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Diversity in Mating Behavior of Hermaphroditic and Male–Female Caenorhabditis Nematodes

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ABSTRACT

In this study, we addressed why *Caenorhabditis elegans* males are inefficient at fertilizing their hermaphrodites. During copulation, hermaphrodites generally move away from males before they become impregnated. *C. elegans* hermaphrodites reproduce by internal self-fertilization, so that copulation with males is not required for species propagation. The hermaphroditic mode of reproduction could potentially relax selection for genes that optimize male mating behavior. We examined males from hermaphroditic and gonochoristic (male–female copulation) Caenorhabditis species to determine if they use different sensory and motor mechanisms to control their mating behavior. Instead, we found through laser ablation analysis and behavioral observations that hermaphroditic *C. briggsae* and gonochoristic *C. remanei* and Caenorhabditis species 4, PB2801 males produce a factor that immobilizes females during copulation. This factor also stimulates the vulval slit to widen, so that the male copulatory spicules can easily insert. *C. elegans* and *C. briggsae* hermaphrodites are not affected by this factor. We suggest that sensory and motor execution of mating behavior have not significantly changed among males of different Caenorhabditis species; however, during the evolution of internal self-fertilization, hermaphrodites have lost the ability to respond to the male soporific-inducing factor.

EVOLUTIONARY changes in reproductive modes of metazoans require modifications not only in the development of reproductive tissues, but also in neuromuscular-based behaviors that relate to species propagation. In nematodes of the genus Caenorhabditis, hermaphroditism has been proposed to have evolved twice (Cho *et al.* 2004; Kiontke *et al.* 2004). Sex-determining molecules that regulate gametogenesis between different Caenorhabditis species have been studied as targets that direct evolutionary changes in reproductive mechanisms (Haag and Kimble 2000; Baird 2002; Haag *et al.* 2002; Stothard *et al.* 2002; Hill *et al.* 2006; Stothard and Pilgrim 2006). However, the repercussions of reproductive modifications of the evolution of behaviors have not been intensively investigated.

The nematode *Caenorhabditis elegans* contains two genders, self-fertilizing hermaphrodites and males. The predominant sex generated from internal self-fertilization is the hermaphrodite; however, at a frequency of 0.2%, males can be generated via spontaneous nondisjunction events during meiosis. In contrast, when hermaphrodites are cross-fertilized, equal numbers of progeny males and hermaphrodites are produced. The ability of *C. elegans* males to mate with hermaphrodites has been documented to be inefficient in comparison to mating behaviors exhibited by Caenorhabditis species that display a gonochorism mode of reproduction (male–female copulation) (Chasnov and Chow 2002). This has been shown in population dynamics studies, which demonstrate that the frequency of males in androdioecious (male–hermaphrodite) *C. elegans* populations declines within a few generations (Chasnov and Chow 2002; Stewart and Phillips 2002). A likely reason for the reduction of cross-progeny males in androdioecious populations is that most hermaphrodites are not cross-fertilized; alternative postcopulatory, prezygotic explanations are unlikely since male sperm will outcompete the hermaphrodite sperm for fertilization (Ward and Carrel 1979). Thus, some aspect of *C. elegans* male mating behavior might not be optimized for successful cross-fertilization into hermaphrodites.

The first cellular dissection of *C. elegans* male mating behavior was conducted using detailed behavioral observations coupled with laser ablation analysis (Liu and Sternberg 1995). From observations using uncoordinated hermaphrodites as mating partners, *C. elegans* male mating behavior was dissected into several sensory and motor sub-behaviors. Mating behavior initiates when the ray and ventral sensilla of the male tail contact the hermaphrodite cuticle. These sensory neurons signal the male to press his tail against the hermaphrodite cuticle and begin moving backward along his mate, scanning for the vulva. If the male reaches the end of the hermaphrodite without encountering the vulva, then

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the posterior ray sensilla signal the male to make a ventral turn and continue to search for the vulva on the hermaphrodite's opposite side. When the HOA and HOB sensory neurons, located in the hook, and the PCA, PCB, and PCC sensory neurons, located in the postcloacal sensilla, sense the vulva, the male stops its backward locomotion and attempts to insert his copulatory spicules into the hermaphrodite. The spicules will repeatedly thrust at the tightly closed vulva until the tips partially penetrate the vulval slit. The putative proprioceptive SPC spicule neurons then trigger the spicules to penetrate completely through the vulva. After the spicules completely insert, the male gametes are then transferred from the seminal vesicle, through the vas deferens, out the cloaca and into the hermaphrodite.

This stereotyped male mating behavior can be consistently observed in couplings between males and genetically paralyzed hermaphrodites. However, not all of the mating substeps are performed with equal efficiency. Spicule insertion is the most difficult step for males to accomplish (Liu and Sternberg 1995). During matings with young paralyzed adult hermaphrodites, some males spend >10 min attempting to insert their spicules before the vulva is breached. During matings with constantly moving hermaphrodites, anecdotal observations from our laboratory, as well as from others, suggest that the ability of males to complete any defined behavioral substep, including spicule insertion, is variable. Thus many studies of *C. elegans* mating behavior use nonmoving hermaphrodites as the male's partner (Loer and Kenyon 1993; Liu and Sternberg 1995; Barr and Sternberg 1999; Yoda *et al.* 2000; Garcia *et al.* 2001; Lints and Emmons 2002; Carnell *et al.* 2005; Schindelman *et al.* 2006).

For species that display gonochorism, copulation is strictly essential for propagation. In contrast, the *C. elegans* self-fertilization mode of reproduction might relax selection on genes that are used in optimizing male mating behavior. This could contribute to the reduced ability of males to mate with normally behaving hermaphrodites. In this study, we asked why males of gonochoristic Caenorhabditis species are more proficient than their hermaphroditic relatives in performing the behavioral steps of copulation. We focused on spicule insertion behavior, since this step is the most difficult for *C. elegans* males to perform, and probably the major reason they fail to impregnate their hermaphrodite mates.

We found that males from the gonochoristic Caenorhabditis species 4, PB2801 and *C. remanei* species executed the various steps of mating similarly to the hermaphroditic species *C. elegans* and *C. briggsae*. However, the major difference between hermaphrodite–male and female–male couplings was that *C. remanei* and PB2801 males induced rapid paralysis in their virgin female mates. They also triggered the female vulval slit to widen, allowing instant spicule insertion. *C. elegans* and *C. briggsae* hermaphrodites were not affected this way during matings with their conspecific males or with gonochoristic males. Interestingly, *C. briggsae* males, but not *C. elegans* males, induced inactivity in virgin females of both gonochoristic species. We suggest that the nervous system of males from hermaphroditic and gonochoristic species are designed to copulate with nonmoving partners and that the nonoptimal mating efficiency that occurs in hermaphroditic species is due to the loss of male or hermaphrodite gene functions that induce paralysis in the hermaphrodites.

MATERIALS AND METHODS

Strains: Strains were propagated at 20° on NGM agar plates seeded with *Escherichia coli* OP50 (Brenner 1974). We used the laboratory strain *C. elegans* Bristol N2 containing the mutation *him-5(e1490).* The *him-5(e1490)* allele (Hodgkin *et al.* 1979), located on LG V, increases the rate of X nondisjunction during meiosis and consequently increases the frequency of spontaneous males from 0.2 to 30%. The male–female strain of *C. elegans* Bristol (N2) contains the *fog-2(q71)* allele, located on LG V (Schedl and Kimble 1988). We also used the *C. elegans* wild strains, Palo Alto CB4855 and Hawaii CB4856. For *C. briggsae,* we used the strains AF16 VT847 and PB826. For *C. remanei* and Caenorhabditis species 4, PB2801, we used the strains PB4641 and PB2801, respectively. PB4641 and PB2801 are 20 times inbred derivatives of *C. remanei* and Caenorhabditis species 4. To generate males in *C. briggsae* and non-*him-5* hermaphroditic *C. elegans*, late L4 hermaphrodites were heat-shocked at 30° for 5 hr and then shifted to 20°. Spontaneously generated males were then serially propagated with hermaphrodites to maintain androdioecious populations.

Laser ablations: Males were laser operated on agarose pads containing NaN_3 using standard protocols (Bargmann and Avery 1995). For gonad and M-cell ablations, newly hatched L1 animals of mixed sexes were anesthetized using pads that contain 1 mm NaN_3. Ablated animals were then separated by sex when they reached the L4 stage. For Pn.p ablations, L1/L2 animals were anesthetized using pads that contain 5 mm NaN_3. Males were then discarded at the L4 stage. For P9.p, P10.p, cloacal ganglia, and linker cell ablations, males were sexed using a dissecting microscope before being anesthetized on pads containing 10 mm NaN_3. P9.p, P10.p cells were ablated in early L3 males. Cloacal ganglia and linker cell ablations were conducted at mid-L4 stage. At this stage, the tail spike is partially retracted, and the linker cell is ~10–20 μm from the cloacal cavity.

Mating assays: From mixed-staged stocks of nematodes, 10–20 L4 males at the developmental stage of tail spike retraction and 10–20 L4 hermaphrodites or females at the stage where a defined clearing was formed in the vulval region ("the Christmas tree stage" of vulval development) were separated from their opposite sex and placed on separate NGM agar plates. Females and hermaphrodites were assayed for mating behavior 24, 48, and 72 hr later. To make the mating lawns, *E. coli* OP50 was grown overnight in LB media at 37° without aeration. A total of 1 ml of culture was concentrated to 10 μl and then spotted onto an NGM agar plate. The diameter of the bacterial lawn was ~5 mm. Ten 24-, 48-, or 72-hr adult females or hermaphrodites were placed onto the lawn using a platinum wire pick. Then the 24-hr male of interest (nonoperated or laser ablated) was placed onto the lawn.

We did not use older mating lawns since hermaphrodites and females behaved differently on 24 hr or older preformed mating lawns, as compared to freshly made ones. On older

lawns, some hermaphrodites and females crawled to the border of the bacterial lawn and sometimes stopped moving once they reached the edge. In contrast, on freshly made bacterial lawns, hermaphrodites and females continually moved around the lawn.

Great care was taken in manually transferring animals from one plate to another. Harsh manipulations sometime caused a male to immediately crawl off the mating lawn. In those cases, the wandering male was removed and another male was added. Mating observations lasted for 10 min or until the male inserted his spicules into a partner; timing started when the male was placed on the lawn. The number of partners that a male attempted to mate with during the observation window was recorded. If the male tail lost contact with a mate during the mating sequence, the concentration of moving hermaphrodites or females was high enough to allow the male to quickly contact a different mate, but if the male recontacted the same hermaphrodite or female, we still recorded that as a new mating encounter. Males and mated hermaphrodites/females were removed from the lawn after the observation was completed. A new hermaphrodite/female was then added to the lawn.

In the course of this study, we noted some interesting observations regarding *C. briggsae* and *C. remanei* mating behavior. *C. briggsae* AF16 and VT847 males appeared to have no ability to discriminate themselves from their mates. Between 12 to 24 hr after L4 molt, 95–100% of *C. briggsae* adult males contained a copulatory plug deposited over their excretory pore. This phenomenon is similar to what has been reported for the wild *C. elegans* strain AB2 (Gems and Riddle 2000). We observed that when the *C. briggsae* male's tail contacted any part of his own body, he immediately initiated mating behavior on himself. This would ultimately result in the insertion of his spicules, transferring of sperm, and depositing of a copulatory plug over his own excretory pore. During mating observations with *C. briggsae* males, if a male started to mate with himself, we gently prodded the male with a pick or an eyelash hair to disrupt the self-mating behavior. In contrast, males from the *C. briggsae* strain PB826 did not show this self-plugging behavior. Also, PB826 males did not show much behavioral interest in mating, either with hermaphrodites from their own strain or with conspecific hermaphrodites. Stocks of PB826 that contained males were serially maintained by setting up mating crosses consisting of five to six PB826 males and one 48- to 72-hr PB826 hermaphrodite. To maximize the probability that the males would contact the hermaphrodite, matings were conducted on plates that contained a 1-cm bacterial lawn.

We also noted that virgin *C. remanei* females were strongly attracted to copulating *C. remanei* males. When a *C. remanei* male induced behavioral inactivity in a mate, the other females would quickly crawl toward the pair and clump around the couple. During mating observations with *C. remanei*, we gently prodded females that moved toward the mating pair with a pick to keep them from disrupting copulation of the mating couple.

RESULTS

Caenorhabditis gonochoristic females behave differently from hermaphrodites during mating: We initiated this study to understand under what conditions the neuromuscular circuit that controls *C. elegans* spicule insertion is designed to function. Previous studies have shown that N2 *C. elegans* males insert their spicules into older, rather than younger, genetically paralyzed hermaphrodites (Garcia *et al.* 2001; Garcia and Sternberg 2003; Gruninger *et al.* 2006). However, the mutations that produce paralysis in hermaphrodites could additionally cause defective vulval musculature that disrupts the males' ability to insert their spicules. To address if males can insert their spicules into young wild-type hermaphrodites efficiently, we observed mating behavior of 10 24-hr virgin N2 males coupled to 24- and 72-hr virgin N2 hermaphrodites for 10 min or until the males inserted their spicules (Figure 1A). We found that the constant hermaphrodite movement caused males to lose contact easily with their mates before the mating sequence was completed. The inability of males to maintain contact led to a greater number of mating attempts with different hermaphrodites. On average, males attempted to mate with 7 ± 3 (mean $\pm$ SD) 24-hr hermaphrodites and 3 ± 2 (mean $\pm$ SD) 72-hr hermaphrodites during the 10 min of observation. Males contacted fewer 72-hr than 24-hr hermaphrodites ($P = 0.001$, Mann–Whitney test) because 90% of males inserted their spicules into the older virgins, whereas 10% of males inserted into the younger hermaphrodites ($P = 0.001$, Fischer's exact test).

One possible explanation for better spicule insertion into older hermaphrodites is that older hermaphrodites have depleted much of their self-sperm, which might trigger some mechanism to allow the vulva to be more accessible. To test this possibility, we observed matings between males and *fog-2(lf)* females. The *fog-2* mutation does not affect males, but inhibits sperm production in hermaphrodites, causing them to become reproductively female (Schedl and Kimble 1988). Results of matings between males and *fog-2(lf)* females were similar to males and hermaphrodites; males penetrated older females more easily than younger ones (Figure 1B). Therefore, the presence of sperm does not inhibit male spicule insertion into young hermaphrodites. Some other age-related factor, whether it is physiological or behavioral, affects how efficiently the vulva can be breached. Although not a central focus of this study, we additionally observed that young *fog-2(lf)* females did not appear to move away from males at the same frequency as young hermaphrodites. On average, males attempted to mate with 2 ± 1 (mean $\pm$ SD) 24-hr females ($P < 0.001$ *vs.* 24-hr hermaphrodites, Mann–Whitney test) during the observation window. This is consistent with reported observations that the *fog-2(lf)* mutation alters behaviors in females that promote copulation (G. A. Kleemann, personal communication; Lipton *et al.* 2004) and that sperm-depleted wild-type hermaphrodites are more behaviorally receptive to males than their self-sperm-containing cohorts (Kleemann and Basolo 2007).

Inefficient spicule insertion could be due to relaxed selection for genes that regulate motor functions used for mating behavior in hermaphroditic species. To determine if *C. elegans* males lost some ability for mating, we asked how spicule insertion behavior of *C. elegans* compares with males of the related Caenorhabditis

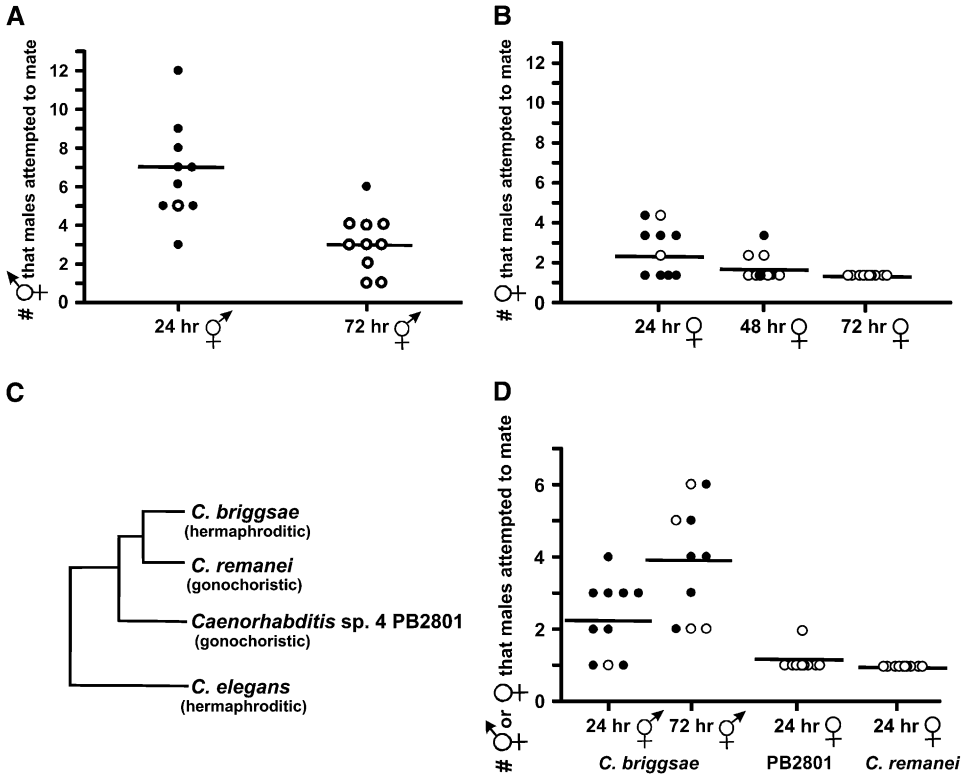

FIGURE 1.—Distribution of spicule insertion ability in different virgin Caenorhabditis males. Each circle represents the behavior of a single male. Males that inserted their spicules into their mates are represented as an open circle. Those that did not insert their spicules within the 10-min assay window are represented as solid circles. The number of mates with which the males attempted to mate are listed on the vertical axis. The horizontal bar denotes the average number of mates with which the males attempted to mate. $n = 10$ males for each distribution. (A) *C. elegans* males mated with 24- and 72-hr virgin hermaphrodites. (B) *C. elegans* males mated with 24-, 48-, and 72-hr virgin *fog-2(q71)* virgin females. (C) Phylogeny of Caenorhabditis species adapted from KIONTE *et al.* (2004). (D) *C. briggsae* males mated with 24- and 72-hr virgin *C. briggsae* hermaphrodites. PB2801 males mated with 24-hr virgin PB2801 females. *C. remanei* males mated with 24-hr virgin *C. remanei* females.

species 4, PB2801, *C. remanei* and *C. briggsae*. Phylogenetic relationships between these nematodes have been determined through analyzing *par-6*, *pkc-3*, RNA polymerase II, and large and small subunit ribosomal RNA sequences. Like *C. elegans*, *C. briggsae* is a hermaphroditic nematode. However, it is more closely related to *C. remanei* and to PB2801 than to its hermaphrodite relative (Figure 1C), indicating that hermaphroditism had evolved independently in the two species (CHO *et al.* 2004; KIONTKE *et al.* 2004). When spicule insertion behavior was observed in *C. briggsae* AF16 males coupled to 24- and 72-hr AF16 *C. briggsae* hermaphrodites, males had difficulty inserting into both ages of hermaphrodites (Figure 1D). The efficiency of inserting into 72-hr *C. briggsae* hermaphrodites was different when compared to *C. elegans* males inserting into their 72-hr hermaphrodites (4/10 *C. briggsae* AF16 hermaphrodites were penetrated, whereas 9/10 *C. elegans* N2 hermaphrodites were penetrated; $P < 0.001$, Fischer's exact test), suggesting that behavioral changes that occur in older *C. elegans* might be specific to their physiology. In contrast, *C. remanei* and PB2801 males instantaneously inserted their spicules into their own 24-hr virgin females (Figure 1D). This demonstrated that, unlike *C. elegans* and *C. briggsae*, males of the two gonochoristic species are more efficient at penetrating and impregnating their mates.

C. remanei, *C. briggsae*, and *C. elegans* males have been shown to transfer sperm into different heterospecific partners after extended periods of mating (HILL and L'HERNAULT 2001). This suggests that Caenorhabditis females and hermaphrodites display common physical and chemical cues that can be recognized by male tail sensilla. Since *C. remanei* and PB2801 males were efficient at penetrating their own 24-hr females, we asked if they could penetrate young heterospecific mates with similar proficiency. We found that, similar to *C. elegans* and *C. briggsae* males, males of the two gonochoristic species do not insert their spicules very efficiently into young hermaphrodites, although they can insert their spicules into females of their heterospecific relatives (Table 1). Surprisingly, although *C. briggsae* AF16 males do not insert their spicules into their own hermaphrodites easily, they instantly penetrated both PB2801 and *C. remanei* females, indicating that the inability to insert their spicules into their conspecific mate is partly due to the *C. briggsae* hermaphrodite (Table 1). *C. briggsae* AF16 was originally isolated from Ahmedabad, India (FODOR *et al.* 1983). To determine how males from different *C. briggsae* strains behave, we analyzed the spicule insertion behavior of males from the *C. briggsae* strain VT847, isolated from Hawaii, and from the *C. briggsae* strain PB826, isolated from Ohio (KIONTKE and SUDHAUS 2006). Similar to AF16 males, we found that VT847 males inserted their spicules into both PB2801and *C. remanei* females better than into *C. briggsae* AF16 or *C. elegans* N2 hermaphrodites (Table 1). In contrast, *C. briggsae* PB826 males did not insert their spicules very efficiently into any of the hermaphrodites or females tested. This was because none of the observed 40 PB826 males showed interest in executing mating behavior when their tails contacted their mates. However, two PB2801 and *C. remanei* females and one *C. elegans*

TABLE 1

Efficiency of spicule insertion during heterospecific matings

Males	No. of males that inserted their spicules into 24-hr mates			
	C. briggsae AF16 hermaphrodites	*C. remanei* females	PB2801 females	*C. elegans* N2 hermaphrodites
C. briggsae				
AF16 (Ahmedabad, India)	1/10[a]	9/10	6/10	0/10
VT847 (Hawaii)	1/10	8/10	7/10	1/10
PB826 (Ohio)	0/10	2/10[b]	2/10[b]	0/10
C. remanei				
PB2801	0/10	10/10[a]	9/10	0/10
	0/10	9/10	10/10[a]	0/10
C. elegans				
N2(Bristol, UK)	0/10	3/10	1/10	1/10[a]
CB4856 (Hawaii)	2/10	1/10	0/10	0/10
CB4855 (California)	0/10	3/10	1/10	2/10

[a] Data were taken from Figure 1.

[b] PB826 males that inserted their spicules also induced behavioral inactivity in the *C. remanei* and PB2801 females.

hermaphrodite were penetrated during the observation period (Table 1).

Locomotor activity of young hermaphrodites is one potential factor that reduces the efficiency of *C. elegans* and *C. briggsae* male spicule insertion behavior. During matings, young *C. elegans* and *C. briggsae* hermaphrodites continue to move around the bacterial lawn as males try to breach the vulva with their spicules. The hermaphrodite movements generally cause the male tail to move off the vulva, and in many instances the male will also lose contact with the hermaphrodite (Kleemann and Basolo 2007). In contrast, we noted that when the cloacal region of *C. remanei* PB2801 and *C. briggsae* AF16 VT847 and also PB826 males contacted the virgin female vulva, the males immediately insert their spicules, and the females simultaneously stop locomotion, defecation, and reduce pharyngeal pumping behavior (supplemental video S1 at http://www.genetics.org/supplemental/). In *C. remanei*, this striking behavioral inactivity continues for ~64 ± 38 sec (mean ± SD $n = 15$ females), which is sufficient time for the male to transfer sperm and deposit a copulatory plug over the vulva. *C. remanei* and PB2801 virgin females do not display this soporific behavior when *C. elegans* N2 males attempt to insert; instead, the virgin females behave like *C. elegans* and *C. briggsae* hermaphrodites by moving off the copulating male (supplemental video S2 at http://www.genetics.org/supplemental/). Similarly, *C. elegans* and *C. briggsae* hermaphrodites do not display this behavioral inactivity during matings with *C. briggsae*, *C. remanei*, and PB2801 males. This demonstrates that virgin hermaphrodites and females behaviorally respond to males differently.

Since the 1950s, *C. elegans* Bristol N2 have been grown in the laboratory under various culture methods (propagation in liquid culture containing axenic media, liquid monoxenic media containing *E. coli*, and surface propagation on *E. coli*-containing NGM plates) (Hodgkin and Doniach 1997). The lack of selection for mating due to these laboratory culture conditions could contribute to the loss of certain N2 male behaviors. We tested *C. elegans* males from the Palo Alto CB4855 isolate and the Hawaii CB4856 isolate to determine if males of other *C. elegans* strains can induce the soporific behavior in virgin gonochoristic females. Unlike N2 males, males from both of these *C. elegans* strains have the ability to deposit a copulatory plug over the vulva after sperm transfer. In addition, Palo Alto CB4855 males have been reported to sire progeny up to 12 days of adulthood, whereas N2 males are impotent after 4 days (Hodgkin and Doniach 1997). However, despite the additional mating attributes that these males display, we found that CB4855 and CB4856 males do not induce behavioral inactivity in *C. remanei* and PB2801 virgin females, nor do they insert their spicules into 24-hr adult *C. briggsae* AF16 or N2 hermaphrodites better than N2 males (Table 1). Thus *C. elegans* males do not display the ability to modify their mate's behavior to promote spicule insertion.

Contact between the *C.remanei* female vulva and the male tail induces female behavioral inactivity: To determine how gonochoristic females respond to males during mating, we narrowed our analysis to determining what aspects of *C. remanei* male mating behavior trigger *C. remanei* virgin females to attenuate locomotion, defecation, and pharyngeal pumping. *C. remanei* virgin females display the soporific behavior when the male contacts the vulva, inserts his spicules, and ejaculates. To determine if the female vulva was required for the soporific behavior, we ablated the Pn.p cells: P4.p, P5.p, P6.p, P7.p, P8.p, and P9.p in 10 late L1 to mid-L2

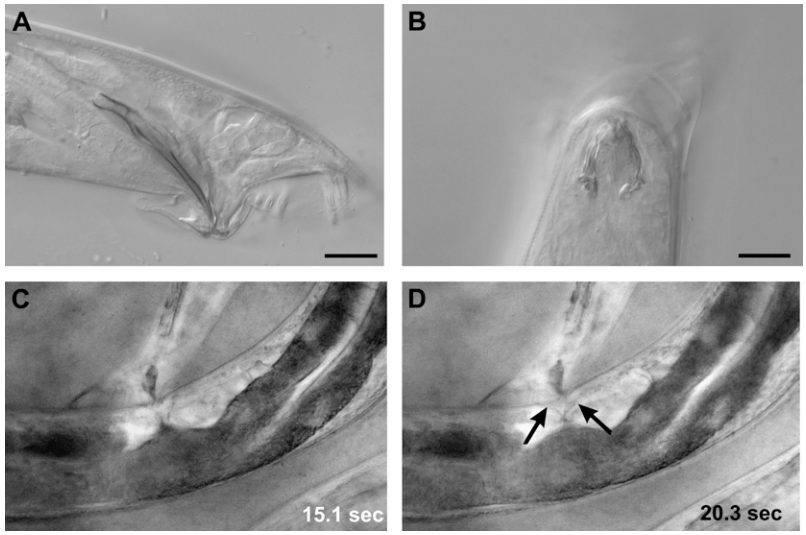

FIGURE 2.—M-cell-ablated male. (A) DIC photomicrograph of the left lateral region of an adult *C. remanei* male tail. Anterior to the left, dorsal to the top. Bar, 10 μm. (B) DIC photomicrograph of a ventral view of the tail region of an M-cell-ablated *C. remanei* adult male. Spicule morphology is abnormal due to the absence of spicule muscles. Posterior to the top. Bar, 10 μm. (C and D) Extracted images from supplemental video S3 (at http://www.genetics.org/supplemental/) of an M-cell-ablated *C. remanei* adult male attempting to mate with a *C. remanei* female. Numbers at the bottom right are time indicators for the video. Image in D occurs 5 sec after the image in C. Arrows in D point to the anterior and posterior sides of the vulval slit. In C, the vulva slit is closed; in D, the male induces the vulval opening to widen.

females. In *C. elegans*, this ablation removes not only the putative vulval precursor cells, but also other Pn.p cells that might adopt a vulval precursor fate (KIMBLE 1981). We found that 0/10 vulvaless females displayed behavioral inactivity as the males continually moved backward along the female.

To rule out the possibility that ablating nonvulval Pn.p cells might indirectly affect the female soporific behavior, we tested the requirement for the vulva in a different way; we ablated the somatic gonad, which is necessary for the Pn.p cells to adopt a vulval fate. Z1, Z2, Z3, and Z4 are gonadal precursor cells in newly hatched *C. elegans* larvae. Descendants of Z1 and Z4 give rise to the somatic gonad, and descendants of Z2 and Z3 give rise to the germline (KIMBLE and HIRSH 1979). Ablating the equivalent cells of Z1, Z2, Z3, and Z4 in five L1 *C. remanei* females results in adults that do not contain a somatic gonad, a germline, or vulval tissue, similar to *C. elegans*. In addition, 0/5 operated animals displayed behavioral inactivity as *C. remanei* males attempted to mate with them. When Z2 and Z3 were ablated in two *C. remanei* females, operated adult animals contained a somatic gonad and vulval tissue, but no germline. In contrast to Pn.p-and gonad-ablated females, both germline-ablated females displayed behavioral inactivity when males contacted their vulva. These experiments suggest that the *C. remanei* vulva can act as a sensor to modify female behavior. However, since the *C. remanei* female does not display the soporific effect when the cloaca of *C. elegans* males touches the vulva, the *C. remanei* vulva must sense something more specific than mechanical contact.

Neurons associated with the *C.remanei* male cloaca are required to induce the soporific behavior in females: The soporific behavior that virgin *C. remanei* females display occurs when males contact the vulva, insert their spicules, and transfer sperm. Since these male mating substeps occur very quickly, we laser ablated various cells in *C. remanei* males that, on the basis of analogy to *C. elegans* male mating behavior, should uncouple vulva location, spicule insertion, and ejaculation behavior (Figures 2 and 3). We then asked if the operated males could attenuate locomotion, pharyngeal pumping, and defecation for at least 20 sec in their female mates.

We first removed the spicule protractor muscles to ask if complete spicule insertion triggers female inactivity during normal mating. To do this, the M cell was laser ablated in L1 males. In *C. elegans*, M-cell-ablated males lack the M-lineage-derived body wall muscles, coelomocytes, and all sex muscles, including the spicule muscles. During normal development, the spicule muscles are required for the copulatory spicules to elongate into their characteristic blade-like appearance (Figure 2A). Without these muscles, the spicules do not elongate and consequently form stunted structures (SULSTON *et al.* 1980). Ablation of the M cell in *C. remanei* resulted in males that lack their sex muscles and also develop crumpled spicules (Figure 2B). During mating behavior, the M-cell-ablated males show variable reduction in the efficiency of backing and turning behavior, but all of the operated males located and stopped at the vulva on the first contact. Because the males lack the muscles that control spicule movements, none of the observed males inserted their crumpled spicules; however, 90% of operated males induced the *C. remanei* females to display the soporific behavior (Table 2). Interestingly, although locomotion, pharyngeal pumping, and defecation were reduced, contact between the male cloaca and the vulva caused the vulva muscles to contract periodically in all paralyzed females (Figure 2, C and D) (supplemental video S3 at http://www.genetics.org/supplemental/).

Since the M-cell ablation result demonstrated that spicule movements were not necessary to induce the soporific behavior, we asked if sustained vulval contact between the male cloaca and the female vulva was

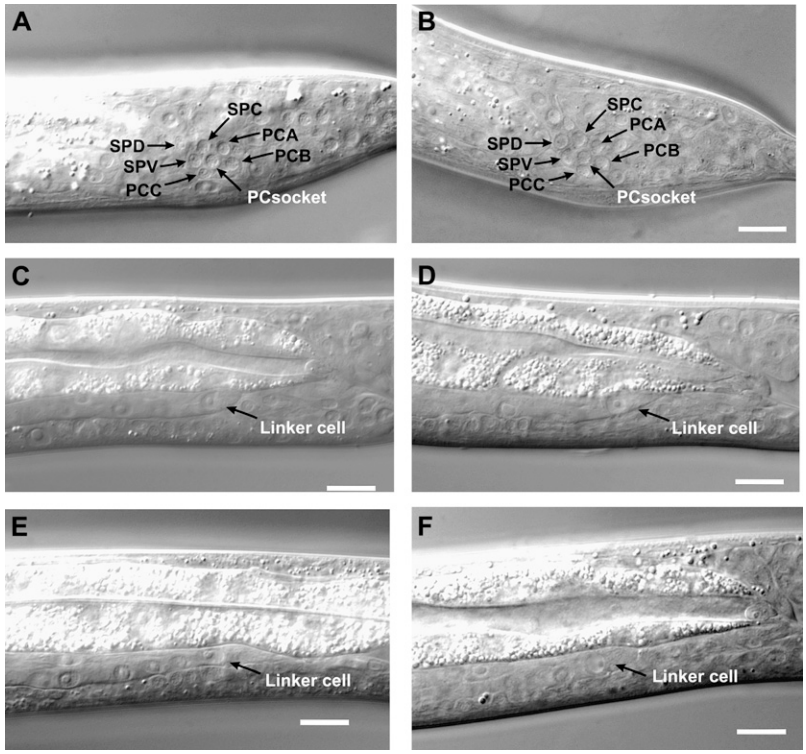

Figure 3.—Positions of cloacal ganglia cells and linker cell in Caenorhabditis males. Anterior to the left, dorsal to the top. Bars, 10 μm. DIC photomicrographs of the left lateral cloacal ganglia region of (A) a mid-L4 *C. elegans* male tail and (B) a mid-L4 *C. remanei* male tail. In C–E, DIC photomicrographs of the posterior ventral region of (C) *C. elegans*, (D) *C. remanei*, (E) PB2801, and (F) *C. briggsae*.

required. In *C. elegans*, the male senses the vulva using the hook sensillum neurons HOA and HOB and the postcloacal sensilla (p.c.s.) neurons PCA, PCB, and PCC. We ablated the blast cells P9.p and P10.p in L3 *C. remanei* males, which in *C. elegans* results in the loss of the HOA and HOB neurons in adults. Similar to *C. elegans*, all P9.p- and P10.p-ablated *C. remanei* males fail to locate the vulva efficiently; however, 90% of the males induced the soporific behavior either when their cloaca transiently passed over the vulva or when they inserted their spicules (Table 2). When the cells analogous to the *C. elegans* PCA, PCB, and PCC were ablated (Figure 3, A and B), all operated males hesitated in the general region of the vulva; however, none of the males positioned their cloaca over the vulval slit for longer than 2–3 sec, and, consequently, very few of the males inserted their spicules (Table 2). This behavioral defect is similar to that displayed by PCA-, PCB-, and PCC-ablated *C. elegans* males (Liu and Sternberg 1995), indicating that these *C. remanei* cells have functions in sensing the vulva similar to those of the *C. elegans* p.c.s. neurons. Similarly, like the P9.p- and P10.p-ablated males, 7/10 PCA-, PCB-, and PCC-ablated *C. remanei* males also induced inactivity in the females when their cloaca transiently contacted the vulva.

In *C. elegans*, the p.c.s. and hook neurons not only function redundantly to sense the vulva (Liu and Sternberg 1995), but also act to initiate spicule insertion behavior (Garcia *et al.* 2001). When the spicules partially breach the vulval slit, the SPC motor neurons then promote complete spicule protraction and ejaculation. In *C. remanei*, ablating the p.c.s. and hook neurons did not strongly reduce the males' ability to induce the soporific affect. However, ablating the p.c.s. neurons did reduce spicule insertion and ejaculation, suggesting that the *C. remanei* SPC and the p.c.s. neurons might share some redundant functions. To test if these neurons might have some functional overlap during mating, we ablated the SPC cells alone and with the p.c.s. neurons. In contrast to *C. elegans*, where the SPC motor neurons are essential for complete spicule penetration, 10/11 SPC-ablated *C. remanei* males induced the soporific behavior and inserted their spicules completely, but of the 10 males that inserted, only 1 transferred sperm (Table 2). Thus, the SPC cells in both *C. remanei* and *C. elegans* are required for sperm transfer; however, their functions in spicule insertion behavior have diverged between the two species. When the *C. remanei* SPC cells were ablated with the PCA, PCB, and PCC cells, 0/10 males induced the soporific effect or inserted their spicules (Table 2). Also, the females would crawl back and forth trying to shift the males off their cuticle, reminiscent of how 24-hr *C. briggsae* and *C. elegans* adult hermaphrodites behaved with males. Since 70 and 91% of p.c.s. and SPC-ablated males, respectively, induced the soporific effect in comparison to 0% of the combination-ablated males, the SPC and the p.c.s. neurons in intact *C. remanei* males must act redundantly to trigger some process that ultimately results in the attenuation of female behavior.

A soporific-inducing factor from *C. remanei*, PB2801, and *C.briggsae* males requires a connection between the somatic gonad and the cloacal opening: Partial EM reconstructions of the *C. elegans* male indicate that the

TABLE 2

Ablation of *C. remanei* male structures that affect inactivity in females

Ablation	Gross mating defects[a]	No. of males that induced female paralysis
None	None	10/10
M cell in L1 males. Removed: sex muscles, M-derived coelomocytes, M-derived body-wall muscles.	Inconsistent ventral turns. No spicule insertion.	9/10
P9.p and P10.p in early L3. Removed: hook structural cells, HOA, HOB, and PVZ.	Males passed over the vulva four or more times before stopping at the vulva; 6/10 inserted their spicules and ejaculated.	9/10 (5/10 when they transiently passed over the vulva; 4/10 when they inserted their spicules).
Y.prppd(l/r), Y.prpa(l/r), and B.a(l/r)paaa in late L4. Removed: PCA, PCB, and PCC neurons.	Males could not position their cloaca over the vulval slit; 2/10 males inserted their spicules, but did not ejaculate.	7/10 (5/10 when they transiently passed over the vulva; 2/10 when they inserted their spicules).
B.a(l/r)paap in late L4. Removed the SPC neurons.	Ten of 11 males inserted their spicules; only 1/10 transferred sperm.	10/11
Y.prppd(l/r), Y.prpa(l/r), B.a(l/r)paaa, and B.a(l/r)paap in late L4. Removed: PCA, PCB, PCC, and SPC.	Males could not position their cloaca over the vulval slit, insert spicules, or transfer sperm.	0/10
Z1, Z2, Z3, and Z4 in early L1. Removed: somatic gonad and germline.	Males behaved like PCA-, PCB-, PCC-, and SPC-ablated males.	0/10
Z2 and Z3 in early L1. Removed the germline.	Males transferred spermless seminal fluid and deposited a copulatory plug.	9/10
Linker cell in mid-L4. Somatic gonad does not connect to proctodeum.	Males behaved like PCA-, PCB-, PCC-, and SPC-ablated males.	0/10

[a] Observations were cursory.

p.c.s. neurons PCB and PCC and the spicule neuron SPC also synapse the proximal somatic gonad (S. W. Emmons, D. H. Hall and M. Xu, personal communication; http://www.wormatlas.org). The relevance of these connections has not been determined; however, they might be used to coordinate vulva location and spicule insertion behavior with ejaculation (Gower *et al.* 2005). Although *C. elegans* males do not induce the soporific effect, the connections between these neurons and the somatic gonad made us ask if, in *C. remanei*, the gonad might act with the p.c.s. and SPC neurons to induce the soporific behavior in females.

When Z1, Z2, Z3, and Z4 of *C. remanei* males were ablated, none of the adults contained a somatic gonad or a germline, but development of other male structures appeared to be superficially normal. Ablation of the gonad eliminated the ability of all operated males to induce the soporific effect (Table 2). In addition, none of the males were able to hold their position on the vulva for >5 sec or to insert their spicules, a defect that is similar to that caused by ablating the p.c.s. and the SPC neurons.

Since the gonad was required, we then asked if the germline provided any contribution to the soporific effect. Z2- and Z3-ablated males lacked a germline, but they still contained the somatic gonad, which is connected to the cloaca. A total of 9/10 operated males induced female inactivity, inserted their spicules, transferred spermless seminal fluid, and deposited a copulatory plug. This indicated that the germline is not necessary for the males to sedate the females.

We then asked if a connection between the proximal somatic gonad and cloaca was required for the soporific effect. The proximal end of the male somatic gonad consists of the vas deferens, which is the conduit between the seminal vesicle and the cloacal cavity; the cloaca opens to the environment. In *C. elegans*, these somatic gonadal cells are guided to the cloacal cavity via the linker cell (Figure 3C). When the linker cell reaches the cloaca, cells associated with the cloacal cavity kill the linker cell, thus facilitating the lumen of vas deferens in connecting with the cloacal opening (Kimble and Hirsh 1979; Sulston *et al.* 1980). If the linker cell is laser ablated before it reaches the cloaca, then no connection is made between the vas deferens and the outside. In mid-L4 males, when the tail spike begins retraction, we ablated the cell that shares similar morphology to the *C. elegans* linker cell (Figure 3, C and D). During mating, all operated males, despite containing a somatic gonad and germline, behaved similarly to Z1-, Z2-, Z3-, and Z4-ablated males; all linker-cell-ablated males failed to sedate their mates, maintain their position over the vulva for >5 sec, or insert their spicules (Table 2) (supplemental video S4 at http://www.genetics.org/supplemental/). Taken together, these results suggest that, prior to spicule insertion, the somatic gonad

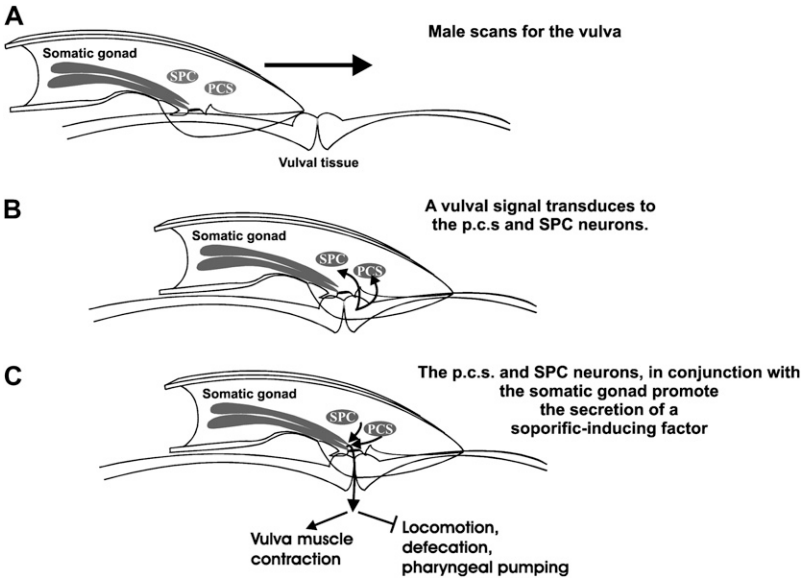

FIGURE 4.—One possible model of how gonochoristic males immobilize their mates. (A) The male moves backward along the female cuticle scanning for the vulva. The female is also moving during this period. (B) The hook and postcloacal sensilla neurons sense the vulva and signal backward locomotion to stop. (C) The vulva also signals the postcloacal sensilla neurons, the SPC neurons, and the somatic gonad to promote the secretion of a substance that induces vulval muscle contraction and behavioral inactivity in the female.

or some substance that it might release requires access to the cloacal opening when the male cloaca contacts the female vulval lips.

Since PB2801 and *C. briggsae* males also induce the soporific effect in gonochoristic females, we ablated the linker cell in PB2801 and *C. briggsae* L4 males (Figure 3, E and F) and then asked if the operated adults could still induce behavioral inactivity in virgin PB2801 females. We found that 0/10 PB2801 and 0/10 *C. briggsae* males can induce the soporific effect. This suggests that the cellular mechanism used to sedate virgin females might be common to *C. remanei*, PB2801, and *C. briggsae* males.

Components of the male germline suppress the soporific behavior in nonvirgin females: We casually noted that *C. remanei* and PB2801 females that contain a mating plug and eggs in their uterus behaved like *C. elegans* and *C. briggsae* hermaphrodites during male mating attempts. To test this more carefully, we mated 10 virgin *C. remanei* females with males. We allowed the males to transfer sperm and then deposit a mating plug. One hour later, we retested the nonvirgin females with virgin males. We found that only 1/10 nonvirgin female displayed the soporific effect. In parallel, we mated 10 virgin *C. remanei* females with germline-ablated males. Each of the germline-ablated *C. remanei* males ($n = 10$) induced behavioral inactivity, transferred spermless fluid from their somatic gonad, and then deposited a copulatory plug. We then retested the females with virgin *C. remanei* nonoperated males. Interestingly, although all 10 females had a copulatory plug covering their vulva, they all displayed behavioral inactivity when males passed over or around the plug (supplemental video S5 at http://www.genetics.org/supplemental/). Eventually, the males displaced the copulatory plug, presumably via their hook structure, inserted their spicules, and transferred sperm. Therefore, some component of the germline, perhaps sperm, can change the physiology of females so that they either become refractive to or inhibit the production of the soporific factor produced by the male somatic gonad.

DISCUSSION

In the species *C. remanei* and in the Caenorhabditis species 4, PB2801, male–female copulation is essential for propagating the species. This necessity predicts that various requirements must be fulfilled to ensure that mating behavior is successful. One requirement is to bring individuals of opposite genders in close proximity to initiate copulation. In *C. remanei* and *C. elegans*, this is accomplished by secreted substance(s) that attract and retain males close to females and hermaphrodites (CHASNOV and CHOW 2002; SIMON and STERNBERG 2002; LIPTON *et al.* 2004). Another requirement is for the mating couple to remain in contact until sperm transfer is complete. Our study provides a mechanism that explains a major behavioral difference between hermaphrodites and gonochoristic females during this stage of mating (Figure 4).

The behavioral steps of copulation leading up to spicule insertion are similar among the males of the Caenorhabditis genus used in this study. However, upon vulval contact, copulation behaviors between the species differ. For *C. elegans* and *C. briggsae*, males repetitively prod the vulval slit of virgin hermaphrodites with their spicules before they completely insert. Depending on the age and the locomotor activity of the hermaphrodite, duration of spicule prodding can range from seconds to minutes. In contrast, when *C. remanei* and PB2801 males contact the vulva, their female mates stop moving, and spicule insertion occurs instantly.

We found that, upon contact with the vulva, a factor associated with gonochoristic males rapidly immobilizes the virgin female, while simultaneously inducing the

vulval opening to widen. Once females are impregnated, they become refractive to the soporific-inducing factor. The soporific-inducing effect is remarkable, considering that topical exposure to the vulva causes multiple muscles used in locomotion, pharyngeal pumping, and defecation behaviors to become simultaneously relaxed, whereas genital muscles that are connected to the vulva contract. This behavior is superficially similar to the behavior that *C. elegans* and other Caenorhabditis males display when they transfer sperm. During sperm transfer, the spicule muscles remain contracted, whereas muscles involved in pharyngeal pumping, locomotion, and defecation are relaxed (Liu and Sternberg 1995; Gruninger *et al.* 2006). Possibly, mating-induced inactivity in females and ejaculation behavior in males might be controlled by the same underlying mechanism.

The male soporific-inducing factor might be a structural component of the male cloaca or a substance that is released from the cloacal opening upon vulval contact. We favor the latter since the effectiveness of the soporific-inducing factor requires the somatic gonad, which produces seminal fluid; the postcloacal sensilla neurons, which facilitate sensing the vulva and inducing spicule insertion; and the SPC neurons, which facilitate the transfer of seminal fluid and sperm from the gonad. Our experiments do not differentiate between whether the soporific-inducing factor is directly secreted by the SPC and the p.c.s. neurons or by the somatic gonad. Nothing is known about the development of the male SPC and the p.c.s. neurons in the gonochoristic Caenorhabditis species. In *C. elegans*, the somatic gonad is not required for SPC and the p.c.s. functions during mating behavior. However, in *C. remanei*, laser ablation of the somatic gonad mimics laser damage to the p.c.s. and the SPC neurons. It is possible that the SPC and the p.c.s neurons directly secrete the soporific-inducing factor through the cloacal cuticle and out the cloacal opening, but this function requires interactions with the somatic gonad. An alternative possibility is that, upon contact with the vulva, the p.c.s. and SPC neurons trigger the somatic gonad directly to release the soporific-inducing factor through the gonadal–cloacal junction and out the cloacal opening (Figure 4). In *Drosophila melanogaster*, peptides found in male seminal fluid have been shown to affect behavior, physiology, and gene expression of impregnated females (Lawniczak and Begun 2004; McGraw *et al.* 2004). Therefore, substances in nematode pre-ejaculation fluid might similarly cause behavioral changes in virgin female nematodes.

The hermaphroditic *C. elegans* and *C. briggsae* species are believed to have evolved independently from gonochoristic ancestors (Braendle and Felix 2006). For *C. briggsae*, this is consistent with our observation that *C. briggsae* males can induce behavioral inactivity in both *C. remanei* and PB2801 females. The putative soporific-inducing factor must be vestigial in *C. briggsae* males, since *C. briggsae* hermaphrodites do not respond to their males in the same way as *C. remanei* or PB2801 females do. Presumably, over evolutionary time, the hermaphroditic mode of reproduction either relaxed the maintenance of genes used in transducing the effects of the male factor or selected for activated alleles of genes that normally inhibit the effects of the male factor. In *C. elegans*, neither hermaphrodite nor male displays any obvious aspect of this behavior. By analogy with *C. briggsae*, either one or both genders lost this behavioral trait. Because the genome of *C. elegans* is more divergent relative to *C. briggsae*, *C. remanei*, and PB2801, it is possible that the gonochoristic ancestor of *C. elegans* might have used a different mechanism to promote copulation. However, since *C. elegans* males can execute all steps of mating behavior with heterospecific partners (Hill and L'Hernault 2001), it is likely that its ancestors probably also shared the ability to induce and respond to the same type of soporific factor.

Mating behavior of *C. elegans* males is not efficient with young moving hermaphrodites. The probable reason is that the *C. elegans* male nervous system, like that of males of the other Caenorhabditis species, was not designed to mate with mobile partners. Additionally, spicule insertion is difficult for *C. elegans* males since the young hermaphrodites do not actively facilitate spicule penetration. *C. elegans* males do not induce behavioral inactivity in young hermaphrodites, but are more efficient in inserting their spicules and transferring sperm into older partners. The 72-hr hermaphrodites used in this study are probably an extreme case of what is the most efficient copulation partner for *C. elegans* males, since cross-fertilized hermaphrodites at that age are not as fertile as younger cross-fertilized hermaphrodites (L. R. Garcia, unpublished observation). The age of the hermaphrodite mate, after L4 molt, that is optimized for copulation and fecundity should be between 24 and 72 hr. The reasons for the correlation between age and copulation efficiency is not obvious. The quantity of self-sperm is likely involved (Kleemann and Basolo 2007), but perhaps in addition, the mechanism that induces mating receptivity in gonochoristic virgin females partially functions constitutively in older *C. elegans* hermaphrodites.

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