Isolation and characterization of the female sex pheromone of the sugar beet cyst nematode, *Heterodera schachtii*, and an analysis of male precopulatory behaviour

by

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Abstract

The sugar beet cyst nematode, *Heterodera schachtii*, is a major agricultural pest. The disruption of the mating behaviour of this plant parasite in the field may provide a means of biological control, and a subsequent increase in crop yield. The *H. schachtii* female sex pheromone, which attracts homospecific males, was collected in an aqueous medium and isolated using high performance liquid chromatography. Characterization of the male-attractive material revealed that it was heat stable and water soluble. The aqueous medium conditioned by female *H. schachtii* was found to be biologically active and stimulated male behaviour in a concentration dependent manner. The activity of the crude pheromone was specific to males of *H. schachtii* and did not attract second stage juveniles. Results indicated that vanillic acid, a putative nematode pheromone, is not an active component of the *H. schachtii* sex pheromone. Male *H. schachtii* exhibited stylet thrusting, a poorly understood behaviour of the male, upon exposure to the female sex pheromone. This behaviour appeared to be associated with mate-finding and was used as a novel indicator of biological activity in bioassays. Serotonin, thought to be involved in the neural control of copulatory behaviour in nematodes, stimulated stylet thrusting. However, the relationship between stylet thrusting induced by the sex pheromone and stylet thrusting induced by serotonin is not clear. Extracellular electrical activity was recorded from the anterior region of *H. schachtii* males during stylet thrusting, and appeared to be associated with this behaviour. The isolation of the female sex pheromone of *H. schachtii* may, ultimately, lead to the structural identification and synthesis of the active substance for use in a novel biological control strategy.
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Literature Review

1.1 Introduction

Nematodes form a diverse group of animals that occupy a wide range of environments. Free living nematodes exist in marine or terrestrial habitats, while parasitic forms are obligated to inhabit tissues of a plant or animal host. The following sections describe *Heterodera schachtii*, an important plant parasitic nematode, and review relevant works from the literature that discuss the life, behaviour and physiology of this organism. It will be demonstrated that the reproductive physiology of *H. schachtii* has not been fully elucidated, and that a further understanding of the communication between individuals of this parasitic species may, ultimately, lead to a novel biological control strategy of *H. schachtii*.

1.2 Life cycle of *Heterodera schachtii*

The first two developmental stages of *H. schachtii* (Schmidt) occur within the egg. Hatching and emergence of the second stage juvenile (J2) has been shown to be stimulated by diffusates produced by the root system of the host plant (Perry, 1987; Perry, 1989). Stimulation of hatching caused by root diffusates has been demonstrated in other *Heterodera* spp. (Williams and Beane, 1979; Beane and Perry, 1983). In addition, root diffusates stimulate stylet thrusting behaviour in *H. schachtii* J2s (Grundler et al., 1991). The stylet is a spear-like structure located within the stoma, and is a typical structure of plant parasitic nematodes. The newly emerged J2 is the infective stage of the cyst nematode and travels toward a potential host while performing searching movements with the head and probing activity with the stylet (Doncaster and Seymour, 1973). Once
a suitable site has been located on the host, such as a growing root tip or where lateral roots emerge (Baldwin and Mundo-Ocampo, 1991), the juvenile penetrates the epidermis of the root with the stylet. Dickinson (1959) demonstrated that suction produced at the anterior lip region of *H. schachtii* J2s enabled the infective juveniles to adhere to a hydrophobic nitrocellulose surface and aided in stabilization during stylet penetration. It was suggested that suberized plant roots fulfill a similar role of the hydrophobic substrate *in vivo*, and allow infective juveniles to anchor themselves to the root surface.

After the *H. schachtii* J2 penetrates the epidermal layer of root tissue, the juvenile settles in the region of the vascular bundle and penetrates an adjacent cell with the stylet and exchanges materials with the host cell (Baldwin and Mundo-Ocampo, 1991). This action stimulates hypertrophy of the plant cell, resulting in the formation of a modified, multinucleated feeding structure, a syncytium, that is characteristic of a parasitic infection by cyst-forming genera (Baldwin and Mundo-Ocampo, 1991). It is at this site that the juvenile *H. schachtii* feeds and continues to develop (Wyss, 1992) into third and fourth juvenile stages, and finally into the adult stage.

The entire life cycle, from egg to adult, may be completed in less than 30 days, although generation time is greatly affected by environmental conditions (Baldwin and Mundo-Ocampo, 1991), such as temperature, moisture and host species. At maturity, females enlarge to form a white, lemon-shaped cyst (Mai and Mullen, 1996) and remain attached to the host root at the anterior region of the cyst. The remaining portion of the sedentary female is exposed to the soil environment. Female cyst nematodes retain their eggs in the body or may release them through a terminal vulva into a gelatinous matrix, or egg sac, that, presumably, provides a certain degree of protection for the eggs from
predators and desiccation (Bird and Soeffky, 1972; Baldwin and Mundo-Ocampo, 1991; Bird and Bird, 1991). A high degree of sexual dimorphism is apparent upon comparison of male and female cyst nematodes, including *H. schachtii*, in the adult stage. While females become sedentary cysts, adult males retain the J2 vermiform morphology and emerge from root tissues as free living, mobile nematodes (Mai and Mullen, 1996).

1.3 Agricultural importance *Heterodera schachtii*

The agricultural importance of *H. schachtii* as a plant parasite was realized during the late 1800s when it caused the devastation of sugar beet fields in Europe (Thorne, 1961). The sugar beet cyst nematode was first identified in 1859 by the presence of female cysts on host plants, and was later considered a new species in 1871 (Raski, 1949; Thorne, 1961). Although *H. schachtii* was taxonomically described on the sugar beet, *Beta vulgaris* L., and is primarily associated with this host species, the host range of *H. schachtii* also includes cabbage (*Brassica*), spinach (*Spinacea*) and radish (*Raphanus*) (Baldwin and Mundo-Ocampo, 1991).

1.4 History of pheromones

The term “pheromone” was proposed by Karlson and Lüscher (1959) to describe substances that are secreted by an organism to the external environment and received by a second organism of the same species, producing a “definite behaviour or a developmental process”. Law and Regnier (1971) introduced the more general term, “semiochemicals”, to describe substances, including pheromones, released by an organism and received by another organism of the same or different species as a means of communication. Pheromones would henceforth be considered semiochemicals having
intraspecific activity, and would be distinguished from other semiochemicals having interspecific activity. Since the inception of the term as a scientific construct, several categories of pheromones have been proposed to classify chemicals into specific groups based on the reactions they induce in the receiving organism, such as alarm, aggregation, dispersion and sexual behaviour (Shorey, 1973). Of particular interest to the present work are the sex pheromones that attract prospective mating partners to the source and may stimulate copulatory behaviour in the receiver (Regnier and Law, 1968; Law and Regnier, 1971; Shorey, 1973). The prevalence of these pheromones as a means of communication in insects is well documented, and most tend to be volatile substances capable of diffusing over great distances to exert a behavioural or developmental effect in the receiver (Regnier and Law, 1968; Law and Regnier, 1971; Shorey, 1973; Howse, et al., 1998).

The first sex pheromone from an insect was isolated by Butenandt et al. (1959) from the silk moth, Bombyx mori. Males were attracted to volatile extracts of female scent glands and responded with a characteristic wing fluttering behaviour. Subsequent studies have demonstrated the presence of sex pheromones in other moth species using other techniques of pheromone collection. Female sexual attractants from Heliothis spp. (Klun et al., 1979) and Cryptophlebia leucotreta (Bestmann et al., 1988) were collected from ovipositor washings and scent gland aeration, respectively. Sex pheromones are the primary means by which female moths broadcast their sexual receptiveness to homospecific males, inducing male behaviours, such as arousal, patterned upwind flight, orientation and mating (Hildebrand, 1995; Howse et al., 1998). Insects and their
pheromones have provided a useful model for behavioural and neurobiological research and have been studied extensively (Hildebrand, 1995).

Schneider (1957) demonstrated, for the first time, that olfactory receptors were present on the antennae of insects and developed the electroantennogram, a means of recording the summed electrical activity of these olfactory receptors after stimulating them with a puff of volatile material (Howse et al., 1998; Chapman, 2000). Slifer (1957) studied the structure of the antenna receptors under the transmission electron microscope and discovered the presence of small pores in the cuticle, allowing the exposure of sensory neurons to the external environment. This finally answered the question of how odour molecules, such as pheromones, could reach the dendrites of these neurons through what seemed to be an impermeable cuticle and be detected by the male (Chapman, 2000). It is now known that insects have both gustatory and olfactory chemoreceptors densely arrayed in the epithelia and exposed to the external environment that allow for the transduction of chemical stimuli into meaningful information regarding the insect’s surroundings (Mullin et al., 1994; Hildebrand, 1995).

The occurrence of sex pheromones as a means of stimulating mate finding and copulatory behaviour is not unique among the insects. Sex pheromones have been shown to be released from other arthropods, such as mites (Tetranychus urticae; Cone et al., 1971), ticks (Amblyomma americanum; Berger, 1972), crayfish (Procambarus clarkii; Ameyaw-Akumfi and Hazlett, 1975), copepods (Temora longicornis; Weissburg et al., 1998; Yen et al., 1998) and from animals of other phyla, such as molluscs (Aplysia brasiliana and A. californica; Painter et al., 1998) and nematodes (Panagrolaimus
rigidus; Greet, 1964). Of interest to the present work are the sex pheromones of nematodes. These are discussed further in forthcoming sections.

1.5 Chemical attraction

Much of the early work that has provided fundamental evidence for the existence of nematode pheromones (Green, 1966; Jones, 1966; Green, 1967; Greet et al., 1968; Green and Plumb, 1970; Green and Greet, 1972) has involved the use of bioassays based on the phenomenon of chemical attraction in a semiaqueous, agarose medium. In a gradient of diffusing chemical attractants, nematodes display klinotaxis, the movement up the gradient accomplished by successively sampling stimulus intensities with swinging motions of the body, and chemotaxis, the movement up the gradient in response to specific chemicals (Ward, 1973). Bone and Shorey (1978) felt that such insensitive bioassays were partly responsible for the superficiality of nematode pheromone work, and stressed the need for the development of rapid and sensitive bioassay techniques. Nevertheless, bioassays based on chemical attraction on agar coated surfaces, coupled with techniques of chemical separation, continue to be used (Bone and Bottjer, 1984; Riga et al., 1997; Aumann et al., 1998a; Aumann et al., 1998b) and have provided information regarding the chemical and physical properties of nematode pheromones. Due to the predominance of studies involving chemical attraction in bioassays in the literature, and the use of these assays in the present work, the concept of chemical attraction in nematodes will be iterated.

It is assumed that the anterior, paired structures of the nematode, the amphids, fulfill a major chemosensory role and allow for the detection of chemical gradients in
vitro (Wright, 1983; Ashton and Schad, 1999). The amphids have also been shown to be chemosecretory (Riga et al., 1995). Ultrastructural reconstructions of sections of the free living nematode, Caenorhabditis elegans, indicated the presence of synaptic connections between nerve endings of the amphidial sensilla and the nervous system (Ward et al., 1975; Ware et al., 1975; White et al., 1986) confirming that the amphids were morphologically characteristic of sensory organs. The nerve endings of the amphidial sensilla penetrated the cuticle and were exposed to the external environment, indicating that the amphids were likely to be chemoreceptive; by contrast, the inner labial, outer labial and cephalic sensilla had nerve endings embedded within the cuticle and were considered mechanoreceptive (Ward et al., 1975). Jones et al. (1994) reported ultrastructural evidence that the structure of the amphids of a related plant parasitic nematode, Globodera rostochiensis, were conserved throughout development after hatching, and suggested that the amphids may be responsible for chemoreception throughout the entire nematode life cycle.

Ward (1973) determined that sensory receptors in the head alone, presumably the amphids, mediated the orientation response of C. elegans to chemical gradients of cyclic nucleotides, anions and cations in agar coated Petri dishes. He was able to demonstrate defects in klinotaxic behaviour by the analysis of responses of mutants in bioassays. Caenorhabditis elegans nematodes selected for “head-covering blisters” of the cuticle could not orient themselves when exposed to a chemical gradient, and behaved as if they were in a stimulus-free environment. The orientation-deficient behaviour was attributed to a defect in the functioning of the amphids. However, the most striking observation reported was the defect in orientation behaviour of “bent-headed” nematodes, selected
for a low penetrating mutation causing either dorsal or ventral bends at the tip of the head. When placed on a solid surface, these bends appeared laterally, since nematodes must travel on their sides to allow for the dorso-ventral undulatory movements required for locomotion. Observations revealed that “bent-headed” nematodes travelled toward the centre of the Petri dish in a complex spiral pattern, but always oriented themselves so that their heads pointed directly up the chemical gradient toward the source. Additional defective nematodes, those with a “shortened head” and “defective head muscles”, did not perform orientation responses as well as the wild type (Ward, 1973), emphasizing the importance of the anterior receptors in chemical attraction. Ward (1973) concluded that the length of the head, and its movements, are critical for chemical orientation because of the location of the amphids.

The underlying neural mechanism of chemical attraction has been investigated and results indicated that the amphids are the primary chemosensory organ of the nematode. Bargmann and Horvitz (1991) selectively killed amphidial neurons in live C. elegans by laser ablation and tested the sensory capacity of operated nematodes in Petri dish bioassays. Their findings revealed that only one pair of chemosensory neurons, ASE\(^1\), was unique in its importance to chemotaxis. Nematodes in which the ASE neurons were killed were defective in their chemotaxic behaviour when compared to the wild type. When all chemosensory neurons were killed, nematodes were chemotaxis defective; but when only ASE neurons were allowed to survive, chemotaxic responses were significantly more efficient (Bargmann and Horvitz, 1991).

\(^1\)Originally designated “e” (Ward et al., 1975) but renamed for its association with the amphidial sensilla by White et al. 1986.
The phasmids are paired structures in the tail region of secernentean nematodes and are believed to be sensory organs. Nematodes of the class Secernentea are, generally, distinguished from those of Adenophorea by the presence of the phasmids (Bird and Bird, 1991). White et al. (1986) described the phasmidial sensilla of *C. elegans* as being exposed to the external environment, an arrangement similar to that of amphidial sensilla (Ward et al., 1975), and suggested that the phasmids fulfill a chemosensory role similar to that of the amphids. However, Ward (1973) had previously demonstrated that the phasmids of *C. elegans*, unlike the amphids, were not necessary for klinotaxis. Mutants selected based on their "tail-blistered" appearance oriented themselves normally in a chemical gradient toward the source. The blisters of the cuticle in these nematodes covered the region of the tail containing the phasmids (Ward, 1973). These results suggest that the phasmids do not perform the same function as the amphids since only the function of the amphids was disrupted by the presence of the cuticle blisters. However, the blisters occurring on the cuticle of mutant *C. elegans* may not have affected the amphids and phasmids equally since the amphids are much larger and have a prominent pore open to the external environment (Bird and Bird, 1991) which may have left the amphids more susceptible to disruption by the cuticle blisters than the phasmids. Despite this, Ward's (1973) findings that the phasmids were not essential for klinotaxis were confirmed by Bargmann and Horvitz (1991) who demonstrated that *C. elegans* worms bearing mutations in the gene, *lin-17*, responded as well as the wild-type to attractive substances. Mutations of *lin-17* in *C. elegans* cause defects in the formation of the phasmid opening, preventing the exposure of phasmid neurons to the external
environment (E. Hedgecock, personal communication, as cited by Sternberg and Horvitz, 1988).

Wang and Chen (1985) speculated that the phasmids of the male plant parasitic nematode, *Scutellonema brachyurum*, may serve as receptors for sexual attractants released by females. Their results from electron microscopy indicated that the orientation of the electron-dense plugs, which are contained within the ampullae of the phasmids, and the associated ducts leading away from the ampullae differ between male and female *S. brachyurum*. Wang and Chen (1985) suggested that the morphological variation of phasmids between the sexes may have indicated a difference in function. Carta and Baldwin (1990a), however, rejected the idea of the phasmids of male plant parasitic nematodes functioning to detect sexual attractants released from females. They demonstrated the absence of the phasmids in adult males of three plant parasitic nematode species, *Verutus volvingentis*, *Cactodera eremica* and *H. schachtii*, and ruled out the possibility of functional phasmids in adult stages of these species. In fact, Carta and Baldwin (1990a) determined that the phasmids of *H. schachtii* show signs of degeneration during the J2 stage, confirming a previous report (Baldwin, 1985) that *H. schachtii* J2s taken from the same population were found to have two distinct types of phasmid morphologies: one more diminutive than the other. That *H. schachtii* males lacking phasmids (Carta and Baldwin, 1990a) can successfully locate females, or attractive substances released from females (Green, 1966; Green, 1967; Greet et al., 1968; Green and Greet, 1972; Aumann and Hashem, 1993; Aumann et al., 1998a, b) indicates that mate finding behaviour is likely achieved by a different mechanism,
possibly involving the anterior sensilla. The phasmids remain poorly understood sensory organs.

Stephenson (1942) showed a unique reaction of the phasmid sensilla of the free living nematode, *Rhabditis terrestris*, to changes in the osmotic pressure of the surrounding medium. When *R. terrestris* were placed in a medium that was hypotonic, relative to the culture medium, an increase in body length and width was observed following an increase in internal water volume. Conversely, when the nematodes were placed in a hypertonic medium, the nematodes lost water and were reduced in size. Curiously, both phasmids hypertrophied during treatment in the hypertonic medium. It was suggested that the phasmids may aid in the elimination of osmotically active materials and regulate the internal volume of the nematodes (Stephenson, 1942). Further, more recent evidence of the enlargement of the phasmids in hypertonic media has been demonstrated in the larvae of free living (Poinar, 1965) and animal parasitic (Muller and Ellis, 1973) nematodes, and in adult *S. brachyurum* (Wang and Chen, 1985) and supports the tenant that the phasmids may act as osmoregulators (Stephenson, 1942; Poinar, 1965; Wang and Chen, 1985; Carta and Baldwin, 1990b).

1.6 Pheromones of nematodes

Mate finding among amphimictic nematodes, such as *H. schachtii*, is one of several types of behaviour that is accomplished, presumably, because of the presence of chemical cues in the environment that play a role in nematode survival (Huettel, 1986). Greet *et al.* (1968) commented on the rarity of mating in nematodes if it were to rely on random contacts between sexes, especially where nematode populations are sparse. The
present understanding of pheromone communication in insects suggests that other invertebrates, such as nematodes, which must seek prospective mates, may have a similar means of communication. Greet (1964) reported the first evidence of a nematode sex pheromone. Sexes of *Panagrolaimus rigidus* were separated by a porous cellophane barrier in troughs constructed from polyvinyl tubing. After 17 hours, males and females had congregated on either side of the barrier, suggesting that the sexes were attracted to each other because of the presence of a diffusing, water soluble chemical through the barrier. The effects of this attractant, however, served only to bring the sexes together, as the act of copulation was dependent on physical contact between males and females (Greet, 1964). By 1980, sexual attraction had been demonstrated in at least 30 nematode species (Green, 1980). The present section reviews past and recent contributions in nematology that have elucidated information regarding the female sex pheromone of *H. schachtii*.

The sedentary lifestyle of *H. schachtii* females requires that mobile males be the active partner in mate pairing. Green (1966) studied the orientation behaviour of *H. schachtii* males to their females, and hypothesized that the male is guided by attractive stimuli released by the female. Males exhibited klinotaxic behaviour on agar coated plates in the presence of a diffusing chemical gradient of female pheromone. Green (1966) concluded that the presence of materials released by females stimulated an attractive, chemotaxic response in males; and that the attraction of males to females was not the result of the random meeting between the sexes and the retention of the male by the female because of tactile stimuli. The association between tactile stimuli and copulatory behaviour, however, was not investigated.
Green (1966) suggested that a labile pheromone with low chemical stability would be advantageous in vivo by preventing the saturation of the soil environment with the male attractant, which would otherwise destroy chemical gradients and cause fatigue of male sensory receptors. An unstable pheromone would degrade faster and be eliminated from the environment, allowing the attractant to be continually renewed from its source, a live female, and intensify male responses to a particular female in the group. The male-attractive pheromone of H. schachtii, however, was found to be stable in solution, diffused through an aqueous medium, and was not detected by males in a gaseous gradient (Green, 1967). That the pheromone substance released from H. schachtii females was not volatile, was further argued, as Green (1967) indicated that drying and redissolving secretions from females did not reduce the male-attractive properties of the material.

Prompted by the discovery that female “extracts enclosed in the same vessel contaminated each other”, Greet et al. (1968) provided evidence contrary to earlier findings by Green (1967) and reported the existence of an attractive, volatile component of the H. schachtii female pheromone. Blocks of agar were placed in a sealed container with an aqueous source of the pheromone such that only volatile materials could be transferred from the pheromone source to the agar blocks. Although the agar blocks were attractive to males in bioassay experiments, results indicated that males were less attracted to the volatile materials than they were to the aqueous standard. Greet et al., (1968) suggested that the volatile component of the pheromone likely plays an indirect role in vivo, possibly by arousing inactive males until they come into contact with a stronger, aqueous pheromone gradient.
The aqueous, male-attractive pheromone of *H. schachtii* was found to be primarily released from the tail of the female (Green and Greet, 1972). Materials were collected directly from the vulvae of females and bioassayed for pheromone activity. The finding that males were attracted to materials collected from female vulvae implicated the vulva as the site of release of the *H. schachtii* pheromone, and it was suggested that the gelatinous matrix surrounding the vulva acts as a carrier of the pheromone (Green and Greet, 1972). This hypothesis seemed likely since the release of the pheromone is correlated with the secretion of the gelatinous matrix (Green and Greet, 1972), and it has been suggested that these actions may be triggered by the same gonadotrophic hormone (Green, 1980). In addition to their findings described above, Green and Greet (1972) reported that males were attracted to materials collected from the heads of *H. schachtii* females, although to a lesser degree, and pointed out the difficulty presented to the male in locating the vulva during copulation if this behaviour were to rely on the same sensory mechanism (*i.e.* pheromone detection) that initially attracts the male to the female. Perhaps a tactile stimulus would allow *H. schachtii* males to locate the vulvae of females after initial contact. Evidence of the dependence of copulatory behaviour on physical contact between sexes, possibly because of tactile stimuli, exists in studies of other nematode species (Greet, 1964; Chin and Taylor, 1969), and may provide insight into the reproductive behaviour of *H. schachtii*.

Recently, several groups have undertaken the task of characterizing nematode pheromones of *Heterodera* spp. Aumann and Hashem (1993) collected materials released by *H. schachtii* females in several solvents, including water, and reported biological activity of pheromone components possessing both polar and nonpolar
properties. Characterization of a water soluble component of the *H. schachtii* sex pheromone was later reported (Aumann *et al.*, 1998b), but no water soluble substance suspected of possessing sex pheromone activity was isolated.

A case for a water soluble pheromone of *H. schachtii* has been established. Intuitively, the pheromone should be soluble in water since nematodes inhabiting a soil environment would have to release and diffuse their pheromone through a partially aqueous medium. The possibility of a volatile component of the pheromone has also been investigated. Using methods of gas chromatographic separation, coupled with behavioural bioassays, Aumann *et al.* (1998a) attempted to characterize a volatile substance extracted from the gelatinous matrices of *H. schachtii* females in pentane and methanol. They reported to have partially isolated two volatile components of the *H. schachtii* pheromone in both solvents. However, no details of statistical analysis were reported and no control was used. The authors did not investigate the possibility that the pentane and methanol solvents used to isolate materials from females might have been biologically active. There was also a substantial variation in the responses of males to fractions not containing the pheromone during bioassays. For example, in separate bioassay trials of fractionated female materials collected in pentane, between 0% and 27% of males responded to fractions not containing the putative pheromone, while between 20% and 40% of males responded to fractions that did contain the putative pheromone. Fractions of female materials collected in methanol containing the putative pheromone yielded more convincing results when compared to methanol extracts not containing the putative pheromone, although, both of these fractions attracted 33% of the males in one trial (Aumann *et al.*, 1998a). Although the results presented by Aumann *et
were not compelling, it appears as though they may have isolated a weakly attractive, volatile substance that may subserve an indirect role in sexual attraction, as suggested by Greet et al. (1968).

To date, only one substance, vanillic acid, has been structurally identified as a putative nematode pheromone (Jaffe et al., 1989). Males of *H. glycines* responded to the volatile vanillic acid, at concentrations ranging from $10^{-7}$ M to $10^{-5}$ M, by coiling their bodies. However, *H. schachtii* males have been shown not to respond to the same concentrations of vanillic acid in chemotaxis bioassays (Aumann and Hashem, 1993). It is possible that vanillic acid is not an active component of the *H. schachtii* female sex pheromone since males of this species do not appear to react to the authentic compound (Aumann and Hashem, 1993). Furthermore, a number of vanillic acid analogues have been shown to exhibit nematicidal activity in the filarial nematode, *Litomosoides carinii*, and cause sterilization of surviving worms (Varma et al., 1993). In addition, vanillic acid extracts from the China berry tree, *Melia azedarach* (Varma et al., 1993), possessed significant anthelminthic¹ activity against the cestode, *Taenia canina*, and the trematode, *Paramphistomum cervi*, *in vitro* (Neogi et al., 1963). The broad spectrum activity of vanillic acid and its analogues against the survival of helminths (Neogi et al., 1963; Varma et al., 1993) suggests that these substances would not be advantageous to *Heterodera* spp. as a sex pheromone. Furthermore, the coiling response of *H. glycines* males to vanillic acid (Jaffe et al., 1989) may have been induced by the nematicidal (Varma et al., 1993), not pheromonal, properties of vanillic acid.

¹The term "anthelminthic" or "vermifuge" describes a drug which destroys intestinal worms.
1.7 Control of plant parasitic nematodes

It is thought that during opportune phases of the nematode life cycle, such as during mate finding, male sensory perception and reproductive ability may be susceptible to disruption (Perry, 1994; Bone, 1987). The exploitation of this idea to reduce nematode pest populations using nontoxic or natural substances has justifiably focused attention on studies of behavioural responses to chemoattractants. The banning of synthetic nematicides for control of plant parasitic nematodes has placed importance on the search for naturally occurring, behaviour modifying, biocontrol agents (Heuttel, 1986). If the sex pheromone of *H. schachtii* is isolated and structurally identified, the substance has the potential to be synthesized and used as a biocontrol agent to saturate the environment that the nematodes inhabit (Bone, 1987). Similar techniques have been employed with insects and have successfully allowed for the control of pest populations (Cardé and Minks, 1995; Howse *et al.*, 1998). Such a strategy may decrease the probability of successful mate finding and reduce populations of *H. schachtii* in the field, leading to crop protection and an increase in crop yield.

1.8 Nematode behaviour

Male nematodes exhibit several distinct behaviours that are associated with copulation. These behaviours, and their potential as indicators of male sexual stimulation, are discussed in the present section. The spicules are posterior, ventral structures of the male, occurring in pairs in most nematodes, that are used during copulation (Bird and Bird, 1991). The rigid blade-like spicules of *Heterodera* spp. consist of a hard, sclerotized cuticle and interlock with each other to form a tube-like
structure forming an extension through the cloaca during sexual stimulation of the male (Clark et al., 1973). Early reports of copulatory behaviour in nematodes indicated that when males of *P. rigidus* (Greet, 1964), *Pelodera teres* (Jones, 1966) and *Cylindrocorpus* spp. (Chin and Taylor, 1969) encountered homospecific females, a coiling of the male tail occurred, accompanied by eversion of the spicules. The spicules aid in anchoring the male to the female during copulation and ensure the safe transfer of sperm from the vas deferens through the cloaca to the female (Clark et al., 1973). Furthermore, evidence of sensilla contained within each spicule extending from the base to a small, distal pore suggests that the spicules may serve a sensory role as well (Clark et al., 1973; Bird and Bird, 1991). Curling of the male tail, also referred to as coiling, and spicule eversion have been observed in *Heterodera* as precopulatory behaviours in root explant cultures and on agar plates in the presence of materials produced by females (Huettel and Rebois, 1986). However, these same behaviours have been observed to be dependent on physical contact between sexes in other species (Greet, 1964; Chin and Taylor, 1969; Loer and Kenyon, 1993) and may be dependent on the presence of tactile stimuli provided by the female mating partner. Coiling behaviour of the male, in the absence of females, was used as an indicator of sexual stimulation and to identify vanillic acid as the putative sex pheromone of *H. glycines* (Jaffe et al., 1989).

An additional accessory reproductive structure in some nematodes is the male copulatory bursa. In many secernentean nematodes, including *C. elegans* and strongyloids, such as *Heligmosomoides* spp. where this structure is very pronounced, the cuticle of the tail is modified to form a fan-like bursa, containing several sensillary rays that clasp the female near the site of the vulva and aid in the insertion of the spicules.
prior to insemination (Bird and Bird, 1991). Marchant (1970) demonstrated that a "flare" of the male copulatory bursa in *H. spiralis* (formerly *Nematospiroides dubius*) could be used to identify water soluble sexual stimulants produced by females. Males were reported to have displayed bursal flaring when placed in a drop of solution with a female and in a medium that had previously contained females. After studying the behaviour of over 220 individuals of *Heligmosomoides* spp., Croll and Wright (1976) were unable to confirm Marchant's (1970) observations. Flaring of the male bursa occurred spontaneously and, contrary to earlier findings (Marchant, 1970), was more characteristic of an inactive male than one that was sexually stimulated (Croll and Wright, 1976) which suggests that bursal flaring can not be used as a means of bioassay.

There has not been the development of a behavioural bioassay, with the exception of those involving chemotaxis, that has provided a means of identifying female sex pheromones by observing the male. A male behaviour that is not dependent on tactile communication with a female, and that is observed during or before copulation, would be an ideal indicator of the presence of a sex pheromone and may provide information regarding the physiological and behavioural state of the male. This behaviour must occur independently of the presence of females and would, therefore, demonstrate a response of the male to other stimuli, such as sex pheromones. Thrusting of the adult male stylet is reported to occur in *H. schachtii* and *G. rostochiensis* as the males probe the vulval area of female cysts (Green and Greet, personal communication, as cited by Clark et al., 1973), but this behaviour has not been quantified or further investigated. The purpose of this behaviour is presently not understood, since males of Heteroderidae reportedly do not feed as adults (Franklin, 1965; Shepherd, 1965). Stylet thrusting in *H. schachtii*
males may, potentially, serve as an indicator of sexual stimulation by female pheromones if this behaviour can be induced upon exposure of males to these substances in the absence of the female and possible confounding tactile stimuli.

The involvement of the stylet in the process of mate finding or copulation has never been proposed in the literature, but its function as a feeding structure is well documented. Linford (1941) first observed the feeding process of plant parasitic juveniles to include the forward movement of the stylet into a host cell and an associated period of "sucking out cell contents". The removal of cell contents by the nematode was, presumably, achieved by the negative pressure created within the pharyngeal lumen produced by pharyngeal pumping contractions, as has been demonstrated in Ascaris (Saunders and Burr, 1978). Wyss (1992) has also observed the coordinated use of the stylet with contractions of the pharynx in H. schachtii during feeding. Stylet-bearing parasitic nematodes, such as H. schachtii, appear to have undergone a modification of the anterior pharyngeal muscles into the stylet protractor muscles (Bird and Bird, 1991). Ultrastructural studies have shown the presence of three non-contractile cell bodies of stylet protractor muscle cells in the procorpus of Meloidogyne incognita, H. glycines (Baldwin and Hirschmann, 1976; Baldwin et al., 1977; Endo, 1984), Sarisodera hydrophila (Baldwin, 1982) and Ditylenchus dipsaci (Shepherd and Clark, 1983). Endo (1984) determined that the procorpus of H. glycines contained three nuclei of cells associated with stylet shaft tissue. The close association between pharyngeal and stylet protractor muscle tissue suggests that the stylet protractors did derive from primitive pharyngeal musculature, and may account for the intimate, functional association of the stylet with the pharynx (Baldwin and Hirschmann, 1976; Wright et al., 1983; Bird and
Bird, 1991). In addition, Baldwin and Hirschmann (1976) indicated that the basal lamina, which lines the lumen of the pharynx, encompasses secondary muscle cells of the stylet protractors and that the shaft of the stylet is more closely related to the pharynx than the stoma.

The complete anatomy of the pharynx of *C. elegans* has been described in detail from serial section electron microscopy (Albertson and Thomson, 1976). Pharyngeal neurons were found to synapse with muscle *en passant* in a manner similar to that of ventral cord neurons with somatic muscles, as described by White *et al.* (1976). Although processes of nerve cells contained within the pharynx of plant parasitic nematodes have been described (Baldwin *et al.*, 1977; Baldwin, 1982; Shepherd and Clark, 1983; Endo, 1984), a detailed record of neuromuscular synapses in these nematodes is lacking. This problem has been addressed elsewhere (Brownlee *et al.*, 1994). It is not known from where stylet protractor muscles receive innervation from the nervous system or where these neuromuscular synapses occur. Baldwin *et al.* (1977) described small cell processes surrounded by numerous junctional complexes\(^1\) in the anterior procorpus of *Heterodera* and *Meloidogyne*, and speculated that these may be neurons that innervate the stylet protractors. Shepherd and Clark (1983) reported that similar cells in *D. dipsaci* appeared to be neuronal but did not comment on their function or possible sites of synaptic innervation. That the noncontractile cell bodies of the stylet protractors lie in the anterior procorpus suggests that the protractors may receive contractile stimuli from neurons in this region. In all nematodes, somatic\(^2\) muscle cells

\(^{1}\)Junctional complexes support and surround the pharyngeal lumen, and probably reflect the amount of stress applied to this region during stylet and pharyngeal activity (Baldwin *et al.*, 1977). These and cells adjacent to the junctional complexes were collectively referred to as the "lumen complex" by Yuen (1968).

\(^{2}\)It should be noted that the protractor muscles are not considered somatic (Bird and Bird, 1991) but their
unanswerable questions and answers, and answers to common questions. In some cases, the double ended
names may be better known among communities in those communities, the

cases of the latter. In the examples above, community id B in various instances all

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names may be better known among communities in those communities, the
are composed of a noncontractile region which receives synaptic input from the nervous system required for contraction (White et al., 1986; Bird and Bird, 1991). Albertson and Thomson (1976) determined that the M1 motor neuron traverses the entire length of the pharynx to innervate cells of three muscle groups of the procorpus. Although a pharyngeal neuron similar in morphology to M1 in stylet-bearing plant parasitic nematodes has not been reported, it is likely that a neuron similar to M1 exists in these nematodes, and may innervate the protractor muscle cell bodies located in the procorpus and stimulate protractor muscle contraction.

Extrusion of materials through the stylet orifice, another behaviour typical of feeding juveniles, has been observed to occur in conjunction with stylet thrusting activity (Wyss, 1992; Sobczak et al., 1999). McClure and von Mende (1987) were able to stimulate stylet movement in *M. incognita* with catechol, and reported that this behaviour was correlated with stimulation of the subventral pharyngeal glands and the release of materials from the tip of the stylet. The simultaneous actions of the protractor muscles moving the stylet and the stimulation of the subventral glands may be explained by ultrastructural evidence obtained from *C. elegans*. Albertson and Thomson (1976) found that a pair of motor neurons each synapse with muscle and gland cells of the pharynx, and speculated that the function of the gland cells may be to secrete digestive enzymes during contraction of the pharynx. The coordinated stimulation of pharyngeal gland secretion and stylet thrusting in plant parasitic nematodes may be the result of an innervation pattern similar to that of the *C. elegans* pharynx, which supplies secretory

somatic-like arrangement warrants this comparison.
and contractile stimuli from the nervous system to the pharyngeal glands and procorpus region, respectively.

The observation that adult male plant parasitic nematodes are capable of stylet thrusting behaviour (Green and Greet, personal communication, as cited by Clark et al., 1973) suggests that the associated pharyngeal musculature may be functioning in concert. The purpose of such a behaviour of nonfeeding adults is unclear, but evidence of possible gustatory receptors in the stoma associated with the stylet of plant parasitic nematodes (Robertson, 1975; Shepherd et al., 1980) suggests that males may be capable of detecting chemical stimuli in the stomal cavity. Contractions of the pharyngeal muscles would be necessary for the nematode to ingest fluids from the surrounding environment and use the putative gustatory receptors to detect chemical substances. Gustatory receptors are known to occur in insects to detect nonvolatile, waterborne nutrients and pheromones (Schneider, 1969; Mullin et al., 1994; Balakireva et al., 1998).

The monoamine, serotonin\(^1\), has been shown to have a profound effect on the copulatory behaviour of nematodes under experimental conditions, and has been beneficial in the determination of the neural control of behaviours in the nematode nervous system. The exogenous application of serotonin may exert its effect on nematodes through absorption of the drug, as serotonin has been shown to cross the semipermeable cuticle of *Ascaris* (Chaudhuri et al., 1988). However, since the concentration of any drug required to produce an effect on a living nematode is, typically, as much as a thousandfold higher that concentrations required when working with exposed cells (Rand and Johnson, 1995), serotonin concentrations of \(10^{-2}\) M have been

\(^1\)5-hydroxytryptamine (5-HT).
used (Horvitz et al., 1982; Raizen and Avery, 1994; Avery et al., 1995). The activity of the pharynx, though not previously reported to be related to copulation, is susceptible to the effects of serotonin. Contraction, or pumping, frequency of the pharynx has been shown to increase in *C. elegans* after serotonin treatment (Horvitz, *et al.*, 1982; Raizen and Avery, 1994; Ségalat *et al.*, 1995; Avery *et al.*, 1995). Second stage juveniles of *M. incognita* responded to serotonin by movement of the stylet (McClure and von Mende, 1987): a behaviour produced by the contraction of the protractor muscles associated with the pharynx. The presence of serotonin activity in the pharynx of several nematode species has been demonstrated (Anya, 1973; Horvitz *et al.*, 1982; Johnson *et al.*, 1996; Brownlee *et al.*, 1994), and it appears that serotonin may serve as a neurotransmitter or neuromodulator in the control of the pharynx during feeding behaviour of *C. elegans* (Albertson and Thomson, 1976; Horvitz *et al.*, 1982) and *Ascaris* (Johnson *et al.*, 1996).

Serotonin has also been shown to be required for tail curling behaviour in *C. elegans* (Loer and Kenyon, 1993), to stimulate tail curling in *Ascaris* (Reinitz and Stretton, 1996), vulval muscle contractions leading to egg-laying (Horvitz *et al.*, 1982; Waggoner *et al.*, 1998) and to inhibit defecation in *C. elegans* (Ségalat *et al.*, 1995). Defecation is relevant in this discussion because the muscles responsible for this behaviour, the anal depressor and sphincter muscles, are modified in males during the fourth juvenile stage to function in spicule eversion and ejaculation, respectively, during copulation of mature nematodes (Sulston *et al.*, 1980). Motor neurons of *C. elegans* controlling tail curling in males, and egg-laying in hermaphrodites, have been identified as serotonergic (Loer and Kenyon, 1993; Ségalat *et al.*, 1995).
1.9 Electrophysiology

Electrophysiological bioassays have proven to be useful in measuring the responses of stimulated insects in the study of pheromone communication (Howse et al., 1998). Responsive activity in male insects has been recorded directly from the antennae, which contain olfactory receptors (Schneider, 1957), from individual sensilla (van der Pers and Minks, 1993) and from the thoracic musculature during wing fanning behaviour (Obrecht and Hanson, 1989) upon exposure of the male to homospecific female pheromone. The successful application of electrophysiology to the study of pheromone communication in insects has provided a system by which similar work with nematodes may be modelled. It is conceivable that the present understanding of the simple nervous system of the nematode (Ward et al., 1975; Ware et al., 1975; Albertson and Thomson, 1976; White et al., 1986) and the exploitation of electrophysiological methods may lead to the isolation of a nematode pheromone.

Studies in nematode electrophysiology have primarily involved the large animal parasite, *A. suum*, and the microscopic, free living nematode, *C. elegans*. These nematodes are taxonomically related only by the presence of the posterior phasmid receptors, typical of the Secernentea (Maggenti, 1991), but otherwise differ from each other in terms of morphology and life history. *Ascaris* spp. were first used in electrophysiological experiments by Jarman (1959), who exploited their potential for studies in neuromuscular physiology, and have since been an ideal animal model because of the relatively large size of the majority of their neurons in comparison with those of other nematode species (Davis and Stretton, 1992). Motor neurons in *A. suum* range from 10-35 μm in diameter (Davis and Stretton, 1989a, 1996) and have allowed for
intracellular and extracellular recordings of neuronal activity (Davis and Stretton, 1989a, 1992).

*Caenorhabditis elegans* is a substantially smaller nematode, approximately 1.3 mm in length and 80 μm in diameter (White et al., 1986), and has received considerable attention in neurobiology. The structure of the entire nervous system of *C. elegans* has been revealed by the reconstruction of serial sections of electron micrographs (Ward et al., 1975; Ware et al., 1975; Albertson and Thomson, 1976; White et al., 1986) and has provided a foundation for the study of the nervous system of all nematode species. Due to the small size of its neurons, typically 2 μm in diameter at the cell body (Avery et al., 1995), neuronal activity of *C. elegans* has, so far, only been recorded using patch-clamp techniques. Intracellular recordings of such small neurons would not be feasible without causing considerable damage to the cell. Goodman et al., (1998) demonstrated that an amphidial neuron, ASE, did not generate classical Na⁺ action potentials under whole-cell patch-clamp recording *in situ*. Their report confirmed earlier findings that classical action potentials could not be recorded from neurons of *A. suum* (Davis and Stretton, 1989a; Davis and Stretton, 1989b). These findings, and the similarity in neuronal structures of *A. suum* and *C. elegans* (Davis and Stretton, 1996), allows one to draw a parallel between the nervous systems of animal parasitic and free living nematodes, such as these, and suggests that plant parasitic nematodes may share similar neuronal characteristics.

The paucity of reports concerning the neurobiology of plant parasitic nematodes in the literature may be a reflection of the technical difficulties encountered when working with such an organism. *Heterodera* spp. in the adult stage may only be 1.25 mm
in length and 34 μm wide (Mai and Mullen, 1996) which is less than half the typical diameter of an adult *C. elegans*. Immobilization and whole-cell recording techniques used with *C. elegans* (Goodman *et al.*, 1998) would, in all likelihood, be more difficult with *H. schachtii*. Furthermore, recent advances afforded by the investigations of *C. elegans*, such as the ultrastructural descriptions of the nervous system (Ward *et al.*, 1975; Ware *et al.*, 1975; Albertson and Thomson, 1976; White *et al.*, 1986) and the completion of the entire genome sequence (*C. elegans* Sequencing Consortium, 1998) have already provided researchers with an ideal nematode model, and have made plant parasites an unlikely target of neurobiological study.

Investigations of the behavioural and physiological effects of nematode sex pheromones have recently been extended into the field of electrophysiology. Using extracellular recording techniques, Riga *et al.*, (1996) were able to record a reversible increase in electrical activity from the cephalic region of male potato cyst nematodes, *Globodera* spp., when the males were exposed to homospecific female pheromones. The increased activity was thought to be the result of a cellular response induced by the application of the pheromone. The electrophysiological assay was subsequently used to demonstrate biological activity of two isolated fractions of the *G. rostochiensis* sex pheromone after high performance liquid chromatographic separation (Riga *et al.*, 1997). However, the cellular responses (Riga *et al.*, 1996) were not determined to be myogenic or neurogenic. The close association between chemosensory structures, such as the amphids, and the three stylet protractor muscles within the cephalic region may bring difficulty to the task of distinguishing between muscular and neuronal activity during extracellular recording. Davis and Stretton (1992) commented on the complications
involved with the interpretation of extracellular recordings from the nerve cords of *A. suum* because of the close association of nerve and muscle tissues. Using a dissected preparation, they were able to distinguish between muscle and neuronal activity in *A. suum* by recording from surgically isolated neuronal and muscle tissues. In addition, only myogenic activity was abolished after the addition of curare to the preparation (Davis and Stretton, 1992). It is not likely that the same criteria developed by Davis and Stretton (1992) can be used to distinguish neuronal activity from activity caused by muscle contraction in plant parasitic nematodes because of the small size of these organisms.

The nearly autonomous nervous system of the *C. elegans* pharynx provides a model system for the study of neural networks and membrane excitability (Albertson and Thomson, 1976; Raizen and Avery, 1994). Raizen and Avery (1994) demonstrated that the contraction of pharyngeal muscles, induced by the application of serotonin, was directly correlated with the recording of extracellular signals from the head of an intact *C. elegans*. They showed that these electrical transients were myogenic, and were the result of the contraction and relaxation of the corpus and terminal bulb of the pharynx. The successful recording of myogenic activity from *C. elegans* represents the potential for the application of techniques similar to those of Raizen and Avery (1994) that may be useful for recording similar muscular events in *H. schachtii*, such as the contractions of the pharynx and associated protractor muscles of the stylet.

1.10 Objectives

The objectives of the present thesis were to confirm the presence of a male-attractive, water soluble sex pheromone released from *H. schachtii* females, and to
isolate and characterize a biologically active component of the pheromone using high performance liquid chromatography. A novel bioassay was developed to demonstrate the biological activity of the *H. schachtii* pheromone on a poorly understood male behaviour. In addition, this bioassay was used to determine if vanillic acid, a putative nematode pheromone, possessed properties similar to those of the isolated *H. schachtii* sex pheromone. The effects of serotonin on the induction of behaviours typically associated with copulation were investigated to confirm the significance of serotonin and copulation reported in other nematode taxa. Finally, extracellularly recorded electrical activity of the male was investigated to determine its potential as an indicator of biological activity in a novel electrophysiological bioassay to indentify the presence of nematode sex pheromones.
Materials and Methods

2.1 Rearing and collection of nematodes

All nematodes originated from a stock of stonehead cabbage, *Brassica oleracea*, L. var. *capitata* (Stokes Seeds Ltd., St. Catharines, Ontario), maintained in fertilized soil under consistent greenhouse conditions at 18°C and a 16 hour photoperiod. Soil and root materials from these cultures were removed as needed no earlier than 8 months after inoculation with *H. schachtii* second stage juveniles (J2s). This amount of time allowed for the propagation of several generations of nematodes. Mobile stages of *H. schachtii* were collected following a modification of the methods of Whitehead and Hemming (1965). Collection procedures involved the use of 20 cm aluminum pans fitted with 18 cm nylon filtering screens. A 50 g sample of soil was taken from stock cabbage cultures and was gently compressed and wrapped in a single piece of tissue (Irving, Dieppe, New Brunswick) which served as an inexpensive, coarse filter. The wrapped soil was then placed on the filtering screen inside the aluminum pans. A volume of 80 ml of water was added in each pan to draw nematodes out of the soil and into the bottom of the aluminum pans for collection. The tissue and filtering screen prevented the unwanted soil and debris from collecting in the pans, and allowed for the separation of nematodes from host roots and soil. Thirty to 40 pans were typically set up in this manner, and were kept in plastic bags in the dark at 23°C to reduce evaporation. After 7 days, water containing only nematodes was collected from pans and stored in an Erlenmeyer flask at 2°C until needed. Male and J2 nematodes could be collected by pipette after two hours when materials had settled to the bottom of the flask and much of the water had been siphoned.
away. Nematodes were manually collected from the water and manipulated using a fine glass hook that was constructed using glass capillary tubes (1.55 mm x 0.86 mm, A-M Systems Inc.) pulled on a micropipette puller (David Kopf Instruments), and flame polished to a fine hook approximately 25-40 μm in thickness.

In order to produce females of similar age, and presumably similar physiological state, females were reared under controlled conditions. Cabbage seedlings were placed in transparent seed growth pouches (Mega International of Minneapolis) 7 days after germination, and grown in the greenhouse, as above. Seedlings were treated only with dechlorinated tap water and were not fertilized, as the development of female cysts and the production of pheromone during rearing may have been adversely affected. After 14 days, when a substantial root system had developed, cabbage seedlings were infected by pipetting 200 *H. schachtii* J2s per seedling. Progress of female development could be observed in the growth pouches throughout the rearing period. Virgin females reached maturity at approximately 3 weeks postinoculation when the gelatinous matrix was produced, and were removed for pheromone collection.

### 2.2 Collection of *Heterodera schachtii* pheromone

An aqueous suspension containing *H. schachtii* female pheromone was produced by carefully removing mature cysts from the host with fine forceps. Since cyst size was correlated with the thickness of the vascular bundle (see Appendix 1), large cysts were consistently selected from large roots, at the proximal region of the the root system, to collect a consistent and maximum amount of pheromone. Twenty females, with gelatinous matrices, were placed into a stoppered vial containing 1 ml of ultrapure water
(Ultrapure Organic Cartridge, Millipak 4.0, 0.22 μm, Millipore) for 3 days to collect any materials the females had released. The aqueous female conditioned medium (FCM) containing pheromone was then separated from the cysts with a pipette and stored at -20°C until used. Initially, several different concentrations of FCM were produced by altering the number of females incubated per ml of ultrapure water, and the number of days of incubation, to determine the incubation methods necessary to collect optimal amounts of pheromone that would yield significant results in bioassays (described below). Lots of 5, 10, and 20 females were each incubated for 2, 3 and 4 days at 23°C. Once optimal methods had been established (Appendix 2), all subsequent amounts of FCM were produced by incubating 20 females for 3 days. Amounts of FCM were produced in this manner as needed, and always used within 3 days of collection.

2.3 Chemical analysis

Absorption spectrophotometry was performed to determine the absorptive properties of FCM in the ultraviolet and visible regions (200-800 nm). This information was necessary for the determination of optimal detection wavelengths to be used during chromatographic analysis (described below). Volumes of 150 μl crude FCM were pipetted into a quartz cuvette (QS 1.0 Hellma, Germany) and placed inside a Beckman DU Spectrophotometer for UV-VIS detection. A 150 μl volume of 10⁻³ M vanillic acid (Sigma), a material believed to have pheromone-like properties (Jaffé et al., 1989) was also tested for absorbance activity.

Reverse-phase partition high performance liquid chromatography (HPLC) was performed using a Hewlett Packard 1090 Liquid Chromatograph to fractionate FCM.
Ultrapure water was the primary component of the mobile phase, and acetonitrile (190, Caledon) was added following a linear gradient from zero to 80% v/v over 12 minutes. Volumes of 150 µl FCM were injected at a rate of 833 µl/min and travelled through a 30 x 3.2 mm 5 µ Spherisorb ODS-2 filter (Phenomenex) and a C-18 250 x 3.2 mm 5 µ Spherisorb ODS-2 column (Phenomenex) at 0.6 ml/min. Column temperature and pressure were maintained below 29°C and 180 bar (1.80 x 10^7 Pa), respectively. Solvents were continuously sparged with helium to remove dissolved gases. The activity of samples eluting between 0 and 12 minutes was monitored using a diode array detector at wavelengths determined by absorption spectrophotometry. Retention time and peak absorbance data were collected and stored using HP ChemStation (Hewlett Packard). Fractionated samples of FCM were collected directly into separate glass concentrator tubes (Kontes) in sequence as they eluted from the column (see below), and were evaporated to near dryness at 23°C using nitrogen gas (Praxair Canada Inc.) to remove mobile phase solvents. Nitrogen gas was regulated at 138 kPa (20 lb/in^2) pressure and discharged through an evaporating unit (Reacti-Vap, Pierce Chemical Company) directly into concentrator tubes. Samples were reconstituted with ultrapure water back to the original injection volume. Evaporation and reconstitution was repeated once more to ensure the absence of organic solvents in FCM fractions. Ultrapure water was fractionated, collected and concentrated as above to serve as control. A 150 µl volume of 10^-4 M vanillic acid (Sigma) was analyzed, by HPLC, in a separate experiment under the conditions described above, but the eluent was not collected.
2.4 Petri dish and stylet activity behavioural bioassays

Materials were assayed for biological activity using a modified version of the Petri dish bioassay described by Riga and Webster (1992). A thin layer of hot 1.5% purified agar (Bactoagar, Difco Laboratories) was poured into 60 mm Petri dishes (Pyrex) and cooled for 1 hour. A 10 μl volume of the test substance was introduced onto the agar surface at the centre (target) of the dish and allowed to diffuse and establish a concentration gradient. After 2 hours, one male *H. schachtii* was placed 15 mm away (at the origin) from the target in each dish, and allowed to move freely over the agar-coated surface. The location of the males could be found at any time during the bioassay by following the tracks left in the soft agar by the movement of the male. The distance between the male and the target was measured under a stereo microscope (Leica, MZ6) at intervals of 30 minutes for 2 hours. Values were converted to represent a straight line distance travelled by the male toward the target by subtraction from the original distance of 15 mm. Ultrapure water was tested as above for control. The responses of 15 males were recorded in each test and control treatment. All experiments were performed at 23°C and dishes were covered with glass lids to reduce evaporation. A Mann-Whitney *U* test was performed for statistical analysis to compare mean responses between treatments (*α*≤0.05).

The biological activity of some test substances was also examined using an additional behavioural assay. Ten *H. schachtii* males were placed in a cavity slide (Fisher) containing 150 μl of the test material. After 1 hour, males were observed under a light microscope (Dialux 20, Leitz) at 100× magnification, and the proportion of males exhibiting stylet thrusting behaviour was scored. Activity of the stylet could be visually
identified by the forward movement of the three-lobed basal knob of the stylet, which is
normally situated at the posterior region of the stomal cavity when not active. Stylet
activity could also be identified by the contractile motion, or shortening, of the stylet
protractor muscles which form attachments between the stylet knob and the cephalic
framework. Males placed in ultrapure water served as control. Each experiment was
performed at 23°C a total of 3 times. The Fisher exact test of proportions was
appropriately used to compare test and control treatments of small sample size and with a
low frequency of observations (Zar, 1996); otherwise, the Chi-squared ($\chi^2$) test was
employed ($\alpha\leq0.05$).

2.5 Female conditioned medium

After crude samples of FCM were shown to attract males in Petri dish bioassays,
FCM was separated into three fractions using HPLC. Materials were collected as they
emerged from the column and divided into fractions based on retention time as follows:
0-4 minutes, 4-8 minutes and 8-12 minutes. Three-fold concentrated samples of these
fractions were also obtained by injecting FCM 3 times and pooling eluted materials into
respective fractions. All fractions were screened for biological activity using Petri dish
bioassays. The fraction that stimulated attractive behaviour in males was further
separated into 4 subfractions: 4-5 minutes, 5-6 minutes, 6-7 minutes and 7-8 minutes. As
above, these subfractions, and the additional 8-9 minute subfraction, were tested for
biological activity using Petri dish bioassays. The testing of crude and fractionated FCM
was performed a total of 6 times.
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Stylet activity bioassays were also used to test crude FCM at original and 10-fold concentrations. Crude FCM was concentrated from a volume of 1.5 ml to 150 µl in Kontes tubes by nitrogen evaporation as described above. In addition, fractionated FCM materials eluting between 6 and 8 minutes after 10 HPLC injections were collected and concentrated by lyophilization at -50°C (Sentry, Virtis Company Inc., Gardiner, New York) and reconstitution with ultrapure water. The 6-8 minute fraction was not concentrated by nitrogen evaporation because of the large volume of the sample.

The effect of the absence of the female gelatinous matrix on the activity of FCM was investigated. Matrix material was separated from the female cyst with fine forceps. The following materials were each incubated in 1 ml ultrapure water for 3 days, producing conditioned media that were tested using Petri dish bioassays: 20 females with gelatinous matrices, 20 females without gelatinous matrices and 20 gelatinous matrices alone.

To determine if FCM was attractive to other stages of *H. schachtii*, the materials released by female nematodes into FCM was tested by exposing *H. schachtii* J2s to FCM in Petri dish bioassays. In addition, a male conditioned medium was produced by incubating 20 males in ultrapure water for 3 days, and tested for behavioural effects on other males in Petri dish bioassays. Male conditioned medium was also concentrated by nitrogen evaporation (as described above) to produce a 10-fold concentrated suspension that was tested for activity in stylet bioassays.

To further characterize substances released by females into FCM, volumes of 150 µl FCM were subjected to a boiling water bath for 1, 5, 10 and 50 minutes to test the heat stability and volatility of FCM. Ultrapure water heated in the same manner for 50
minutes served as control. The media were tested for their effect on male behaviour in Petri dish assays. The volatile, vanillic acid was tested in $10^{-3}$ M and $10^{-5}$ M concentrations in stylet assays to determine if its effect on male behaviour was similar to that of FCM.

Materials from uninfected growth pouch cultures were tested for biological activity to determine if they were a source of contamination in FCM samples. In Petri dish bioassays, males were exposed to water taken directly from cultures, and to media conditioned by incubating, separately, the entire root system of a whole cabbage seedling, and 5 mg (the approximate wet weight of 20 female cysts) of injured root material in 1 ml of ultrapure water for 3 days.

**2.6 Effects of serotonin on male behaviour**

The stylet activity bioassay (described above) was employed to determine the effects of serotonin on male behaviour; however, in these experiments nematode saline (Goodman *et al.*, 1998) containing either $10^{-2}$ M or $10^{-4}$ M serotonin was used instead of ultrapure water. The saline consisted of 145 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 5 mM MgCl$_2$, 10 mM Hepes and 20 mM d-glucose (pH 7.2) and has been shown to support pharyngeal pumping in *C. elegans* (Goodman *et al.*, 1998). Serotonin was added to the saline at the expense of NaCl. Fresh males were incubated in each concentration of serotonin, and saline, as control, for 15 minutes and examined for stylet thrusting behaviour. Previous experience had shown that this was the optimal amount of time for observing stylet thrusting. Experiments were repeated in the same manner with fresh juveniles (J2s).
The males used in the above experiments were observed until a total elapsed time of 45 minutes, as determined from preliminary experiments, in both concentrations of serotonin and saline, as control, to investigate the effects of serotonin on spicule activity. The activity of the spicules could be observed by the obvious protrusion of the blades through the cloaca. Second stage juveniles were not tested for spicule activity in serotonin due to the absence of the spicules. All experiments were performed at 23°C.

2.7 Electrophysiology

A preparation was developed by which extracellular electrical activity in the anterior region of *H. schachtii* males could be recorded during stylet thrusting activity. Males were anaesthetized with 0.25% 2-phenoxyethanol (Sigma) (Townshend, 1983) in nematode saline (Goodman *et al.*, 1998) for 20 minutes until their movement became slow. Movement of the nematodes had to be reduced to facilitate successful immobilization. The following immobilization procedures were adapted from Goodman *et al.* (1998). A droplet of hot agar (Bactoagar, Difco Laboratories) was placed on a sterile microscope slide (Fisher) and immediately pressed against the slide with a coverslip (Corning). The coverslip was slowly drawn away by sliding it off the long edge of the microscope slide. The resulting film of agar coating the slide was very thin and would evaporate within 2 or 3 minutes. The anaesthetized male was immediately placed on the thin film of agar and glued into position. Glass pipettes were fitted with capillary tubes (A-M Systems Inc.) made (as described above) with an open tip diameter of approximately 15-20 μm, to apply the glue to the preparation. The glue was a cyanoacrylate compound (Nexaband, Veterinary Products Laboratories, Phoenix,
Arizona) which, upon contact with an alkaline environment, polymerized in less than 6 seconds to form a thin bond between the nematode and the agar. Minute volumes of glue, less than 1 µl, were placed at three positions on the agar film on either side of the nematode: near the cephalic, middle and tail regions. After application, the glue droplets diffused on the agar and made contact with the nematode cuticle. The preparation was quickly washed with M9 buffer (Sambrook et al., 1989) to prevent uncontrolled polymerization and damage to the cuticle of the nematode. The immobilization process was completed in less than 2 minutes to avoid evaporation of the agar and dehydration of the nematode. Excess agar was trimmed away from the slide with a razor blade to allow for the placement of a circular ring of wax (Surgident, Miles Inc. Dental Products, South Bend, Indiana), approximately 20 mm in diameter and 5 mm thick, which held 400 µl of saline in which the nematode was maintained during recording experiments. Males, still under the anaesthetic effects of 2-phenoxyethanol, were washed with 20 ml saline until the nematode regained movement in areas that were not immobilized.

The slide preparation was placed on the stage of a compound microscope (Dialux 20, Leitz), under 160x magnification, inside a Farraday cage. A glass microelectrode (A-M Systems Inc.) was pulled on a micropipette puller (David Kopf Instruments) and filled with 1.5 M NaCl. Using a micromanipulator (Narishige, Japan), the electrode tip was driven through the cuticle of the nematode and placed near the posterior, glandular, region of the pharynx to avoid contact with the pharyngeal musculature. The indifferent electrode was placed in the recording medium near the recording electrode. Both positive and negative leads were silver wires plated with AgCl. Signals were amplified with an AC amplifier (model 1800, A-M Systems Inc.) with low and high cut filters set at
1.0 Hz and 5kHz, respectively, and monitored on an oscilloscope (Nicolet Instrument Corp., Madison, Wisconsin). Potentials were recorded (model B instrument recorder, A.R. Vetter Co., Rebersburg, Pennsylvania) and analyzed with a data acquisition programme (Sensory Neuron, Brock University) with a sampling rate of 1 ms/pt. The recording medium consisted of nematode saline and $10^2$ M serotonin, known to induce pharyngeal pumping (Horvitz et al., 1982; Raizen and Avery, 1994; Avery et al., 1995). Control recordings were obtained from nematodes in normal saline.

2.8 Identification of spicular extrusions

Male nematodes incubated in $10^2$ M serotonin for over 1 hour began to extrude materials through the extension of the cloaca formed by the interlocking spicules. The possibility of this material being spermatids was investigated. A flow chamber was constructed, following the methods of Shakes and Ward (1989), to identify the presence of spermatids by the activation of amoeboid movement. A glass coverslip was elevated above the surface of a microscope slide by the application of petroleum jelly (Vaseline, Chesebrough Pond's Inc., Markham, Ontario) along the long edges of the slide, allowing the flow of solution through the chamber. Spermatids undergo a transformation, spermiogenesis, from sessile, spherical bodies to motile, amoeboid spermatozoa during mating, as reviewed by Bird and Bird (1991). Spermiogenesis is, evidently, induced in vivo by endogenous activating factors produced by the glandular vas deferens during sexual stimulation (Foor and McMahon, 1973; Foor, 1976).

Males (n=4) previously incubated in $10^2$ M serotonin for 1 hour and having extruded materials collected at the spicule tip were individually placed in the flow
chamber in sperm medium. Sperm medium (Shakes and Ward, 1989) contained the following: 50 mM NaCl, 25 mM KCl, 5 mM CaCl₂, 1 mM MgSO₄, 5 mM Hepes and 60 mM triethanolamine. Triethanolamine activates spermiogenesis by causing a rapid increase in spermatid intracellular pH (Ward et al., 1983). Fresh sperm medium was continuously applied to one end of the flow chamber by pipette, and taken up at the other end by a wedge of filter paper, allowing a constant flow through the chamber. The extruded material at the tip of the spicules was observed for spermatozoa-like activity after 10 minutes. In addition, the flow chamber was used for the application of Wright’s stain, commonly used to stain sperm cells of invertebrates (Weesner, 1963), to the males to determine if sperm cells had been extruded through the spicules. Both procedures, sperm activation and sperm staining, were also performed, as above, using spermatids removed directly from the testis by dissection of the male through the gonad. The contents of nematodes are under pressure and readily dissociate once the cuticle has been breached. This provided an opportunity to demonstrate the activating and staining abilities of triethanolamine and Wright’s stain, respectively, on *H. schachtii* spermatids for comparison with materials extruded through the spicules.

2.9 Photography

All images were captured using a compound microscope (Dialux 20, Leitz) coupled with a digital camera (HV-C20M, Hitachi Denshi Ltd., Japan) and a capturing programme (KS 400 v2.0, Kontron Elektronik, GmbH).
Results

3.1 Biological activity of the female conditioned medium

The biological activity of the crude female conditioned medium was demonstrated in both Petri dish and stylet activity bioassays. Males travelled a significantly greater distance \((P<0.01)\) toward the target when the target contained FCM than when it contained ultrapure water (Fig. 1A). This indicated the presence of a male-attractive substance contained in FCM. Male attraction to FCM was evident as early as 30 minutes into the assay and increased in magnitude over the 120 minute period (Fig. 1A). The biological activity of crude FCM was demonstrated in this manner a total of 6 times \((i.e. \, with \, 90\, different\, males)\) before each HPLC fractionation experiment, utilizing fresh FCM in each experiment and yielding similar results (data not shown). In addition, the proportion of males that displayed stylet thrusting behaviour when incubated in concentrated FCM, unconcentrated FCM or ultrapure water was significantly dependent \((P<0.0001)\) on the FCM concentration (Fig. 1B). The proportion of males with active stylets was highest in concentrated FCM. The concentration dependent effect of FCM on male behaviour is also evidence of a male-attractive substance in the FCM.

Materials collected from initial HPLC fractionation procedures were assayed and a biologically active fraction of FCM was found. Males travelled a significantly greater \((P<0.05)\) distance toward the target when the 4-8 minute fraction had been introduced to the agar medium than when the corresponding control fraction of ultrapure water was assayed (Fig. 2B). Males did not exhibit a response that was significantly different from
Figure 1. Response of *Heterodera schachtii* males to crude female conditioned medium (FCM). *(A)* Movement of *H. schachtii* males toward the target in the presence of FCM (●) and ultrapure water as control (○) over a period of 120 minutes in Petri dish bioassays. Means (n=15) and ±SEM are reported. Asterisks denote a significant difference from control according to the Mann-Whitney *U* test: *=P<0.01; **=* *P<0.001.* *(B)* Percentage of males exhibiting stylet thrusting behaviour after incubation in 10× concentrated FCM (n=30), unconcentrated FCM (n=30) and ultrapure water as control (n=60). Proportions are significantly different from each other: $\chi^2$=39.49, *P*<0.0001.
A

Mean distance travelled toward target (mm)

Time (min.)

B

Males with active styliets (%)

Incubation media

FCM x10
FCM
Control
Figure 2. Response of *Heterodera schachtii* males to fractionated female conditioned medium (FCM) and corresponding ultrapure water fractions as control. Movement of *H. schachtii* males toward the target in the presence of the FCM fraction eluting between (A) 0 and 4 minutes (●) and control (○), (B) 4 and 8 minutes (●) and control (○), (C) 8 and 12 minutes (●) and control (○) over a period of 120 minutes in Petri dish bioassays. Means (n=15) and ±SEM are reported. Asterisks denote a significant difference from control as indicated by the Mann-Whitney *U* test: *=*P<0.05; **=*P<0.01.
controls when the 0-4 minute and 8-12 minute FCM fractions were assayed (Fig. 2A, C). This experiment was repeated a second time, with different males and fresh FCM, and similar results were obtained (data not shown). In addition, males travelled significantly further \( (P<0.01) \) toward the target when the target contained the concentrated 4-8 minute fraction of FCM than when it contained the concentrated 4-8 minute fraction of ultrapure water (Fig. 3B). This response was not evident in assays of other concentrated FCM fractions (Fig. 3A,C). A concentration dependent difference in male behaviour, associated only with the active 4-8 minute FCM fraction, is illustrated in Figure 4. Males had travelled a significantly further distance \( (P<0.05) \) toward the target after a time of 90 minutes when exposed to the concentrated 4-8 minute FCM fraction than when exposed to the unconcentrated 4-8 minute FCM fraction. The distance travelled toward the target in concentrated and unconcentrated control treatments (ultrapure water) did not differ significantly. The Petri dish assay results of the above fractions at two concentrations indicated that males responded to the 4-8 minute fraction in a concentration dependent manner.

The active 4-8 minute fraction was further separated into 4 one-minute subfractions. Bioassay results indicated that males were not significantly attracted to the 4-5 minute FCM subfraction (Fig. 5A). Although males did respond to the 5-6 minute subfraction in a significantly different manner \( (P<0.05) \) than in controls, this was not apparent until after 60 minutes into the assay (Fig. 5B). This observation was not typically seen when assaying the crude FCM. Males had normally travelled a significantly further distance \( (P<0.01) \) toward the target, in the presence of crude FCM, than had the controls as early as 30 minutes into the assay (Fig. 1A). This indicates that
Figure 3. Response of *Heterodera schachtii* males to 3× concentrated fractions of female conditioned medium (FCM) and corresponding concentrated ultrapure water fractions as control. Movement of *H. schachtii* males toward the target in the presence of the concentrated FCM fraction eluting between (A) 0 and 4 minutes (●) and control (○), (B) 4 and 8 minutes (●) and control (○), (C) 8 and 12 minutes (●) and control (○) over a period of 120 minutes in Petri dish bioassays. Means (n=15) and ±SEM are reported. Asterisks denote a significant difference from control as indicated by the Mann-Whitney U test: *P<0.01; **P<0.001.
Figure 4. Summary of the response of *Heterodera schachtii* males to two different concentrations of fractionated female conditioned medium (FCM). Data were taken from Fig. 2B and 3B and displayed for clarity. Movement toward the target after 90 minutes in the presence of 3× concentrated, and unconcentrated FCM fraction (solid bars) and control (open bars) eluting between 4 and 8 minutes. Asterisks indicate that the values are significantly different from each other according to the Mann-Whitney *U* test (*P*<0.05).
Figure 5. Response of *Heterodera schachtii* males to female conditioned medium (FCM) subfractions and corresponding ultrapure water subfractions as control. Movement of *H. schachtii* males toward the target in the presence of the FCM subfraction eluting between (A) 4 and 5 minutes (●) and control (○), (B) 5 and 6 minutes (●) and control (○), (C) 6 and 7 minutes (●) and control (○), (D) 7 and 8 minutes (●) and control (○), (E) 8 and 9 minutes and control (○) over a period of 120 minutes in Petri dish bioassays. Means (n=15) and ±SEM are reported. Asterisks denote a significant difference from control as indicated by the Mann-Whitney *U* test: *=*P<0.05; **=*P<0.005.
the 5-6 minute subfraction may not have contained the same concentration of the attractive substance contained in the crude FCM, and suggests that the active component of FCM may have been in other fractions as well. Males were, however, significantly attracted \((P<0.05)\) to the 6-7 minute and 7-8 minute subfractions as early as 30 minutes into the assay (Fig. 5C, D), as seen in assays of crude FCM (Fig. 1A). This suggests that the majority of the active substance initially detected in the crude FCM (Fig. 1A) and the 4-8 minute FCM fraction (Fig. 2B) was contained in the latter two subfractions, and eluted between 6 and 8 minutes. The 8-9 minute FCM subfraction was assayed, and no significant attraction to the target was observed (Fig. 5E), demonstrating that materials eluting after 8 minutes were not biologically active (Fig. 2C, 3C, 5E). This experiment was repeated twice more, with fresh FCM and different nematodes, and similar results were obtained (data not shown). In one repetition, males were not significantly attracted to the 5-6 minute and 6-7 minute FCM subfractions until after 90 minutes and 120 minutes, respectively, emphasizing the relatively smaller quantity of the active substance eluting between 5 and 7 minutes. Figure 5A-E illustrates that the active substance of FCM, after HPLC fractionation, was most likely to elute between 6 and 8 minutes. However, the pooling of the 6-7 minute and 7-8 minute subfractions yielded no significant results in stylet activity bioassays (Fig. 6), suggesting that the substance stimulating stylet thrusting behaviour does not elute between 6 and 8 minutes.

Absorption spectrophotometry of a sample of FCM indicated the presence of absorbance activity between 260 nm and 275 nm \((n=4)\) (see Appendix 3). In one trial, a distinct absorbance peak was recorded at 260 nm. Absorption spectrophotometric analysis of vanillic acid consistently indicated distinct absorbance peaks at 204 nm, 252
Figure 6. Effect of fractionated female conditioned medium (FCM) on stylet activity of male *Heterodera schachtii*. Percentage of males exhibiting stylet thrusting behaviour after incubation in the 10× concentrated FCM fraction eluting between 6 and 8 minutes (n=10) and corresponding ultrapure water fraction as control (n=10). Values are not significantly different from each other according to the Fisher exact test.
nm and 286 nm (n=3), which indicated that the spectral signature of vanillic acid is markedly different from that of FCM (Appendix 3).

Figure 7A is a representative chromatogram of a sample of FCM during HPLC fractionation. Six different FCM samples, and ultrapure water as control, were fractionated by HPLC in total (see also Appendix 4) and absorbance at 260 nm was measured. In all fractionation experiments, a relatively large (5 mAU, in Fig. 7A) absorbance peak at approximately 2 minutes (Fig. 7A) was detected that was not detected in controls (Fig. 8A), indicating a substance of relatively high concentration eluting during the collection of the 0-4 minute FCM fraction. This fraction, however, was not biologically active in Petri dish bioassays (Fig. 2A, 3A). A series of lower magnitude (1.5 mAU to 2.5 mAU, in Fig. 7A) absorbance peaks was detected between, approximately, 5.5 and 7 minutes in FCM that was not detected in control (Fig. 8A). The response of males to FCM subfractions and controls in bioassays after 90 minutes are summarized (Fig. 7B, 8B), and correspond to respective chromatogram retention times (Fig. 7A, 8A). Materials of FCM eluting between 5.5 and 7 minutes were characterized by absorbance activity, indicated by absorbance peaks (Fig. 7A), and significantly attracted (P<0.0001) males (Fig. 7B). By contrast, ultrapure water subfractions were not characterized by a series of small absorbance peaks between 5.5 and 7 minutes and did not attract males (Fig. 8A, B). The lower magnitude absorbance peaks in FCM were not as consistent between HPLC fractionation experiments as the large absorbance peak recorded at 2 minutes (Fig. 7A, Appendix 4). Although absorbance activity between 5.5 and 7 minutes was always present in FCM samples, the retention times of these peaks varied substantially. The large biphasic spikes at approximately 5.5 minutes were system peaks
Figure 7. Chromatographic analysis and attractiveness of the female conditioned medium (FCM) to *Heterodera schachtii* males. (A) Representative chromatogram of reverse-phase HPLC analysis of FCM containing *H. schachtii* female sex pheromone. Absorbance activity was detected at 260 nm and is displayed as a function of time (min.). (B) Summary of the response of *H. schachtii* males (n=15) to FCM subfractions in Petri dish bioassays after 90 min. Abscissa of the histogram corresponds with that of the chromatogram above for clarity. Means ±SEM are reported. A significant difference exists among the responses of males to FCM subfractions according to the Kruskal-Wallis test ($P<0.0001$).
Figure 8. Chromatographic analysis and attractiveness of ultrapure water as control to *Heterodera schachtii* males. (A) Representative chromatogram of reverse-phase HPLC analysis of ultrapure water. Absorbance activity was detected at 260 nm and is displayed as a function of time (min.). (B) Summary of the response of *H. schachtii* males (n=15) to ultrapure water in Petri dish bioassays after 90 min. Abscissa of the histogram corresponds with that of the chromatogram above for clarity. Means ±SEM are reported. No significant difference exists among the responses of males to control subfractions according to the Kruskal-Wallis test.
A

Elution time of subtractions (min.)

B

Mean distance travelled (mm)

Elution time of subfractions (min.)
Figure 9. Chromatographic analysis of vanillic acid, and the effect of vanillic acid on *Heterodera schachtii* male behaviour. (A) Representative chromatogram of reverse-phase HPLC analysis of $10^{-5}$ M vanillic acid. Absorbance activity was detected at 260 nm and is displayed as a function of time (min.). (B) Percentage of males with active stylets after incubation in $10^{-3}$ M vanillic acid (VA) (n=30), $10^{-5}$ M VA (n=20) or ultrapure water as control (n=50). $\chi^2$ analysis indicated no significant difference among proportions of male responses.
caused by the absorbing properties of the organic solvents, and were present in FCM and control chromatograms. Occasionally, small peaks were recorded in ultrapure water eluting between 4 and 8 minutes that were, presumably, the result of the column or solvent impurities (Fig 8A, Appendix 4). Illustrated in Figure 9A is a chromatogram of a sample of $10^{-5}$ M vanillic acid during HPLC separation. Analysis revealed a large absorbance peak at, approximately, 2 minutes and several small absorbance peaks between 5.5 and 9.5 minutes. These characteristic peaks are not unlike those seen in FCM HPLC analysis (Fig. 7A). Although the HPLC elution profile of vanillic acid resembled that of FCM, *H. schachtii* males did not respond to vanillic acid (Fig. 9B) as they did to FCM (Fig. 1B) in stylet activity bioassays. The proportion of males with active stylets did not differ significantly among those incubated in $10^{-3}$ M vanillic acid, $10^{-5}$ M vanillic acid or nematode saline. In addition, males incubated in $10^{-3}$ M vanillic did not display any stylet thrusting activity (Fig. 9B).

The biological activity of FCM was not attenuated when the gelatinous matrices were removed from females prior to incubation in ultrapure water. Males were significantly attracted ($P<0.05$) to FCM and to FCM without gelatinous matrix material in the incubation medium when compared to ultrapure water and to gelatinous matrix conditioned medium (Fig. 10). There was no significant difference between the response of males to FCM and the response of males to FCM without matrices; nor was there a significant difference between the response of males to matrices only and the response of males to ultrapure water (Fig. 10).
Figure 10. Effects of the gelatinous matrix on *Heterodera schachtii* male behaviour. Movement of *H. schachtii* males toward the target in the presence of media conditioned by whole females (FCM) (●), females with gelatinous matrices removed (○), gelatinous matrices only (■) and ultrapure water as control (□) over a period of 120 minutes in Petri dish bioassays. Means (n=15) and ±SEM are reported. Asterisks denote a significant difference between the activity of materials from females (*i.e.* ●, ○) and from gelatinous matrices or control (*i.e.* ■, □) according to the Mann-Whitney *U* test (*P*<0.05).
Figure 11. Response of *Heterodera schachtii* juveniles (J2s) to the female conditioned medium (FCM). Movement of *H. schachtii* J2s toward the target in the presence of FCM (●) and ultrapure water as control (○), over a period of 120 minutes in Petri dish bioassays. Means (n=15) and ±SEM are reported. Values are not significantly different from controls according to the Mann-Whitney *U* test.
The response of *H. schachtii* J2s to FCM was investigated. Juveniles did not travel significantly further toward the target when the target contained crude FCM than when it contained ultrapure water (Fig. 11), suggesting that the activity of FCM may be male-specific. Males did not respond significantly to the male conditioned medium in bioassays when compared with controls (Fig. 12A). This experiment was repeated with similar results (data not shown). In addition, the proportion of males with active stylets after incubation in concentrated male conditioned medium did not differ significantly from that of males incubated in ultrapure water (Fig. 12B). This demonstrates that males do not produce the same male-attractive substance as the females.

Bioassays indicated that the crude FCM contained a relatively stable active component. After subjecting samples of FCM to a boiling water bath for either 1, 5, 10 or 50 minutes, males were significantly more attracted (*P*<0.05) to all heated FCM samples than they were to ultrapure water that had been placed in a boiling water bath for 50 minutes (Fig. 13). The activity of the crude FCM was not reduced after the samples were heated.

Materials taken from uninfected growth pouches did not elicit an attractive response from males in bioassays. Males did not travel significantly further toward the target when the target contained ultrapure water than when it contained water taken directly from cultures, or when it contained media conditioned by a whole cabbage seedling or 5 mg of injured root material (Fig. 14). These results indicate that the biological activity of FCM was not due to contamination from seed growth pouch cultures.
Figure 12. Response of *Heterodera schachtii* males to male conditioned medium. (A) Movement of *H. schachtii* males toward the target in the presence of male conditioned medium (●) and ultrapure water as control (○), over a period of 120 minutes in Petri dish bioassays. Means (n=15) and ±SEM are reported. Values are not significantly different from controls according to the Mann-Whitney *U* test. (B) Percentage of males exhibiting stylet thrusting behaviour after incubation in 10× concentrated male conditioned medium (n=10) or ultrapure water as control (n=10). Values are not significantly different from each other according to the Fisher exact test.
A

![Graph showing mean distance travelled toward target (mm) over time (min).]

B

![Bar chart showing percentage of males with active styles in MCM and Control incubation media.]

- MCM
- Control
Figure 13. Chemical stability of the female conditioned medium (FCM). Movement of *H. schachtii* males toward the target in the presence of FCM heated for 1 minute (○), 5 minutes (□), 10 minutes (■) 50 minutes (●), or ultrapure water heated for 50 minutes as control (○), over a period of 120 minutes in Petri dish bioassays. Means ±SEM are reported. Asterisks denote a significant difference from control as indicated by the Mann-Whitney *U* test: *P*<0.05.
Figure 14. Effects of materials removed from uninfected growth cultures on *Heterodera schachtii* male behaviour. Movement of *H. schachtii* males toward the target in the presence of water removed from growth pouches (●); or media conditioned with 5 mg of injured root material (■), the root system of a seedling (○) or ultrapure water as control (□). Means ± SEM are reported. Values are not significantly different from control according to the Mann-Whitney *U* test.
3.2 Effects of serotonin on male behaviour

Male and juvenile *H. schachtii* displayed vigorous stylet thrusting behaviour, approximately 1 to 2 thrusts per second, after 15 minutes of incubation in $10^{-2}$ M serotonin (Fig. 15A, B). In both male and juvenile groups, the proportion of nematodes with active stylets was significantly dependent ($P<0.0001$) on the concentration of serotonin in the saline (Fig. 15A, B). Extracellular recordings from the anterior region of the male demonstrated that electrical activity coincided with stylet thrusting behaviour. Males incubated in $10^{-2}$ M serotonin displayed stylet thrusting and generated spike activity at a frequency of, approximately, 1 thrust per second (Fig. 16A), while control males in normal saline did not display stylet thrusting and produced no electrical activity (Fig. 16B). Although spike activity appeared to be associated with stylet thrusting behaviour, the origin of the electrical potentials could not be determined.

After the males had been exposed to $10^{-2}$ M serotonin for 45 minutes, the cessation of stylet thrusting was common (some stylets remained motionless in the protruded position), and tail curling and spicule eversion occurred (Fig. 17). Spicule eversion behaviour included the slow protruding and retracting motion of the spicules (Fig. 17) and was significantly dependent ($P<0.0001$) on the concentration of serotonin (Fig. 18). In fact, only males incubated in $10^{-2}$ M serotonin displayed spicule eversion behaviour (Fig. 18). In addition, several males with active spicules extruded materials through the tube-like extension of the cloaca formed by the interlocking blades of the spicules. The extruded material from males ($n=10$) usually consisted of a homogeneous substance (Fig. 19A, B, C, D; 20C) and, occasionally, males ($n=2$) released spherical bodies less than 5 μm in diameter that may have been spermatids (Fig. 19C). These
Figure 15. Effect of serotonin on stylet activity of *Heterodera schachtii* males. (A) The percentage of males exhibiting stylet thrusting behaviour after incubation in $10^{-2}$ M serotonin (5-HT) ($n=30$), $10^{-4}$ M 5-HT ($n=30$) or nematode saline as control ($n=60$) for 15 minutes. Proportions are significantly different from each other: $\chi^2=91.02$, $P<0.0001$. (B) The percentage of juveniles exhibiting stylet thrusting behaviour after incubation in $10^{-2}$ M 5-HT ($n=30$), $10^{-4}$ M 5-HT ($n=30$) or nematode saline as control ($n=60$) for 15 minutes. Proportions are significantly different from each other: $\chi^2=101.61$, $P<0.0001$. 
Figure 16. Representative extracellular recordings of electrical activity from the anterior region of *Heterodera schachtii* males. Males were placed in *(A)* nematode saline containing $10^{-2}$ M serotonin and *(B)* normal nematode saline. Electrical potentials in *(A)* coincided with stylet thrusting behaviour. Scale bars apply to both traces.
Figure 17. Representative photograph of spicule eversion behaviour of male *Heterodera schachtii* caused by serotonin. The male was contained in a cavity slide and was exposed to $10^2$ M serotonin for 45 minutes. Arrow indicates the tip of the everted spicules. Note the curling of the tail. Scale bar = 20μm.
Figure 18. Effect of serotonin on spicule activity of *Heterodera schachtii* males. Percentage of males exhibiting spicule eversion behaviour after incubation in $10^{-2}$ M serotonin (5-HT) (n=30), $10^{-4}$ M 5-HT (n=30) or nematode saline as control (n=60) for 45 minutes. Proportions are significantly different from each other: $\chi^2=114.73$, $P<0.0001$. 
Incubation media

Males with active spicules (%)

$10^{-2}$ M 5-HT  $10^{-4}$ M 5-HT  Saline
spherical bodies, however, dissociated quickly in saline and could not be collected for analysis. The extrusion of materials through the spicules was a rapid process that involved the release of materials in bursts (presumably caused by the intermittent release of internal pressure) and the collection of materials at the tip of the spicules (Fig. 19A, B, C, D). The homogeneous materials that were extruded appeared to originate from the vas deferens of the nematodes (n=4).

Spermatids obtained directly from the testis of *H. schachtii* males (n=4) were successfully activated with triethanolamine after 10 minutes and underwent the process of spermiogenesis to become motile spermatozoa, as indicated by the amoeboid morphology of the cells and the formation of pseudopodia (Fig. 20), which are typical characteristics of *Heterodera* spermatozoa (Shepherd et al., 1973). In addition, spermatids obtained directly from the testis of males (n=3) could be identified by their tendency to take up Wright’s stain and turn pink, or purple, in colour (Fig. 20B). However, both methods of identifying the presence of spermatids (*i.e.* activation and staining) indicated that the homogeneous materials collected at the tip of the spicules after incubation in 10⁻² M serotonin were not spermatids or spermatozoa. Extruded material from males did not become activated (n=4) upon exposure to triethanolamine (Fig. 20C), and did not take up Wright’s stain (n=3) (not shown).
Figure 19. Representative photograph of the extrusion of materials through the spicules of male *Heterodera schachtii* caused by serotonin. The male was contained in a cavity slide and was exposed to $10^2$ M serotonin for 60 minutes. Each frame was photographed in sequence (A, B, C, D) and separated by 5 seconds. Square brackets in each frame indicate increasing amounts of the material being extruded from the tip of the spicules. Arrow in C indicates a small, spherical body that resembled a spermatid. Scale bar in D = 25μm.
Figure 20. Identification of materials being extruded from the tip of the spicules of male *Heterodera schachtii* caused by serotonin. All materials were photographed in a microscope slide flow chamber. (A) Spermatozoa exhibiting pseudopod formation (indicated by arrows) induced by 60 mM triethanolamine in sperm medium after 10 minutes. Other spherical cells are spermatids that had not undergone spermiogenesis. Scale bar = 5 μm. (B) Spermatids removed directly from the male gonad and stained pink / purple with Wright’s stain. Several spermatids had dissociated from the male, while others remained attached to the rachis of the testis. Scale bar = 10 μm. (C) Extrusions (indicated by square bracket) from the tip of the spicules of the male after treatment with 60 mM triethanolamine in sperm medium of 10 minutes. Scale bar = 20 μm.
Discussion

4.1 The *Heterodera schachtii* female sex pheromone

The present results confirm the existence of a male attractive, water soluble sex pheromone that is released from homospecific *H. schachtii* females. The active component of the pheromone, contained in the female conditioned medium, was partially isolated and characterized. The male-attractive material stimulates chemotaxis and stylet thrusting behaviour, in a concentration dependent manner, in its crude form but only chemoattractive behaviour after HPLC fractionation. This suggests that the active component of FCM that stimulates chemotaxis may not be the same component that causes stylet thrusting behaviour. Only males were attracted to materials released by females, presumably through the vulva, and the gelatinous matrix alone did not contain any detectable amounts of male attractive substances. There is evidence that implicates the vulva as the site of release of the pheromone. Marchant (1970) demonstrated that the attraction of male *Heligmosomoides* (formerly *Nematospiroides*) to homospecific females was abolished when the vulval region of the females was covered with silicone grease. The present findings differ from those of a previous study, where it was reported that males were equally attracted to gelatinous matrices as they were to females (Aumann and Hashem, 1993). This discrepancy may be due to the fact that Aumann and Hashem (1993) obtained matrix extracts by incubating 12 gelatinous matrices in 200 µl of water, producing a suspension 3 times more concentrated than that used in the present work. Nonetheless, these results demonstrate that males were more attracted to females than they were to the gelatinous matrix at lower concentrations, and provide support for the
idea that the gelatinous matrix, when intact, may act as a carrier of the pheromone (Green and Greet, 1972; Aumann and Hashem, 1993). Bird and Soeffky (1972) examined the ultrastructure of the gelatinous matrix of *M. javanica*, and determined that the matrix consisted of a fine meshwork that may serve to inhibit water loss from the eggs. One function of the gelatinous matrix may be to regulate, or slow, the diffusion of the pheromone released through the vulva into the surrounding medium. The maintenance of a highly concentrated source of pheromone would ensure the stability of a concentration gradient *in vivo* and create an environment conducive to mate finding behaviour of males.

Aumann *et al.* (1998b) reported characterization of a water soluble component of the *H. schachtii* sex pheromone. However, aqueous females extracts were collected by incubating entire 3 to 4 month old monoxenic nematode cultures on turnip or white mustard in water (Aumann *et al.*, 1998b), and the possibility that the cultures and nutrient media contributed additive, or contaminating, substances to the female extracts was not considered. The present methods involved the culturing of *H. schachtii* females on cabbage, without the use of nutrients or fertilizer, and the collection of an aqueous medium conditioned by females that did not contain contaminants from growth cultures capable of affecting male behaviour. In addition, Aumann *et al.* (1998b) did not use an appropriate control in their bioassay experiments. Males were reported to have been significantly more attracted to female materials extracted in water than to female materials extracted in diethyl ether (Aumann *et al.*, 1998b), but the attraction of males to either of the pure solvents was not investigated for comparison.
Results reported here, that the biologically active substance of FCM did not degrade in a boiling water bath and was not volatile, indicate that the active substance of FCM was stable in solution. The observation that the biologically active properties of FCM did not decrease after nitrogen evaporation of HPLC-fractionated samples also suggests that the active substance was not volatile. Green and Plumb (1970) have reported the stability of the pheromone and found that attractants from females of several *Heterodera* spp. remained active for several months when stored at 5°C. Aumann and Hashem (1993) demonstrated that the concentration of active substances in extracts from *H. schachtii* females was not reduced after incubation with pronase, a broad spectrum proteolytic enzyme, or leucine aminopeptidase at 37°C for 20.5 hours, and suggested that the *H. schachtii* pheromone was not a polypeptide.

Several lines of evidence indicate that vanillic acid, the proposed pheromone of *H. glycines* (Jaffe et al., 1989), is not an active component of the *H. schachtii* sex pheromone. Males have been shown not to respond to vanillic acid in Petri dish bioassays (Aumann and Hashem, 1993), and the present work has demonstrated that vanillic acid did not elicit stylet thrusting behaviour in *H. schachtii* males. By contrast, males were attracted to FCM in Petri dish bioassays and displayed stylet thrusting behaviour when exposed to FCM. This suggests that vanillic acid and the active component of FCM do not have similar effects on *H. schachtii* males; therefore, vanillic acid is not likely to be an active component of the *H. schachtii* sex pheromone. The reported "paralytic-like" effect that vanillic acid, at concentrations greater than $10^5$ M, had on *H. glycines* males (Jaffe et al., 1989) may account for the observation that no males exhibited stylet thrusting behaviour when incubated in $10^3$ M vanillic acid in the
present experiments. It is unclear why a putative pheromone substance would have such a potent effect at high concentrations, but Jaffe et al. (1989) speculated that it may have been the result of an “overload of pheromone receptor(s)”. In addition, the difference between the absorption spectra of vanillic acid and FCM indicates a difference in physical properties between the two substances. However, the HPLC elution profiles of FCM and vanillic acid share similar characteristics, which is consistent with previous findings, that the elution profile of vanillic acid and the pheromone of H. glycines were identical (Jaffe et al., 1989). The presence of vanillic acid in FCM is suggested by the large absorbance peak recorded at, approximately, 2 minutes in FCM and in vanillic acid. However, the FCM material eluting between 0 and 4 minutes was not biologically active. The likelihood of vanillic acid being an active component of the H. schachtii sex pheromone is further diminished when one considers the nematicidal properties of vanillic acid analogues (Varma et al., 1993) and the efficacy of such a volatile substance diffusing in a moist soil environment.

Green and Plumb (1970) investigated the attractiveness of female secretions from 10 Heterodera spp. to heterospecific males. Males of H. schachtii and H. glycines were attracted to materials released by females of both these species, and it was postulated that females of H. schachtii and H. glycines released a common attractive substance, termed delta (Green and Plumb, 1970). This evidence, and the fact that hybrid offspring have been produced by the mating of H. schachtii and H. glycines (Potter and Fox, 1965), indicates a close relationship between these two species and suggests that they may share a common sex pheromone component. The present observation, that H. schachtii males do not respond to vanillic acid, challenges the evidence that vanillic acid is an active
component of the *H. glycines* sex pheromone (Jaffe *et al.*, 1989). Unfortunately, Jaffe *et al.* (1989) did not present the results of their bioassay experiments or data analysis in any detail, as they reported that vanillic acid was "extremely active" with 60% of the males responding to $10^{-7}$ M vanillic acid (Jaffe *et al.*, 1989).

The efficacy of vanillic acid, and its analogues, in the reduction of *H. glycines* populations has been tested in the field (Meyer *et al.*, 1997) and in greenhouse experiments (Meyer and Huettel, 1996), but the effects of these substances could not have been distinguished between those of a pheromone and a nematicide in these studies. Theoretically, both a pheromone and a nematicide would be expected to decrease nematode populations on the host plant by the disruption of mating, or the direct lethal action on individual nematodes, respectively. The same nematicidal activity of vanillic acid analogues (Varma, 1993) may have obscured previous reports of the pheromonal properties of vanillic acid (Meyer *et al.*, 1997; Meyer and Huettel, 1996; Jaffe *et al.*, 1989). Furthermore, the potent effects of vanillic acid and syringic acid as antifungal agents (Aziz *et al.*, 1998) may account for the lack of synergistic effects observed in reducing *H. glycines* populations with *Verticillium lecanii*. The fungus, *V. lecanii*, reduces *H. glycines* populations on its own, but its efficacy decreases when combined with vanillic acid or its analogue, syringic acid (Meyer and Huettel, 1996). Meyer and Huettel (1996) may have demonstrated the nematicidal action of vanillic acid and vanillic acid analogues on plant parasitic nematodes.

Behaviours typically observed before or during nematode copulation are elements that may be useful in bioassays. Huettel and Rebois (1986) demonstrated the coiling of male *H. glycines* as a means by which biologically active substances could be identified.
However, it appears that coiling, or tail curling behaviour, may be dependent on tactile stimuli. During collection, the sex of the animal parasitic nematode, *A. suum*, can be easily distinguished by stroking the cuticle. Males respond to this stimulus by curling their tails (L'Hernault and Roberts, 1995). Early observations of nematode copulation indicated that attraction and copulation were independent behaviours provoked by separate stimuli (Greet, 1964), and that coiling of the male tail appeared to be the result of direct contact with the female. Greet (1964) first reported that, although sexes were brought together by chemical attractants, copulation of *P. rigidus* occurred only when the posterior third of the male came into contact with the female; and when the male touched the female, there was an immediate ventral coiling of the tail. Chin and Taylor (1969) stated that copulation in *Cylindrocorpus* spp. was only initiated when the posterior halves of nematodes of the opposite sex made contact with each other. After contact, the posterior region of the male curved ventrally around the female. Jaffe et al. (1989) identified vanillic acid as the putative sex pheromone of *H. glycines* by inducing coiling behaviour of the male with vanillic acid, in the absence of females and sexual stimuli. This suggests that the coiling behaviour induced by vanillic acid (Jaffe et al., 1989) was not due to sexual stimulation, which appears to be dependent on tactile stimuli (Greet, 1964; Jones, 1966; Chin and Taylor, 1969; Loer and Kenyon, 1993; L'Hernault and Roberts, 1995), but may have resulted from other effects of vanillic acid. Tail curling in *H. schachtii* males was observed in culture only when males were inside of the gelatinous matrix, in direct contact with the female, and wrapped around the vulval cone. Eversion of the spicules, a sign of sexual stimulation, could also be seen. Tail curling was not induced by FCM. The absence of tail coiling in FCM bioassays was, presumably,
because of the lack of tactile stimulation from the female. However, stylet thrusting behaviour was observed in culture as the male approached the female, and in bioassays. The action of the stylet occurred independently of the presence of females, and proved to be a very useful indicator of the biological activity of FCM.

4.2 Serotonin and precopulatory behaviour of *Heterodera schachtii*

The present work has demonstrated the effects of exogenous serotonin on the stylet protractor muscles, leading to stylet thrusting behaviour in both juvenile (J2) and male *H. schachtii*. Although serotonin-induced stylet activity has previously been observed in J2s (McClure and von Mende, 1987), this is the first report of this phenomenon in adult male plant parasitic nematodes. The effects of serotonin on the pharyngeal musculature of *C. elegans* have been described by others, and include the contraction of the procorpus and metacorpus of the pharynx (Horvitz *et al.*, 1982; Raizen and Avery, 1994; Ségalat *et al.*, 1995; Avery *et al.*, 1995). That serotonin induces pharyngeal contraction in *C. elegans* suggests that the pharynx of *H. schachtii* may also be active during serotonin treatment. The coordinated activity of the stylet and pharynx has been observed in *H. schachtii* J2s (Wyss, 1992), and the stylet protractor and pharyngeal muscles appear to be phylogenetically and morphologically related in plant parasitic nematodes (Baldwin and Hirschmann, 1976; Baldwin *et al.*, 1977; Baldwin, 1982; Shepherd and Clark, 1983; Endo, 1984; Bird and Bird, 1991). The possibility of pharyngeal contractions occurring during stylet thrusting behaviour in *H. schachtii* males is relevant because the former may result in the ingestion of materials from the surrounding environment; but since males of *H. schachtii* do not feed as adults (Franklin,
1965; Shepherd, 1965), such a coordinated behaviour has not been investigated. This work has demonstrated that *H. schachtii* males respond to FCM, containing a female sex pheromone, with stylet thrusting behaviour, and suggests that the coordinated actions of the stylet and pharynx may be necessary to allow for the ingestion of the female sex pheromone by the male during mate finding behaviour. Several authors have reported evidence of nerve endings behind the stylet and in the cuticle of the pharyngeal lumen in other nematodes, and have speculated that these may function as gustatory receptors (Robertson, 1975; Shepherd *et al.*, 1980) or mechanoreceptors (Albertson and Thomson, 1976; Robertson, 1979) capable of detecting chemical stimuli or rates of flow of ingested materials, respectively. In comparison, insects are known to detect chemical stimuli, such as water soluble nutrients, with gustatory receptors (Schneider, 1969; Mullin *et al.*, 1994). It is proposed, here, that *H. schachtii* males may be able to detect the water soluble female sex pheromone through similar receptor mechanisms.

Albertson and Thomson (1976) reported evidence of multifunctional cells in the pharynx of *C. elegans*. The neurosecretory motor neurons (NSMs), having large secretory vesicles, were positioned at the periphery of the pharynx and appeared to have mechanoreceptive nerve endings beneath the cuticle of the pharyngeal lumen. The authors speculated that these cells may release humoral factors into the pseudocoelom upon stimulation of their sensory endings by ingested materials in the pharyngeal lumen. Immunocytochemical techniques have established the presence of neurosecretory cells, resembling NSMs in *C. elegans*, and a nerve plexus on the dorsal surface of the pharynx in *Ascaris* that were serotonin-immunoreactive (Brownlee *et al.*, 1994; Johnson *et al.*, 1996). Reinitz and Stretton (1996) suggested that these cells release serotonin into the
pseudocoelomic fluid to be transported to serotonin-sensitive sites. Tail curling, a typical copulatory behaviour of male nematodes, has been shown to be defective in males lacking serotonin expression (Loer and Kenyon, 1993) and to be induced by exogenous serotonin (Reinitz and Stretton, 1996). The present work has also reported observations of tail curling in male *H. schachtii* induced by serotonin. Tail curling, and other behaviours associated with copulation, may be regulated by the release of serotonin from stimulated secretory cells of the pharynx into the pseudocoelomic fluid.

Serotonin stimulated spicule eversion behaviour and the extrusion of a homogeneous mass of material through the tube-like structure formed by the paired spicules in male *H. schachtii*. Sulston *et al.* (1980) reported that the anal depressor and sphincter muscles of juvenile *C. elegans*, which control defecation, become modified to function in copulation during the adult stage. The anal depressor is reoriented and forms attachments with the spicule protractor muscles, while the sphincter muscle enlarges and develops attachments to the body wall, allowing for the closing and displacement of the gut during sphincter contraction and ejaculation (Sulston *et al.*, 1980). This suggests that the eversion of the spicules and the extrusion of materials from *H. schachtii* may have been caused by the action of serotonin on the anal depressor and sphincter muscles, respectively. Serotonin is reported to inhibit defecation (Ségalat *et al.*, 1995), presumably by causing contraction of the sphincter and closing of the gut, and to induce egg-laying, a reproductive behaviour in *C. elegans* hermaphrodites (Horvitz *et al.*, 1982; Waggoner *et al.*, 1998). Although it was determined that the extruded material did not consist of spermatids or spermatozoa, it may have been an ejaculatory substance. In *A. suum*, an endogenous sperm activator, produced by the glandular vas deferens, coalesces
to form a homogeneous mass inside the lumen of the vas deferens that is released with spermatozoa during copulation (Foor and McMahon, 1973; Foor, 1976). The extruded material observed in the present work may have been secretory products of the vas deferens. The observation that serotonin elicits spicule eversion and apparent extrusion of materials from the reproductive tract indicates that exogenous serotonin induces copulatory behaviours in male *H. schachtii*. The results also suggest that these behaviours (spicule eversion and extrusion of the homogeneous mass) may be naturally regulated by endogenous serotonin.

The possibility that stimulation of sensory-neurosecretory cells, such as NSMs, results in the release of serotonin into the pseudocoelom and the subsequent induction of copulatory behaviours is speculation at this point. That FCM did not induce the same behaviours as serotonin (*i.e.* spicule eversion and extrusion) suggests that another mechanism may be involved. Stylet thrusting behaviour of males in FCM was not as vigorous as that of males in serotonin. Perhaps excessive amounts of ingested fluids caused by vigorous pharyngeal pumping in the serotonin group resulted in spicule activity and extrusion. An increase in pressure within the gut is thought to cause defecation (Crofton, 1971; Lee, 1975) and may have stimulated activity of copulatory muscles in *H. schachtii*. Jairajpuri and Azmi (1977) observed that the rate of pharyngeal pumping in female *Acrobeloides* spp. was correlated with the number of eggs being laid by the female, and concluded that egg laying behaviour was due to an increase in pressure inside the gut. Such a mechanism may be beneficial in male *H. schachtii* since all male secernentean nematodes lack ejaculatory muscles (Maggenti, 1991). That ejaculation could not be induced in male *H. schachtii* in liquid culture has been observed in *C.*
elegans, and may be a reflection of the lack of tactile stimuli in the liquid medium (Ward et al., 1983; L'Hernault and Roberts, 1995).

Extracellular recording from the anterior region of *H. schachtii* males appeared to be associated with stylet thrusting behaviour. However, the origin of the electrical potentials, whether myogenic or neurogenic, was not determined. Nevertheless, the electrophysiological preparation presented in this work may be useful as a technique of bioassay of nematode pheromones, since FCM has been demonstrated to induce stylet thrusting behaviour, and stylet thrusting behaviour (induced by serotonin) was recorded from *H. schachtii*.

### 4.3 Conclusions

The present work has documented the characterization and isolation of a water soluble, male specific, component of the female sex pheromone of the nematode, *H. schachtii*, using HPLC techniques, and the biological activity of this substance *in vitro*. Furthermore, the stylet of male *H. schachtii* has been shown to be functional, and its activity can be used as a novel indicator of biological activity in behavioural bioassays to identify the existence of nematode sex pheromones. Thrusting of the stylet, like other precopulatory behaviours, was induced by exogenous serotonin. This suggests that stylet thrusting behaviour may be essential for successful mate finding and copulation to occur in *H. schachtii*. 
References


Appendix I
The effects of host infection site on female *Heterodera schachtii* development

If a high degree of morphological variation exists among females selected for pheromone collection, then quantities of pheromone produced by the females may vary accordingly. To prevent such a phenomenon from interfering with the outcome of the experiments described in the text, the relationship between the thickness of the vascular bundle of the cabbage root at the infection site and cyst size was investigated to determine if the site of infection affected female development and, possibly, pheromone production. Rearing of female *H. schachtii* in transparent growth pouches (Mega International of Minneapolis) allowed for the observation of female development under the light microscope throughout the rearing period. Growth pouches containing cabbage seedlings and sedentary females were placed directly onto the stage of a light microscope (Dialux 20, Leitz) and investigated under 100× magnification. A calibrated micrometer placed in the ocular barrel of the microscope was used to measure the dimensions of 30 mature cysts and the thickness of the vascular bundle at corresponding sites of infection. The vascular bundle could be seen traversing the length of the roots because the roots were transparent once illuminated on the microscope stage. The overall thickness of the root, including epidermal tissue, was not measured because small roots tended to have a disproportionately thick layer of epidermis, when compared to larger roots, which prevented reliable measurements of the differences in root capacity. The radius of the vascular bundle of the root was measured 0.5 mm distal to the infection site of the root to avoid measuring lesioned root material enlarged by the action of the infective nematodes. This phenomenon was commonly seen in thinner roots. Values were squared and multiplied by \( \pi \) to express results as an estimation of the cross sectional area (mm\(^2\)) of
the vascular bundle near the infection site. The length of the female cyst was taken as the straight line distance from the posterior extremity of the vulval cone to the opposite end of the cyst, which was not the anterior most extremity. Females of *H. schachtii* take on a curved shape as they mature into cysts and, as a result, the cephalic region is shifted to one side rather than being aligned with the longitudinal axis, as in males. The width of the cyst was measured at the thickest region of the nematode. The product of cyst length and width provided a value, in units of area (mm$^2$), that gave a useful estimate of cyst size. The cross-sectional area of the vascular bundle 0.5 mm distal to the infection site was compared to the size of the cyst. Data were analyzed using a linear regression model with 95% confidence.

As it was discovered that large cysts develop on large roots, and small cysts develop on small roots, female cysts were selected from large roots to maximize the amounts of pheromone being collected in the female conditioned medium. It was assumed that larger cysts would produce greater quantities of pheromone. Care was taken not to collect females that varied greatly in size to ensure that consistent amounts of pheromone were collected in the FCM.
*Heterodera schachtii* female cyst size is correlated with cross sectional area of the root vascular bundle near host infection site. Each point (n=30) in the accompanying figure represents one female cyst. Cyst size was estimated by the product of length and width. Variables are correlated, as determined by a linear regression analysis with 95% confidence: \( F=47.95, r^2=0.62, P<0.0001. \)
Appendix II
Optimal incubation methods for producing the female conditioned medium (FCM)

The following are the results of a preliminary experiment to establish methods for obtaining optimal amounts of *H. schachtii* female pheromone in the female conditioned medium (FCM). Males were, predominantly, most attracted to FCM produced by incubating 20 females in water (*A, C*). The, apparently, low attraction of males toward FCM produced from 20 females after 3 days of incubation (*B*) might be explained by a high concentration of pheromone contained in the FCM after 3 days of incubation. This explanation is possible because if the concentration of a substance diffusing through the agar medium is high enough, a uniform distribution of that substance will occur (Ward, 1973), preventing the formation of a chemical gradient that can be detected by the nematode. Assuming this was the case, and that the highest concentration of pheromone was obtained by incubating 20 females for 3 days (*B*), the attraction of males toward FCM produced after 2 or 4 days incubation (*A, C*) must have had a lower concentration of pheromone in the medium since the nematodes were not disoriented, but were attracted to the centre of the dish. For these reasons, incubating 20 females in 1 ml of ultrapure water for 3 days was deemed an optimal method of collecting the *H. schachtii* female sex pheromone.
Attraction of male *Heterodera schachtii* to female conditioned medium (FCM) produced following different incubation methods. Movement of *H. schachtii* males toward the target in the presence of FCM produced from 5 (■), 10 (○) and 20 (□) females, or ultrapure water as control (○), over a period of 120 minutes in Petri dish bioassays. Females were previously incubated in concentrations of 5, 10 or 20 per 1ml of ultrapure water for (A) 2, (B) 3 or (C) 4 days to produce FCM. Means ±SEM are reported. All points are significantly different (*P*<0.05) from control, as indicated by the Mann-Whitney *U* test, except where indicated by asterisks.
Absorption spectrophotometry. The following data are the absorption spectra of vanillic acid ($10^{-5}$ M) and 4 different samples of female conditioned medium (FCM). The wavelength and relative absorbance of the major peaks in each spectrum are reported in an accompanying table. The figures illustrate a variable degree of absorbance activity near 260 nm in FCM samples (see text).
Vanillic acid ($10^{-5}\text{M}$)

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<th>Absorbance</th>
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<tbody>
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<tr>
<td>252</td>
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<td>287</td>
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Female conditioned medium: sample 1

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<td>383</td>
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![Graph showing absorbance vs. wavelength](chart.png)
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<th>Cost/Benefit</th>
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<td>0</td>
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Female conditioned medium: sample 2

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Female conditioned medium: sample 3

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Female conditioned medium: sample 4

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Appendix IV
High performance liquid chromatography (HPLC). The following are HPLC chromatographs of 5 different samples of female conditioned medium (FCM) and respective samples of ultrapure water as control. All FCM samples were tested in bioassays and were biologically active. The following figures illustrate the variability in absorbance activity between FCM samples (see text).
Sample 1
FCM

H₂O
Sample 2
FCM

H₂O
Sample 3
FCM

\[ \text{Time (min.)} \]

\[ mAU \]

\[ H_2O \]

\[ \text{Time (min.)} \]

\[ mAU \]
Sample 4
FCM

\[ \text{mAU} \]

Time (min.)

\[ \text{H}_2\text{O} \]

\[ \text{mAU} \]

Time (min.)
Sample 5
FCM

H₂O