THE NERVOUS SYSTEM OF PLANT-PARASITIC NEMATODES AND THEIR BEHAVIORS

BY

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DISSERTATION

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ABSTRACT

Nematodes are the most abundant animals on earth. The majority of nematodes are free-living which feed on bacteria and fungi in the soil. However, some nematodes are parasitic to other animals and plants and have a huge impact on human health and agriculture worldwide. The nervous system of the non-parasitic nematode *Caenorhabditis elegans* has been studied extensively. Based on *C. elegans* and a few other species, nematode nervous systems were thought to be highly conserved. However, using a comparative neuroanatomy approach, I found unexpected variation in the number and structural properties of neurons among ten species across four clades. To further study the nervous system of plant-parasitic nematodes, I investigated how the neurotransmitter serotonin regulates their behaviors. Neurotransmitters are endogenous molecules used by neurons for signal transmission. Serotonin regulates feeding and reproductive behaviors in *C. elegans*, but its role in plant-parasitic nematodes remains unknown. In the root-lesion nematode *Pratylenchus penetrans*, I detected serotonergic neurons in cells adjacent to feeding and reproductive structures. I observed that exogenous serotonin induced *P. penetrans* feeding and reproductive behaviors. Also, using pharmaceutical compounds that disrupt serotonin signaling, my data suggest that these neurons regulate feeding and reproductive behaviors through endogenous serotonin. The soybean cyst nematode *Heterodera glycines* has a distinct life cycle: both juvenile males and females lose their mobility when feeding is initiated; however, the adult males regain their mobility while females never regain mobility. As a part of a collaborative study with other lab members, I have studied the change in the mobility of *H. glycines* from a neuronal aspect. GABA is the most prominent inhibitory neurotransmitter in nematodes. I have constructed a map of GABAergic neurons and cloned the gene encoding the key enzyme in GABA synthesis (*hg-unc-25*) in *H. glycines*. I have used heterologous rescue in a *Caenorhabditis elegans* mutant and validated that HG-UNC-25 is indeed the GABA synthesizing enzyme in *H. glycines*. Also, I have found the sedentary stages of *H. glycines* is associated with a reduction of GABAergic neurons in the ventral nerve cord.

Together, my research has provided evidence that the nervous systems of nematodes are not as conserved as we thought and there is a need to further investigate the nervous system. A better understanding of the nervous system of plant-parasitic nematodes may be important to understand the evolution of these parasitic nematodes. More importantly, the knowledge of the
how the nervous system regulates the specific behaviors of plant-parasitic nematodes may provide insights into new control strategies.
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CHAPTER 1 LITERATURE REVIEW: THE NERVOUS SYSTEM OF PLANT-PARASITIC NEMATODES

Impact of nematodes worldwide

Nematodes are the most abundant animals by number and volume on the earth (Decraemer and Hunt 2006). Nematodes belong to the phylum Nematoda and are pseudocoelomate and unsegmented worms. Nematodes live almost everywhere and represent a wide range of diversity in their body size and structure. The size of nematodes can be from a few hundred microns up to a few meters in length. A million or more nematode species have been estimated and around 30,000 species have been described (Kiontke and Fitch 2013). Despite a large number of species in this phylum, most nematodes are free-living, feeding on fungi or bacteria in the soil. A small group of nematodes are parasitic which feed on plants or other animals. The parasitic nematodes have a great impact on world food production and human health. Plant-parasitic nematodes cause a considerable loss of food production every year. Over 4100 species of plant-parasitic nematodes have been described (Decraemer and Hunt 2006) and 80 billion dollars of crop loss is caused by plant-parasitic nematodes annually (Nicol et al. 2011). For almost every major crop there is at least one nematode species which can cause yield loss (Jones et al. 2013). Meanwhile, over one third of the world population is infected by parasitic nematodes; nematode-associated diseases are especially severe in tropical regions (Ghedin et al. 2007). For example, at least 25 million people are estimated to be infected by the nematode *Onchocerca volvulus* causing blindness or visual impairment in over 1 million people (https://www.cdc.gov/parasites/onchocerciasis/gen_info/faqs.html). This review will focus on plant-parasitic nematodes.

From the nematode neuroanatomy to behaviors

Nematode behaviors are regulated by the nervous system. The knowledge of neuroanatomy is critical to understand the nervous system and to further understand the neuronal circuits regulating behaviors. Most knowledge of nematode neuroanatomy comes from studies of the free-living nematode *Caenorhabditis elegans* and the animal-parasite *Ascaris suum*. *C. elegans* is the best studied nematode for neurobiology because it has both a completely described cell-
lineage and connectome (Sulston et al. 1983) as well as a well-annotated genome (C. elegans Sequencing Consortium 1998). A C. elegans adult hermaphrodite has 302 neurons, while an adult male has 385 neurons (Sulston, Albertson, and Thomson 1980; Sulston et al. 1983; Sammut et al. 2015). With relatively simple neuroanatomy, C. elegans can perform diverse behaviors including locomotion, foraging, feeding, and reproduction. Many C. elegans behaviors have been characterized in detail to the level of neuronal regulation.

Numerous studies have been conducted on the feeding behavior of C. elegans and the neural regulation is well understood (Avery and You 2012). The pharynx is a tubular neuromuscular pump that connects the mouth and the intestine. The pharyngeal muscle of C. elegans is divided into three parts, the corpus, the isthmus and the terminal bulb. The corpus is further divided into procorpus and metacorpus. In C. elegans, two motions are involved in the feeding behavior, pumping and isthmus peristalsis. Pumping is the motion to absorb the food into the mouth of the nematode. It starts with a near-simultaneous contraction of the muscles of the corpus, anterior isthmus, and terminal bulb. The radially oriented pharyngeal muscles contract and open up the lumen which takes in liquid and suspended bacteria from the outside. The nematode expels liquid by doing a near-simultaneous relaxation. By doing so, the nematode traps the bacteria from the liquid.

The amount of food taken is determined by the rate of pumping. Although C. elegans can maintain the base pumping conduct without nervous system input, during the normal rapid feeding, the pumping rate is regulated by the MC motorneurons. MCs are a paired of cholinergic excitatory neurons in the anterior pharynx bulb on the dorsal site (Raizen, Lee, and Avery 1995). The firing of MC initiates pharyngeal muscle contraction. Mutants that are defective in MC signaling to pharyngeal muscle result in a malfunction of feeding. EAT-2 is a nicotinic acetylcholine receptor subunit functioning in the pharyngeal muscle localized to MC and a small-pass transmembrane protein EAT-18 is likely required for other nicotinic receptors in the pharynx (McKay et al. 2004). These two mutants are severely starved and have retarded growth due to the slow pumping rate. Also, if MC is killed, the ability to increase pumping rate disappears in wild-type C. elegans (Avery and Horvitz 1989). Food sensing and serotonin send signals to MC for the fast pharyngeal pumping. In the absence of food, exogenous serotonin can mimic the effects of food that causes increased activities of MC. The serotonin receptor in
pharyngeal muscle SER-1 (Tsalik et al. 2003) and another SER-7 receptor in MC have been identified. The MC neurons in ser-7 mutant do not respond to exogenous serotonin indicating that SER-7 is an MC serotonin receptor (Hobson et al. 2006). Overall, MC is the master neuron response for pumping. Among all other pharyngeal neurons, only I1 has shown a synaptic connection to MC and receives direct inputs from the nerve ring. It is thought that I1 might mediate fine tuning of MC firing and maintain the basal pumping rate. When I1 is ablated, food and serotonin can still stimulate MC. However, without I1 and in the absence of food, the basal pumping rate is significantly decreased. Killing MC also decreases basal pumping. Killing both MC and I1 has the same result as killing either alone. These results suggest I1 stimulating MC to maintain the basal pumping.

While the MC neurons regulate when the contraction starts, the M3 neurons regulate when the contraction ends. M3 are paired neurons located in the posterior pharynx bulb. The M3 neurons control the timing of pharyngeal relaxation (Avery 1993). When M3 is ablated, the pump duration increases due to a slower relaxation and results in ineffective bacteria trapping. The second feeding motion is isthmus peristalsis, which carries the food from the posterior part of the corpus to the terminal bulb for grinding and eventually carries to the intestine (Avery and Horvitz 1989). The anterior half of the isthmus contracts with every pump, but in contrast, the posterior isthmus peristalsis always begins following a pump, but does not occur after every pump. Only about one contraction of posterior isthmus occurs out of four pumps. Isthmus peristalsis seems to be an all-or-none event, and solely depend on the neuron M4. It is thought that isthmus peristalsis occurs only when M4 fires a potential (based on the slo-1 mutant). When the M4 is killed, the posterior isthmus peristalsis is disappeared, but the anterior isthmus peristalsis is not affected (indicate there are subcellular specializations between the anterior and posterior isthmus peristalsis). M4 is cholinergic neuron and acetylcholine agonist arecoline can stimulate uncoordinated posterior contractions when M4 is killed. This suggests that at least, acetylcholine is released from M4 onto pharyngeal muscle. However, neuropeptides have been identified in M4 and it is likely the neuropeptides also contribute to the isthmus muscle contraction.

There is a total of 20 neurons of 14 types in the pharyngeal nervous system (Alberton and Thomson 1976). Three of the 14 types, MC, M3 and M4 are essential for the regulation of
feeding. And, the three types of neurons alone are sufficient to complete the normal feeding under the lab condition. MC neurons control the timing when a contraction starts, whereas M3 controls when a contraction ends. M4 neurons control the posterior contraction of isthmus peristalsis. The ablation of the rest 11 types of neurons still allows the nematode have nearly normal feeding behavior but a “slippery phenotype” under lab condition (Avery 1993).

Chemosensation is also studied in detail in *C. elegans*. The head region of nematodes generally contains sensory neurons involved in multiple behaviors. There are 32 neurons presumed as chemosensory neurons (Hart and Chao 2010). Most of those neurons have ciliated endings that are exposed to the environment directly or indirectly through gilia cells open to the environment. The chemosensory neurons sense volatile and water-soluble cues which allow nematodes to sense the surrounding environment for finding food and avoiding toxic areas. The amphid neurons ASE are known to be water-soluble chemosensory neurons. When the pair of ASE neurons are ablated, the nematode is less attractive to attractants such as Na, Cl and Serotonin. However, when other amphid neurons are ablated but ASE, the nematode is still attractive to those water-soluble cues which indicate that ASE alone regulates water-soluble chemotaxis. Interestingly, the two ASE neurons although are bilaterally symmetric anatomically, are asymmetric in their function which each of the neurons senses different chemicals (Yu et al. 1997). The amphid neurons AWA, AWB and AWC sense volatiles. It is known those neurons can sense compounds naturally produced from bacterial metabolism and asymmetry in the function of the paired neurons also exists. However, different from water-soluble chemicals, volatiles travel rapidly through the air and these neurons are thought to guide long distance locomotion of *C. elegans*, whereas the water-soluble neurons ASE guide short distance locomotion.

Besides chemosensation, there are other sensory neurons called mechanoreceptor neurons which detect external force to regulate the locomotion, feeding, egg laying, defecation, and mating of *C. elegans* (Goodman 2006). Unlike the chemosensory neurons, the endings of mechanoreceptor neurons can be either ciliated (which can be exposed to the environment) or nonciliated. When a forward moving *C. elegans* is genital touched in the anterior half, it will reverse; when the posterior half is touched, it will accelerate. The same principle also applies when the nematode is moving backwards. Six mechanoreceptor neurons, ALML, ALMR, AVM, PLML, PLMR, PVM
along the body of *C. elegans* sense external force and regulate locomotion. ALML, AMLR and AVM responses to anterior touches, while PLML and PLMR response to posterior touches.

The ventral nerve cord (VNC) consists of motor neurons between the head region (retro-vesicular ganglion) and the tail (pre-anal ganglion) and regulates nematode locomotion. In *C. elegans*, there are 57 VNC motor neurons innervating the body muscles in both ventral and dorsal sides (White et al. 1976, Sulston 1976). Within the VNC, the motor neurons are designated into eight classes. Type A and B motor neurons (VA, VB, DA, DB, AS) are cholinergic and stimulatory. Type D motor neurons (VD, DD) are GABAergic and inhibitory. The VC4 and VC5 neurons are serotonergic and likely received serotonin from the hermaphrodite specific neurons (HSNs). However, not all the motor neurons in the VNC are essential for locomotion. Using network control principles, Yan et al. (2017) predicted and validated that ablation of the ventral neurons DD02 and DD03 did not affect the locomotion behavior.

The nematode nervous systems are considered highly conserved. For example, the nematodes *C. elegans* and *Ascaris suum* are distinct in morphology and habitat. However, both nematodes have a very similar number of neurons. Less is known regarding the neuroanatomy of nonmodel nematode species. Previously, a few papers using electron microscopy examined the nervous system. These studies provided the foundation for the nervous system of plant-parasitic nematodes, but lacked a systematic comparison to *C. elegans* and identifications of orthologues of individual neurons (Endo, Zunke, and Wergin 1997; Endo and Wergin 1977; Endo 1984). In Chapter 2, we examined the dye-filling pattern of head sensory neurons in different nematode species and discovered unexpected variation (Han, Boas, and Schroeder 2016). Together, we concluded that the nematode neuroanatomy was not as conserved as previously assumed. Variation of neuroanatomy may be related to their specific behaviors.

**The role of neurotransmitters in nematode key behaviors**

The neurocircuits that regulate nematode behaviors are of great interest. In the nervous system, neurotransmitters are small molecules transmitting signals between cells and the major modulators in regulating nematode behaviors. *C. elegans* is extensively used to study the role of neurotransmitters. Acetylcholine (ACh), dopamine, GABA, glutamate and serotonin are among
the best characterized neurotransmitters in *C. elegans*. Among those, Ach, GABA and glutamate are considered as fast-acting neurotransmitters (Gendrel, Atlas, and Hobert 2016). ACh was the first discovered and functions as an excitatory neurotransmitter. ACh is involved in the regulation of locomotion, egg laying, pharyngeal pumping, defecation cycling and male mating in *C. elegans*. Over half of *C. elegans* neurons have been found to be cholinergic through the detection of acetyltransferase (ChAT) expression (Pereira et al. 2015). The majority of cholinergic neurons are motor neurons and regulating important behaviors. For example, the cholinergic neuron MC in the head regulates pharyngeal pumping. The cholinergic neurons in the ventral nerve cord regulate the muscle contraction.

In *C. elegans*, GABA is the most prominent inhibitory neurotransmitter and is mainly expressed in motor neurons. About 50 neurons from 9 neuron classes are GABAergic in *C. elegans* (Gendrel, Atlas, and Hobert 2016). In *C. elegans*, a typical sinusoidal move is performed by contracting on one ventral or dorsal side through Ach signals, while relaxing on the other side through GABA signals. Without sufficient GABA, *C. elegans* will have a few behavioral deficiencies. The *C. elegans* mutant (unc-25) lacks the gene for GABA synthesis. Therefore, no GABA can be detected in this mutant. The most obvious phenotype of this GABA- mutant is shrinking. Shrink is caused by the hypercontraction on the body wall muscles caused by lack of GABA for relaxation, when GABA- mutants are touched in the head, instead of performing a proper reverse, the head of the mutant will contract, whereas the majority of the body will remain unmoved. The nematode looks like shortened, which is defined as shrinking.

Glutamatergic neurons are defined by the present of vesicular glutamate transporter (Serrano-Saiz et al. 2013). In *C. elegans*, the gene *eat-4* encodes the vesicular glutamate transporter and this gene is expressed in 78 neurons which fall into 38 neuron classes. Most of the glutamatergic neurons are sensory neurons and interneurons except two motor neurons in the pharynx. For example, the GLR-1 is a glutamate receptor and expressed in motor neurons and interneurons including AVA, AVD, AVB and PVC. The *C. elegans* mutant *glr-1* is not able to respond to light touch in the nose or to the body (Maricq et al. 1995).

Exogenous serotonin is known to stimulate feeding and reproductive behaviors while inhibiting locomotion in *C. elegans* (Sze et al. 2000; Waggoner et al. 1998). When *C. elegans* encounters
food, the neurosecretory motor neurons (NSMs) sense food in the lumen of pharyngeal and might use serotonin to signal the rest of the body (Chase and Koelle 2007). Serotonin signaling slows the movement of C. elegans to remain in a favorable food environment and stimulates the feeding behavior. More recently, it has been shown that the food stimulated pumping is regulated by the serotonergic NSM, the hermaphrodite-specific neurons (HSN), but the ADF (Lee et al. 2017). In addition, serotonin is released in the HSN to stimulate egg laying in C. elegans. For the mutants (tph-1) that lack serotonin, the hermaphrodites cannot effectively lay eggs resulting in the young larvae hatch in the hermaphrodites’ bodies, this phenotype is called “bag of worms”. In Chapter 3, we studied the role of serotonin in the root-lesion nematode Pratylenchus penentrans and found that endogenous serotonin regulate the feeding and reproductive behaviors of this nematode.

Regulation of octopamine in nematodes is less clear compared to other neurotransmitters. One obstacle to understanding the function of octopamine is that the precursor of octopamine, tyramine is also a neurotransmitter. The two compounds are synthesized in the same cells which makes it difficult to assess the function of either. It is known that octopamine is synthesized in the RIC neurons and gonadal sheath cells (Alkema et al. 2005). Octopamine is an antagonist of serotonin. C. elegans has reduced egg laying rate in the presence of food and kinked movement, when exogenous octopamine is applied (Horvitz et al. 1982).

Dopamine is made in eight neurons in the C. elegans hermaphrodite, and dopaminergic neurons are thought to be mechanosensory. Dopaminergic neurons sense the environment and slow down C. elegans locomotion when encounters food source. This “basal slowing response” prevents C. elegans from leaving a good food source.

However, less is known about the roles of neurotransmitters in plant-parasitic nematodes compared to C. elegans. Neurotransmitters were applied to plant-parasitic nematodes and, changes in feeding and locomotion behaviors were found (McClure and Von Mende 1997). Several neurotransmitters were applied exogenously to the cyst and root-knot nematodes, and behavioral changes were examined (Masler 2007; McClure and Von Mende 1997; Jonz et al. 2001; Masler 2008). The author found that in the soybean cyst nematode Heterodera glycines and the root-knot nematode Meloidogyne incognita exogenously applied neurotransmitters
dopamine, octopamine, serotonin and neuropeptide could affect locomotion, egg laying, and stylet thrusting of these two nematodes. ACh was detected in *Meloidogyne* spp using HPLC (Chang and Opperman 1991). Furthermore, an acetylcholinesterase gene *ace*-2 was cloned from the cyst nematodes *H. glycines* and *Globodera pallida* (*Gp-ace*-2) (Costa et al. 2009). Acetylcholinesterase is an enzyme terminating ACh in the synaptic cleft and malfunctions of acetylcholinesterases result in excess ACh, which is toxic to the organism. The authors used a heterologous rescue approach in a *C. elegans* mutant and validated the function of *Gp-ace*-2 as the gene encoding an acetylcholinesterase. Endogenous GABA was also detected in the ventral nerve cord of *G. rostochiensis* and *M. incognita* and the GABAergic neurons were thought to be motor neurons (Stewart, Perry, and Wright 1994). However, no further research was followed to investigate the role of those neurotransmitters at a molecular level.

The FMRFamide-like peptides (FLPs) which are short chain peptides are considered as neurotransmitters and neuromodulators in nematodes (Walker, Papaioannou, and Holden-Dye 2009). They are probably the best studied neuromodulators in the plant-parasitic nematodes. The FLPs were first detected in the nervous system of *H. glycines* through immunohistochemistry (Atkinson et al. 1988). Later on, FLPs were also detected in the potato cyst nematodes (Kimber et al. 2001) and *flp* genes that encode FLPs were cloned. To understand the functions of FLPs in plant-parasitic nematodes, the authors examined the *flp* expressions through in situ hybridization (reviewed in Kimber and Fleming 2006). For example, the *G. pallida* FLP RNKFEFIRamide which is thought to be a homologue of *C. elegans* FLP-12, was detected in motor neurons and might regulate locomotion. The patterns of *flp* expression are not identical to their homologs in *C. elegans*, which suggests differences in the role between plant-parasitic nematodes and *C. elegans*. With the advantage of whole genome sequencing of *M. incognita*, more *flp* genes were predicted (Abad et al. 2008). In the potato cyst nematode *G. pallida*, the gene (*Gp-flp*-32) encoding a conserved neuropeptide in nematodes was found expressed in the ventral nerve cord (Atkinson et al. 2013). When this gene was knocked down in *G. pallida* J2s, surprisingly the migration distance increased compared to the control. Furthermore, the recently published genome of *G. pallida* revealed in-depth descriptions of genes involved in its nervous system (Cotton et al., 2014).
Plant-parasitic nematode behaviors

The behaviors of plant-parasitic nematodes are mostly studied in root-knot and cyst nematodes because of their economic importance. Nematodes feed inside the plants, which significantly increases the difficulty of observation. Also, with limited knowledge of their nervous system, the neuronal circuits for specific behaviors are not clear. However, behaviors associated with feeding are probably best characterized among all behaviors in plant-parasitic nematodes. The feeding behaviors of the plant-parasitic nematodes are significantly distinct from the bacterial-feeding nematode *C. elegans*. The feeding behavior of cyst nematodes has been described in great detail. For example, freshly hatched *Heterodera schachtii* second stage juveniles (J2s) move towards the roots (Wyss and Grundler 1992) and the stylet thrusts to explore the area. When *H. schachtii* encounters the differentiation zone of the roots, it uses the stylet to create holes and rupture the root cell wall, and migrates intracellularly inside the root tissue towards the vascular cylinder. The migration pattern to the vascular cylinder is destructive by causing a line of holes to the cells. However, once it arrives at the vascular cylinder, the nematode switches to a subtle exploration pattern, seeking the initial syncitial cell. Once the cell is chosen, the stylet carefully penetrates the cell wall and stays protruded. For the following 6-8 hours, instead of feeding, *H. schachtii* secretes proteins through the stylet from the subventral glands to the targeted cell. The nematode initiates the feeding site through inducing the initial syncitial cell to widen some of the plasmodesmata (Gheysen and Jones 2006). It results in the fusing of the neighboring cells to the initiate syncytial cell. The secretions from the nematode manipulate the growth of the syncytium and increase the size of the nucleus and metabolism. After the establishment of feeding sites, the nematode starts to feed in cycles involving three distinct phases. During the phase I, the stylet inserts shallowly (3-4 µm) into the syncytia. The metacorpus pumps continuously at a rate of 5-7 per minute to take in cell materials through the feeding tube. This phase usually lasts for one hour. In phase II, the nematode retracts the stylet and reinserts into the syncytia deeply (4-11 nm) while the metacorpus pumps. In phase III, the stylet reinserts shallowly (3-4 µm) without active metacorpus activities. This last phase usually lasts 20 minutes, and the nematode repeats phase I. Although some behaviors have been recorded in detail, how these behaviors are regulated at a neuronal level is not well understand.
Traditional nematode control and how the knowledge of the nervous system may improve nematode control

Chemical control has been one of the most important control methods traditionally. Chemicals are usually applied in one of the two ways, fumigant or non-fumigant. Fumigation is a pre-planting application that eliminates soil pathogens and weeds. It is usually applied by injecting volatile compounds that have a nematicidal activity into the soil profiles. These compounds evaporate and diffuse through the soil air spaces and results in effective contact with soil pathogens (Lembright 1990). However, soil fumigation is highly affected by soil conditions such as soil texture, temperature, moisture, and soil organic matter. Thus, varied results in nematode suppression are not uncommon. More importantly, one of premier fumigant for controlling plant-parasitic nematodes methyl bromide has been found to cause the depletion of the ozone (Zasada et al. 2010). Because of the public concerns to the environment and human heath, applications of soil fumigation have become more strictly regulated. This makes soil fumigation a less available practice to growers. Beside soil fumigation, nematicides can also be applied to soil or plants to kill nematodes. The most widely used compounds for nematode control belong to organophosphorus and carbamates (Gupta 2006). Those compounds inhibit acetylcholinesterases and cause excess acetylcholine which eventually leads to the death of nematodes. Compounds with high acetylcholinesterase-inhibition are oftentimes used as pesticides. Although effective as nematicides, organophosphorus and carbamate are also toxic to vertebrates. In severe cases, respiratory depression is the main symptom. The nematicides have been heavily used for agriculture practices and large amount residuals can accumulate in the environment. For example, the nematicide residuals were detected in the aquatic environment of a banana plantation and might be chronically toxic to aquatic organisms (Castillo et al. 2000).

Nevertheless, the nervous system is a great target for control of plant-parasitic nematodes. Neurotransmitters regulate behaviors that are essential for plant-parasitic nematodes to complete their life cycles (Kimber and Fleming 2006; Holden-Dye and Walker 2011). Disturbing the neurotransmitter signaling pathway could result in effective control. However, finding more nematode-specific targets in the nervous system may reduce the potential danger to vertebrates. The nervous system of nematodes is far less conserved than it is generally considered and variation exists in the structure of neurotransmitter receptors between invertebrates and vertebrates, and even among nematodes. Therefore, investigating the neurobiology of plant-
parasitic nematodes may bring insight into the control strategies. In this dissertation, chapters 3 and 4 mainly focus on the important neurotransmitters serotonin and GABA, respectively. These two neurotransmitters are found to be important in behaviors of plant-parasitic nematodes. In Chapter 4, the GABA and GABAergic neurons were also studied from a developmental aspect in the soybean cyst nematode.
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CHAPTER 2 UNEXPECTED VARIATION IN NEUROANATOMY AMONG DIVERSE NEMATODE SPECIES

ABSTRACT

Nematodes are considered excellent models for understanding fundamental aspects of neuron function. However, nematodes are less frequently used as models for examining the evolution of nervous systems. While the habitats and behaviors of nematodes are diverse, the neuroanatomy of nematodes is often considered highly conserved. A small number of nematode species greatly influence our understanding of nematode neurobiology. The free-living species *Caenorhabditis elegans* and, to a lesser extent, the mammalian gastrointestinal parasite *Ascaris suum* are, historically, the primary sources of knowledge regarding nematode neurobiology. Despite differences in size and habitat, *C. elegans* and *A. suum* share a surprisingly similar neuroanatomy. Here, we examined species across several clades in the phylum Nematoda and show that there is a surprising degree of neuroanatomical variation both within and among nematode clades when compared to *C. elegans* and *Ascaris*. We found variation in the numbers of neurons in the ventral nerve cord and dye-filling pattern of sensory neurons. For example, we found that *Pristionchus pacificus*, a bacterial feeding species used for comparative developmental research had 20% fewer ventral cord neurons compared to *C. elegans*. *Steinernema carpocapsae*, an insect-parasitic nematode capable of jumping behavior, had 40% more ventral cord neurons than *C. elegans*. Interestingly, the non-jumping congeneric nematode, *S. glaseri* showed an identical number of ventral cord neurons as *S. carpocapsae*. There was also variability in the timing of neurodevelopment of the ventral cord with two of five species that hatch as second-stage juveniles showing delayed neurodevelopment. We also found unexpected variation in the dye-filling of sensory neurons among examined species. Again, sensory neuron dye-filling pattern did not strictly correlate with phylogeny. Our results demonstrate that variation in nematode neuroanatomy is more prevalent than previously assumed and recommend this diverse phylum for future “evo-devo-neuro” studies.

**Keywords:** invertebrate, amphid, phasmid, *Pratylenchus*, *Meloidogyne*, *Heterodera*, *Heterorhabditis*, heterochrony
INTRODUCTION

For the past 200 years, nematodes received significant attention from neurobiologists due to their relatively simple anatomy (reviewed in Chitwood and Chitwood, 1938). The nervous system of the nematode *Caenorhabditis elegans* consists of only 302 neurons in the adult hermaphrodite and remains the only nervous system to be completely reconstructed (White et al., 1986). In addition to *C. elegans*, the neuroanatomy of several parasitic and free-living (non-parasitic) species has been examined using both light and transmission electron microscopy (TEM).

The phylum Nematoda is currently divided into 12 Clades (Figure 1.1) (Holterman et al., 2006; van Megen et al., 2009). Significant divergence in neuroanatomy exists between nematodes in basal clades (class Enoplea; formerly Adenophorea) and those in higher clades (class Chromadorea; formerly Secernentea) (Sulston and Horvitz, 1977; Gans and Burr, 1994; Malakhov, 1994). However, within the higher clades (clades 8–12), which include *C. elegans* and other intensely studied species, the neuroanatomy is often considered highly conserved (Angstadt et al., 1989; Martin et al., 2002; Burr and Robinson, 2004; Kimber and Fleming, 2005; Hallem and Sternberg, 2008; Srinivasan et al., 2008).

A classic example supporting a high degree of conservation of neuroanatomy among nematodes is the similarity in structure between the ventral nerve cords (VNC) of *C. elegans* (Clade 9) and the gastrointestinal parasitic nematode *Ascaris suum* (Clade 8). The VNC consists of a series of motor neurons that innervate body-wall muscles and regulate movement (White et al., 1976). While *Ascaris* adults are hundreds of times larger than *C. elegans* and inhabit an extremely different environment, the number of ventral cord neurons is remarkably similar. In *C. elegans*, 57 neurons in the VNC innervate 95 body-wall muscles (Sulston, 1976; White et al., 1976). In *A. suum*, 55 neurons in the VNC innervate approximately 50,000 muscle cells (Stretton, 1976; Stretton et al., 1978).

While the VNC anatomy of other nematode species has received little attention, several studies have examined the anatomy of anterior sensory neurons and pharyngeal neurons (Ward et al., 1975; Ashton and Schad, 1996; Endo, 1998; Li et al., 2001; Bumbarger et al., 2009; Ragsdale et al., 2009). In *C. elegans*, there is one pair of amphid sensilla each containing 12 sensory neurons. The anterior nervous system of *C. elegans* also contains six inner labial, six outer labial and four
cephalic sensilla each with an invariant number of neurons (Ward et al., 1975). While the pattern of sensilla and underlying neurons is typically conserved among examined species, variations in the number, position and ultrastructure of the anterior nervous system are well documented (Endo, 1998; Ashton et al., 1999; Bumbarger et al., 2009; Ragsdale et al., 2009). Similarly, recent data demonstrated extensive differences in neuronal connectivity between the pharynxes of *C. elegans* and *Pristionchus pacificus* (Bumbarger et al., 2013), a Clade 9 nematode frequently used for evo-devo studies. These anatomical differences may underlie functional differences in feeding behavior between the two species (Chiang et al., 2006).

To elucidate the evolution of nematode nervous systems, we utilized Differential Interference Contrast (DIC) and fluorescence microscopy to examine the neuroanatomy of the VNC and sensory neurons in nematodes from clades 9 to 12 (Holterman et al., 2006; van Megen et al., 2009). We found unexpected variation in the number of putative neurons in the VNC as well as the dye-filling pattern of chemosensory neurons among several species of parasitic and free-living nematodes. The variability was found both within and among nematodes clades suggesting a dynamic evolution of nematode neuroanatomy. Furthermore, we found variation in the developmental timing of the VNC among nematode species. Our results suggest that nematodes represent a valuable resource for understanding the evolution of nervous systems.

**MATERIALS AND METHODS**

**Nematode Cultures**

*Meloidogyne hapla* was isolated by the senior author from infected tomato plants and identified using morphological characters. *Pratylenchus penetrans* was isolated by Dr. Terry Niblack (formerly University of Illinois). Both *M. hapla* and *P. penetrans* were cultured on monoxenic excised corn and tomato root cultures, respectively (Lauritis et al., 1983). Seeds for monoxenic cultures were surface sterilized and germinated on water agar. After germination, roots were excised and transferred to Gamborg’s agar (Gamborg et al., 1968; Rebois and Huettel, 1986). *Heterodera glycines* was received from the plant clinic at University of Illinois and maintained in a sandy loam soil on the soybean variety ‘Lee’ in the greenhouse. *Aphelenchus avenae* was originally isolated by the senior author from soil surrounding garlic plants and identified using
morphological characters. *A. avenae* was cultured on 1/4 strength Potato Dextrose Agar with the fungus *Botrytis cinerea* as previously described (De Soyza, 1973). The entomopathogenic species (*Steinernema* sp. and *Heterorhabditis* sp.) were received from Dr. Albrecht Koppenhöfer at Rutgers University and reared on living greater wax moth larvae *Galleria mellonella* (Carolina Biological Supply Company, Burlington, NC, USA; Kaya and Stock, 1997). Infective juveniles (IJs) of the four species were collected using White traps (White, 1927) and stored in cell culture flasks with water before DAPI staining. Two methods were used to collect non-IJ and adult stages of entomopathogenic nematodes. For *S. carpocapsae*, IJs were induced to recover and complete development on lipid agar plates as previously described (Wouts, 1981). For other EPNs, non-IJs were collected by dissecting open *Galleria mellonella* approximately 9 days after inoculation. This allows for sufficient time for IJs to recover and develop into mixed stages of non-IJs and adults. *Acrobeles* sp. (strain PS1156), *C. elegans* (strain N2), and *P. pacificus* (strain PS312) were received from the Caenorhabditis Genetics Center and cultured on NGM agar with *Escherichia coli* OP50 using standard methods (Brenner, 1974).

*Pratylenchus penetrans*, *A. avenae*, and *M. hapla* were extracted from Petri dishes using a Baermann funnel and washed three times with distilled water before fixation. Second stage juveniles (J2s) of *H. glycines* were extracted from soybean roots using sugar centrifugation and washed three times with distilled water (Jenkins, 1964). *C. elegans, Acrobeles* sp., and *P. pacificus* were washed from the Petri dishes and rinsed three times with M9 buffer (Brenner, 1974) to remove adhering bacteria before fixation. IJs of *H. bacteriophora*, *H. megidis*, *S. carpocapsae*, and *S. glaseri* were washed three times with distilled water before fixation.

**DAPI Staining**

Nematodes were fixed in 4% formaldehyde at 4 °C overnight in microcentrifuge tubes. Following formaldehyde fixation, nematodes were washed three times with Phosphate buffered saline with Triton X-100 (PBST; 0.1% Triton) and incubated in methanol for at least 4 h. Nematodes were then washed three times with PBST and incubated in 0.2–0.5 µg/ml of 4’, 6-diamidino-2-phenylindole (DAPI; Life technologies, Carlsbad, CA, USA) overnight in dark at room temperature. Nematodes were store at 4 °C prior to examination. We were unable to distinguish the sex of *H. glycines, M. hapla* or young juveniles of *P. penetrans*. The gender of
Steinernema was identified based on the shape of the gonad. Only hermaphrodites of C. elegans and P. pacificus, and females of Acrobeles sp. were examined. Between 10 and 30 animals were examined for each species. Putative neurons in the VNC were identified based on the size and morphology of the nuclei (Sulston, 1976; White et al., 1976). Counts of neuronal nuclei were made from immediately posterior of the retrovesicular ganglion (RVG) to immediately anterior of the preanal ganglion (PAG) (Figure 1.2). In cases where a cell could not be unambiguously identified as a neuron an independent count was made by a researcher blind to the species. If the cell identity was still in doubt, it was excluded from the total neuron count.

A separate microwave fixation method was developed for the staining of J2 M. hapla. Nematodes were recovered from tomato root cultures and transferred to 0.2X Finney-Ruvkun buffer with 5% methanol and 2% formaldehyde (http://www.wormatlas.org/EMmethods/ Antibodystaining.htm; Finney and Ruvkun, 1990). The fixation solution was placed in a 1 L ice bath in a household microwave with rotating turntable. A separate 1 L beaker of H2O was included as a heat sink. Nematodes were exposed to three separate 1 min irradiations at 30% power with a 30 °C maximum temperature. Following fixation, nematodes were washed three times with PBST and then irradiated nine times in 0.2 μg/ml DAPI for 3–4 min at 30% power with a maximum temperature of 39 °C.

Dye-filling

Dye-filling was adapted from previously described methods (Tong and Bürglin, 2010). All nematodes were transferred into centrifuge tubes and prewashed 3 times with distilled water. Nematodes were incubated in 10 μg/ml of DiI (1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Life technologies, Carlsbad, CA, USA) and wrapped with aluminum foil on an orbital shaker for at least 2 h. Excess liquid was removed from the centrifuge tubes and nematodes were transferred onto 1.5% water agar for at least 1 h covered with foil to remove excess dye. Animals were then picked to agar pads amended with 20 mM levamisole for imaging with DIC and fluorescent microscopy (Shaham, 2006). For each species, more than 30 animals were examined. Images were acquired using a Zeiss M2 AxioImager with mechanized stage and Zen software. Z-projections were created using FIJI.
Development of the VNC in *A. avenae*

Synchronized *A. avenae* eggs were obtained by picking gravid *A. avenae* females into 5% M9 buffer (Stiernagle, 2006) for 1 h to lay eggs. Adults were then removed and the remaining eggs stored at 22 °C until hatching (~48 h). Immediately after hatching, J2s were transferred to 1/8 strength Potato Dextrose Agar with the fungus *Phomopsis logicolla* at 22 °C. *A. avenae* nematodes were then examined at specified time points after hatching by mounting on a 5% agar pad with 20 mM levamisole and observed using DIC microscopy.

**RESULTS**

**Surprising variation in the number of putative VNC-neurons among nematode species**

The VNC is an easily recognized series of neurons lying along the ventral cord of nematodes (Figure 1.2). In *C. elegans*, the VNC contains a series of 57 motoneurons lying between the RVG and the PAG (Sulston, 1976; White et al., 1976). Using DIC optics, neuronal nuclei in *C. elegans* are typically small, granular in appearance and lacking obvious nucleoli (Sulston, 1976; Yochem, 2006). Neurons are seen as highly condensed round fluorescent puncta following DAPI staining (Sulston, 1976).

The free-living nematode, *P. pacificus* (Clade 9) is used as a satellite nematode species for evolutionary studies (Sommer, 2005, 2006). Similar to *C. elegans*, *P. pacificus* typically feeds on bacteria. However, *P. pacificus* is also capable of predatory behavior toward other nematodes (Bumbarger et al., 2013). We found that the *P. pacificus* ventral cord contained approximately 20% fewer VNC neurons than *C. elegans* (Table 2.1; Figure 2.3 A) suggesting that the number of neurons does not strictly correlate with phylogeny. Furthermore, this data indicates that evolved behaviors such as predation do not necessarily require an increase in the number of motoneurons. To test if other, more distantly related bacterial feeding nematodes, show even greater divergence in ventral cord neuron number, we examined the Clade 11 bacterial feeding nematode *Acrobeles* sp. Interestingly, *Acrobeles* sp. showed a nearly identical number of VNC neurons as *C. elegans* (Table 2.1; Figure 2.3 F).
The entomopathogenic nematode genera *Heterorhabditis* sp. (Clade 9) and *Steinernema* sp. (Clade 10) infect a wide range of insect hosts. Though phylogenetically distinct, the two genera have similar lifestyles. *Heterorhabditis* sp. is more closely related to *C. elegans* than to *Steinernema* sp. (Clade 10). We found that two species of *Heterorhabditis* had 59 ventral cord neurons, similar to *C. elegans* (*Table 2.1; Figure 2.3 B*). However, the ventral cord of *S. carpocapsae* contained 76 neurons (*Table 2.1; Figure 2.3 D*); approximately 40% more than *C. elegans* and the largest number of VNC neurons among all examined species. *S. carpocapsae* is capable of an unusual jumping behavior wherein it stands on its tail, curls to form a loop and quickly extends to jump into the air (Reed and Wallace, 1965). We hypothesized that the increased number of neurons in *S. carpocapsae* evolved to allow for jumping behavior. To test this, we examined *S. glaseri*, another entomopathogenic species that does not exhibit jumping behavior. While slightly longer than *S. carpocapsae*, *S. glaseri* also had 76 neurons in the VNC (*Table 2.1; Figure 2.3 E*). This data suggests either that jumping behavior does not specifically require additional motor neurons or that an ancestor to *S. glaseri* could jump and the additional neurons in *S. glaseri* are remnants of this ancestor. *Steinernema* sp. are male-female species. During the IJ stage sexes are easily distinguishable based on the shape of the gonad. However, we did not observe major differences in the number of VNC nuclei between sexes (data not shown).

Clade 12 contains nematodes with diverse life histories including fungal-feeding, plant-parasitic, and insect-parasitic species (van Megen et al., 2009). We examined four species in Clade 12 including one fungal-feeding species and three plant-parasitic species. There was no obvious correlation between the number of VNC neurons and food source or phylogeny. The fungal-feeding nematode *Aphelenchus avenae* and the plant-parasitic nematodes, *Heterodera glycines* and *Meloidogyne hapla* each had approximately 65 VNC neurons (*Table 2.1; Figures 2.3 G, H, J*). However, the plant-parasitic nematode *P. penetrans* ventral cord contained fewer neurons than any other Clade 12 species (*Table 2.1; Figure 2.3 I*). *Pratylenchus* is considered basal to *Meloidogyne* (van Megen et al., 2009). This data suggests either that there were multiple events leading to an increase in VNC number or that *Pratylenchus* underwent a loss in VNC neurons during evolution.
Neuronal heterochrony has evolved at least twice among nematodes

*Caenorhabditis elegans* does not hatch with a full set of VNC neurons. Following embryogenesis, *C. elegans* hatches as a J1 (equivalent to L1 in *C. elegans* nomenclature) with 15 VNC neurons (Sulston, 1976). During J1 development, additional precursor cells (P0–P12 cells) migrate into the ventral cord followed by multiple rounds of cell division to produce the final complement of 57 VNC neurons (Sulston, 1976). This post-embryonic development occurs entirely within the J1–J2 developmental period. To determine if post-embryonic VNC development during J1 development is conserved, we examined the VNC of three species that hatch as J2s rather than J1s. We examined the VNC of both newly hatched J2s and adult nematodes of *P. pacificus* (Clade 9), *A. avenae* (Clade 12) and *P. penetrans* (Clade 12). Both *P. pacificus* and *A. avenae* showed delayed development in the VNC. *P. pacificus* hatched as a J2 with 20 VNC neurons while the adult hermaphrodite has 45 VNC neurons (Table 2.1). Similarly, *A. avenae* hatched as a J2 with 26 VNC neurons, while the adult female has 66 (Table 2.1). Interestingly, the delayed development of the VNC was not conserved among all species that hatch as J2s. We found no apparent difference in the number of VNC neurons between newly hatched J2 and adult *P. penetrans* (Clade 12) (data not shown). These data suggest an independent evolution of neuronal heterochrony in nematodes.

The delayed development seen in *A. avenae* and *P. pacificus* may be due to a shift in development from the *C. elegans*-like J1 VNC development to a J2 VNC development. Alternatively, the delayed development may be due to a progressive increase in VNC neuron number from J1 to the adult stage. To test these options, we collected time-series data on the development of the VNC in *A. avenae* using DIC microscopy. *Aphelenchus avenae* is an easily cultured fungal feeding nematode, closely related to, and a possible transitional model for, plant-parasitic nematodes. We found that eggs developed from single-cell embryos to hatched J2s in approximately 48 h. We observed 26 neurons in the VNC of newly hatched J2s using DIC microscopy. Cell division occurs after hatching and during feeding. Immediately prior to the J3 molt (25–35 h after hatching), we observed the most intensive increase of neurons in the VNC. By 48 h after hatching, *A. avenae* had a fully developed VNC with 66 neurons (Table 2.1; Figures 2.3 and 2.4). Thus, the delayed development of the VNC in *A. avenae* is a shift from a *C. elegans*-like J1 developmental sequence to a J2 post-hatching developmental sequence.
Sensory neuron dye-filling varies among and within nematode clades and among developmental stages

We were interested if a similar divergence in neuronal properties could be detected using a dye-filling protocol common to *C. elegans* research. Specific ciliated sensory neurons in the head and tail of *C. elegans* will fluoresce following exposure to the lipophilic compound DiI (Hedgecock et al., 1985; Collet et al., 1998). In *C. elegans*, six pairs of amphid neurons in the head and two pairs of phasmid neurons in the tail can be stained using fluorescent dyes (DiI, DiO and FITC; Figure 2.5 A; Hedgecock et al., 1985; Collet et al., 1998). Staining patterns can vary depending on the method. For example, under certain conditions six inner-labial sensory neurons can also be stained (Tong and Bürglin, 2010). While the precise mechanism is unknown, dye-filling is frequently used to indicate the structural integrity of specific sensory neurons in *C. elegans* (Perkins et al., 1986). For example, Srinivasan et al. (2008) used a comparative dye-filling approach to identify and investigate the function of a homologous sensory neuron among six free-living nematodes in Clades 9 and 10. They found a nearly identical dye-filling pattern among all tested nematodes, suggesting a high degree of conservation of nematode neuroanatomy. We expanded upon these results by testing additional species in Clades 9–12.

We initially attempted dye-filling on the entomopathogenic nematodes *Heterorhabditis bacteriophora* (Clade 9) and *Steinernema carpocapsae* (Clade 10). These entomopathogenic nematodes infect their hosts as IJs, a non-feeding developmentally arrested stage analogous to the *C. elegans* dauer stage. Following infection, the nematodes will resume development and feed until resources are depleted. The nematodes then reenter the IJ stage and disperse to find a new host. Interestingly, we did not observe any dye-filling in IJs of either species. Therefore, we isolated non-infective stages of these species using both *in vitro* and *in vivo* methods. We observed dye-filling in six putative IL2 orthologs in non-IJs and adults of *S. carpocapsae* (Figure 2.5 B). Two pairs of putative phasmid neurons also dye-filled in the tail of males of *S. carpocapsae* (Figure 2.5 B). Surprisingly, dye-filling of amphid neurons was only periodically observed in both of these insect-parasitic species. Based on these results, we examined the dauer stage of *C. elegans*. As previously shown, five of the six amphid neurons routinely dye-filled in *C. elegans* dauers (Figure 2.5 A) (Peckol et al., 2001). However, we never observed IL2 dye-
filling in C. elegans dauers using modified dye-filling protocols that routinely result in IL2 dye-filling in non-dauers. This lack of dye-filling may be due to modifications to cilia structure during dauer as previously shown by TEM (Albert and Riddle, 1983). Together these results demonstrate that development has a marked influence on sensory neuron properties.

Similar to our VNC data, we found significant variability in dye-filling among nematode clades. As discussed above, H. bacteriophora (Clade 9) and S. carpocapsae (Clade 10) showed different dye-filling patterns than C. elegans. However, the bacterial-feeding nematode Acrobeles sp. (Clade 11) showed an identical dye-filling pattern to C. elegans (Figure 2.5 C). The fungal-feeding nematode A. avenae (Clade 10) also displayed a similar dye-filling pattern as C. elegans. Specifically, six putative IL2 orthologs and twelve putative amphid orthologs dye-filled in A. avenae (Figure 2.5 D). In A. avenae, an additional four neurons were stained anterior to amphid neurons that we identified as cephalic neurons. Previous TEM data demonstrated that A. avenae females have two cephalic neurons (CEP1 and CEP2) in each sensillum (Ragsdale et al., 2009). The cilia of CEP1 in A. avenae are exposed to the environment, whereas CEP2 is embedded in the cuticle. Therefore, the dye-filling of cephalic neurons in A. avenae corresponds to the EM data. In C. elegans hermaphrodites, there is one cephalic neuron in each sensillum that is not exposed to the external environment and does not dye-fill (Hedgecock et al., 1985). However, C. elegans males, similar to A. avenae females, have two cephalic neurons. While these male-specific cephalic neurons (CEM) in C. elegans are exposed to the external environment, they do not dye-fill (Perkins et al., 1986).

Unlike the fungal feeding Clade 12 nematode A. avenae, the Clade 12 plant-parasitic nematodes displayed restricted dye-filling. In P. penetrans, only one pair of putative amphid neurons stained (Figure 2.5 E). Based on axon morphology and nuclear position we identified these neurons as likely ADL orthologs. We observed only occasional and weak dye-filling in P. penetrans phasmid neurons (data not shown). Two plant-parasitic nematodes, H. glycines and M. hapla, did not show dye-filling in any neuron suggesting a possible modification of these neurons.

**DISCUSSION**

Nematodes have made substantial contributions to our understanding of the molecular basis of evolution. These studies have generally focused on non-neuronal structures such as vulva
development and male-tail morphology (Fitch, 1997; Sommer, 2000). Fewer studies have utilized nematodes for examining the evolution of neurodevelopment and neuroanatomy. The lack of nematode “evo-neuro” studies may be due, in part, to the assumption that the neuroanatomy of nematodes is highly conserved. The few comparative studies have primarily utilized low-throughput TEM to dissect ultrastructural differences in the sensory or pharyngeal nervous systems of a handful of species. Here, we demonstrated that light microscopy can be used to observe differences in neuroanatomy and neurodevelopment across multiple species. Furthermore, we showed that neuroanatomical and neurodevelopmental differences among nematodes are more abundant than previously assumed.

We found differences in the number and developmental timing of the VNC among various nematode species. The VNC of C. elegans and A. suum consist of a series of motor neurons that regulate movement through coordinated excitation and inhibition of body wall muscles. Amazingly, these phylogenetically and ecologically separate species show a nearly identical number and pattern of cholinergic and GABAergic VNC neurons (Stretton, 1976; White et al., 1976; Stretton et al., 1978; Johnson and Stretton, 1985, 1987; McIntire et al., 1993; Duerr et al., 2001). As we found that several species diverge from the C. elegans and Ascaris neuroanatomy, it will be useful to examine the neurotransmitter identity of VNC neurons in these species for conservation of the basic patterning.

While identification of neuronal nuclei in the VNC is relatively straightforward using DAPI fluorescence and DIC microscopy there are two potential causes of ambiguity and resulting variation. In some species, a clear demarcation did not exist between the VNC and the retrovesicular and PAGs. This may have led to some of the variation in the number of VNC neurons recorded among individuals within a species. A similar variability in the position of individual VNC neurons is seen in C. elegans (White et al., 1976). An additional source of variability within species may be due to misidentification of cell type. While cell types were usually unambiguous, occasionally nuclei could not be strictly categorized as neuronal or non-neuronal. This ambiguity occurred most frequently in earlier larval stages where nuclei were often immediately adjacent to one another. In these cases, we used a second observer, blind to the species, to categorize the nucleus. In cases where the nucleus type was ambiguous to both researchers, the nucleus in question was excluded from the count. Therefore, it is possible that
some of our counts were underestimates of the true number of VNC neurons. It will be interesting to confirm these counts and conduct comparative connectomics on select species with TEM.

The variation in nematode neuroanatomy did not strictly correlate with phylogeny. While more closely related to *C. elegans* (Clade 9) than *A. suum* (Clade 8), *P. pacificus* (Clade 9) had 20% fewer VNC neurons than either species. Similarly, *P. penetrans* had 20% fewer VNC neurons than all other examined Clade 12 species (van Megen et al., 2009). The number of VNC neurons does appear to be conserved within individual genera as shown by *Steinernema* sp. and *Heterorhabditis* sp. Similarly, sensory neuron dye-filling did not correlate with phylogeny. While *C. elegans* (Clade 9) and *Acrobeles* sp. (Clade 11) show a nearly identical dye-filling pattern, the entomopathogenic nematode *Heterorhabditis bacteriophora* (Clade 9) showed striking differences. This variability in dye-filling is consistent with previous results showing only a single pair of amphid neurons dye-fill in the Clade 10 mammalian-parasitic nematode *Parastrongyloides trichosuri* (Zhu et al., 2011). Although the precise mechanism of dye-filling is unknown, we hypothesize that differential dye-filling patterns indicate ultrastructural or biochemical differences among sensory neurons. Alternatively, the sensilla pores may be blocked or filled with secretions in certain species (Perry, 1996). However, we think this alternative hypothesis less likely. Previous TEM studies on *Heterodera glycines* and *Meloidogyne* sp. clearly indicate that the amphids are exposed to the environment (Wergin and Endo, 1976; Endo, 1980). *Heterodera glycines* (Clade 12), which showed no dye-filling with Dil, was previously shown to undergo amphid dye-filling with fluorescein isothiocyanate (Winter et al., 2002). In *C. elegans*, certain environmentally exposed amphid neurons do not dye-fill (Hedgecock et al., 1985). Again, this implies that ultrastructural or biochemical differences in individual neurons may underlie species-specific dye-filling in our study. Finally, we demonstrate that there are developmental differences in dye-filling within individual species. In *C. elegans* dauers, changes in the ultrastructure of individual neurons likely result in altered dye-filling during this developmental stage (Albert and Riddle, 1983; Peckol et al., 2001; Schroeder et al., 2013).

In *C. elegans*, post-embryonic development of the VNC requires the migration and subsequent cell division of 12 sub-ventral precursor cells (P cells). Following migration, the P cells divide
to form an anterior neuroblast cell (Pn.a) and a posterior cell (Pn.p) which either forms a hypodermal nucleus or a vulval precursor cell. Certain ancestors of the *C. elegans* Pn.a cell undergo programmed cell death eventually leading to the defined 57 VNC neurons. Previous lineage analysis in *P. pacificus* demonstrated that the non-vulva forming Pn.P cells undergo apoptosis prior to hatch (Sommer and Sternberg, 1996). As *P. pacificus* and *C. elegans* have an identical number of Pn.a neuroblast cells (Félix et al., 2000), it seems likely that additional apoptotic events occur in the *P. pristionchus* Pn.a lineage following migration. Similarly, with species showing additional neurons, it will be important to find if this results from the embryonic development of additional P cells or through extra rounds of cell division.

Heterochrony is defined as differences in the timing of developmental events and may play an important role in evolution (Gould, 1977; Alberch et al., 1979; Smith, 2003). The delayed development of neurons in the VNC of *A. avenae* (Clade 12) and *P. pacificus* (Clade 9) is suggestive of neuronal heterochrony. As *P. penetrans* (Clade 12), which also hatches as a J2, shows no post-hatch VNC development we propose that the heterochronic developmental events in *A. avenae* and *P. pacificus* arose independently. In *C. elegans*, several mutants have been isolated that result in altered timing of development events (Ambros and Horvitz, 1984; Ambros, 1988; Lee et al., 1993). The homologs of these heterochronic genes and microRNAs in *C. elegans* are present throughout the animal kingdom and may play roles in altered developmental timing (Moss and Tang, 2003).

Our data suggest that the neuroanatomy of nematodes is not as highly conserved as previously described. The evolution of nematode nervous systems has been relatively neglected, in part, due to previous assumptions of high anatomical conservation. It will be valuable to use higher resolution imaging techniques and antibody staining to define the neuronal subtypes found in these species. For example, Stretton et al. (1978) found that the VNC of *A. suum* consists of five repeating sets of neurons each containing 11 cells. Among these cells, they found seven neuron types based on synaptic connectivity (Stretton et al., 1978). In our study, we observed a possible repeating pattern in the VNC of *M. hapla* (**Figure 2.3 H**). However, our methods do not allow for the identification of individual neuron types. TEM could be used to study the neuron types in order to classify repeated segments on select species. In addition, immunohistochemistry may be utilized to distinguish the neurotransmitter identity of individual cells. Using
immunohistochemistry, Loer and Rivard (2007) found variation in the expression of serotonin among the VNC of male Rhabditid nematodes. It will be interesting to examine the VNC for immunoreactivity to neurotransmitters such as serotonin and GABA. Together with previous data demonstrating large-scale rewiring between the pharyngeal nervous systems of *C. elegans* and *P. pacificus* (Bumbarger et al., 2013), we propose that the phylum Nematoda represent a bountiful source of data for understanding the evolution of nervous systems. A close comparative examination of the nervous systems of parasitic nematodes may also lead to the development of species–specific control strategies.
### TABLE AND FIGURES

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</tr>
<tr>
<td>11</td>
<td><em>Acrobeles sp.</em></td>
<td>57</td>
<td>10</td>
<td>54-59</td>
<td>Mixed</td>
</tr>
<tr>
<td></td>
<td><em>Aphelenchus avenae</em></td>
<td>66</td>
<td>10</td>
<td>63-69</td>
<td>Post J2s</td>
</tr>
<tr>
<td>12</td>
<td><em>Meloidogyne hapla</em></td>
<td>65</td>
<td>10</td>
<td>62-68</td>
<td>J2</td>
</tr>
<tr>
<td></td>
<td><em>Pratylenchus penetrans</em></td>
<td>57</td>
<td>20</td>
<td>53-58</td>
<td>Mixed</td>
</tr>
<tr>
<td></td>
<td><em>Heterodera glycines</em></td>
<td>66</td>
<td>14</td>
<td>62-69</td>
<td>J2</td>
</tr>
</tbody>
</table>

Table 2.1. **Ventral cord neuron cell bodies in nematode species.** IJ, infective juveniles and J2, second stage juvenile stage. \(^1\)Species not examined in this study, number of neurons obtained through light microscopy (Stretton et al., 1978). \(^2\)Species not examined in this study, numbers of neurons obtained through electron and light microscopy (Sulston, 1976; White et al., 1976). \(^3\)Young J2s of *P. pacificus* have 20 neurons in the VNC. \(^4\)Young J2s of *A. avenae* have 26 neurons in the VNC (11 nematodes observed using Differential Interference Contrast).
Figure 2.1. Phylogeny of the genera of nematode discussed in this study. The phylum Nematoda is currently divided into 12 clades as discussed in van Megen et al., 2009. Branch lengths do not represent distance.
Figure 2.2. DAPI staining of wild-type *Caenorhabditis elegans* (Clade 9). The ventral nerve cord (VNC) consists of a line of motor neurons extending along the ventral midline from the retrovesicular ganglion (RVG) to the pre-anal ganglion (PAG). (A) Ventral view of DAPI stained animal. (B) Region surrounding end of RVG and anterior portion of VNC (arrow). (C) Part of the ventral nerve cord showing neuronal (arrowheads) and hypodermal (arrow) nuclei. (D) Division between pre-anal ganglion and VNC (arrow). Inset scale bars = 5
Figure 2.3. The VNC of nematodes in Clades 8–12 is highly variable. Fluorescent micrographs of individual nematode species fixed in formaldehyde and exposed to DAPI followed by imaging under fluorescent light. Species examined include: (A) *Pristionchus pacificus* (Strain PS312) hermaphrodite. (B) *Heterorhabditis bacteriophora* infective juvenile. (C) *Heterorhabditis megidis* infective juvenile. (D) *Steinernema carpocapsae* infective juvenile. (E) *Steinernema glaseri* infective juvenile. (F) *Acrobeles sp.* (Stain PS1156) adult female. (G) *Aphelenchus avenae* J3. (H) *Meloidogyne hapla* J2. (I) *Pratylenchus penetrans* adult female. (J) *Heterodera glycines* second juvenile stage. Scale bar = 20 µm
Figure 2.3. (cont.)
Figure 2.4. *Aphelenchus avenae* undergoes a post-hatch increase in the number of VNC neurons during J2. The number of VNC nuclei was examined with DIC microscopy in synchronized *A. avenae* nematodes at various time-points following hatch. The molt from J2 to J3 occurs at approximately 36 h after hatching. Each data point represents an individual animal.
Figure 2.5. DiI-filling is highly variable among nematodes. Live nematodes were exposed to DiI for 2 h followed by repeated washes in water or buffer and then imaged with fluorescent microscopy. (A) Left Anterior of the Clade 9 nematode *Caenorhabditis elegans* dauer, arrowhead indicates amphid neurons. (A) Right Posterior of a *Caenorhabditis elegans* dauer, two pairs of phasmid neurons are shown. (B) Left Anterior of the Clade 10 nematode *Steinernema carpocapsae* female, arrow indicates inner labial neurons. (B) Right Posterior of a *S. carpocapsae* male, arrow indicates phasmid neurons and arrowheads indicate unidentified neurons. (C) Left Anterior of the Clade 11 nematode *Acrobeles* sp. female, arrow indicates amphid neurons and arrowhead indicates inner labial neurons. (C) Right Posterior of an *Acrobeles* sp. female, two pairs of phasmid neurons are shown. (D) Left Anterior of the Clade 12 nematode *Aphelenchus avenae*, pentagon indicates amphid neurons, arrow indicates cephalic neurons, and arrow head indicates inner labial neurons. (D) Right Posterior of an *Aphelenchus avenae* female, two pairs of phasmid neurons are shown. (E) Anterior of the Clade 12 nematode *Pratylenchus penetrans* female (ventral view), one pair of amphid neurons in the anterior of the nematode is shown. Scale bar = 10 μm for all images.
Figure 2.5. (cont.)
REFERENCES


CHAPTER 3 SEROTONIN REGULATES THE FEEDING AND REPRODUCTIVE BEHAVIORS OF *PRATYLENCHUS PENETRANS*

ABSTRACT

The success of all plant-parasitic nematodes is dependent on the completion of several complex behaviors. The lesion nematode *Pratylenchus penetrans* is an economically important parasite of a diverse range of plant hosts. Unlike the cyst and root-knot nematodes, *P. penetrans* moves both within and outside of the host roots and can feed from both locations. Adult females of *P. penetrans* require insemination by actively moving males for reproduction and can lay eggs both within and outside of the host roots. We do not have a complete understanding of the molecular basis for these behaviors. One candidate modulator of these behaviors is the neurotransmitter serotonin. Previous research demonstrated an effect of exogenously applied serotonin on the feeding and male mating behaviors of cyst and root-knot nematodes. However, there are no data on the role of exogenous serotonin on lesion nematodes. Similarly, there are no data on the presence and function of endogenous serotonin in any plant-parasitic nematode. Here, we establish that exogenous serotonin applied to *P. penetrans* regulates both feeding and sex-specific behaviors. Furthermore, using immunohistochemistry and pharmacological assays, our data suggest that *P. penetrans* utilizes endogenous serotonin to regulate both feeding and sex-specific behaviors.

INTRODUCTION

Plant-parasitic nematodes utilize sophisticated behaviors for both feeding and reproduction. Although efforts to elucidate the biochemical, cellular, and genetic regulation of plant-parasitic nematode behavior are increasing and ongoing (Atkinson et al. 2013, Cotton et al. 2014; Gheysen and Mitchum 2011; Haegeman et al. 2012; Manosalva et al. 2015; Rehman et al. 2016), our knowledge of these mechanisms is still relatively limited. Plant-parasitic nematodes use a protractible stylet for infection and feeding (Decraemer and Hunt 2006). In the Tylenchida plant-parasitic nematodes, the needle-shaped stylet is attached to three stylet protractor muscles that contract to produce a forward thrust of the stylet (Baldwin and Hirschmann 1976; Endo 1983).
Reproductive behaviors, including egg laying and male mating, are also likely under neuronal control. Although feeding and reproductive behaviors have been extensively studied in the bacterial-feeding nematode *Caenorhabditis elegans*, there are striking anatomical differences between the structures mediating these behaviors in *C. elegans* and plant-parasitic nematodes (Albertson and Thomson 1976; Endo 1984; Endo et al. 1997).

The neurotransmitter serotonin (5-hydroxytryptamine) is a conserved regulator of various behaviors in animals. In *C. elegans*, serotonin signaling regulates feeding, egg laying, and male mating behaviors (Chase and Koelle 2007; Loer and Kenyon 1993; Sze et al. 2000). Application of exogenous serotonin induces stylet thrusting in several cyst and root-knot species in the absence of a host (Hu et al. 2014; Jonz et al. 2001; Masler 2007). Similarly, exogenous serotonin application can induce spicule eversion behavior in male cyst nematodes (Jonz et al. 2001). However, previous studies examined the effects of exogenously applied serotonin on plant-parasitic nematodes, rather than the role of endogenous serotonin.

Lesion nematodes (*Pratylenchus* spp.) are important migratory endoparasites of numerous crops worldwide (Jones et al. 2013). Lesion nematodes display several behaviors that may be mediated through serotonin signaling. Unlike the sedentary cyst and root-knot nematodes, lesion nematodes move throughout development and feed both endo- and ectoparasitically (Zunke 1990). Upon reaching adulthood, the females lay eggs both within and outside of the roots (Rebois and Huettel 1986). Many species of lesion nematodes are gonochoristic and likely require male-specific mating behaviors.

The primary objective of this study was to elucidate the role of serotonin in *Pratylenchus penetrans* behavior. Therefore, we tested how exogenous serotonin affects feeding and reproductive behaviors of male and female *P. penetrans*. To investigate the role of endogenous serotonin in *P. penetrans*, we used immunohistochemistry to identify putative serotonergic neurons. We tested several pharmacological compounds that affect the endogenous serotonergic signaling pathway. Our results suggest that *P. penetrans* uses endogenous serotonin signaling to regulate specific behaviors. The nervous system is a well-established target for several nematicides and anthelmintics (Holden-Dye and Walker 2011). A better understanding of the neurobiology of plant-parasitic nematodes may lead to novel control strategies.
MATERIALS AND METHODS

Nematodes culturing

*P. penetrans* nematodes were obtained from Dr. Terry Niblack and cultured on monoxenic corn root cultures grown on sterile Gamborg’s (1.5% agar) media (Rebois and Huettel 1986). Briefly, sweet corn seed were surface sterilized in 95% ethanol for 3 min followed by 10% bleach for 10 min, and rinsed with sterile water. Sterile seed were placed on Gamborg’s media for germination at room temperature. After approximately 7 days, the root was excised from the seed and placed back onto the media. A block of agar containing numerous *P. penetrans* nematodes was then transferred from an old culture onto the fresh media using sterile technique. Cultures were incubated at 20 °C for at least 2 weeks before use.

Behavioral assays

To test the effect of serotonin on behavior, a two-factorial (sex and treatment) completed randomized design was used. Each experimental unit contained 20 to 30 nematodes. Nematodes were extracted from cultures by transferring a portion of the agar into sterile 5% M9 buffer (22 mM KH₂PO₄, 42 mM Na₂HPO₄, 85.5 mM NaCl, and 1 mM MgCl₂) (Brenner 1974). Actively moving adult males and females were picked under a dissecting microscope and incubated in 200 μl of 5 mM serotonin in 5% M9 buffer (treated) or 5% M9 buffer alone (control) for 15 to 20 min at room temperature (20 to 22 °C). This concentration of serotonin was previously shown to induce stylet thrusting in cyst and root-knot nematodes (Masler 2007). To ensure that all nematodes were exposed for a similar time, only 5 to 10 mixed male and female *P. penetrans* nematodes were incubated in the serotonin or control as a batch, and multiple batches were tested for the assay. The duration for one batch from the beginning of incubation to the end of the observation did not exceed 40 min. We did not observe any obvious decrease in stimulated behavior in the various compounds that would suggest habituation during the exposure period. The batch of nematodes from the incubation solution was placed immediately into a drop of either 5 mM serotonin in 5% M9 buffer (for treated) or 5% M9 buffer alone (for control) on an 8 to 10% agarose pad made by dissolving agarose in water (Sulston 1976). A coverslip was then gently placed onto the agarose pad with the nematodes. The high concentration of agarose
effectively restrained the nematodes for observation while still allowing sufficient movement to ensure that the handling had not killed the nematodes. The number of stylet thrusts in both females and males and vulva muscle contractions in females were counted in each nematode for 1 min at room temperature. We define a stylet thrust as a forward movement of the stylet knobs, cone, and shaft due to contraction of the stylet protractor muscles. Males and females were examined in a random order based on their position on the agarose pad. Males were also examined for the presence of spicule eversion. All behaviors were observed under differential interference contrast microscopy at ×630 or ×1,000 magnification at room temperature. An experimental design similar to the serotonin assay was used to test the behavior of P. penetrans following exposure to the following compounds: 2 mM fluoxetine, 10 mM imipramine, 100 mM imipramine, 10 mM octopamine, and 26 mM phentolamine. All chemicals were dissolved in 5% M9 buffer, with the exception of 100 mM imipramine, which was insoluble in 5% M9 and, therefore, dissolved in sterile H2O. For the imipramine assay, we had only 20 nematodes for each treatment. The concentrations of compounds were based on previous experiments with C. elegans and other plant-parasitic nematodes, with the exception of 100 mM imipramine, which was used to test whether a higher concentration would affect vulva contractions (Dempsey et al. 2005; Horvitz et al. 1982; Schroeder and MacGuidwin 2010).

Data from the stylet thrusting assays were compared using a two-way analysis of variance. Because of the lack of a significant main or interaction effect due to sex, data from both sexes were pooled for the figure. Vulva muscle contraction data were analyzed using Student’s t test. Spicule eversion was never observed in control males and, therefore, was not subjected to statistical analysis.

**P. penetrans antiserotonin staining**

*P. penetrans* nematodes were extracted from monoxenic cultures and washed with distilled water in a microcentrifuge tube. The antibody staining procedure was modified from previous methods (Hussey 1989). Briefly, nematodes were fixed in 4% formaldehyde at 4°C for 8 to 11 h. After washing with phosphate-buffered saline with Triton X-100 (PBST: 8 mM Na2HPO4, 150 mM NaCl, 2 mM KH2PO4, 3 mM KCl, and 0.05% Triton X-100, pH 7.4), nematodes were transferred into a glass well. A razor was used to slice the nematodes into small segments. Nematode
sections were incubated in 100 mM Tris (pH 7.5), 1 mM CaCl₂, and proteinase K (2 mg/ml) for 30 min at room temperature to facilitate antigen retrieval, then washed three times with PBST. Next, nematode segments were incubated in prechilled methanol at 20 ºC for 1 min followed by 1 min in prechilled acetone at 20 ºC, then washed three times in PBST. Nematode segments were blocked in 1% bovine serum albumin (BSA)/PBS buffer (catalog number A9418; Sigma-Aldrich, St. Louis) overnight at room temperature or 4 ºC. After blocking, nematode segments were incubated in 1:100 rabbit-derived antiserotonin (catalog number S5545; Sigma-Aldrich) overnight at either room temperature or 4 ºC, then washed three times with PBST. Finally, nematode segments were incubated in 1:100 goat-derived fluorescein isothiocyanate (FITC)-labeled antirabbit serum (catalog number F9887; Sigma-Aldrich) in 0.1% BSA/PBST for 2 h at 37 ºC. Samples were washed three times in PBST before observation. Images were acquired using a Zeiss M2 AxioImager and Zen software. Overlay images and scale bars were created using ImageJ.

RESULTS

Similar to studies in sedentary endoparasitic nematodes (Hu et al. 2014; Jonz et al. 2001; Masler 2007; Rolfe and Perry 2001; Schroeder and MacGuidwin 2010), we found that application of serotonin induced stylet thrusting in the migratory parasite *P. penetrans* (Figure 2.1). We rarely (<10%) observed serotonin-induced pumping of the metacorpus in *P. penetrans*. Although not statistically significant (*P* = 0.0966), we noted a trend of greater sensitivity to serotonin-induced stylet thrusting in female compared with male *P. penetrans* (data not shown).

To investigate the role of serotonin further, we tested the effect of exogenous serotonin on sex-specific structures. We observed continuous spicule eversion in 29 of 31 *P. penetrans* males following exposure to serotonin. Control males never showed spicule eversion (*n* = 30). In females, we found that application of serotonin induced contractions of vulva muscles (Figure 2.2).

To determine the presence and localization of endogenous serotonin in *P. penetrans*, we used an antiserotonin antibody. We detected several serotonin-immunoreactive cells in *P. penetrans* (Figure 2.3). The brightest and most consistent staining was found in a pair of neurons in the
head of the nematodes immediately posterior of the nerve ring (Figure 2.3A). Based on their position and serotonin immunoreactivity, we speculate that these cells are homologous to the C. elegans ADF amphid neurons (Figure 2.4). Anterior to the nerve ring, we detected at least one pair of neurons that have no known positional homolog among the serotonergic neurons in C. elegans. Within the esophagus, we only observed occasional and faint staining in a cell located on the ventral side of the metacorpus that is likely homologous to the C. elegans NSM neurons. We also detected serotonin-immunoreactive cells in the ventral nerve cord of both sexes. In females, two serotonin-immunoreactive cells were reliably detected in the ventral cord located immediately anterior and posterior of the vulva (Figure 2.3B). These cells extended processes that came into close contact with the vulva muscles. These cells appear homologous to the C. elegans VC4 and VC5 neurons (Figure 2.4). In one P. penetrans female, we found a serotonin-immunoreactive neuron anterior to the vulva that we suspect is homologous to the C. elegans HSN neuron (data not shown); however, this observation was not consistent. In males, we observed several serotonin-immunoreactive neurons in the ventral cord (Figure 2.3C). We suspect that these are homologous to the male serotonergic CP neurons in C. elegans (Figure 2.4). Because of the requirement to cut the animal to achieve adequate fixation and staining, we could not obtain an accurate count of these male-specific neurons.

To further probe the mechanism of endogenous serotonin signaling in P. penetrans, we tested the effect of the serotonin reuptake inhibitors fluoxetine and imipramine on serotonin-responsive behaviors in P. penetrans. Serotonin signaling is negatively regulated through reuptake (Dempsey et al. 2005). Reuptake inhibitors should increase the level of endogenous serotonin available for signaling. Therefore, application of these reuptake inhibitors should produce results similar to those from the application of exogenous serotonin alone. As expected, both fluoxetine and imipramine caused increased stylet thrusting in P. penetrans compared with control nematodes (Figure 2.1). Interestingly, however, these compounds did not affect sex-specific behaviors identically to exogenous serotonin. Although fluoxetine increased the frequency of vulva muscle contractions compared with nontreated nematodes \( P = 0.0342 \), the effect was noticeably reduced compared with that seen with exogenous serotonin (Figure 2.2). Although not statistically significant \( P = 0.0705 \), 10 mM imipramine caused a trend of increased vulva contractions; however, similar to fluoxetine, the effect of imipramine was reduced compared with exogenous serotonin. However, exposure to 100 mM imipramine caused a significant
increase in vulva contractions compared with the control that was similar in magnitude to that seen with 5 mM serotonin. Conversely, exogenous fluoxetine application resulted in no apparent spicule eversion in males whereas application of 10 mM imipramine resulted in spicule eversion in 25 of 30 males examined. Interestingly, the spicule eversion behavior of imipramine-exposed males differed from males exposed to serotonin. Although males exposed to serotonin had continuously everted spicules, approximately half of the imipramine-exposed males showed repeated spicule eversion and retraction.

Several studies in *C. elegans* suggest that octopamine acts as a physiological antagonist to serotonin (Chase and Koelle 2007; Horvitz et al. 1982). In our initial serotonin behavioral assays, we observed low levels of spontaneous stylet thrusting in control animals. We hypothesized that, if octopamine antagonizes endogenous serotonin signaling, then exposure to octopamine would reduce spontaneous stylet thrusting in *P. penetrans*. As expected, exposure to 10 mM octopamine significantly reduced spontaneous stylet thrusting ([Figure 2.1](#)). However, exposure to octopamine did not affect vulva contraction ([Figure 2.2](#)). To confirm our octopamine results, we tested the effect of phentolamine, an antagonist of octopamine signaling, on stylet thrusting (Evans and O’Shea 1978). Exposure to phentolamine increased stylet thrusting in *P. penetrans* ([Figure 2.1](#)), suggesting that endogenous octopamine acts to suppress serotonin signaling for stylet thrusting. Interestingly, however, phentolamine had no apparent effect on vulva muscle contraction or spicule eversion ([Figure 2.2](#)).

**DISCUSSION**

Our results suggest that endogenous serotonin signaling regulates stylet thrusting behavior in *P. penetrans*. Similar to previous results in sedentary plant-parasitic nematodes (Hu et al. 2014; Jonz et al. 2001; Masler 2007), we found that exogenously applied serotonin induces stylet thrusting in *P. penetrans*. Similar to *C. elegans*, we identified serotonin-immunoreactive cells within and adjacent to the esophagus. However, the specific pattern of staining differed from that found in *C. elegans* and other bacterial feeding nematodes (Horvitz et al. 1982; Loer and Rivard 2007).
In *C. elegans*, the two strongest serotonin immunoreactive neuron classes are NSM and ADF (Horvitz et al. 1982; Loer and Rivard 2007) (Figure 2.4). The NSM neurons are located within the metacorpus of the esophagus whereas the ADF neurons are located outside the esophagus (Albertson and Thomson 1976; Ward et al. 1975). NSM serotonin-immunoreactive homologs are also found in a wide range of bacterial-feeding and animal-parasitic nematode species (Johnson et al. 1996; Loer and Rivard 2007). Interestingly, we found only weak and inconsistent immunoreactivity within the metacorpus of *P. penetrans* where an NSM homolog is expected; however, we cannot completely rule out that our antibody staining was ineffective at penetrating the esophagus. Recent research in *C. elegans* suggests that serotonin produced by the ADF neurons is sufficient to modulate feeding behavior (Cunningham et al. 2012; Song et al. 2013). Therefore, it is possible that serotonin released from outside of the *P. penetrans* esophagus leads to stylet thrusting behavior. In addition to the putative ADF homologs, we consistently found serotonin-immunoreactive cells outside of the esophagus in a position just anterior of the nerve ring in *P. penetrans*. There are no known serotonergic cells at this position in *C. elegans*. One caveat to our antibody staining is that very little is known about the neuroanatomy of *P. penetrans* (Endo et al. 1997; Han et al. 2016; Trett and Perry 1985). Although our data demonstrate the presence of serotonin in *P. penetrans*, our assignment of individual cells as homologs to *C. elegans* is based solely on serotonin immunoreactivity, the position of the cell body, and, when available, the morphology of associated neuronal processes. Exogenous serotonin stimulated sex-specific behaviors in *P. penetrans*. Similarly, we found immunoreactive cells that may serve as a source of endogenous serotonin in proximity to these sex-specific structures. In *C. elegans* hermaphrodites, the serotonergic HSN neurons innervate the vulva muscles and regulate egg laying behavior (Figure 2.4). The VC4 and VC5 motor neurons of *C. elegans* hermaphrodites are serotonin immunoreactive but do not express the gene required for serotonin biosynthesis (Sze et al. 2000). Therefore, the VC4 and VC5 neurons of *C. elegans* are thought to take up serotonin produced by HSN (Anderson et al. 2013). Interestingly, in *P. penetrans*, we found serotonin immunoreactivity in the putative VC4 and VC5 neurons even in the absence of apparent serotonin immunoreactivity in an HSN homolog (Figure 2.3). We also observed serotonin immunoreactivity in the ventral cord of male *P. penetrans*. We speculate that these serotonin-immunoreactive cells are homologous to the serotonergic CP motor neurons found in *C. elegans* and several other free-living nematodes (Loer and Rivard 2007; Sze et al.
Ablation of CP neurons in *C. elegans* results in males defective for turning during mating (Loer and Kenyon 1993). Similar to results in *Heterodera schachtii* and *Panagrellus redivivus*, we found that serotonin induced spicule eversion in Pratylenchus penetrans (Croll 1975; Jonz et al. 2001). Unlike in *H. schachtii* males (Jonz et al. 2001), we did not observe any ejaculation from *P. penetrans* following exposure to serotonin. Given the proximity of the *P. penetrans* CP neurons to the spicule, it is reasonable to assume that serotonin released from these neurons modulates spicule insertion during mating.

Our experiments with serotonin reuptake inhibitors also provided evidence that sex-specific behaviors in *P. penetrans* are regulated by endogenous serotonin. However, unlike the stylet-thrusting assays, application of imipramine and fluoxetine did not produce a size effect similar to that of exogenous serotonin. One possible explanation is that the dose of fluoxetine and imipramine required to induce stylet thrusting is lower than that required for vulva muscle contraction or continuous spicule eversion. Although both fluoxetine and imipramine inhibit the reuptake of serotonin, they can also act outside of the serotonin reuptake pathway to regulate *C. elegans* egg laying (Dempsey et al. 2005; Weinshenker et al. 1995). Therefore, the inconsistent effects of fluoxetine and imipramine in *P. penetrans* sex-specific behaviors compared with *C. elegans* might indicate that *P. penetrans* uses a partially distinct neuronal signaling pathway.

A better understanding of the mechanisms regulating behaviors in plant-parasitic nematodes could lead to novel control strategies. The anthelmintic ivermectin is safe for mammals due, in part, to its affinity for invertebrate-specific glutamate neurotransmitter receptors (Geary 2005). The application of synthetic peptides designed to inhibit acetylcholinesterase in the head sensory neurons disrupted the cyst nematode host-sensing behavior (Wang et al. 2011). Despite the conservation of the serotonin biosynthesis pathway among animals, serotonin receptors can vary among different animal species (Komuniecki et al. 2004). Our results demonstrate the presence of endogenous serotonin in *P. penetrans*. The identification of specific serotonin receptors in plant-parasitic nematodes may provide new targets to selectively interfere with serotonin signaling.
Figure 3.1. Serotonin and octopamine signaling affect stylet thrusting behavior in *Pratylenchus penetrans*. Adult male and female *P. penetrans* were exposed to either a 5% M9 buffer solution (white bars) or one of the following compounds: 5 mM serotonin, 2 mM fluoxetine, 10 mM imipramine, 10 mM octopamine or 26 mM phentolamine (black bars). The number of stylet thrusts was counted for 1 min under a compound microscope; n >30 per treatment; *** and ** indicate *p*<0.001 and <0.01 respectively; error bars =stand error of the mean.
Figure 3.2. Serotonin induces vulva contractions in *Pratylenchus penetrans*. 20 to 30 adult female *P. penetrans* nematodes were exposed to either a control solution (white bars, 5% M9, with the exception of the 100 mM imipramine, which was dissolved in H$_2$O) or one of the following compounds: 5 mM serotonin, 2 mM fluoxetine, 10 mM imipramine, 100 mM imipramine, 10 mM octopamine, or 26 mM phentolamine (black bars). The number of vulva contractions per min were counted for 1 min under a compound microscope. Data from each assay were analyzed using an unpaired t test; ***, **, and * indicate $P < 0.001$, 0.01, and 0.05, respectively; error bars = standard error of the mean.
Figure 3.3. Antibody staining reveals endogenous serotonin in *Pratylenchus penetrans*.

Fluorescence (left) and differential interference contrast overlay (right) images of antiserotonin staining. All images are arranged anterior to the left and dorsal aspect to the top. Arrowheads indicate serotonin immunoreactive cells. (A) Anteriors of both sexes have two pairs of serotonin immunoreactive cells. Putative ADF homologs and one unidentified pair (question mark) were consistently and strongly immunoreactive. Unidentified neurons are anterior to the nerve ring whereas ADF are posterior to the nerve ring. The putative NSM homologs in the metacorpus are weakly and inconsistently immunoreactive. (B) In the vulva region of adult females, the putative VC4 and VC5 neuron homologs are strongly immunoreactive. (C) In the ventral nerve cord of the male, several putative CP neuron homologs are strongly immunoreactive.
Figure 3.4. Schematic of antiserotonin immunoreactivity in *Pratylenchus penetrans* and *Caenorhabditis elegans* as determined by this study and previous research, respectively (Duerr et al. 1999; Loer and Kenyon 1993; Loer and Rivard 2007).
REFERENCES


CHAPTER 4 WIDESPREAD REMODELING OF NEUROMUSCULAR TISSUE IN A PARASITIC NEMATODE SUGGESTIVE OF METAMORPHOSIS

ABSTRACT

A single unified definition of metamorphosis is lacking; however, one of the most common criteria of metamorphosis is large-scale morphological changes during post-embryonic development including remodeling of neuromuscular systems (Bishop et al. 2006; Tissot and Stocker 2000; Kollros 1981; Brown and Cai 2007). Nematodes are generally not considered to undergo metamorphosis. However, several species of parasitic nematodes have morphologically distinct larval and adult stages and exhibit sex-specific alterations to mobility. Here, we use a combination of light and electron microscopy to demonstrate that degeneration and sex-specific renewal of neuromuscular tissue in the parasitic nematode *Heterodera glycines* are associated with changes to mobility. We found that while both female and male nematodes undergo muscle degeneration during immobile stages of post-embryonic development, male *H. glycines* undergo somatic muscle renewal prior to molting into a mobile adult. In addition, developmental changes to the number of putative motor neurons are correlated to changes in mobility. To further examine neuronal changes associated with development, we characterized the GABAergic nervous system of *H. glycines* and found a reduction in gene expression of *hg-unc-25*, which encodes the primary GABA synthesis enzyme, as well as a reduction of GABA-immunoreactive neurons during developmental stages associated with immobility. Finally, we found evidence of similar developmentally associated muscle degeneration in the phylogenetically diverged parasitic nematode *Meloidogyne hapla* suggestive of independent evolution of neuromuscular degeneration. Together, our results suggest an expansion of animal phyla that undergo metamorphosis and present a new comparative species to examine mechanisms of neuromuscular degeneration and renewal.

**KEYWORDS:** evo-devo; ventral nerve cord; muscle atrophy; *Caenorhabditis elegans*; *Ascaris suum*
RESULTS AND DISCUSSION

Following infection and the initiation of feeding, several species of “sedentary” Tylenchomorpha plant-parasitic nematodes become immobile. The sedentary nematode *Heterodera glycines* infects as a second stage juvenile (J2) and then establishes a feeding site where it remains immobile throughout the remainder of juvenile development (Figure 3.1A). Unlike most nematodes, sedentary nematodes, including *H. glycines*, grow proportionally more in width than length. During J4, females remain immobile and continue to grow in width, eventually molting into immobile lemon-shaped adults. However, male J4s remodel into a more typical vermiform-shaped nematode capable of full movement following the molt into the adult stage (Turner and Rowe 2006).

*H. glycines* body wall muscles degenerate and undergo sex-specific renewal during development

The immobility of sedentary nematodes was hypothesized to occur due to degeneration of body wall muscles (Bird 1971; Elsea 1951; Klink et al. 2007). To test this hypothesis, we first analyzed the somatic muscle tissue in the mobile J2 stage of *H. glycines* using both the F-actin binding fluorescent probe phalloidin as well as transmission electron microscopy (TEM). Mobile J2 *H. glycines* somatic muscle comprise rhomboid-shaped cells longitudinally arranged in four quadrants along the length of the body (Figures 3.2 and 3.3). Each quadrant contains two rows of overlapping muscle cells and each cell contains striations obliquely oriented to the longitudinal axis. Using TEM on transverse sections following high-pressure freezing and freeze substitution, we found that individual muscle cells in J2 *H. glycines* comprise multiple sarcomeres separated from the cuticle by a thin basal lamina and epidermis (Figure 3.4A). Individual J2 muscles are arranged into obvious A- and I-bands with thick and thin filaments (Figure 3.5). The non-contractile region of body wall muscles is enriched with mitochondria. Overall, our light and electron microscopy images correspond to previous data from mobile stages of *H. glycines* and indicate a similar muscle structure to *C. elegans* (White et al. 1976; Waterston, Thomson, and Brenner 1980; Baldwin and Hirschmann 1976; Endo 1983).
Within six days following infection, *H. glycines* molts to a J3 (Lauritis, Rebois, and Graney 1983). We found that phalloidin-stained J3 *H. glycines* somatic muscles are disorganized and smaller than in mobile J2s (Figure 2). The body wall muscles lose their rhomboid shape and the pattern of two-row cells in each quadrant is no longer recognizable. We found that the body wall head muscles and esophageal muscles of immobile stages are intact (Figure 3.6) suggesting that muscle degeneration is cell-specific and not due to a generalized atrophy as seen in sarcopenia of *C. elegans* (Herndon et al. 2002). Our TEM examination of immobile J3s also demonstrated a shrinkage of muscle with disorganization of sarcomeres (Figure 3.4B). We did not observe recognizable A- and I-bands in immobile J3s. Furthermore, the number of thick and thin filaments are reduced in J3s compared with J2s. Sexual differentiation of *H. glycines* is first visible in late-J3 (personal observation and (Raski 1950)); however, we did not observe any obvious sex-based differences in muscle structure at this point. In addition to structural changes to the muscle itself, we observed that the body wall muscles are displaced internally away from the cuticle in J3 *H. glycines*. This displacement is correlated with a corresponding thickening of the epidermis. In *C. elegans*, the force of body wall muscle contraction is transduced through a thin epidermal layer via attachment to the outer cuticle (Francis and Waterston 1991; Hresko, Williams, and Waterston 1994). Loss of mobility in *H. glycines* may be due to a combination of muscle degeneration as well as an inability to transduce contractile force to the outer cuticle.

J4 *H. glycines* females continue to feed and grow in width, while J4 males begin remodeling back into a vermiform shape (Figure 3.1). In phalloidin-stained J4 females (Figure 3.2B), we detected faint stripes underlying the epidermis suggestive of muscle filaments. Interestingly, nuclear staining revealed muscle-like nuclei immediately underlying the phalloidin stripes (data not shown). These data suggest that rather than a programmed cell-death, *H. glycines* muscle undergoes atrophy. J4 males renew body wall muscle. While lacking the typical organization of mobile J2s or adult males, J4 male phalloidin-stained muscle is substantially larger compared with J3s and J4 females. Based on TEM, J4 male muscles contain identifiable sarcomeres, albeit less organized than in mobile J2s (Figure 3.4C). Like J3 and J4 females, the muscle tissue of J4 males were located more internally than in mobile J2s indicating that muscles had not reattached to the body wall. When males reach adulthood, the muscles are positioned directly adjacent to the body wall (Figure 3.4E). The relative size of body wall muscles in adult males is larger compared to J3s or J4 females and comprises well-defined sarcomeres (Figures 3.2, 3.4D and 3.4E).
E). Together, our light and electron microscopy data suggest that *H. glycines* undergoes cell-specific muscle degeneration and sex-specific muscle regrowth during development.

Besides, the expressions of several muscle related genes were also examined among different life stages of *H. glycines*. The genes *act-1* and *unc-87* are known involved in the muscle generation and maintenance (Klink et al. 2007). Using RT-qPCR, we found that during sedentary stages, these genes were down-regulated (Figure 3.7), which indicated a reduced metabolic activity of muscle maintenance and generation.

**Muscle degeneration evolved independently in parasitic nematodes**

Among Tylenchomorpha nematodes, sedentary behavior has evolved multiple times. The root-knot nematodes (*Meloidogyne* spp.) also become immobile soon after infecting the plant host (Bird 1967). However, Meloidogynidae are phylogenetically diverged from *Heterodera glycines*. Basal lineages to both Meloidogynidae and Heteroderidae are mobile during all post-embryonic stages (van Megen et al. 2009; Holterman et al. 2006). We examined mobile and immobile developmental stages of *Meloidogyne hapla* to determine if changes in muscle structure are correlated with sedentary behavior in phylogenetically diverged species. Similar to *H. glycines*, mobile J2 *M. hapla* have well developed body wall muscles that degenerate following infection and the onset of sedentary stages (Figure 3.8). The independent evolution of muscle degeneration suggests a substantial selection pressure for the evolution of sedentary behavior.

**H. glycines motor neurons degenerate during development**

Remodeling of motor neurons can occur independently of muscle remodeling during insect metamorphosis (Weeks and Truman 1985; Tissot and Stocker 2000). In *C. elegans*, contraction and relaxation of most body wall muscles are regulated by motor neurons within the ventral nerve cord (VNC) (Chalfie et al. 1985; White et al. 1976). We, therefore, examined the VNC during *H. glycines* development. We previously found that mobile J2 *H. glycines* contain 66 VNC neurons (Han, Boas, and Schroeder 2016). Here, using DAPI staining we found that the number of VNC neurons is reduced between the mobile J2s and the immobile J3s with a further reduction in J4 females (Figure 3.9A-C). In addition, the overall pattern of the VNC deviates from a completely linear pattern during sedentary stages. Some nuclei of the VNC are located
several microns away from the ventral midline (Figure 3.9B). Examination of the VNC by TEM also suggested a loss of fasciculation and separation from the nearest muscle during J3. Interestingly, TEM data suggested that J4 males have a properly fasciculated VNC with synaptic vesicles in several processes (data not shown). Strikingly, we found 73 neurons in the adult male VNC and a reorganization of the cord into a linear arrangement. It appears that similar to muscle, the motor neurons in the VNC of both sexes of *H. glycines* degenerate during sedentary stages of development; however, males undergo neuronal remodeling which includes adding additional VNC neurons beyond the mobile J2 stage. Similar to *C. elegans*, the males of *H. glycines* may include sex-specific motor neurons in the VNC (Loer and Kenyon 1993; Sulston and Horvitz 1977).

**GABAergic neurons decrease in the VNC of female *H. glycines* during development**

To further examine motor neuron degeneration in the VNC of *H. glycines* females during development, we characterized the GABAergic nervous system. In *C. elegans*, GABAergic neurons comprise approximately one-fourth of the VNC neurons (McIntire et al. 1993; Gendrel, Atlas, and Hobert 2016). GABA was previously shown in the mobile J2 stage of the sedentary nematode *Globodera rostochiensis* (Stewart, Perry, and Wright 1994). In *C. elegans*, GABA is produced from glutamate by glutamate acid decarboxylase (GAD) UNC-25 (McIntire, Jorgensen, and Horvitz 1993). We cloned the ortholog of *C. elegans* unc-25 from *H. glycines* (*hg-unc-25*). The amino acid sequence of *hg*-UNC-25 is over 60% identical to *C. elegans* UNC-25 (Figure 3.10). To validate the function of *hg*-UNC-25 we used a heterologous rescue approach. The *C. elegans* mutant *unc-25(e156)* is negative for GABA immunoreactivity (McIntire, Jorgensen, and Horvitz 1993) (Figure 3.11). We found that *hg*-unc-25 cDNA driven by the *C. elegans* unc-25 promoter rescues the *C. elegans* unc-25(e156) mutant for anti-GABA immunoreactivity (Figure 3.11) confirming the function of *hg*-UNC-25 in GABA synthesis. We hypothesized that GABAergic VNC neurons were among those that degenerated during development. Therefore, we expected a reduction of *hg*-unc-25 expression in sedentary stages compared to the mobile J2s. Using RT-qPCR, we found that expression of *hg*-unc-25 is significantly decreased in J3s and J4 females compared to J2s (Figure 3.9D). These data suggest a reduction in GAD synthesis and possibly a concomitant reduction in GABA production.
A thorough description of the number and position of GABAergic neurons associated processes is lacking in Tylenchomorpha nematodes. We used immunohistochemistry to characterize the GABAergic nervous system of *H. glycines*. We found that the pattern and morphology of GABA-immunoreactive neurons in J2 *H. glycines* were similar, but not identical to *C. elegans* (Gendrel, Atlas, and Hobert 2016). We detected several GABA-immunoreactive cells in the head of *H. glycines* surrounding the nerve ring and in the retro-vesicular ganglion that we identified as likely orthologs of the motor neurons RMEs and the polymodal neuron AVL, respectively (Figure 3.9E). In addition, we identified a pair of GABA-immunoreactive neurons in the head with no known GABAergic positional homolog in *C. elegans*. At this time, we cannot differentiate true GABAergic neurons from those that simply take up GABA. We found 21 GABA-immunoreactive cells in the VNC of J2s (Figure 3.9E). Similar to *C. elegans*, the commissures derived from these GABA-immunoreactive neurons in *H. glycines* run to the dorsal side (White et al. 1976). However, unlike the VNC commissures of *C. elegans* and *Ascaris suum*, which usually run along the right side, we found that the majority of GABA-immunoreactive commissures in *H. glycines* travel dorsally along the left side of the animal suggesting a complete reversal in neuronal handedness (Stretton et al. 1978; White et al. 1976).

In the J4 females, we found fewer GABAergic neurons in the VNC compared with the J2 (Figure 3.12), while the pattern of GABAergic neurons remains similar to J2s in the head region (data not shown).

**CONCLUSION**

We demonstrate that the *H. glycines* neuromuscular system remodels extensively during development. Neuromuscular remodeling is a common component to metamorphosis in insects and amphibians. Most nematodes are not considered to undergo metamorphosis (Bishop et al. 2006). For example, the remodeling of *C. elegans* neuromuscular tissue during development is not correlated with large-scale changes in gross morphology. Combined with the well-described changes in overall morphology of sedentary nematodes, our data provide evidence that sedentary nematodes undergo metamorphosis (Bird and Bird 1991). Indeed, we found that similar to holometabolous arthropods, *H. glycines* males are able to rebuild a degenerated neuromuscular system. Intriguingly, the fossil record suggests the earliest holometabolous arthropods induced
plant galls, similar to the hypertrophy of plant roots induced by sedentary nematodes (Labandeira and Phillips 1996).

Despite their evolutionary separation, both arthropods and chordates utilize hormone signaling through nuclear hormone receptor (NHR) signaling to mediate metamorphosis (Brown and Cai 2007; Truman and Riddiford 2002). The molecular mechanism regulating neuromuscular remodeling in H. glycines are unknown; however, NHRs make an appealing class of candidate genes to regulate this process. While sedentary nematodes do not appear to have undergone the expansion of NHRs found in C. elegans (Robinson-Rechavi et al. 2005; Opperman et al. 2008; Cotton et al. 2014; Abad et al. 2008), RNAseq data from the sedentary nematode Globodera pallida indicate differential expression of several NHR encoding genes between mobile and immobile stages (Cotton et al. 2014). It will be interesting to determine if NHR signaling is a conserved mechanism for regulating metamorphosis-like remodeling in nematodes. Sedentary nematodes are among the most damaging of plant-parasitic nematodes and able to establish a long-term parasitic relationship with the host (Jones et al. 2013). The degeneration of muscle and motor neurons may assist with the adoption of a sedentary lifestyle through catabolism of muscle protein. Insight into the mechanisms regulating muscle remodeling in sedentary nematodes may provide insight into new targeted control strategies.

This chapter was a collaboration of multiple people from Schroeder lab. Conceptualization, Z.H. and N.E.S.; Methodology, Z.H., U.R.C., S.T., K.N.L, and N.E.S; Investigation, Z.H., U.R.C., S.T., H.R., M.G.; Writing, Z.H. and N.E.S.; Supervision, K.N.L and N.E.S.
Figure 4.1. *H. glycines* includes both sedentary and mobile stages. *H. glycines* hatch as mobile second-stage juveniles (J2s). J2s migrate to the host roots, infect and initiate feeding. Following the initiation of feeding the body diameter increases and the nematodes become sedentary. J3s remain sedentary and continue feeding. During late J3, we are able to distinguish sexes. Following the molt to J4, males begin remodeling into a vermiform morphology while females continue to feed and grow in width. Following the final molt, adult males are fully mobile and seek out females to inseminate. Adult females remain immobile and continue to feed. Each life stage is not represented to scale.
Figure 4.2. *H. glycines* body wall muscles degenerate and undergo sex-specific renewal during development. Fluorescent micrographs of phalloidin-stained *H. glycines*. The J2 body wall muscles contain actin-enriched striated filaments obliquely oriented to the longitudinal axis. In J3, the diameter of muscle filaments is smaller than in J2 and lacks a well-defined organization. The phalloidin stained body wall muscle filaments are thicker in J4 males compared with J3. In the adult male, the body wall muscles contain additional filaments, but are otherwise similar to J2 body wall muscles. In J4 females, the size of the actin-enriched muscle filaments is further reduced compared to J3s. Scale bars, 5 µm.
Figure 4.3. Cartoon of J2 *H. glycines* bisected and with the cuticle and epidermis removed from the subventral quadrant to show the longitudinal organization of body wall muscles.
Figure 4.4. *H. glycines* body wall muscle undergoes ultrastructural changes during development. Transverse TEM sections of body wall muscle in the midbody of *H. glycines* following high-pressure freezing and freeze substitution fixation (left) and with pseudocolor overlay (right). (A) Mobile J2 *H. glycines* have intact muscle separated from the cuticle by a thin layer of epidermis. The sarcomeres of mobile J2s comprise well-defined A- and I-bands with numerous thick and thin filaments (also see Figure S2). (B) Sedentary J3 muscle shows signs of degeneration including a reduction of thick and thin filaments and a thickening of epidermis. (C) J4 male muscle comprises distinct sarcomeres, but with reduced numbers of thick filaments and an epidermal thickness similar to J3s. (D) J4 females undergo further degeneration of sarcomeres and increased thickening of the epidermis beyond that seen in J3s. (E) The muscles of adult males are similar in orientation and size to J2 muscle. Scale bar, 1 µm.
Figure 4.5. TEM cross section of a J2 *H. glycines* body wall muscle demonstrating a well-defined sarcomere with A- and I-bands. Arrowheads indicate the mitochondria in the non-contractile region. Scale bar, 1 µm.
Figure 4.6 Fluorescent micrograph of phalloidin stained J3 H. glycines demonstrating that head (arrow) and esophageal (arrowhead) muscle do not degenerate. Scale bar, 10 μm.
**H. glycines** developmental stages

Figure 4.7. Relative expressions of the muscle related genes *hg-act-1* and *hg-unc-87* compared to *hg-far-1* at different developmental stages of *H. glycines*. RT-qPCR was performed based on the previous study (Klink et al. 2007). Expressions of *hg-act-1* and *hg-unc-97* were compared to an internal control *hg-far-1* (Bekal et al. 2014; Prior et al. 2001). Different letters indicate significant differences between stages and mean separations were conducted using one-way ANOVA (α=0.05).
Figure 4.8. Fluorescent micrographs of phalloidin stained *Meloidogyne hapla* mobile J2s (A) and sedentary post-infection stage (B). Similar to *H. glycines*, *M. hapla* undergoes cell specific muscle degeneration. Scale bar, 10 µm.
Figure 4.9. Motor neurons degenerate in the ventral nerve cord during H. glycines development.

Lateral and sub-ventral micrographs of DAPI-stained mobile J2 (A) and J4 female (B), respectively. VNC neuronal nuclei (arrowheads) are highly condensed fluorescent puncta. VNC nuclei in immobile J4 females deviate from the linear pattern seen in mobile J2s. (C) Table of number of VNC neuronal nuclei during H. glycines development. Adult females have fewer VNC nuclei than mobile J2s, while adult mobile males have more VNC nuclei than mobile J2s.

Data for mobile J2s were taken from (Han, Boas, and Schroeder 2016) (D) Expression of hg-unc-25, based on RT-qPCR, is reduced in immobile J3 and J4 females compared with mobile J2s. hg-far-1 is used as an internal control (Bekal et al. 2014; Prior et al. 2001). * indicates statistical significance between stages based on ANOVA (α=0.05). (E) Lateral left view cartoon (top) and representative fluorescent micrographs of GABA immunostained J2 H. glycines sections (bottom). GABA immunostaining was conducted on bisected animals to facilitate penetration. The location of individual neurons was determined by their position relative to landmarks such as the esophagus (purple) and the primordial gonad (blue). Several GABA immunoreactive neurons (green) are located surrounding the nerve ring located between the metacorpus and the esophageal glands. Twenty-one GABA immunoreactive neurons are found in the VNC that send commissures, usually, along the left body wall to the dorsal cord.
The *hg-unc-25* from *H. glycine* is highly identical compared with *unc-25*, the *C. elegans* ortholog (isoform a; shown as *ce-unc-25*). The amino acid sequence alignment between *unc-25* (isoform a) and *hg-unc-25* is performed using multiple sequence alignment in Clustal Omega. In *C. elegans*, *unc-25* encodes the sole enzyme glutamate acid decarboxylase for GABA synthesis. *hg-unc-25* has a 69% identity compared to *unc-25*. 
Figure 4.11. The *hg-unc-25* encodes the sole enzyme glutamate acid decarboxylase for GABA synthesis in *H. glycines*. The *hg-unc-25* cDNA from *H. glycines* partially rescues the *C. elegans unc-25(e156)* mutant. In wild-type *C. elegans*, GABAergic neurons are detected in the head and ventral nerve cord (arrowheads) using anti-GABA staining. In the *C. elegans unc-25(e156)* mutant, GABA is not produced and GABAergic neurons cannot be detected using anti-GABA staining. A plasmid construct containing *hg-unc-25* cDNA driven by the *C. elegans unc-25* promoter partially rescues the *C. elegans unc-25(e156)* mutant and GABAergic neurons are detected (arrowheads). Arrows indicate the expression of *coel::RPF* used as a co-injection marker. Scale bars, 10 µm.
Figure 4.12. The anti-GABA staining of an *H. glycines* J4 female reveals twelve neurons in the ventral nerve cord. The anterior is oriented to the left. Scale bar, 10 µm.
REFERENCES


CHAPTER 5 CONCLUSION AND FUTURE DIRECTION

Plant-parasitic nematodes cause significant losses to the world crop production. Due to multiple reasons, there is a need for novel control strategies: increased regulation of traditional nematicides because of environmental concern from the general public, high costs for the application of nematicides, and the lack of natural resistance in the germplasm for breeding programs. The nervous system of nematodes has been the primary target for nematode control (Holden-Dye and Walker 2011). However, knowledge of the nervous system of plant-parasitic nematodes is relatively limited compared to the model nematode, C. elegans. Research in this area would be a first step towards discovering new nematicides. In Chapter 2, I have shown that the nervous system is varied among nematode species in the motor system and potentially in the sensory system. Those systems regulate locomotion and feeding behaviors, which are essential to the parasitism of plant-parasitic nematodes. It has been shown that disturbing neuropeptides in G. pallida could alter their movement and infection (Atkinson et al. 2013). Synthetic peptides have also been applied to plant-parasitic nematodes to disrupt chemosensation as one method of control (Wang et al. 2011). More recently, more neuropeptides have been identified and used through transgenic microbials for control (Warnock et al. 2017). Therefore, characterizing the sensory neurons and the host-finding mechanism can be beneficial for new target-development.

In Chapter 3, I have found that serotonin signaling regulates feeding and reproductive behaviors in the root-lesion nematode Pratylenchus penetrans. Disturbing the serotonin signals has the potential to decrease the aggressiveness of this nematode. With the increasing assemblies of new genomes, it will be interesting to identify serotonin receptors in Pratylenchus spp. and other plant-parasitic nematodes, and compare with the C. elegans genome.

In Chapter 4, I have looked at the development of H. glycines from a neuronal aspect. It appears that the losing mobility is part of the adaptation for cyst and root-knot nematodes resulting in a more sophisticated host-pathogen relationship. Although difficult, it will be interesting to figure out the mechanism which triggers the dramatic body change during the development of H. glycines. One potential way to address this question is to use RNA-Seq. Examining the gene expression at different developmental points and identifying the genes significantly changed through the development might provide insights into the mechanism. Further, validations of the
candidate genes from *H.glycines* in *C. elegans* may be convincing evidence. From a control point of view, the male *H. glycines* need to regain the mobility when reaching adulthood to complete mating. Disturbing the development signal of males can potentially control this nematode. Together, I have shown that the nervous system of plant-parasitic nematodes is of interest to study. A better understanding of the neural regulation of plant-parasitic nematodes will provide new insights into control strategies.
REFERENCES


APPENDIX A DNA SEQUENCES

>hg-unc-25 (cloned and sequenced)
ATGAAATTTAAAGGAGCATAAAAAGAATCAATTTGACACAAAACAAATGGTGAGCAAG
AACACGGACGACAGCAACTGGATGGACCAACCGGACCAACCGGACGACGACGAGAGAGAAAT
AACCGATTTGAAGCAACCGATTTGCTTCCACATAATCTGACTGGATGGGACAACAC
TCAGCAATTCTTGAGAGCAATGCAAAAATTTTACTGAATTACATTTCGGGAAGAGAA
CGACCGTCTCCACGAAAATGTCTTGATTTTACCACCAACCAGAACAAATGGCACAACATCAT
TGACCTGACACATTCACGAGAAACAAAATGAAACTGGGCGAACTTTTAAATGCTTGTG
CGAAGTGCTGCTGCTATGGGTGGCTGACAGGACATCCGCGCTTTCTTTAACCACATTC
GTGCGGATTTGGAATTTGGAATGGCAGGCGAATGGACTCACGTCTCAGGCAACATGCAACA
CGAATATGTGTCACCTACGAAATTTGCAACTGCTTGTCTCAGTCAGTGAGGAAGAAGAGTT
CAGAGAATGGTGAACTGATCGGCTGGCAGGGGGGCGGTGATGCCATCTTTTCG
CCCGGCGGTCCATCGCAATATGTCAGCGATGAACGCACGCACCGCATTTCCTCATTT
CCCGCTGCGAAGCCGCGATCAAGCCGCAATTGGCGGAAGGCGATCAATGGGAAGGT
GAAGAGTGCATAATAATATGAATAATGCAAAAGATGAAAGTCTTCCACCCATTTTTGGTTG
ACGGAAGGTACACTGGTTTACGGTGCTTGGGACCCGATCCCGCAAATGGCTGACATTT
TGACACGCGCCATAAGGCTTTGCGATGTTGGAGTTGGGAGTTGGGACTTGGT
TTGAGCCCTGAAATCAGTCATATAATTGGCTGGAATTTGGAAGGCTGATTCAGTCTCACA
TGGAAACACACAAACTGATGTTGGGACCTGGCGTCTTGGGAGTTGGGACTTGGT
AGAGAAGGCTTTTCTTCTTCAAAACCAATCAAATGTCAGCTGATTATCTGTTCTCTCCAACAG
GACAAAACATATGTATGTCGACTACGATACCAGGGGACAAAGGCGATGCAATGGGACG
ACACAAACGACATTCTTTCAAGTGGTGCTTATATGTGGCGTCTCACAAGGGAATGGAAGGT
TGGCGATGCAAATTATGGACCTTGGCAAAATATTTCAGTAAAAATCAA
ACGCACGTGAAGGCCTTTGGAATGTTGCTGCGACGAACCGGAAATCTTACACACATTTGCTT
CTGGTACATTTCCCGAAAGGCTCTGAAACAAATGTCACAACAAGAAAAAGATGGC
GTTGGAGAAGGGTGCGACGAAACAAAATTCAGTCAATGGGACGAGTCAGTGCTG
ATGGGCTACAAAACCTGACACCCGAAACTTCCTTACACACATTTGCTT
AACCGGCAATTGCGAGAGTCGATTGGAAGGTAGTCGACACACTG
GGAGAAGGTTTGTGA
>hg-tph-1 (From Dr. Lambert’s database. Multiple isoforms from the cDNA are seen)

ATGGCTTCCGGAATGAAATTCTCTATTACAACAAAAGGCACCAGGCTCGGAGGAC
AATGTCAACATCAATGAGCGACCAATCGCTGGAGGAGCTGAACGCAGGCTTTCCAC
GCACCGGAAATTTGGGACTTCTCTCCTCCGAGGAGGACGACGACGCAATGGAGCCTG
AAGAAGGAACTGACCAGCAGGAGACGAGTCGCTGGAGGAGGCACCAGGCGCC
CGCTTTCACAGTTGTCTGCCAGAGCACAGAAAGGCGTTCATCGGCTGGACCAAAATGT
TGTCGGCACTTTCAAGAAAAGGGTCAAAACTGAAATTTTCGAGGTCGCTGACGAC
GACGGACAGTGCAGCAGCAATGGGAAGATCTGCTCAGGATTTGAGGGGAGGCGC
CGGCGGCAACGATGCTAGCAGGACATCCAACGGCTGGGTTGTCGCTGTCATGAAAC
GACGCGAATTCGACGTCGTGAGATGATTTTGCGGAATTGGCGATGAAATTCAAA
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AACATTCTACGCCAAATTCGCCAGACTCCACGCAGAAGGAGGTCTGAGAATATTTT
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TGCACTCAGTGATCATTGCAACCGCAGGCTCTTTTACTACGCCCAAGGCAGACACC
GGCCCTTCTGGTCTTTCGCTGAGCTCTGTTGCTGAGCAGACGGTGGTGCAGCAAG
CTACGCTCTATTTCTCTTCACCAATTTTGCTAGGCTTCTCTTGCTGACTGAGACC
AAAAATGGACCACATGAATTTGGAAACCGGAGGACGAAATGGCAAATGGATGC
CGGCGCAGCAAAAGATAAGCAGAAAGACGCCGGAAATTCAGGCAAATTTAGGAGCG
ACTTCTTTCAAGTGCAAGGCACACTTCACGCAGCAGCGTTGGAGGAAAATTCGCGCAT
GCCGCTTCCGGCAACAGAGTGCTGTGACGACAGGATGTGCTGATCCACACCTGCCAG
CCGCCTACTTCTTCACGGCAAAATTTGGAAGGTTCAGCAAAGCTCTCGACACATCA
CTTCCAAATAGAACCAGCCCTTGTCGCTGACAAATCCGTACAGAATCCGAGTGG
AAGTGCTGAACAAACAAACGTTCTCTGATGTTGTCGCCGTCATTCACACCTTCCTG
TCAATCTGTTGGCCTCCTCCCAACACATCCTTCTG

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