Virgin *Caenorhabditis remanei* females are attracted to a coital pheromone released by con-specific copulating males

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The gonochoristic soil nematode *Caenorhabditis remanei* strictly requires copulation for species propagation. Males of this species are sexually promiscuous with females of other species; therefore, we asked in this study whether virgin *C. remanei* females display evidence of mate choice. We digitally recorded and measured the locomotor behaviors of one or more virgin females in the presence of a single male on a 5 mm diameter mating lawn. We observed that initially only the male modifies his locomotor trajectory to another animal on the mating lawn; the virgin females showed no locomotor bias toward the mate-searching male. However, once a male started to copulate, females in the vicinity altered their movement trajectories toward the copulating couple. Newly inseminated females are refractive to the coital signal, but partially regain their attraction to copulating males after 24 h. We found only copulating males with an intact gonad can attract females, and that the coital signal can be broadcasted at least 1.5 mm through the air. Unlike males, which are also attracted to hetero-specific females, virgin *C. remanei* females will only crawl toward a copulating con-specific male. We suggest that *Caenorhabditis* females use the coital signal as a pheromone to identify a vigorous male of their own species.

Introduction

Mate selection is a process that allows an individual to discriminate or choose a suitable sexual partner from an available pool. However, studies of *Caenorhabditis* nematodes in the laboratory have not yet revealed overt behaviors that contribute to mate selection. In hermaphroditic nematodes of the genus *Caenorhabditis*, such as *C. elegans*, *C. briggsae* and *C.* sp 11 (JU1373), copulation is optional and under standard laboratory settings, the self-fertilizing hermaphrodite does not display any overt locomotor behaviors to seek out rare males for mating. In the wild, outcrossing for *Caenorhabditis* hermaphroditic nematodes does occur, but the frequency is low.[1-5] Unlike hermaphrodites, males of the hermaphroditic *Caenorhabditis* species display strong chemotactic behavior toward secretions of con- and hetero-specific hermaphrodites and females.[6-9] After physical contact is made with a hermaphrodite or a female, regardless of the species, the hermaphroditic male will attempt copulation and even transfer their gametes into their partners.[10,11] Thus, the male's strong drive to mate appears to supersede any restraint for strict mate selection.

In contrast to hermaphroditic *Caenorhabditis* nematodes, gonochoristic (male-female) *Caenorhabditis* nematodes require copulation for species propagation. The mating behaviors of gonochoristic *Caenorhabditis* nematodes are less studied than

their hermaphroditic relatives.[8,10,12] But they have been shown to be promiscuous with their sexual partners and display many similar copulatory sensory and motor steps with their more intensely studied hermaphroditic cousin, *Caenorhabditis elegans*. A gonochoristic *Caenorhabditis remanei* or *Caenorhabditis brenneri* male will crawl toward any available female. After contact, he will press his tail firmly against the female's body and move backward until his tail contacts her vulva. If upon reaching the female's head or tail without locating the vulva, he will turn to her opposite side and continue to scan for the vulva. When the male contacts the vulva, behaviors diverge between hermaphroditic and gonochoristic males. For the hermaphroditic male, because his partner is likely moving, he must constantly adjust his position to stay over the vulval slit. Also, the hermaphroditic male must repetitively thrust his spicules to breach the tightly closed vulval lips. In contrast to a hermaphroditic male, at the vulva, the gonochoristic male will release a factor that immobilizes the female's movements. Simultaneously, the vulval slit widens, and the gonochoristic male will completely insert his copulatory spicules and transfer his sperm into the female. This ability to sedate the female's movements allows the gonochoristic male to be more effective at copulation than hermaphroditic males; but again, like hermaphroditic males, the gonochoristic males will attempt copulation and waste their sperm on females of other

Caenorhabditis species, indicating that even though con-specific copulation is essential, there is little indication for mate discrimination. In other well-studied laboratory model animals, such as *Drosophila* and rodents, the female's active role in mate selection has been intensively studied;[13,14] thus, in this study, we asked if the *Caenorhabditis remanei* female plays any role in choosing a productive mate, or if her reproductive state is determined solely by the con- or hetero-specific male that contacts her.

Results

Virgin *Caenorhabditis remanei* females crawl toward a copulating male-female pair. Wild-type gonochoristic *Caenorhabditis remanei* strictly requires copulation for the propagation of the species; therefore, we were interested in determining if both sexes (female and male) display attractive behavior toward each other. Three possibilities could affect directional locomotion: males could crawl toward females, females could crawl toward males or both males and females could crawl toward each other. To address this, we observed for 15 min the behaviors of a single virgin male and two virgin females on a ~5 mm diameter lawn of *E. coli* OP50 (n = 14 trials). We initially noticed that in all cases, the females do not display any obvious locomotor directionality preference to males. Conversely, when a male approaches within ~3 mm of a female, he will actively follow behind her until contact is made, or she moves too far from him (**Fig. 1A**). We measured the time required for the first mating pair to form and found the average time for the mating pair formation was ~4 min post-introduction to the mating lawn. We plotted the time that the aggregate forms against fraction of aggregate formation and found that the curves had a general hyperbolic shape (**Fig. 1B'**). We then re-plotted the data on a log 2 time scale, so that differences in the rise of the two curves can be visualized better (**Fig. 1B''**). We modeled mating aggregate formation as a simple binding reaction and fitted the curves to a Michaelis-Menten-like equation: Fraction of mating aggregates $Fr_A = A_{max}[time/(K_A + time)]$; where A_{max} is the maximal aggregation fraction, and K_A is the mating aggregation constant (**Fig. 1B**). During copulation, very little movement was seen from either sex.

Interestingly, once the initial mating pair was formed, the second female immediately changed her behavior and directed her locomotion toward the copulating couple (**Fig. 1C**). When she contacted the couple, she localized her movements to stay in their vicinity. We measured when the second female joined the mating group, and found that the average time for a second female to join the group was ~2 min after the initial formation of the group (**Fig. 1B**). Plotting 1/time relative to $1/Fr_A$ (**Fig. 1D**), similar to a Lineweaver-Burk plot, and using linear regression to fit a line, we found that slopes of the two fitted lines are statistically different and had different K_a values (p < 0.001, ANCOVA test). The K_a values (the negative reciprocal of the X-intercept) for the initial and subsequent aggregate are ~385 and ~196, respectively. The difference between the K_a values indicates that the rate of the second interaction is faster than the formation of the initial copulatory pair, likely because the copulatory pair is stationary.

Males direct the trajectory of females toward the mating aggregate. We then asked if the female and/or male in a copulatory aggregate modify the behavior of additional females. To address this question, we introduced groups of one (27 trials), two (14 trials), four (five trials) and 12 (three trials) females onto ~5 mm diameter lawns of *E. coli* containing a single male. If the females are the source of the attraction, we asked if more females were present in the group, will they cause additional females to join at faster rates. On the other hand, if the male is the cause, the number of females in the group will not affect the rate at which additional females join. When a male encountered a single female, they began mating and additional females aggregated to the group (**Fig. 2A**). Plotting this data, we saw that the Fr_A deceased as more females joined the group, because as more females joined the group, the group became unstable (**Fig. 2B**). Re-graphing the data as 1/time relative to $1/Fr_A$ (**Fig. 2C**) showed that A_{max} steadily decreases as female numbers increase (the reciprocal of the Y-intercept); simply because as the group gets larger, females start to move in and out of the group. However, for trials containing greater than one female, the K_A values remain similar across the different female densities (ranging from ~167 to ~175) (p > 0.05, ANCOVA test), and are faster than the K_A of trials containing only one male and one female (~850). Thus, the mechanism of attraction is likely not attributed to females calling more females toward the group, but rather the copulating male and or female might be responsible for the attraction.

We had previously shown that the male gonad contributes to the efficacy of a soporific factor that blunts female behavior. Ablating the gonad removes the ability of the male to sedate his mate during the mating sequence. We therefore asked if the male gonad was also required to attract additional females during copulation. We removed the germline and somatic gonad via laser ablation, in males or females, and asked if the operation affected the male's ability to attract a mate. Conversely we also asked if the operation affected the female's response to a copulating couple using the assays previously described. We observed that mock-ablated females, females with ablated gonads, mock-ablated males and virgin females will all form mating aggregates (**Fig. 2D**). In contrast, we observed that gonad-ablated males search out females and attempt to mate, but fail to sedate the female or perform a successful copulation. Furthermore, additional females do not show attraction toward the male's fruitless copulation attempts, which indicates that a copulating male plays an active role in attracting additional females (**Fig. 2D**).

Grouping response varies with the egg-laying. After showing that the male gonad played a prominent role in attracting additional females, we asked whether the reproductive state of the female also affects her behavior. Since removing the female gonad does not affect her attraction to a copulating couple, we ask if another major change in gonadal state, such as egg-production, will increase or decrease her response. We predicted that upon impregnation, a female will not crawl toward a mating group. Trials were conducted using either females that had been pregnant for 20 min or 24 h (**Fig. 3A**). We noticed a graded response to the coital signal; newly pregnant females (n = 14 trials) showed little interest in grouping, as compared with virgin females (n =

14 trials). Contrasting this, females that had been pregnant for 24 h showed a rate of grouping midway between the two (n = 13 trials). Graphing the data as 1/time vs. 1/Fr$_A$ and extrapolating a line confirms that in this set of experiments, K$_A$ increases from virgin females (~175), to 24 h pregnant females (~250), to 20 min pregnant females (~434) (p < 0.001, ANCOVA test) (**Fig. 3B**). We suggest that while newly impregnated females display reduced grouping response, as they use up sperm stores, they gradually regain attraction to mating pairs.

The coital signal can be broadcasted through aerosolization. *C. remanei* males could employ two methods for dispersing the coital signal: diffusion through the surrounding media and/or aerosolization. The mating lawns used in our studies were sufficiently small, such that a factor can diffuse through the agar and be sensed by the locally confined females. However, we did notice in some of our digital recordings that exceptional females can sense the coital factor from longer distances; for example, the female in **Figure 2C** appeared to detect the coital signal from greater than 7 mm away. To determine if aerial transmission was possible, we designed a sandwiched experimental setup in which an *E. coli* lawn containing 10 gonochoristic females was suspended ~1–1.5 mm above a smaller *E. coli* lawn, where copulations would occur (**Fig. 4A**). If the lower lawn was vacant, females showed no preference for any specific location on the upper lawn during the observation period (**Table 1**). However, after introducing a mating pair onto the lower lawn, we observed in 16 experimental trials that once copulation began, females on the top lawn moved more frequently over the copulation area; in eight of the 16 trials, females sustained their positions over the mating pair at an incidence statistically higher than in other areas (**Fig. 4B** and **Table 1**). Repeating the experiment using a single male or female in place of the mating pair produced responses in the top lawn females similar to those seen in the control experiment, indicating that the females were not simply attracted to the presence of another animal on the lower lawn (**Table 1**). Interestingly, we noticed that when two males were placed together on the lower lawn, they gave the appearance of following the female's trajectories on the upper lawn. Nonetheless, when the two males accidentally collide, they will attempt to copulate with each other. Before the occurrence of the same-sex coital attempts, females on the top lawn displayed no preference for specific areas on their lawn; but after the males began to copulate with each other, the top lawn females began to coalesce over the males; however, because of the low number of females used on the top lawn, only one of the trials gave statistical significance (**Table 1**). These observations indicate that the *C. remanei* male coital signal can be broadcasted through the air.

Previous work has shown that *Caenorhabditis* males are not selective with their female/hermaphrodite mating partners.[11] Specifically, males of *C. remanei*, *C. brenneri, C. briggsae* and *C. elegans* will seek out and attempt to mate with hetero-specific females or hermaphrodites.[10] Therefore, we asked if the coital signal released by *C. remanei* will attract hetero-specific females. To address this question, we placed a *C. remanei* male and female on one side of our sandwiched experimental setup and 10 *C. brenneri* females on the opposite side (n = four trials). If the cue was non-species specific, we expected the *C. brenneri* females to aggregate over the *C. remanei* pair, similar to *C. remanei* females. Instead, we found that *C. brenneri* females showed no preference for the *C. remanei* mating pairs (**Fig. 4C** and **Table 1**). The *C. brenneri* female's lack of response indicates that the broadcasted *C. remanei* male factor serves as a coital pheromone that attracts available virgin and sperm-limited con-specific females. We then conducted a much simpler version of the experiment, by putting one *C. brenneri* male directly on a ~5 mm diameter lawn containing 10 *C. remanei* females (n = three trials). The *C. brenneri* males attempted copulation and immobilized *C. remanei* females within 4 min after introduction, but none of the remaining *C. remanei* females responded to the copulating couple. This simple observation indicates that virgin *C. remanei* females do not display the same level of promiscuity as their male siblings.

Discussion

Under standard laboratory conditions, commonly studied *Caenorhabditis* nematodes spend most of their activity foraging for food and reproducing. In hermaphroditic nematodes, such as *C. elegans* and *C. briggsae*, reproduction can be accomplished as a solitary behavior. For example, a *C. elegans* hermaphrodite develops self-sperm, which will internally fertilize her own oocytes. As long as the hermaphrodite contains a store of sperm, she does not display any overt motor behaviors that promote sexual copulation, and in fact responds to an interested male as a noxious stimulus.[15] However, when a hermaphrodite depletes her sperm store, she is behaviorally, anatomically and physiologically more permissive toward the male.[15-17] Unlike the hermaphrodite, the male exhibits mate searching behavior[18] and is the individual that initiates sexual copulation.[19] In contrast to hermaphroditic *Caenorhabditis* species, gonochoristic species such as *C. remanei* and *C. brenneri* require sexual copulation for reproduction. In a previous work, we showed that when a single *C. remanei* or *C. brenneri* male is introduced to a single con- or hetero-specific female, upon contact with his mate, he will execute motor responses similar to males of hermaphroditic species. The major difference is that when the gonochoristic male contacts the vulva, he will release a soporific factor, which immobilizes the female and allows him to intromit. Once copulation is complete, the male and female separate and resume food foraging behavior.[10] Because copulation is essential for reproduction in gonochoristic species, we asked in this study, focusing on *C. remanei*, if both sexes actively search out a potential mate, or do the males of these species determine which female gets mated into.

We found that when a single male and females are introduced together on a mating lawn, the females exhibit no obvious attraction toward the male. However, the behavior of the females changes when the copulating male immobilizes one of the females; the remaining females on the lawn move toward and congregate with the copulating pair. After the male finishes his initial copulation, he contacts another female in queue and repeats the mating sequence. We suggest that this group behavior increases the potential that a virgin female will be in the proximity of an established mating competent male. This finding
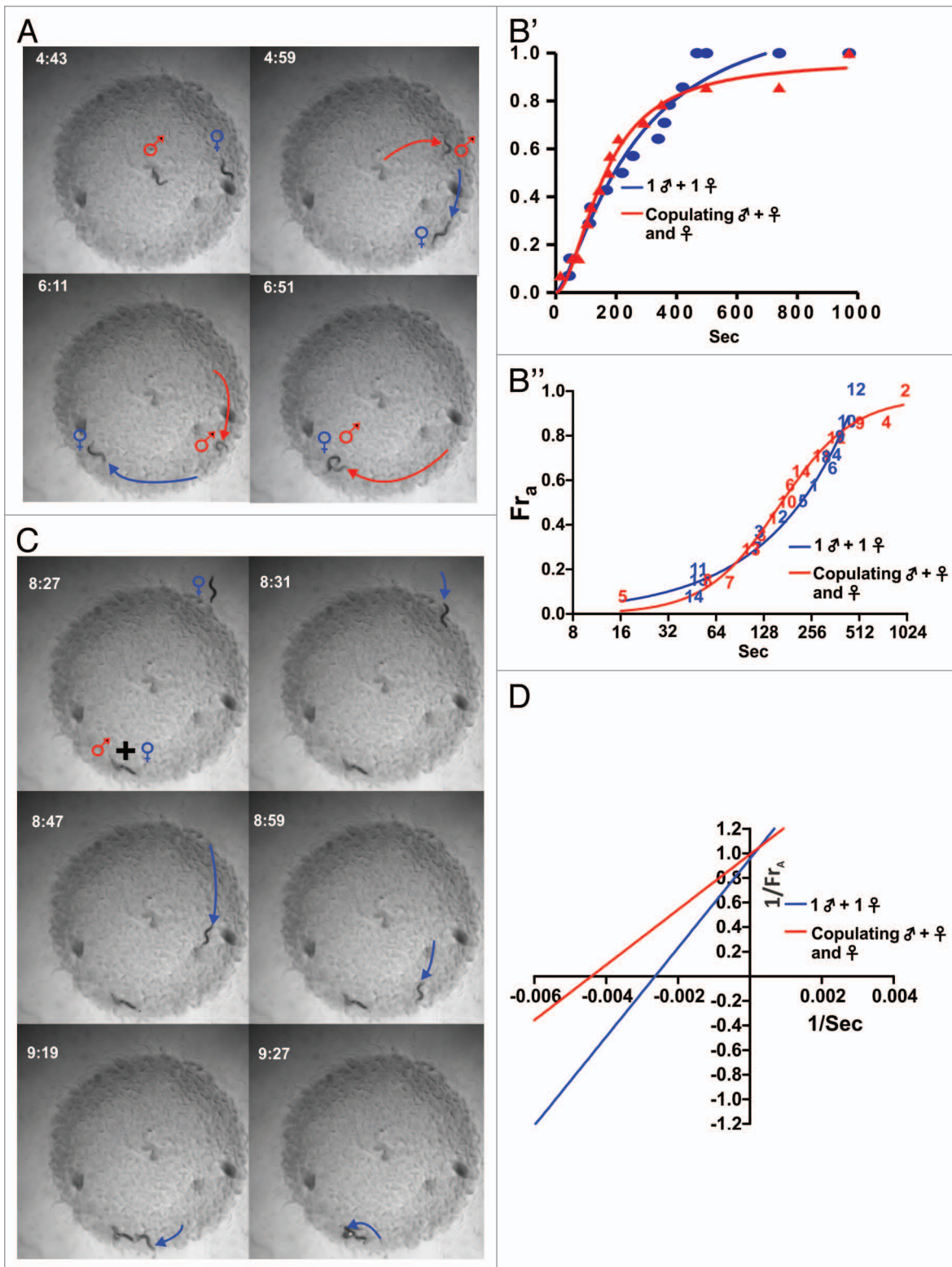
Figure 1. For figure legend, see page 5.

indicates that, similar to males, the females also utilize a neural circuit that actively promotes their reproductive success.

From measuring the virgin females' grouping kinetics, we found under our experimental conditions, the rate virgin females approach a mating pair is independent of the number of animals on the mating lawn, and the female mate-searching rate is faster, but not grossly different from the rate a male searches and contacts a female. In contrast to the male, who actively searches for

Figure 1. Virgin males crawl toward a virgin female, and virgin females crawl toward a copulating couple with different kinetics. **Figure 1A** depicts a video montage showing the path of the male as he approaches a crawling virgin female. The duration (minutes and seconds) of the video is displayed at the top left of each pane. The colored arrows (red for male and blue for female) show the path taken by the animals. The mating lawn had two females and one male; one of the females temporarily crawled off the bacterial lawn and is out of the viewing field. The **Figure 1B'** graph shows the fraction of females grouped with the male over time (seconds), averaged from 14 trials. **Figure 1B″** graph shows the data from **Figure 1B'** with time in the X axis arranged on a log 2 scale. Each point displays the trial number of when a female and male comes into contact (blue) or when a female contacts the copulating couple (red). For the first mating aggregate (blue line), the time is relative to when the animals were introduced to the mating lawn. For the addition of the second female to the preexisting mating aggregate, the time is relative to the formation of the first mating aggregate. For example, 226 sec after animals were introduced to each other (blue), 50% of the trials had a copulating couple; 153 sec after the first mating aggregate formed (red), in 50% of the trials, a second female was associated with the couple. The lines were fitted through the points using the equation of $Fr_A = A_{max}[(time)/(K_A + time)]$; where A_{max} is the maximal aggregation fraction, K_A is the mating aggregation constant and Fr_A is the fraction of mating aggregates. **Figure 1C** depicts a video montage showing the path of the second female as she approaches the copulating couple. The video is of the same trial (trial 10) as shown in **Figure 1A and B**. The female that was off-video crawled back into the viewing area and directed her movement (blue arrow) toward the mating couple. **Figure 1D** depicts the data in **Figure 1B** re-plotted as 1/time relative to $1/Fr_A$. Lines were fit using linear regression, with X and Y intercepts representing $(-1/K_A)$ and $(1/A_{max})$, respectively.

any female, the activity of the female mate searching circuit is targeted toward a male engaged in mating attempts. Eliminating the female vulva and germ line, by laser-ablating her gonad, does not affect either the male or female's response to the mating process. The males will fruitlessly attempt to mate with a gonad-ablated female, while other gonad-ablated females will continue to amalgamate into the mating group. This indicates that successful spicule penetration or sperm transfer is not required to attract neighboring females. However, the fruitless mating attempts of a gonad-ablated male elicit no obvious response from a neighboring female. Thus, the transmission of this coital signal requires an activity from his gonad and the fertility of a copulating female or the number of "waiting" virgin females in the group does not affect the rate of subsequent virgin female attraction. However, the congregation of females around the male is not static; when a female becomes impregnated, she will move away from the group.

Newly impregnated females show a reduced attraction to copulating males. When a copulating pair is present, most pregnant females show little change in their food foraging behavior, although exceptional pregnant females will show some attractive behavior to a mating pair. A pregnant female's refractory response to the coital signal is not permanent; pregnant females isolated from males for 24 h partially display an attractive behavior toward a mating pair. At present, we do not know the mechanism for the changes in the female's behavior, prior to and after insemination, but we favor the hypothesis that the female's dynamic response to the coital signal is coupled to her levels of egg production. We suggest that there might be similarities between *C. remanei* females' reproductive behaviors and the phenomenon ascribed for the compliant responses of sperm-depleted *C. elegans* hermaphrodites to mating-interested males.[15,17]

The chemical nature of the coital signal is not known, but our experiments demonstrate that it can be dispersed, at least 1–1.5 mm, through the air. The coital signal could be a single molecule or mixture, either sprayed/broadcasted by the copulating male or locally secreted by the male and then volatilized. In the *Caenorhabditis* genus, there exists a class of compounds called ascarosides, sugar-containing lipids differentially secreted from both sexes.[6,20,21] From social/antisocial signaling to mating cues, ascarosides have been demonstrated to induce different developmental and behavioral responses across species.[22,23]

Currently, we do not rule out ascarosides as contributing to the potency of the coital signal. But if they are the sole component, then there are constraints to their mode of activity. Ascarosides are not volatile, as demonstrated by how they are purified. Thus, if the coital signal is an ascaroside or an ascaroside blend, it must be aerially sprayed by the copulating male. In contrast, if the coital signal is volatile, then it could be a volatile component of ascaroside breakdown. However, it is also possible that the coital signal is distinct from ascarosides. Nonetheless, expression through the air allows the signal to disperse rapidly and over a broad area. But public transmission has a potential disadvantage as well. By traveling quickly and covering a large area, copulating males might inadvertently attract nematodes of different species.

Caenorhabditis nematodes in the *Elegans* super group have been isolated from rotting fruits, decaying plant stems, leaves, flowers and nuts, snails and terrestrial isopods in different temperate and tropical environments.[24-27] In some cases, different species were found to cohabit the same geographic region and even rotting substrates.[24,28,29] One can conceive that if the nematode's food source contains a mixed population with sufficient number of males, then promiscuous copulations between males and females of different species in the *Elegans* super group could result in large scale fertilization interference, sex-specific and sex-independent F1 lethality, F1 sterility and F2 hybrid breakdown, as has been demonstrated in the laboratory by many studies.[11,26,30-33] Thus, if females responded in mass to the broadcasted signals of a hetero-specific copulating male, then the occurrence of non-productive copulations would be greater than if an individual male had to chase down females. However, we show in a limited example that at least in the laboratory, this is not the case. Although diffusible mating cues released by *Caenorhabditis* females can attract hetero-specific males, and the soporific factor released by gonochoristic males can immobilize hetero-specific females, the coital signals released by *C. brenneri* or *C. remanei* males do not effectively call virgin females of hetero-specific species. Thus we suggest that the coital signal acts as a species mating pheromone, rather than a generalized mating cue for attracting females. This mechanism of mate choice might contribute to which different gonochoristic species might be compatible to stably cohabit and propagate in the same nutrient environment.
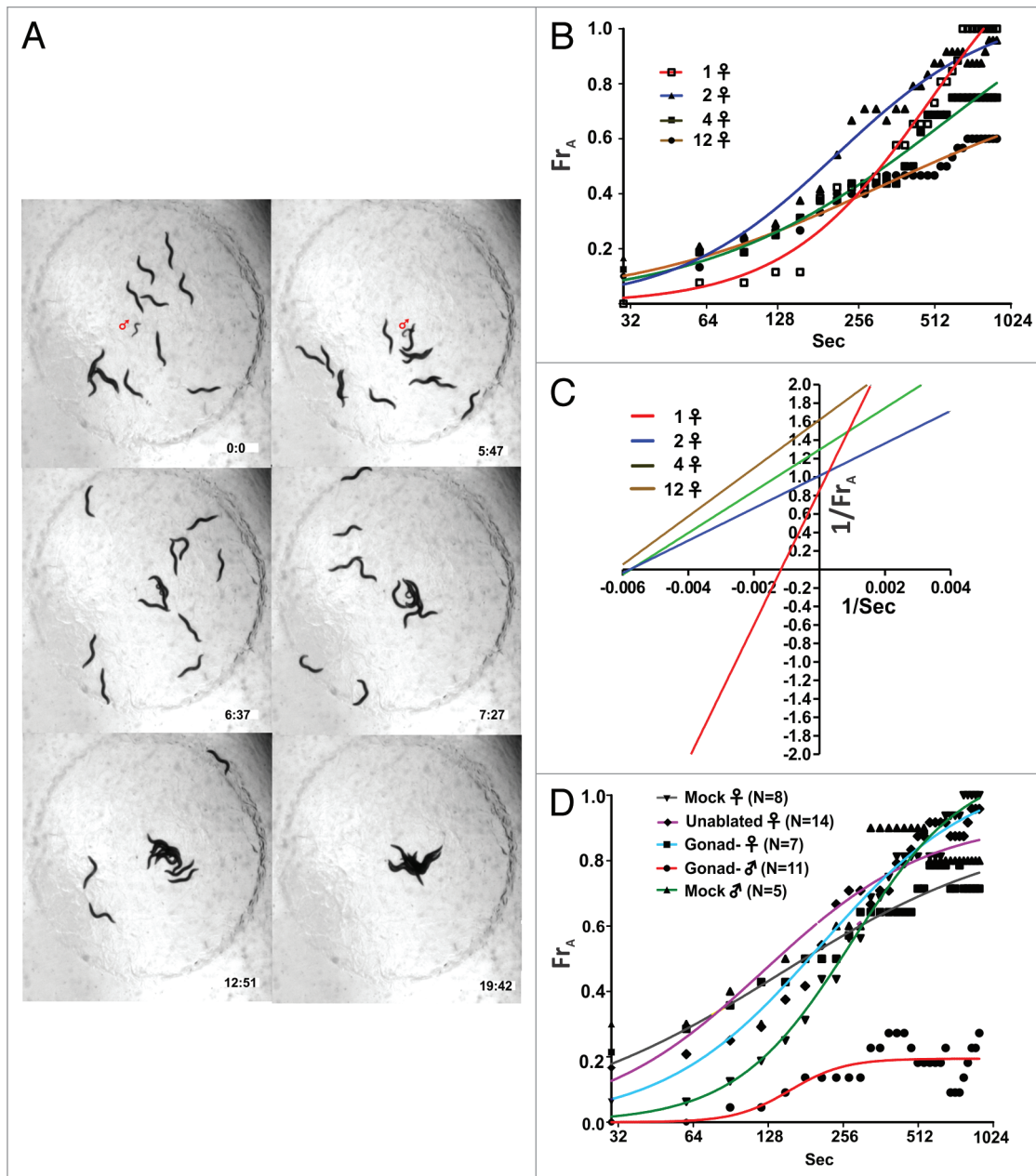
Figure 2. The male and not females affect the kinetics of aggregation. **Figure 2A** shows a sequence of frames taken from a single video depicting a large group of females coalescing onto a male. The time relative to male's placement on the lawn is shown in the upper left corner in each frame. The mating lawn consists of one male, marked with a red gender symbol and 12 females. **Figure 2B** shows the fraction of females grouped with the male over time (seconds). Tests containing one female, two females, four females and 12 females were averaged over 27, 14, five and three trials, respectively. Each point shows the average fraction of females contacting a male at that time point. All times are relative to the males placement onto the plate; e.g., for plates containing one male and two females at 186 sec, 50% of the females across all trials were grouped with a male; at 890 sec greater than 95% of the females were in contact with a male. Similar to **Figure 1B"**, the lines were fitted through the points using the equation of $Fr_A = A_{max}[X/(K_A + X)]$; where A_{max} is the maximal aggregation fraction, and K_A is the mating aggregation constant. Time on the X axis is arranged on a log 2 scale. The data from **Figure 2B** is re-graphed in **Figure 2C** as 1/time relative to $1/Fr_A$. Lines were fit using linear regression, with X and Y intercepts representing $(-1/K_A)$ and $(1/A_{max})$, respectively. **Figure 2D** represents the Fr_A over time (seconds) of gonad-ablated and mock-ablated animals. Time on the X axis is arranged on a log 2 scale. As with **Figures 2B**, each point shows the average fraction of females contacting a male at that time point, with lines fitted using the equation $Fr_A = A_{max}[X/(K_A + X)]$.

Materials and Methods

Animal husbandry. *Caenorhabditis remanei* (strain PB4641) and *Caenorhabditis brenneri* (strain PB2801) were grown at 20°C on nematode growth media (NGM) agar plates seeded with *E. coli* OP50.[34] Strains were serially propagated by transferring 10–20 gravid females to fresh *E. coli* plates. For all experiments, both sexes were segregated at L4 stage the night before, and placed

onto fresh OP50-containing NGM agar plates.

Mating assay. A single adult male and one or more females were placed on an NGM agar plate containing a ~5 mm diameter lawn of OP50. The animals were observed and digitally recorded, generally for 11–15 min (sometimes up to 20 min) using an Olympus SZX16 stereomicroscope mounted with a Hamamatsu ImagEM CCD camera. Frames were recorded every second for the one male and one/two female matings, and recorded every 4 sec for higher density matings. Recordings were then analyzed using the Hamamatsu SimplePCI (version 6.6.0.0) software. Data was then plotted and statistically analyzed using GraphPad Prism 5. Time from when the male was placed on the lawn, until first and subsequent sustained contacts were measured and described as the length of time necessary for two or more separate units (ranging from a single worm to a group) to coalesce. Only sustained contacts exceeding 30 sec in duration were measured.

Laser ablation. The somatic gonad and the germline were eliminated in adult females and males by laser-ablating the Z1, Z2, Z3 and Z4 gonadal precursor cells at L1 larval stage. Laser ablations were performed using a Spectra-Physics VSL-337ND-S Nitrogen Laser attached to an Olympus BX51 microscope via the Micropoint laser focusing system (Andor Technology). Mixed-sex L1 worms were mounted on 5% agar pads containing 5 mM sodium azide. Each ablated *C. remanei* had a mock-ablated cohort that was mounted onto a pad for the same length of time, but was not subjected to laser irradiation. After the operation, subjects were placed onto an NGM agar plate containing OP50 and allowed to grow up to the L4 stage. At L4, males and females were separated onto different NGM plates containing OP50. The following day, two ablated females were placed with a single non-ablated male onto an NGM plate containing a ~5 mm diameter lawn of OP50. All animals were digitally recorded and analyzed as described above. This was repeated with the following combinations: two virgin females and single virgin male, two mock-ablated virgin females and single virgin

male, two virgin females and single mock-ablated virgin male, two virgin females and a single ablated virgin male.

Non-virgin female assays. Virgin females were incubated with males and assayed 20 min or 24 h later. The non-virgin female was placed on a ~5 mm mating lawn with a single virgin male and virgin female. Animals were digitally recorded for 15 min. A frame was recorded every 4 sec. The times from the initiation of mating between the virgin male and virgin female, and when the
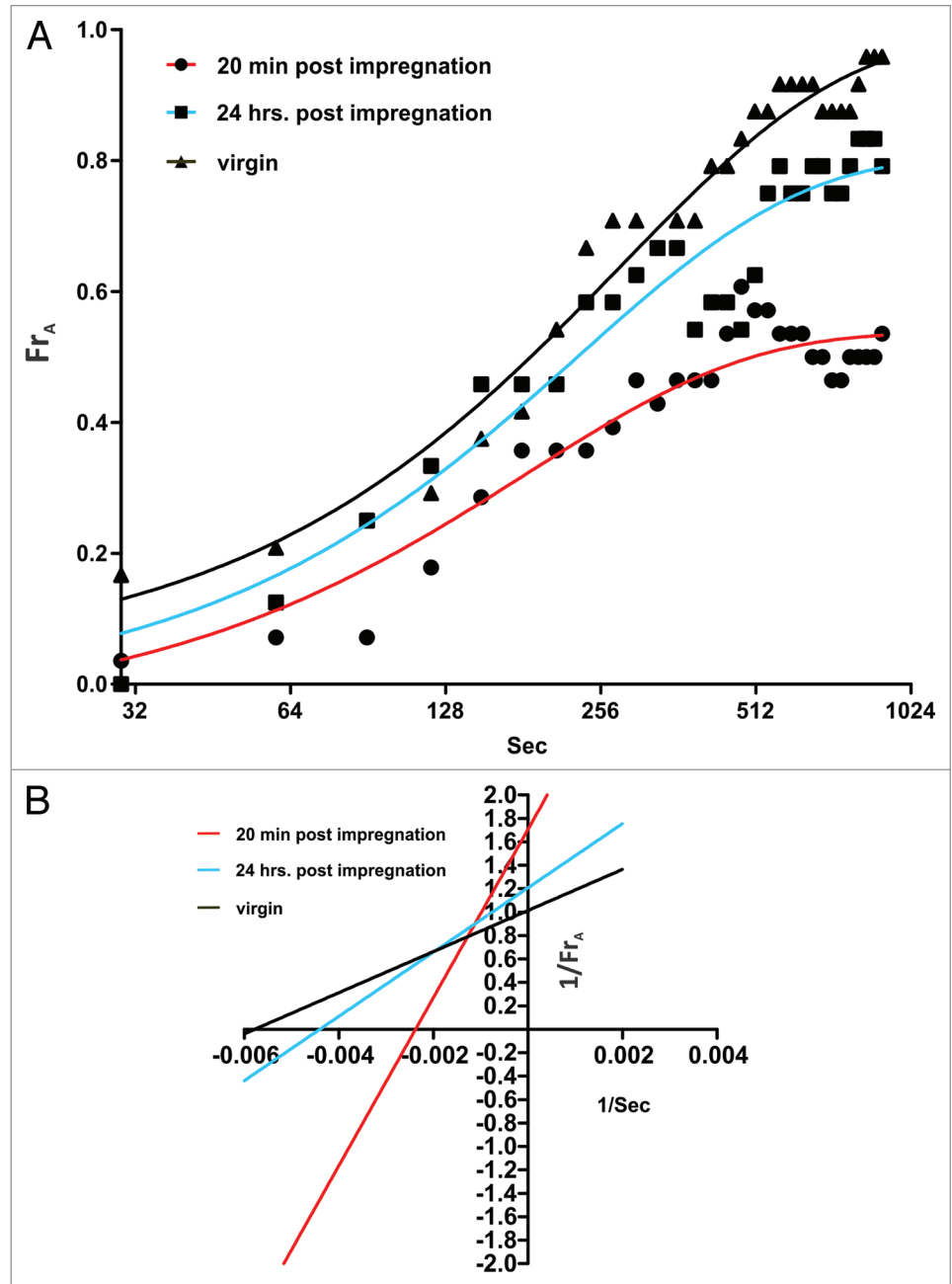

Figure 3. Aggregation rate is reduced in newly inseminated females. **Figure 3A** depicts Fr_A over time (seconds) of virgin females and females pregnant for 20 min or for 24 h. Each point shows the average fraction of females contacting a male at that time point. The line fitted through the points follows the equation $Fr_A = A_{max}[X/(K_A + X)]$. Time on the X axis is arranged on a log 2 scale. In **Figure 3B**, the data are shown using $1/Fr_A$ relative to $1/time$, with X and Y intercepts representing $(-1/K_A)$ and $(1/A_{max})$, respectively.
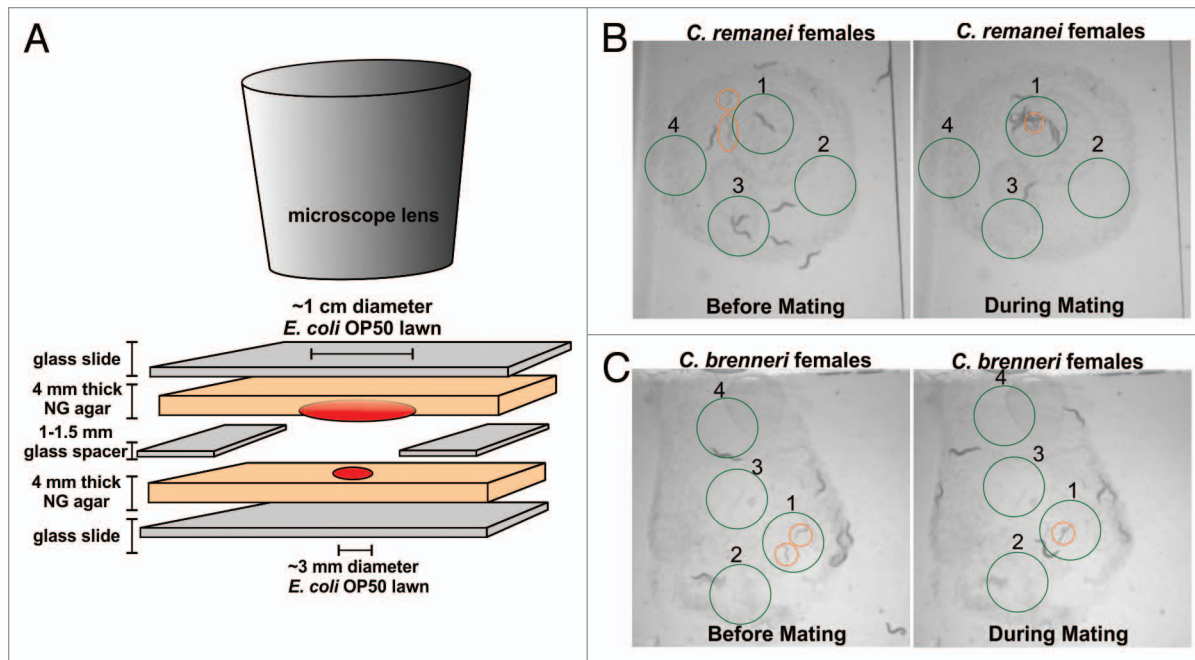
Figure 4. Females react to aerial transmission of the coital signals. **Figure 4A** shows a schematic of the sandwiched mating apparatus used to view the females' behavioral response to a copulation event on the opposite lawn. **Figure 4B and C** show the response changes in 10 *C. remanei* and C. *brenneri* females, respectively, in the 1 cm diameter top mating lawn, before and during, a copulation event on the ~5 mm diameter bottom mating lawn. The copulating pair is marked with an orange circle. The green circles denote the areas that were used to monitor the occupancy of females on the top lawn.

non-virgin female joined the pair were measured to the nearest 4 sec. Recordings in which males initiated mating with the gravid female first, rather than the virgin, were not analyzed.

Assaying aerial transmission of the coital signal. NGM agar slabs were placed onto two microscope glass slides, slide A (bottom slide) and slide B (top slide). Slide A was seeded with a ~5 mm diameter lawn of *E. coli* OP50, whereas slide B was seeded with a ~1–1.5 cm diameter lawn of *E. coli* OP50. On the lawn of slide A, we left it blank or placed onto it one of the following combinations: one male, one female, two males or one male and one female. Onto the lawn of slide B, we placed 10 virgin female *C. remanei* or *C. brenneri*. We then placed slide B upside down and above slide A, using two ~1–1.5 mm thick glass plates as spacers to separate the two NG agar slabs; this allowed the ~1–1.5 cm diameter lawn to be suspended directly above the ~5 mm diameter lawn, separated by air. We placed the sandwiched setup on an Olympus SZX16 stereomicroscope mounted with a Hamamatsu ImagEM CCD camera, focused through the glass of slide B and onto the lawn containing the 10 females. We recorded their responses for 15 min, scoring each test based on how many females, located on the slide B lawn, were present in one of four specified circular areas. Each

monitoring area had a diameter of 2 mm. One of the areas (area 1) was defined, post hoc, over the copulating pair; the three other areas were defined as random regions range 0.5–1.75 mm away from area 1 (the area over the copulatory act). All four monitoring areas were contained within the 1 cm top mating lawn. The recordings were then analyzed using the Hamamatsu SimplePCI (version 6.6.0.0) software. The numbers of slide B females located in each of the areas were counted during the entire copulatory act. In some of the experiments, the copulatory act was shorter than others, which likely affected the distribution of females that accumulated over the pair. The average numbers of animals in each area were statistically compared with each other, using GraphPad Prism 5 to calculate the results of the chi-square test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Table 1. Average number of females over an area during the remote copulation period

Top lawn/bottom lawn	Trial	Area 1 mating area	Area 2	Area 3	Area 4	p value (Chi-square)[a].
Vacant	1	1	0	2	1	0.5276
10 *C. remanei* Females/*C. remanei* Mating Pair	1	5	0	1	0	0.004**
	2	3	0	0	1	0.0833
	3	3	0	0	0	0.021*
	4	2	0	0	0	0.0972
	5	5	0	0	0	0.0007***
	6	3	0	0	0	0.021*
	7	1	1	1	0	0.7816
	8	3	1	0	0	0.0833
	9	3	0	0	1	0.0833
	10	2	0	0	0	0.0972
	11	5	0	0	0	0.0007***
	12	1	0	0	0	0.3799
	13	2	0	0	1	0.2654
	14	7	0	0	0	< 0.0001***
	15	6	0	0	0	< 0.0001***
	16	4	0	0	0	0.004**
10 *C. remanei* Females/One *C. remanei* Male	1	1	0	1	1	0.7816
	2	0	0	1	2	0.308
	3	0	1	0	1	0.5508
	4	1	0	0	1	0.5508
10 *C. remanei* Females/One *C. remanei* Female	1	0	1	1	1	0.7816
	2	0	2	2	1	0.4727
	3	1	1	2	1	0.8766
	4	1	0	1	0	0.5508
10 *C. remanei* Females/Two *C. remanei* Males	1	3	1	1	0	0.2267
	2	4	1	0	1	0.07
	3	6	1	0	0	0.0007***
	4	3	1	1	0	0.2267
10 *C. brenneri* Females/*C. remanei* Mating Pair	1	0	0	0	0	N/A
	2	0	0	0	0	N/A
	3	0	0	0	0	N/A
	4	0	0	0	0	N/A

[a]P values: * < 0.05; ** < 0.01; *** < 0.001.

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