Males, Outcrossing, and Sexual Selection in *Caenorhabditis* nematodes

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Abstract

Males of *C. elegans* provide a crucial practical tool in the laboratory, but, as the rarer and more finicky sex, have not enjoyed the same depth of research attention as hermaphrodites. Males, however, have attracted the attention of evolutionary biologists who are exploiting the *C. elegans* system to test longstanding hypotheses about sexual selection, sexual conflict, transitions in reproductive mode, and genome evolution, as well as to make new discoveries about *Caenorhabditis* organismal biology. Here, we review the evolutionary concepts and data informed by study of males of *C. elegans* and other *Caenorhabditis* . We give special attention to the important role of sperm cells as a mediator of inter-male competition and male-female conflict that has led to drastic trait divergence across species, despite exceptional phenotypic conservation in many other morphological features. We discuss the evolutionary forces important in the origins of reproductive mode transitions from males being common (gonochorism, females and males) to rare (androdioecy, hermaphrodites and males) and the factors that modulate male frequency in extant androdioecious populations, including the potential influence of selective interference, host-pathogen coevolution, and mutation accumulation. Further, we summarize the consequences of males being common versus rare for adaptation and for trait divergence, trait degradation, and trait dimorphism between the sexes, as well as for molecular evolution of the genome, at both micro-evolutionary and macro-evolutionary timescales. We conclude that *C. elegans* male biology remains underexploited and that future studies leveraging its extensive experimental resources are poised to discover novel biology and to inform profound questions about animal function and evolution.

An easy one-hour train ride outside of Paris one can find the picturesque little village of Santeuil, whose town center is dominated by a 12th century church high on a hill that overlooks the surrounding countryside. On the edge of town next to the railroad tracks there is a small stream where one can readily find an important species once thought to be very elusive in the wild: the nematode *Caenorhabditis elegans*. First thought to be denizens of soil and compost heaps, it turns out that *C. elegans* is easy to collect in rotting fruit such as apples and their apparent "natural" habitat is rotting vegetation in general (FRÉZAL AND FÉLIX 2015; SCHULENBURG AND FELIX 2017). In Santeuil, this rotting vegetation means the large hollow stems of decomposing hogweed (*Heracleum sphondylium*) and comfrey (*Symphytum officinale*) along the moist banks of the wooded stream. And if one collects hundreds or even hundreds of thousands of individuals from these populations, virtually no males are to be found. In fact, if you use methods from molecular population genetics to study these populations over a period of a decade, there is little evidence that they ever have sex at all (BARRIÈRE AND FÉLIX 2005; RICHAUD *et al.* 2018). This is because the dominant member of these worm populations is the hermaphrodite, which first produce sperm early during sexual maturity and then switch to the production of eggs that are subsequently fertilized by the sperm (KUWABARA AND KIMBLE 1992). So, technically, the worms do have sex—with themselves (autogamy)—but do not outcross. The overall outcome of this self-fertilization is separation of reproductive lineages that end up having independent evolutionary histories until a rare outcrossing event occurs. This unusual mode of reproduction has without question dominated much of the evolution of *C. elegans* as a natural organism. This evolutionary history should be used to inform the way we think about this species as a model system for questions ranging from epigenetics to neurobiology to aging. It is the

evolutionary consequences and potential functional roles of the oft overlooked members of this story—the males—that are the focus of this review.

In the lab, of course, it is the self-same hermaphrodites that have become the workhorse of *C. elegans* genetics. The ability to quickly generate self-propagating homozygous lines is one of the major benefits of the worms as a model system. A lesser appreciated benefit of this system is that very severe mutations, even those leading to nearly complete paralysis, can still be maintained genetically because strong deficiencies in mating ability do not preclude reproduction as they might, say, in *Drosophila* or mice. As long as sperm and eggs can be produced and migrate through the reproductive tract of a hermaphrodite, reproduction can take place. Even in cases in which the eggs cannot be laid, hatching can proceed internally, with offspring eventually bursting out from within their parent (the "bag of worms" phenotype; (TRENT *et al.* 1983)). Yet even in the laboratory, males are critically important, as they allow genetic crosses to be made. Conveniently, because the chromosomal sex determination system of this group of nematodes is XX (hermaphrodites) and XØ (males), males can be generated by non-disjunction of the X chromosome (Box 1), a process that in the lab is often encouraged by a quick shock at high temperatures (FAY 2013). Non-disjunction occurs spontaneously as well, at a rate of 1/1,000 for the N2 lab strain and as high as 1/250 for some natural isolates (TEOTONIO *et al.* 2006). So, males are not strangers to *C. elegans* lab populations, making their apparent rarity in natural populations something of a conundrum.

Importantly, the story of males is very different in other closely related species. It is now clear that the vast majority of *Caenorhabditis* are male-female (gonochoristic) species (FELIX *et al.*

2014). Surprisingly, hermaphrodites (androdioecy) have evolved three times independently in the genus: in *C. elegans*, in *C. briggsae*, and in *C. tropicalis* (Figure 1). The evolution of hermaphrodites also appears to be fairly common in other nematodes (DENVER *et al.* 2011), such as the closely related genus *Oscheius* (Felix et al. 2001). So whatever controls the balance between the retention of males and their loss to very low frequencies appears to have generated a common theme across the group. Indeed, this makes *C. elegans* and its relatives ideal models for understanding the causes and consequences of outcrossing, changing sex ratios, and the evolution of male-specific function *per se*. In many respects, the relationship between the sexes, the role of males, and the genetics and evolution of the transition to hermaphroditism is *the* question that *C. elegans* raises from the point of view of its organismal biology.

In this chapter, we focus on the major themes that emerge from the presence and absence of males within *Caenorhabditis* populations. First, we discuss how differences in male frequency lead to variation in the opportunity for sexual selection and sexual conflict. Second, we highlight studies that have built upon the unique biology of *C. elegans* to test some of the major theories of the evolution of sex and outcrossing. Finally, we highlight recent results from comparative and population genomics that reveal unmistakable signals of the role that males have played—and continue to play—within these species. The rapid increase in both species diversity and genomic resources within *Caenorhabditis* provides a rich context for examining each of these questions (Box 1).

While our growing knowledge of genomic variation is important, in the end, the beauty of *C. elegans* is its strength as an experimental system. This is as true for evolutionary biology as it is

for developmental and molecular genetics. Researchers interested in the genetics of adaptation, outcrossing, and the evolution of intra- and intersexual interactions have been increasingly utilizing the many genetic tricks available in *C. elegans* to conduct experiments and test hypotheses that would be very difficult to perform in other species. Understanding the evolutionary implications of reproductive transitions in *C. elegans* also provides a bridge to other organisms that have enjoyed intense study in their own right to explore androdioecy (e.g. *Eulimnadia* clam shrimps (CHASNOV 2010; WEEKS 2012) *Pristionchus* nematodes (SOMMER 2006), *Mercurialis* plants (PANNELL 1997), mechanisms of sexual conflict (e.g. *Drosophila melanogaster* (AVILA *et al.* 2011), water striders (KHILA *et al.* 2012), and the genome implications of selfing (e.g. plants like *Arabidopsis* and *Capsella* (BARRETT *et al.* 2014)). While it is impossible to provide a comprehensive review of all of these topics and systems, in each section we aim to highlight a few studies focused on *Caenorhabditis* that exemplify the core questions at stake and illustrate the cutting edge of the *C. elegans* field. We do not review many aspects of the functional biology of *C. elegans* males, as fortunately a number of excellent recent reviews cover these topics (BARR *et al.* 2018; EMMONS 2018). Our focus is on evolutionary biology, and overall this is still a very young field of study for *C. elegans*, with a great deal of work still ahead. With this in mind, we also point to areas in which more work is needed or where unresolved controversies still remain. The rapid accumulation of genomic information, genome engineering, and deepening insights into the basic biology of an ever-growing circle of *C. elegans* relatives suggests that the field as a whole is poised for very rapid progress over the next few years.

SEXUAL SELECTION AND SEXUAL CONFLICT

Although trivial on its face, the presence of males within worm populations means that there is more than one predominant phenotypic class within the population, i.e., *C. elegans* is sexually dimorphic (Box 1). This dimorphism is of course driven by the functional requirements for sex-specific reproduction. Perhaps the most fundamental consequence of the two sexes having different roles in reproduction is that males and females/hermaphrodites have very different reproductive strategies and that these differences can lead to potential fitness conflicts both within and between the sexes (CHAPMAN 2006). When different individuals—usually males—display large differences in mating success, there is an opportunity for sexual selection to operate, leading to the evolution of traits specifically geared toward increasing reproductive success in terms of individual attractiveness (think peacock's tail) or male-male competition (think ram's horns). While there appear to be few males within natural populations of *C. elegans*, even if they were numerous, observations of mating dynamics would still be difficult to observe. Indeed, in the laboratory, *C. elegans* males are notoriously poor at mating (GARCIA *et al.* 2007). In contrast, the intense mating vigor of males from gonochoristic species such as *C. remanei* manifests as a distinct tendency to swarm over females when raised on plates (Figure 2), strongly suggesting the opportunity for sexual selection within these species.

Despite what looks to be fairly intense competition for mates among males of gonochoristic species, there is little evidence in terms of morphology for the presence of exaggerated secondary sexual characteristics within *Caenorhabditis* . Most of the dimorphism that is evident between males and females/hermaphrodites, such as the structure of the gonad and morphology of the tail (Figure 2), appears to be directly tied to sex-specific reproductive function. Instead,

there are a number of puzzling features of reproductive interactions within and between the sexes that may be clues to possible sexual selection and sexual conflict hidden within the unseen world of gametic interactions and chemical signaling.

Reproductive context of sexual selection

One of the many conundrums regarding males within *C. elegans* is that it is clear that hermaphrodites are strongly sperm limited. An individual self-fertile hermaphrodite can only produce ~300 offspring, with that number being determined by the number of self-sperm generated by the hermaphrodite before its gonads transition to oocyte production (WARD AND CARREL 1979). When mated with a male, however, hermaphrodites can produce upwards of 900 offspring over their lifetime (HUGHES *et al.* 2007). Indeed, even after self-sperm have been depleted, the hermaphrodite gonad can "wake up" and become rejuvenated in the presence of male sperm late in life (HUGHES *et al.* 2007; MENDENHALL *et al.* 2011). Unmated hermaphrodites therefore represent a largely untapped pool of reproductive output.

Based on these facts, one might expect males to be common such that (1) hermaphrodites would be subject to intense mate competition among males and (2) the fitness interests of hermaphrodites would favor attraction of males to nearly triple their reproductive output (although as discussed below this benefit is discounted by the fact that the outcrossed offspring come late in the reproductive cycle (HODGKIN AND BARNES 1991)). Surprisingly, neither of these factors appear to be central driving elements of *C. elegans* biology. Late-life reproductive capacity of hermaphrodites in the wild may be a pipe-dream, however, given that mortality

curves are likely more severe in nature than in benign lab conditions (VAN VOORHIES *et al.* 2005). Instead, there is a premium on early-life reproduction, made more acute from the colonization of ephemeral resource patches to be exploited and dispersed from before they disappear, giving greater reproductive value to the first offspring produced in what becomes a mass of overlapping generations (CUTTER 2015; FRÉZAL AND FÉLIX 2015). Consequently, it appears that the evolutionary transition from outcrossing to self-fertilization has allowed another critical feature of mating interactions—sexual conflict—to dominate the evolution of both males and hermaphrodites.

Hallmarks of sexual conflict

While mating has the obvious benefit of fertilization, it also comes with some serious risks, especially for females/hermaphrodites. A number of studies have demonstrated that mating can lead to early mortality in both males and hermaphrodites (VAN VOORHIES 1992; GEMS AND RIDDLE 1996), and that excessive mating reduces a female's lifetime reproductive success (DIAZ *et al.* 2010). The effect on hermaphrodites seems clear, as males continually harass hermaphrodites in their attempt to mate and, in particular, the insertion of the male spicule—especially when ill placed—seems to hold the potential for direct damage to the cuticle (WOODRUFF *et al.* 2014).

The potential for harm has been demonstrated most clearly in matings between closely related species in which one species contains hermaphrodites (e.g., *C. briggsae*) and the other is gonochoristic or male/female (e.g., *C. nigoni*; (TING *et al.* 2014)). Here, sperm from the

obligately outcrossing species appear to have evolved such intense competitive ability that male sperm cells actually break out of the spermatheca of hermaphrodities, to later be seen wandering throughout the rest of their bodies (TING *et al.* 2014; TING *et al.* 2018). The fact that females from the outcrossing species rarely suffer this fate, whereas hermaphrodites from the selfing species usually do, suggests that males and females co-evolve with one another in a type of reproductive arms race that results from sexual conflict. Thus, despite having very similar outcomes of outcross reproduction (many offspring), the reproductive dynamics that are actually generated within natural populations of hermaphrodites versus gonochoristic species appears to be shaped strongly by the opportunity for (or avoidance of) sexual conflict (CHASNOV 2010; PALOPOLI *et al.* 2015).

The presence of sexual conflict in *C. elegans'* outcrossing ancestor predicts the likely evolution of mating-related traits subsequent to the transition to reproduction primarily by self-fertilization within a single sex. In particular, we expect the loss of traits that induce a cost to 'female' fitness and the exaggeration of traits that confer a benefit to 'female' fitness. In many cases, that evolution will involve trait loss, as we outline below. Three factors could be responsible such trait change: 1) degeneration via genetic drift of loss-of-function mutations to loci subject to relaxed selection in the new sexual context, 2) direct selection on the trait to eliminate male-induced costs or promote benefits to hermaphrodite self-fitness (i.e. adaptive evolution to a selfing lifestyle), or 3) indirect selection on traits due to pleiotropy or linkage with other directly-selected traits. For any given trait or molecular feature, it can be a challenge to distinguish among these possibilities.

One of the clearest signals pointing to a history of sexual conflict in *Caenorhabditis* comes from the fact that hermaphrodites appear to avoid mating with males in the first place. There are several lines of evidence for this male avoidance, which is especially apparent in contrast to gonochoristic species that provide a view of the likely ancestral state. Females, especially virgin females in gonochoristic species, behave quite differently from hermaphrodites with respect to mating interactions. In species such as *C. remanei*, previously unmated females become quiescent (still) during mating. The female vulva seems to act as a sensor to facilitate mating, which appears to be induced upon contact of the male cloaca with the female vulva prior to spicule insertion by a germline-independent seminal factor produced by the male somatic gonad (GARCIA *et al.* 2007). *C. elegans* males fail to induce facilitated mating behavior in this way, and hermaphrodites of *C. elegans* and *C. briggsae* fail to respond to males of their own or other species (GARCIA *et al.* 2007); results for androdioecious *C. tropicalis* await study. Interestingly, mated females will actively run away from males until they become sperm depleted. In contrast, virgin females instead actively seek out males if they detect their presence (GARCIA *et al.* 2007; BORNE *et al.* 2017). In *C. elegans*, however, hermaphrodites nearly always actively move away from males, or at least do not slow down during mating (GARCIA *et al.* 2007), and may expel sperm from their uterus (KLEEMANN AND BASOLO 2007). Moreover, hermaphrodites of *C. elegans* and *C. briggsae* secrete less-potent sex pheromone relative to virgin and sperm-depleted females of gonochoristic species (CHASNOV *et al.* 2007; BORNE *et al.* 2017). There is some evidence that sperm-depleted hermaphrodites are more receptive to males (KLEEMANN AND BASOLO 2007), although the effects are not large when compared to the behavioral differences seen for females from gonochoristic species.

Taken together, these observations suggest that hermaphrodites actively avoid mating for most, if not all of their lives, probably because assured reproduction via self-reproduction tends to outweigh the mortality risks of mating with males, at least early in life (CHASNOV AND CHOW 2002; CHASNOV 2010).The X/Ø sex determination system within this group means that the most direct way of making new males is via mating with existing males. So the most direct consequence of hermaphrodite avoidance of male mating is a rapid decline of the frequency of males within the population (STEWART AND PHILLIPS 2002). This, in turn, almost assuredly is the major proximate cause of the rarity of males within natural populations. While the fact that hermaphrodites have maintained "avoidance" traits while losing "attraction" traits suggests that these processes have been under direct selection, it is also likely that there has been a general degradation of inter-sexual mating behavior in hermaphrodites, just as there appears to be in males, as discussed below. Any additional degradation of function in hermaphrodites would serve to accelerate the loss of males from *C. elegans* populations.

Degradation of male function within self-fertilizing species

The apparent fickleness of hermaphrodites means that the frequency of males (and male mating) can be very low, which in turn means that the opportunity for selection on male function should also be very low. Direct sampling of male individuals and population genetic inference indicate males typically being present in nature at frequencies of 1% or much less (BARRIÈRE AND FÉLIX 2005; HABER *et al.* 2005; SIVASUNDAR AND HEY 2005; BARRIÈRE AND FÉLIX 2007; FELIX AND BRAENDLE 2010; ANDERSEN *et al.* 2012; SCHULENBURG AND FELIX 2017; RICHAUD *et al.* 2018) (but see (SIVASUNDAR AND HEY 2005)). Moreover, such extreme male rarity means that *C.*

elegans males will almost never encounter one another, making male-male competition largely absent as a mode of sexual selection. Thus, the likely explanation for the relative ineptness of *C. elegans* males (at least as compared to gonochoristic species) is that they are simply not called to duty frequently enough to maintain their faculties at a high level in the face of either the relentless accumulation of deleterious mutations or, more intriguingly, by selection against an inherent trade-off between a given gene's contribution to male and hermaphrodite fitness that generates a negative pleiotropic intralocus conflict (something that is often anticipated in theories of sexual conflict; (CHAPMAN 2006)). Nevertheless, as discussed below, over 10% of the genome is devoted to male-biased function and males themselves have more cells, more neurons, specialized morphology, and sex-specific behavior, and so it is likely that there must be enough direct selection on *C. elegans* males, or indirect selection on males due to the pleiotropic effects of genes with shared activity in hermaphrodites, to maintain these aspects of male function. Interestingly, there is actually substantial variation in male mating ability across different natural isolates of *C. elegans* (TEOTONIO et al. 2006), which suggests that different populations might experience different patterns of selection on male function.

There are two strong sources of evidence that selection on males has dramatically declined independently in several lineages during the transition to self-reproduction. First, there has been substantial loss of male-specific genes, such as the *mss* genes discussed below, within the genomes of hermaphroditic species. The disrupted allele of *plg-1*, which disables copulatory plug deposition (Figure 2) and which occurs at high frequency among wild isolates of *C. elegans* (BARKER 1994; PALOPOLI *et al.* 2008), provides another striking example of male-specific gene loss of function (AJIE *et al.* 2005). Second, male mating vigor is poor, compared to outcrossing

species (GARCIA *et al.* 2007), and *C. elegans* has ample natural genetic variability conferring the potential for male sexual function to improve: it is actually quite simple to rapidly select for increased male function by manipulating the level of male-male competition within a population using experimental evolution. The pioneering work by LAMUNYON AND WARD (2002) showed how enhanced male reproductive function evolves when several *C. elegans* natural isolates are mixed together, using a common *spe-8* mutant background that renders hermaphrodites self-sterile. They found that sperm size and competitiveness rapidly increased after a few generations of maintaining populations at a 50:50 sex ratio (see below). A similar result was found by PALOPOLI *et al.* (2015), who used 16 *C. elegans* natural isolates to create a base population in a feminized *fog-2* genetic background to increase intrasexual competition (Box 1). Similar to LAMUNYON AND WARD (2002), they also found a rapid increase in sperm size. Most interestingly, they also found that males also rapidly evolved a "female-harm" phenotype that led to increased mortality in mated females, most likely because of increased copulation times and spicule insertion rates. This study is particularly valuable because it simultaneously links together the loss (and subsequent recovery) of male mating ability to the sexual conflict that likely drove the decrease in male frequency in the first place.

Evolution of sperm competition

Like *C. elegans*, male-female species of *Caenorhabditis* also appear to be sperm-limited in their reproductive output (TIMMERMEYER *et al.* 2010; PALOPOLI *et al.* 2015). Sperm limitation means that females must mate multiply in order to maximize reproductive output. Unlike *C. elegans*, this opportunity for sexual selection appears to have had real consequences within gonochoristic

species. LAMUNYON AND WARD (1999) noted tremendous variation in sperm size among nematode species. *C. elegans* males actually have relatively small sperm for a nematode (~20μm^2 cross-sectional area of spermatids). In contrast, *C. remanei* sperm are more than twice this size. These size differences are particularly relevant here because when it comes to fertilization success, bigger really is better. Larger sperm outcompete smaller sperm. Because of the amoeboid nature of the sperm themselves, it seems likely that direct physical interactions between the sperm within the spermatheca are a major part of this large sperm advantage, in addition to their greater speed (LAMUNYON AND WARD 1998). Indeed, within *C. elegans*, male sperm are used "preferentially" for fertilization, but, rather than active "cryptic female choice" of sperm, this is almost certainly caused by the fact that male sperm are as much as 50% larger than sperm of hermaphrodites (LAMUNYON AND WARD 1998; LAMUNYON AND WARD 1999).

But neither *C. elegans* nor *C. remanei* male sperm hold a candle to recently discovered species that display sperm gigantism (Figure 1). These species have sperm that can exceed 200 μm^2 in cross-sectional area (VIELLE *et al.* 2016). *C. inopinata*, which is the closest known relative to *C. elegans*, has sperm that is six times larger than its cousin (WOODRUFF *et al.* 2018). It is important to note that *C. elegans* sperm are roughly the same size as the cell body of human sperm, despite the stark differences in overall animal body size, so each worm sperm cell is a substantially larger physiological investment than that seen in most animals. However, sperm competition theory generally predicts that greater sperm competition risk will lead to the evolution of more and smaller sperms cells (PARKER AND BEGON 1993). Therefore, it remains something of an enigma as to what conditions of sperm competition would favor the evolution of fewer sperm per ejaculate, as species with gigantic sperm transfer fewer of them (VIELLE *et al.* 2016).

These giant sperm cells can represent as much as 5% of the initial volume of the fertilized embryo, so their existence represents a significant loss of anisogamy (disparate size of male and female gametes) that is fairly unique in the animal world (VIELLE *et al.* 2016). Species with giant sperm also have males with greater body width and experimental evolution populations that evolved larger sperm also evolved larger males (LAMUNYON AND WARD ; VIELLE *et al.* 2016), likely an indicator of testis size and investment in gamete production given that most of the male body is comprised of gonad. While competition may be the major driver of the evolution of sperm size, the gigantic sperm found in some species begs the question as to whether the sperm's "soma" has some other role to play. For example, some fruit flies make sperm that are several times longer than the male himself (PITNICK *et al.* 1995). They may be used to clog up the female reproductive tract or they may actually serve as a nuptial gift to provide nutrition to the females and/or egg. Small RNAs are important in sperm fertility (CONINE *et al.* 2009), and their paternal transfer to the zygote also could conceivably influence embryonic development. There may be a similar role for gigantic sperm within *Caenorhabditis* , although little work has been done yet to test these ideas.

Despite the important role of sperm size in sperm competition, the number of sperm transferred per ejaculate and the remating rate also represent crucial components of fertilization success (MURRAY *et al.* 2011; GIMOND *et al.* 2018). Unfortunately, it remains unclear what, genetically, is responsible for natural variation in sperm size and number. However, disruption of the NURF-1/ISW-1 chromatin remodeling complex appears to drive small sperm size in *C. elegans* domestication to a liquid environment, and RNAi knockdown of *nurf-1* reduces sperm size in

other species as well (GIMOND *et al.* 2018). Mutations to the *nath-10* acetyltransferase also likely are involved in *C. elegans*' adaptation to the lab environment, with its pleiotropic effects including increased hermaphrodite self-sperm number (DUVEAU AND FELIX 2012), and artificial mutants that perturb the sperm-oocyte switch also alter the number of sperm that hermaphrodites make (HODGKIN AND BARNES 1991; MURRAY *et al.* 2011). While many genes involved in the hermaphrodite-specific spermatogenesis pathway have been characterized for *C. elegans* (L'HERNAULT 2006), the genetics of male-specific spermatogenesis remains largely unknown.

Equally mysterious are the proteins that are likely transmitted along with the sperm during insemination. Work in *Drosophila* has shown that seminal fluid proteins play an important role in mediating competition among sperm (SIROT *et al.* 2015). More intriguingly, some of these fly proteins mediate female-specific behaviors such as egg laying rate and susceptibility to remating. We still know very little about seminal fluid proteins for *C. elegans*. The proteins identified thus far, such as TRY-5 and SWM-1, are necessary for sperm activation, a critical step in the fertilization process (STANFIELD AND VILLENEUVE 2006; SMITH AND STANFIELD 2011). The PLG-1 mucin protein also gets transferred during copulation to form a copulatory plug on the vulva, which partially inhibits subsequent male mating attempts and may aid in sperm retention in the uterus (BARKER 1994; PALOPOLI *et al.* 2008). The sperm themselves secrete some proteins via specialized vesicles upon activation (KASIMATIS *et al.* 2018b) and secretion of MSP is important in triggering ovulation (MILLER *et al.* 2001), although it is not clear whether any of these proteins play an important part in sperm competition. Interestingly, the SWM-1 sperm activation protein is actually produced by muscle cells before it migrates to the gonad (CHAVEZ *et al.* 2018) and gut-derived compounds migrate to the female germline for use by oocytes to

secrete prostaglandins as sperm chemoattractant (KUBAGAWA *et al.* 2006), suggesting that proteins important for mediating sexual conflict could be recruited from tissues spread across the bodies of both males and hermaphrodites/females. These proteins almost assuredly influence the competitive environment among the sperm (HANSEN *et al.* 2015). The likely complex chemical environment that serves as the context for post-mating interactions within and between the sexes remains a mostly open frontier and is virtually guaranteed to yield some interesting and unexpected outcomes when more fully explored.

The role of sperm cells and seminal fluid components in reproductive success necessarily follows copulation, the most complex behavior performed by *C. elegans*. Upon contact with hermaphrodites/females, male *Caenorhabditis* slide their tail along her cuticle, presumably facilitated by the dense set of male-specific neurons in the tail, some of which form the finger-like projections of the rays (FITCH 1997). Once he locates the vulva, successful copulation depends on insertion of the paired spicules that guide transfer of sperm and seminal fluid (LIU AND STERNBERG 1995; SMITH AND STANFIELD 2011). How might sexual selection or sperm competition influence the evolution of these various traits and behaviors? Comparative phylogenetic analysis shows substantial trait variation across species in features like ray number and positioning, presence versus absence of a pronounced tail fan, size and shape of spicules, as well as parallel versus spiral mating position (KIONTKE *et al.* 2011). However, these traits correlate strongly with the phylogenetic distance between species (KIONTKE *et al.* 2011), and some traits are nearly indistinguishable between species (e.g. *C. brenneri* and *C. remanei*, (SUDHAUS AND KIONTKE 2007)). For example, spiral mating orientation appears to associate perfectly with males having a reduced fan, though phylogenetically restricted to only those

species most closely-related to *C. angaria* (KIONTKE *et al.* 2011; STEVENS *et al.* 2019) the evolution of which could even reflect natural selection pressures on mating due to the particular habitat matrix that such species typically encounter. Thus, despite male tail traits being among the most disparate organismal phenotypes between *Caenorhabditis* species, their phylogenetic-dependence argues against sexual selection driving rapid, lineage-specific, co-evolutionary arms race evolution that targets these structures perpetually for innovation and novelty in form. This contrasts with the repeated independent evolution of sperm size across the phylogeny (VIELLE *et al.* 2016). A caveat to this conclusion is that no studies have yet formally tested for coevolution of male tail morphology traits with characteristics that might be indicative of the strength of sexual selection and sexual conflict, such as male mating vigor, female re-mating latency, copulation duration, ejaculate size, and sperm size.

Genomic persistence of male-related genes

The evolutionary transition from an ancestral population with ~50% males to a derived population with <0.5% males represents a drastic shift in the selection pressures on sexually-dimorphic traits and the genes that encode them. Unique male traits all must be encoded by genes with sex-limited expression or by sexually-dimorphic regulation of genes that are expressed in both sexes. On the one hand, the rarity of male contributions to reproduction mean that purifying selection will be weaker against deleterious mutations to such genes (CUTTER 2008; GLEMIN AND RONFORT 2013). As a result, selection will be less capable of weeding out mutations, leading to accumulation by genetic drift of changes to protein sequences, including loss-of-function mutations and gene deletions. Genetic drift, however, is a slow process, and the genomic and phenotypic degradation implicit in relaxed selection on male function also ought to

be slow. The effects of transmission ratio distortion (see above 'Non-Mendelian byproducts of mixed selfing and outcrossing') on deletions affecting male-related loci in *Caenorhabditis* do, however, provide one selectively neutral force that could accelerate loss of genes that have male-biased activity (WANG *et al.* 2010; YIN *et al.* 2018). Despite the disproportionate genomic loss of genes with male-related function (THOMAS *et al.* 2012; FIERST *et al.* 2015; YIN *et al.* 2018), theory predicts that some male-specific loci can be retained even with exceptionally rare mating (CHASNOV AND CHOW 2002).

On the other hand, the novel reproductive environment of females (now as self-capable hermaphrodites) creates opportunity for selection to optimize traits to this new context (SLOTTE *et al.* 2012). For sexually-dimorphic traits, selection thus ought to favor trait values that maximize hermaphrodite fitness at the expense of males, even eliminating male-specific traits that confer a cost to hermaphrodites due to negative pleiotropy of loci with intralocus sexual conflicts (CHAPMAN 2006). The genes that contribute to sexual conflict in the ancestor would thus disproportionately feel the influence of selection favoring hermaphrodites as they adapt to become 'better' hermaphrodites, potentially accelerating the degradation and loss of male traits and their genetic encoding (CUTTER 2008; GLEMIN AND RONFORT 2013; SHIMIZU AND TSUCHIMATSU 2015). Regardless of the process (neutral or adaptive), all three known *Caenorhabditis* species with selfing hermaphrodites show convergent evolution in sex-related traits and genome features indicative of an animal manifestation of the 'selfing syndrome' that is well-known in plants (ORNDUFF 1969; CUTTER 2008; FIERST *et al.* 2015; SHIMIZU AND TSUCHIMATSU 2015).

A key genomic consequence of the transition to selfing is the convergent evolution of reduced genome size compared to the genomes of nearest non-selfing relatives (THOMAS *et al.* 2012; FIERST *et al.* 2015; YIN *et al.* 2018), though even smaller genomes are now known for a number of non-selfers in the more distantly-related *Japonica* and *Drosophilae* groups of *Caenorhabditis* (STEVENS *et al.* 2019).This genome shrinkage involves loss of both non-coding sequence and coding genes, disproportionately genes with male-biased expression (THOMAS *et al.* 2012; FIERST *et al.* 2015; YIN *et al.* 2018). Spermatogenesis-related are especially prone to rapid protein sequence evolution and gene family size turnover, in addition to loss (CUTTER AND WARD 2005; ARTIERI *et al.* 2008; YIN *et al.* 2018). However, it is not entirely clear how much of the rapid sequence evolution in these retained genes is due to the consequences of 1) greater genetic drift under selfing, 2) sexual selection-driven divergence leftover from the outcrossing ancestors of selfing species, or 3) generally weaker selective constraint on such genes regardless of sexual mode (MANK AND ELLEGREN 2009; DAPPER AND WADE 2016). An important consequence of genome shrinkage following selfing is the irreversibility of the loss of singleton genes. Presuming that at least some of the genomic degradation is driven by selective pressures for hermaphrodite adaptation, 'adaptation by loss of function' could constrain subsequent responses to selection (CUTTER AND JOVELIN 2015).

A striking example of gene loss related to male-specific function that arose independently in *C. elegans*, *C. briggsae* and *C. tropicalis* is the case of the *mss* genes that confer improved sperm competitive ability when functional (YIN *et al.* 2018). These short glycoproteins form a multi-gene family encoded on autosomes in those species with obligatory male mating, localizing to spermatocyte and sperm membranes (YIN *et al.* 2018). While ablation of *mss* function does not

yield infertility, it does depress the ability of sperm cells to outcompete the sperm from other males for oocyte access in fertilization and, impressively, experimental re-introduction of *mss* expression enhances sperm competitive ability (YIN *et al.* 2018). The disrupted function of the *plg-1* locus by a retroelement in many wild isolates of *C. elegans* also provides a well-characterized example of the consequences of male rarity through genetic disruption of a sex-specific gene (HODGKIN AND DONIACH 1997; PALOPOLI *et al.* 2008). Deposition of a copulatory plug by males onto the vulva of their mate confers benefits to males in terms of fertilization assurance most strongly when females mate with multiple males, leading to selection conserving plugging in most species. Interestingly, the *plg-1* mucin-like protein contains a large repetitive peptide sequence region with low sequence identity across species (PALOPOLI *et al.* 2008). Natural allelic disruption of *plep-1*, which alters male mating behavior, also may represent a byproduct of relaxed sexual selection in *C. elegans* (NOBLE *et al.* 2015). The highly-expressed proteins encoded by the *msp* (major sperm protein) family play important roles in sperm cell motility and cell-cell signaling (SMITH 2006). Their molecular evolution, in contrast to many other sperm-associated genes, is highly conserved and appears strongly influenced by gene conversion that leads to concerted evolution among the family members within a species (KASIMATIS AND PHILLIPS 2018).

How many male-specific genes are there, what do they do, and where are they located? Estimates suggest that about 270 of the 19,050 genes with detectable expression in the *C. elegans* genome are male-specific and lack hermaphrodite expression, with over 2400 genes having highly male-biased expression (THOMAS *et al.* 2012). Separate experiments quantifying differential expression in gonads identified over 2700 genes to have enriched expression in

spermatogenic gonads relative to about 1700 enriched in oogenic gonads (ORTIZ *et al.* 2014). Microarray analysis found 430 genes with enriched expression in male soma, which was about one third the number of genes with spermatogenesis enrichment in that study (REINKE *et al.* 2004). Lower throughput proteomics analysis further supports the presence and abundance of a subset of these genes (KASIMATIS *et al.* 2018b). These male-biased genes are enriched for membrane and kinase/phosphatase gene ontology terms (REINKE *et al.* 2004; THOMAS *et al.* 2012). Thus, sperm development in particular provides an abundant source of differential gene expression, though it remains unclear how many genes have sex-biased or sex-specific activity in larval development. Because hermaphrodites also make sperm, those genes indispensable for spermatogenesis are shielded from loss. In the contrast of the *C. briggsae* and *C. nigoni* genomes, however, multi-gene families are smaller in *C. briggsae* to account partly for the 6854 (23.5%) difference in gene count between the species (YIN *et al.* 2018), providing one means by which male-biased genes might be lost without total eradication of functional capacity.

Genes with male-biased expression are rare on the X-chromosome, likely resulting from the fact that most male-biased genes are associated with gonad expression rather than somatic expression (REINKE *et al.* 2004; ALBRITTON *et al.* 2014; ORTIZ *et al.* 2014). More specifically, meiotic sex chromosome inactivation (MSCI) in males (KELLY *et al.* 2002; REUBEN AND LIN 2002; BEAN *et al.* 2004; BESSLER *et al.* 2010) should act as a potent selective agent against the encoding on the X-chromosome of genes important in spermatogenesis. Indeed, sperm genes are nearly absent from the X-chromosome (REINKE *et al.* 2004; ALBRITTON *et al.* 2014; ORTIZ *et al.* 2014). Genes with sperm-related functions also are exceptionally rare in operons across all chromosomes (REINKE AND CUTTER 2009), likely due to the unusually promoter-dependent regulation of

spermatogenesis gene expression relative to other germline genes (MERRITT *et al.* 2008). In addition to the protein function of coding genes, the 22G- and 26G-small-RNA derivatives of coding sequence transcripts appear to be important in maintaining sperm fertility (CONINE *et al.* 2009; LI *et al.* 2016), implicating important post-transcriptional regulatory mechanisms on male-biased traits and those sperm genes that have shared activity in hermaphrodites. Genes with somatic male-biased expression tend to have lower magnitudes of sex-bias than do spermatogenesis genes (ALBRITTON *et al.* 2014), suggesting the potential for differences in sexual conflict over expression levels for somatic versus gametic traits.

Intersexual communication

The contrast in the effects of inter-sexual communication between outcrossing and selfing species provides another strong indicator that sexual selection mediates the role of males within *Caenorhabditis* populations. The last decade has seen a dramatic unveiling of insights into the rich chemical milieu in which these nematodes exist and how they use a complex set of chemical signals to mark the state of the environment and to communicate with one another (IZRAYELIT *et al.* 2012; BUTCHER 2017). Within *C. elegans*, the classic inter-individual communication system of study has been environmental conditioning that trips a developmental switch in young larvae, leading to the dauer resting/migratory stage. Initially identified as a "pheromone" via treatment with crude nematode exudate (GOLDEN AND RIDDLE 1984), the dauer response is now known to be generated by a balance between food availability and a set of nematode-specific lipid derivatives known as ascarosides (JEONG *et al.* 2005; BUTCHER *et al.* 2007; BUTCHER *et al.* 2009). But the role of ascarosides is not limited to dauer induction. Instead, they seem to be the

very language that nematodes use to communicate with one another (IZRAYELIT *et al.* 2012). Most important for the current discussion, ascarosides are used by males and hermaphrodites to detect the presence of one another (CHUTE AND SRINIVASAN 2014; BARR *et al.* 2018).

Early experiments looking at inter-sexual communication demonstrated that males—and often hermaphrodites—are attracted to media that have been pre-conditioned by the presence of hermaphrodites (SIMON AND STERNBERG 2002; WHITE *et al.* 2007). A great deal of clever protein biochemistry (solid phase extraction chromatography and NMR spectroscopy) comparing males and hermaphrodites in wildtype and *daf-22* ascaroside-deficient backgrounds revealed that there are actually multiple fractions of hermaphrodite exudate that are attractive to males (SRINIVASAN *et al.* 2008), with ~4 ascarosides involved specifically in male attraction and another ~4 involved in hermaphrodite "aggregation" (CHUTE AND SRINIVASAN 2014). These pheromones appear to target a subset of the male-specific neurons (BARR *et al.* 2018). Interestingly, sexually-attractive signals appear to be fairly well conserved across species (CHASNOV *et al.* 2007), so whether or not they can serve as targets for sex-specific mate recognition within a species remains to be seen. This pattern holds for sperm-oocyte chemical signals as well (HILL AND L'HERNAULT 2001; MILLER *et al.* 2001; TING *et al.* 2018). Recently, BORNE *et al.* (2017) developed a microfluidic device that allows males and females/hermaphrodites to interact with one another chemically while being physically separated, which is a paradigm more akin to the majority of studies of chemical interactions in behavioral ecology. Interestingly, they found that *C. remanei* females showed attraction to virgin males, but only when they themselves are virgins. Consistent with the discussion on mating avoidance above, *C. elegans* hermaphrodites showed no real attraction to males from either species.

But lack of attraction of *C. elegans* hermaphrodites to males does not mean that they are not paying attention to the presence of males. One of the most bizarre discoveries related to intersexual communication is the observation that the mere smell of a male can be enough to generate early death within hermaphrodites. As discussed above, it has long been known that direct interactions between males and hermaphrodites during mating can be harmful to the hermaphrodites. MAURES *et al.* (2014) and SHI AND MURPHY (2014) found that at least some of these harmful effects are caused by chemically mediated interactions during insemination, as mated females have greatly reduced lifespans relative to unmated females in a manner that strongly depends on the actual transfer of sperm (as opposed to mating *per se*). MAURES *et al.* (2014) also demonstrated that at least some consequences to hermaphrodites occur via "spooky action at a distance." Specifically, they found that male-produced compounds left on male-conditioned plates led hermaphrodites to have reduced lifespans, even if they never actually mated. SHI *et al.* (2017) built upon this paradigm in the opposite direction, showing that hermaphrodites also secrete a signal that decreases the longevity of males, even if hermaphrodites are not in contact with the males. Interestingly, the male-produced signal only appears to be present within androdioecious self-fertilizing species, leading Shi et al. to speculate that it might serve as a mechanism of eliminating males from a population after the benefits from outcrossing had been achieved (see below).

Although initially described as a "male-pheromone mediated killing" phenotype, it is difficult to see exactly why males would want to kill hermaphrodites in such a manner or why hermaphrodites would not rapidly become resistant to such an effect if deleterious. A more likely

explanation is that the presence of males leads to a physiological change in hermaphrodites—most likely related to a change in reproductive state such as mobilization of fat for egg production—and that it is these changes that lead to changes in hermaphrodite longevity. In other words, it is likely not direct harm imposed by males on hermaphrodites/females but a hermaphrodite/female response based on their own reproductive interests. It is actually difficult to formulate tests that cleanly distinguish between male-focused and hermaphrodite-focused explanations for the fitness consequences of reproductive interactions, and SHI AND MURPHY (2014) note no obvious increases in fecundity as a potential tradeoff for the longevity effects. Of course, these experiments were conducted in the laboratory and in a strain that is adapted to the laboratory (N2), and so expansion on this topic will benefit tremendously by discovering how to relate these fascinating observations to the actual ecological circumstances in which the worms have evolved. Part of the challenge for future research is to take the exquisite precision of functional analysis that *C. elegans* allows as a model genetic system and link it more directly to evolutionary causation, which has been a significant barrier within this system until fairly recently.

OUTCROSSING AND ADAPTATION

Reproductive assurance, inbreeding depression, and outbreeding depression

Gonochoristic *Caenorhabditis* in nature live on the brink, repeatedly forced to emigrate from one ephemeral habitat patch of rotting vegetation to the next, potentially with few colonizers arriving

at a given patch to reap the rewards of a few generations of booming reproduction—if those original patch pioneers are lucky enough to find a mate (FELIX AND BRAENDLE 2010; CUTTER 2015; FRÉZAL AND FÉLIX 2015; SCHULENBURG AND FELIX 2017). RICHAUD *et al.* (2018) found for *C. elegans* that most habitat patches in nature are likely colonized by at least 3 to 10 individuals, consistent with the potential for a small number of founders; *C. japonica* likely colonizes patches with just tens of individuals (YOSHIGA *et al.* 2013). This natural history context in which *Caenorhabditis* worms often find themselves may predispose them to experiencing selection favoring the evolution of self-fertilization as a means of reproductive assurance (WOLF AND TAKEBAYASHI 2004; DORNIER *et al.* 2008). Developmental genetics experiments in *C. remanei* demonstrate that it is possible for a small number of mutations to confer on a female the ability to 1) make sperm in her gonad and 2) self-activate those sperm to enable self-fertilization (BALDI *et al.* 2009), thus providing an evolutionary route to the origin of the hermaphrodite phenotype (ELLIS AND GUO 2011)(HAAG *et al.* 2018). The idea of reproductive assurance favoring the increase of such mutations that enable selfing is well-appreciated in the plant literature, in which reproductive transitions to selfing also are a common theme, with the reproductive assurance advantage to self-fertile colonizing individuals known as Baker's Law (BAKER 1955; STEBBINS 1957; PANNELL *et al.* 2015). Experimental evolution studies assessing the invasion of mutations that confer selfing into obligately outcrossing populations of *C. elegans* support the idea of reproductive assurance in the evolution of selfing (Box 2) (THEOLOGIDIS *et al.* 2014). The essential idea required for reproductive assurance to favor selfing is that mate availability limits reproduction. An alternate perspective is that some circumstances fundamentally change the 'cost of males' as a wasted resource investment in

fitness maximization (MAYNARD SMITH 1978; LIVELY AND LLOYD 1990), shifting the balance from favoring biparental to uniparental reproduction (see 'The cost of males' below).

Another concept from botanical studies of the evolution of selfing, however, does not apply to the nematode case: the fact that hermaphrodite worms cannot inseminate one another eliminates the "automatic selection" advantage to selfing that applies to hermaphroditic flowers that gain the advantage of being able use pollen for both selfing and crossing (GOODWILLIE *et al.* 2005; BUSCH AND DELPH 2012). Thus, from this broad-brush perspective, selection for reproductive assurance or to avoid the cost of males provides the basic rationale for why obligatorily outbreeding species with abundant males evolved into species like *C. elegans* with exceptionally rare males.

But why has *C. elegans* and other self-fertile *Caenorhabditis* evolved such an extreme degree of selfing, shouldn't a little go a long way? Interestingly, under many circumstances relevant to *Caenorhabditis* , selfing can reinforce itself to favor even greater levels of self-fertilization with extreme selfing often expected to be a stable evolutionary outcome (WOLF AND TAKEBAYASHI 2004; DORNIER *et al.* 2008). Theoretically-speaking, inbreeding depression is one of the major impediments to self-fertilization actually conferring a fitness advantage over outcrossing (LANDE AND SCHEMSKE 1985; CHARLESWORTH AND CHARLESWORTH 1987; UYENOYAMA AND WALLER 1991). Because persistent selfing increases the levels of homozygosity within a single lineage, selfing tends to expose recessive deleterious mutations to selection and to purge them; elevated genetic drift due to smaller genetic effective population sizes also can lead weakly deleterious mutations to become fixed. Both of these effects will act to diminish inbreeding depression and

thus diminish the fitness cost of selfing relative to outcrossing (LANDE AND SCHEMSKE 1985). Additionally, persistent selfing maintains linkage disequilibrium so that different loci are stuck in the same genomic context and co-evolve. When genomes evolve as cohesive units, rather than each locus evolving semi-independently, epistatic interactions are maintained over long periods of time making loci adapted to their specific genomic context (CHARLESWORTH AND WRIGHT 2001). As a result, outcross progeny may actually suffer fitness deficits, with recombination inducing outbreeding depression in F2 and later generations by breaking linkage disequilibrium and disrupting coadapted gene complexes (NEI 1967). Although evidence is still somewhat limited, natural populations of *C. elegans* (as well as selfing *C. briggsae* and *C. tropicalis*) do exhibit exceptionally strong linkage disequilibrium as well as evidence of intra-genomic adaptation and outbreeding depression, rather than inbreeding depression (DOLGIN *et al.* 2007; ANDERSEN *et al.* 2012; GIMOND *et al.* 2013; THOMAS *et al.* 2015). These factors appear to have been important in fostering the rarity of males and outcrossing in *C. elegans* populations.

When did selfing hermaphroditism and male rarity originate?

It is valuable to know how long extreme self-fertilization and male rarity has persisted in *C. elegans'* history as a species in order to place phenotypic and genomic evolution in proper context. Two types of data commonly applied to the question of timing in other taxa are, unfortunately, little help for *C. elegans*: fossils and phylogeny. While nematode fossil forms for the family Rhabditidae are known from preservation in amber, they do not include *Caenorhabditis* species (POINAR 2011). And, despite the recent discovery of *C. inopinata* as the closest-known relative of *C. elegans*, molecular divergence shows it to be nearly as distantly-

related to *C. elegans* as *C. elegans* is to any other member of the genus (Figure 1) (KANZAKI *et al.* 2018; WOODRUFF *et al.* 2018). Population genetic data and molecular evolutionary patterns in the genome (codon usage bias decay, *fog-2*/*ftr-1* duplication), however, have been useful to provide loose upper- and lower-bound estimates on the time since *C. elegans* evolved selfing (CUTTER 2008; CUTTER *et al.* 2008; RANE *et al.* 2010; THOMAS *et al.* 2015). In particular, they suggest a range between 0.35 Mya and 7.2 Mya for the origin of selfing in *C. elegans*. *C. nigoni* as sister species to *C. briggsae* provides some phylogenetic help in dating the origin of selfing in *C. briggsae*, for which estimates place the time between 0.20 Mya and 3.5 Mya (THOMAS *et al.* 2015). All of these numbers come with substantial assumptions and caveats about mutation rates and generation times in the wild. Regardless of the timing, selfing species have not diversified phylogenetically: they are restricted to individual tip lineages on the *Caenorhabditis* tree (Figure 1). Consequently, androdioecy with extreme selfing may tend to be evolutionarily short-lived, as it appears to be in plants (GOLDBERG *et al.* 2010; GLEMIN AND GALTIER 2012). Future research that is able to refine the timing for the origin of selfing will help to illuminate how rapidly phenotypes and genome architecture have diverged, and the relative influence of natural selection and non-adaptive forces in that process.

The cost of males: Why have any males at all?

Why would a species produce males at all when it could reproduce asexually or by self-fertilization? After all, the production of male offspring that are not capable of bearing offspring themselves seems like a waste of 50% of a female's resources: the so-called two-fold "cost of males" (MAYNARD SMITH 1978). This "cost of males" limits the rate at which outcrossing

lineages can grow relative to selfing lineages, at the expense of restricting the opportunities of genetic exchange to generate novel genotype combinations through recombination. A second cost of outcrossing is the dilution of the genetic contribution of each parent to their offspring: the "cost of meiosis" (WILLIAMS 1975). This "cost of meiosis" reduces the genetic contribution of each outcrossing parent by 50% relative to a selfing parent. The order of resource allocation decisions defined by the life history of androdioecious *Caenorhabditis* , however, means that the cost of biparental reproduction in *C. elegans* should be due to the "cost of males" and not the "cost of meiosis" (LIVELY AND LLOYD 1990). In any case, simple evolutionary theory predicts that outcrossing should be rare. And yet, outcrossing pervades animal and plant reproduction in nature, including the 95% of outcrossing species within the *Caenorhabditis* genus (Figure 1). The question of what offsets the cost of biparental reproduction is still very much a hot topic in evolutionary biology (HARTFIELD AND KEIGHTLEY 2012; LIVELY AND MORRAN 2014), with much experimental work aiming to test the plausibility and relative importance of the possible answers that have been proposed.

C. elegans has proven very useful for testing hypotheses on the evolution and maintenance of both obligate outcrossing and mixed mating systems under androdioecy. Despite theory predicting mixed mating systems with intermediate outcrossing rates to be generally unstable (LLOYD 1979; LANDE AND SCHEMSKE 1985), empirical work indicates that many plant mating systems maintain intermediate outcrossing rates (GOODWILLIE *et al.* 2005), partly due to "delayed selfing" as a common plant mechanism of individual reproductive assurance. Further, small amounts of outcrossing may be sufficient to gain many of the benefits of outcrossing at a fraction of the two-fold cost of obligate outcrossing. In *C. elegans*, however, mortality profiles

and the greater reproductive value of early-produced offspring in nature may preclude effective "delayed outcrossing" as a means of producing intermediate selfing rates in populations.

C. elegans has undoubtedly evolved an extreme rate of self-fertilization, perhaps facilitated by the developmental constraint of complete "pollen discounting" (i.e. hermaphrodites cannot inseminate one another, as hermaphrodite flowers can). The genetic tools and manipulability of the system (Box 1, Table 1, Table 2), however, permit explicit experimental tests of the balance of forces to characterize the roles of inbreeding depression and reproductive assurance. What is the threshold level of outcrossing necessary to facilitate adaptation to a novel environment? Why does obligate outcrossing evolve if small amounts of outcrossing yield substantial benefits? Does the combination of outcrossing and self-fertilization facilitate adaptation while also minimizing the mutation load in mixed mating populations? Here, we focus on three of the major hypotheses for the evolution and maintenance of outcrossing (Hill-Robertson interference, Red Queen hypothesis, deleterious mutation load), discuss the use of *C. elegans* to test these hypotheses, and highlight questions for further investigation.

The speed of adaptation and Hill-Robertson interference between selected loci

The answer to the riddle of the widespread prevalence of outcrossing lies in identifying the advantages of outcrossing, relative to selfing, that more than offset the inherent costs accompanying outcrossing. One likely advantage of outcrossing is facilitating more efficient natural selection. This benefit accrues from the potential to generate novel offspring genotypes

and break linkage disequilibrium via genetic exchange, with subsequent recombination between genetically diverse lineages (FISHER 1930; MULLER 1932; HILL AND ROBERTSON 1966; FELSENSTEIN 1974). By breaking linkage disequilibrium, outcrossing can increase the efficacy of selection on individual alleles relative to selfing, which tends to maintain linkage. Strong linkage between selected loci reduces the efficacy of selection on each locus individually, thus impeding evolutionary change (HILL AND ROBERTSON 1966). This process is known as Hill-Robertson interference. Outcrossing thus loosens Hill-Robertson interference, whereas selfing maintains interference. As a result, outcrossing is predicted to 1) facilitate more rapid adaptation to novel or rapidly changing conditions than self-fertilization, 2) increase the mean fitness of populations by disassociating beneficial from linked deleterious alleles, and 3) more effectively eliminate deleterious mutations from the genome.

C. elegans researchers have measured the rate of adaptation under different novel environments or conditions to compare obligately outcrossing populations to mixed mating or obligately selfing populations (Box 2). Overall, these studies have converged on a remarkably consistent result. As predicted by theory, outcrossing facilitates more rapid adaptation to novel conditions than selfing (LOPES *et al.* 2008; WEGEWITZ *et al.* 2008; MORRAN *et al.* 2009b; WEGEWITZ *et al.* 2009; ANDERSON *et al.* 2010; MORRAN *et al.* 2011; TEOTONIO *et al.* 2012; MASRI *et al.* 2013; PARRISH *et al.* 2016; SLOWINSKI *et al.* 2016; LYNCH *et al.* 2018). For example, MORRAN *et al.* (2009b) found that fitness increased ~150% in obligately outcrossing populations after 40 generations of exposure to a novel bacterial parasite, whereas fitness increased by 50% in mixed mating populations and obligately selfing populations did not adapt (~0% increase in fitness).

Although obligately outcrossing and obligately selfing populations have fixed mating strategies, rates of outcrossing can evolve in mixed mating *C. elegans* populations in response to selection (Box 2). Exposure to novel conditions tends to favor increased outcrossing and male frequency in experimental androdioecious populations (reviewed in ANDERSON *et al.* (2010)). However, the benefits of outcrossing, relative to selfing, often appear to be short-lived: in most cases exposure to novel parasites only temporarily favors outcrossing over self-fertilization (MORRAN *et al.* 2009b; MORRAN *et al.* 2011; LYNCH *et al.* 2018). The androdioecious populations in MORRAN *et al.* (2009b) evolved outcrossing rates approaching the maximum value of 100% within 20 generations of exposure to the parasite. However, male frequencies and outcrossing rates abruptly dropped to control levels within five generations thereafter. Further, alleles conferring selfing began to invade obligately outcrossing populations of *C. elegans* after about 10 generations of exposure to a novel parasite (SLOWINSKI *et al.* 2016). Presumably, the temporary benefits of outcrossing reflect the consequences of Hill-Robertson interference. Several generations of outcrossing and subsequent recombination likely generate a locally optimal genotype from standing genetic variation that drives adaptation to the novel parasite. Then, after adaptation, the benefits of outcrossing no longer offset the inherent costs, making selfing again favored by selection. So, while Hill-Robertson interference seemingly can favor outcrossing over selfing, outcrossing's advantage generally appears to be short-lived in the absence of a dynamic source of selection (LIVELY AND MORRAN 2014).

There are notable exceptions to the pattern of male frequency decline over time in androdioecious *C. elegans* populations. Multiple experiments using strains generated by hybrid crosses or funnel crossing schemes found that males were maintained at elevated levels for the

duration of experiments lasting from 47 (ANDERSON *et al.* 2010) to 100 generations (TEOTONIO *et al.* 2012). However, the degree to which outcrossing was maintained due to inbreeding depression induced by the genetic composition of the starting population versus the breakdown of Hill-Robertson interference is currently unclear. The composition of base populations for experimental evolution presents the general issue of how such studies can be interpreted relative to the natural context (TEOTONIO *et al.* 2012; TEOTONIO *et al.* 2017). Regardless of the source of selective pressure, it is clear that males can be maintained at moderate to high levels in androdioecious lab population under some conditions. Going forward it will be critical to determine the role of standing genetic variation and genome architecture in the maintenance of males and outcrossing in *C. elegans*.

From a broader perspective, outcrossing's ability to break down Hill-Robertson interference is, unfortunately, not a completely sufficient explanation for the widespread prevalence of outcrossing in nature. Apart from increasing the efficacy of selection, Hill-Robertson interference alone does not provide a mechanism to impose persistent selection on populations, and it appears that persistent selection is necessary to maintain outcrossing. Selective pressures with the ability to favor the long-term maintenance of outcrossing may require dynamic selection, as opposed to a singular shift in the environment. Two of the most prominent sources of selection predicted to favor the long-term maintenance of outcrossing are coevolving parasites and deleterious mutations (see below). Importantly, the ability of outcrossing to reduce Hill-Robertson interference has not been tested directly in *C. elegans*. Rather, studies have tested predictions based on the assumption that outcrossing can break Hill-Robertson interference. These studies strongly support the prediction that outcrossing can confer advantages relative to

selfing by breaking Hill-Robertson interference. Direct tests would require specifically linking recombination events at multiple loci to increased fitness, a goal that is readily attainable with the current tools available in *C. elegans* (Table 1, Table 2).

Red Queen model of host-parasite co-evolution

Interactions between species are predicted to provide an ecological source of dynamic selection favoring outcrossing over selfing. In particular, the Red Queen model proposes that host-parasite coevolution creates negative frequency-dependent selection that favors the maintenance of outcrossing in host populations (JAENIKE 1978; HAMILTON 1980; BELL 1982). Parasites are thought to adapt to infect the most common host genotypes, so selection favors hosts with rare or novel genotypes. Outcrossing has the potential to produce offspring with diverse genotypes, whereas selfing severely limits the genetic diversity of offspring and populations. Therefore, selfing lineages are predicted to suffer disproportionately from coevolving parasites, which can offset the cost of males (or the cost of meiosis). Non-nematode field studies provide the majority of empirical evidence supporting the Red Queen model (HARTFIELD AND KEIGHTLEY 2012; LIVELY AND MORRAN 2014), but many field systems are ill-suited to direct manipulative tests of its predictions. Utilizing *C. elegans* as a host of parasites, including bacteria, viruses, microsporidia and fungi (reviewed in GIBSON AND MORRAN (2017) and SCHULENBURG AND FELIX (2017)), provides researchers the opportunity to use experimental evolution to test directly diverse predictions and assumptions of the Red Queen model.

Thus far, researchers have coevolved *C. elegans* host populations with bacterial parasites to provide direct experimental support for several predictions of the Red Queen hypothesis. First, multiple studies found that coevolving parasites provide conditions that can maintain males and outcrossing in *C. elegans* populations (MORRAN *et al.* 2011; MASRI *et al.* 2013; SLOWINSKI *et al.* 2016). For example, MORRAN *et al.* (2011) exposed mixed mating *C. elegans* hosts either to coevolving bacterial parasites or to homogenous non-coevolving parasites to test the role of coevolving parasites in the maintenance of host outcrossing. They found that host-parasite coevolution conditions maintained outcrossing rates of ~80% after 30 generations of selection, whereas hosts exposed to non-coevolving parasites also produced elevated rates of outcrossing initially, but then dropped to only ~ 20% outcrossing after 30 generations. MASRI *et al.* (2013) found that selection imposed by coevolving parasites favored host outcrossing so strongly that elevated levels of *C. elegans* males and outcrossing continued to persist in the presence of a parasite that imposed greater virulence against males than hermaphrodites. These findings strongly indicate that the benefits of outcrossing outweigh its costs in the presence of virulent coevolving parasites. Second, not only do coevolving parasites favor the maintenance of outcrossing, but greater outcrossing rates have been directly linked to decreased host mortality rates (MORRAN *et al.* 2013).

Finally, obligate self-fertilization is an evolutionary dead end in the presence of virulent coevolving parasites. MORRAN *et al.* (2011) found that obligately selfing *C. elegans* populations were driven to extinction within 20 generations by coevolving parasites, whereas mixed mating and obligately outcrossing populations persisted throughout a 30 generation experiment. Collectively, these *C. elegans* experiments and numerous field studies on several different host

species (LIVELY 1987; MORITZ *et al.* 1991; LIVELY AND DYBDAHL 2000; DECAESTECKER *et al.* 2007; JOKELA *et al.* 2009; KING *et al.* 2009; VERHOEVEN AND BIERE 2013)demonstrate that coevolving parasites can contribute to the persistence of biparental reproduction. However, coevolving parasites are far from established as an important factor for maintaining outcrossing in *Caenorhabditis* in nature. Further, the overall role of coevolving parasites in the maintenance of outcrossing across the tree of life is also unresolved. Nevertheless, *C. elegans* provides the means to address some of the key questions that remain. How virulent must parasites be to favor outcrossing? Is there a role for parasite co-infection in the maintenance of host outcrossing? Are coevolving parasites also under selection favoring genetic exchange?

Mutation accumulation and the load of deleterious mutations

Deleterious mutations are relentless, and provide another selective force predicted to favor outcrossing over selfing. The deterministic mutational hypothesis predicts that, under specific assumptions about mutation rates and effects, outcrossing will be favored over self-fertilization (KONDRASHOV 1984; KONDRASHOV 1985; CHARLESWORTH 1990). Generally, selfing can effectively purge deleterious mutations because their greater homozygosity exposes recessive deleterious mutations to selection, which results in purging (LANDE AND SCHEMSKE 1985). However, beyond a threshold mutation rate, recessive deleterious mutations can accumulate in the genomes of selfing populations; recombination in heterozygote outcrossers empowers selection to avoid this problem. When selection against deleterious mutations is weak or the effect size of each deleterious mutation is small (or mutations interact synergistically), then selfing lineages are at risk of fixing deleterious mutations at greater rates than outcrossing lineages, leading to their extinction (GABRIEL *et al.* 1993; LYNCH *et al.* 1995). Therefore, the

influx of deleterious mutations are predicted to favor outcrossing over selfing, and potentially act as a persistent source of selection capable of maintaining males and outcrossing.

Mutation accumulation studies in *C. elegans* are especially powerful, compared to other study systems, because of the ability to not only assess the impact of mutation accumulation on the fitness of outcrossing versus selfing populations, but also to determine whether the influx of deleterious mutations offsets the cost of males and directly favors the maintenance of outcrossing. Several studies have utilized experimental evolution in *C. elegans* for this purpose, yielding incredibly consistent conclusions. Populations exposed to elevated mutation rates (via either mutagen exposure or disabled mismatch repair) experience either slower declines in male frequency and outcrossing (CUTTER 2005) or the maintenance of moderate to low outcrossing rates (~60% to less than ~10%, depending on the genetic background) (MANOEL *et al.* 2007; MORRAN *et al.* 2009b). Under these conditions, obligately selfing populations rapidly lose fitness, some to the point of extinction (MORRAN *et al.* 2009b; MORRAN *et al.* 2010), as also anticipated by theory (LOEWE AND CUTTER 2008). Mixed mating populations have exhibited varying degrees of fitness loss during periods of elevated mutation rates (MANOEL *et al.* 2007; MORRAN *et al.* 2009b), and obligately outcrossing populations have maintained fitness despite increased mutation rates (MORRAN *et al.* 2009b). Additionally, increased outcrossing rates have evolved as populations recovered fitness after periods of mutation accumulation (WERNICK *et al.* 2019). Therefore, as predicted, outcrossing can reduce the fixation of deleterious mutations under high mutation rates or facilitate recovery from previously accumulated mutations, relative to selfing, favoring the persistence of outcrossing over time.

Despite the advantages of outcrossing under mutation accumulation, selection imposed by deleterious mutations does not appear to fully offset the inherent costs of outcrossing. Rather, apart from unnaturally high mutation rates, the influx of deleterious mutations only maintains males at relatively low levels that are likely insufficient to explain the widespread prevalence of obligate outcrossing. Further, the parameters required for the mutational deterministic hypothesis to favor outcrossing greatly restrict the applicability of the hypothesis in most natural populations (HARTFIELD AND KEIGHTLEY 2012). Therefore, the accumulation of deleterious mutations is unlikely a general explanation in nature for the widespread maintenance of outcrossing across *Caenorhabditis* , provided that mutational properties are similar to *C. elegans* across species (DENVER *et al.* 2004; BAER *et al.* 2006; DENVER *et al.* 2009; SALOMON *et al.* 2009).

Mutation accumulation alone may not be sufficient to offset the costs of outcrossing, but the 'pluralistic hypothesis' proposes that selection imposed by both mutation accumulation and coevolving parasites may together serve as a general explanation for the maintenance of outcrossing (WEST *et al.* 1999; NEIMAN *et al.* 2017). Importantly, fitness loss via mutation accumulation reduces the threshold level of parasite virulence required to maintain outcrossing. Further, the accumulation of recessive deleterious mutations in a predominantly outcrossing population will result in the evolution of inbreeding depression. If coevolving parasites maintain outcrossing for extended periods of time, then mutation accumulation under outcrossing may impose substantial fitness costs on individuals that self-fertilize. In other words, a combination of coevolving parasites and mutation accumulation may prevent or substantially impede the invasion of selfing alleles into an outcrossing population. Given our ability to manipulate the

mutation rate of *C. elegans* as hosts, coupled with a diverse selection of bacterial parasites, *C. elegans* presents a unique opportunity to conduct some of the first experimental tests of pluralistic theory.

What good is outcrossing for *C. elegans*?

The irony of *C. elegans* experimental evolution is that it has produced definitive answers about outcrossing in general, but less definitive answers about *C. elegans* males and outcrossing in nature. Future studies could aim to more closely mimic the natural context, perhaps using "macrocosms" rather than Petri dishes and not imposing lab-convenient transfer protocols across generations. Tests of the potential influence of higher male than hermaphrodite survival in the dauer stage also could help connect to a natural context (MORRAN *et al.* 2009a). We anticipate that clever nature-inspired experiments with *C. elegans* will help to test whether or not males may be evolutionary relics (see "Are males evolutionary relics?" below).

GENOME EVOLUTION AND POPULATION GENETICS

Genome evolution starts as a new mutation to a single copy of DNA in a population, a mutation that then rises in frequency to become fixed, creating divergence between species, or that instead goes extinct, resulting in sequence conservation. Males influence the micro-evolutionary process of such allele frequency changes in natural populations in predictable ways and, correspondingly, shape its outcome that accumulates as the degree of divergence observed in inter-species genome comparisons. Some of the key predictable effects of outcrossing via males relative to self-

fertilization include: increased heterozygosity and population variation, increased genetically effective recombination (reduced linkage disequilibrium), more effective direct selection on fitness-affecting alleles (weaker linked selection effects), stronger natural selection and sexual selection on male-related gene function, facilitation of selfish genetic element activity. The repeated evolution of highly self-fertilizing species with a rarity of males, coupled with empirical accessibility, has made *Caenorhabditis* an important system for testing these predictions with population genetics and comparative genomics methods.

Micro-evolutionary consequences of male outcrossing vs. selfing

When females evolved the ability to fertilize themselves in *C. elegans*' history, the stage was set for a cascade of micro-evolutionary consequences that we can quantify with analyses of natural genetic variation. First, the homozygosity that results from self-fertilization makes meiotic recombination leave no genetic trace from parent to offspring. We measure this lack of recombination between distinct genotypes in the population overall as linkage disequilibrium, the non-random representation of distinct combinations of alleles at different loci. Linkage disequilibrium (LD) is so high in the *C. elegans* genome that it creates haplotype blocks that span 20% of a chromosome (2.5Mb) on average (BARRIÈRE AND FÉLIX 2005; HABER *et al.* 2005; CUTTER 2006; BARRIÈRE AND FÉLIX 2007; ANDERSEN *et al.* 2012), with similarly strong LD also holding true for *C. briggsae* and *C. tropicalis* (CUTTER 2006; GIMOND et al. 2013; THOMAS et al. 2015). As BARRIÈRE AND FÉLIX (2007) and RICHAUD *et al.* (2018) have shown in one of the few natural time series samples of *Caenorhabditis* , individual genomic haplotypes can be remarkably stable over time in a given locality. The LD is so pervasive that it occurs even

between polymorphisms that occur on different chromosomes, a fact that has been used to estimate the genetically effective rate of outcrossing between males and hermaphrodites in recent generations to be <0.1% (THOMAS *et al.* 2015).

Another byproduct of high homozygosity in a highly selfing population is that overall genetic variability is predicted to be 2-fold lower than outcrossing species with the same number of individuals, due to a halving of the effective population size (N_e) (CHARLESWORTH AND WRIGHT 2001; GLEMIN AND GALTIER 2012). Genome-wide single nucleotide polymorphism (SNP) is indeed lower in the selfing *C. elegans*, *C. briggsae*, and *C. tropicalis* than in all other known non-selfing *Caenorhabditis* (GRAUSTEIN *et al.* 2002; JOVELIN *et al.* 2003; LI *et al.* 2014). The measured values of polymorphism for outcrossing species includes *C. brenneri* with the highest known for any animal (CUTTER *et al.* 2013), implying that effective population sizes (N_e) can exceed 10 million (DEY *et al.* 2013), compared to mammals with N_e typically ranging from 10^2-10^4 (PALSTRA AND FRASER 2012). However, the difference in diversity between selfing and non-selfing *Caenorhabditis* generally is >10-fold rather than just 2-fold, implying that factors other than just the influence of homozygosity on N_e must be important. At least two additional processes are thought to reduce population variation further in *C. elegans* and other selfers: selection at linked sites (recurrent genetic hitchhiking and background selection; see below) and metapopulation dynamics (extinction-recolonization of habitat patches). The boom-and-bust life history of *Caenorhabditis* , as individuals colonize ephemeral rotting vegetal substrates, sets up a scenario conducive to local extinctions exerting a strong influence on patterns of polymorphism (CUTTER 2015; FRÉZAL AND FÉLIX 2015). Extinction-recolonization dynamics in a metapopulation tend to reduce species-wide genetic variation (PANNELL 2003), and is likely to be

disproportionately strong in selfing species as founder effects exaggerate haplotype frequency differences among local patches.

This patchiness of habitats and inability of recombination to mix genotypes ought to yield low gene flow and high genetic differentiation among patches. The reality, however, appears more nuanced. In *C. elegans*, genomic haplotypes appear "well-mixed" at global scales, with little broad-scale separations among genotypes, implying long-distance dispersal (CUTTER 2006; ANDERSEN *et al.* 2012). At local scales, distinct genomic haplotypes can co-occur (BARRIÈRE AND FÉLIX 2005), despite both local and global measures of differentiation with F_{ST} giving similarly high values, often with $F_{ST} > 0.5$ (BARRIÈRE AND FÉLIX 2005; CUTTER 2006). Genetic differentiation among localities for the large ranges of outcrossing species like *C. brenneri*, *C. remanei* and *C. sinica* is several fold lower by comparison (CUTTER *et al.* 2012; DEY *et al.* 2012; DEY *et al.* 2013). *C. briggsae*, by contrast, shows striking geographic differentiation across latitudes, with most wild genomic haplotypes corresponding to so-called "Temperate" or "Tropical" phylogeographic groups (CUTTER 2006; FELIX *et al.* 2013; THOMAS *et al.* 2015). Other genetically distinctive isolates of *C. briggsae* tend to be constrained geographically to one or a few local regions (CUTTER 2006; FELIX *et al.* 2013; THOMAS *et al.* 2015), a finding that will be interesting to compare with ongoing extensive global sampling of *C. elegans*. *C. tropicalis* is known predominantly from tropical locations, and shows strong genetic differentiation between different Caribbean islands (GIMOND *et al.* 2013). The patterns of genetic differentiation for *C. briggsae* and *C. tropicalis* thus suggest that they experience either stronger dispersal limitation than *C. elegans* or stronger post-dispersal selection that eliminates maladapted genotypes in a given local environment to then reinforce the genetic differentiation across space. If humans provide a recent means of dispersal to explain global distributions (CUTTER 2015; FRÉZAL AND

FÉLIX 2015), then perhaps anthropogenic activity is more conducive to spread of *C. elegans* genotypes.

Despite high LD overall, population genomic analyses of *C. elegans* and *C. briggsae* both clearly demonstrate that recombination has occurred between distinct genotypes and therefore that males do contribute genetically to population variation to some extent (ANDERSEN *et al.* 2012; THOMAS *et al.* 2015). The signal of this male influence is most obvious by looking along chromosomes, such that the higher meiotic and population recombination rates on chromosome arms makes them about 10-times more polymorphic than the chromosome centers (ANDERSEN *et al.* 2012; THOMAS *et al.* 2015) (see below). If there truly were zero males and zero outcrossing, then SNP density ought to be uniform along chromosomes, provided that recombination does not generally increase the mutation rate (current data are consistent with this assumption in *Caenorhabditis* (DENVER *et al.* 2009; DENVER *et al.* 2012; THOMAS *et al.* 2015). This disparity in polymorphism among chromosome domains is true for both neutral polymorphisms (e.g. SNPs in intergenic, intronic, and synonymous sites) as well as for polymorphisms that likely have a functional effect that could influence fitness (ROCKMAN *et al.* 2010; THOMAS *et al.* 2015). Thus, even rare outcrossing via males in highly selfing species affects the potential for adaptation in a way that depends on the genomic location of loci.

The recent high-quality *de novo* assembly of the Hawaiian CB4856 *C. elegans* genome sequence complements the reference genome for the classic strain Bristol N2, and led to the discovery of at least 61 islands of extreme sequence divergence between them (THOMPSON *et al.* 2015) (Figure 3). SNP variation between these allelic sequences can be as high as 16% of sites (vs. just 0.2% of sites for most genomic regions) (THOMPSON *et al.* 2015), comparable to the magnitude of allelic difference seen in the hyperdiverse outcrossing species *C. brenneri* (DEY *et al.* 2013).

The leading hypothesis holds that these divergent regions in *C. elegans* reflect allelic haplotypes from the pre-selfing ancestor of modern *C. elegans* that have persisted in different wild isolates into the present day, known as retained ancestral polymorphism. The persistence of these divergent regions as population polymorphisms raises the possibility that some form of balancing selection has favored their persistence. These divergent sequences occur disproportionately on autosomal arms, being rare in autosome centers and the X-chromosome (THOMPSON *et al.* 2015). Such ancestral polymorphism in the genome also hints that *C. elegans*' proto-hermaphrodite ancestor might have experienced a protracted duration of 'mixed mating' with males occurring and crossing at non-negligible frequency within populations. A better understanding of the duration of such a period in *C. elegans'* history would help to determine the importance of indel transmission ratio distortion in the evolution genome size and gene composition (see below 'Non-Mendelian byproducts of mixed selfing and outcrossing') (WANG *et al.* 2010). Moreover, broader analysis of ancestral polymorphism is required to determine how much the divergent regions may be able to explain functional differences among wild isolates and to reveal about *C. elegans* evolutionary history, as has been explored for selfing plants (BRANDVAIN *et al.* 2013).

One intriguing divergent region in *C. elegans* includes the *peel-1/zeel-1* loci on Chromosome I that encodes a selfish genetic element with a toxin-antidote mode of action (SEIDEL *et al.* 2008; SEIDEL *et al.* 2011). This locus has no obvious effect unless an isolate containing intact *peel-1/zeel-1* loci crosses with another isolate lacking the *peel-1/zeel-1* element. When that happens, 25% of the selfed offspring from the F1 hermaphrodites will arrest in embryogenesis, due to a sperm-derived toxin that fails to get degraded by ZEEL-1 in zygotes that lack the *peel-1/zeel-1* element because ZEEL-1 doesn't get made (SEIDEL *et al.* 2008; SEIDEL *et al.* 2011).

Interestingly, an analogous maternal-effect toxin and antidote system comprised of *sup-35* and *pha-1* also has been characterized in *C. elegans* (BEN-DAVID *et al.* 2017). These well-characterized and striking cases of incompatibility between wild strains may just represent the tip of the negative epistasis iceberg, however, as other multi-locus incompatibilities that affect fitness only upon male-mediated crossing have been mapped across the genome (SNOEK *et al.* 2014). These genetic interactions with negative epistatic effects fit the criteria for Dobzhansky-Muller incompatibilities that form the basis of models of speciation (ORR 1995), and equivalently are often discussed in terms of outbreeding depression in literature on *Caenorhabditis* (DOLGIN *et al.* 2007; DOLGIN *et al.* 2008b; GIMOND *et al.* 2013) (see above). These negative epistatic interactions likely further reduce the genetic effectiveness of male-mediated crossing in *C. elegans* and, like mating-avoidance in hermaphrodites, are part of the positive feedback loop that likely accelerated the rate of loss of males within *C. elegans* populations (PHILLIPS 2008).

Genetic linkage and selection in genome evolution

It is simple to think about selection on alternate alleles of a single gene, but in fact the linkage of that gene to the rest of the genome is important for understanding the response to such selection and for predicting patterns in genome evolution (CUTTER AND PAYSEUR 2013). In particular, any genetic variants that happen to be nearby on the same haplotype as a favorable mutation will get dragged along toward fixation in the population, a process termed genetic hitchhiking (MAYNARD SMITH AND HAIGH 1974). What counts as "nearby" depends on what the effective population recombination rate is, which depends positively on the meiotic recombination rate,

the amount of outcrossing with males, and the size of the population. As a consequence, parts of genomes with less recombination ought to have less polymorphism if positive selection and recurrent genetic hitchhiking pervades genomes (STEPHAN 2010); similarly, species like selfers with less effective recombination ought to have less polymorphism (CUTTER AND PAYSEUR 2013). And yet, negative selection can create a similar pattern: so-called background selection against deleterious mutations also acts to reduce polymorphism in low-recombination regions (CHARLESWORTH *et al.* 1993; CHARLESWORTH 2012). Moreover, selection at one locus can interfere with the efficacy of selection on another linked locus, thus slowing down an adaptive response, if recombination has not put both beneficial alleles of the loci on the same haplotype (HILL AND ROBERTSON 1966). These forms of 'linked selection' all represent instances of the general feature that selection on one locus can affect or interfere with selection elsewhere in the genome.

The low-recombination center regions of *C. elegans* autosomes contain nearly 10-fold lower density of SNPs than do high-recombination arm regions (Figure 3) (KOCH *et al.* 2000; CUTTER AND PAYSEUR 2003; ANDERSEN *et al.* 2012). Those SNPs that do occur in center regions tend to be singleton or low frequency variants in the species, reflecting a skewed site frequency spectrum toward an excess of rare variants in low recombination regions. These patterns do not seem to reflect differences in mutational input, but instead the byproduct of the combined effects of genetic hitchhiking and background selection (KOCH *et al.* 2000; CUTTER AND PAYSEUR 2003; ROCKMAN AND KRUGLYAK 2009; ANDERSEN *et al.* 2012). *C. elegans* chromosomes I, IV and V in particular show evidence of large-scale selective sweeps in recent history that created striking differences in polymorphism across the genome (ANDERSEN *et al.* 2012). *C. briggsae*'s genome

shows a remarkably similar pattern and for the same reasons (CUTTER AND CHOI 2010; THOMAS *et al.* 2015).

Two features of these species contribute to such radical differences in the density of polymorphisms in different parts of the genome. First, chromosome centers are especially dense with coding sequences, so new mutations are more likely to have a fitness effect in exactly the parts of the genome that also have low recombination; this genomic feature is opposite to that of most other organisms studied for the effects of linked selection, like *Drosophila* and humans. Second, both *C. elegans* and *C. briggsae* have very low rates of outcrossing, which drastically decreases the genetically effective recombination rate across the population and so increases the width of genomic regions that will feel the influence of linked selection. In a broad phylogenetic study from plants to vertebrates, these two species of *Caenorhabditis* show a stronger impact of linked selection than most other species analyzed (CORBETT-DETIG *et al.* 2015).

These highly selfing species show profound genomic trends due to linked selection. What should we expect in outcrossing species of *Caenorhabditis* ? That is, how important are males in defining whether or not arm vs center regions of chromosomes differ in patterns of polymorphism? The overall karyotype and chromosome fidelity of gene orthologs appears unusually strong across *Caenorhabditis* species (HILLIER *et al.* 2007; FIERST *et al.* 2015; KANZAKI *et al.* 2018; REN *et al.* 2018; YIN *et al.* 2018), raising the possibility that arm vs center domains of recombination also are widely conserved (ROSS *et al.* 2011). Addressing these issues awaits population genomic analysis of outcrossing species of *Caenorhabditis* .

These patterns of polymorphism are usually quantified for SNPs considered to be selectively neutral so that they can relate most easily back to evolutionary theory about linked selection. But SNPs associated with functional variation also show strong genomic differences between low-recombination center and high-recombination arm regions, in both *C. elegans* and *C. briggsae* (ROCKMAN *et al.* 2010; THOMAS *et al.* 2015). Specifically, eQTL are underrepresented in center regions compared to arms (ROCKMAN *et al.* 2010) and replacement-site SNPs that alter protein coding sequences are disproportionately rare in center regions (THOMAS *et al.* 2015). Thus, linked selection has purged functional variation in the genomes of *C. elegans* and *C. briggsae*, not just 'inconsequential' alleles. These observations imply that the region a gene happens to reside in affects its potential to contribute to adaptation from existing functional variation within the species, independently of what functional role the gene might play.

C. elegans' sex chromosome distinguishes itself in several evolutionarily-relevant ways in addition to being a hemizygous X-chromosome in males, with these features generally being shared with *C. briggsae*. It remains to be demonstrated, however, what is the full extent of generality across *Caenorhabditis* for distinctive X-chromosome features. The X-chromosome experiences meiotic sex chromosome inactivation (MSCI) in males, reflected in distinctive chromatin marking and absence of transcription in sperm cells (KELLY *et al.* 2002), potentially predisposing males to sterility in the genetically-perturbed state of interspecies hybrids (LI *et al.* 2016; CUTTER 2018). However, the X-chromosome is underrepresented for genes with male-biased and sperm-biased expression (REINKE *et al.* 2004; ALBRITTON *et al.* 2014; ORTIZ *et al.* 2014). Rates of recombination are more uniform along its length than seen for autosomes (ROCKMAN AND KRUGLYAK 2009; ROSS *et al.* 2011), as is the intrachromosomal distribution of

polymorphisms (ANDERSEN *et al.* 2012; THOMAS *et al.* 2015), coding genes and other genomic features (*C. ELEGANS* SEQUENCING CONSORTIUM 1998). Moreover, the lack of recombination on the X-chromosome due to its hemizygosity in males means that the population recombination rate will be reduced compared to autosomes in obligatorily outcrossing species, but not in highly selfing hermaphrodite species in which males are unusually rare.

Deleterious and adaptive genome evolution

By mediating genetically effective recombination and population size, males allow natural selection to operate more efficiently on the fitness effects of alleles at each locus independently of other loci (see above). In addition to the chromosomal patterns of linked selection, this role of males also leaves a genomic signature in the accumulation of slightly deleterious mutations in species where male-mediated outcrossing is rare. One way to quantify accumulation of deleterious mutations is to contrast the ratio of polymorphisms at replacement sites (which often ought to be deleterious) relative to polymorphisms at synonymous sites as a neutral reference (π_N/π_S). We expect that selection against new deleterious mutations will be relaxed in species with small effective population sizes, as for species with high selfing rates. This scenario should cause π_N/π_S to be especially high in selfing species because the deleterious mutations haven't been weeded out effectively by purifying selection. In the outcrossing species *C. remanei* and *C. brenneri*, π_N/π_S averages ~0.025 implying that over 97% of mutations to replacement sites get weeded out or fixed and so are unobservable as polymorphisms at any given time (DEY *et al.* 2012; DEY *et al.* 2013). In selfing *C. elegans* and *C. briggsae*, the equivalent ratio is roughly 10-fold higher ($\pi_N/\pi_S \sim 0.25$) (THOMAS *et al.* 2015). This higher ratio implies that a much larger

fraction of slightly deleterious mutations are able to remain as polymorphisms due to the less effective selection in these species, and that a larger fraction of those deleterious mutations will actually get fixed eventually in the selfing species compared to species with obligatory male mating.

We can also contrast the polymorphism ratio for replacement : synonymous sites to the analogous ratio for divergence (d_N/d_S or, equivalently, K_A/K_S), which reflects the mutations that accumulate as fixed differences between species. The value of d_N is usually less than d_S because most mutations to non-synonymous sites are deleterious and get eliminated by purifying selection in the polymorphic phase, and so never contribute to divergence between species. In the closest species pair available for analysis within *Caenorhabditis* (*C. briggsae* vs. *C. nigoni*), median d_N/d_S across orthologous genes is 0.07 (THOMAS *et al.* 2015) and this value is similar to deeper-time comparisons (e.g. 0.075 for *C. briggsae* vs. *C. elegans* (CUTTER AND WARD 2005)), which tells us that on average only about 7% of mutations that alter the amino acid sequence in proteins eventually get fixed. The d_N/d_S metric reflects the long-term evolutionary outcome in the shared history of those species being compared. Most of this history would have occurred as an obligatorily outcrossing population because high selfing with rare males is thought to have evolved relatively recently (CUTTER 2008; CUTTER *et al.* 2008; RANE *et al.* 2010; THOMAS *et al.* 2015).

The value for d_N/d_S is higher than for π_N/π_S in outcrossing *Caenorhabditis* species, which implies that many of the mutations to non-synonymous sites that did manage to get fixed likely did so as

a result of adaptive evolution (SMITH AND EYRE-WALKER 2002). Few studies thus far have aimed to estimate the rate (ω_a) and fraction (α) of such substitutions that get fixed by positive selection for *Caenorhabditis* . One study that included *C. brenneri* suggested that over 80% of non-synonymous substitutions were fixed by positive selection, a value among the highest observed in the animal kingdom (adaptive substitution rate estimated to be 0.16) (GALTIER 2016). Similar calculations have so far been avoided for selfing species, because selfing violates assumptions of the methods used in estimation of these evolutionary quantities. However, theory predicts that the lack of mating via males in selfing species would yield lower per-site rates of adaptive evolution, due to the selective interference effects of linkage and smaller effective population size (GLEMIN AND GALTIER 2012).

A challenge for understanding the relative incidence (α) and rate (ω_a) of adaptive molecular evolution is that background selection against deleterious mutations reduces the true rate of adaptation at linked sites as well as interfering with our ability to estimate that true rate (URICCHIO *et al.* 2019). In particular, simulations using a McDonald-Kreitman test framework (MCDONALD AND KREITMAN 1991) show that we may often underestimate α in the face of background selection when selection coefficients tend to be small for adaptive alleles, as likely is the case for selection on individual loci in polygenic traits or for loci underpinning traits well-matched to the environment (URICCHIO *et al.* 2019). This problem will be especially acute for highly selfing species with strong linkage, like *C. elegans* and *C. briggsae*, unless adaptation proceeds primarily from large-effect beneficial mutations; recognition of this challenge has led researchers to avoid estimating metrics like α for these species. Interestingly, contrasts of

genomic regions with high versus low recombination, as for *C. elegans* chromosome arms versus centers, might be exploited to better infer details about adaptive molecular evolution (URICCHIO *et al.* 2019).

Non-Mendelian byproducts of mixed selfing and outcrossing

While meiotic mechanisms usually enforce the fair segregation and transmission of DNA copies to gametes, selfish genetic elements like transposable elements (TEs) can evade cellular controls to enable their own proliferation and transmission. Even though TE insertions are usually deleterious, selection generally cannot eradicate them from genomes (DOLGIN AND CHARLESWORTH 2008). Reproductive mode, however, influences the balance of forces that favor versus disfavor high TE activity: mating with males acts as a facilitating conduit for these sexually transmitted parasites (WRIGHT AND SCHOEN 1999; MORGAN 2001; BOUTIN *et al.* 2012). Very high rates of self-fertilization favor low TE transposition rates (WRIGHT AND SCHOEN 1999; MORGAN 2001; BOUTIN *et al.* 2012), as the fitness of the TE becomes tethered to the genomic haplotype in which it resides, thus eliminating the conflict of fitness interests between TE and organism. Low rates of outcrossing, however, can maintain TE activity (BOUTIN *et al.* 2012). The fact that the genomes of outcrossing species, from maize to humans, commonly are comprised of >40% TEs testifies to the potential for TE activity to shape genome size and structure (ELLIOTT AND GREGORY 2015). Unlike some plant genomes, however, it does not appear that TE activity differences between selfing and non-selfing species of *Caenorhabditis* provides the dominant reason for selfing species tending to have smaller genomes (FIERST *et al.* 2015). This observation of a consistent 10-15% TE composition in genomes across

Caenorhabditis suggests that the low outcrossing rates in selfing *Caenorhabditis* might be sufficient to preclude TE domestication, or that high selfing is sufficiently recent that TE domestication does not yet show up as a strong signal in the genome. Novel TE insertions into the genome are abundant in different wild isolates of *C. elegans* (LARICCHIA *et al.* 2017). Analysis of population frequencies of TEs suggests that selection against TEs in *C. elegans* is weaker than in *C. remanei* (DOLGIN *et al.* 2008a), but more thorough genomic analyses are required to determine generality across TE families and with respect to reproductive mode.

C. elegans chromosomes exhibit another form of non-Mendelian inheritance mediated by males: transmission ratio distortion (TRD) of autosome homologs that differ in size (WANG *et al.* 2010). Specifically, in males, a shorter autosome copy will segregate disproportionately to the sperm cell that has the X-chromosome, with the longer copy segregating to the sperm cell that lacks a sex chromosome (Figure 3). This phenomenon appears to be common in *Caenorhabditis* (LE *et al.* 2017), and has greater magnitude of effect the bigger the size differences between chromosome copies (Figure 3) (WANG *et al.* 2010). When males are rare-but-not-too-rare in a population, the consequence of this TRD is that genome size is predicted to decline over time (WANG *et al.* 2010). This process could have operated in the proto-hermaphrodite populations that gave rise to modern day highly selfing species, potentially contributing to their smaller genomes compared to non-selfing relatives (FIERST *et al.* 2015; YIN *et al.* 2018). This process should only influence the size of autosomes and not the X-chromosome, however, so the fact that the X-chromosome also appears to be shorter in selfing species than related non-selfing species implicates other factors, as well, in the genome shrinkage of species that lack abundant males (FIERST *et al.* 2015; YIN *et al.* 2018). Even in outcrossing species, the primary sex ratio may

often have slightly but consistently <50% males (KANZAKI *et al.* 2018), suggesting that TRD might operate throughout the genus as a force counteracting genome expansion.

ARE MALES EVOLUTIONARY RELICS?

Why keep males around after the evolution of self-fertilization? One hypothesis predicts that selection has pushed males to the brink of elimination within the species, but that the genetics of sex determination in *C. elegans* allows for low levels of male persistence (CHASNOV AND CHOW 2002) (CUTTER *et al.* 2003; CHASNOV 2010). In its strongest form, the "evolutionary relic hypothesis" posits that males exist in populations at a balance between male input by X-chromosome non-disjunction and loss by selection, contributing no real functional or evolutionary role in *C. elegans* populations. Thus, while selection on males certainly favors outcrossing (it is their only form of reproduction), selection favoring hermaphrodite selfing is undoubtedly stronger because *C. elegans* predominantly reproduce via selfing. This asymmetry is exacerbated by the fact that there appears to be selection on hermaphrodites to avoid mating with males and outbreeding depression in outcrossed offspring, as outlined above. So, do males actually have a functional role in present-day populations of *C. elegans* and other androdioecious *Caenorhabditis* , or are they simply a kind of vestigial organ, leftover from a bygone male-female ancestor?

There are at least four counterarguments to the 'males as relics' view. First, mutation accumulation studies in *C. elegans* have demonstrated that behavioral traits degrade nearly as quickly as fitness-related traits in the absence of natural selection, with an overall rate of decline

of 2-10% per generation (AJIE *et al.* 2005). Given the large average effect sizes of mutations in *C. elegans*, we would expect specialized male behavior to be completely lost within a few hundred generations, although this testable hypothesis has yet to be investigated. Similarly, LOEWE AND CUTTER (2008) found that pure selfing in *C. elegans* ought to persist for only short periods evolutionary time (on the order of thousands of years) due to the accumulation of deleterious mutations. Complete loss of males is a likely path to species extinction.

Second, as outlined above, a great deal of male-specific molecular function persists within the *C. elegans* genome. This is essentially the molecular analog of the mutation accumulation argument, but at a per-locus basis. While it is clear that some aspects of male function have been lost (YIN *et al.* 2018), the abundance of male-specific gene expression, the large number of male-specific neurons (BARR *et al.* 2018), etc. suggest that direct selection on males contributes at least partly to the maintenance of molecular function (CUTTER *et al.* 2003). Nevertheless, it is challenging to completely rule out inter-sexual pleiotropy of the genetic architecture of these male-specific features such that purifying selection in hermaphrodites leads to indirect perseverance in males.

Third, males in predominantly self-fertilizing species display clear differentiation of function within the context of their hermaphrodite siblings. Male sperm are larger than hermaphroditic sperm within self-fertile species (LAMUNYON AND WARD 1998; LAMUNYON AND WARD 1999; VIELLE *et al.* 2016), consistent with the idea that selection on male reproductive function might sustain greater sperm competitive ability. Developmental bias toward small sperm cell size in the origin of the hermaphrodite phenotype, as has been induced in *C. remanei* and *C. nigoni* (BALDI

et al. 2011), provides an alternate, neutral possible explanation for the origin of sex differences in sperm size. Regardless, the present-day smaller hermaphodite sperm indicates that there is phenotypic space for male sperm size to have declined to be as small as hermaphrodite sperm in the absence of opposing selection. *C. elegans* males also still retain the ability to detect female pheromones produced by other species (LAMUNYON AND WARD 1998; LAMUNYON AND WARD 1999; CHASNOV AND CHOW 2002; BORNE *et al.* 2017). And no matter how the mystery surrounding some of the male-specific longevity effects generated by chemical signaling described above turns out, it does appear that there is differential sensitivity in males from self-fertilizing species relative to those from outcrossing species (SHI *et al.* 2017).

Fourth, chromosomal patterns of genomic polymorphism require at least some mixing of genomes from periodic outcrossing: chromosomal recombination environment should not influence patterns of polymorphism in strictly self-fertilizing species, because recombination exerts no effect when the entire genome is homozygous. And yet, polymorphism is strongly reduced within the central sections of chromosomes that have low recombination rates (Fig. 3). Moreover, the strong signals of selective sweeps in chromosomes suggests that recombination might have facilitated adaption (ANDERSEN *et al.* 2012). Thus, while there is not much evidence for genetic exchange among selfing lineages in ecological time (RICHAUD *et al.* 2018), there is strong genomic evidence for historical incidents of genetic exchange among lineages (ANDERSEN *et al.* 2012).

Despite these signals of selection on males, it remains unresolved whether males are still important in nature. While males clearly have played a decisive role in structuring patterns of

genetic variation and have been under selection for maintained function in the past, it is possible that we are observing a residual ghost of each of these features, which are no longer relevant within the current ecological and evolutionary setting of the species. This is especially relevant if sexual conflict represents the major driver of interactions within and between the sexes for outbreeding *Caenorhabditis* species, including *C. elegans'* ancestor, as we have discussed above. Any sort of negative pleiotropy between the sexes that generates a tradeoff between female and male function will tend to tilt strongly toward the female side of the equation within the androdioecious species, at the expense of males. Coupled with potential selection for reduced mating interactions within hermaphrodites, this dynamic has the potential to create a ratchet of decline in male function with high self-fertilization: hermaphrodites avoid mating, which decreases male frequency in the short-term so that populations experience weakened selection to retain male-related functions, in turn potentially leading to a diminished ability of males to maintain long-term representation within the population.

How do we distinguish between these alternatives? It is actually surprisingly difficult to devise a critical test of the relic hypothesis, although the answer must ultimately lie in observations in nature. For example, the frequency at which males can be maintained within the lab varies substantially among wild isolate strain genotypes, partly due to male reproductive traits, partly due to hermaphrodite reproductive traits, and partly due to differences in X-chromosome non-disjunction (TEOTONIO *et al.* 2006; ANDERSON *et al.* 2010). It remains unclear whether this genetic variation in the potential for male persistence might reflect differences in the functional role of males in the populations from which they were derived, or stochasticity among genetic backgrounds in the decline of male-related function. These natural populations—and the

potential presence of males within them—probably hold the most direct clues to the answer. Further, the rate of meltdown of male function under mutational pressure is readily testable. In the end, we still need a great deal more information from natural populations to understand how laboratory observations connect to the evolution of males in nature.

CONCLUSIONS

Reproduction via outcrossing in *Caenorhabditis* requires males, which, when they are abundant as in most *Caenorhabditis* species, sets the stage for sexual selection and sexual conflict to act as major drivers of the selective regime of both sexes, affecting organismal traits and the genome. Male traits involving gamete size and number, and perhaps seminal fluid, appear to be particularly important targets of such selective pressures. This evolutionary arena changed radically for those species like *C. elegans* that evolved extreme male rarity due to the evolution of selfing hermaphroditism, with striking consequences for the evolution of traits in both sexes as well as for genomic features of the species overall. The balance of selection on organismal function shifted in *C. elegans* toward females, leading to declines in male reproductive function and changes to "female" traits like mating receptivity. Reduced male function over the course of *C. elegans* history likely results from multiple related but distinct factors: relaxed selection and genetic drift, indirect selection due to linkage and the pleiotropic effects of selection for improved hermaphrodite fitness, and direct selection against male traits involved in sexual conflict.

At the genomic level, male rarity has led to drastic loss of genes with male-related activity along with overall shrinkage of the genome, stark reductions in population genetic variation and individual heterozygosity in nature, and potentially a more limited capacity for adaptive evolution for the species as a whole. Convergent evolutionary changes are found in all three species of *Caenorhabditis* that have each evolved selfing hermaphroditism independently. Despite these profound changes in male traits and their genomic basis, many male-specific genes persist and continue to control ontogeny of the male phenotypic form as a competent reproductive outcome of development, conferring clear evidence of successful outcrossing in the genome.

We now enjoy an exceptionally rich set of resources—from experimental techniques to genome sequences to phylogenetic biodiversity—to test diverse evolutionary and functional hypotheses about *Caenorhabditis* male biology. Topics especially ripe for the picking include conceptual issues about sexual selection and sexual conflict, as well as the genetic and developmental mechanisms underlying associated phenotypes and the resulting genome-scale molecular evolutionary consequences. Because *C. elegans* will continue to serve as the workhorse for most studies of male biology, it is important to consider the generality of discoveries made with the N2 genetic background, a strain known to harbor numerous adaptations to laboratory conditions having pleiotropic effects (ZHAO *et al.* 2018), and yet with little understanding of their implications for male traits. More generally, a challenge for *C. elegans* laboratory experimental power remains: how to link exciting lab discoveries to the more complex natural environment, which includes both biotic and abiotic heterogeneity (GIBSON AND MORRAN 2017; ZHANG *et al.* 2017). With abundant questions at the ready, both evolutionary and mechanistic, future studies of

C. elegans males that leverage the system's extensive experimental resources are poised to discover novel biology and to inform profound questions about animal function and evolution.

TABLES, BOXES & FIGURES

Table 1. Virtues and resources for studying *C. elegans* male biology.

Life history virtues	Short generation time (2-6 d, depending on temperature) Ability to cryopreserve strain genotypes and populations Ability to rear in solid media or liquid environments Simple food resources (E. coli or other bacteria, axenic media)
Genomic virtues	Small genome (100Mb), Low repeat content (10-15%), Exceptional reference genome assembly/annotation, Genomes of ~700 non-reference wild isolates (CENDR), Genomes of ~20 *Caenorhabditis* species, modENCODE functional genomic datasets
Experimental resources	Advanced intercross recombinant inbred line collections (AI-RILs), genotyped strains for GWAS (CENDR), experimental evolution populations (e.g., CeMEE)
Mechanism resources	Gene knock-out collection, large mutant allele collection, RNAi knockdown libraries, efficient CRISPR/Cas9 gene editing, multiple transgenic methods, inducible phenotype systems (e.g. auxin, light-activated)
Dataset resources	neuronal connectome, sex-biased gene expression profiles
Male-related experimental tricks	Genetic manipulation of sex-determination pathway and sperm/oocyte germline switch, Auxin-inducible hermaphrodite self-sterility, Live fluorescent male sperm cell imaging

Table 2. Merits of experimental evolution as a powerful tool in the *C. elegans* system (see GRAY AND CUTTER (2014) and TEOTONIO *et al.* (2017) for general reviews).

Living fossil record	*C. elegans* can be cryopreserved for long periods of time and re-animated to compare ancestral populations with evolved populations.
Short generation time	*C. elegans* reproduce in three to four days under standard lab conditions, meaning researchers can detect evolution in real time within several months.
Scale of assessment	*C. elegans* phenotypes can be easily assessed at the both the individual and population level, permitting a wide range of questions to be addressed.
Interspecific interactions	Researchers can readily manipulate *C. elegans* interactions with other species to answer questions regarding a wide range of interspecific interactions (parasitic, commensalistic, and mutualistic).
Small genome size	*C. elegans* have a relatively small genome size compared to most other animal model systems, thus permitting genetic characterization of multiple replicate populations.
Available mutants	Numerous mutant strains exist which allow researchers to manipulate *C. elegans* mating system, immune system, rate of aging, and development. These mutant alleles can be wielded to isolate specific variables and test hypotheses via experimental evolution.
Competitive fitness assays	Similar to bacterial competition assays, *C. elegans* fitness can be measured via competitive fitness assays that can account for the survival, reproduction, and competitive ability of specific populations relative to others.

Genetic manipulation	Using numerous tools for genetic transformation, researchers can map and functionally test specific alleles that arise during experimental evolution.

1

2

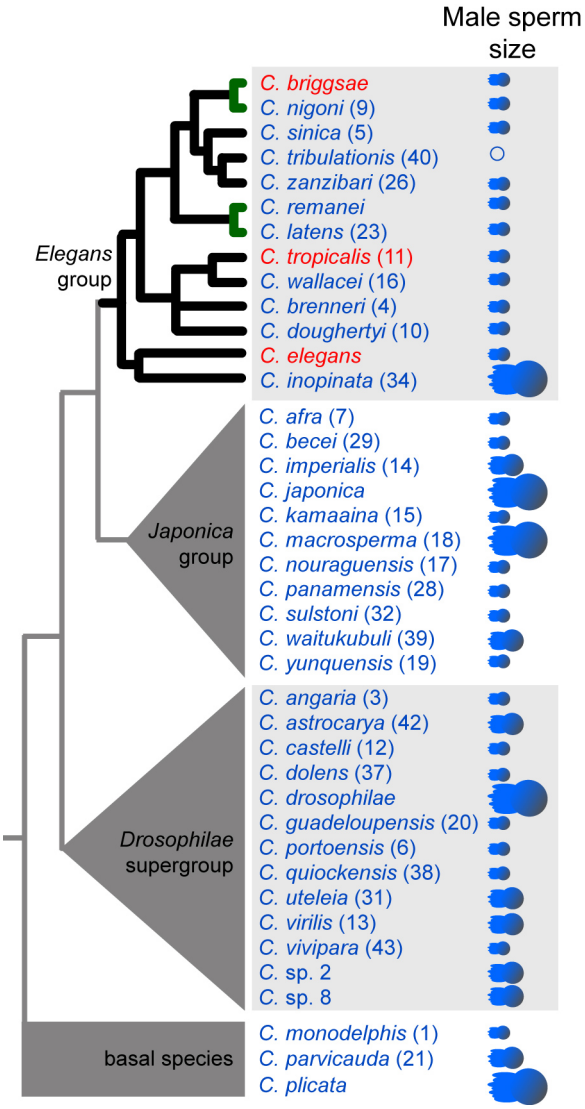


Figure 1. *Caenorhabditis* phylogeny and mating system evolution. Diagrammatic representation of the major phylogenetic groups within *Caenorhabditis*, with current topology for species within the *Elegans* group (other species arranged alphabetically). Note that recent work suggests that relationships among some members of the *Drosophilae* supergroup might make this set of species polyphyletic (STEVENS *et al.* 2019); the three species shown as "basal" also comprise a polyphyletic group. Numbers following species names refer to deprecated numerical identifiers prior to species naming. Names in red text indicate species that independently evolved androdioecy (selfing hermaphrodites and rare males), all other species are gonochoristic (females and males). Lineage pairs with green lines show partial hybrid compatibility. Male sperm size phenotypes shown by spermatozoa cartoon (large = average spermatid

cross-sectional area >100um^2, medium = area 50-100 um^2, small = area <50um^2, unfilled = no data) (VIELLE *et al.* 2016); sperm information for 11 species courtesy R. Salle, A. Vielle and C. Braendle. Within each androdioecious species, hermaphrodite sperm are smaller on average than male sperm.

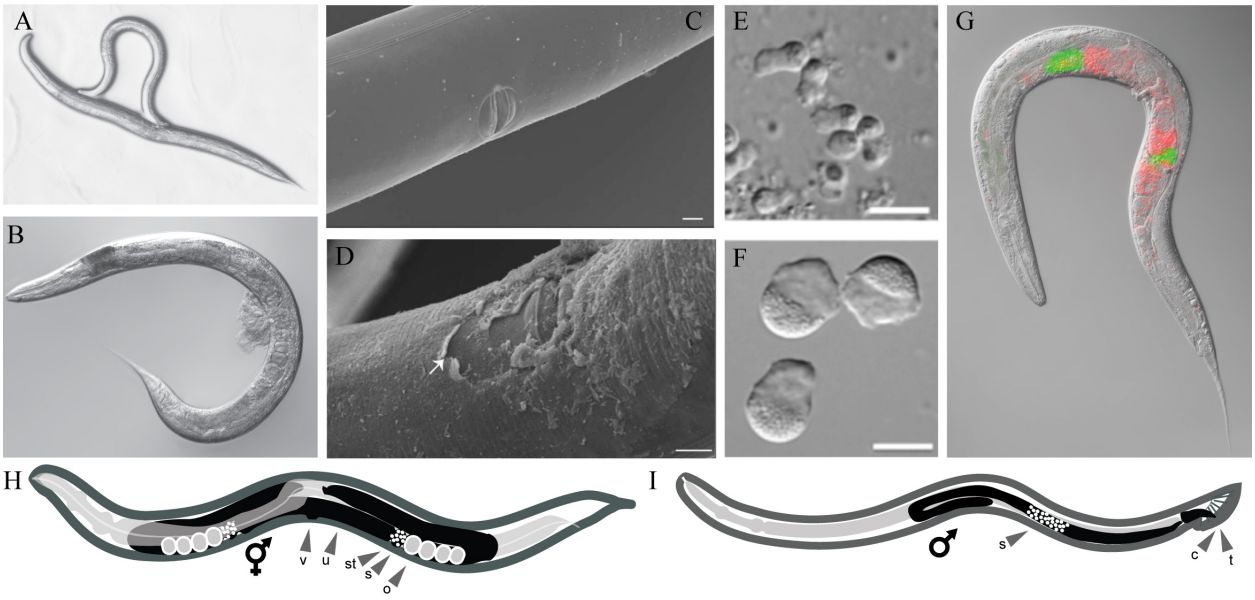


Figure 2. *C. elegans* traits associated with sexual selection and sexual conflict. (A) Male and female *C. nigoni* mating. (B) Copulatory plug deposited by a *C. nigoni* male over the vulva of a female upon mating. (C,D) Cuticular damage around the vulva of a mated *C. elegans* hermaphrodite (D), induced by spicule scraping from male mating attempts (WOODRUFF *et al.* 2014); scale bars = 10μm. (E) Spermatozoa from males of *C. elegans* and (F) *C. macrosperma*, showing divergence in sperm cell size (VIELLE *et al.* 2016); scale bars = 10μm. (G) Ectopic sperm from male *C. nigoni* (stained red with MitoTracker CMXRos) having invaded the gonad of a *C. elegans* hermaphrodite (strain DZ325 expressing GFP in spermathecae). (H) Diagram of hermaphrodite (or female) and (I) male *Caenorhabditis*. Gonad in black and gut in gray, with sperm as white dots and oocytes as gray ovals outlined in white (v=vulva, u=uterus, st=spermatheca, s=sperm, o=oocyte, c=cloaca, t=tail spicules). Sperm in *C. elegans* hermaphrodites develop from the first ~150 germ cells in each gonad arm, then reside in the spermatheca, with all subsequent germ cells developing as oocytes. Sperm transferred from males to hermaphrodites or females migrate through the cloaca into the uterus, facilitated by insertion of spicules into the vulva, after which sperm crawl to the spermatheca to fertilize mature oocytes that enter the spermatheca.

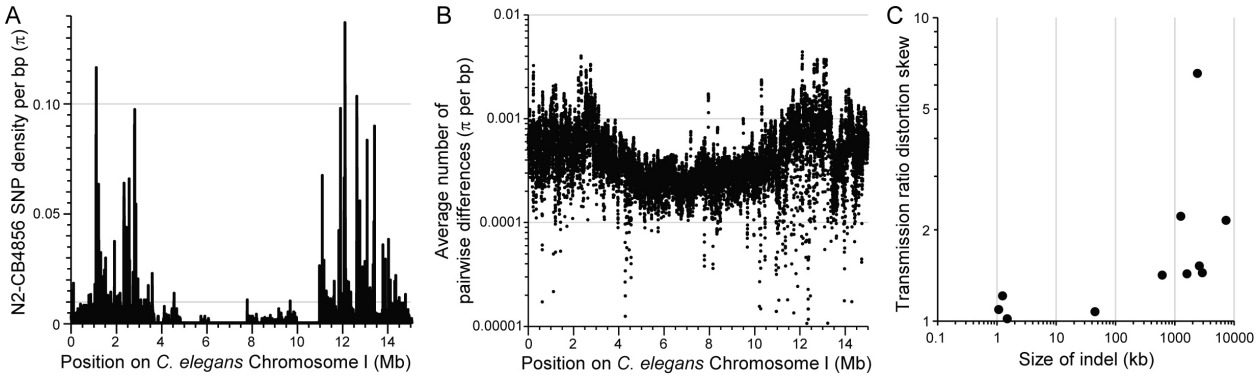


Figure 3. (A) "Islands" of high divergence >5% in the *C. elegans* genome likely reflect retained blocks of ancestral polymorphism (redrawn from data for all sites in File S2 of (THOMPSON *et al.* 2015) using 2kb non-overlapping windows; average across windows $\pi = 0.0024$). (B) Central portions of chromosomes, which have lower recombination rates and higher gene density, have less population genetic SNP variation. Data courtesy E. Andersen and S. Zdraljevic shows the average number of pairwise differences per kb for 330 wild isolate genomes of *C. elegans* based on 10kb windows of all sites, with a 1kb step size along Chromosome I; note log scale of polymorphism axis; windows with highly divergent sequence and $\pi < 0.00001$ excluded for visual clarity. (C) Larger autosomal indels show stronger transmission ratio distortion when transmitted through sperm from males (data from (WANG *et al.* 2010) redrawn courtesy J. Wang).

Box 1. Making a male.

One of the great strengths of using *C. elegans* to test the role of males in evolutionary processes is that there are multiple ways of manipulating the nematode sex determination system in order to control mating systems dynamics. As outlined in the figure below, the sex determination pathway was one of the first systems investigated in depth in *C. elegans* and so has been reviewed multiple times (KUWABARA AND KIMBLE 1992; ZARKOWER 2006; ZANETTI AND PUOTI 2013). For the purposes of this review, we are particularly interested in illustrating how this knowledge can be used to manipulate the sex determination system to allow experimental tests of consequences of mating system variation and the role of males with a level of precision that is impossible in any other species.

Like many animals, *Caenorhabditis* sex is determined by X-autosome balance, which in this case means that XX individuals become hermaphrodites (or females) and XØ individuals (i.e., those actually missing an X chromosome, but otherwise diploid) become males (NIGON 1951); other aberrant ratios are possible and have been used to test the fine tuning of the system. Most crucial here is the dosage compensation system of the X chromosome, in which worms down regulate genes on both copies in XX individuals (MEYER 2005). This process is initiated by the XOL-1 GHMP kinase, which is a critical regulator of dosage compensation and cell-specific sex determination (LUZ *et al.* 2003). *xol-1* mutants inappropriately down-regulate the X in males, leading to male lethality (XOL stands for XO Lethal). So, in effect, populations fixed for a *xol-1* knockout become obligate selfers, which is useful, for instance, to completely exclude males when testing whether males play an important role in determining the rate of adaptation to a new environment and/or in eliminating deleterious mutations from the population (MORRAN *et al.* 2009b; MORRAN *et al.* 2011).

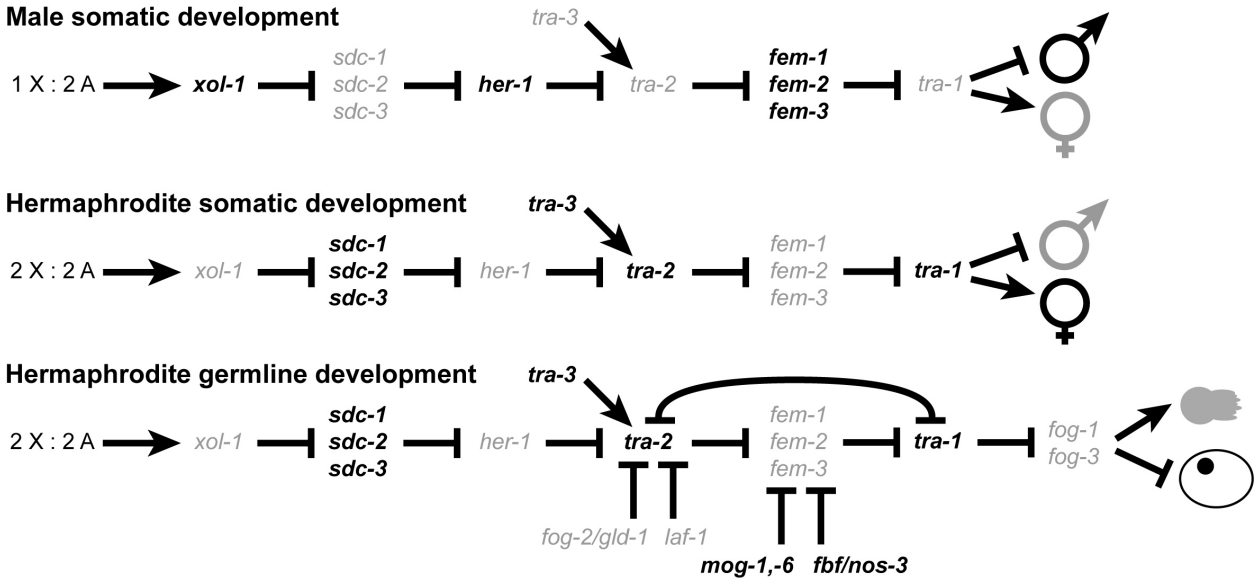
On the opposite end of the spectrum, *C. elegans* hermaphrodites undergo a protandrous transition from producing sperm early during sexual maturity to producing exclusively eggs during young adulthood. Therefore, any means of blocking sperm production in hermaphrodites that does not influence egg

production, or sperm function in males, effectively transitions the mating system from primarily selfing to being obligate outcrossing, though hermaphrodites that lose sperm production are not exactly true females. The FOG-2 F-box protein fits the bill perfectly here, as it normally binds and inactivates *tra-2* mRNA within the developing gonad, briefly masculinizing it so that hermaphrodites can make some sperm; loss-of-function alleles of *fog-2* thus eliminate hermaphrodite sperm production (CLIFFORD *et al.* 2000). Populations fixed for a *fog-2* mutation are therefore dioecious (male-female) against the native androdioecious background (STEWART AND PHILLIPS 2002). A similar effect can be achieved using hermaphrodite-specific sperm knockouts (CUTTER 2005). The ability to readily switch populations between a hermaphrodite-dominated to male-female mating system has yielded a wide variety of interesting experimental approaches within the field, as highlighted in the main text.

The sex determination system lends itself to other tricks that have been somewhat less utilized to ask evolutionary questions. Because of its central role in flipping sex determination, the transmembrane signaling protein TRA-2 is particularly important, and a number of interesting allelic variants have been characterized (HODGKIN 2002). For example, a temperature-sensitive mutation of *tra-2* can be used to titrate the frequency of males within a population (JANZEN AND PHILLIPS 2006) and has been used in experimental evolution to examine the evolution of specialized male-specific gene expression (CHANDLER *et al.* 2009; CHANDLER *et al.* 2012). Males generated in this fashion do not to tend to be particularly virile (even on a *C. elegans* scale of function) and actually perform a bit better in a *xol-1* background (HODGKIN 2002). An interesting related technological development is the ability to manipulate the sex determination system within somatic tissue to independently masculinize or feminize a given part of the body. For instance, masculinizing all of the neurons within a hermaphrodite has recently been used to identify which neurons are important for generating male attraction to hermaphrodites (FAGAN *et al.* 2018). Basically, researchers systematically generated male-specific gene expression within a specific neuron using *tra-1* and then used this to determine which neurons are necessary and sufficient to convert hermaphrodite-typical responses to male-typical responses in the presence of hermaphrodite

ascaroside secretions. As of yet, no one has applied this approach to address specific evolutionary questions, although the potential to analyze any within-locus effects on sexual conflict in a cell-by-cell manner would seem to be an exciting frontier.

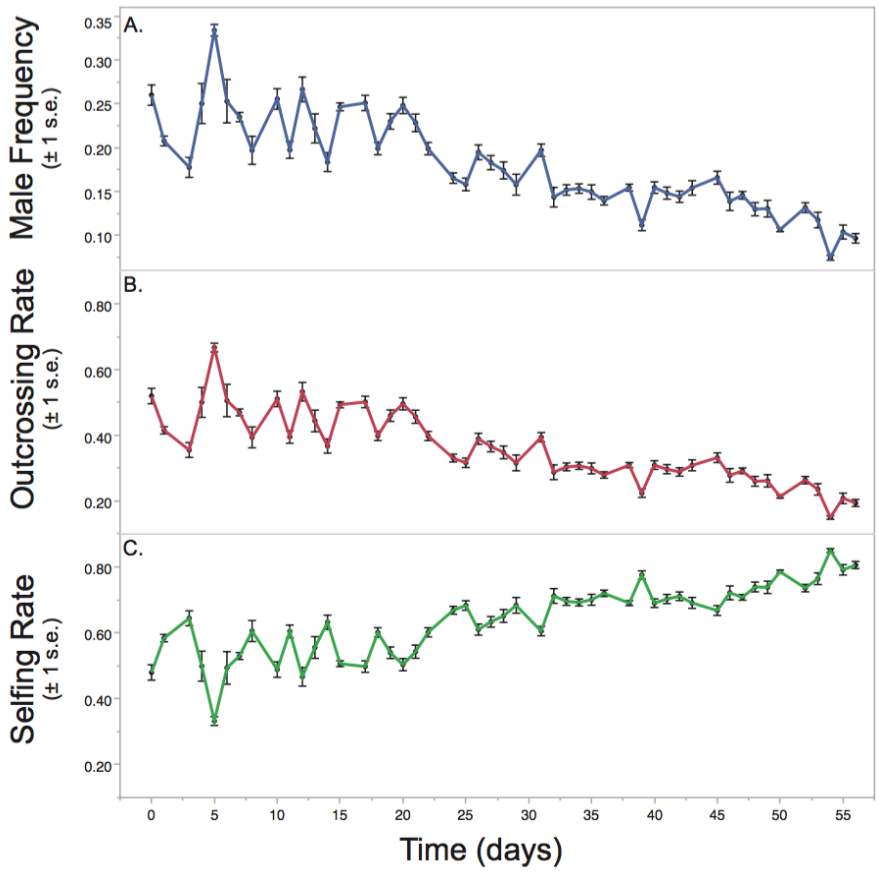
To date, most of these pathway manipulations have been achieved using mutations, which makes it difficult to switch the effects on and off. There are a growing number of different approaches for controlling gene expression and/or genomic state within *C. elegans* (ARAYA *et al.* 2014; DICKINSON AND GOLDSTEIN 2016; MUNOZ-JIMENEZ *et al.* 2017). The recently-created auxin-inducible degradation (AID) system holds particular promise in this area because it allows a tagged protein to be specifically degraded when worms are grown in the presence of the plant hormone auxin (ZHANG *et al.* 2015). The first application of this technique in an evolutionary context has been to knockout sperm production in both hermaphrodites and males in a switchable manner (KASIMATIS *et al.* 2018a). This approach is useful for aging assays and studies of reproduction *per se*, but also sets the stage for manipulating the intensity of sperm interactions within and between both hermaphrodites and males. Overall, we are at the very earliest stages of truly leveraging the full genetic toolkit available in *C. elegans* to address difficult long-standing questions in evolutionary biology.



Box 1 Figure summarizing *C. elegans* sex determination pathway redrawn from KELLEHER *et al.* (2008), primarily defined by negative regulatory interactions (bars; arrows indicate positive regulation). The developmental fate in black (male vs female, sperm vs oocyte) represents the phenotypic output of high activity of genes indicated by bold text along the pathway (low activity in gray text).

Box 2. **Evolution of selfing rates.**

C. elegans researchers have developed two approaches to determine whether specific selective pressures favor outcrossing over selfing during experimental evolution. First, using longitudinal studies that track outcrossing rates in mixed mating populations over the course of experimental evolution, researchers can gauge the selective benefit of outcrossing relative to selfing in real-time. When outcrossing is favored by selection, outcrossing rates increase relative to selfing and vice versa when selfing is favored. This method has been employed to determine that outcrossing is favored over selfing as populations adapt to parasitic bacteria, novel temperatures, and chemical exposure. The second approach works similarly to the 'longitudinal' approach, but specifically tests the maintenance of obligate outcrossing by introducing a threat of invasion by a selfing genotype. A mutant *fog-2* allele is used to generate obligately outcrossing populations of *C. elegans* and the wildtype *fog-2* allele, which confers mixed mating, is then introduced into the obligately outcrossing population. If selfing is favored by selection, then the mixed mating allele and self-fertilization increase in frequency. However, if obligate outcrossing is favored then selfing rates do not increase in the population over time. Both methods track outcrossing and selfing rates in populations by measuring male frequencies at multiple time points throughout experimental evolution. The male frequency (A) is then converted to outcrossing rate $2(m - \mu)$ (B) or selfing rate $1 - o$ (C), where m is the frequency of male offspring, μ is the rate of X chromosome nondisjunction, and o is the outcrossing rate. Using male frequency data from an invasion experiment in STEWART AND PHILLIPS (2002), panels A, B, and C, display the male frequency, outcrossing rate, and selfing rate for the same data set as an example of tracking the selective advantages of selfing versus outcrossing populations in real time. These populations were passaged under standard lab conditions. Male frequencies (A) and outcrossing rates (B) declined over time, while selfing rates increased (C). Therefore, as explained by STEWART AND PHILLIPS (2002), selfing is favored in genetically uniform populations maintained under standard lab conditions.



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