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Finding females: pheromone-guided reproductive tracking behavior by male *Nereis succinea* in the marine environment

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SUMMARY

Pheromones trigger reproductive responses of many marine organisms, but little is known about how pheromones mediate mate-finding behavior in the marine environment. This paper investigates whether the tetrapeptide nereithione (cysteine-glutathione disulfide), known to be released by females of the polychaete *Nereis succinea* to trigger spawning in male *N. succinea*, can also be used at lower concentrations to guide males to the females. Low concentrations of pheromone elicited increased swim speed and turning left or right 84% of the time. Animals sometimes weaved back and forth, or in other cases swam straight along the trails an average of 8.1±1.2 cm before veering off. At higher concentrations, the males circled frequently, often encountering 10–20 cm of pheromone trail before swimming away. Male responses to nereithione were modeled by computer simulation, taking into account arousal of swim speed, activation of turning, speed of response and its decay, etc. In the model, low concentrations ($<10^{-8}$ mol l^{-1}) of pheromone significantly increased the number of encounters with the pheromone trail, an average following of simulated trails of 10.5±3.6 cm, and a significant increase in the frequency of encountering a virtual female on the trail (ANOVA, $P<0.001$). The model supports the hypothesis that a pheromone can have a dual function, with low concentration pheromone trails being used by male *N. succinea* to find females and increase their likelihood of mating whereas high concentrations of the same pheromone trigger the spawning behavior itself.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/5/757/DC1

Key words: Annelida, computer simulation, cysteine-glutathione disulfide, polychaete, reproduction.

INTRODUCTION

For marine animals that release gametes for external fertilization, assuring that male and female gametes have a reasonable chance of encountering one another to initiate fertilization is not a trivial problem. Large numbers of sperm and the use of sperm attractants released by oocytes (Miller et al., 1994) can facilitate fertilization; however, such gamete-level mechanisms are helpful only over short distances (<1 mm, the distance sperm may swim before being dispersed by bulk flow or turbulence). Of greater importance is making sure that males release gametes in close proximity to the females so that gamete interactions have a chance to occur. Chemical attractants (pheromones) may play a role in attracting mates to one another. However, an important gap in our knowledge is that not many marine pheromones have been characterized chemically, and of those that have been characterized the pheromone-release and stimulus–response patterns that mediate close encounters have not been described. The marine worm *Nereis succinea* Frey and Leuchart 1847 (Annelida, Polychaeta, Aciculata, Nereididae; synonyms: *Neanthes succinea* and *Nereis limbata*), which is well known for its dramatic reproductive swarming behavior, is an excellent model system in which to investigate the mechanisms that mediate pheromone-guided reproductive behaviors (Hardege, 1999). Not only is its behavior easily observable in the field (Costello and Henley, 1971) and in the laboratory (Ram et al., 1999), but also the structure of the female pheromone that triggers male spawning (cysteine glutathione disulfide, CSSG) has been determined (Zeeck et al., 1998b) and is readily synthesized and isolated (Hardege, 1999).

N. succinea ripens into mature male and female heteronereids while residing in sediments of brackish tidal areas and emerges to spawn, synchronized by the influence of circadian, lunar and annual cycles (Lillie and Just, 1913). *N. succinea* spawn only during summer months, especially around the new moon or full moon, and for just a few hours beginning shortly after dusk. Upon emergence from the sediments, male and female worms swarm (swim actively) at the water surface, searching for the opposite sex. If male and female *N. succinea* approach each other within about 3 cm they exhibit a 'nuptial dance' in which males and females circle rapidly around one another releasing clouds of sperm and eggs. Following the nuptial dance, males swim off in search of other females, while the females, having released all of their oocytes, stop swimming and appear to die, as they sink from the surface.

The nuptial dance and gamete release behaviors of *N. succinea* are triggered by sex-specific pheromones, with sperm release being triggered in males by CSSG excreted by females (Hardege, 1999; Hardege et al., 2004; Zeeck et al., 1998b), and a bouquet of inosine and amino acids released by males with the sperm (Zeeck et al., 1998a) triggering egg release in females. CSSG has the threefold effect on males: arousing swim speed, activating circling behavior, and rapidly eliciting ejaculation (Ram et al., 1999). However, an important part of this encounter that has not been worked out is what mechanisms mediate the approach within 3 cm to enable the pheromone-mediated nuptial dance to occur.

Although the density of some swarming polychaete species may be high enough for encounters to occur by random chance, additional

mechanisms may facilitate these encounters in *N. succinea*. The use of mechanisms other than random encounters in *N. succinea* is suggested by observed *N. succinea* swarming densities, anecdotal observations of *N. succinea* swarming behavior, and quantitative observations of swimming speeds and CSSG release by swarming animals. The appearance of a female on the surface, recognizable by its greener color (Fig. S1 in supplementary material) and slower swim speed than males, has often been followed by a seemingly higher density of males than before and a resultant nuptial dance and spawning, suggesting that males somehow sense females from a distance, are attracted to them, and can track them down.

By what mechanism might males detect the presence of females and how might these cues mediate the close approach necessary for spawning to take place? In this paper we propose the hypothesis that the pheromone CSSG has a dual function, with low concentration pheromone trails being used by male *N. succinea* to find females and increase their likelihood of mating, whereas high concentrations of the same pheromone trigger the spawning behavior itself. The spawning function of relatively high concentrations of CSSG (~10^{-7}–10^{-6} mol l^{-1}) is already well established (Ram et al., 1999), so we have focused here on the hypothesized search behavior in response to lower concentrations of pheromone. For behavioral experiments, we devised a unique, heart-shaped aquarium to facilitate observations of male worm responses to linear pheromone trails. Computer simulation was then used to show that male responses to low concentrations of pheromone can increase the frequency of finding female *N. succinea* on pheromone trails.

MATERIALS AND METHODS

Animals

N. succinea Frey and Leuchart 1847 were collected from Roath Basin (Cardiff, UK) by scraping immature specimens off the concrete walls of Roath Basin. Animals were cultured in the laboratory at 20°C in 140 l aquaria filled with a 10 cm gravel plus 15 cm sand bed in seawater diluted to a salinity of 20 g l^{-1} (DSW, ~57% seawater), and fed with Tetra Marine™, frozen mussel and live *Corophium volutator* twice a week. Moonlight was simulated with a 4 W lamp for four consecutive nights every month (Hardege, 1990). Animals that emerged and began swimming, indicating their ripening into mature animals, were removed and stored in separate containers in DSW at 8°C until use. Under such conditions, ripe male *N. succinea* resume active swimming upon warming to ambient temperature (22°C) and are responsive to stimulation by CSSG. Any animals not swimming spontaneously within 30 min of re-warming were discarded.

Behavioral testing

Initial tests were conducted in a standard rectangular aquarium filled to a depth of 1.0 cm; however, worms often swam almost all of the time along the walls and would get 'stuck' in corners (head-in, with continued swimming motion) and only relatively infrequently head out into the open field where they might encounter the stimulus trails. Animals also usually swam along the periphery of round tanks. Accordingly, we designed a unique 'rounded heart-shape' test apparatus (Fig. 1; see also Movie 1 in supplementary material), which had no corners, so that upon reaching the internal apex, animals swimming along the wall would be directed into the open field. The test apparatus was filled to a depth of 1.0 cm with DSW.

The stimulus was a 'line' of stimulant (usually CSSG) with an included dye to ascertain its extent and mixing. CSSG, glutathione or control solutions were injected into the tank with a 5 ml syringe fitted with a 25 G5/8 needle. Glutathione is a mimic of CSSG, and is able to stimulate both arousal and spawning behavior but at concentrations 10–100 times that of CSSG (Ram et al., 1999). All solutions contained a dye [0.03% fluorescein sodium salt or black ink (Parker Quink)] that preliminary experiments showed had no behavioral effects at the concentrations used. Trails were often laid down perpendicular to and just below the internal apex of the heart apparatus (Fig. 1 and subsequent data) but occasionally at an oblique angle or criss-crossing one another. A typical trail was created by ejecting approximately 0.2 ml of solution to produce a pheromone plus dye trail approximately 20 cm long. Trails were 5–10 mm wide, judging by the spread of the dye. Assuming an approximately cylindrical trail of diameter 5–10 mm and length 20 cm (a volume of ~4–16 ml), then the solution in the syringe is diluted ~20- to 80-fold in the pheromone trail. Since the exact dilution is unknown, concentrations are given in the text as 'estimated' (~) concentrations, and the exact concentration in the syringe is given in figure captions to make the source of the estimate apparent.

Behavioral responses were recorded by video, digitized, and then played back frame-by-frame with Sony Movie Studio, plotting animal positions every 0.2 s.

Simulation modeling

The movement of male worms was modeled in two dimensions with respect to swim speed, turns, and the effect of encountering a chemical stimulus on these variables. The chemical stimulus was modeled as a linear trail of given concentration and width. To simulate mechanical dispersion and mixing of the chemical by the modeled worm swimming through it, the chemical at a particular location could be dispersed into adjacent locations with no loss of total chemical, thereby reducing its concentration in its original location and spreading it to adjacent areas. The amount of dispersion caused by the modeled worm was a user-adjustable variable. In the simulation, the movement of the worm over a time, *dt*, is determined according to the swim speed and turning angle calculated from pheromone concentration–response and behavioral arousal curves described in the Results; the worm is then moved to the new calculated position, and the process is repeated, keeping track of the worm's previous positions, turning angle and speed. The simulations in this paper used a *dt* of 50 ms. The model also includes small spontaneous direction changes that occur according to whether a random number generated each '*dt* cycle' exceeds a value set by the user. Sources of quantitative values (swim speeds, turning, CSSG sensitivity) are given with the model, in the Results.

The simulation was programmed in JAVA and has been placed on the internet for testing and general access. The URL is http://paris.cs.wayne.edu/~aw6056/Simulation/Simulation.html.

Since the model disperses the chemical in the trail, the length of trail over which the simulated chemical was dispersed (i.e. of lower concentration after passage of the simulated worm) was measured. For simple crossing of the trail, this would be the width of the path through the trail. Where the simulated worm traveled along the trail for some distance, this is the contiguous length of simulated trail disturbed by the simulated animal before veering off in another direction.

RESULTS

Behavioral observations

Spontaneous behavior

In aquaria, *N. succinea* often swam along the periphery. As described in Materials and methods, animals were tested in a unique

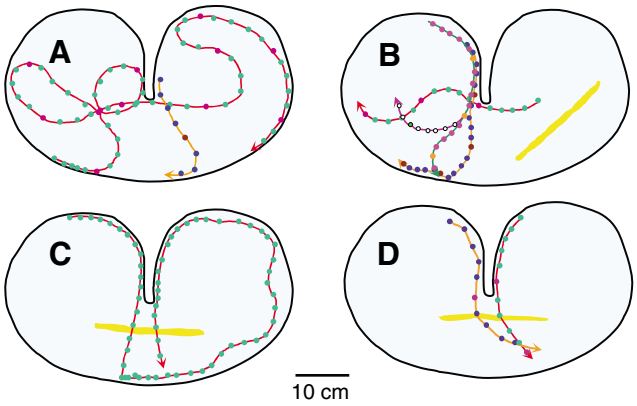

Fig. 1. Spontaneous swimming and responses of male *N. succinea* to low concentrations of CSSG. Points show animal positions at 0.2 s intervals. (A) No CSSG present. (B) CSSG present in a remote part of the tank (yellow strip). The image shows three different paths swum down the left side of the internal apex and one 'cross-tank' path. (C) Lack of response to low concentration of CSSG. The trail was created with 0.2 ml of 10^{-8} mol l^{-1} CSSG and therefore has an estimated trail concentration of $<10^{-9}$ mol l^{-1}. (D) Responses to a trail made with 10^{-7} mol l^{-1} CSSG (estimated trail concentration of $<10^{-8}$ mol l^{-1} CSSG).

'rounded heart-shape' tank in which animals swimming along the wall inevitably swam into the open field upon reaching the internal central apex (Fig. 1). Worms also occasionally swam into the open field spontaneously.

Fig. 1 illustrates representative swim tracks of male worms with no pheromone trail present (Fig. 1A), a pheromone trail present in a distant part of the tank (Fig. 1B), or a pheromone trail present at a very low concentration (Fig. 1C; $<10^{-9}$ mol l^{-1}, estimated as described in Materials and methods). In summary, spontaneous swimming behavior of *N. succinea* is characterized by (1) frequently swimming along edges, (2) when swimming in the open field, swimming straight (e.g. Fig. 1C), or (3) when turning in the open field, swimming in broad, gradual curves with occasional changes in direction.

Deflection and trail-following upon encountering a pheromone trail

With a moderately higher concentration of pheromone, the usual response of male worms upon encountering the trail was a deflection of the swim path (Fig. 1D). The deflection occurred about equally often to the left as to the right. For nine animals crossing trails at relatively low concentrations (CSSG between 10^{-9} mol l^{-1} and 10^{-8} mol l^{-1} or glutathione 10- to 100-fold higher), deflections of the swim path averaged 44±7% to the left, 39±6% to the right and 16±3% no turn (mean ± s.e.m., N=9 animals).

When crossing a low-concentration trail at right angles the deflection was usually not large enough to bring the stimulated worm back to the trail. However, encounters at an oblique angle often resulted in encountering the trail again, which again deflected the direction of swimming. The resultant 'weaving' behavior by a representative animal is illustrated in Fig. 2.

Animals were also able to respond to trails by swimming directly along them for some distance. An example of a male worm swimming 18 cm along a trail after encountering a low-concentration pheromone trail at an oblique angle is illustrated in Fig. 3. For 15 instances of animals swimming directly along a pheromone trail, the average distance before veering off the trail was 8.1±1.2 cm.

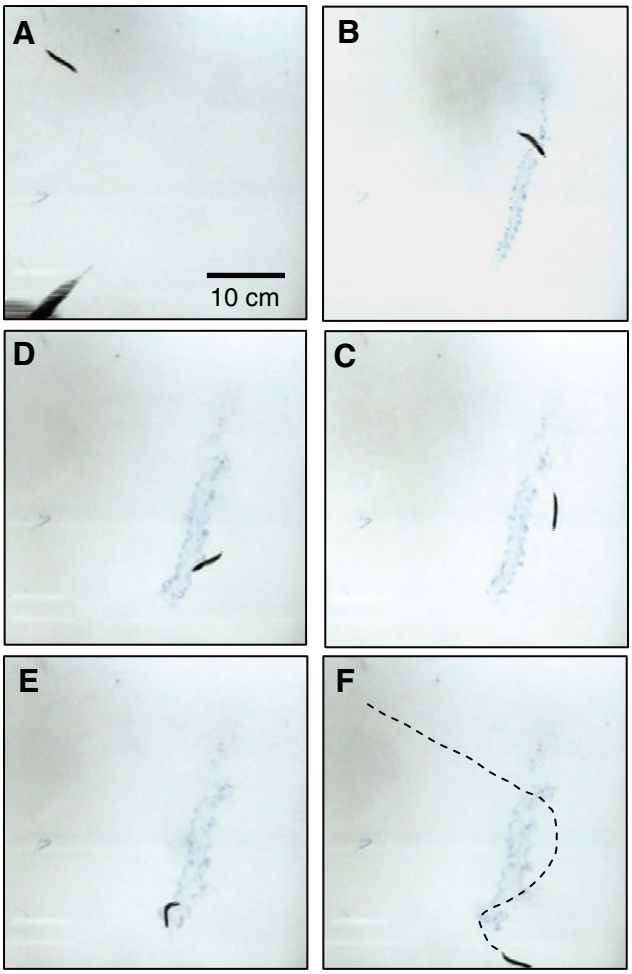

Fig. 2. Weaving pattern of *N. succinea* upon encountering a trail of glutathione at an oblique angle. The concentration of glutathione in the syringe was 10^{-5} mol l^{-1}, yielding a trail (gray streak) concentration somewhat less than 10^{-6} mol l^{-1}. (A–F) Successive images of the path swum by the animal (indicated by the broken line in F). The total time between images A and F was 3 s. The 10 cm calibration applies to all parts.

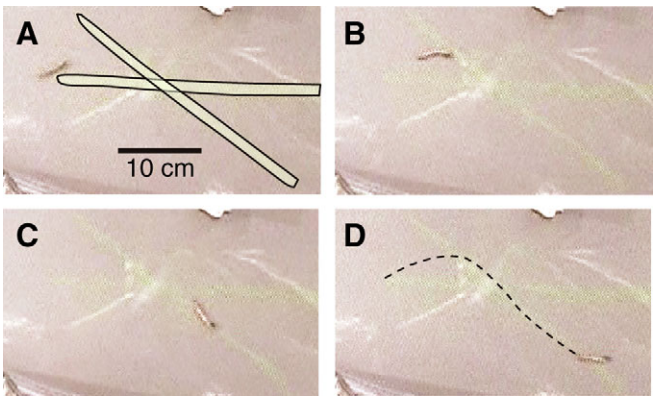

Fig. 3. Trail-following swimming pattern of *N. succinea* upon encountering a trail of CSSG at an oblique angle. (A) Two CSSG trails were present, as outlined. The concentration of CSSG in the syringe was 10^{-7} mol l^{-1}, yielding a trail concentration $<10^{-8}$ mol l^{-1}. (A–D) Successive images of the path swum by the animal (indicated by the broken line in D). The 10 cm calibration in A applies to all parts.

Circle-swimming along the trail

Finally, when male worms encountered pheromone trails at high enough concentrations that the worms were stimulated to turn at least a semicircle to bring them back to the trail, the animals exhibited extensive circling behavior that resulted in relatively long sections of the pheromone trail being encountered. In the example illustrated in Fig. 4, the male worm immediately began circling and speeding up upon encountering a trail at a concentration above 10^{-8} mol l^{-1}. Prior to encountering the pheromone, the worm had been swimming along the aquarium wall at a moderate speed (~85 mm s^{-1}; Fig. 4A) and with some hesitation. Fig. 4B,C shows how the pheromone stimulated rapid circling. A further effect was that the worm dispersed the trail by swimming through it multiple times, so that eventually the worm responded to the decreased concentration of pheromone with larger circles and occasional changes in the direction of circling (Fig. 4D). However, when the worm again swam into undisturbed pheromone at the left end of the trail (Fig. 4E), it resumed swimming in the smaller circles characteristic of the responses of male worms to higher concentrations of pheromone. Eventually, the trail became so dispersed that the worm swam through it without making a sustained turning movement, and thus swam away from the trail with, a now, aroused higher swimming speed (~170 mm s^{-1}; Fig. 4F). The behavior of this animal in response to the pheromone trail is also shown in Movie 1 in the supplementary material. Typically, animals encountering pheromone trails at this concentration circled back to the trail an average of seven times (±1 time, N=17 instances) and usually encountered 10–20 cm of pheromone trail before swimming away.

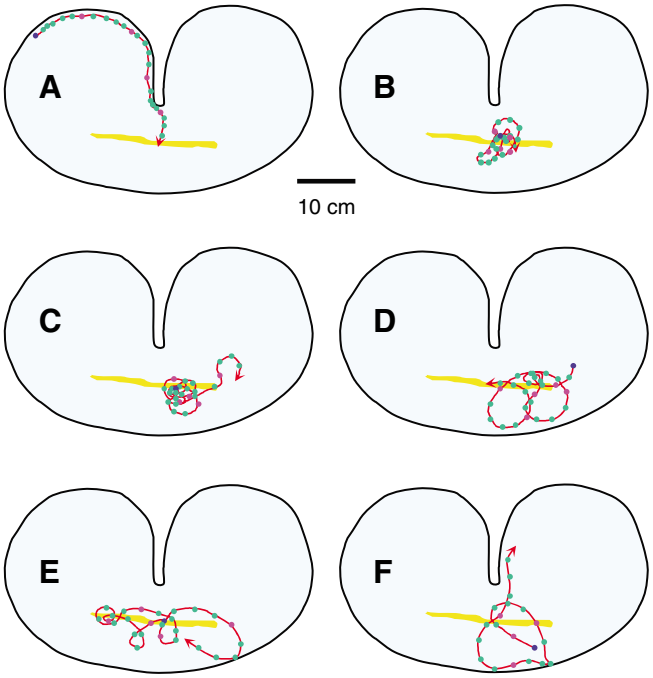

Fig. 4. Response of a male *N. succinea* to a moderate concentration of CSSG. Images show the swim track, plotted every 0.2 s. The concentration of CSSG in the syringe was 10^{-6} mol l^{-1}, yielding a trail concentration <10^{-7} mol l^{-1}. The drawings show the position of the original CSSG trail and do not show the dispersion of the chemical as the worm swims through it (to observe dispersion of the trail, see Movie 1 in the supplementary material). Swim tracks are sequential recordings of 5 s (A–E) and 4 s (F). (A) Typical 'wall-swimming' behavior prior to encountering CSSG. (B,C) CSSG stimulates circling behavior. (D–F) Larger circles result as the worm encounters more dilute dispersed CSSG.

Thus, laboratory experiments demonstrate that the turning and circling responses of male worms to pheromone encountered in linear trails of the chemical stimulate worms to encounter more of the trail. In order to determine whether this behavior would help male worms encounter females that could have produced such trails, the behavior was simulated in a computer model.

Simulation parameters

Parameter values for modeling the sensitivity, rate and duration of arousal of the circling and swim speed behavior of male worms in response to pheromone were determined from information in previous publications, as described below, in order to avoid 'circular reasoning' that would result from choosing parameter values from the behavior to be modeled. Dimensions of the pheromone trail were chosen on the basis of previous measurements of the rate at which females excrete pheromone, and the assumption that the trail was a cylinder of uniform concentration and width that would become dispersed as a result of mixing when animals swam through it. The model was used initially to see if that information alone would yield the trail-following behavior seen in the above experiments. Subsequently, the model was tested for effects of pheromone on the frequency of simulated male encounters with simulated females.

Responses of males to CSSG

Accelerated swim speed

CSSG arouses the swim speed of male *N. succinea* to two- to fourfold pre-arousal speeds. Ram et al. [(Ram et al., 1999) see fig. 4] showed that a representative male accelerated from 50 mm s^{-1} to 180±30 mm s^{-1} (mean ± s.d., average speed 3–5 s after CSSG stimulation) in response to a spawning dose (10^{-6} mol l^{-1}) of CSSG. Similarly, Ram and Hardege [(Ram and Hardege, 2005) see fig. 2)] showed that a male accelerated from ~80 mm s^{-1} to 200 mm s^{-1} in response to 10^{-6} mol l^{-1} CSSG.

The threshold concentration for swimming arousal is one to three orders of magnitude lower than needed to induce spawning. In Woods Hole, males that did not spawn until stimulated with 10^{-6} mol l^{-1} CSSG exhibited accelerated swimming (a bit less than doubling the speed) at 10^{-7} mol l^{-1} (Ram and Hardege, 2005). At Isefjord, males were more sensitive, showing 30–40% increases in swim speeds in response to 10^{-10} mol l^{-1} CSSG (Hardege et al., 2004) and significant 10–20% increases in response to 10^{-10} mol l^{-1} CSSG (Ram and Hardege, 2005). Threshold concentrations for arousal by CSSG varied among animals collected from different locations (J.D.H., unpublished observations). In the model that we illustrate, we used a 'standard'-looking dose–response curve that had a reasonably good match to the previous experimental data: a baseline speed of 50 mm s^{-1}, a 12% increase in speed in response to a concentration of 10^{-9} mol l^{-1}, a doubling of speed at 10^{-7} mol l^{-1} and a 2.6-fold increase in speed at 10^{-6} mol l^{-1} (Fig. 5A).

Latency and duration of the speed arousal response

Figure 4 in our previous study (Ram et al., 1999) also showed that in response to a high concentration of CSSG, male swim speed took ~4 s to accelerate from 50 mm s^{-1} to 180 mm s^{-1} and then slowed to its original level over the next minute. Loss of swimming arousal over the course of about 1 min was also reported for a second animal. The corresponding time course of arousal and relaxation of *N. succinea* swim speed in response to CSSG in the model is illustrated in Fig. 5B.

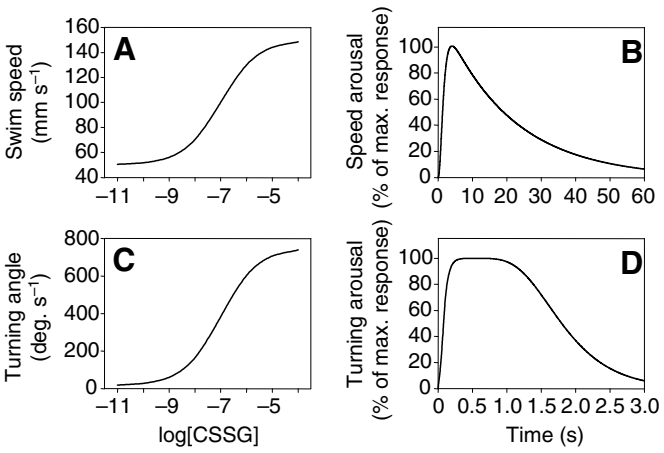

Fig. 5. Dose–response and arousal time-courses used for simulation modeling of male *N. succinea* swimming responses to CSSG.
(A) Dose–response relationship for speed arousal. The equation used was speed=50+(180–50)/{1+10^[(–7–log[CSSG])*0.6]}. (B) Time-course of arousal and duration of the response, as a percentage of the maximum response to the encountered CSSG stimulus. The equation used was %=130*[1–EXP(–time)]^3*EXP(–time/20). (C) Dose–response relationship for turning arousal. The equation used was angle s^{-1}=(750)/{1+10^[(–7–log[CSSG])*0.6]}. (D) Time course of arousal and duration of the turning response, as a percentage of the maximum response to the encountered CSSG stimulus. The equation used was %= 100*[1–EXP(–time/0.05)]^3–[1–EXP(–time/0.05)]*[1–EXP(–time/0.5)]^25. The preceding formulae are written with Excel spreadsheet functions and operators, where EXP() means e raised to the power in the parentheses, ^ means exponential and * means multiplication.

Circling behavior

Circling behavior and aroused swimming speed are independent components of the response to CSSG, with the accelerated swimming out-lasting the circling behavior in time. Ram et al.'s fig. 4 (Ram et al., 1999), showed that upon encounter with pheromone, the male worm initially made a semi-circle ~10 mm in diameter with little increase in speed, and then as the worm accelerated, swam in a circle ~20 mm in diameter. The angle turned per second was ~500° s^{-1}, with the larger diameter circle resulting mainly from the increased speed. Upon leaving the area of the dish in which the pheromone was applied, the animal's speed was maintained at a highly aroused level while the worm curved its swimming path only as much as required because of the perimeter of the dish. Upon re-entry into the 'cloud' of CSSG, the worm resumed circling, with a rate of 600–700° s^{-1}. A video of another animal (http://sun.science.wayne.edu/~jram/N_succinea.htm), shows similar behavior with a maximum turning rate in response to CSSG of ~625° s^{-1}. Previous experiments have not looked closely at turning angles for lower concentrations of pheromone, so we have simply assumed a similar dose–response curve to the one used for speed (Fig. 5C). This assumption seemed reasonable in view of the larger arcs at lower concentrations in experiments reported here (e.g. Fig. 2 and after dispersion of pheromone in Fig. 4).

Arousal, duration and direction of the circling response

At high CSSG concentrations, animals responded quickly, within ~0.2 s of encountering CSSG. If their trajectory did not take them back into the CSSG, their path began to straighten out in less than a second. When a worm is circling rapidly in response to CSSG and re-enters the CSSG location, it usually continues circling in the same direction; however, worms can change their direction of circling, though usually only after its path has begun to straighten out. With low concentrations of CSSG, passing through the CSSG usually produced a brief deflection of their path. Accordingly, this behavior was modeled with a fast rise in arousal of circling, a decrease of circling arousal beginning within a second, and a 50% chance of changing direction of circling when the circling rate was less than a given level (in the illustrated simulations, only at circling rates of <100° s^{-1}). The curve used to model the arousal and duration of circling behavior over time is illustrated in Fig. 5D. The initial direction of turning for any particular response and the possible change in direction at low turning angles were determined probabilistically by comparison with random numbers, with responses occurring equally often to the left as to the right. Since worms also spontaneously turn, although never at such rapid rates as when stimulated with pheromone, the model also includes spontaneous slow turns in either direction in the absence of pheromone.

Dimensions and concentration of the pheromone trail

An estimate of the concentration of CSSG expected to be in a trail is based on data and anecdotal information in previous publications, as follows.

Average excretion rates of CSSG by female *N. succinea* ranged from 20 μg h^{-1} to 150 μg h^{-1} with an average of 68.5±12.5 μg h^{-1} (Hardege et al., 2004).

Swim speeds of females have not been quantitatively reported; however, female *N. succinea* have been reported to swim slower than the non-aroused males. For example, Townsend (Townsend, 1939a) comments "The mechanics of the spawning response appears to be entirely similar in the two sexes but may be more readily observed in the female because of the slower swimming." Lillie and Just's original publication on spawning in these animals (Lillie and Just, 1913) says: "males...dart rapidly through the water...The much larger females then begin to appear, usually swimming laboriously through the water." We have made similar observations (J.L.R., unpublished data). Since the non-aroused males swim an average speed of 50 mm s^{-1} (the parameter used in the simulation model), for purposes of estimating a 'typical' pheromone concentration, we use here an estimated female swim speed of 25 mm s^{-1}, i.e. half that of the non-aroused males.

Swarming females typically range from 3 to 8 mm in diameter (a representative female, with a diameter of 6 mm, is shown in supplementary material Fig. S1). If a female leaves a cylindrical trail of approximately the same diameter, the concentration of CSSG ([CSSG]) in the trail can be calculated as follows:

Length of trail in 1 min: 60×25 mm s^{-1} = 1500 mm

Volume of a 1500 mm cylinder 6 mm in diameter =
1500 mm × $3.14\times(6/2)^2$ = ~0.4 l

70 μg h^{-1} = ~1.1 μg min^{-1} =
(1.1 μg/426.5 g mol^{-1}) min^{-1} = ~2.5×10^{-9} mol min^{-1}

(2.5×10^{-9} mol)/(0.4 l) = ~0.6×10^{-8} mol l^{-1}.

Thus for a 'typical' female, [CSSG] in a trail 6 mm in diameter, excreted at a rate of 70 μg h^{-1} while swimming at 25 mm s^{-1}, would be in the range of 10^{-9} to 10^{-8} mol l^{-1}. Owing to uncertainties in this calculation, the model includes user-adjustable variables for pheromone concentration, trail width, and dispersion of the pheromone in response to simulated worms passing through it.

Simulation results

General observations of the simulated behavior

The movement of male worms with and without a simulated pheromone trail present was simulated. As described in Materials and methods, the speed and direction of swimming are calculated at each interval, *dt*, based on whether simulated pheromone is present at the location of the worm and their associated concentration–response and arousal curves (Fig. 5). Without pheromone present, the simulated worm made occasional small angle turns (Fig. 6A) but rarely turned where the trail would be simulated when pheromone is present.

With a low concentration (log[CSSG]=–8.5), the swim path always showed a deflection when the simulated male crossed the trail. If the simulated worm encountered the trail at right angles (Fig. 6B), such deflections resembled the brief deflections exhibited by real worms (Fig. 1D). If the simulated worm encountered the trail at an oblique angle, the small deflection sometimes caused the simulated worm to cross back over the trail and turn again, as illustrated in Fig. 6C, comparable to the weaving behavior in Fig. 2. In other simulations, with the same starting parameters, the simulated worm might turn away from the trail or alternatively follow right along the trail for some distance (e.g. in the illustrated example, in Fig. 6D, for a distance of about 20 cm), similar to the tracking behavior of the real animal illustrated in Fig. 3. Each run of the simulation produced behavior that is different in detail but similar in the amount of turning upon encountering pheromone. The variability in response is due to the fact that at low turning angle the simulation allows the worm to change direction and it does so with 50:50 probability in each cycle of the simulation.

Higher concentrations of simulated CSSG yielded complex circling behavior, comparable to behaviors observed with real worms encountering higher concentrations of pheromone: multiple turns, dispersion of the pheromone trail, many encounters with the trail as the simulated worm circles back, etc. Examples of complex circling responses in response to log[CSSG]=–8.0 and –7.5 are illustrated in Fig. 6E and F, respectively. In performing these multiple circles, the worm can 'explore' a considerable length of trail even though it may not 'track' along the pheromone trail for as long a distance at each encounter as occurs with lower concentrations of pheromone. In Fig. 6F, although the pheromone becomes dispersed further from the trail than in Fig. 6E, the animal's actual swim trajectory, represented by the thin black line, generally consists of smaller, faster circles than in Fig. 6E, but the higher concentration of pheromone must be spread more broadly before the simulated animal can 'escape' from the cloud of pheromone.

After observing the general properties of the behavior in response to simulated pheromone trails, the simulated behavior was examined quantitatively to determine whether pheromone responses led to significant increases in encountering the trails, following the trails, and finding females on the trails.

Multiple encounters with the pheromone trail

To compare the number of times that the simulated worm crossed the pheromone trail with and without pheromone present, the behavior of the male worm was simulated for [CSSG]=0 (i.e. no trail width), and log[CSSG]=–8.5, –8.0 and –7.5. For each test, the male worm was launched at an angle of 30° from a distance of 5 cm (perpendicular distance) from the pheromone trail. In ten trials at each concentration the number of trail crossings were: no pheromone, 1 (no variation); –8.5, 1.7±0.4; –8.0, 14.3±2.8; and –7.5, 42.5±6.4 (P<0.001, ANOVA on ranks).

Trail-following and length of trail encountered

At each concentration, the longest distance that the simulated worm traveled along the trail before veering off in another direction was measured. The maximum length of simulated trail-following observed in all simulation experiments was ~37 cm. In ten trials, the average maximum tracking lengths were: no pheromone (taken as the width of dispersion from a single crossing, if a trail had been present), 2 cm; –8.5, 10.5±3.6 cm; –8.0, 4.8±0.3 cm; and –7.5, 5.3±0.4 cm (P<0.001, ANOVA on ranks).

The relatively shorter maximum tracking lengths at higher pheromone concentrations is due to the fact that the simulated worms usually circled and hence did not 'track' along the chemical trail. However, since they often circled back onto the trail many times, they also often encountered a considerable length of the trail overall. Adding up the length of 'disturbed pheromone', the total length of trail encountered (out of a total possible length of 75 cm) was: no pheromone (only a single crossing of the trail, equivalent length that would have been dispersed), 2 cm; –8.5, 11.7±3.7 cm; –8.0, 15.9±2.8 cm; and –7.5, 26.9±5.3 cm (P<0.001, ANOVA on ranks).

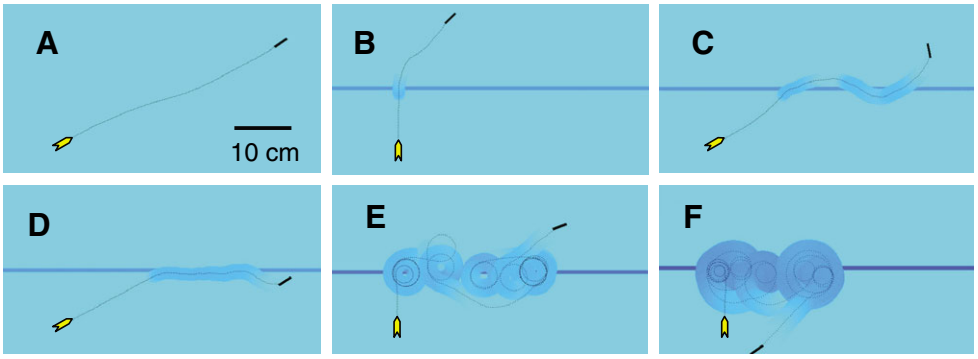

Fig. 6. Simulated behavior of male *N. succinea* in response to various concentrations of CSSG. The direction and starting point of the worm in the simulation is shown by the yellow arrow in each illustration; the trajectory of the worm is indicated by the narrow black line; the simulated final position of the worm when the simulation was terminated is shown by the short black line; and the dispersion of pheromone by the simulated swimming of the worm through it is shown by the spread of the blue color of the initial line of pheromone (initially approximately 7 mm wide). Concentrations of pheromone in the simulated 'trail' initially were (A) 0 (control, no pheromone), (B–D) log[CSSG]=–8.5 (i.e. 3.16×10^{-9} mol l^{-1}), (E) log[CSSG]=–8.0 (i.e. 10^{-8} mol l^{-1}), and (F) log[CSSG]=–8.5 (i.e. 3.16×10^{-8} mol l^{-1}). The 10 cm calibration applies to all parts.

Probability of encountering a female on the trail

To determine whether the search strategy simulated in this model would result in a more likely encounter with a female on the trail, simulated males were 'launched' at various angles from a start position of 5 cm (shortest distance of a perpendicular path) from the CSSG trail and ~20 cm to the left of a virtual female on the trail (see inset of Fig. 7). The virtual female was thus at an angle of about 15° from the male's starting position (where 0° is parallel to the pheromone trail towards the right). Simulations without a pheromone trail present (trail of width '0') placed the simulated female in the same relative position with respect to the male.

In order to analyze the result for males starting off in a wide range of directions, simulated males were launched 20 times at 5° increment of the starting angle, from 0° (heading directly to the right) to 180° (heading directly to the left). As illustrated in Fig. 7A, having the pheromone present spread out the angles at which a random swim of the male would result in encountering the female. All mating encounters under control conditions occurred for simulated worms starting at angles between 0° and 30°, i.e. heading in the direction of the female to start with, plus a little variation due to spontaneous turns. A simulated male starting off at an angle of 45° under control conditions *never* encountered the female in the absence of the pheromone, but with pheromone present the male turns appropriately to find her about 10% of the time. At the highest concentration tested, even males initially heading in the wrong direction (in some cases starting out at 160°) still managed to turn to the right direction and explore enough of the pheromone trail to encounter the female a significant percentage of the time.

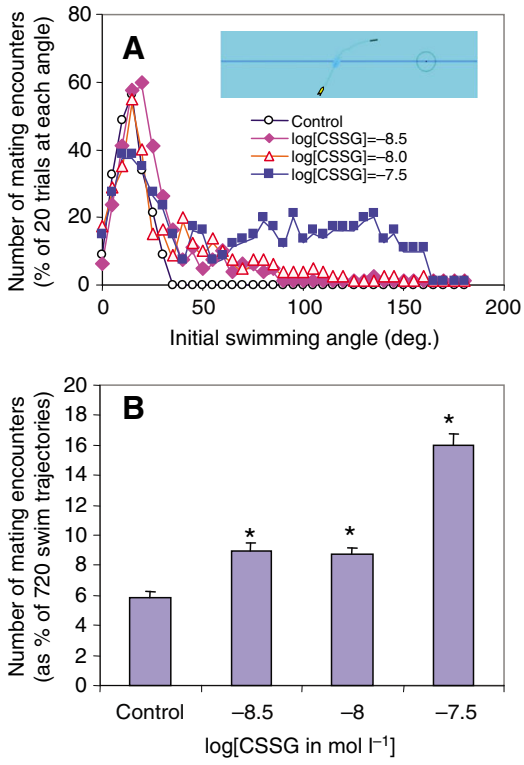

Fig. 7. Percentage of simulated male trajectories resulting in a mating encounter with a female, i.e. coming within 3 cm of a virtual female located on the pheromone trail. The female is located ~20 cm to the right of the closest point of the trail from the simulated male. The male starts 5 cm from the trail, so that the female is at an angle of ~15° from the male (see inset), where 0° is parallel to the pheromone trail, to the right. The simulated male was 'launched' 20 times at each angle, in 5° increments. (A) Percentage of trajectories out of 20 at each launch angle resulting in a mating encounter. (B) Overall percentage of trajectories (out of a total of 720 at all angles) resulting in mating encounters. ANOVA, $P<0.001$; *$P<0.05$, pairwise Tukey's test, compared to control.

Overall, considering all 720 swim trajectories, under control conditions (i.e. no pheromone trail present), the simulated male encountered the female only 6% of the time. With pheromone present, the percentage of trials that resulted in encountering the female increased significantly, approximately doubling at log[CSSG]=–8.5 and increasing approximately threefold at log[CSSG]=–7.5 (ANOVA, $P<0.001$; all concentrations significantly greater than control, $P<0.05$, Tukey's test; Fig. 7B).

Thus, the model not only mimics many aspects of the worm's real behavior in response to pheromone trails, it also shows that this pheromone-guided behavior can help male worms find their mates.

DISCUSSION

This paper supports the hypothesis that the female *N. succinea* pheromone CSSG has a dual function as a trail to guide males to find females at low concentration and as an activator of male spawning at high concentration. A combination of behavioral measurements and computer simulation enabled us to show that male *N. succinea* can respond to linear trails of pheromone and that such responses can increase the frequency with which males encounter females on the trails.

Behavioral data in this paper show that linear distributions of pheromone can stimulate turning and increase swim speed of male worms. This stimulus geometry differs from all previous studies of *N. succinea*, which tested males either with a point stimulus (Hardege et al., 2004; Ram and Hardege, 2005; Ram et al., 1999; Zeeck et al., 1998b), or with a uniform 'bath' concentration (Ram et al., 1999; Zeeck et al., 1998b). By developing the 'rounded heart' aquarium configuration, we were able study encounters of animals with linear trails. Upon encountering low concentration trails of pheromone at an oblique angle in the present study, male worms sometimes swam directly along the trail, an average distance of 8 cm. At higher concentrations of pheromone, albeit still lower than the amount needed to elicit spawning, circling behavior stimulated by the pheromone results in even longer sections of the pheromone trail being explored.

The use of computer simulation enabled us to test hypotheses that would have been difficult to investigate otherwise. This study used computer simulation to examine whether the responses of male *N. succinea* would facilitate mating encounters of male worms with females. Computer simulation enabled the repetition of experiments and the testing of a large range of pheromone concentrations, swimming directions, etc. that would have been well-nigh impossible to accomplish with the animals themselves because of some of their biological characteristics. As noted in the Introduction, once males get close enough to dance with the females, the females 'spawn out' and die, which would prevent a repeat of the experiment with the same animal under the same or different conditions. Although males can respond to the pheromone repeatedly, they too weaken after repeated spawning (Ram and Hardege, 2005). Moreover, the need for filtering and/or changing of water when working with pheromones may cause disturbance of animals from transfer or turbulence. The need for calm weather for the nuptial dances to occur has been noted anecdotally (Costello and Henley, 1971). Computer simulation is, of course, not affected by the vagaries of weather and can readily re-run experiments.

Computer simulation of the responses of male worms to pheromone, based on previous observations of female excretion

of pheromone and of male turning and acceleration aroused by the pheromone, approximately reproduced the behavioral responses to linear trails of pheromone observed in the present study. By setting parameters using data from prior publications, the model was created without the circular reasoning that would result from choosing parameters from the behavior to be modeled. Quantitative estimates of pheromone sensitivity do, however, differ somewhat among the various previous publications, possibly because they were obtained with animals from different global locations (Woods Hole, MA, USA and Isefjord, Denmark) that may differ in salinity, temperature, early or late season of experiments, etc. The sensitivity and arousal curves used in the present model therefore produce only a representative set of responses. Variations in these parameters can be used in future studies of the robustness of the simulated behavior over a range of sensitivities, and effects of salinity and seasonality on reproductive behavior and the sensitivity to pheromone can be compared to predictions from the model.

The simulation model was further applied to answer the question of whether this behavior would be likely to increase the likelihood that a male would encounter a female. For example, there is no guarantee upon following a trail that the male would be heading in the right direction. Would heading in the right direction along the trail only about half of the time still result in more frequent encounters with females than ignoring the pheromone trail altogether? Application of the simulation model to this question demonstrated that simulated male worms do indeed encounter virtual females on the trail significantly more frequently than would occur by chance.

The above studies support the hypothesis that male *N. succinea* can find females at the water surface by following pheromone trails excreted by the females. Previous descriptions of *N. succinea* mating behavior have been little more than anecdotal. Lillie and Just (Lillie and Just, 1913) commented that "When a female appears she is soon surrounded by several males... which swim rapidly in narrow circles about her." Townsend (Townsend, 1939b), commenting on the lack of detail regarding the spawning behavior of another species, the Atlantic Palolo worm, by Mayer (Mayer, 1908) says that he "gave no fuller account of the swarming reactions... than that it swam westward, spiraling in a clockwise direction." A more recent commentary (Costello and Henley, 1971) on *N. succinea* notes only that "males will appear first.…The females appear later; fewer in number and swimming more slowly than the males.…males which approach within a certain orbit will deviate from their original spiral paths to swim actively around her in rapidly narrowing circles…" Quantitative videographic studies of the behavior prior to spawning would help to determine whether details of the behavior match that predicted by the model.

A constant release of CSSG by females is assumed in the pheromone trail hypothesis, but the moment-to-moment pattern of excretion is actually unknown. Townsend (Townsend, 1939b) and others (Hardege et al., 2004) have described the large amounts of pheromone released by females over periods of hours. For example, Townsend (Townsend, 1939b) measured the accumulated amounts of 'spawning inducing material' excreted into a bowl of seawater by a swimming female in successive 2 h time periods. Although such measurements document the large total amounts of pheromone released by female worms, measurements of cumulative amounts over long periods give no details as to whether the release may be pulsatile, directional or constant. Measurements with a finer time and spatial resolution are needed. Ultimately, simulations modeling both male and female behavior would provide the best test of the roles of pheromone trails in reproductive success.

This paper demonstrates how a pheromone may have a dual use, eliciting one type of behavior at high concentration and short range, and a different, though related, behavior at longer distance and lower concentrations. CSSG has previously been clearly demonstrated to be a male spawning pheromone for *N. succinea* at high concentrations, above 10^{-7} mol l^{-1} (Ram et al., 1999). This paper shows that the 'non-spawning' swim arousal effects of lower concentrations of CSSG can be exploited by females as a seductive signal to attract and guide male worms to their spawning encounters. Previously, Painter et al. (Painter et al., 2003) investigated attraction and mating effects of attractin, a waterborne peptide pheromone in the marine gastropod *Aplysia*, and concluded that they could not distinguish multiple activities of the pheromone from an alternative interpretation in which the main effect is to increase the 'desire to mate'. In fact, attractin alone does not cause significant attraction of animals but only facilitates turning in t-maze tests when other attractive factors (e.g. a potential mate) are present (Painter et al., 2003). The present study is the first to show that the pheromone alone is enough to cause both the spawning response (Ram et al., 1999) and the behavioral responses that can result in mate-finding.

Overall, the function of the CSSG pheromone is interesting for future studies on many levels. Although Ram et al. (Ram et al., 1999) reported that electrophysiological responses could be activated by CSSG applied near the anterior of semi-intact male worms, the sensory receptors for the pheromone have yet to be clearly identified. The 'decision-making' process by which electrophysiological activity in the male results in left or right turns and maintains that direction above a certain stimulus level in response to the pheromone, as described in the simulation model, is unknown. The molecular receptors and transduction mechanisms for glutathionergic compounds used as extracellular informational molecules [in the case of CSSG as a pheromone, and in hydra as an activator of ingestion behavior (Sakaguchi et al., 1991)] are also completely unknown. Moreover, in the female, regulatory mechanisms controlling the synthesis and release of the pheromone are almost completely unexplored. Thus, the multiple behaviors elicited by CSSG to mediate reproduction in *N. succinea* are an excellent area in which new molecular, neurophysiological, and reproductive mechanisms may be discovered.

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