

## The evolutionary role of males in *C. elegans*

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**A**lthough the nematode worm *Caenorhabditis elegans* reproduces primarily as a self-fertilizing hermaphrodite, males are maintained in natural populations at low frequency. In this commentary, I discuss the evolutionary forces that maintain males and the role males might play in this mating system.

The hermaphroditic nematode worms of *C. elegans* can reproduce either by self-fertilization or by mating with males. A self-fertilizing hermaphrodite can produce around 300 offspring before sperm depletion; an outcrossed hermaphrodite supplied with plentiful virile males can produce as many as 1,400 offspring.<sup>1</sup> Yet despite their sperm-limited fecundity, hermaphrodites may have been selected to self-fertilize rather than outcross with males.<sup>2</sup> Hermaphrodites in comparison to the females of related *Caenorhabditis* species are less attractive to males and less receptive to mating.<sup>3</sup> To wit, females, but not hermaphrodites, have been shown to secrete a volatile sex pheromone that readily attracts males from a distance.<sup>4</sup> And females, but not hermaphrodites, have been shown to respond to a male sporific-inducing factor when mating.<sup>5</sup>

The *Caenorhabditis* sex pheromones have been shown to be secreted by females of the dioecious (male-female) species *C. remanei* and *C. brenneri* and not by hermaphrodites of the androdioecious (male-hermaphrodite) species *C. elegans* and *C. briggsae*.<sup>4</sup> As would be expected for a true sex pheromone, production of the attractant by a female, and the corresponding male response, begins soon after worms reach adulthood and are ready to mate. Similar to pheromonostasis observed in moths and other insects,<sup>6</sup>

females also cease sex pheromone production immediately after mating, taking several hours to restart production. Somewhat surprisingly, the female sex pheromones are not strictly species-specific, and the males of the androdioecious species are more attracted to heterospecific females than they are to their own conspecific hermaphrodites.<sup>3</sup> Androdioecy in *Caenorhabditis* is thought to have evolved independently from common dioecious ancestors,<sup>7</sup> so it seems likely that a not-too-distant female ancestor of modern hermaphrodites must have once secreted a pheromone similar to the pheromones secreted by modern females.

The lack of a volatile sex pheromone in androdioecious *Caenorhabditis* species does not mean that males find their conspecific hermaphrodites unattractive. Displaying what is apparently a mate-searching behavior, *C. elegans* males will tend to leave a food source in the absence of conspecific hermaphrodites, but will stay when hermaphrodites are present.<sup>8,9</sup> And close observations of male behavior have shown that males can find regions of agar pre-conditioned by either hermaphrodites or by hermaphrodite metabolites, and that once males find these regions, they tend to crawl backward and forward to remain there.<sup>10,11</sup>

Yet, make no mistake: there is a qualitative difference in the male response to conspecific females compared with the male response to conspecific hermaphrodites. A now standard chemoattraction assay for *C. elegans*<sup>12</sup> places test worms between two spots containing either a potential volatile attractant or a control, and this assay was used to elucidate the nature of the *Caenorhabditis* female sex pheromone.<sup>4</sup> In one such series of 20

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independent assays—each assay testing the attraction of 20 *C. remanei* adult males to supernatant obtained by soaking several *C. remanei* virgin females—an overwhelming number of males were attracted to the supernatant: 376 males were attracted vs. 14 to the control spot and 18 to neither. Males were also observed to crawl rapidly toward the supernatant spot: the one-millimeter-sized worms traveled 2 cm in about 2 min.

In contrast, determining that *C. elegans* males were indeed attracted to conspecific hermaphrodites<sup>10</sup> required the design of a new more sensitive assay that measured how long males stay in regions previously conditioned by hermaphrodites. Test males were videotaped for 15 min and scored for the time spent on a conditioned compared with a control region. In one such series of assays,<sup>13</sup> adult males spent just under 5 min on the conditioned region compared with about 1.5 min on the control region.

At first, the weak male attractant was termed a “mate-finding cue.”<sup>10</sup> It was later re-characterized, however, as “a chemical signal that strongly attracts males,” and as a “mating pheromone.”<sup>13</sup> Since this male attractant has now been chemically identified as a blend of at least four ascarasides,<sup>13-15</sup> and others have used these ascarasides to further explore *C. elegans* biology,<sup>16</sup> it has become important to accurately characterize these chemicals so that their significance can be placed within the proper evolutionary perspective.

The standard definition of a pheromone is that of a chemical or chemicals externally secreted by an animal to directly influence the behavior or physiology of another conspecific animal.<sup>17</sup> In particular, a sex (or mating) pheromone is a pheromone that is secreted by individuals of one sex to attract individuals of the opposite sex for the purpose of mating. Most commonly, females secrete sex pheromones to attract males.

The secretion of a true sex pheromone should be adaptive. Because females that secrete sex pheromones can rapidly attract males, natural selection would in many situations favor fertile females that secrete sex pheromones over those that do not. Given that hermaphrodites no longer secrete a volatile sex pheromone similar to

one secreted by related females, it seems difficult to argue that hermaphrodites instead secrete the much weaker ascarasides to attract males, and that these ascarasides are true sex pheromones. Males, however, do need to find and mate with hermaphrodites to pass their genes on to the next generation. So natural selection should strongly favor males capable of detecting the presence of hermaphrodites, whether by sensing ascaraside metabolites or otherwise.

Equally strong independent support of the notion that hermaphrodites have been selected to self-fertilize rather than outcross is provided by the *Caenorhabditis* male sporific-inducing factor. This factor immobilizes females during mating by stopping female locomotion and defecation, and reducing female pharyngeal pumping. It also widens the female vulva slit, which apparently enables easier insertion of the male copulatory spicules. The females of the dioecious species *C. remanei* and *C. brenneri*, but not the hermaphrodites of the androdioecious species *C. elegans* and *C. briggsae*, have been observed to sporifically respond to their conspecific males.<sup>5</sup> And suggestive of pheromonostasis, just mated females no longer respond to the male sporific-inducing factor.

Males of the androdioecious species *C. briggsae* (but not *C. elegans*) can also cause sporific behavior in heterospecific *C. remanei* and *C. brenneri* females. Again, it seems likely that a female ancestor of modern hermaphrodites at one time responded to the sporific-inducing male factor and that this behavior has been subsequently lost by modern hermaphrodites.

Not only does this male sporific-inducing factor not affect hermaphrodites, but hermaphrodites sometimes initiate rapid backward and forward movement upon male contact, and crawl with higher than normal speed away from males.<sup>18</sup> And even after successful insemination, hermaphrodites on occasion eject some or all of the male seminal mass.<sup>18,19</sup>

As long as hermaphrodites are self-fertile, selection seems to have favored hermaphrodites that self and males that outcross, resulting in a sexual conflict over mating.<sup>2</sup> However, selection on hermaphrodites to avoid mating may be expected to be much weaker than selection on

males to successfully mate. A hermaphrodite that either self-fertilizes or outcrosses produces viable offspring; a male that fails to outcross produces none. When males are rare, as they seem to be in natural populations,<sup>20,21</sup> most hermaphrodites may never encounter a mate and selection on hermaphrodites for mate avoidance should be weak. Males, on the other hand, must find and mate with hermaphrodites to reproduce, and selection on males to do so rapidly and efficiently should be strong. This asymmetry in selection on the two sexes may result in males that express behaviors aimed at finding and successfully mating hermaphrodites, and in hermaphrodites that appear to be ambivalent about mating.<sup>22</sup>

Males, even when rare, should be continuously selected to improve their mating efficiency with hermaphrodites. In fact, genetic variation in male-mating ability has been found among *C. elegans* natural isolates.<sup>23-25</sup> Some isolate strains can maintain males at higher frequencies in laboratory culture, and this seems to be mainly due to the ability of males from these strains to obtain more copulations, resulting in the fathering of more offspring.

Long after sperm depletion when self-fertilization is no longer an option, hermaphrodites still remain fertile<sup>26</sup> and can resume the production and laying of eggs when mated as a response to the presence of allosperm in their uterus.<sup>27-29</sup> It is self-evident that when hermaphrodites are sperm depleted, selection on hermaphrodites must favor outcrossing even though this selection may be weak, acting as it does on aged hermaphrodites.

So do sperm-depleted hermaphrodites behave in ways that facilitate outcrossing? One study<sup>18</sup> suggests that they may. Sperm-depleted and self-fertile hermaphrodites were compared: males mated more efficiently and faster with sperm-depleted hermaphrodites; sperm-depleted hermaphrodites were less likely to sprint away from males and sperm-depleted hermaphrodites were less likely to expel the male seminal mass after mating.

A second study<sup>30</sup> examined mating-defective males from *C. elegans* polycystin mutants, which were originally discovered by their lack of a robust response to contact with (self-fertile) hermaphrodites.<sup>31</sup>

Surprisingly, these polycystin-mutant males responded almost normally to sperm-depleted hermaphrodites. Although wild-type males can respond normally to both self-fertile and sperm-depleted hermaphrodites, wild-type males too were shown to demonstrate a distinct preference for sperm-depleted hermaphrodites. Apparently, males can only sense self-fertile hermaphrodites by way of a signaling pathway requiring polycystin genes, but are able to sense sperm-depleted hermaphrodites via another signaling pathway.

Also suggestive of pheromonostasis, polycystin-mutant males no longer respond normally to sperm-depleted hermaphrodites that have just mated with males. Indeed, although polycystin-mutant males can also respond normally to heterospecific virgin *C. remanei* females, these males no longer respond normally to just-mated females.

Although these aforementioned studies suggest that sperm-depleted hermaphrodites may be more receptive to mating than self-fertile hermaphrodites, sperm-depleted hermaphrodites are still not as receptive as virgin females.<sup>5</sup> Most notably, sperm-depleted hermaphrodites, in contrast to virgin females, still do not secrete a volatile sex pheromone.<sup>4</sup>

What are the selection pressures on females that can result in the evolution of self-fertile hermaphrodites? Previously, I have mathematically modeled the evolution from females to hermaphrodites as a two-step process.<sup>2</sup> First, if the benefit from reproductive assurance exceeds the cost, females may evolve the ability to self-fertilize in the absence of males. Second, if by successive self-fertilization deleterious mutations are purged and inbreeding depression is reduced, hermaphrodites may evolve to be less attractive and receptive to males.

Interestingly, a mutational path that can turn females into hermaphrodites was recently discovered by considering RNAi mutants of the dioecious species *C. remanei*.<sup>32</sup> Two independent mutations were required to make a hermaphrodite: (1) a mutation in the sex-determination pathway that enabled females to make spermatids as well as oocytes, and (2) a mutation in the sperm-activation pathway that activated the spermatids. The mutation in

the sperm-activation pathway appeared to be nearly selectively neutral. Provided this mutation occurred first, a female carrier incurring a second mutation in its sex-determination pathway could become selectively favored. When overcrowded, starved or heat stressed, *C. elegans* larvae may molt to a specialized dauer stage<sup>33</sup> that apparently facilitates dispersion. A singly dispersed hermaphrodite with the ability to self-fertilize and without a readily available mate could have a substantial selective advantage over a singly dispersed female.

Furthermore, inbreeding depression in modern-day *C. elegans* is thought to be absent.<sup>34</sup> Although inbred lines of the dioecious species *C. remanei* exhibit reduced fitness relative to outcrossed lines, pure isolate strains of self-fertilizing *C. elegans* hermaphrodites exhibit higher fitness than crosses between isolate strains.<sup>35</sup> During the evolutionary transition from females to hermaphrodites, the diminishment of inbreeding depression can cause a reversal in selection: from favoring hermaphrodites that strongly attract males and are highly receptive to mating, to favoring less attractive and receptive hermaphrodites.

My mathematical models<sup>2</sup> of the evolution from females to hermaphrodites considered a population without age structure, perhaps missing some fundamental biology. For example, a celebrated theory<sup>36</sup>—now commonly called the antagonistic pleiotropy hypothesis, or more picturesquely the “Pay Later” theory—postulates that senescence evolved as the consequence of selection for genes that increase fitness when young but decrease fitness when old.

We have already met three plausible pay-later genes: the first results in sperm-limited fecundity; the second in the loss of a sex pheromone; and the third in the loss of the soporific behavior upon mating. Within an age-structured model, it is known that early-born offspring increase parental fitness more than late-born offspring.<sup>37</sup> Sperm-limited fecundity increases fitness by ceasing sperm production so that egg production can start. But sperm-limited fecundity also decreases fitness after hermaphrodites run out of self-sperm and stop producing eggs. That self-fertilizing hermaphrodites

nevertheless attain a net fitness gain by limiting sperm production has been shown both experimentally and theoretically.<sup>38–41</sup> Also, hermaphrodites, by being both less attractive and responsive to males when they are self-fertile, presumably gain more fitness as young adults from self-fertilizing rather than outcrossing. But older sperm-depleted hermaphrodites lose fitness from being less attractive and responsive to males that can provide needed allosperm. Presumably, here too, the fitness gain to hermaphrodites as young adults outweighs the fitness loss later in life.

The previously mentioned pheromonostasis-like behavior of the *Caenorhabditis* females and hermaphrodites poses two similar, yet distinct, puzzles. Consider the first.<sup>4,5</sup> Females immediately after mating no longer secrete a sex pheromone or exhibit a soporific response to males. Yet when sperm depleted, females do recover both these outcross-favoring behaviors. Hermaphrodites when self-fertile also do not secrete a sex pheromone or exhibit a soporific response. However, in contrast to females, hermaphrodites do not seem to gain these behaviors when sperm depleted. Why do sperm-depleted hermaphrodites behave differently than sperm-depleted females? The solution to this first puzzle is to suppose that the ancestors of modern hermaphrodites incurred mutations that irreversibly eliminated the production of both the volatile sex pheromone and the soporific-response to mating.

Now consider the second puzzle concerning the pheromonostasis-like response of *C. elegans* polycystin-mutant males to females and hermaphrodites.<sup>30</sup> Both recently mated females and self-fertile hermaphrodites do not elicit a normal mating response from *C. elegans* polycystin-mutant males. But both females and hermaphrodites can elicit a normal response: females when either virgin or sperm-depleted, and hermaphrodites when sperm-depleted. Here, why do sperm-depleted hermaphrodites behave the same as sperm-depleted females? It would seem that the presence of sperm alone, or some associated factor, is sufficient to eliminate the cue that is sensed by polycystin-mutant males.

In this regard, it might be informative to observe the behavior of the new hermaphroditic mutants<sup>32</sup> of *C. remanei*

toward male mating. When self-fertile, do these proto-hermaphrodites still secrete a sex pheromone and exhibit a soporific response to males? And can polycystin-mutant males respond normally to these self-fertile hermaphrodites? Finally, how do these behaviors change when these proto-hermaphrodites become sperm depleted? The behaviors of modern hermaphrodites suggest that the self-fertile proto-hermaphrodites might still secrete a sex pheromone and exhibit a soporific response to males, having not yet incurred any additional mutations that eliminate these behaviors. In contrast, the behavior of polycystin-mutant males with modern hermaphrodites suggests that self-fertile proto-hermaphrodites, by virtue of the presence of internal sperm, will not elicit a normal response from polycystin-mutant males, but that sperm-depleted proto-hermaphrodites will.

Finally, some laboratory evolution experiments have considered the advantages of outcrossing over selfing in *C. elegans*.<sup>42</sup> Different populations of *C. elegans* were evolved under elevated mutation rates and under strong selection. Worm strains were constructed that were either obligate selfing or obligate outcrossing. The obligate outcrossing strains were observed to evolve to a higher mean fitness than the obligate selfing strains, and the wild-type strains that could both self-fertilize and outcross increased their outcrossing over the course of evolution. The proximate reasons for increased outcrossing, however, were unknown. These evolution experiments suggest that under certain conditions, increased outcrossing can confer some benefit to a worm population, in agreement with previous theoretical arguments for the evolution of sex.<sup>43</sup>

The potential benefit of outcrossing to a population, however, is not exactly what is at issue here. What is at issue is whether or not selection acts to maintain rare males in reserve for the purpose of bestowing the benefits of outcrossing when environmental conditions worsen and the population finds itself under duress. To suggest that selection can in fact act in this way<sup>44</sup> seems to be tantamount to arguing for a type of group selection.<sup>45,46</sup> It may be true that in certain stressful environments, a population of self-fertilizing hermaphrodites

with rare males will evolve increased outcrossing and enhance its survival relative to a population of self-fertilizing hermaphrodites absent males. But despite this population-wide benefit, selection may still on average favor self-fertilizing over outcrossing hermaphrodites, and ultimately reduce the frequency of males, even possibly to zero.

Laboratory experiments have also demonstrated an elevated male frequency in some *C. elegans* natural isolates after passage through a stress-resistant dauer-larvae stage.<sup>47</sup> The elevated male frequency might have been caused by more males than hermaphrodites surviving the dauer stage, and by increased outcrossing. Post-dauer males of some strains seemed able to mate more efficiently with hermaphrodites, and post-dauer hermaphrodites of some strains seemed to be mated more efficiently by males.

The interpretation of the results of these laboratory experiments in terms of natural selection, however, seems problematic. Males, because they can only reproduce by outcrossing, should always be under selection for their high mating efficiency. It seems incorrect to argue that post-dauer males compared with males that do not pass through dauer could be selected for a higher mating efficiency because of the plausible benefit to the population as a whole. Post-dauer hermaphrodites, however, might conceivably be selected to change their behavior so as to facilitate mating. For instance, selection might favor post-dauer hermaphrodites that secrete a volatile sex pheromone or respond to the male soporific factor. Previous experiments to determine if post-dauer hermaphrodites from the canonical N2 strain secrete a sex pheromone have in fact been negative.<sup>4</sup>

To summarize, I have argued that selection on self-fertile hermaphrodites has favored self-fertilization rather than outcrossing, and that selection on sperm-depleted hermaphrodites for more efficient outcrossing may be weak. Selection on males, however, always favors efficient outcrossing. The evolutionary endpoint of this sexual conflict between hermaphrodites and males would likely have been the extinction of males if not for their rare spontaneous production

due to X-chromosomal non-disjunction occurring during meiosis in self-fertilizing hermaphrodites.

Although in some sense *C. elegans* males can be viewed as relics of their ancestral dioecious mating system,<sup>3</sup> one can also view males as having a profound effect on the current and future evolution of the species. A purely self-fertilizing species is predicted to suffer a mutational meltdown and eventual extinction due to the accumulative effects of Muller's ratchet.<sup>48</sup> The continuing maintenance of rare *C. elegans* males and their heroic efforts to mate with ambivalent hermaphrodites may therefore act to ensure the long-time stability of this species.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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