

The species, sex, and stage specificity of a *Caenorhabditis* sex pheromone

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Four species in the ELEGANS group of the subgenus *Caenorhabditis* are distinguished by two very different mating systems: androdioecy in *C. elegans* and *Caenorhabditis briggsae* with males and self-fertilizing hermaphrodites and dioecy in *Caenorhabditis remanei* and *Caenorhabditis* sp. strain CB5161 with males and females. Using chemotaxis assays, we demonstrate that females secrete a potent sex pheromone that attracts males from a distance, whereas hermaphrodites do not. The female sex pheromone is not species-specific, with males of all four species attracted to both the *C. remanei* and *Caenorhabditis* sp. female sex pheromones. The pheromone is, however, sex-specific, with only females secreting the pheromone and attracting only males. Furthermore, the sex pheromone is stage-specific, with female secretion and male detection of the pheromone beginning near adulthood. Females lose their attractiveness immediately after mating but regain it several hours after mating ceases. Finally, the female somatic gonad is required for sex-pheromone production, and the male-specific cephalic neurons (CEM) are required for male response.

chemotaxis | cephalic neuron | sex attractant | nematode mating

Direct observations show that the mating efficiency of the androdioecious species *Caenorhabditis elegans* is poor compared with the related dioecious species *Caenorhabditis remanei* and that both *C. remanei* and *C. elegans* males find *C. remanei* females more attractive than *C. elegans* hermaphrodites (1). Perhaps during evolution *C. elegans* hermaphrodites lost the ability to secrete a sex pheromone that is still secreted by *C. remanei* females and this pheromone still attracts *C. elegans* males. The focus of the present study is to definitively determine the presence of a sex pheromone in dioecious *Caenorhabditis* that is absent in related hermaphroditic species.

Although *C. elegans* is a well-studied organism, it is yet debatable whether there is a true sex pheromone in this species, although apparently males can sense a hermaphrodite-derived chemical (2, 3). If hermaphrodites do indeed secrete a true sex pheromone, it is surprising that males do not rapidly and consistently chemotax to hermaphrodites, because *C. elegans* is capable of chemotaxing to both water-soluble and volatile chemicals (4). In fact, detection of sex pheromones secreted by other nematode species has been fairly straightforward (5), with the secreting female commonly establishing a gradient of attractant readily followed by conspecific males.

Females, in contrast to self-fertilizing hermaphrodites, must mate with males to reproduce, and natural selection should favor those females that do so rapidly and consistently after reaching adulthood. Here, we demonstrate that females from the dioecious species *C. remanei* and *Caenorhabditis* sp. strain CB5161 attract conspecific males from a distance, whereas hermaphrodites from the androdioecious species *C. elegans* and *Caenorhabditis briggsae* do not. We then obtain, by soaking females, a supernatant solution that can attract males from a distance. Using this solution and standard chemotaxis assays (4), we explore the species, sex, and stage specificity of the *Caenorhabditis* sex pheromone.

Results

Females Attract Conspecific Males. Individual virgin females and hermaphrodites were assayed for their ability to attract conspecific males. Results are summarized in Fig. 1, for which the chemotaxis index (CI) is defined as the difference between the number of worms scored on the test and the control spots, divided by the total number of animals tested. Evidently, females from the dioecious species *C. remanei* and *Caenorhabditis* sp. attract conspecific males from a distance, whereas no evidence for a similar potent attraction is observed for the hermaphroditic species (*C. elegans* and *C. briggsae*).

Only Females Produce Attractant, and Only Males Respond. Supernatant solutions obtained from soaking of virgin *C. remanei* (Fig. 2a) and *Caenorhabditis* sp. (Fig. 2b) females were assayed for pheromone activity. Males of all four species are attracted to both the *C. remanei* and *Caenorhabditis* sp. pheromones. Experiments in which the *C. remanei* pheromone was placed on the Petri dish cover instead of the agar surface still attracted *C. remanei* males (200 males tested, $CI = 0.87 \pm 0.02$), indicating that the pheromone is volatile and can be sensed through the air.

To determine whether production of, and response to, the attractant is sex-specific, supernatant solutions were obtained from overnight soaking of both sexes of *C. elegans* and *C. remanei* to obtain four different supernatant solutions. The results are presented succinctly by using the $CI \pm SEM$ in Table 1. The supernatant solutions obtained from *C. elegans* males, *C. elegans* hermaphrodites, and *C. remanei* males have only weak or no attraction. In contrast, the supernatant from *C. remanei* females strongly attracts *C. remanei* and *C. elegans* males.

To test further for the possibility that *C. elegans* hermaphrodites secrete both attractive and repulsive signals, we have mixed supernatant solutions obtained from *C. remanei* females and *C. elegans* hermaphrodites in a 1:1 ratio. Both *C. remanei* and *C. elegans* males were still attracted to the mixed-supernatant solution (*C. remanei* males had a CI of 0.96 ± 0.02 versus 0.97 ± 0.02 for pheromone mixed with M9; *C. elegans* males had an identical CI of 0.73 ± 0.04 in test and control experiments), indicating the absence of any *C. elegans* hermaphrodite secretions that can significantly degrade the attractiveness of the *Caenorhabditis* sex pheromone or repel males.

Plausibly, hermaphrodites secrete pheromone only under very specific environmental or physical conditions. We have tested *C. elegans* hermaphrodite supernatant obtained under the following conditions and from the following strains and have found no significant attraction: hermaphrodites starved for 1 or 2 days;

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Abbreviations: CI, chemotaxis index; CEM, male-specific cephalic neuron; CEP, cephalic neuron.

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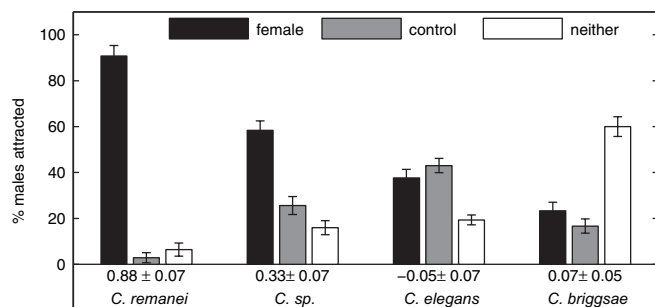


Fig. 1. Only females attract conspecific males. Shown is the percentage of males attracted to females (a bacterial spot on which there is a conspecific virgin female or hermaphrodite), control (a bacterial spot with no worm), or neither (neither of the spots). The CI is shown below the grouped bars. One hundred twenty to 300 males were assayed from each species. Only the conspecific male attraction to the *C. remanei* and *Caenorhabditis* sp. female is significant at $P < 0.0001$.

hermaphrodites incubated at the high temperature of 30°C for 3 h; sperm-depleted hermaphrodites; hermaphrodites exiting from the dauer stage (using *daf-4* temperature-sensitive dauer mutants); CB4108:*fog-2(q71)V*, which is a mutation that blocks hermaphrodites from producing self-sperm, essentially converting hermaphrodites into females; and the Hawaiian wild-type race CB4856, distinguished by its ability to maintain a large number of males in a population (M. Hammarlund and E. M. Jorgensen, personal communication).

Attractant Production and Response Begin at Sexual Maturity. *C. remanei* worms were synchronized at the L1 larval stage and tested at various time points after release from L1 arrest to determine the life stages at which females produce and males are attracted to the sex pheromone. The data for female secretion (Fig. 3a) and male detection (Fig. 3b) of the pheromone demonstrate that secretion and detection are stage-specific, with maximum production and attraction occurring at adulthood, when the worms are ready to mate.

Mated Females Lose Their Attractiveness. We tested young-adult *C. remanei* males for their attraction to supernatant solution ob-

tained from either (i) 20 virgin adult *C. remanei* females on their first, second, fourth, and seventh days of adulthood or (ii) 20 virgin adult *C. remanei* females mated with 40 *C. remanei* males for 3 h on the same days. In Fig. 4, the circles and crosses indicate the CI of the supernatants obtained from the virgin and mated females, respectively. We observe only a slight decline in attractiveness as the females aged. The arrows drawn on the figure emphasizes our main result: although virgin females of adult age up to 7 days old secrete sex pheromone, mating with males at any age immediately results in females losing their attractiveness to males.

Mated Females Regain Attractiveness After Cessation of Mating.

Twenty virgin adult *C. remanei* females were mated with 40 *C. remanei* males for 3 h. Starting from between 0 and 24 h after mating, a supernatant solution was obtained by soaking the mated females for 6 h. In Fig. 5, we observe that females begin to regain their attractiveness several hours after the cessation of mating, with the supernatant solution regaining maximum attractiveness 12–18 h postmating. Twelve hours postmating, females still have male sperm in their spermatheca and continue to lay fertilized eggs.

Female Somatic Gonad Is Required for Pheromone Production.

To ascertain which female organ is required for production of the sex pheromone, *C. remanei* females were subject to laser ablation and, as virgin young adults, individually soaked in 20 μ l of M9 at 25°C for 6 h so that the resulting supernatant could be tested for its ability to attract *C. remanei* males. We ablated the Z1 and Z4 cells (required for development of somatic gonad, germ line, and vulva), the anchor cell (required for vulval induction but not gonad or germ-line development), or the Z2 and Z3 cells (germ-line precursors) (6, 7).

In Fig. 6, we observe that females with ablated Z1 and Z4 cells were significantly less attractive than the wild type ($P < 0.0001$, two-sample *t* test), implicating the gonad, germ line, or vulva as the female-specific cells required for sex-pheromone production. Females with ablated Z2 and Z3 cells or ablated anchor cell are still strongly attractive to males, ruling out the germ line and vulva as being required for female attractiveness. Females with ablated Z1 cells lack the anterior gonad arm, whereas females with ablated Z4 cells lack the posterior gonad arm. To further confirm the somatic gonad as the origin of pheromone production, additional experiments individually ablating the Z1 or Z4 cells were performed and resulted in females that were still attractive (data not shown), suggesting that the pheromone can be produced by somatic tissue of either gonad arm. Interestingly, females with a laser-ablated anchor cell are not significantly less attractive than the wild type, suggesting that the release of pheromone does not require the presence of a vulval channel.

Male Cephalic Neurons (CEMs) Are Required for Pheromone Detection.

We assayed males with mutations that affect the male-specific CEMs. Fig. 7 shows the results from attraction assays for three *C. elegans* male mutant strains: *pkd-2*, *lov-1*, and *ceh-30*, together with *C. elegans* wild-type males. The proteins PKD-2 and LOV-1 are known to be expressed in the male CEMs (8), and the *ceh-30* males used here lack all four CEMs without any other obvious mutant phenotype (H. Schwartz and H. R. Horvitz, personal communication). All three mutant strains are significantly less attracted to the pheromone than wild type ($P < 0.0001$, two-sample *t* test). Furthermore, the *ceh-30* mutants that lack all four CEMs, as confirmed by loss of *pkd-2::gfp* expression in the head, are less attracted to the pheromone than are the male mutants *pkd-2* and *lov-1* males ($P < 0.0001$, two-sample *t* test) that have intact CEMs. The CI value of 0.12 ± 0.03 for *ceh-30* is comparable to the value obtained for *C. remanei* males to *C. remanei*

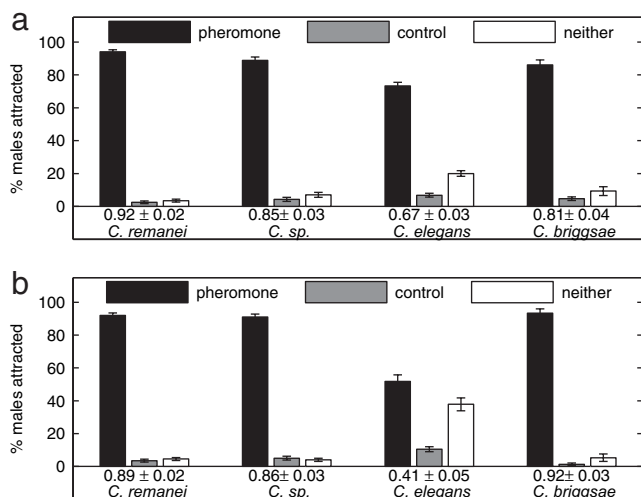


Fig. 2. Female pheromone attracts males of all four species. Shown is the percentage of males scored on the pheromone spot, control spot, or neither spot. The CI is shown below the grouped bars. Three hundred to 400 males were assayed from each species. All attractions are significant at $P < 0.0001$. (a) *C. remanei* pheromone; (b) *Caenorhabditis* sp. strain CB5161 pheromone.

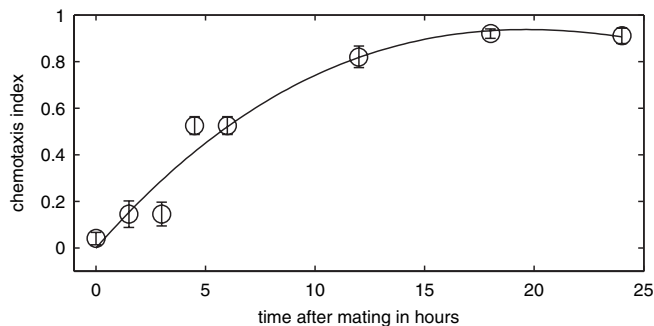


Fig. 5. Mated females regain attractiveness after cessation of mating. *C. remanei* males are tested for their attraction to a supernatant solution obtained from mated *C. remanei* females from 0 to 24 h after mating. Two hundred males were assayed for each data point. The circles indicate the CI of the supernatants obtained from the mated females. The data are fit by a least-squares cubic polynomial.

adulthood, as expected for a sex pheromone that facilitates immediate mating.

We also showed that virgin adult females continue to secrete pheromone as they age but are no longer attractive immediately after mating. Females, however, regain attractiveness soon after mating ceases. Indeed, it is well known that female behavior and physiology in many insect species change dramatically immediately after mating, and that secretions produced by the accessory reproductive glands of male insects, when transferred to females, trigger these changes (12). In a process called pheromonostasis, females in a number of moth species cease production of a sex pheromone after mating with males. In the corn earworm moth, *Helicoverpa zea*, pheromonostatic action has been attributed to a 57-aa pheromone-suppression peptide (13) that is present in the male seminal fluid. Indeed, the fruit fly, *Drosophila melanogaster*, has emerged as an important model for understanding the nature and specific function of a large number of male accessory-gland protein genes (*Acps*) that are required for this pheromonostatic control (12).

We have further demonstrated that *C. remanei* females that lack somatic gonads have significantly reduced attraction to males, implicating the gonad in the production of the female sex pheromone. Females missing only a vulva are still attractive to males, suggesting that a pheromone of small molecular size could be released by diffusion through the hypodermis or from the intestinal tract through excretion.

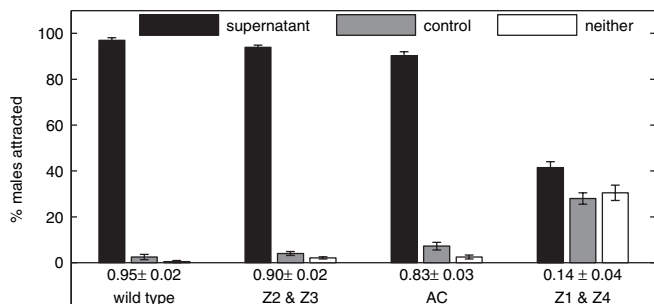


Fig. 6. Laser ablation implicates female somatic gonads in pheromone production. Supernatant solutions are obtained from individual *C. remanei* females and *C. remanei* females with the specified cells ablated. Shown is the percentage of *C. remanei* males scored on the supernatant spot, control spot, or neither spot. The CI is shown below the grouped bars. Four hundred to 800 males were assayed for each ablation type. Supernatant obtained from females with cells Z1 and Z4 ablated are significantly less attractive at $P < 0.0001$ than that obtained from wild type.

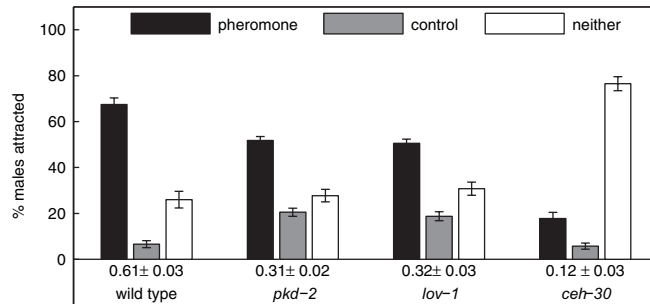


Fig. 7. Sensory mutants implicate male CEMs in pheromone detection. *C. elegans* wild-type, *pkd-2*, *lov-1*, and *ceh-30* mutant males are tested for their attraction to the *C. remanei* pheromone. Shown is the percentage of males scored on the pheromone spot, control spot, or neither spot. The CI is shown below the grouped bars. Four hundred males of each mutation were assayed. Mutant responses differ from wild type at $P < 0.0001$.

Previous behavioral analysis of worms in which head amphid neurons were laser-ablated showed that these neurons are necessary for chemotaxis behavior, with specific neurons detecting either water-soluble or volatile odorants (4). *C. elegans* males, compared with hermaphrodites, have 79 additional neurons, of which there is one male-specific sensory neuron in each of the four cephalic sensilla in the head (14). These four male-specific CEMs are similar in structure to the amphid sensory neurons, and their processes extend into the cephalic openings and possess ciliated endings (4). It was previously speculated that these CEMs might be used to sense a sex pheromone released by the *C. elegans* hermaphrodite (15), although no evidence for a sex pheromone yet existed. Our experiments with male-specific mutants and males without CEMs demonstrate that these neurons indeed are required for males to respond to a volatile sex pheromone of a related *Caenorhabditis* species. It is not clear whether the four CEMs in *C. elegans* males are used for any other function, but it would be surprising if they are only used to detect a sex pheromone that is not secreted by conspecific hermaphrodites. Of course, the dioecious ancestor of *C. elegans* may have secreted a female sex pheromone, and its continued detection by *C. elegans* males may be an evolutionary relic. Although *C. elegans* diverged from *C. remanei* ≈ 100 million years ago (16), which perhaps is a long time to maintain functional CEMs in the absence of purifying selection, *C. elegans* hermaphrodites may

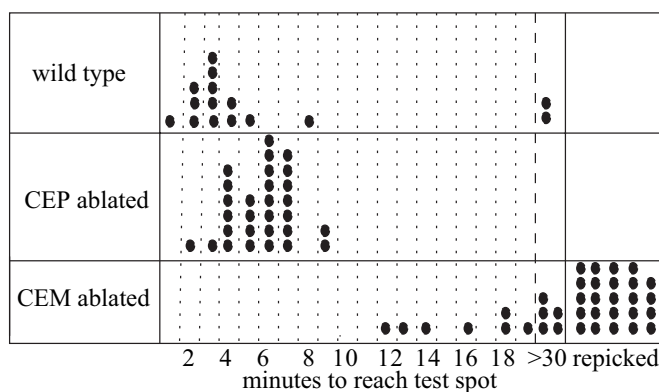


Fig. 8. Laser ablation implicates male CEMs in pheromone detection. Wild-type *C. elegans* males and *C. elegans* males with their CEPs or CEMs ablated are scored by the time required to reach a test spot containing *C. remanei* sex pheromone. Each single-worm attraction assay is indicated by a circle, and the arrival times are rounded to the nearest minute. Males that could not reach the spot within 30 min are scored as ">30"; males that were about to crawl off the slide were repicked to the start position and scored as "repicked."

have only lost the ability to secrete a sex pheromone in the more recent past.

It was previously demonstrated (2, 3) that *C. elegans* males can detect a hermaphrodite-derived chemical. Simon and Sternberg (2) observed that males reverse direction of movement more frequently in regions of agar conditioned with hermaphrodites and tend to stay longer in these regions. Furthermore, they determined that males introduced at a distance found hermaphrodites somewhat more readily when these hermaphrodites were placed on previously hermaphrodite-conditioned regions. The hermaphrodite vulva is not required for male response. Lipton *et al.* (3) demonstrated that *C. elegans* exhibits a leaving behavior that is both sex- and stage-specific. Whereas juveniles and hermaphrodites rarely wander away from food, adult males leave food to wander unless adult hermaphrodites are present.

It is important, however, to distinguish between a hermaphrodite-derived chemical sensed by males and a female-derived chemical that is actively secreted to attract males from a distance. To further clarify the qualitative difference between these chemicals, we have repeated the “attraction assay” of Simon and Sternberg (2) (see their materials and methods section for further details). Ten *C. remanei* or *C. elegans* males were individually tested for their ability to find *C. remanei* females or *C. elegans* hermaphrodites placed on regions that were previously conditioned for 24 h by uncoordinated mutant females or hermaphrodites. All 10 *C. remanei* and *C. elegans* males were able to find the *C. remanei* female within 100 min, with a median finding time of 5 min for *C. remanei* males and 30 min for *C. elegans* males. In contrast, only 5 of 10 *C. elegans* males and 7 of 10 *C. remanei* males were able to find the *C. elegans* hermaphrodite within 100 min. Among those males that did find the hermaphrodite, the median finding time was 43 min for *C. elegans* males and 53 min for *C. remanei* males. The consistency and rapidity with which *C. remanei* males find their conspecific females clearly distinguishes the *C. remanei* female sex pheromone studied here from the previously studied Simon and Sternberg (2) “mate-finding cue” of *C. elegans*.

The secretion of a sex pheromone by females is only the first step in the mating process that enables females to attract males from a distance. Our results suggest that male–hermaphrodite couplings lack this first step: certainly, hermaphrodites no longer secrete a sex pheromone of potency comparable to that secreted by females. Still present in the androdioecious species, however, are the subsequent steps in the male–hermaphrodite mating process involving both mechanical and chemical sensory inputs at short range, including the characteristic male behaviors of searching, turning, and location of vulva.

We now speculate about the evolutionary trajectory on which hermaphrodites lost the ability to secrete a sex pheromone. The *C. elegans* and *C. briggsae* hermaphrodites are thought to be descendant from a common dioecious ancestor shared with *C. remanei* and *Caenorhabditis* sp. strain CB5161 females (17). The most fundamental step in the evolutionary transition from female to hermaphrodite must have been a mutation in which females gained the ability to produce internal sperm. Self-fertilized offspring from females of a previous obligate outcrossing species, however, most likely suffered high inbreeding depression, and natural selection would presumably still favor females that outcrossed in the presence of males. Yet, the ability of females to self-fertilize in the absence of males certainly has a selective advantage to animals colonizing new habitats. Presumably this advantage must have outweighed the disadvantage of accidental inbreeding, and the initial mutation, transforming females into protohermaphrodites, must have been fixed. Perhaps additional mutations that followed fine-tuned the physiology and mechanisms associated with sperm production in these protohermaphrodites. One can imagine that at some point in the

evolutionary history of *C. elegans* and *C. briggsae* there existed hermaphroditic nematodes that still secreted a sex pheromone to attract males yet were able to self-fertilize when males were absent. Outcrossing may still predominate over selfing, but there may be a critical level of inbreeding depression at which the advantage of a selfing population, namely the elimination of the 2-fold cost of males (18), balances the disadvantage of inbreeding. When inbreeding depression falls below this critical level, perhaps by the purging of recessive deleterious mutations over many generations, any new mutation resulting in the loss of sex-pheromone production by hermaphrodites could become fixed. The ultimate loss of sex-pheromone production transformed *C. elegans* and *C. briggsae* into essentially self-fertilizing hermaphroditic species, with relatively low outcrossing rates (19, 20). Whether the continuing presence of males in these species is adaptive (21) or males are evolutionary relics (1) is debatable and directly relevant to the long-standing and important problem as to why sexual reproduction and outcrossing is so widespread in nature (22).

Materials and Methods

Worm Strains. For wild-type *C. elegans*, we have used the N2 CB4088:*him-5(e1490)V* strain with a high incidence of male progeny. We have also tested the mutants EM401:*daf-4(m63)III*; *him-5(e1490)V*, which enters dauer stage at 25°C and exits at 20°C; CB4108:*fog-2(q71)V*, which blocks hermaphrodites from producing self-sperm; and the Hawaiian wild-type race CB4856. The strain EM464 was used for *C. remanei*; CB5161 was used for *Caenorhabditis* sp.; and AF16 was used for *C. briggsae*. Males from AF16 were induced by heat shock and were maintained as a mating stock. In tests of *C. elegans* CEM mutants, we have made use of PS3151:*lov-1(sy552)II*; *him-5(e1490)V*; PT8:*pkd-2(sy606)IV*; *him-5(e1490)V*; MT13261:*nIs133I*; *him-5(e1467ts)V*; and *ceh-30(n3714,n4111)X*. To aid in laser ablation of the CEMs and CEPs, KC818:*unc-119(e2498)III*; *him-5(e1490)V*; *wxEx64[ppkd-2::gfp + M142XbaIselfL]*; and BZ555:*eglIs1[Pdat-1::GFP]* were used.

Single Female or Hermaphrodite Attraction Assay. To assay the attractiveness of a single virgin female or hermaphrodite, two rubber O-rings of 1-cm diameter are placed on opposite sides of a 5-cm agar plate and 5 μ l of OP-50 bacterial solution dropped inside the O-rings. A virgin young female (or hermaphrodite) is trapped inside one of the O-rings on the bacterial spot and stored overnight with attraction assays to be run the following day. For the assay, the female and both O-rings are removed, and a 2- μ l drop of 1 M sodium-azide solution is added to both bacterial spots to paralyze males that reach these spots. Ten previously picked males, which were isolated on single plates for several hours, are then placed on the midline between the two bacterial spots, and the plate is covered and stored for \approx 30 min. The males are then scored for their residence. Males not found on either spot because they have crawled off the plate or are still wandering are recorded as “neither spot.”

Pheromone-Attraction Assay. To simplify the attraction assay further, five young virgin *C. remanei* or *Caenorhabditis* sp. females were put in 100 μ l of M9 buffer for 6 h at 25°C. The supernatant solutions were then removed and assayed for pheromone activity. The assay was performed on a microscope slide with a layer of 1.5% agar placed on top of an empty plate. Two 2- μ l drops of 1 M sodium-azide solution were separated by 3 cm and allowed to dry. Then, a drop of 2 μ l of supernatant and 2 μ l of M9 buffer were separately added on top of the sodium-azide spots. Twenty young-adult males from the species to be tested were placed on the midline between the spots. After 30 min, the paralyzed male worms were scored on the basis of their location. The remaining worms were scored as “neither spot.” To test the

