem 2(m27)</i>	<i>Cbr-fem 2(nm27/+)</i>	256 (31.6)	22.8 (0.87)	8	2051
<i>Cbr-fem 2(m27/+)</i>	Self	182 (9.4)	0.0 (0.3)	5	912
<i>Cbr-fem 2(m27/+)</i>	AF16	195 (31.7)	36.8 (2.19)	8	1560
<i>Cbr-fem 2(m27/+)</i>	<i>Cbr-fem 2(nm27/+)</i>	302 (41.0)	33.2 (2.85)	7	2112
<i>SyIs[myo-2::GFP]X/+<sup>†</sup></i>	Self	57 (13.6)	31.5 (1.87)	7	400
<i>SyIs[myo-2::GFP]X/+;</i> <i>Cbr-fem-2(nm27)III<sup>†</sup></i>	Self	80 (14.1)	0.0 (0.9)	4	319
<i>Cbr-fem-3(nm63)</i>	Self	234 (2.7)	0.0 (0.1)	10	2341
<i>SyIs[myo-2::GFP]X/+;</i> <i>Cbr-fem-3(nm63)IV</i>	Self	n.d. <sup>¶</sup>	0.0 (1.8)	n.d. <sup>¶</sup>	169
<i>Cbr-fem-2(nm27);</i> <i>Cbr-fem-3(nm63)</i>	self	136 (7.9)	0.0 (0.4)	6	816

\* Scored only if hermaphrodite remained on plate for at least 48 hours.

\*\* A high degree of embryonic lethality was seen, which is somewhat alleviated by growth at 15<sup>o</sup> C. This may be due to enhancement of the *syIs802*-mediated meiotic non-disjunction in the *nm63* background .

\*\*\* Cross and data collection performed by Eric Haag.

\$ 95% upper confidence bound computed from the Poisson distribution as per Hanley's rule.

### 3.2.2. Method to generate XX *Cbr-fem-2(nm27);Cbr-fem-3(nm63)*

A double *Cbr-fem-2;Cbr-fem-3* mutant was generated as detailed in Figure 12. *Cbr-fem-2 (nm27)* hermaphrodites were mated to AF16 (wild type) to generate *Cbr-fem-2(nm27)/+* males. These males were then crossed to *Cbr-fem-3 (nm63)* hermaphrodites. One half of the offspring from this cross will be *Cbr-fem-2 (nm27)/+; Cbr-fem-3(nm63)/+*. The offspring were singled, allowed to lay for about two days, then removed for genotyping via PCR assay. The PCR assay was performed as per method detailed in the Appendix I with the following modification. Two PCR assays must be performed on each worm to determine the genotype of the potential double mutants (outer reactions for both *fem-2* and *fem-3*), so each individual worm lysis solution is increased to 10ul, mixed well, and then split into two 5ul aliquots for the PCR assays. A large number of animals (at least 96 to ensure isolating a double mutant) were singled from the plates founded by double *fem* heterozygotes, allowed to lay for two days, then removed for PCR assay to determine genotype. Approximately 1/16 will be *Cbr-fem-2(nm27);Cbr-fem-3(nm63)* individuals.

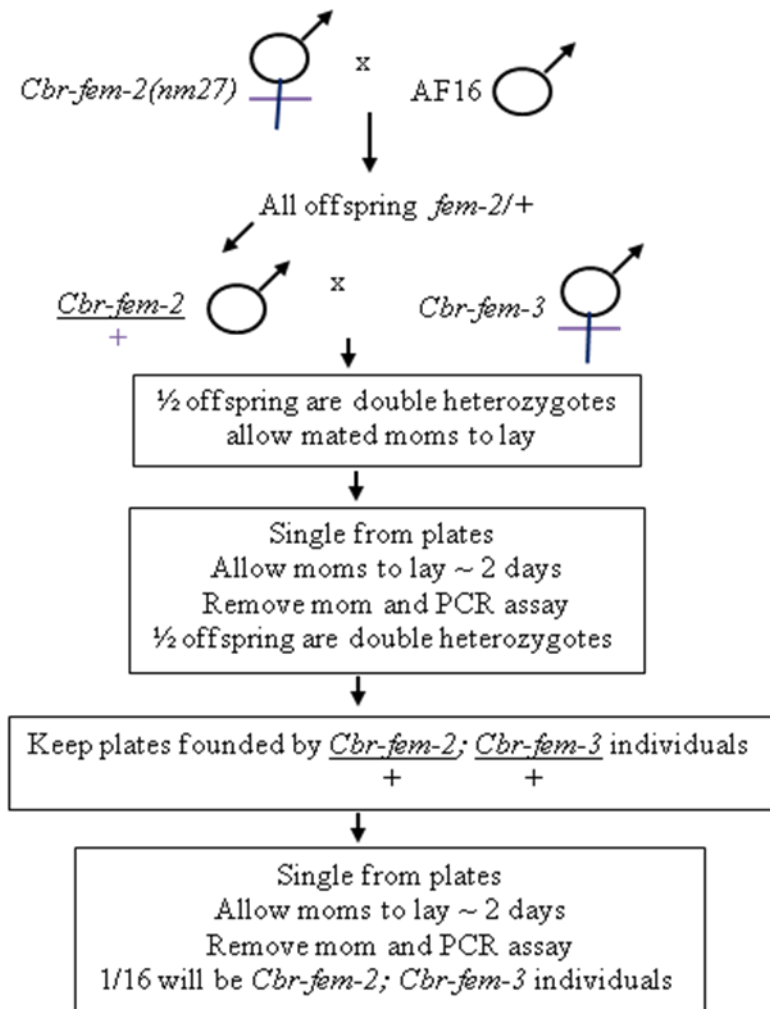


Figure 12. Scheme for generating  $Cbr-fem-2;Cbr-fem-3$  double mutants

### 3.2.3. Method to characterize the $Cbr-fem$ XO phenotype

Three methods were developed to determine the  $Cbr-fem$  XO phenotype.

#### I. Crude sex ratios

$Cbr-fem-2(nm27)$  hermaphrodites were crossed to AF16 (wild type) males to generate  $Cbr-fem-2(nm27)/+$  males. The  $fem-2$  heterozygous males were then crossed to  $Cbr-fem-2 (nm27)$  hermaphrodites (Figure 13), following the same mating scheme described above, with the single mated hermaphrodites placed on large (95x15mm)



NGM plates, and moved to fresh plates every 8-10 hours to make counting offspring easier and decrease to possibility of the hermaphrodite leaving. Brood sizes and

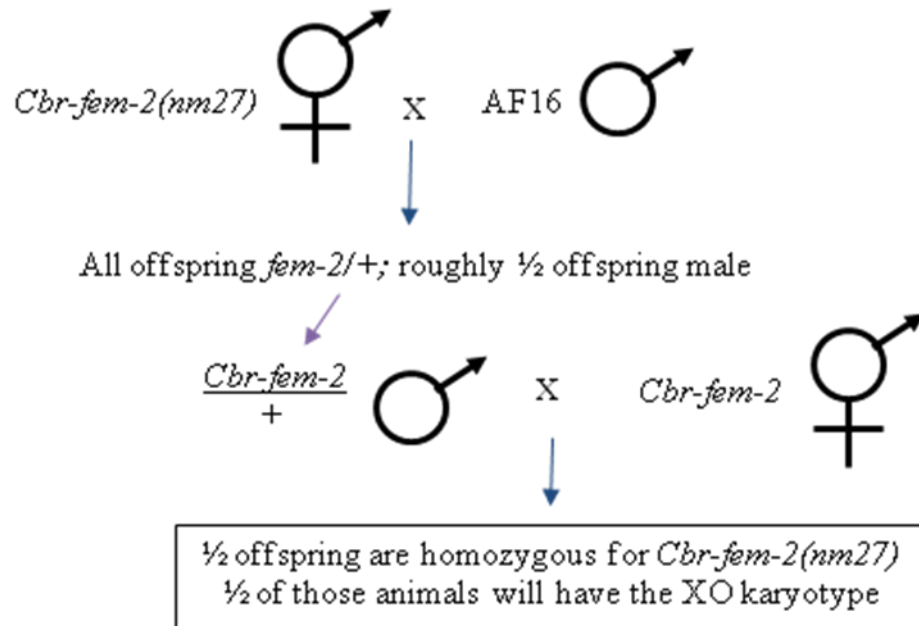


Figure 13. Scheme for generating XO animals homozygous for *Cbr-fem-2(nm27)* percentage of male offspring were determined for hermaphrodites who laid embryos for at least two days. Crosses of *Cbr-fem-2(nm27)/+* males and *Cbr-fem-2(nm27)/+* hermaphrodites were also performed using the above mating scheme. Approximately  $\frac{1}{4}$  of the offspring would be homozygous for the *Cbr-fem-2(nm27)* deletion; roughly  $\frac{1}{2}$  of the offspring should be male. Brood sizes and percentage of males were determined for hermaphrodites who laid embryos for at least two days.

## II. Him suppression

*syIs802 [myo-2::GFP]X* hermaphrodites were crossed to AF16 (wild type) males to generate *syIs802[myo-2::GFP]X* males (Figure 14). Animals were checked

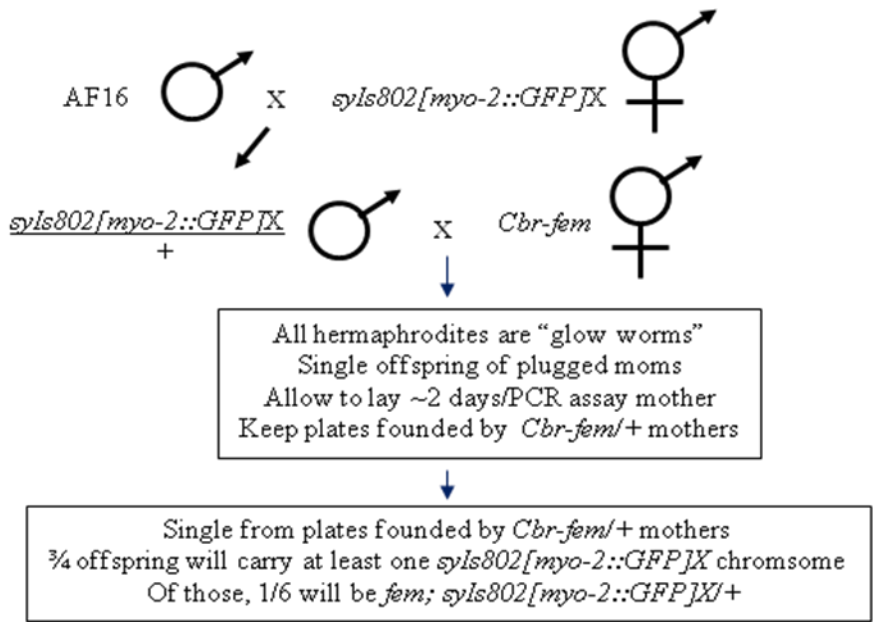


Figure 14. Generation of strain used in Him Assay

by epifluorescence microscopy for the presence of the integrated GFP transgene. *Cbr-fem* hermaphrodites were mated to the *syIs802 [myo-2::GFP]X* males. Multiple hermaphrodites and males were placed on the same small (60x15mm) NGM plate, and then plugged hermaphrodites were singled to small (60x15mm) NGM plates. Hermaphrodites were singled from these plates onto small (60x15mm) NGM plates and allowed to lay. The offspring were singled onto small (60x15) NGM plates and allowed to produce progeny, then PCR assayed (see Appendix I for details of PCR assay) for the *Cbr-fem* gene deletion. From the plates founded by *syIs802 [myo-2::GFP]X*/+; *Cbr-fem*, brood size and the percentage of males was determined as described earlier.

### III. Definitive crosses with X-linked and outcross markers

Two strains were generated (see Figure 15) to perform the definitive cross.

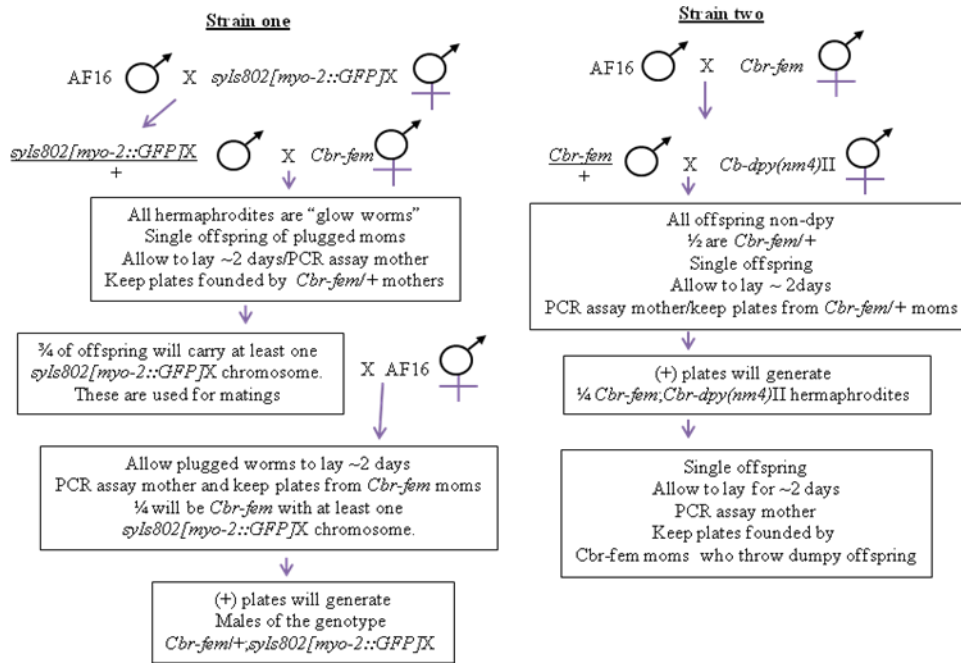


Figure 15. Generation of strains used in definitive cross for characterization of *Cbr-fem* XO phenotype

Strain one generated the *Cbr-fem*/+; *syIs802 [myo-2::GFP]X* males, which was used to identify karyotype. The crossing scheme of the Him suppression was followed, but the hermaphrodite offspring of the cross between *Cbr-fem* hermaphrodites x *syIs802 [myo-2::GFP]X* male were crossed to AF16 males to generate *Cbr-fem*/+; *syIs802[myo-2::GFP]X* males. All crosses were performed with multiple males and hermaphrodites on a single small (60x15mm) NGM plate. Following mating, plugged hermaphrodites were singled to small (60x15mm) NGM plates and allowed to lay

offspring. PCR assays to confirm the genotype of the mother were performed as per method described in Appendix I, and were conducted per the steps described in Figure 15.

The second strain generated was the *Cbr-fem;dpy(nm4)II* hermaphrodite, which was used to identify self progeny from outcross progeny. As with the generation of the first strain, all crosses were performed with multiple males and hermaphrodites on a single small (60x15mm) NGM plate. Following mating, plugged hermaphrodites were singled to small (60x15mm) NGM plates and allowed to lay offspring. PCR assays to confirm the genotype of the mother were performed as per method described in Appendix I, and were conducted at the steps described in Figure 15.

AF16 (wild type) males were crossed to *Cbr-fem* hermaphrodites to generate males heterozygous for the *Cbr-fem* deletion. These males were crossed to *Cbr-dpy(nm4)II* hermaphrodites. Mated hermaphrodites were singled and allowed to lay for roughly two days, then PCR assayed to identify *Cbr-fem/+* mothers. Hermaphrodites were singled from the plates founded by *Cbr-fem/+* mothers. Approximately ¼ of the single offspring would be of the genotype *Cbr-fem;Cbr-dpy(nm4)II*. These offspring were allowed to lay for about two day, then PCR assayed to identify homozygous worms. The dumpy phenotype was visually determined. Plates founded by *Cbr-fem;Cbr-dpy(nm4)II* were retained; the remaining were discarded.

#### 3.2.4. Method for In situ hybridization

Eric Haag generated the digoxigenin (DIG)-labelled DNA probes used in the hybridizations by asymmetric PCR. A description can be found in Hill et al (2006). All methods were performed as in Jones et al (1996) and described in detail at [http://www.genetics.wustl.edu/tslab/Protocols/gonad\\_in\\_situ.html](http://www.genetics.wustl.edu/tslab/Protocols/gonad_in_situ.html), except the concentration of Proteinase K was increased to 100 µg/ml to improve signal. Eric Haag performed all *Cbr-fem-2* gonad dissections and hybridizations. Both Eric Haag and Robin Hill performed *Cbr-fem-3* gonad dissections. Robin Hill performed all *Cbr-fem-3* hybridizations. DNA staining was by 0.5 µg/ml Hoechst 33258 in PBST.

### 3.3. Results

#### 3.3.1. *Cbr-fem* XX characterization results

##### *XX Cbr-fem-2 (nm27)*

This was the first mutant isolated. The *Cbr-fem-2* PCR assays for deletion and wild-type chromosomes was used to verify a homozygous strain, which was assigned the strain designation CP36. This strain was used for all subsequent crosses requiring a homozygous *Cbr-fem-2* strain.

*Cbr-fem-2(nm27)* XX animals are self-fertile (Figure 16a), with brood sizes approximately 60% that of AF16 (wild-type) hermaphrodites (Table 1). Homozygous XX animals produce the same proportion of males as AF16 wild type XX animals when crossed with wild-type males (Table 2). Brood size of the *Cbr-fem-2(nm27)* hermaphrodite x AF16 male cross is approximately 72% of wild type. The self-fertile

phenotype is unlike the *C. elegans* phenotype, akin to feminization (*fem*) of the germline (Hodgkin 1986).

It is more like the *C. elegans her-1* mutation (Hodgkin 1980). Unlike *Ce-fem-2*, there is no maternal effect, as there is no mutant phenotype to “rescue”.

#### XX *Cbr-fem-3* (nm63)

PCR assays were also used to verify a strain homozygous for *Cb-fem-3*(nm63), which was assigned strain designation CP48. This strain was used for all subsequent crosses requiring a *Cbr-fem-3* homozygous strain. *Cb-fem-3* XX mutants are self-fertile hermaphrodites (Figure 16b), with a robust brood size over many generations (Table 2). No further genetic crosses were done in the characterization of the *Cb-fem-3* XX hermaphrodites. As with *Cbr-fem-2* (nm27), there is no maternal effect as there is no mutant phenotype to “rescue”.

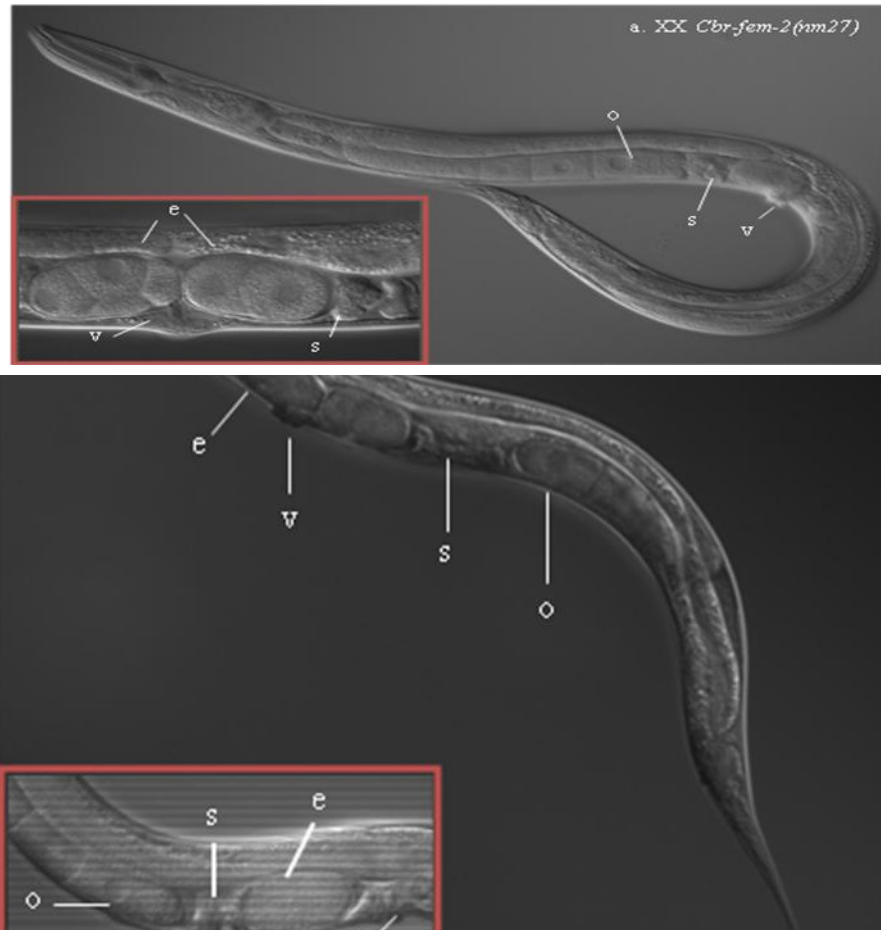


Figure 16. Phenotypes of *Cbr-fem* XX hermaphrodites. a. *Cbr-fem-2(nm27)* b. *Cbr-fem-3(nm63)*. Labels. e, embryo; s, sperm with spermatheca; v, vulva; o, oocyte.

### XX *Cbr-fem-2(nm27);Cbr-fem-3(nm63)*

The double XX hermaphrodite is also a self-fertile (Figure 17 and Table 2), with a brood size roughly 68% that of *C. briggsae* wild type (AF16).

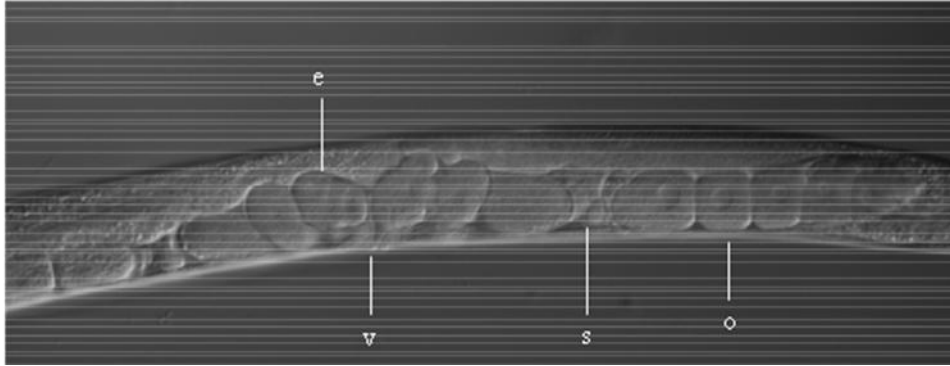


Figure 17. Phenotype of *Cbr-fem-2(nm27); Cbr-fem-3(nm63)* hermaphrodites. Labels: e, embryo; v, vulva; s, spermatheca; o, oocytes

### 3.3.2. *Cbr-fem* XO characterization results

#### Crude Sex Ratios

Genetic crosses were performed which would produce *Cbr-fem* XO offspring. These crosses would result in less than the expected 40% males if there were male to hermaphrodite transformation. In *C. briggsae*, male sperm have a competitive advantage over hermaphrodite sperm and X-bearing male sperm have a competitive advantage over nullo-X male sperm, resulting in less males from outcrossing than in seen in *C. elegans* (LaMunyon and Ward 1997). They note the initial offspring of a mated *C. briggsae* hermaphrodite are predominantly hermaphrodite and the percentage of males increases over time, reaching about 40% by day 2 post-mating. This pattern was observed in the wild-type crosses conducted in this experiment (data not shown). Given the fact that in *C. elegans* both sexes have the same germline phenotype, this



was a possibility. Transformation of males should result in approximately half the expected male offspring. Crosses of *Cbr-fem/+* XX with *Cbr-fem/+* XO animals should also result in less than expected proportion of males if sexual transformation occurs. This assay was only employed for *Cbr-fem-2(nm27)*, as by the time the *Cbr-fem(nm63)* was generated, more definitive assays had been developed.

#### *Cbr-fem-2(nm27)* XO animals

Genetic crosses not resulting in *Cbr-fem-2 (nm27)* homozygotes produced male progeny in the wild-type range (~40%). If *Cbr-fem-2 (nm27)* can feminize XO animals, crosses between *Cbr-fem-2(nm27)* hermaphrodites and *Cbr-fem-2(nm27)/+* males should produce approximately ½ of the normal number of male offspring, or roughly 20%, and they do (see Table 2). Crosses of *Cbr-fem-2/+* XX hermaphrodites X *Cbr-fem-2/+* XO males also resulted in less than the expected percentage of male offspring (Table 2). This is suggestive of complete transformation of males into hermaphrodites even in the presence of wild-type maternal contribution.

To more definitively investigate maternal rescue, *Cbr-fem-2(nm27)/+III; dpy(nm4)/+ II* hermaphrodites were crossed to *Cbr-fem-2(nm27)/+ III; syIs802 X males* (Hill et al 2006, supplemental data). XO animals from this cross were non-Dpy and non-GFP, and ½ of these animals were homozygous for the *fem-2* mutation. These results show that unlike *C. elegans*, *C. briggsae fem-2* sex determination function is not maternally provided or temperature-sensitive.

### *Cbr-fem-3(nm63)* XO animals

The phenotypic similarity of *Cbr-fem-2* and *Cbr-fem-3* XX hermaphrodites suggested the phenotype of *Cbr-fem* XO animals would also be the same, therefore genetic crosses to determine sex ratios were not employed to establish the phenotype of *Cbr-fem-3(nm63)*.

For investigating possible maternal rescue in *Cbr-fem-3(nm63)*, crosses between *Cbr-fem-3(nm63)/+* hermaphrodites and males were performed. The *fem-3* phenotype in *C. elegans* is lower brood size and intersexual XO *Cbr-fem-3(nm63)* offspring. The mated *fem-3* heterozygous mothers were allowed to lay and the offspring visually examined for an intersexual phenotype (incomplete male tail, vulva, etc.). Brood size was determined by the previously described method, but mothers were moved once a day rather than every eight hours.

### Him Suppression

This particular assay was developed by chance. During the generation of the strains for crosses using X-linked and molecular markers for our first deletion, *Cbr-fem-2(nm27)*, it was observed (initially by Danielle Kelleher) that hermaphrodites heterozygous for the X-chromosome integrated green fluorescent protein (GFP) reporter transgene *syIs802* produce small broods with approximately 32.5% males. Hermaphrodites of the genotype *Cbr-fem-2(nm27);syIs802/+* produced no males. Although it was possible that this result was unique to *Cbr-fem-2*, we decided to employ this test for the XO phenotypic characterization of *Cbr-fem-3*.

*Cbr-fem-2(nm27)* XO animals

*Cbr-fem-2(nm27)* was able to suppress the production of males in a high incidence of males (Him) strain; Hermaphrodites of the genotype *Cbr-fem-2(nm27);syIs802/+* produced no males (Table 1). This, again, was suggestive of sexual transformation.

*Cbr-fem-3(nm63)* XO animals

*Cbr-fem-3(nm63)* was also able to suppress male production in *syIs802/+* hermaphrodites (Table 1); again suggestive of sexual transformation of males within this normally Him strain.

Definitive crosses with X-linked and outcrossed markers

In order to positively establish XO male transformation and identify individual transformed males, *syIs802*, an X chromosome-integrated GFP (green fluorescent protein) reporter transgene, and the dumpy marker *dpy(nm4) II* were employed for outcrossing and karyotyping. The rationale for this cross can be found in figure 18.

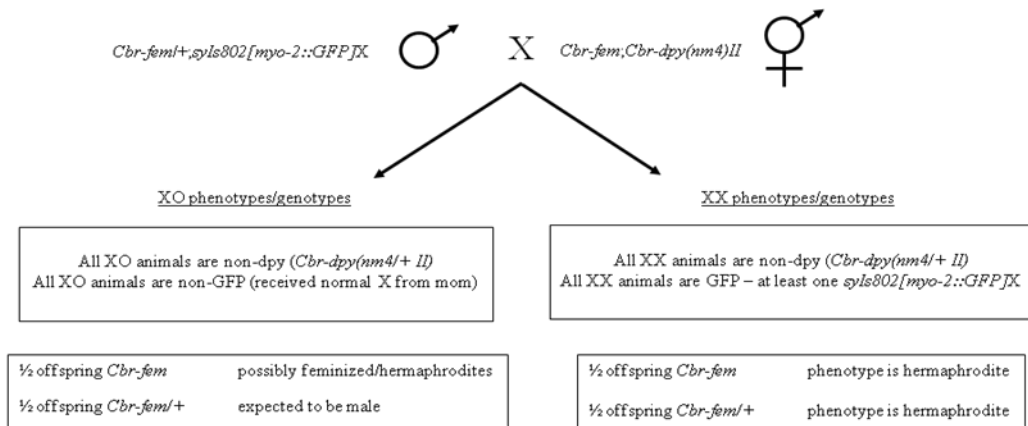


Figure 18. Outcomes of crosses with X-linked and outcrossed markers.

### XO *Cbr-fem-2(nm27)*

In crosses utilizing X-linked (GFP) and outcross (DPY) markers, 59% of non-Dpy, non-GFP progeny (N=90) were somatically feminized; the remainder were normal males. PCR assay determined that the somatically feminized worms were *Cbr-fem-2* homozygotes.

*Cbr-fem-2 (nm27)* XO animals are transformed into fertile hermaphrodites (Fig.19a). Unlike *C. briggsae fem-2 (nm27)* XX hermaphrodites, *nm27* XO hermaphrodites have very small brood sizes and minor defects in somatic gonad development (Fig.19b). The small brood size is at least partially the result of karyotype. Despite the transformation, they still produce either X or nullo gametes. The process of selfing would generate approximately half double nullo zygotes. However, they do produce viable offspring and they are able to mate.

Work done by a collaborator, Carlos Carvalho, at the University of Alberta, Edmonton, determined *Cbr-fem-2/+* males have a late-onset germline feminization. This is similar to *Cbr-fem-2* (RNAi) results (Stothard, et al. 2001).

### XO *Cbr-fem-3(nm63)*

The same genetic marker crossing scheme employed for the *Cbr-fem-2 (nm27)* characterization was used to characterize *Cbr-fem-3(nm63)*. As in *Cbr-nm27*, 59% of *Cbr-fem-3 (nm63)* non-Dpy, non-GFP XO progeny (N=88) were feminized.

*Cbr-fem-3 (nm63)* XO mutants are self-fertile hermaphrodites (Fig.19c). Similar to *nm27*, *Cbr-fem-3(nm63)* XO hermaphrodites have low brood sizes and somatic gonad defects (not shown).

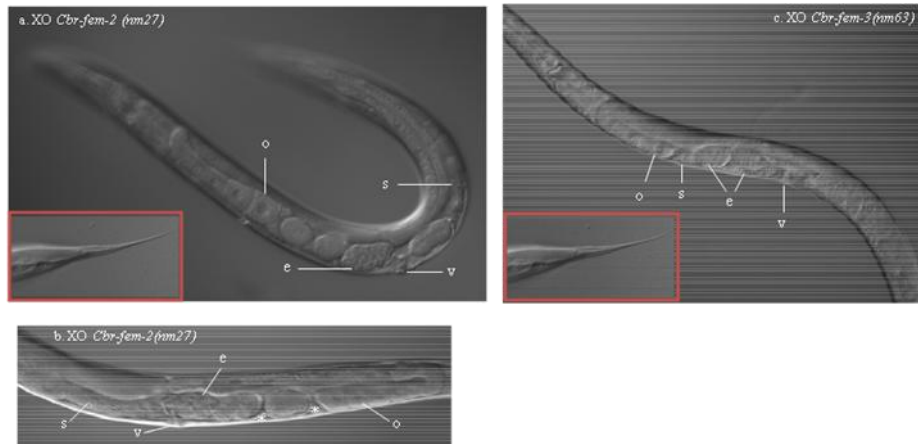


Figure 19. Phenotypes of *Cbr-fem* XO hermaphrodites. **a.** XO *Cbr-fem-2(nm27)*. Insert is well formed hermaphrodite tail. **b.** XO *Cbr-fem(nm27)* hermaphrodite with somatic defects. There are two spermatheca in the posterior gonad and none in the anterior gonad. **c.** XO *Cbr-fem-3(nm63)* hermaphrodite. Insert is well formed hermaphrodite tail. Labels: e, embryo; o, oocytes; s, sperm with spermatheca; v, vulva; s+ sperm loose in anterior gonad arm; \* two spermatheca in the posterior gonad

### 3.3.3. Germline expression of the *Cbr-fem* genes

Given the differences between the *C. elegans* and *C. briggsae* germline phenotypes, it was possible that difference was due to changes in germline mRNA expression. Rosenquist and Kimble (1988) demonstrated that in *C. elegans* mutants lacking germlines, somatic expression of *Ce-fem-3* is much lower than in *Cbr-fem-3*. To address this issue, in situ hybridizations were performed. As seen in Figure 20 A-E, hermaphrodites in both species produced *fem-2* mRNA at comparable levels. Expression was absent at the distal tip, but could be seen in oocytes as they begin gametogenesis, and was particularly strong in mature diakinesis oocytes.

*Cbr-fem-3* mRNA was expressed in a similar pattern that that of *Cbr-fem-2*. No staining was observed at the distal tip, but staining increased as oocytes move in a proximal direction. *Cbr-fem-3* staining in oocytes was often perinuclear, and was also observed in somatic tissues, including the gut (Figure 20 F-G).

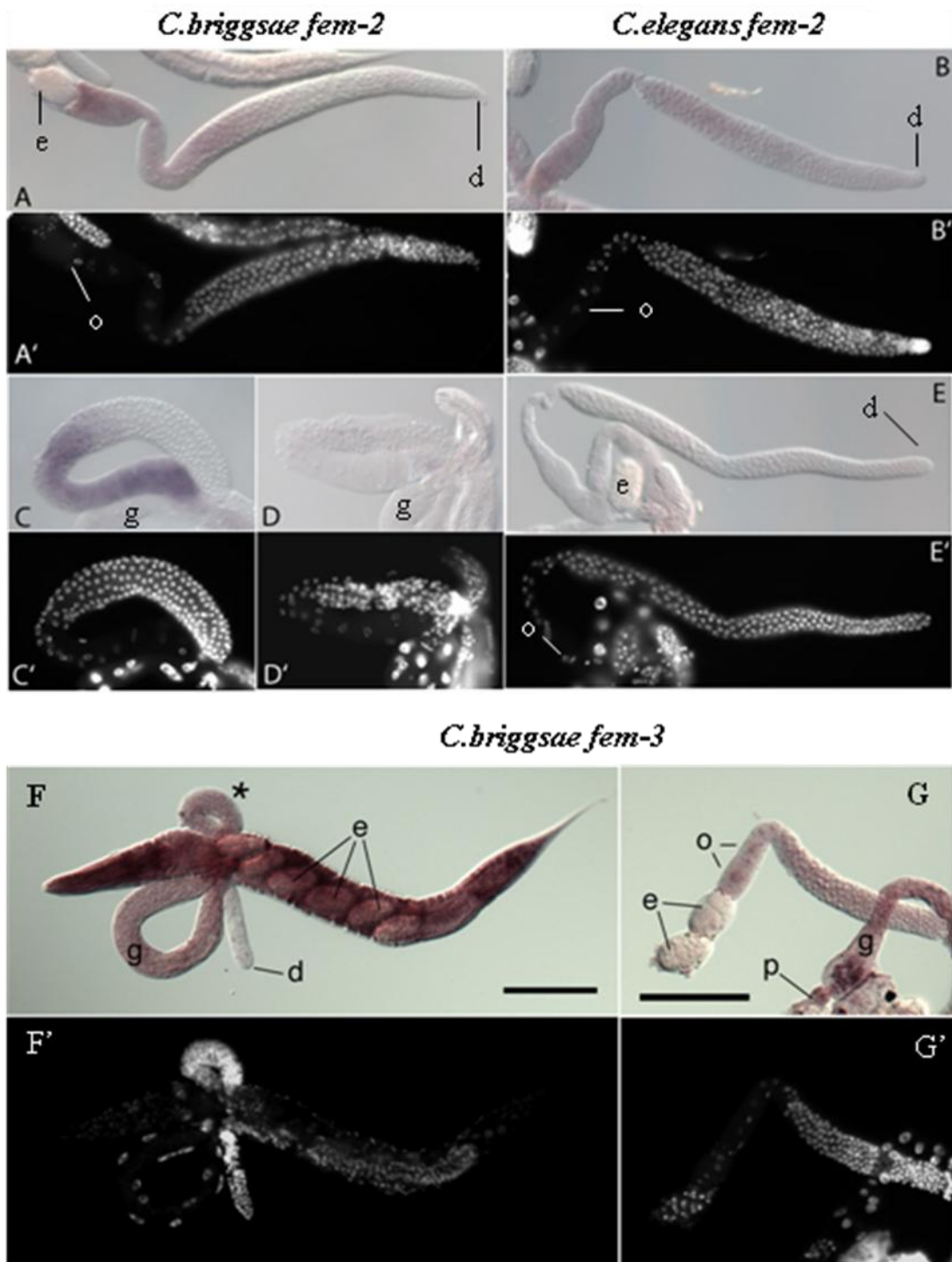


Figure modified from Cook Hill et al 2006. *fem-2* work by E.Haag

Figure 20 (previous page). mRNA expression of *fem-2* and *fem-3* in the *C.briggsae* germline. **A.** Antisense hybridization of *Cbr-fem-2* cDNA probe to extruded wild-type (AF16) *C.briggsae* gonad. **B.** Antisense hybridization of *Ce-fem-2* cDNA probe to extruded wild-type *C.elegans* gonad. **C.** *C.briggsae* AF16 extruded gonad probed with antisense *Cbr-fem-2* cDNA. A higher (double) concentration of Proteinase K was used to enhance permeability, but this resulted in more fragile gonad arms and only partially extruded arms could be tested. **D.** *Cbr-fem-2* gonad arm treated as in (C) and probed with antisense *Cbr-fem-2* cDNA probe. **E.** *Ce-fem-2* cDNA sense probe control hybridization to extruded wild-type (N2) *C.elegans* gonad. **F.** Partially extruded gonad arm and (G) completely extruded arm from *C.briggsae fem-3* hermaphrodite. Both are probed with *Cb-fem-3* antisense cDNA. **A' – G.** Hoechst 33258-stained DNA images of the sample above each fluorescent image. Orientation of images: Proximal gonad end to the left in **A, B, E, G**. Proximal gonad the bottom of loop in **C** and **D**. **Labels:** d, distal tip of germline; e, embryo; g, gut; o, oocyte; d, diakinesis oocyte; p, pharynx; \* loop of gonad arm.

### 3.4. Conclusions

These results generally support the earlier RNAi data (see Table 2). Thus, although the phenotype generated by the core sex determination system in *C. elegans* and *C. briggsae* is similar, the modifications of the core pathway in the germline appear distinct in the two species (see Table 4 for comparison of phenotypes). In *C. elegans*, the *fem* genes, in particular the negative regulation of *fem-3*, is required for the sperm to oocyte switch (Ahringer and Kimble 1991; Kuwabara and Perry 2001). The lack of mutant phenotype in *C. briggsae* hermaphrodites indicates neither *Cbr-fem-2* nor *Cbr-fem-3* is required for hermaphrodite spermatogenesis, although the smaller brood size of *Cbr-fem-2* (*nm27*) could indicate some minor role in spermatogenesis, or a particular effect of this allele. It was noted in Hodgkin (1986) that there are phenotypic differences among the *Ce-fem-2* alleles (Hodgkin 1986). There are currently no additional alleles of *Cbr-fem-2* for comparison.

	<i>C.elegans</i>		<i>C.briggsae</i>	
	XX	XO	XX	XO
<i>fem-2</i>	Fertile female if mom homozygous.  Hermaphrodite if mom is heterozygous.  Non-ts	@ 20° C Fertile female if mom homozygous.  Abnormal male if mom heterozygous.  @ 25° C Fertile female if mom homozygous.  Abnormal male if mom heterozygous	Hermaphrodite	Hermaphrodite with minor somatic gonad defects
<i>fem-3</i>	Fertile female	Fertile female if mom homozygous	Hermaphrodite	Hermaphrodite with minor somatic gonad defects

Table 4. Comparison of *C.elegans* and *C.briggsae fem-2* and *fem-3* loss of function mutant phenotypes. Data on *C.elegans* from Wormbase (<http://www.wormbase.org/>).

It was possible that the lack of XX phenotype could be the result of redundancy among the *Cbr-fem* genes. However, the phenotype of the *Cbr-fem2 (nm27);Cbr-fem-3(nm63)* mutant is a self-fertile hermaphrodite, suggesting there is no redundancy between at least *fem-2* and *fem-3*. The lower brood size of *Cbr-fem-2(nm27)* hermaphrodites could again indicate some minor effect on spermatogenesis. Interestingly, however, the *Cbr-fem2 (nm27);Cbr-fem-3(nm63)* double mutant has essentially wild-type fertility. It is thus also possible the lower *Cbr-fem-2* brood size was an artifact of the counting method used. Mothers were only allowed two days to lay offspring, as after two days the incidence of “leaving” the plate increased dramatically. If they had a reduced rate of fertilization, they may have been picked relatively earlier in their reproductive careers, thus lowering their apparent brood size.



Although the *Cbr-fem-2* (*nm27*) allele is a probable null, it is part of a large, 11-member PP2C phosphatase family (Cook Hill et al 2006). It was possible the lack of XX feminization was due to redundancy with another phosphatase. It was also possible that the *Cbr-fem-3* (*nm63*) mutation could produce a truncated protein with some activity. This would also lead to a lack of feminization in XX hermaphrodites. The XO phenotype indicates that *Cbr-fem-2* and *Cbr-fem-3* are required for male somatic development and to maintain male spermatogenesis. Therefore, there does not appear to be redundancy with other PP2C phosphatases. Additionally, if a *fem-3* truncated protein is produced, it does not appear able to produce male somatic or germline identity.

Our collaborator, Carlos Carvalho, determined *Cbr-fem-2*/+ males have a late-onset germline feminization. This was not a phenotype I observed. The crosses employed in the characterization of the XO *Cb-fem* phenotype used young males, which are fertile. Additionally, multiple males were plated with each hermaphrodite to increase successful mating, so the smaller brood sizes that would most likely occur with a single sperm-limited male siring progeny were not observed.

Both *Ce-fem-2* and *Ce-fem-3* mutants exhibit maternal effects, though the actual phenotypes differ. *Ce-fem-2* also exhibits a temperature dependent maternal effect; *Ce-fem-3* does not. In the test for maternal effect in *Cbr-fem-2* (*nm27*) XO transformed progeny were hermaphrodites. Furthermore, in *C. briggsae* the transformation is complete at 20°C (the transformation is complete at 25°C in *C. elegans*). This indicates that *C. briggsae fem-2* function is not provided by the mother or subject to temperature dependency. *Ce-fem-3*/+ XX mutants display a reduced brood size; XO mutants

exhibit an intersexual phenotype. Neither of these defects were observed for *Cbr-fem-3* (*nm63*) mutants.

The results of in situ conducted by Eric Haag indicate the germline expression patterns of both *Ce-fem-2* and *Cbr-fem-2* is similar. Although *Ce-fem-3* germline expression was not assessed in this research, personal communication with A.Puoti suggests the *Ce-fem-3* and *Cbr-fem-3* expression patterns are similar as well. Therefore, the differences in *fem-2* and *fem-3* function between the two species cannot be the result of differences in germline transcription.

It is possible that the lack of feminization of both the *C.briggsae* hermaphrodite germline and the male soma and germline is the result of wild-type *fem-1* activity. A deletion mutant was not successfully generated for *Cbr-fem-1*, therefore it is active in the *fem-2* and *fem-3* mutants. As stated earlier, in *C.elegans* FEM-1 is the substrate recognition subunit for a CUL-2 ubiquitin ligase complex that regulates TRA-1 activity via proteolysis of full-length TRA-1A (one of three isoforms of TRA-1) (Starostina et al 2007). Complete degradation of full-length TRA-1A requires all three FEM proteins, but partial proteolysis is seen with just FEM-1. If this mechanism is conserved in *C.briggsae*, then it is possible a functional FEM-1 protein allows the production of sperm in both XX and XO animals mutant for the other *fem* genes. This possibility can be addressed by generating a *Cbr-fem-1* deletion mutant. Additionally, a TRA-1 antibody would allow the determination of TRA-1 levels in the *Cbr-fem* mutants.

## Chapter Four: Comparative Analysis of the *tra-1/fem* germline interaction in *C. elegans* and *C. briggsae*.

### 4.1. Introduction

In *C.elegans*, most mutant alleles of the *fem* genes were identified as suppressors of *tra-2* and *tra-3* alleles (Hodgkin 1986). John Salogiannis, an undergraduate in the lab, conducted a large screen for mutants that suppress the XX masculinization of a conditional *Cbr-tra-2* mutation. A similar screen was conducted by Dave Pilgrim's lab, together totaling 810,000 haploid genomes. However, all of the 75 total suppressors found were self-fertile (Hill et al 2006). Additionally, a double *Cbr-tra-2(nm9ts); Cbr-fem-2(nm27)* mutant was generated and was also self-fertile. The double mutant results indicated the epistatic relationship between *tra-2* and *fem-2* has been maintained in *C.briggsae*, and suggest the *Cbr-fem* genes can be used in epistasis analysis to assist in determining the location of other genes in the pathway and to help determine the location of the sperm to oocyte transition relative to the *Cbr-fem* genes.

In *C .elegans*, double *Ce-fem; Ce-tra-1* mutants display a male soma (the phenotype of the single *Ce-tra-1* mutants), but a completely feminized germline (the phenotype of the *Ce-fem* genes) (Schedl et al 1989). This result is suggestive of either a reversed epistatic relationship between the *Ce-fems* and *tra-1* in the germline, or possibly an independent role of the *fem* genes in the control of spermatogenesis (Chen and Ellis 2000). Given the lack of concordance in the *fem* phenotype between the two

species, an additional goal of this part of the project was to see if the underlying epistatic relationship between *tra-1* and the *fems* are different as well.

*Cbr-tra-1(nm2)* was the allele employed to generate *Cbr-tra-1; Cbr-fem* double mutants for epistasis analysis. This allele contains a glutamine-to-nonsense mutation at codon 512, which is predicted to eliminate approximately 50% of the full-length TRA-1A protein, however is it unclear if *nm2* is a true loss of function allele. Animals with mutations in this region of *C.elegans* TRA-1 protein have a fully transformed soma but continue to produce both sperm and oocytes. Additionally, when the transformation is unaltered when placed in trans over a *tra-1* deficiency (Schedl et al 1989). However, *nm2* is a strong loss of function allele, producing complete somatic transformation. The mutants exhibit robust mating behavior and if mated with true females (no self sperm), young XX *nm2* males can sire cross-progeny, although at much lower levels than wild-type males (Kelleher et al 2008).

An important caveat to this epistasis analysis is the lack of distinct phenotypes between the single mutants. The single *Cbr-fem* mutants have a well-defined hermaphroditic germline, producing both sperm and oocytes in a double-armed gonad. The single *Cbr-tra-1 (nm2)* mutants have a poorly defined hermaphroditic germline. Sperm is produced, as are oocytes. However, the quality of the oocytes is questionable, and hermaphroditic germ cells are produced in a one armed male gonad. Therefore, the differences between the single mutants are more qualitative; a “good: vs. “bad” hermaphrodite germline. The lack of distinct phenotypes, combined with a lack of a true *Cbr-tra-1* null allele makes definitive statements regarding epistasis between the genes problematic.

The phenotypic description of *C. elegans tra-1* mutant animals is similar to the description of *C. briggsae tra-1(nm2)* mutants, with the exception of a granular, acellular matrix found in the germline of mutant worms. This phenotype is routinely observed in *Cbr-tra-1* mutants. However, a review of the literature in *C. elegans* did not find a description of this phenotype. The appearance of the matrix suggests the possibility of germ cell deterioration, and it therefore could contain DNA. To help clarify the identity of the matrix, Hoechst 33258 was employed to stain DNA contained in the germ line. As this phenotype has not been investigated in *C. elegans*, and there is germline phenotypic variation among the *C. elegans tra-1* alleles, two strains of that species were also investigated for this phenotype.

*Ce-tra-1(e1099)*, a strong loss-of-function allele, is an ochre nonsense mutation prior to the zinc fingers (Zarkower and Hodgkin 1992). Roughly 17% of e1099 mutants produce sperm then oocytes; 36% have abnormal germlines (Schedl et al 1989). *Ce-tra-1(e1781)* is an amber nonsense mutation located after the zinc fingers and in the same general location of the TRA-1 protein as the *Cbr-tra-1(nm2)* allele (Zarkower and Hodgkin 1992). Roughly 95 % of e1781 mutants produce sperm then oocytes; 2 % have abnormal germlines (Schedl et al 1989).

As noted by Schedl et al (1989), the *Ce-tra-1* alleles do not display a single phenotype and cannot be ordered in an allelic series, suggesting *Ce-tra-1* may produce multiple products and/or is auto regulated. Given the overall similarity between the *Ce-tra-1* and *Cbr-tra-1* phenotypes, it was important to determine if there are also germline phenotypic differences in *C. briggsae*. Our lab currently has

two alleles of *Cbr-tra-1* that have been outcrossed at least four times, and therefore could be examined for phenotypic differences.

As discussed earlier, the mutation in the *Cbr-tra-1(nm2)* allele is predicted to eliminate approximately 50% of the full-length TRA-1A protein. These mutants have a perfectly formed male soma and exhibit normal mating behavior. Preliminary analysis by Danielle Kelleher, a former graduate student in the lab, indicated XX *Cbr-tra-1 (nm2)* males produce sperm for the first day of adulthood, and then begin to produce oocyte-like cells during day two of adulthood. Our lab has isolated the *Cbr-tra-1(nm30)* allele. This allele has a GT → GA mutation in the 5' splice site of intron 2, and is characterized by an incomplete male tail and well formed oocytes within a male somatic gonad (Kelleher et al 2008).

## 4.2. Methods

### 4.2.1. Differential interference microscopy (DIC)

DIC microscopy was used to assess phenotypes. Worms were immobilized with NaN<sub>3</sub> (500mM) in M9 salts and mounted on 2% agar pads.

### 4.2.2. Mutant strains investigated

Mutants used in this study include *Ce-tra-1(e1099) III*, *Ce-tra-1(e1781) III*, *Cbr-tra-1(nm2) III*, *Cbr-tra-1(nm30) III*, *Cbr-fem-2(nm27) III*, and *Cbr-fem-3(nm63) IV*.

### 4.2.3. Categorizing phenotypes

Initial phenotypic categories for *Cbr-tra-1 (nm2)* and *Cbr-tra-1(nm2);Cbr-fem-2(nm27)* were determined based on observations of commonly occurring phenotypes. Later phenotypic categories for other strains examined were based on

those in Schedl et al (1989), as they had conducted an extensive phenotypic analysis of the *C. elegans tra-1* alleles.

4.2.4. Generation of *Cbr-fem*; *Cbr-tra-1(nm2)* double mutants

The mating scheme to produce double *Cbr-fem*; *Cbr-tra-1 (nm2)* mutants is detailed in Figure 21. Briefly, *Cbr-tra-1(nm2)/+* hermaphrodites were crossed to AF16. One half of males produced are *Cbr-tra-1 (nm2)* heterozygotes. The males from this cross were mated to homozygote *Cbr-fem* mothers. All the offspring from this cross are *Cbr-fem/+*; one-half will be *Cbr-tra-1 (nm2)/+*. F1 worms are singled, allowed to lay then genotyped to confirm their *Cbr-fem* heterozygosity. If the mother was also *Cbr-tra-1 (nm2)/+* she will produce approximately 1/4 pseudo-males, and can be identified by the presence of males on the plate. Offspring from plates founded by double heterozygous mothers are singled, allowed to lay and then genotyped for the *Cbr-fem* mutation. Approximately 1/6 of the plates (1/4 of plates with *tra* pseudo-males) will be founded by a *Cbr-fem*; *Cbr-tra-1(nm2)/+* mother.

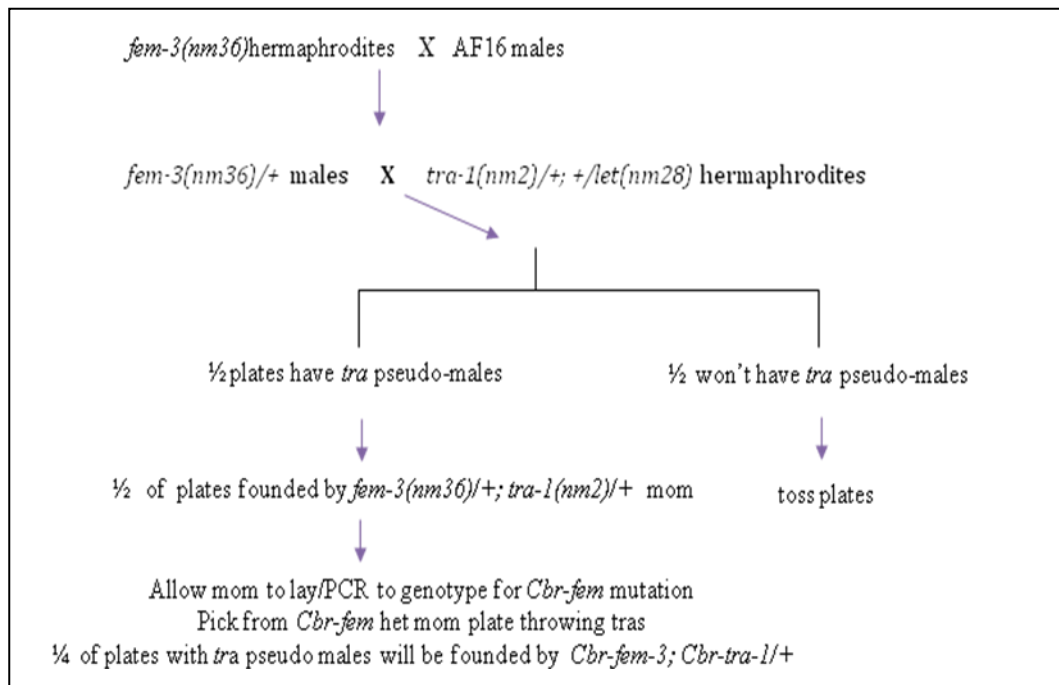


Figure 21. Crossing scheme for generating *Cbr-fem;Cbr-tra-1(nm2)/+* strain

#### 4.2.5. Hoechst 33258 staining of worms

Worms are individually collected into M9 salts. They are then rinsed three times with M9 salts, with centrifugation for three minutes at 3400 rpm to collect to collect the worms between each rinse. Approximately 400  $\mu$ l of  $-20^{\circ}\text{C}$  methanol is added to the worms, which are then incubated at  $-20^{\circ}\text{C}$  for a minimum of 10 minutes. They are collected by centrifugation and rinsed as stated above. After the last rinse most of the M9 buffer is removed, a 200  $\mu$ l solution of M9 containing 1.5  $\mu$ l of 1mg/ml Hoechst 33258 is added and the worms are incubated at room temperature in the dark for roughly 45 minutes. Following incubation the worms are rinsed as described above and brought up in a final volume of about 30  $\mu$ l of M9 salts. To maintain fluorescence, 10  $\mu$ l of vectashield (Vector Laboratories) is added to the solution, and the worm solution is distributed onto 3-4 agar pads (2%) for visualization with DIC and fluorescence microscopy.

### 4.3. Results

#### 4.3.1. Phenotypic characterization of *Cbr-tra-1(nm2)*

To become familiar with the single mutant phenotype and confirm the initial observations by Danielle Kelleher, an extensive analysis of the *Cbr-tra-1(nm2)* phenotype was undertaken. 211 *Cbr-tra-1(nm2)* mutants were examined by DIC microscopy (Figure 22a and c). While the majority of *XX Cbr-tra-1(nm2)* mutants followed the general pattern of sperm production through day one followed by



oocytes (the oocytes often have abnormal morphology) on subsequent days, the timing of the transition between germ cell fates varied more than previous indicated.

A small portion of L4 mutants exhibited oocyte production (Figure 23), while some older worms had no observable oocytes. Some mutants exhibited poorly organized germlines, consisting of a granular, acellular matrix. The extent of the granular matrix region varied in location and extension, sometimes occurring between regions of sperm and oocytes. Other worms exhibited the acellular matrix throughout most of the entire gonad arm, and often the gonad arm was enlarged relative to the width of wild-type worms (Figure 24). Mutants with this phenotype were classified as “bad”. It was observed that older worms (day 2 or greater adults) had a higher percentage of the “bad” phenotype.

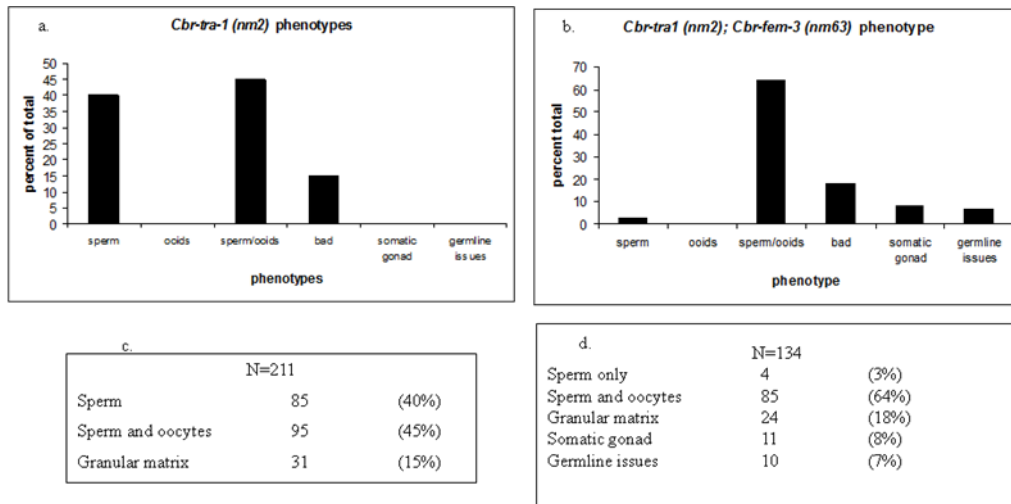


Figure 22. Summary of *Cbr-tra-1(nm2)* and *Cbr-fem-3(nm63); Cbr-tra-1(nm2)* somatic gonad and germline phenotypes. **a.** Percentages of phenotypic categories of *Cbr-tra-1(nm2)* mutants. **b.** Percentages of *Cbr-fem-3(nm63); Cbr-tra-1(nm2)* double mutants. **c.** Totals and breakdown of totals for each phenotypic category of *Cbr-tra-1(nm2)*. **d.** Totals and breakdown of totals for each phenotypic category of *Cbr-fem-3(nm63); Cbr-tra-1(nm2)* double mutants. The somatic gonad phenotype is composed of abnormally shaped gonad arms as well as double gonads oriented in the same direction. The germline issues phenotypes include a change in the location and number of sperm, spermatocytes, oocytes and germline stem cells. Sperm cells may occur in both the proximal and distal ends of the gonad.

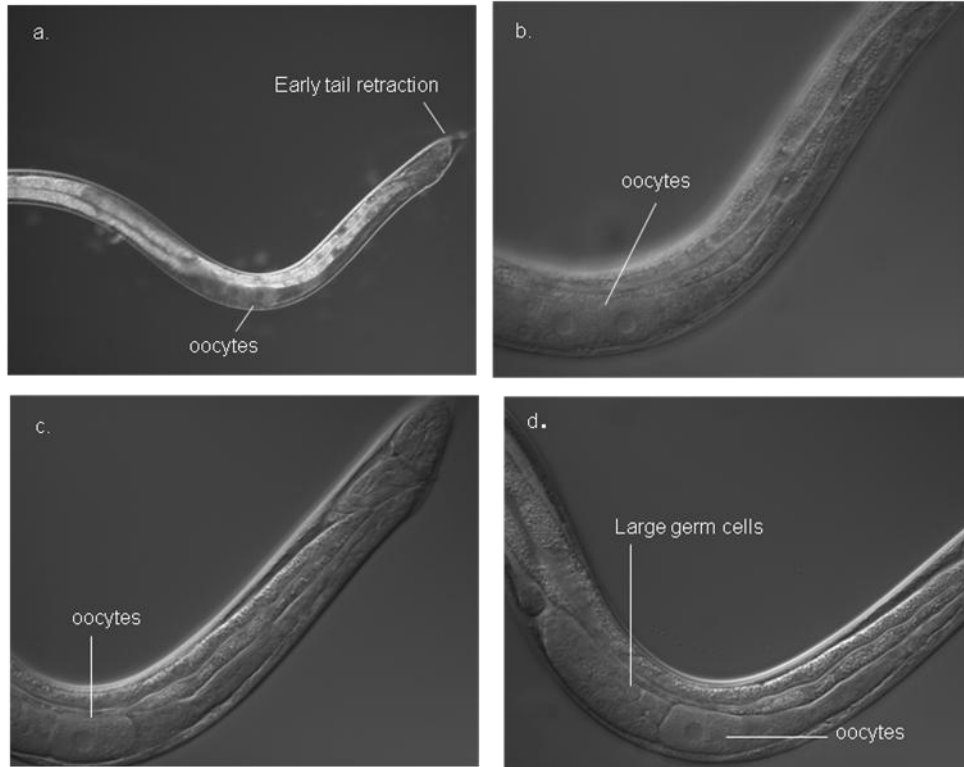


Figure 23. *Cbr-tra-1(nm2)* XX L4 pseudo-males. **a.** early tail retraction with oocytes in the gonad arm. **b.** 63X of L4 mutant showing multiple oocytes within the gonad arm. **c.** Another L4 animal in early tail retraction with gonad arm containing oocytes. **d.** 63X magnification of (c) showing large germ cells prior to oocytes.

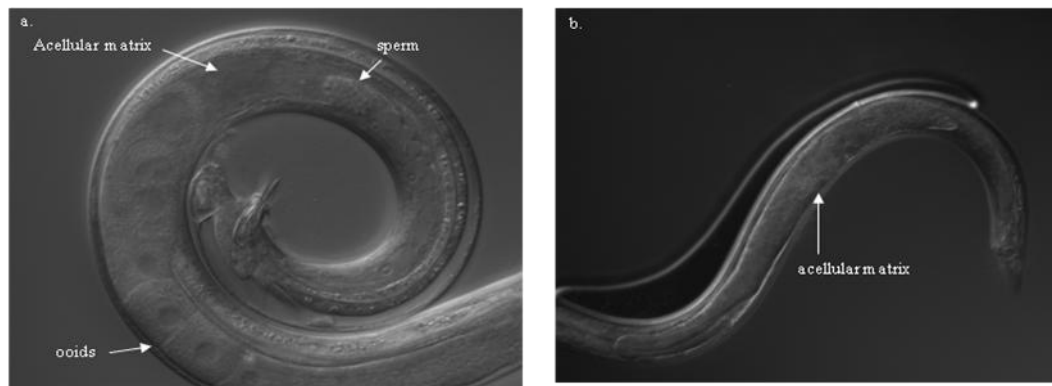


Figure 24. *Cbr-tra-1(nm2)* mutant. **a.** Note the region of acellular matrix between sperm at the proximal end of the gonad (where it is normally found) and oocytes at the distal end (where it is normally found). **b.** The gonad arm of this mutant is enlarged relative to normal AF16 XO males, and the acellular matrix occupies most of the interior.

#### 4.3.2. Phenotypic characterization of *Cbr-fem*; *Cbr-tra-1(nm2)* mutants

The first double mutant strain generated was *Cbr-fem-3(nm63);Cbr-tra-1(nm2)*. The general phenotypes of this mutant was determined by examining 134 *Cbr-fem-3(nm63); Cbr-tra-1(nm2)* mutants by DIC microscopy (Figure 22b and d). The general phenotypes found in *Cbr-tra-1(nm2)* single mutants are also found in *Cbr-fem-3(nm63);Cbr-tra-1(nm2)* double mutants (Figure 25). Many fewer mutants exhibited sperm only (3% vs. 40%) compared to the single *tra-1(nm2)* mutant.

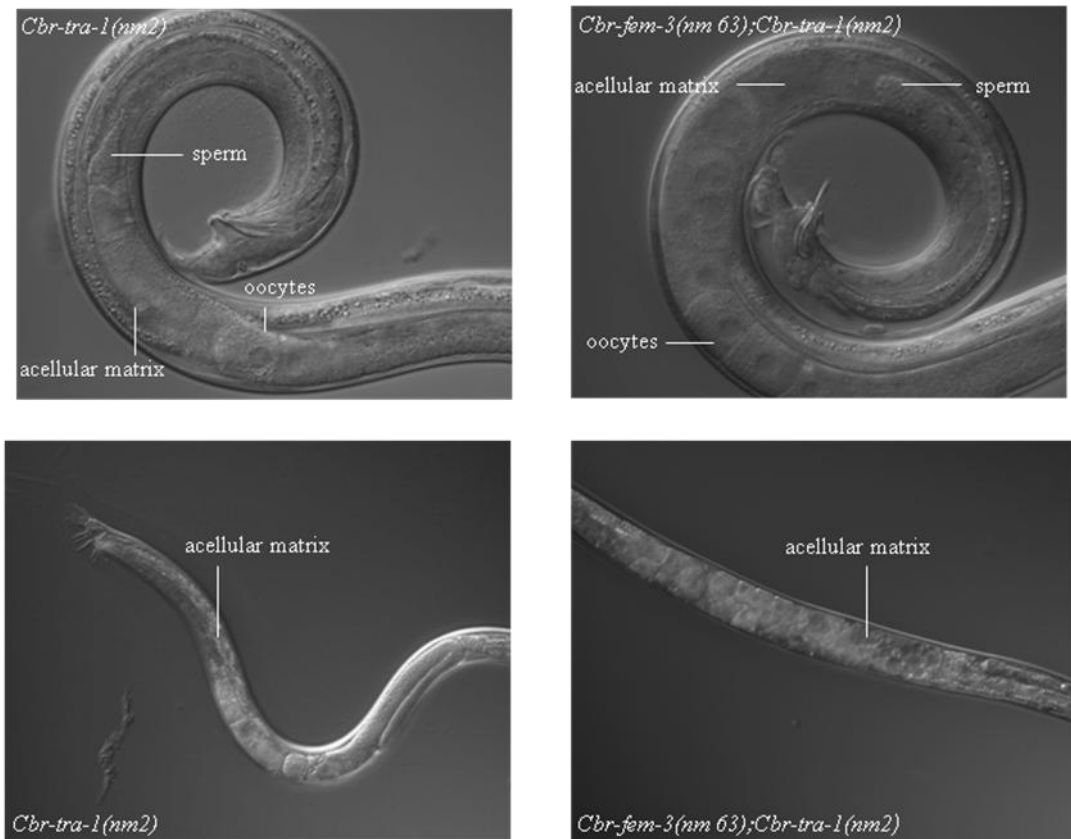


Figure 25. Comparison of *Cbr-tra-1(nm2)* and *Cbr-fem-3(nm63);Cbr-tra-1(nm2)* mutant phenotypes. Note the similarity in germ cell identity and granular matrix within the gonad arm.

Two additional phenotypes were observed. The somatic gonad category was composed of either an irregularly shaped gonad (n=6) or what appeared to be a double gonad in the same orientation (n=5) (Figure 26). The germline issues category was composed of changes in the location of germ cells. Some mutants had sperm and spermatocytes mixed together; others had spermatocytes and oocytes mixed together in the distal part of the gonad arm, and a few had what appeared to be undifferentiated cells in the entire gonad arm (Figure 27).

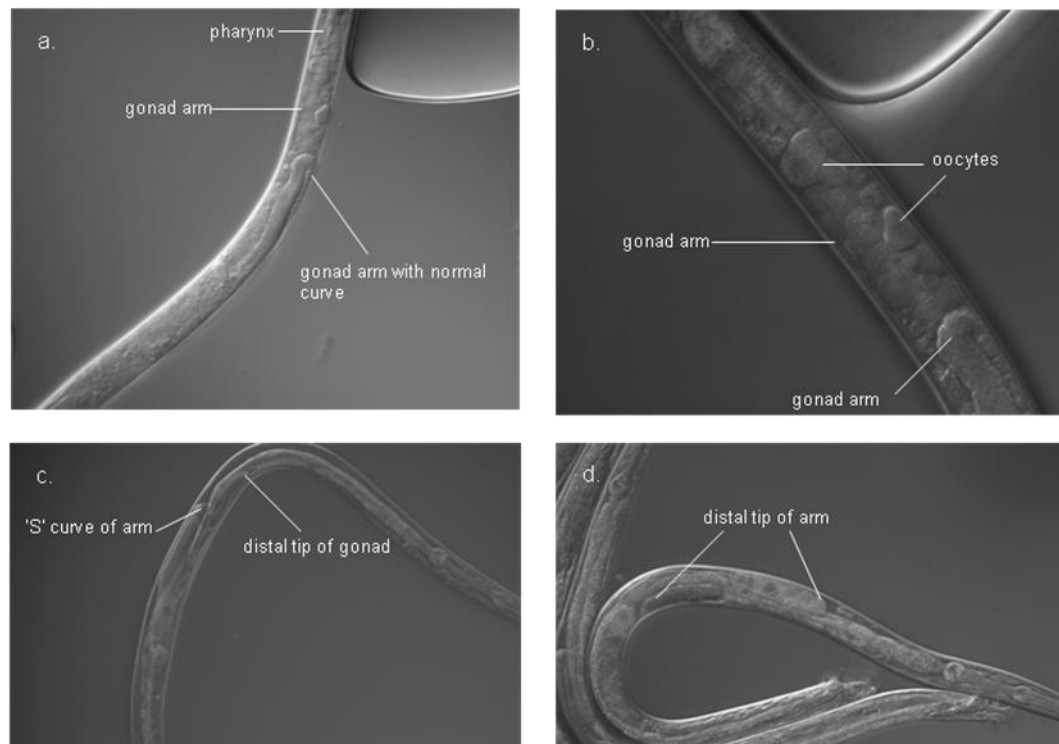


Figure 26. *Cbr-fem-3(nm63);Cbr-tra-1(nm2)* double mutant unique somatic germline phenotypes. **a.** Double gonad arm oriented in the same direction. One has the normal ‘U’ shape, the other is straight, continuing to the pharynx. **b.** 63X of the distal end of gonad in (a). **c.** gonad arm with an ‘S’ rather than the normal ‘U’ shape. **d.** Another double gonad arm oriented in the same direction. You can clearly observe the distal end of both gonad arms.

Only eight L4 worms were observed and not included in the data. Of the eight L4 mutants, five contained only sperm, one contained sperm and spermatocytes

mixed together and one appeared to have an abnormally shaped gonad arm (small rounded ball with undifferentiated cells).



Figure 27. *Cbr-fem-3(nm63);Cbr-tra-1(nm2)* mutant. The entire gonad arm is filled with undifferentiated germ cells.

#### 4.3.3. Hoechst Phenotypic characterization

##### *Cbr-tra-1(nm2)*

This was the first mutant examined with Hoechst staining (Figure 28). The staining confirmed the range of phenotypes identified by standard DIC microscopy (Figures 22 and 29). A problem with this method is the distortion of the somatic gonad as a result of methanol fixation. This made the determination of abnormal somatic gonad problematic. A surprising result of the staining was approximately 26% of the mutants exhibited an Emo (endomitotic) (Iwasaki et al 1996) phenotype within both the granular matrix and oocytes (Figure 28a and b; Figure 30). The Emo phenotype occurs when oocytes undergo multiple rounds of endomitotic DNA replication, resulting in polyploid cells. This phenotype has been identified in *C.*

*C. elegans* mutations impacting ovarian muscle contractions (Ono and Ono 2004), germline cytokinesis (Kuwabara et al 2000), as well as the meiotic cell cycle (Hajnal and Berset 2002). It also occurs in wild-type hermaphrodites that have exhausted their sperm supply (Ward and Carrol 1979). *C. elegans tra-1* mutant alleles vary in the degree of germline masculinization; in most cases some oocyte-like cells are produced (Schedl et al 1989), however I could not find any reference to an Emo phenotype for *Ce-tra-1* mutations in the literature.

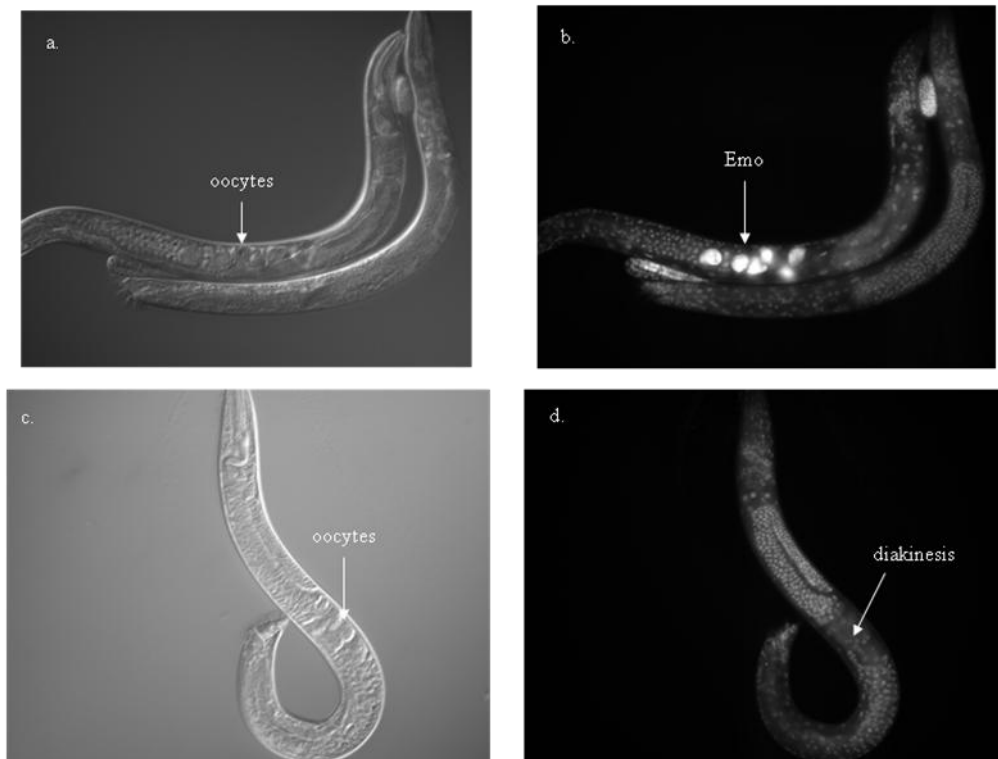


Figure 28. *Cbr-tra-1(nm2)* mutants. **a** and **c** are normal DIC microscopy, **b** and **d** are fluorescence microscopy with Hoechst staining. **a** and **b** are the same worm; **c** and **d** are the same worm. A range of DNA states were observed, from germ cells in diakinesis to numerous multinucleated cells.

#### *Ce-tra-1 (e1099)*

The proportions of mutants in each category were in rough agreement with the Schedl et al (1989) results (Figure 29). Approximately 2.5% of the mutants exhibited

the Emo phenotype (Figure 30; Figure 31 a and b). A high proportion (36%) exhibited abnormal germlines, which is defined as a change in number or normal location of germ cells (Schedl et al 1989). A normal male germline would have mature sperm in the proximal portion of the single gonad arm. Distal to the sperm, cells normally undergo spermatogenesis, with the distal tip of the gonad arm containing germline stem cells. Almost 30% exhibited an abnormally shaped somatic gonad, most often rounded with no connection to the tail (Figure 32).

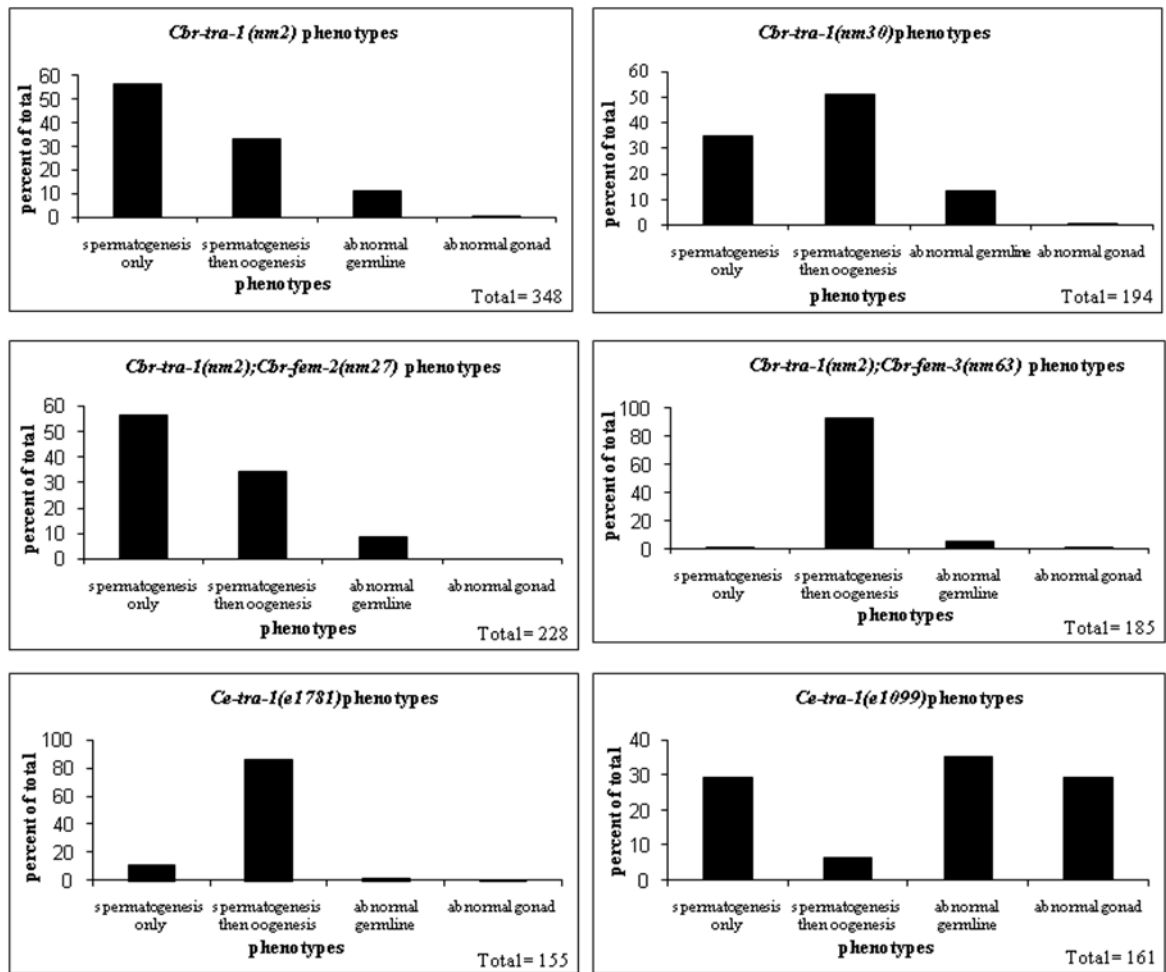


Figure 29. Hoechst phenotypic analysis. Phenotypic categories as percentages of total mutants examined for alleles of *Cbr-tra-1*(n, dom2) double *Cbr-tra-1(nm2);Cbr-fem* mutants and alleles of *Ce-tra-1*. The Emo category is subsumed under the sperm then oocytes category for comparison with Schedl et al (1989) and the previous phenotypic

analysis (see Figure 22). Separate analysis of the Emo category for these mutants is in figure 30.

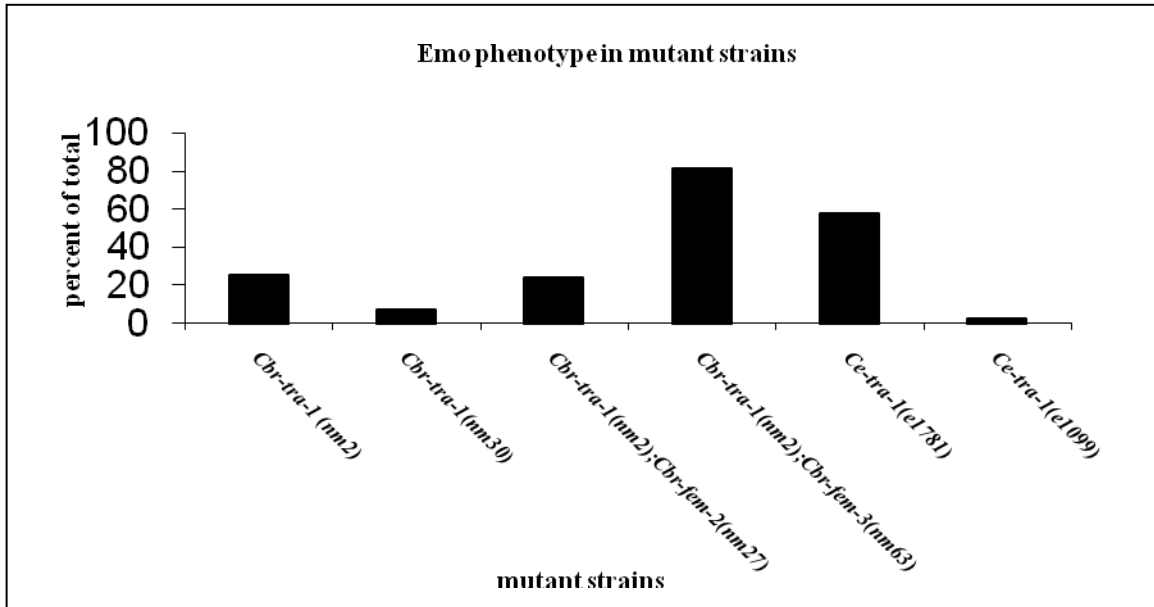


Figure 30. Hoechst phenotypic analysis. Emo phenotype as percentage of total mutants examined for alleles of *Cbr-tra-1(nm2)*, double *Cbr-tra-1(nm2);Cbr-fem* as well as alleles of *Ce-tra-1*. *Cbr-tra-1(nm2)* Emo = 89, total examined = 348; *Cbr-tra-1(nm30)* Emo = 15, total examined = 194; *Cbr-tra-1(nm2);Cbr-fem-2(nm27)* Emo = 55, total examined = 228; *Cbr-tra-1(nm2); Cbr-fem-3(nm63)* Emo = 150, total examined = 185; *Ce-tra-1(e1781)* Emo = 90, total examined = 155; *Ce-tra-1(e1099)* Emo = 4, total examined = 161.



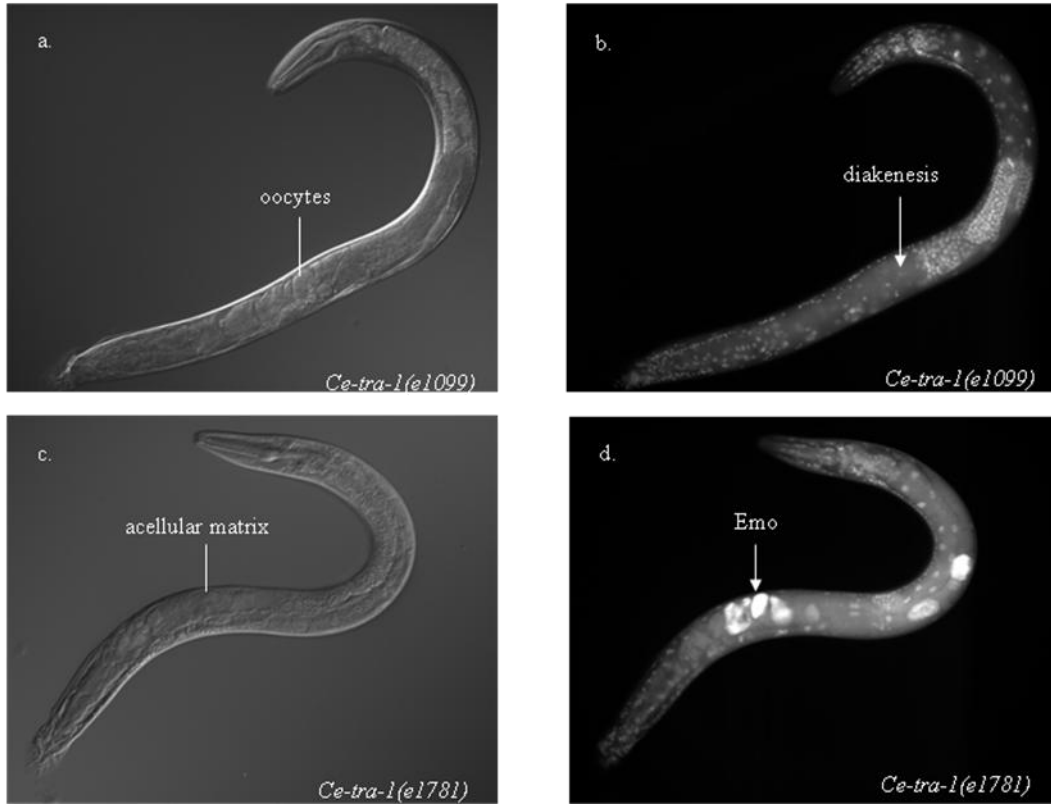


Figure 31. *Ce-tra-1* mutants. **a** and **c** are normal DIC microscopy, **b** and **d** are fluorescence microscopy with Hoechst staining. **a** and **b** are *Ce-tra-1(e1099)*; **c** and **d** are *Ce-tra-1(e1781)*. No mature sperm are present in either mutant.



Figure 32. *Ce-tra-1(e1099)* pseudomale. **a**. and **b**. are same mutant. **a**. is normal DIC microscopy. **b**. is fluorescent microscopy with Hoechst staining. Note the rounded gonad arm in the central portion of the worm. The arm is circled in **b** and contains only undifferentiated germ cells

### *Ce-tra-1 (e1781)*

These mutants exhibited a much higher proportion of spermatogenesis than oocytes (28.4% vs. 3.7%) and Emo (58% vs. 2.5%) compared to *e1099* (Figures 29 and 30). The Emo phenotype was particularly pronounced, with many worms containing a high number of Emo cells (Figure 31c and d). Very few mutants had either abnormal germlines or somatic gonads. This mutation is located in the same general location of the TRA-1 protein as the *Cbr-tra-1(nm2)* allele, yet the percentages of animals in each category differ. Fewer mutants in the *e1781* strain produce only sperm compared to *C.briggsae nm2* allele. This result was also seen in Schedl et al (1989). The published analysis of *e1781* indicates 95% of mutants make sperm then oocytes. This is comparable to our analysis if the categories of sperm then oogenesis and Emo are combined (86%).

### *Cbr-tra-1 (nm30)*

A much larger proportion (43.4%) of *nm30* mutants exhibited sperm then oocytes compared to the *Cbr-tra-1 nm2* allele (7.2%) (Figure 29). Conversely, many fewer *Cbr-tra-1(nm30)* mutants exhibited the Emo phenotype (7.7%) compared to *nm2* mutants (25.6%) (Figure 30).

### *Cbr-fem-2(nm27);Cbr-tra-1(nm2)* (Figure 33)

The single *Cbr-tra-1* and double *Cbr-fem-2;Cbr-tra-1* mutants show strong concordance in percentages of mutants in each category (Figure 29). Statistical analysis found no significant difference between the strains for the sperm and sperm then oocyte categories (two-tailed P value = 0.8530, Fisher's exact test). In both

mutants, roughly 56% produce only sperm. The percentage of Emo phenotype (24% vs 25.6%) is also similar (Figure 30). No abnormal somatic gonads were observed.

*Cbr-fem-3 (nm63); Cbr-tra-1 (nm2)* (Figure 34)

The range and percentages of phenotypes is similar to those seen with DIC microscopy alone, and markedly different from the single *Cbr-tra-1(nm2)* mutant phenotypes in the sperm and sperm then oocyte categories. Statistical analysis found a significant difference (two-tailed P value = < 0.0001, Fisher's exact test) between the strains for these phenotypes. The double *Cbr-tra-1;Cbr-fem* mutants also differed in the range and percentages of phenotypes. Where almost 57% of *fem-2; tra-1* mutants make sperm only, just over 1% of *fem-3; tra-1* mutants produce only sperm (See Figure 29). The overwhelming majority of worms display the Emo phenotype (81%). A small percentage (1.1%) also displays gonad defects; a possible double gonad arm and abnormal gonad shape.

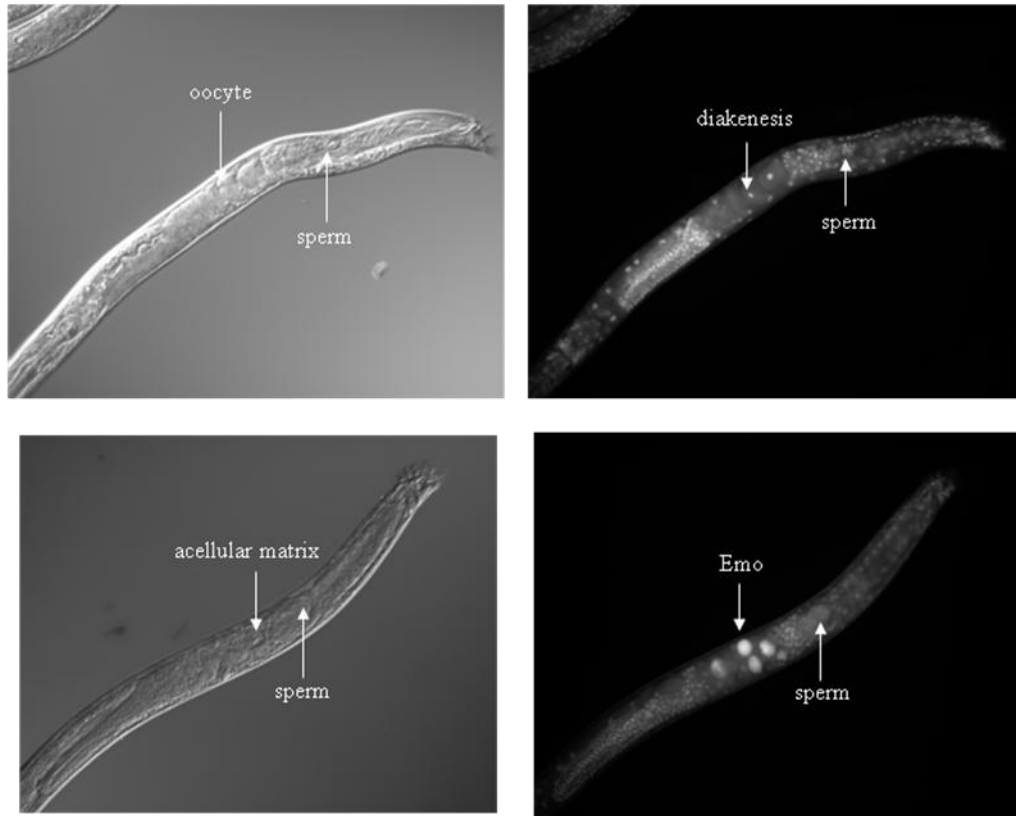


Figure 33. *Cbr-fem-2(nm27); Cbr-tra-1(nm2)* mutants. **a** and **b** are the same worm; **c** and **d** are the same worm. The phenotypes are similar to the single *Cbr-tra-1(nm2)* mutants.

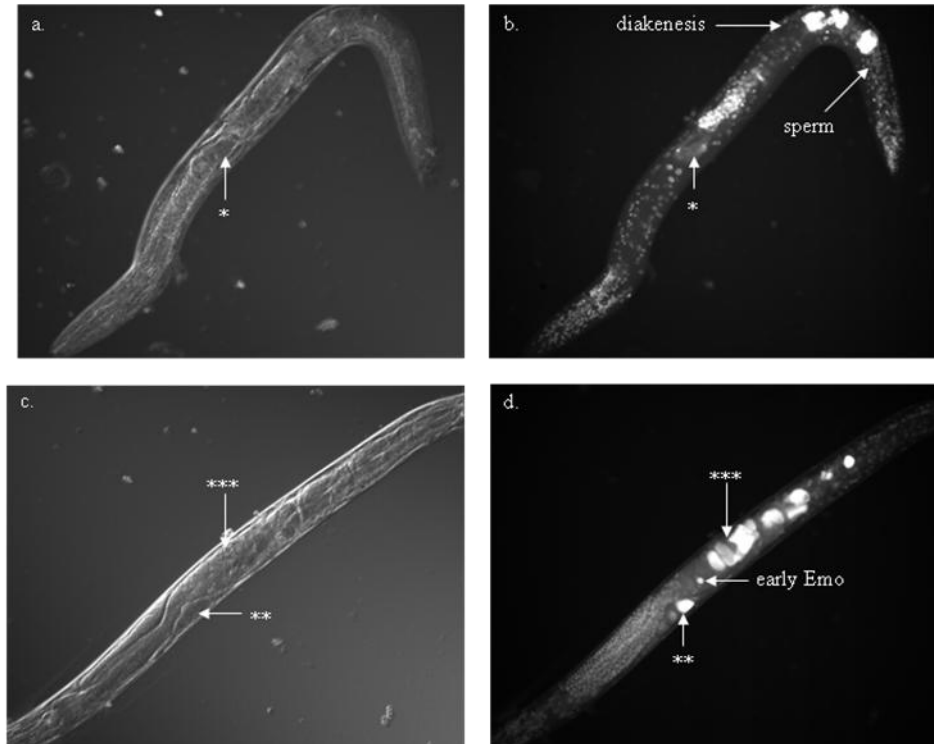


Figure 34. Phenotype of *Cbr-fem-3 (nm63); Cbr-tra-1 (nm2)*. **a** and **b** are the same mutant; **c** and **d** are the same mutant. A range of germ cell stages/types are observed, from sperm to diakenesis oocyte to early Emo oocytes, as well as acellular matrix containing degraded endomitotic oocytes. \* = abnormal distal end of gonad arm; \*\* = possible double gonad arm; \*\*\* = possible endomitotic degraded oocytes within the acellular matrix.

#### 4.4. Conclusions

The double mutant analysis suggests that, unlike *C. elegans*, *Cbr-tra-1* remains epistatic to the *Cbr-fem* genes in the germline sex determination system in *C. briggsae*. Unlike the robust hermaphroditic germline found in *Cbr-fem-2* and *fem-3* mutants, the double *Cbr-fem; Cbr-tra-1(nm2)* exhibits a highly variable period of spermatogenesis, similar to the single *Cbr-tra-1(nm2)* mutants. *Cbr-tra-1* mutants are capable of producing viable sperm and oocytes within the context of a male somatic gonad, evidenced by the ability of *nm2* mutants to sire offspring, and the *nm30*

mutants to produce occasional embryos (Kelleher et al 2008). Although the quality of sperm within the double *C. briggsae* mutants has not been assessed, the presence of the Emo phenotype indicates the oocytes are going through the normal process of maturation. In a normal hermaphroditic gonad arm, maturation is complete just prior to entry into the spermatheca, where fertilization occurs. As noted by Iwasaki (1996), oocytes unable to enter the spermatheca undergo multiple rounds of endomitotic DNA replication. Male gonads do not have spermathecae, thus any oocyte within a male somatic gonad should undergo this process. The Emo phenotype is seen in all the single *tra-1* mutants in both *C. elegans* and *C. briggsae*, as well as the double *Cbr-fem*; *Cbr-tra-1 (nm2)* mutants, suggesting the oocytes in these mutants are at least potentially viable. The Emo phenotype was not observed in either the single *Cbr-fem-2* or *Cbr-fem-3* mutants (data not shown).

Single *Cbr-fem* mutants contain viable oocytes that follow consistent developmental stages; proliferation at the distal end of the gonad, entry and progression through meiotic prophase as the cells move proximal in the gonad arm. In both single *Cbr-tra-1* and double *Cbr-fem*; *Cbr-tra-1 (nm2)* mutants, there is no normal progression. Some oocytes within the distal end display the Emo phenotype; likewise oocytes at the proximal end can be in diakinesis, with endomitotic oocytes on either side. It is possible this is the result of oocytes production in a single male gonad. Recent work from the Kimble lab (Vogel et al 2008) suggests there is sexual dimorphism in the mitotic region of gonad arms. Thus it is possible the germ cells are receiving ‘mixed messages’ as to their state.

Although there is overall similarity in phenotypic categories between *Cbr-fem-2(nm27);Cbr-tra-1(nm2)* and *Cbr-fem-3 (nm63); Cbr-tra-1 (nm2)*, the percentage of mutants within each category is surprisingly different. The single *Cbr-tra-1* and double *Cbr-fem-2;Cbr-tra-1* mutants show strong concordance in percentages of mutants in each category, supporting *tra-1* epistasis.

Very few double *Cbr-fem-3;Cbr-tra-1* mutants produce only sperm, and an overwhelming majority of these mutants produce sperm then oocytes. This result was unexpected significantly different from the percentage of single *Cbr-tra-1* mutants producing only sperm and sperm then oocytes. The identical phenotypes of the single *Cbr-fem* mutants suggested the doubles would also share a similar pattern. The difference in phenotype could be suggestive of *Cbr-fem-3* having a role in regulating *Cbr-tra-1* activity (perhaps promoting sperm production) that is different from the role of *Cbr-fem-2*. There is precedence for this in *C. elegans*. As noted in Starostina et al (2007), both XX and XO *Ce-fem-2 and fem-3* mutants display elevated levels of TRA-1A (relative to wild-type), and that the highest levels in XO animals are in *Ce-fem-3(e1996)* null mutants. They also found both that in *C. elegans*, both FEM-2 and FEM-3 enhance the ubiquitin-mediated proteolysis of TRA-1A compared to the levels of degradation that occur when FEM-1 is alone. *Ce-fem-3(e1996)* also exhibits a stronger feminization phenotype relative to the other *Ce-fem* mutants (Hodgkin 1986; Kimble et al 1984).

Perhaps there is a *tra-1* regulatory pathway in *C. briggsae* that only involves *Cbr-fem-3*. It is also possible that *Cbr-tra-1(nm2)* is not a complete loss of function allele, and the difference in phenotypes between the double mutants is due to

differing regulatory roles between them. *C. briggsae* FEM-2 may be more important in regulating TRA-1A than FEM-3. Therefore, when *fem-2* is mutated, the single tra-1 mutant phenotype is observed. In *fem-3* mutants, *fem-2* is active and you observe a more 'fem-like' phenotype. At present time, the data does not allow us to distinguish between these possibilities.



## Chapter Five: Conclusions

“The most exciting phrase to hear in science, the one that heralds the most discoveries, is not 'Eureka!' (I found it!) but 'That's funny'”

- Issac Asimov

### 5.1. Big questions addressed by this project

This research project began with a ‘That’s funny’. RNAi experiments against the *fem* genes within the species *C. briggsae* suggested that the germline phenotype was different than that found in its sister species, *C. elegans* (Stothard et al 2001; Haag et al 2002). As discussed in the introduction, mutations in any of the three *Ce-fem* genes result in feminization of the hermaphroditic germ line; no self-sperm are produced. The *fem* genes, in particular the regulation of *fem-3*, had been shown to be crucial to the sperm to oocyte switch in *C. elegans* (Puoti et al 2001; Ahringer and Kimble 1991; Ahringer et al 1992). Yet the RNAi results in *C. briggsae* indicated the germline remained hermaphroditic, suggesting the *fem* genes may not be involved in hermaphroditic spermatogenesis in *C. briggsae*. This also suggested the control of the sperm to oocyte transition is elsewhere in the pathway. So, after the “that’s funny”, came the question – are the RNAi results real? As RNAi does not result in complete elimination of gene function, the decision was made to generate true mutations in the *Cbr-fem* genes.

The reverse genetic screens described in this dissertation resulted in deletion mutations in *Cbr-fem-2* and *Cbr-fem-3*, and the characterization of these genes confirmed the RNAi results. Our epistasis analysis of *Cbr-fem;Cbr-tra-1 (nm2)* mutants suggest *Cbr-tra-1* is epistatic to the *Cbr-fem* genes in the germline (See Figure 1). *Cbr-tra-2 (ts)* suppressor screens conducted by Hill et al (2008) isolated no

self-sterile animals. In addition, they determined that *Cbr-tra-2*; *Cbr-fem-2* double mutants are self-fertile hermaphrodites. This work again suggests the regulation of the sperm to oocyte switch is downstream of the *fem* genes.

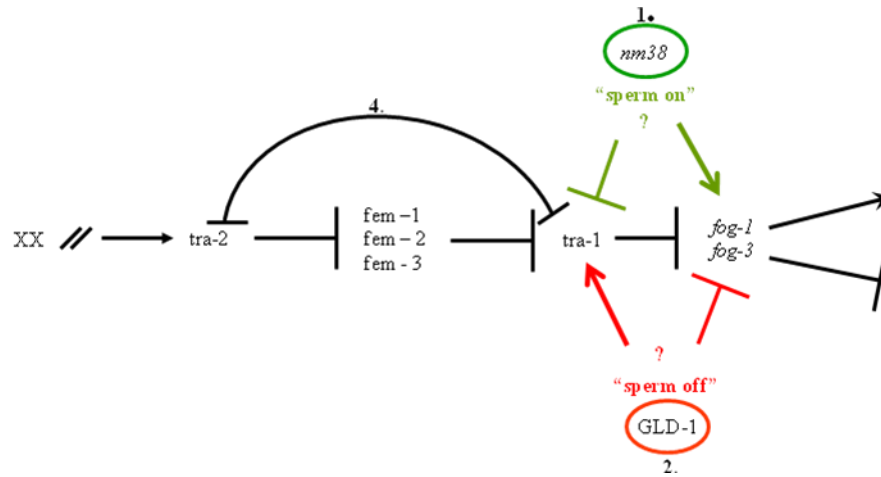


Figure 33. The downstream portion of the *C. briggsae* germline sex determination system, with possible regulatory regions. **1.** Work presented in this dissertation and in the Haag lab suggest control of hermaphrodite spermatogenesis is at the level of *tra-1*. Possible candidate for spermatogenesis is the *nm38* allele isolated by A.Doty (unpublished data). **2.** Possible negative regulation of spermatogenesis could be through GLD-1. Mutants of this gene have a fog phenotype. **3.** The interaction between *tra-1* and *tra-2* is conserved in *C. briggsae*. In *C. elegans*, this interaction appears to promote spermatogenesis in *elegans* (Wang and Kimble 2001).

The question now becomes, “Where is the control of hermaphrodite spermatogenesis and how is it achieved?” It is reasonable to assume that the core sex determination pathway has been conserved in *C. briggsae* relative to *C. elegans* and the ancestral male-female species (see Figure 4, Introduction, for core pathway; Figure 1, introduction, for phylogeny). Mutations in *tra-2* and *tra-1* result in similar phenotypes in both *C. elegans* and *C. briggsae* (Hodgkin and Brenner 1977; Hodgkin 1987; Kelleher et al 2008). There is conservation of function of the *tra-1* gene in the sex determination systems of *C. elegans* and *Pristionchus pacificus*, a species

separated from *C. elegans* by approximately 200-300 million years (Pires-daSilva and Sommer 2004). Wang and Kimble (2001) discovered TRA-1 binds the intracellular domain of the TRA-2 in *C. elegans*, and that this binding is conserved in *C. briggsae*. As detailed in Chen et al (2001), *fog-3*, a germline specific gene, show conservation of function between *C. elegans*, *C. briggsae* and *C. remanei* (See Figure 1, Introduction, for orientation). Stothard and Pilgrim (2005) determined that interactions between FEM-2 and FEM-3 have been conserved between the three species, and that *C. remanei* requires FEM-2 function for proper somatic gonad development.

Given the likely conservation of the core pathway, our work suggests the control is at the level of *Cbr-tra-1*, as well as its targets. *Cbr-tra-1*, a transcription factor, is the next gene (after the *fems*) in the core pathway and the terminal regulator in *C.elegans* (Hodgkin and Brenner 1977; Hodgkin 1987). It is known to control, either directly or indirectly, genes required for sexual development (Yi et al 2000; Conradt and Horvitz 1999; Chen and Ellis 2000). Thus its regulation would impact sex specific development. Additional support for regulation at the level of *tra-1* is the work of Alana Doty, a fellow graduate student in the Haag Lab. She has isolated a fog-like mutant, *nm38*. Epistasis analysis conducted by A.Doty with *Cbr-nm38* and *Cbr-fem-3 (nm63)* indicates *nm38* is epistatic to *Cbr-fem-3*, again suggesting control downstream of *Cbr-fem-2* and potentially at the *tra-1* level (personal communication; unpublished data). However, recent work from the Spence lab (Guo et al 2008) suggests the story is not straightforward, however. They have isolated *Cbr-glf-1*, a germline specific gene. Mutations in this gene cause hermaphrodites to develop as

females, but do not affect males. Suppressor screens of *glf-1* isolated loss-of-function alleles of *tra-2* (Chen et al 2008), suggesting there may be control of spermatogenesis at the level of *Cbr-tra-2* as well.

How might this control be achieved? Just as *C. elegans* is a useful reference for determining the core sex determination pathway, germline control of sex determination in *C. elegans* can give insight into how control might function in *C. briggsae*. Many of the genes that modulate germ cell identity in *C. elegans* (*fbf-1* and 2; *gld-1*; *nos-3*) are also involved in the cell cycle and the mitosis vs meiosis decision (Hansen et al 2003; Crittenden et al 2002). Developmental pathway evolution theories suggest that many genes are recruited into new functions due to being in the “right place at the right time” (Wilkins 2002; Lowe and Wray 1997; Yamamoto et al 2004). There is evidence in *C. briggsae* that this is indeed happening. RNAi results with *Cbr-gld-1* indicate it exhibits a germline phenotype (Mog), which is opposite that of *Ce-gld-1* (Fog) (Nyak et al 2005).

True mutants of *Cbr-gld-1* have also been isolated (A.Doty, unpublished data) which also show a Mog (masculinization of germline) phenotype. In *C. elegans*, *fog-2* is a recent gene duplication within that species; it has no homologue in *C. briggsae*. However, the isolation of both *nm38* and *glf-1* indicate new “fog like” genes have been recruited into the *C. briggsae* germline sex determination pathway. RNAi work with *Cbr-puf-2*, a homologue of the *fbfs* in *C. elegans* also displays a germline phenotype (Fog), and that phenotype is opposite that of the *Cbr-fbf* genes (Mog) (Q. Liu, unpublished data).

Thus it appears that, as in *C. elegans*, the core somatic sex determination pathway is being modified in *C. briggsae* to allow a period of spermatogenesis in an otherwise female germline and somatic body. Some of the genes involved in that modification in *C. elegans* appear to be involved in *C. briggsae* modification as well. However, the method of modification is distinct from that found in *C. elegans* (Figure 33 and Table 3).

Why would this be the case? All of the comparative data, from the work discussed in this dissertation to the research cited, suggests hermaphroditism evolved convergently in *C. elegans* and *C. briggsae*. Recent phylogenetic work supports this viewpoint (Kiontke et al 2004; Kiontke, personal communication). It is possible that that there are many ways to evolve hermaphroditism, Hodgkin (2002) was able to perturb, through various mutations of sex determination genes, the sex determination system of *C. elegans*. However, the phylogenetic analysis suggests a more likely scenario is the initial steps towards hermaphroditism began in different genetic regulatory systems in *C. elegans* and *C. briggsae*. As discussed in the Introduction, *C. elegans* may have evolved hermaphroditism earlier than *C. briggsae* (Cutter et al 2008). If this is the case, *C. elegans* branched from the *C. briggsae/C. remanei* common ancestor and each began their own unique evolution. Therefore, initial conditions in each species were different at the time of hermaphroditism evolution, and distinct modifications would not be surprising.

Why would hermaphroditism have evolved? This is a question difficult to answer with any certainty, but studies of natural populations suggest environment may play a role (Cutter et al 2006; Dolgin et al 2008). *C. remanei* and *C. brenneri* are

male/female species within the Elegans Clade. Natural populations of these species are found North and South of the Tropic of Cancer, respectively (Kiontke, personal communication). *C. elegans* and *C. briggsae* are cosmopolitan species with overlapping geographic distributions. However, as noted in Dolgin et al (2008), they probably occupy different ecological niches. Cutter et al (2006) note the lack of nucleotide variation in temperate populations of *C. briggsae* compared to temperate populations of *C. elegans* suggests a recent colonization and expansion of *C. briggsae* in the northern areas. Hermaphroditism might be a viable reproductive strategy for species colonizing new habitats as solitary individuals.

There is a growing community of researchers interested in *Caenorhabditis* species evolution. *C. elegans* and *C. briggsae* have already been sequenced, and a project to sequence three additional species (Haag et al 2007). Polymorphism maps are being developed for *C. briggsae*, as well as a larger molecular toolkit. Interest has grown in understanding natural populations of *Caenorhabditis* species, and the collection of new strains has rapidly increased (Dolgin et al 2008). As more resources come online to investigate the sex determination system within this genus, questions relating to the origins and evolution of hermaphroditism should finally find some answers.

## Appendix I: Deletion screen protocol

### Deletion Screening in *Caenorhabditis briggsae*

Begin the screen with at least 6 small (60x15mm; Fisher # 08-757-13A) plates confluent with gravid adults and eggs. Bleach to synchronize, determine population size by counting the eggs in your final volume, and plate onto 15-20 large (95x15mm; Fisher # 08-757-14G). If the egg count is ~ 300,000 or greater continue towards mutagenesis. If less than that, allow one generation of growth, repeat the bleaching process and proceed to mutagenesis. All incubations are conducted at 20°C unless otherwise stated. Plates are seeded with OP50. The plates for each step of the screen should be poured at least one week prior to use, and seeded with OP50 at least three days prior to use. It is important to have very dry plates, so the worm/M9 solution distributed on the plates during library plating will be quickly absorbed. Wet plates encourage fungal/bacterial contamination. The plates should be seeded with OP50 at least three days prior to use to allow good bacterial growth. Deletion screens require large amounts of reagents, and it is important to ensure adequate supplies prior to each step so the screen will proceed smoothly. Large NGM plates seeded with OP50 are required on a regular basis during the course of the deletion screen, so it is advisable to have a supply (~50 plates) available for the duration of the screen.

#### NGM media (per liter)

3 grams NaCl

2.5 grams Peptone

22 grams Agar (2.2%)

H<sub>2</sub>O to 1000ml

Autoclave the solution on liquid cycle for 30 minutes. Allow cooling to 50-60°C.

Add the following:

1 ml 1M CaCl<sub>2</sub>

1 ml 1M MgSO<sub>4</sub>

25 mls 1M KH<sub>2</sub>PO<sub>4</sub> (pH6.0)

1 ml Cholesterol (5mgs/ml in EtOH)

1 ml Uracil(2mgs/ml)

M9 buffer (1 liter)

3 grams  $\text{KH}_2\text{PO}_4$  (monobasic)

6 grams  $\text{Na}_2\text{HPO}_4$  (dibasic)

5 grams NaCl

1 ml 1M  $\text{MgSO}_4$

$\text{H}_2\text{O}$  to 1000ml

Mix thoroughly and autoclave on liquid cycle for 20 minutes

Bleaching large populations

Bleaching large populations of worms is best performed in batches. Wash off 4 large plates at a time, using large volumes of M9 and glass Pasteur pipets to pull of worm/egg/M9 solution (worms/eggs stick to plastic). Eggs also tend to adhere to plates despite multiple washings. Using an open flame, bend the thin end of a 9" Pasteur pipet into a "hockey stick" shape. Allow cooling and gently rub the thin end over the surface of a plate covered with M9. This will loosen eggs and assist in a more complete recovery of eggs. Collection of worms/eggs can be done in either 1.5ml eppendorf tubes or 14ml falcon tubes. Centrifugation for collection is 2000rpm, 2 minutes, 4°C for eppendorf tubes and 2-3000rpm, 4 minutes, 4°C for falcon tubes. Remove the supernatant carefully.

The 4 plates of worms/eggs are brought up in 5mls of M9 in a 50ml Nalgene conical tube, and then 2.5mls of hypochlorate solution (60% bleach/40% 4M NaOH) is added and the total solution is vortexed intermittently for 2-3 minutes. To dilute the bleach solution, M9 is added to the tube for a total volume of 12 mls. The worm/M9 solution is mixed thoroughly, and then split into four 14ml falcon tubes or 10 1.5ml eppendorf tubes. The tubes are centrifuged as previously described; the supernatant pulled off, and the worms/eggs are washed 3x with M9. This process is repeated for



all the plates. The eggs are brought up in M9 and plated onto 15-20 large NGM plates seeded with OP50.

### EMS mutagenesis

Late L4 is the optimal time for mutagenesis. At 20°C *C.briggsae* should reach late L4 around 50-54 hours. Even with bleaching synchronization is not perfect, so checking the worms at approximately one hour intervals from about 50 hours is advisable to ensure the correct timing for mutagenesis. It is also likely the original large NGM plates will starve out prior to the mutagenesis, so replate onto fresh OP50 seeded plates as needed. Mutagenesis is conducted in a fume hood. Have ready in the fume hood:

15ml tube containing 4mls M9	gloves
4 14ml tubes(round or conical bottom	parafilm
10ml pipets and pipet bulb	paper towels
waste container w/aluminum foil cover	M9 for rinsing

Rinse worms off the plates with a large volume of M9. Centrifuge to collect the worms, such that the final volume is 12mls worms/M9 in a 50 ml conical tube. Add 85ul EMS to the 15ml tube containing 4mls M9. Mix and add to the 12mls worms/M9. Parafilm the tube cover, then secure onto a nutator in the fume hood. Rock the worm/EMS/M9 solution for 4 hours at room temperature. Split the solution into 4 14ml tubes and spin at 2000rpm for 5 minutes to collect the worms. Rinse with large volumes of (~5mls) M9 4x, combining the tubes for a final volume of 4mls. Plate the worms onto 15-20 large NGM plates seeded with OP50.

Plate 250-500 worms onto two large NGM plates and incubate overnight at 25°C. The remaining plates are incubated at standard temp (20°C) until confluent with embryos and gravid adults (usually 24 hours). The following day assess the

plates incubated at 25°C for dead embryos and obvious mutations (unc, dpy, rol). A kill/mutation rate of 5-10% is optimal. Check these plates again just prior to plating the library. It may take a couple of days for mutated phenotypes to become evident.

Wash the eggs/gravid adults off the plates with M9, estimating total number of eggs by counting one plate and multiplying by the total number of plates. Gravid adults may hold ~5 embryos. There should be at least 600,000 eggs to ensure the roughly one million haploid genomes needed for the screen. Bleach in batches, placing the eggs in 4 50 ml tubes each containing 40mls M9/eggs. Seal the tubes with parafilm and rock overnight on a nutator at 15°C. The worms will begin to hatch and arrest at the L1 stage.

### Library plating

One week prior to library plating, prepare roughly 1300 small NGM plates. It is important to have extra plates, as some contamination will most likely occur and those plates will need to be replaced. Label the plates in 12 groups of 96 according to deep well/PCR plate identities (rows A-H; columns 1-12). For example, the first batch of 96 would be labeled 1-A1 through 1-H12.

Remove the tubes of worms from the 15°C incubator; spin down at 2000rpm, 4°C for 5 minutes. Rinse the worms at least 3x with large volumes of M9 to eliminate dauer pheromone released by the larval worms. Dilute the worms to a concentration of 8-10 worms/ul and plate 50ul of the solution onto each of 1152 labeled seeded small plates. If a plate is contaminated, replace with a fresh plate labeled to correspond to the contaminated plate. Prepare an additional 50-100 plates. This will allow replacement plates should some of the original 1152 become contaminated

during the initial incubation period. Allow the plates to dry thoroughly, place in large plastic containers and incubate for five days.

#### First round of DNA preparation

This stage of the protocol is particularly tedious; best accomplished by a group effort and advanced planning. Be sure to autoclave a sufficient amount of pipet tips and deionized water, and set up a few stations on lab benches with all the reagents needed for the washing of plates to make the process easier.

Reagents at each station include:

- 1000ul pipetman and one box of tips
- 200ul pipetman and one box of tips
- One labeled (1-12) deep-well microplate (Fisher # 12-566-121)
- Set of 96 deletion screen plates – numbers corresponding to the deep-well labeled plate
- 50ml conical tubes containing sterile water with streptomycin (100ug/ml) and nystatin (12.5ug/ml)
- Paper towels
- Deep-well plate cover (Fisher # 08-772-120)
- Colored tape

It is most efficient to wash the plates in groups. Stack the plates in groups of 6. Add 800ul of the water/antibiotic solution to each plate and then gently rock each plate to dislodge some of the worms. Tilt the plate and using the 200ul pipetman, remove 150ul of worm/water solution. Place the solution in the appropriate well of the labeled 96 deep-well microplate. It is important to ensure placement of the worm solution in the correct well of the microplate. This can be ensured in a couple of ways. Pipet tip boxes contain 96 tips – the same number of wells as the microplate. By lining up the tip box in the same orientation as the deep-well plate, and using the tips in the same order as the loading of the samples in the microplate, you can help eliminate missing wells or double loaded microplate wells. Once a row is completed,

cover it with colored tape labeled to correspond to the well beneath it (1-12). Move the tape down the rows as they are filled. Again, this prevents double loading of the wells, and assists in keeping track of well number. When a plate is completed, cover it with the plate cover and store at 4°C until all plates are washed off. Allow the screen plates to completely dry. This is very important. The plates will be stored at 15°C for the duration of the screen. At this temperature condensation forms on the plates and wet plates increase condensation, which in turn increases bacterial and fungal contamination. Place the dry plates in large boxes and store at 15°C.

Once all the deep-well microplates are filled, 150ul of the Proteinase K/lysis solution is added to each well, using a multichannel pipetman or a repeater pipet. The plate covers are taped around the edges to ensure there is no cross-contamination between wells. The plates are thoroughly mixed, centrifuged at 2000rpm for 5 minutes and frozen for 2-3 hours at -80°C. The plates are placed in a 65°C preheated hybrid oven overnight (6-8 hours minimum).

#### Proteinase K/lysis solution

50mM KCl	10mM TrisOHCl (pH 8.3)
2.5mM MgCl <sub>2</sub>	0.45% NP-40
0.45% Tween-20	0.01% gelatin
200 ug/ml Proteinase K	

#### Sample preparation and pooling

Following the overnight incubation, the DNA preparations are centrifuged at 2000rpm, 4°C, 5 minutes. Using a multichannel pipetman, 100ul of DNA preparation is transferred to the corresponding wells of labeled (1-12) 96-well PCR plates. An additional 50ul of preparation is added to a 96 deep-well microplate. This deep well

plate contains the pooled reactions. The individual PCR plates are sealed (Eppendorf thermo-sealing foil #0030 127.605), Proteinase K inactivated in a thermocycler (95°C, 30 minutes), and then stored at -20°C. The pooled reactions are mixed and separated into three 96 well PCR plates (200ul/well). The plates are sealed and Proteinase K inactivated as previously described. One of these pooled plates will be used for the initial PCR reactions. The other 2 are placed in -20°C as back-ups should the original plate become contaminated/damaged.

### First round PCR

#### Outer reactions

5 µl	template DNA*
5 µl	10x ThermoPol buffer
5 µl	dNTP stock (2.5mM stock)
2.5 µl	10mM MgSO <sub>4</sub>
2 µl	100 µl primer mix (0.5µM each outside primer)
0.4 µl	(2 units) Taq polymerase (NEB- M0267L)
<u>30.1 µl</u>	<u>dH<sub>2</sub>O</u>
50 µl	Total volume

#### Inner reactions

5 µl	DNA**
2.5 µl	10x ThermoPol buffer (NEB)
2.5 µl	dNTP stock (2.5mM stock)
1.25 µl	10mM MgSO <sub>4</sub>
1 µl	100 µl primer mix (0.5µM each inside primer)
0.2 µl	(1 unit) Taq polymerase (NEB – M0267L)
<u>12.55 µl</u>	<u>dH<sub>2</sub>O</u>
25 µl	Total volume

\*DNA may be diluted with equal volume of water if reaction quality poor

\*\*DNA concentration ranges from 2ul straight DNA to 1:50 dilution

Nested PCR reactions are performed in 96 well PCR plates (Fisher # 08-408-250). The outer PCR reactions are performed using 5ul of pooled DNA preparations. The inner reactions use 5ul of the outer reaction, diluted to a concentration

determined during primer optimization. The cycling conditions for each primer set are described in Table 1. The PCR plates are sealed with either flexible PCR mats (Perkin Elmer # N801-0550) or thermo-sealing foil. Outer reactions are not analyzed via gel electrophoresis unless the inner reactions failed. In that situation a portion of the outer samples are run for troubleshooting purposes. The inner reactions are run on 1% agarose (BioRad # 161-3101) containing ethidium bromide(0.5ug/ml) for visualization.

Potential deletion populations will show a PCR band smaller than the wild-type PCR product (see Figure 1 appendix for example). It is important to note this protocol has a high false positive rate. Once a candidate population has been identified, nested PCR reactions are performed on the 12 individual populations that comprise the pool. For example, if the lane is identified as well B6, then the individual plate DNA preparations are removed from  $-20^{\circ}\text{C}$  and the DNA from well B6 in each plate is tested via nested PCR. If it is a true hit, one of the samples will contain the deleted product.

The deletion product is gel purified and sequenced to determine the location of the deletion and to enable primers to be designed within the deletion region. These primers are designated “Wild-type only” and will be used to identify heterozygous and homozygous strains during outcrossing and homozygous strain generation.

#### First round of sib selection

While PCR screening is performed, plates are prepared for the next round. Six well Falcon flat-bottomed tissue culture plates (Fisher # 08-772-4J) are filled with NGM media and seeded with OP50 at least three days prior to use, to ensure dry

plates and densely grown OP50 culture. The plates are labeled to correspond to the wells of two 96 well PCR plates (1-A1 through 2-H12). Additional plates are prepared as replacements for contaminated plates.

Once a positive population is identified, the plate is recovered from 15°C storage. It will be starved out, so the entire plate is chunked onto ~ 6 large NGM plates seeded with OP50 and allowed to recover, usually for 1-2 days. The worms are washed off the plates, and distributed at a concentration of 50 worms/plate into the wells of 32 6 well flat-bottomed tissue culture plates (192 total populations). The plates are incubated for 5 days, and then a portion of the worms from each plate are collected for DNA preparations as described previously with the following modifications. Each plate is rinsed with 200ul water containing streptomycin and nystatin at the same concentrations described earlier. Approximately 50ul is collected from each plate and placed in the corresponding wells of 2 96 deep well microplates. The plates are allowed to dry thoroughly, then stored at 15°C. An equal volume of ProteinaseK/lysis solution is added to each well. The following day 50ul of each DNA prep is transferred to the corresponding wells of 2 96 well PCR plates and ProteinaseK inactivated as previously described. There is no pooling of DNA preps at this stage.

Nested PCR reactions are performed as described previously and run on 1% agarose gels to identify positive populations (Figure 1 appendix). At this stage there is often more than one positive population. The plates corresponding to the positive lanes are identified and removed from 15°C storage. Once again they will be starved out, so they are chunked onto large NGM plates seeded with OP50. If there are more

than two positive populations, two of the populations are chosen to continue screening and the remaining are frozen down as insurance should the deletion populations be lost later in the screen.

#### Second round of sib selection

Prepare the plates for the next round of selection while the PCR reactions are being performed. At this point, either small plates or 6 well flat-bottomed culture plates can be used. Each positive population requires 96 plates (or 16 6 well flat-bottomed plates). Additional plates are poured as replacements.

Once the plates have recovered, wash the worms off following the previous protocol. The worms are plated at 10 worms/plate for a total of 96 populations. The plates are incubated for five days and the DNA preparation process is repeated. If small plates are used, 800 ul of water/antibiotic solution is used, pulling off 200ul and placing it in the corresponding wells of a labeled 96 deep well microplate. If tissue culture plates are used, then 200 ul of water/antibiotic is used, with 50ul of worms placed in each well of a 96 deep well microplate. The plates are allowed to dry thoroughly and stored at 15°C.

The DNA is prepared as previously described, and nested PCR is again performed and run on 1% gels to identify positive populations (Figure 1 appendix). Plates are identified, removed from storage and chunked onto large NGM plates seeded with OP50 for recovery of the starved populations. If more than two positive populations are identified, 2 are chosen to proceed with and the rest are frozen for insurance.



### Third round of sib selection

As the previous round of PCR is being performed, the plates for the last round of selection are prepared. Small NGM plates seeded with OP50 are used for this step. Each positive population requires 96 plates, with additional plates prepared for contamination replacement. The plates should be prepared and seeded at least 3 days prior to use to ensure dry plates and good bacterial growth.

Once the plates have recovered, single worms are picked and plated onto 96 small NGM plates. Sometimes it is not possible to obtain 96 single worms from each positive hit, so it may be necessary at this stage to move forward with more than 2 positive populations. The plates are incubated for 5 days, and the DNA preparation is performed as previously stated. Allow the plates to dry thoroughly and store at 15°C.

Nested PCR is performed and run on 1% gels to identify positive populations (Figure 1 appendix). These populations are founded by a single worm carrying the deletion (usually a heterozygote). Once the population(s) has been identified, the plates are recovered from storage, and chunked onto large NGM plates seeded with OP50. At this point a portion of the positive plates should be frozen as insurance.

The next step in the screen is to outcross the strains to eliminate extraneous mutations and then generate (if possible) a homozygous strain.

## Appendix II

Table 1. PCR cycling conditions used in the deletion screens

<u>Primer set</u>	<u>Anneal temp.</u>	<u>Extension time</u>	<u>Total cycles</u>
Fem1-A OR/OL	60° C	3 minutes	35
Fem1-A IR/IL	59° C	2 min. 30 sec	35
Fem1-B OR/OL	55° C	2 min. 15 sec.	35
Fem1-B IR/IL	55° C	2 min. 15 sec.	35
RH01-02	56° C	30 sec.	35
Fem2-A OL/OR	52.5° C	2 min. 30 sec.	35
Fem2-A IR/IL	60° C	2 min. 30 sec.	30
Fem2-B OR/OL	52.5° C	2 min. 15 sec.	26
Fem2-B IR/IL	58° C	1 minute	35
EH21-22	58° C	45 seconds	35
Fem3-A OR/OL	60° C	2 min. 30 sec.	30
Fem3-A IR/IL	59° C	2 minutes	35
Fem3-B OR/OL	60° C	2 min. 30 sec.	30
Fem3-B IR/IL	59° C	2 min. 30 sec.	30
RH09-10	58° C	1 minute	30

DNA is denatured @ 94° C, 2 minutes, initial cycle  
@ 94° C, 30 seconds each subsequent cycle  
Extension performed @ 72° C  
All annealing performed for 30 seconds

Table 2. Nested PCR primers used in deletion screen:

Cbfem-1A

cbfem1A-OR2

5'- CAA AAC CAT CGT GTT AGC GGA CTC- 3'

cbfem1A-OL2

5'- GAT CTT CTT TCC GTC CAA GTG TCG- 3'

cbfem1A-IR2

5'- AAG AGC GTT GGA ATA GTG AGG C- 3'

cbfem1A-IL2

5'- TTT CAA CTC TGC TAC TCA CTG G- 3'

Cbfem-1B

cbfem1B-OR

5'- CGA AGC AAA CTA CCG TAA CAG C- 3'

FEM-1B OR2

5'- GTG CGG GAA GAT GTA GTT TCG G- 3'

Fem-1B OR3

5'- TTC GTT CTC ATC GCG TTG GCA ACC- 3'

cbfem1B-OL

5'- CTC TAC GAA TCT ACA TGT CTG C- 3'

FEM-1B OL2

5'- CAC AAT CAC GTC GAA ATG CAG AC- 3'

Fem-1B OL3

5'- GCT ATC AGA TAG AGC AGT TGG ATG G- 3'

cbfem-1B-OL4

5'- CGA GCT CAA ACT TGA CAG TGT GAA GGC- 3'

cbfem1B-IR

5'- CGA TAT CAG AAA TCG GTC AAC TG- 3'

FEM-1B IR2

5'- AGA GTG ATT AGT TCG AGA GGA CGG- 3'

cbfem1B-IL

5'- TTC GAA GAA GAC ATA TCG GTC C- 3'

FEM-1B IL2

5'- GTT TTC AGT TCC AGC GAG AAC TCG- 3'

Cbfem-2A

cbfem2A-OR

5'- TGT CAC TTT GCG AAC TTC CAG- 3'

cbfem2A-OL

5'- TTG AGC TAC TTT CGC GTT TGA G- 3'

Cbfem-2A-IR

5'- TCT CCC AAC CAA GCA AAT GCC- 3'

Cbfem-2A-IL

5' - GTA AAT CGA CAC GAG CGC TGT C- 3'

Cbfem-2B

cbfem-2B-OR

5' - CGT ATC GAG AAG AGA TCT CG- 3'

cbfem-2B-OL

5' - AGT TTC CAG GAT CTC CAC TTG G- 3'

cbfem-2B-IR

5' - TCC TAA GCC TGT ACT TAA GCC- 3'

cbfem-2B-IL

5' - TCA TGA CGT TTT CGG AGA TGC- 3'

Cbfem-3A

cbfem3A-OR2

5' - CTG CCA AAA GCA ACG ATC GCG AG- 3'

cbfem3A-OL2

5' - GTG GTG ATT CTG CAC AT GGA CG- 3'

cbfem3A-IR2

5' - TGA CTA ACC CTC TTC CAA CAT GGC- 3'

cbfem3A-IL2

5' - GA ATA GTG TGC GAA ACG AGG AGG- 3'

Cbfem-3B

cbfem-3B-OR

5' - AAC CCA CAT GTT AGT CAA TCG C- 3'

cbfem-3B OR2

5' - CAA TTT CGT GGT CCA ATG GGA TTC GG- 3'

cbfem3B-OL

5' - CCA GAC GAA GAA AGC GGT TTC G- 3'

FEM-3B OL2

5' - TCA GAT GGC CAT GTT GGA AGA GGG- 3'

cbfem-3B-IR

5' - CTT GTT CCT ACC AAC CGC AGT G- 3'

FEM-3B IR2

5' - GGT TGA GTG TAC ACT GAA AGT AGC- 3'

Cbfem-3B-IL

5' - GTT CAG TGG CAT CTC TAT AGC AC- 3'

FEM-3B IL2

5' - CTC GCG ATC GTT GCT TTT GC AG- 3'

Wild type only primers

Cbfem-1 primers

RH02

5'- GTC TCA AAT CCG CAA AAG TGA CG- 3'

RH01

5'- CGA ACG AAT TCA ACT TCC ACT GG- 3'

Cbfem-2 primers

EH22

5'- CGA GAT CAT CGG TCG GCC AGG G- 3'

EH21

5'- TGC TCC CAA TAC GCT GCT GGG C- 3'

Cbfem-3 primers

RH09

5'- CAT CGT GAT ACA GTA GTC GAC ACG- 3'

RH10

5'- AGA CGT TCA CGA ACT GAT CTC CAG G- 3'

Table 3. Wild-type and deletion PCR product sizes for the *C.briggsae fem* genes deletion screen

<u>Primer set</u>	<u>PCR product size (in base pairs)</u>		
	outer	inner	deletion
Fem 1A(set 2))	3171	2451	
Fem 1B	2815	2674	399
RH01-02	422		
Fem 2A	2561	2304	
Fem 2B	2480	2048	733
EH21-22	460		
Fem 3A(set 2)	2261	2027	977
Fem 3B(set 1)	2983	2832	
Fem 3B(set 2)	2100	1680	
RH09-10	570		

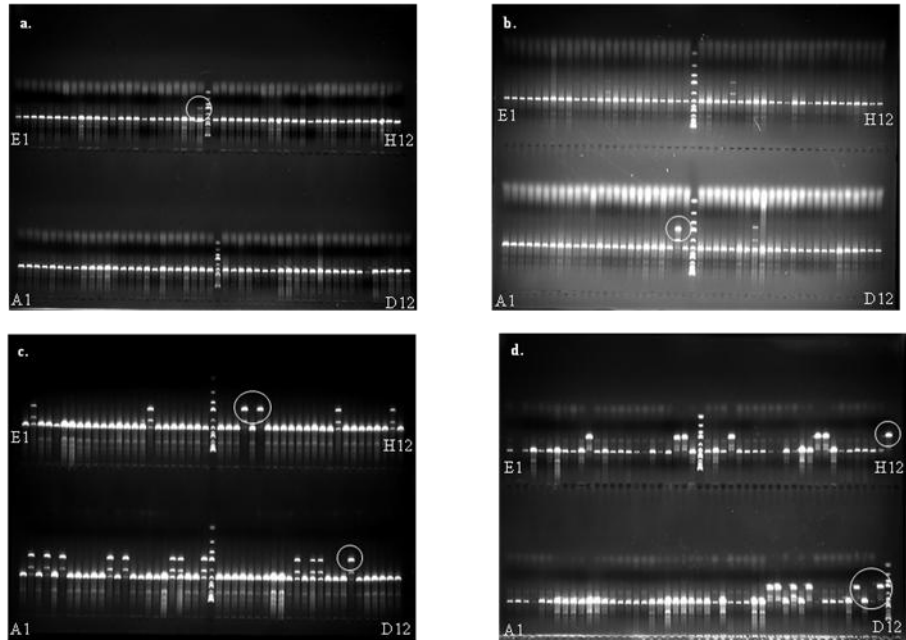


Figure 1. Identification of *C. briggsae fem-3* deletion mutant. Positive hits carried forward are identified by circles. **a.** Pooled populations. Positive lane F12. **b.** First round/ 50 worm sib selection. Positive lane B11. **c.** Second round/ 10 worm sib selection. Positive lanes D6,G4,G6. **d.** Third round/single worm selection. Positive lanes 45 (D9), 48 (D12), 96 (H12).

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