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Sensory Regulation of Male Mating Behavior in Caenorhabditis elegans

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Summary

C. elegans male mating behavior comprises a series of steps: response to contact with the hermaphrodite, backing along her body, turning around her head or tail, location of the vulva, insertion of the two copulatory spicules into the vulva, and sperm transfer. By ablation of male-specific copulatory structures and their associated neurons, we have identified sensory structures and neurons that participate in each of these steps: the sensory rays mediate response to contact and turning; the hook, the postcloacal sensilla, and the spicules mediate vulva location; the spicules also mediate spicule insertion and regulate sperm transfer. Generally, successful completion of each step places the male in a position to receive a cue for the next step in the pathway. However, the high degree of sensory regulation allows the male to execute some steps independently.

Introduction

Understanding the cellular basis of a behavior requires that the neurons mediating sensory input, any internal processing, and the motor output all be identified and delineated in a pathway by both morphology and physiology. This daunting task can be simplified by studying invertebrate systems with their smaller nervous systems and often stereotyped behaviors, which allow better control of measurements and hypothesis testing. The task can be simplified further by focusing on the question of how a response behavior is generated and modified. Two experimental approaches to answer this question are the identification of the parts of the nervous system that mediate this behavior (by means of lesions and electrophysiology) and the elucidation of the mechanisms by which these neurons serve their function (by means of electrophysiology and genetic and molecular analysis). The large, identifiable neurons of such organisms as lobster, leech, and Aplysia have allowed the functional mapping of neuronal circuits. In contrast, whereas electrophysiology is limited in Drosophila and nearly nonexistent in Caenorhabditis elegans (however, see Raizen and Avery, 1994), these two systems are amenable to genetic analysis. For example, Drosophila neurogenetics has shed light on learning, biological clocks, and courtship behavior (Quinn and Greenspan, 1984; Davis, 1993; Dunlap, 1993; Hall, 1994).

In addition to genetic analysis, cell ablations in C. elegans can be used to assign behavioral roles to neurons (developed by Sulston and White, 1980; modified by Avery and Horvitz, 1987, 1989). Candidate neurons are ablated with a laser microbeam, and the behavior of the operated animal is compared with the behavior of intact animals. Behavioral defects, if any, suggest that the ablated neurons are involved in that behavior. As the number of neurons in the animal is small (302 in the hermaphrodite, 381 in the male) and as each neuron is identifiable by lineage and position, systematic ablation of individual cells is feasible. The combination of ablation of identified cell types and genetic studies has led to dissection of a significant part of the worm behavioral repertoire, including response to touch (Chalfie et al., 1985; Kaplan and Horvitz, 1993), chemotaxis (Bargmann and Horvitz, 1991), and pharyngeal pumping (Avery and Horvitz, 1989).

Compared with the above behaviors, male mating behavior, comprising a series of sub-behaviors or steps, is arguably the most complex behavior exhibited by this small nematode. Yet each step in the behavior is highly stereotyped, suggesting an innate motor program. This reproducibility allows description, reported by Hodgkin (1974), Dusenbery (1980), Baird et al. (1992), and J. Sulston (personal communication), and extended by us. When the posterior part of a C. elegans male comes into contact with a hermaphrodite, the male responds by placing the ventral side of his tail against her and proceeding to swim backwards along the length of her body (Figure 1A), turning via a deep ventral flexion around either her head or tail (Figure 1B), until he locates the hermaphrodite vulva. At this point, he stops (Figure 1C), inserts a pair of copulatory structures called the spicules (Figure 1D), and transfers sperm. Hermaphrodites, which are internally self-fertilizing, appear to play little or no active role in this process.

In organisms as distantly related as rats (Breedlove, 1986) and insects (Schneiderman and Hildebrand, 1985), sex-specific behaviors result from sexually dimorphic nervous systems. In C. elegans, one of two classes of hermaphrodite-specific neurons mediates the hermaphrodite-specific behavior of egg laying. Male-specific mating behavior is likely mediated by the male-specific neurons. As a testament to the complexity of the behavior, in contrast with the hermaphrodite, which has only 8 sex-specific neurons, the male has 87 (almost a quarter of the entire male nervous system). All but 4 of these neurons are associated with a set of male-specific structures collected in the posterior part of his body, referred to as the male tail (Figure 2).

The structures of the male tail have been described by Sulston et al. (1980). From anatomical studies, they are all believed to be sensory. Each male is endowed with 9 bilateral pairs of sensory rays that spread out on both sides of the male (Figure 2A); each ray is composed of 2 neurons and a structural cell. On the ventral side, just anterior to the cloaca, is a sensory organ called the "hook," which consists of 2 sensory neurons, 2 support cells, and a structural cell. Just posterior to the cloaca are a pair of bilaterally symmetrical sensilla, called the postcloacal sensilla. Each sensilla contains 3 sensory neurons and 3 support cells. Within the cloaca, there exists a pair of cuticularized structures called the spicules (Figure 2B); each spicule is com-

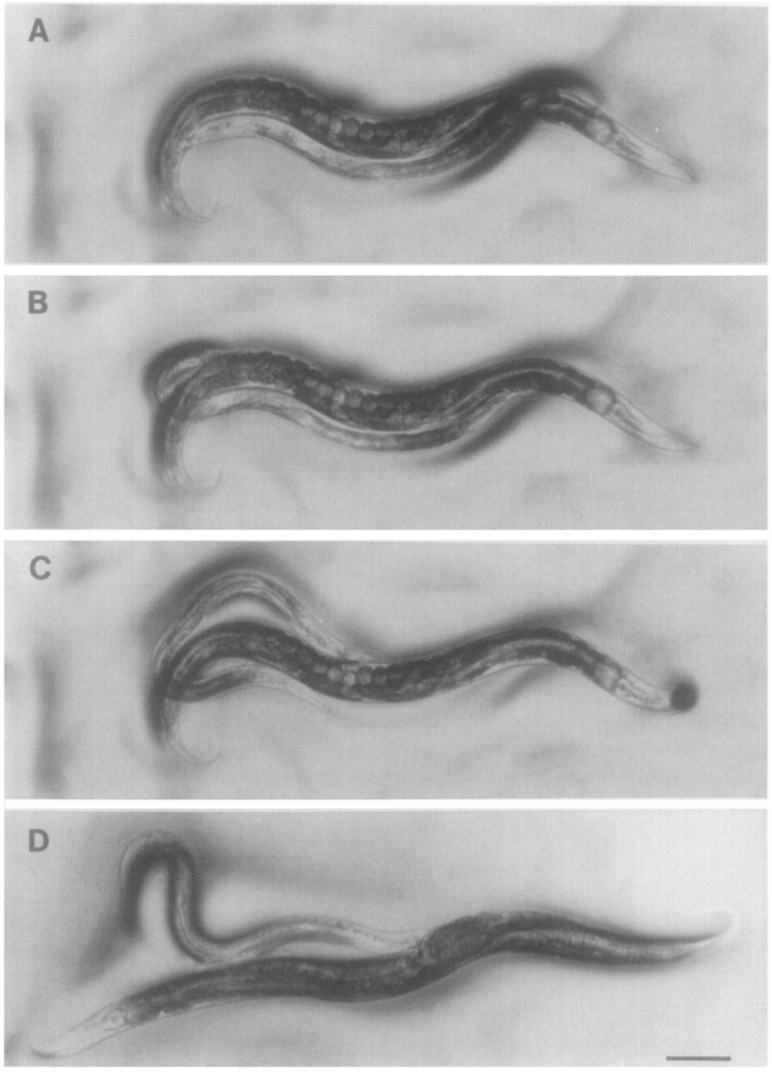


Figure 1. The Sequence of Steps in Male Mating Behavior in C. elegans

(A) Response to contact with the hermaphrodite. The male arches the posterior third of his body such that the ventral side of his tail, containing the copulatory apparati, is apposed to the hermaphrodite.

(B) Male turning from the dorsal side of the hermaphrodite tail to the ventral side. The turn, consisting of a deep ventral flexion of the male's tail, is initiated before he reaches the end of the hermaphrodite.

(C) Male at the hermaphrodite vulva.

(D) Insertion of the copulatory structures called the spicules. Bar, 0.1 mm.

posed of 6 structural cells, 2 sensory neurons, and a motor neuron also thought to be proprioceptive in nature.

In our analysis, we have quantified many of the steps of mating behavior to better understand the behavior in the intact animal and to serve as a basis for determining defective behavior in altered animals. Subsequently, we ablated male-specific structures and associated neurons to infer their roles in mating behavior.

Results

Mating Behavior in Intact Animals

Here we discuss mating behavior in the intact animal and our criteria for behavioral defects. For all measurements given, n = 23, unless otherwise stated.

Response to Contact

When the male tail comes into contact with a hermaphrodite (Figure 1A), the male responds by apposing the ventral side of his tail to her body and swimming backwards along the length of her body. If contact is made with the ventral side of the male tail, he proceeds to swim backwards, keeping the posterior third of his body rigid against her (the rest of the body moves in a sinusoidal motion that is normal for swimming). If contact is made with the dorsal side, response entails swimming backwards with a ventral arching of the posterior sixth of his body until the ventral side comes into contact with the hermaphrodite. Thus, what we call response to contact is composed of three parts: the halting of forward motion, the placement of the ventral side of the male tail against the hermaphrodite, and the start of backwards swimming. To be classified as defective in response to contact, the males must fail to perform the last two substeps.

Turning

As the male approaches either the hermaphrodite head or tail (Figure 1B), he turns around the head or tail to the other side of the hermaphrodite via a sharp ventral arch of his tail. The turn is initiated before he reaches the end of the hermaphrodite, with approximately one-twelfth (estimated by eye from observation and micrographs) of the body length left to travel. Thus, there are two components to a proper turn: a sharp ventral arch (executed by male-

specific muscles and motoneurons [Loer and Kenyon, 1993]) and proper timing of the arching behavior. Even if he completes a turn, a male is considered defective in this step if either one of these components is defective.

Vulva Location

The male continues to swim backwards until he locates the hermaphrodite vulva, where he stops (Figure 1C). More than 95% of the time, males stop backing upon first encounter with the vulva (taking about 8.5 ± 1.5 s). Occasionally, the male will stop beyond the vulva. If he is within 10% of a hermaphrodite body length, he will more likely back up slowly (i.e., swim forwards) to relocate the vulva. Beyond that distance, he will more likely continue to swim backwards until he encounters the vulva again. Once the male locates the vulva, he slows his swimming speed and adjusts his position via a short back and forth motion, covering about 10% of the hermaphrodite body. During this time, the male's spicules are extruded, the posterior third of his body is no longer kept rigid, and his pharyngeal pumping rate decreases dramatically from an average of 180 pumps per minute to 50 ± 16 (n = 16).

Thus, what we call vulva location is composed of two parts: the cessation of backward motion along the hermaphrodite, at the approximate location, and the adjustment of position via a slow back and forth motion in the vicinity of the vulva, to find the precise location. For the purposes of scoring behavioral defects, brief hesitations around the vulva without subsequent adjustment behavior were not considered sufficient for vulva location behavior.

Spicule Insertion

Spicule insertion and adjustment of vulva location are functionally equivalent and behaviorally inseparable except by ablation (Figure 1D). The spicules are extruded while the male searches for the precise location of the vulva, and they can be seen depressing the hermaphrodite cuticle. The male continues to extrude spicules until they are inserted into the vulva, anchoring the male in place.

For reasons that are unclear, spicule insertion is a difficult step for the males to accomplish. In almost three-quarters of the attempts, males swim away after being unable to insert their spicules, after attempting for an average of 210 ± 45 s. Observations indicate that this spicule insertion "problem" is hermaphrodite specific (see Experimental Procedures). If a male does succeed in inserting his spicules, it usually happens quickly, taking an average of 20 ± 2 s.

Sperm Transfer

After spicule insertion, sperm is released from the seminal vesicle, through the vas deferens and cloaca and into the vulva (between 30–180 sperm per mating). The anal sphincter is contracted at this time, presumably to open the cloaca, but the active mechanism, if any, that transports the sperm is not known (Sulston et al., 1980). The time it takes for sperm transfer is about 4 s (from the start of fluid flow to the end). The male continues to keep his spicules inserted after the completion of sperm transfer for about 27 ± 5 s. In other Caenorhabditis species and in the *plg-1* strain, males lay down a copulatory plug over the vulva at this time (J. Hodgkin, personal communications; unpublished data; Barker, 1994). Presumably, the "plugless" laboratory (Bristol) strain has retained the quiescent behavior. After the male "releases" the hermaphrodite, the spicules slowly retract, taking about another 20 ± 3 s. The male slowly begins swimming forwards again and foraging, with pharyngeal pumping returning to normal.

Cell Ablations

Rationale

To determine the possible roles of male-specific sensory structures in male mating, we ablated them individually and in combinations and observed subsequent mating behavior for defects. Our strategy was first to ablate blast cells from which the sensilla are derived, eliminating entire groups of male-specific cells. If a behavioral defect was observed, the progeny of the blast cell were then systematically ablated, following the behavioral defect to the single-cell level. Daughter cells that did not give rise to the behavioral defect serve as internal controls for sisters that did, as these animals were subjected to the same treatment as those that did exhibit the defect. If at any time the defect was not attributable to a single cell, both daughter cells were ablated to determine whether the behavior is mediated by both cells or their progeny. If ablation of a blast cell did not give rise to a behavioral defect, other cells that are candidates for a redundant function were ablated in conjunction. Because of the large number of cells in question, not all combinations could be tested, but many logical combinations (because of proximity, type, lineage, or known function) were. In this way we were able to identify systematically the roles of many of the male-specific neurons in male mating behavior.

This approach addresses another potential problem: when we eliminate a precursor cell, we can be certain that its progeny and their function have been removed. However, it is possible that the targeted cells may be replaced by a neighboring, intact cell. Thus, lack of an observed defect may be due to replacement of a necessary cell. Conversely, a noted defect may be due to loss of a neighboring cell to replace the ablated cell. Ablation of terminal cells in the lineage avoids this problem of possible regulation of cell fates. However, it is conceivable that cell function is not completely eliminated (but see Avery and Horvitz, 1989). We therefore used both protocols when possible.

For the purposes of these studies, we assume these neurons are correctly classified as sensory. Functional studies of hermaphrodite neurons, also classified as sensory by morphology, have verified their classification (Davis et al., 1986).

The Sensory Rays

The blast cells V5L/R.p, V6L/R.p, and TL/R.ap generate 9 bilateral pairs of sensory rays, numbered 1 (anterior) to 9 (posterior) (Figure 2A). Each ray is composed of a single structural cell and 2 sensory neurons, with processes that extend the length of the ray. The sensory neurons are designated RnA and RnB, where n is the number assigned the ray. The neurons have access to the outside via a sensory opening in all ray pairs but ray 6 (Sulston and Horvitz, 1977; see also Baird et al., 1991). Because of the large number of rays, we divided them into subgroups

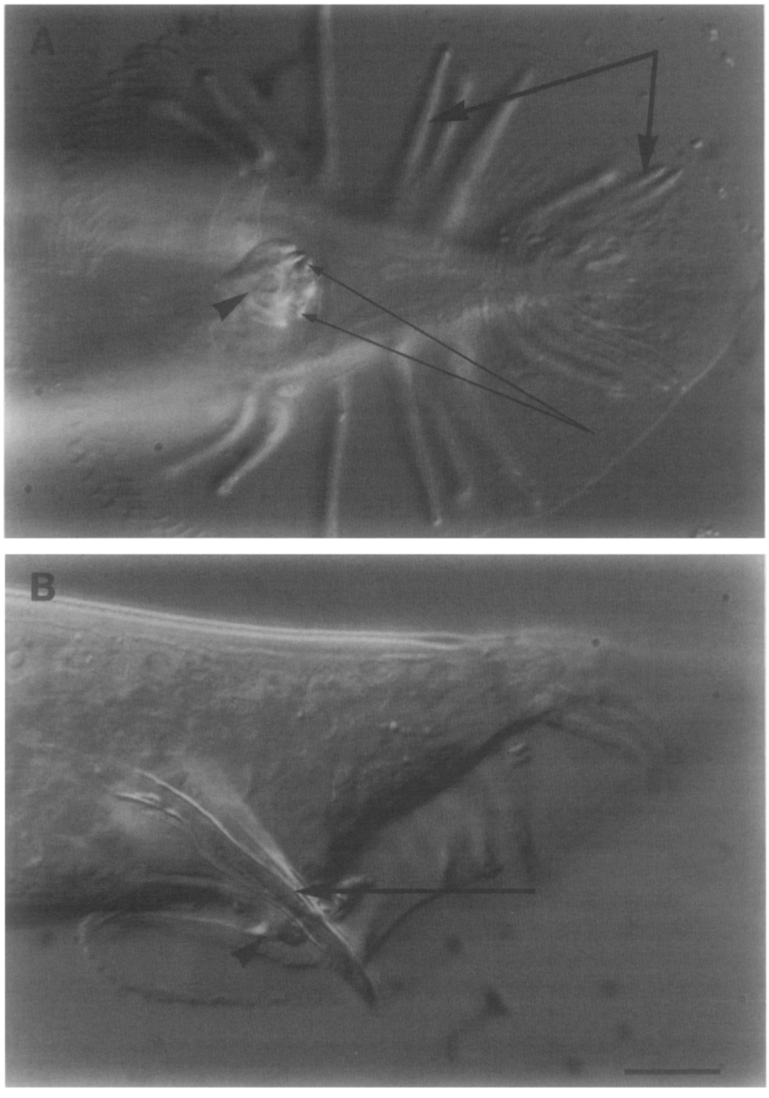


Figure 2. The Male Tail Contains All of the Copulatory Structures of the Male and Most of His Sex-Specific Neurons

(A) Nomarski micrograph of a ventral view of the male tail showing 9 bilateral pairs of sensory rays (thick arrows), the hook and associated sensillum just anterior to the cloaca (arrowhead), and a pair of postcloacal sensilla (thin arrows), named because of their position.

(B) Lateral view of the male tail (ventral is down) showing the left homolog of a pair of extendible cuticular structures called the spicules (thick arrow). The hook (arrowhead) and some rays can be seen as well. Bar, 0.01 mm.

based on location of their sensory openings (Table 1), position of rays along the animal (Table 2), and neurotransmitter phenotype.

The sensory openings of rays 1, 5, and 7 open to the dorsal side of the animal. The sensory openings of rays 2, 4, and 8 open ventrally. Rays 3 and 9 open laterally. Ray 6 has no sensory openings to the external environment. To determine the significance of the positions of these sensory openings, we ablated only the dorsally opening rays. Operated males fail to respond to dorsal contact with the hermaphrodite but respond normally to ventral contact. We infer that the rays require direct contact with the sensory openings to mediate response to the hermaphrodite. Ablation of the ventrally opening rays results in no observable defect. Ablation of the laterally opening rays and ray 6 also leads to no observable defects, either alone or in combination with any rays other than the dorsally opening rays. Ablation of all but the dorsally opening rays gives no observable defect. Thus, the dorsally opening rays are both necessary and sufficient to mediate response to dorsal contact. Also, the lack of a defect after ablation of the ventral rays is not due to redundancy of function with the lateral or closed rays.

To test whether the ability to respond to contact after the ablation of the ventrally opening rays is due to the presence of additional ventral sensory structures, namely the hook, postcloacal sensilla, and spicules, we ablated all ventral organs via their precursors and the ventrally opening rays (see above). These animals do not respond to ventral contact with the hermaphrodite. In response to dorsal touch, the males curl their tails ventrally, reminiscent of contact response behavior, but do not pursue contact (backing) with the hermaphrodite. When only the hook, postcloacal sensilla, and spicules are ablated, leaving the ventrally opening rays intact, the operated males respond to ventral (and dorsal) contact and back along the hermaphrodite, but fail to locate the vulva. This observation suggests that the ventral sensory structures are redundant with the ventrally opening rays for the maintenance of search behavior along the hermaphrodite. However, the presence

Table 1. Sensory Ray Ablations by Position of Sensory Opening

Structures Ablated	Effect (removes)	Response to Dorsal Contact		Response to Ventral Contact	
		By Males	By Trial	By Males	By Trial
None	None	10/10	97/100	10/10	98/100
Rays 1, 5, 7	All dorsal pairs	0/10	0/80	10/10	24/24
Rays 2, 4, 8	All ventral pairs	10/10	64/66	10/10	45/45
Rays 3, 9	Both lateral pairs	10/10	52/52	10/10	24/24
Ray 6	Closed pair	10/10	44/44	10/10	21/21
Rays 2, 3, 4, 6, 8, 9	All but dorsal pairs	7/7	46/48	7/7	35/36
Rays 2, 4, 8 plus hook, p.c.s., spicules	All ventral sensilla	8/8[a]	64/64	0/8	0/22
Hook, p.c.s., spicules	All non-ray ventral sensilla	10/10	99/100	10/10	96/100

p.c.s., postcloacal sensilla.
[a] All males exhibited ventral arching of the tail upon dorsal contact with hermaphrodites but did not pursue hermaphrodites following either dorsal or ventral contact.

of the ventral sensory structures alone is not sufficient to mediate the initiation of backing behavior. Hence, the initiation of backing behavior and its maintenance are mediated by distinct sets of sensilla.

Because the male swims backwards during mating behavior, the most posterior rays precede the rest of the animal. The 3 most posterior pairs of rays are generated by the bilateral pair of blast cells, TL/R.ap (which also generate two hypodermal cells and a non-sex-specific interneuron). The 6 more anterior pairs of rays are generated from the blast cells V5L/R.p and V6L/R.p (which also generate a pair of sensilla called the postdeirids and some hypodermal cells).

Ablation of V5L/R.p and V6L/R.p, which generate the 6 anterior pairs of sensory rays, results in males missing these rays. These males are incapable of responding to contact with hermaphrodites, in that they continue to swim forwards, passing the hermaphrodite. This lack of response is not simply due to insufficient numbers of rays remaining to transmit a signal (the remaining 3 pairs of T-derived rays). Ablation of the T-derived rays along with 3 of the 6 V-derived rays results in males that respond at normal efficiency. This control demonstrates that 3 pairs of rays are adequate for mediating response to contact; however, it does not account for position. It may be that the T-derived rays are not in the position to serve this function, being at the most posterior end of the male. There is no obvious difference in morphology between the T- and V-derived rays; thus, the functional difference between them may result from differences in position and wiring rather than innate differences in the two lineages.

Ablation of TL/R.ap, which gives rise to the 3 most posterior pairs of rays, results in males that are missing the T-derived rays. Whereas intact males initiate a turn (deep ventral flexion of the posterior quarter of the male) at the last twelfth of the hermaphrodite body (Figure 1B), TL/R.ap-ablated males swim past the end of the hermaphrodite, losing contact and ending with their tail in a tight ventral coil (Figure 3B). These observations suggest that the males fail to turn at the appropriate time, while the tight ventral coil remains intact.

The A neurons of rays 5, 7, and 9 express dopamine and are likely dopaminergic (Sulston et al., 1975). Ablation of these neurons results in males that turn at the end of the hermaphrodite but tend to do so in a large, sloppy arc instead of the tight ventral coil seen in intact animals (Figure 3C). These observations suggest that the males have kept the timing of the turn intact but have lost the

Table 2. Sensory Ray Ablations by Position of Rays along Anterior–Posterior Axis

Structures Ablated	Effect (removes)	Response		Turning	
		By Males	By Trial	By Males	By Trial
None	None	10/10	100/100	10/10	100/100
Rays 1–6	6 most anterior pairs (V rays)	0/10	0/100	NA	NA
Rays 7–9	3 most posterior pairs (T rays)	14/14	140/140	0/14	0/140
Rays 1–3, 7–9	All but 3 middle pairs	10/10	58/62	0/10	[a]
Rays 4–6, 7–9	All but 3 most anterior pairs	10/10	49/49	0/10	[a]

NA, not applicable.
[a] Turning defects were not recorded in these experiments, as it had already been demonstrated that ablation of the T-derived rays eliminated turning. These ablations were done to see whether they had any effect on response.

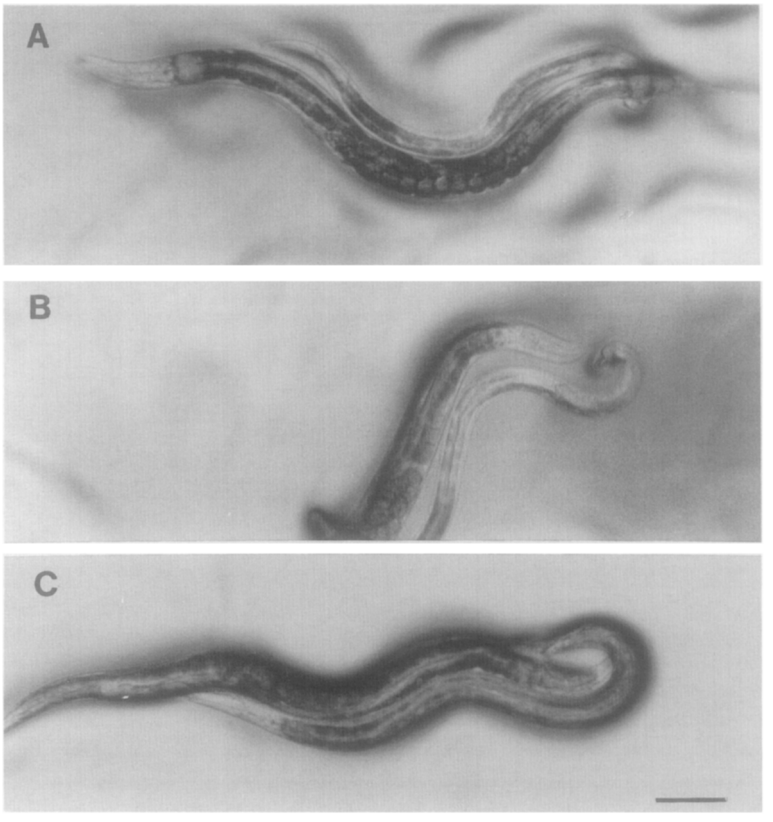


Figure 3. Comparison of Turning Defects Resulting from Sensory Ray Ablations

Note the position of the hermaphrodite head/tail in relation to the male tail.

(A) Turning in the intact male. Males turn via a sharp ventral flexion of the posterior before they reach the end of the hermaphrodite.

(B) Ablation of the posteriorly positioned T-derived rays results in males that fail to turn at the appropriate time. Instead, they swim off the end of the hermaphrodite, coiling ventrally.

(C) Ablation of the dopamine-containing rays (5, 7, and 9) results in males that turn in a wide loop, as opposed to the sharp ventral coil of the intact male. Bar, 0.1 mm.

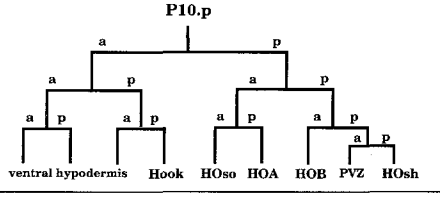
tight ventral coil. Ablation of the corresponding B neurons results in males with no behavioral defects. The RnA ablation defect is similar to the behavioral defect of *cat-2* males (K. Liu, unpublished data), which are dopamine deficient (Sulston et al., 1975).

The Hook Sensillum

The hook sensillum comprises 2 sensory neurons (HOA and HOB) and 2 support cells and is associated with the hook, a single cell–derived, hook-shaped, sclerotic structure. Ablation of P10.p eliminates all of these cells in addition to an interneuron and 3 epidermal cells (Figure 4); morphology is normal, except that the hook and associated sensillum are missing. Operated males fail to stop at the vulva, circling the hermaphrodite many times, yet yield cross-progeny (Sulston and White, 1980; unpublished data). Eventually, the males stop, extrude their spicules, and begin to back along the hermaphrodite at a much slower pace, displacing her cuticle with their spicules as they back, until they locate the vulva by slipping the spicules into the vulva. This alternative form of vulva location behavior is discussed below.

Ablation of P10.pp eliminates both hook-associated sensory neurons, 2 neuronal support cells, and an unrelated interneuron, but leaves the hook structure intact (Figure 4). Operated males pass the vulva as before. Ablation of either hook neuron HOA or HOB alone (P10.ppap or P10.pppa, respectively) results in males that are similarly impaired. These males occasionally hesitate around the area of the vulva but continue swimming and do not try to insert their

A



B

Cell(s) Ablated	Structure Missing	Vulva location by male	Vulva location by trial
none	none	10/10	97/100
P10.p [a]	hook & sensillum +	0/10	0/100
P9.p [b]	none	4/4	13/13
P10.pa	hook +	10/10	33/37
P10.papp	hook alone	11/11	32/35
P10.paa	hypodermal cells	3/3	10/10
P10.pp	sensillum +	0/12	0/120
P10.ppap	HOA	0/10	0/100
P10.pppa	HOB	0/10	0/100
P10.ppap & pppa	both hook neurons	0/7	0/70
P10.ppaa	HOso	6/10	6/62
P10.ppppp	HOsh	3/10	3/88
P10.ppppa	PVZ	10/10	42/44

Figure 4. Role of the Hook and Sensillum

(A) The P10.p lineage from which the hook is derived. The hook is composed of a structural cell and a sensillum, containing 2 neurons and 2 support cells. HOso, hook socket cell; HOsh, hook sheath cell. Lineage from Sulston et al., 1980.

(B) Results of ablations of the P10.p ectoblast and its descendants. The plus sign indicates that additional structure/cells were removed with this ablation.

[a]Since P9.p has been shown to replace P10.p after ablation at this stage (Sulston and White, 1980), both P9.p and P10.p were ablated to remove P10.p functionally.

[b]Analogously, both P8.p and P9.p were ablated.

Table 3. Analysis of Vulva Location Behavior

Cell(s) Ablated	Structure(s) Missing	Vulva Location Behavior		Mating Efficiency
		Approximate	Precise	
Observations with Intact Hermaphrodites				
None	None	Stops at vulva	Slow search using spicules	High
P10.p	Hook	Circles hermaphrodite	Finds vulva via slow search	Low
Y.pl/r	p.c.s. (except PCC)	Stops at vulva	No slow search; loses vulva easily	High
Y.pl/r, B.al/rpaaa	p.c.s.	Stops at vulva	No slow search; loses vulva easily	High
P10.p, Y.pl/r	Hook, p.c.s. (except PCC)	Circles hermaphrodite	No slow search	Very low
P10.p, Y.pl/r, B.al/rpaaa	Hook, p.c.s.	Circles hermaphrodite	No slow search	0
B.al/rpa	SPC, SPD, PCC	Stops at vulva	No slow search	0
B.β	SPV	Stops at vulva	Slow search	Very low
P10.p, B.al/rpapap	Hook, SPD	Circles hermaphrodite	Slow search; no spicules	0
Observations with vulvaless hermaphrodites				
None	None	Circles hermaphrodite	No slow search	NA
P10.p	Hook	Circles hermaphrodite	No slow search	NA

Vulva location behavior is divided into two substeps, here designated "approximate" and "precise," as explained in the description of vulva location behavior in intact animals. Mating Efficiency is explained in Experimental Procedures. For observations with intact hermaphrodites, n = 10; vulvaless hermaphrodites, n = 14 (None) or n = 6 (P10.p). p.c.s., postcloacal sensilla.

spicules at that time. Thus, neither neuron alone is sufficient to mediate vulva location. Ablation of both HOA and HOB is equivalent to ablation of the hook sensillum, indicating that both are necessary for proper hook function. Ablation of the support, socket (P10.ppaa), and sheath (P10.ppppp) cells also impairs vulva location, although to a lesser extent. The socket and sheath cells are thus necessary for proper function or differentiation of the HOA and HOB neurons.

Ablation of P10.pa, which eliminates the cell that forms the hook structure and 3 epidermal cells, results in males that have only a sensillum where the hook is normally located (see also Sulston and White, 1980). These males are capable of locating the vulva, indicating that the structure itself is not necessary for hook sensillum function (Figure 4). This observation supports the idea that the hook functions in a chemosensory manner. However, ablation of the hook structural cell does result in a minor defect, in which the males consistently stop slightly beyond the vulva. Subsequent examination using Nomarski optics shows that the remaining hook sensillum, while intact, is often displaced anteriorly. Therefore, we believe that, though the hook structural cell is not necessary for sensillum function, the position of the sensillum might be important for efficient vulva location.

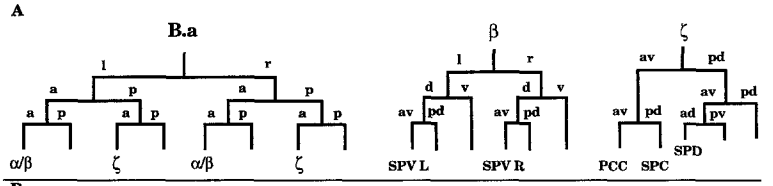
The Postcloacal Sensilla

Each postcloacal sensillum is made up of 3 sensory neurons (PCA, PCB, and PCC) and 3 support cells. The blast cell Y.p generates all of these cells exclusively, with the exception of the PCC neurons, which are generated by the B.a cell. Ablation of the entire postcloacal sensilla (by ablation of Y.pl/r and B.al/rpaaa) results in males with normal morphology and almost normal mating behavior (Table 3). (Since ablation of Y.p in L1 larvae often results in deformed spicules [Chamberlin and Sternberg, 1993], we ablated the progeny Y.pl/r in the L2.) Operated males respond, turn, and readily stop in the general area of the vulva, indicating that they recognize it. However, they tend to lose the vulva while attempting to locate its precise position (normal behavior exhibited in 0/10 males, 6/100 trials). Mating efficiency in these animals is normal (see Experimental Procedures).

Ablation of any 2 of the 3 pairs of postcloacal sensory neurons results in males with a similar but less severe defect (PCA^-PCB^-, 0/8 males, 12/80 trials; PCA^-PCC^-, 0/2 males, 7/20 trials; PCB^-PCC^-, 0/3 males, 9/30 trials). Ablation of any 1 pair results in no observable defect (PCA^-, 6/6 males, 18/19 trials; PCB^-, 7/7 males, 20/24 trials; PCC^-, 6/6 males, 18/20 trials). Therefore, a single pair of postcloacal sensilla sensory neurons is insufficient for the postcloacal sensilla to function; 2 pairs are sufficient but not optimal. Whereas the 3 pairs of sensory neurons seem interchangeable and overlapping in function, their number is important.

Although males in which the hook has been ablated (P10.p) cannot locate the vulva using the usual method, they eventually locate the vulva by extruding their spicules and backing slowly. This slow search behavior is similar to that observed in the vicinity of the vulva in intact animals, during adjustment to find the precise location of the vulva. Ablation of the hook and the postcloacal sensilla together results in males that neither hesitate around the vulva nor adopt the slow search behavior (Table 3). Ablation of the SPD sensory neurons (located in the spicules; see below) along with the hook also impairs vulva location. From this observation, we infer that, whereas the hook is responsible for finding the approximate location of the vulva, the postcloacal sensilla, in conjunction with the spicules, is used to find the precise location. In the absence of cues from the hook, the postcloacal sensilla can be used with the spicules to perform the same function.

A



B

Cell(s) Ablated	Neurons Missing	by male	by trial	Defect
none	none	10/10[a]	30/30[a]	--
B.a	all	0/10	0/100	no spicule insertion
Bζ	SPC, SPD, PCC, +	0/12	0/120	no spicule insertion
Bζaa	PCC	6/6	18/18	--
Bζap	SPC	0/10	0/100	no spicule insertion
Bζpaa	SPD	2/10	2/95	no spicule insertion
Bζpap	SPsh	5/5	15/15	--
Bζpp	SPso	6/6	18/18	--
Bβ	SPV +	1/12	2/112	no sperm transfer (31/112) premature transfer (79/112)
Bβl/rda	SPV	1/10	1/93	no sperm transfer (17/93) premature transfer (75/93)
Bζap, Bζpaa	SPC, SPD	0/4	0/40	no spicule insertion
Bζap, Bβl/rda	SPC, SPV	0/4	0/40	no spicule insertion
Bζpaa, Bβl/rda	SPD, SPV	0/3	0/30	no spicule insertion

Figure 5. Role of the Spicules

(A) The B.a lineage from which the spicules are derived. The spicules each contain 2 sensory neurons and a motor neuron that is thought to act as a proprioceptor as well. (α/β) In a given male, either B.alaa or B.araa becomes α; the other becomes β; (ζ) B.al/rpa. Lineage from Sulston et al., 1980.

(B) Results of ablations of the B.a ectoblast and its descendants. The plus sign indicates that additional structure/cells were removed with this ablation.

[a]Even intact males cannot insert their spicules much of the time. Observations have shown that the reason is hermaphrodite specific (see Experimental Procedures, Observations of Behavior). For these observations, we selected hermaphrodites that allowed spicule insertion by intact males.

The slow search behavior is not seen in intact males paired with hermaphrodites lacking vulvae (either vulvaless *let-23(sy1)* mutants or hermaphrodites where the vulva has been eliminated via ablation; Table 3). Thus, either the slow search behavior is vulva dependent, or it is an aberration resulting from ablation of the hook (and rescued by further ablation of the postcloacal sensilla). Pairing hook-ablated males with vulvaless hermaphrodites also results in no alternative behavior, suggesting that a signal from the vulva must be sensed by the postcloacal sensilla for the male to adopt this alternative behavior. Thus, even though the male cannot find the precise location of the hermaphrodite vulva while circling, he must be responding to some general cue from, or released through, the vulva.

The Spicules

Each spicule contains 2 sensory neurons, SPD and SPV, the processes of which run down the length of the spicule to the sensory opening at the end. Each spicule also contains a motor neuron, SPC, which appears, by morphology, also to be proprioceptive in nature (Sulston et al., 1980). Ablation of the blast cell B.a eliminates all of these neurons plus a pair of sensory neurons from the postcloacal sensilla PCCL/R (Figure 5). In addition, the majority of the structural cells of the spicules are also eliminated. The operated male has no spicules, and as expected, spicule insertion and sperm transfer do not occur. There are no other discernible defects (ablation of PCCL/R has no noticeable effect, as their function is redundant with PCAL/R and PCBL/R; see above).

Ablation of the Bζ cell (nomenclature from Chamberlin and Sternberg, 1993) eliminates only the SPC, SPD, and PCC sensory neurons and 4 support cells (a pair of socket cells and a pair of sheath cells). Despite lacking 2 structural cells each (out of 6), the spicules appear structurally intact. These animals fail to insert their spicules into the vulva (Figure 5). Ablations of the SPC motor neurons (Bζap) or the SPD sensory neurons (Bζpaa) also result in failure to insert spicules. Ablation of the PCCs (Bζaa) has no discernible effect. Neither does ablation of either pair of structural cells (Bζpap/Bζpp), implying that these cells are not necessary for proper neuronal differentiation or function.

Ablation of Bβ eliminates the SPV sensory neurons and 4 spicule support cells (2 socket and 2 sheath) but leaves the spicules intact. With most ablated animals, sperm are occasionally released outside of the vulva (Figure 5), before the spicules have been properly inserted. In all but a few of the remaining trials, the males fail to release sperm altogether once the spicules have been inserted.

Ablation of both the SPC motoneurons and the SPD sensory neurons resulted in males defective in spicule insertion, as expected. Ablation of the SPV sensory neurons, in conjunction with either the SPCs or the SPDs, also resulted in males defective in spicule insertion. No defects in sperm transfer were observed. These results imply that the function of the SPV sensory neurons is dependent upon the function of both the SPD and SPC neurons.

Discussion

We have described in detail the steps in male mating behavior in C. elegans. We then examined the roles of the sensory neurons/structures of the C. elegans male tail, via ablation and observation of the resulting behavioral defects, and assigned most to specific roles in these steps.

Response to Contact

Response to contact with the hermaphrodite is mediated through the sensory rays. For this function, the V-derived rays appear to be more important, as they are capable of mediating contact response in the absence of the T-derived rays; the converse is not true. This functional difference is more likely due to differences in ray position rather than the number of rays. Direct contact of the hermaphrodite with the sensory openings of the rays is necessary, as shown by the dorsally opening rays being both necessary and sufficient to mediate response to contact with the dorsal side of the male. However, response to ventral contact

appears to be mediated through both the ventrally opening rays and the additional sensory organs on the ventral side.

Turning

Once the male is backing along the length of the hermaphrodite body, he might encounter either her vulva or her head or tail first. If he encounters the end of the hermaphrodite before reaching the vulva, he will turn around to her other side. Turning is also mediated through the sensory rays. There are two components to a properly executed turn: timing and ventral coiling. The T-derived rays (rays 7–9) appear to mediate the timing, as operated males coil too late, swimming off the end of the hermaphrodite. The dopamine-containing rays (or the dopaminergic neurons alone), rays 5, 7, and 9, appear to mediate the ventral coil, as these operated males make wide sloppy turns instead of the sharp ventral arches exhibited by intact males.

Vulva Location

Once the male arrives in the vicinity of the vulva, he ceases backing behavior and adjusts his position before spicule insertion. Our observations suggest that this is a two step process involving distinct sensilla. The role of the hook and its associated sensillum is to identify the approximate location of the vulva over the entire area of the hermaphrodite body. Once the male has stopped in the general area of the vulva, the postcloacal sensilla in conjunction with the spicules act locally to determine the precise location of the vulva.

In the absence of the hook, males are still able to locate the vulva through the postcloacal sensilla and spicules, by adopting an alternate, slow search behavior. We hypothesize that the reduced backing rate during this alternate behavior allows these organs, which normally act locally, to detect the vulva as the they pass it. Thus, we do not believe the alternate behavior is a novel one brought on by ablation, but is rather the expansion of a behavior normally restricted to the area around the vulva. Because males do not adopt this alternative behavior with hermaphrodites lacking vulvae, we believe that the postcloacal sensilla also acts by sensing some signal from the vulva.

Spicule Insertion

Spicule insertion is mediated by the SPD sensory neurons. We propose that these neurons sense arrival of the male at the vulva and initiate spicule protraction. Upon receiving that signal, the spicule motor neurons SPCL/R excite the spicule protractors and inhibit the spicule retractors, allowing the spicules to extend into the vulva.

Sperm Transfer

We propose that the spicule sensory neurons SPVL/R serve to inhibit the transfer of sperm until the spicules are properly situated in the vulva. Once the SPVs sense that the spicules are in the uterus, they release sperm transfer from inhibition. Ablation of the SPVs results in release of sperm outside of the vulva. Double-ablation experiments indicate that the function of the SPVs is dependent upon the function of the SPCs and SPDs. This dependence ensures spicule insertion into the vulva before release of sperm. Thus, sperm transfer is regulated not only by some signal inside the vulva acting via SPV, which signals it to release sperm transfer from inhibition, but also by a signal from the SPC neurons (direct or indirect), indicating that spicule insertion has occurred, probably via proprioceptive feedback. The dependence of SPV function upon SPD function can be explained by connection through the SPCs. In addition, another signal must be acting in response to the vulval signal to promote sperm release, as SPV-ablated animals do not release sperm constitutively in the absence of the vulva.

General Conclusions

We have been able to assign a role in male mating to all of the male-specific sensory structures and have identified structures and neurons responsible for each step of mating behavior. The sensory rays mediate both response to contact with the hermaphrodite and turning around the hermaphrodite head or tail; the hook mediates approximate location of the vulva; the postcloacal sensilla, together with the spicules, mediate precise location; and the spicules mediate spicule insertion. Each of the identified steps can be affected by elimination of 1 or more identified neurons. Thus, separable neuronal components mediate each of the steps, indicating that these steps are not identified arbitrarily. Moreover, the observed defects suggest the specific mutant phenotypes to be used for a genetic analysis of this behavior.

As analysis of invertebrate behavior proceeds, it is clear that these systems are far more complicated than originally thought. Several systems have been shown to be capable of both non-associative and associative learning (reviewed by Carew and Sahley, 1986). Analysis of central pattern-generating circuits shows that, owing to modulation, a neural network is capable of generating more than one activity pattern (reviewed by Getting, 1989; Harris-Warrick and Marder, 1991). Genetic analysis of Drosophila behavior reveals both the plasticity described above and a high degree of sensory regulation. For example, as in C. elegans, courtship behavior comprises a series of highly stereotyped acts (reviewed by Quinn and Greenspan 1984; Hall, 1994), yet it is highly dependent upon visual and olfactory cues. In addition, though these cues normally act sequentially, the sensory pathways are partially redundant, the presence of one being able to compensate for the absence of the other.

In C. elegans mating behavior, we have demonstrated that a high degree of sensory regulation also exists, such that each step is regulated by feedback from different sensory organs. Instead of a fixed motor program, this sensory feedback at each step allows the male to adjust his behavior as the environment warrants. Thus, he is capable of responding appropriately to any part of the hermaphrodite with which he comes into contact, as opposed to requiring a specific start site and then executing an invariant motor program. Should the male encounter the hermaphrodite with the ventral side of his tail, he can simply initiate backing rather than beginning the shallow ventral arching he exhibits in response to dorsal contact. Should he contact the vulva before he encounters her head or tail, he is capa-

ble of detecting a signal from the vulva and responding to it by ceasing backward motion, even if he has not executed the usual motor program for turning behavior.

We have also found that redundancy of sensory feedback allows for plasticity in behavior. The male receives information regarding vulva location, not only from the hook sensillum but also from the postcloacal sensilla and the SPD spicule sensory neurons. Information from these structures is normally used sequentially; however, in the absence of normal feedback from the hook sensillum, signaling arrival at the vulva, the male is capable of using feedback from the postcloacal sensilla to determine the location of the vulva. Behaviorally, whereas a slow search is normally seen only in the approximate area of the vulva, in the absence of the hook, the male abandons the fast search and uses a slow search to find the vulva.

Experimental Procedures

Strains and Strain Maintenance

For these studies, four strains, all isolated from N2 Bristol, were used: *him-5(e1490)* (Hodgkin et al., 1979), *unc-31(e169)* (Brenner, 1974), *unc-52(e444)* (Brenner, 1974), and *let-23(sy1)* (Aroian and Sternberg, 1991). Worms were cultured as described by Brenner (1974; Sulston and Hodgkin, 1988) at 20°C, except during behavioral observations that were made at room temperature, around 22°C–23°C.

Nomenclature

Nomenclature for C. elegans cell names is from Sulston and Horvitz (1977) and Sulston et al. (1983). Cells in a lineage are designated by a blast cell name (a combination of a capital letter and possibly a number) followed by an a, p, d, v, l, or r, standing for anterior, posterior, dorsal, ventral, left, or right, respectively. Each lower case letter denotes a cell division and refers to the position of the daughter cell. A period is used to denote the transition from embryogenesis to the larval stages, with all letters to the right of the period referring to postembryonic divisions. Terminal cells that are neurons are also referred to by a combination of three capital letters. Where symmetrical cells exist on the left and right sides, they are denoted by the letters L and R, respectively.

Cell Ablations

C. elegans is primarily hermaphroditic and gives rise to males with a frequency of only 0.2%. The strain *him-5(e1490)* (isolated from N2 Bristol; Hodgkin et al., 1979) gives rise to a higher incidence of males (33%) and has a slightly lower brood size, but is otherwise wild type. Since subsequent genetic analysis will require use of the *him-5* mutation, we chose to characterize this strain.

Males were selected from a mixed population of *him-5(e1490)* worms. The age of the animals selected depended upon the position of the targeted cell along the lineage. The animals were mounted on a glass slide for Nomarski microscopy (Sulston and Horvitz, 1977) on a 5% agar pad with 2–4 mM sodium azide (depending on age of animals) as an anesthetic. The targeted cells were ablated by focusing a laser microbeam on the nucleus of the cell, as described by Sulston and White (1980; Avery and Horvitz, 1987, 1989). Animals were recovered from the slide in M9 buffer and placed onto individual plates with bacterial lawns. A few hours later (about two cell divisions later), the worms were remounted without azide to verify that the proper cell(s) were killed and that no unintentional damage was done.

Animals were allowed to mature into young adulthood. They were then observed individually for behavioral defects in mating. Afterwards, the animals were remounted for observation under Nomarski optics to determine whether unwanted damage had occurred during the ablation procedure or some other part of handling. If damage was detected, the data collected for the animal were not used. (This number was less than 5%.) Finally, the mating efficiency of each male was determined.

Mating Efficiency Tests

This procedure was first described by Hodgkin (1983) to measure the mating efficiency of males. Each male is placed individually on a mating plate with six hermaphrodites carrying a recessive marker. The percentage of cross-progeny it sires is determined by the number of nonmarked progeny divided by the total number of progeny. For our studies, we used hermaphrodites from the marked strain *unc-52(e444)*. The strain was chosen because the mutation is easy to score, and the lack of movement facilitates mating.

Observations of Behavior

The behavioral phenotype of intact and ablated males was determined by observation with young adult *unc-31(e169)* hermaphrodites on a 0.5 cm diameter lawn of Escherichia coli (OP50) bacteria. The use of *unc-31* hermaphrodites allowed for observation with hermaphrodites that were sluggish, making it easier for the male to keep pace with the hermaphrodite. Note that this method will be sensitive to only the more severe mating defects, as males that are only slightly impaired will probably be able to mate with a sluggish hermaphrodite.

Measurements were obtained in the following manner: time was kept with a stop watch, and distances were estimated by eye and confirmed from micrographs taken of the behavior. The number of sperm transferred was determined by counting the number of cross-progeny sired from one successful mating. This method assumes that all the male sperm transferred will be utilized to generate progeny. Thus, the actual numbers of sperm transferred may be higher.

Mating behavior is sensitive to a number of variables, some known, some suspected. For example, males fail to respond to hermaphrodites if the observation plates are too dry. For that reason, plates were not used if they were more than a week old. Other suspected variables are room temperature, concentration of dauer pheromone, and hermaphrodite age. For these reasons, intact control animals, isolated at the same time as their ablated siblings, were observed under the same conditions with each round of ablations. If intact animals had difficulty mating, data for that batch of ablated animals were discarded.

Operated males were determined to be defective if they tried but failed to perform a step a total of at least ten times with at least three different hermaphrodites. The first criterion ensures reproducibility in the male's behavior; the second controls for possible difficulties with specific hermaphrodites. We found, by observation with intact animals, that not all hermaphrodites allowed easy access into the vulva. If one male failed to insert his spicules, another was likely to fail; if one succeeded, another was likely to succeed (about 85%). Thus, this high failure rate is hermaphrodite specific. Similar spicule insertion failures are seen with mating observations done with hermaphrodites of the strain *unc-52* and with wild-type hermaphrodites (*him-5*). Thus, the phenomenon is not specific to the *unc-31* strain used. To control for possible erroneous spicule insertion defect scorings, and other possible hermaphrodite-specific problems, we used at least three hermaphrodites per male. For measurements specifically concerned with spicule insertion, the hermaphrodites were preselected by the ability of intact males to insert their spicules.

Some representative records of behavioral observations are given below for ablations in the P10.p lineage, which generates the hook (Figure 4), which in turn mediates vulva location. The symbols used are as follows: r, responded to hermaphrodite contact; t, turned; v, stopped at vulva; si, inserted spicules; st, transferred sperm; p, passed vulva without stopping; h, hesitated at vulva; b, resumed backing after hesitation; ..., removed hermaphrodite; /., blocked at that step. Intact animals exhibit one of the following behavioral sequences: r/v/si/st, r/t/v/si/st, or r/t/t/v/si/st, depending upon how many times a male encounters the end of the hermaphrodite (and so must turn) before he encounters the vulva. Following ablation of P10.pppa (the HOA neuron), we observed the following sequence: r/t/p/t/t/p/t/t/p/t/t/h/t/t/p/t/t/p...r/t/t/p/t/t/ h/t/t/p/t/t/p/t/t/p...r/t/p/t/t/p/t/t/p..., which we scored as vulva location defective. In the absence of P10.ppppa (PVZ interneuron), we observed: r/t/t/v/./b/t/t/v/./b/t/t/v/...r/t/v/si/st, which we scored as having no mating defect. Ablation of P10.ppaa (HOso) gave mixed results. After ablation, one animal exhibited the following: r/t/t/h/t/t/v/ b/t/t/ v/b/t/t/v/b/t...r/t/h/t/t/v/...r/t/v/si/st, which we interpreted as having no behavioral defect, while another exhibited: r/t/p/t/t/p/t/t/h/t/t/h/t/t/p/

t/t/p... r/t/t/p/t/t/p/t/t/h/t...r/t/t/p/t/t/h/t/t/h/t/t/p..., which we interpreted as vulva location defective. After ablation of P10.papp (the hook structural cell), the animal behaved as follows: r/t/v/./b/t/t/v/./b/t/t/v/... r/t/t/v/./b/t/t/p/t/t/v/...r/t/v/si/st, which we interpreted as normal behavior.

The scoring method we used addressed the question of whether the males are capable of performing a step after ablation. If a male failed to perform a step, we checked for reproducibility of that defect (at least ten times). If a male was able to perform the step, we checked only that he was able to perform that step with more than one hermaphrodite. As a result, the number of trials is lower with males that execute the step normally. Thus, while we can make conclusions as to whether an operated male is capable of performing a step in mating behavior, we cannot make any conclusions about changes in frequency of completed behavior of the males determined to be capable.

Some observations were also made with *let-23(sy1)* hermaphrodites in which 93% lack a functional vulva (Aroian and Sternberg, 1991). Individual animals were examined under Nomarski microscopy to confirm that they were vulvaless. This strain was used for some observations on ablated animals with possible vulva location defects.

Photomicrography

Behaving animals were photographed on Petri plates, through a Wild M420 macroscope, with ILFORD XP2 400 film. Exposures were taken at ASA 800. Nomarski micrographs were taken with a 100× objective.

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