A PHYLOGENETIC EVALUATION OF THE NEMATODE VENTRAL NERVE CORD

BY

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THESIS

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ABSTRACT

Nervous systems are incredibly complex networks that, in order to understand, we rely on models like *Caenorhabditis elegans* to deconstruct. Due to the prevalence of *C. elegans* and other derived nematode research, it was long assumed that nematode nervous systems were highly conserved across the phylum. Previous evidence revealed that within the derived clades, the assumption of conservation was wrong and that the timing of neuronal development varied. As these previous accountings disregarded the basal clades except for some sporadic observations, this research endeavored to corroborate that the nervous systems of basal nematodes are in fact the most complex and there is a nervous system simplification from the basal to the derived clades. My findings did indeed reveal a simplification of the nerve cord, specifically there appears to be a decrease in ventral nerve cord neurons between the basal and derived clades, which adds further evidence that class Enoplea bears most similarity to the nematode ancestor. A potential reason for nervous system simplification could lie in marine pressures as large marine nematodes are adapted to move through sediment and have more neurons in their ventral nerve cords while smaller marine worms can navigate the water column and were observed to have less neurons, though this hypothesis needs to be more thoroughly investigated. In addition, ventral nerve cord development within Mononchidae appears to be consistent with derived clades and despite basal clade indeterminate development, Mononchidae nervous systems are invariant as adults. Considering all instances of recorded ventral nerve cord development, it appears the precise timing of development shifts but there is always a moment of rapid neurogenesis, often preceding a molt, like what was observed within Mononchidae. These

results indicate that while nematode nervous systems may vary in their cell numbers and timing, their general organization and development is highly conserved as previously suggested.

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TABLE OF CONTENTS

CHAPTER 1: A GENERAL INTRODUCTION TO INVERTEBRATE NERVOUS SYSTEMS AND PHYLOGENY

1.1 INTRODUCTION

Nematode nervous systems are one of the most well-studied organ systems on the planet. As simple models, they are used to unravel more complex systems. Even so, there is a great deal unknown about them when you consider most of our understanding comes from a single species. We don't understand differences in the integration of sensory structures between nematodes. We don't understand differences in nervous system developmental rates. Most importantly, we can't even begin to understand nervous system evolution within nematodes because there is a lack of comprehensive knowledge of the nervous system across Nematoda. Unfortunately, due to the focused scope of nervous system data on *Ascaris suum* followed by *Caenorhabditis elegans*, scientists often had to extrapolate this information to other nematodes. Slowly in recent years, evidence has mounted that *C. elegans* nervous system data is not congruent with all nematodes. Nematoda itself is divided into basal and derived clades, the latter of which *C. elegans* belongs to. While it can be argued that basal and derived are empty terms, please note that they are used for effective conveyance to delineate nematode clades 1 through 6 (basal) from 7 through 12 (derived). Evolutionary order is not suggested through these terms, ignoring the fact that the basal clades contain the most similar extant relatives to the nematode ancestor. Fragments of evidence from both groupings—using the nematode ventral nerve cord as a microcosm for the entire nervous system—suggest that the nervous system is not as conserved as assumed in the literature. A more thorough analysis of the derived clades established that there are differences within their group and the results indicate that the basal clades could have a significant increase of neurons in comparison. If this could be confirmed, then there would be proof of a

simplification of the nematode nervous system and our understanding of nervous system evolution beyond nematodes would be augmented. The research presented here endeavors to expand on previous evidence by also including the basal clades, which lack representation in nematology. In addition, this research compares novel *Mononchus laminatus* data on ventral nerve cord neuron developmental rates to previous reports in order to further topple the concept of a conservative nervous system. In essence, the simplification of the nematode ventral nerve cord may act as an important flagship model for nervous system evolution that is reflected in more complex organisms so that perhaps one day humans too could have a complete connectome.

1.2 NEMATODE INTRODUCTION AND DEVELOPMENT

Nematodes are the most ubiquitous animal taxa on the planet. Not only are they unequivocally present in the three major habitat divisions—terrestrial, marine, and freshwater—but they have even been recovered from more extreme environments such as Antarctic dry valleys and arsenic-rich lakes (De Tomasel, Adams, Tomasel, & Wall, 2013; Shih et al., 2019). They are incredibly specious and diverse, with 30,000 species described being only a fraction of their suspected 1 million species (Blaxter, 2016; Hugot, Baujard, & Morand, 2001; Lambshead, 1993). They are considered the most numerous animal on the planet, containing more than seventy-five percent of all animals (Hugot et al., 2001).

Relative to other animals, nematode tissues are much simpler and their life cycles are brief. This makes them prime candidates for study. In addition, many are translucent, enabling easy visualization, and some representatives exhibit eutely, where all cells have a prescribed fate and each animal has a set number of cells. *C. elegans* possesses the above characteristics, plus it

is a free living nematode—easily culturable on Petri dishes with bacterial lawns. Small organisms such as *C. elegans* are ideal for transmission electron microscopy allowing for connectome or organ system reconstruction (Schmidt-Rhasea, 2014). As such, a lot of our modern understanding of nervous systems are rooted in *C. elegans* research (White, Southgate, Thomson, & Brenner, 1986). Not only that, but it is capable of being cryopreserved for ease of research. All of these factors alone make *C. elegans* desirable for study but in addition it is a close relative to numerous significant plant and animal parasites (Holterman et al., 2006). At a time when the Human Genome Project was only midway through, the *C. elegans* genome was the landmark first completed multicellular genome (Blaxter, 1998). Furthermore, nematode anatomy is not a sole interest; more than eighty percent of the *C. elegans* proteome are homologs to human genes, including disease relevant genes, such as those involved with Alzheimer's (Kaletta & Hengartner, 2006; Lai, Chou, Ch'ang, Liu, & Lin, 2000). There is a lot of potential for study and insights within a tiny worm; however, the use of *C. elegans* as a lens into all nematode morphology has resulted in some improper generalizations that this review will discuss.

Nematode life cycles have a simple complexity to them involving six stages. They begin as eggs, proceed through four cuticle molts, and reach adult maturity. These molts are referred to as the juvenile stages, abbreviated as J1 through J4. Depending on species, some nematodes will remain in their eggs through their J1 or possibly even J2 or J3 stages prior to hatch. To identify adults, a reliable method is to observe either the vulva or the spicule, the female and male reproductive structures, respectively. Both distinctive structures are completed by late J4 in *C. elegans* so they are reliable identifiers to determine adults, which was necessary for this research

(Schindler & Sherwood, 2013; Sharma-Kishore, White, Southgate, & Podbilewicz, 1999; Shemer, Kishore, & Podbilewicz, 2000; Sulston, Albertson, & Thomson, 1980).

Acknowledging some of the history behind the two types of embryogenesis—determinate (referred to above as eutely) and indeterminate development—aids in understanding the premise of this thesis as well. Determinate development is characterized by individual cells having fates so growth is specified in an organism. For instance, *C. elegans* adults will have exactly 959 somatic cells whereas indeterminate nematodes are not held to this rigid body planning and individual cells are not universally held to predetermined fates (Sulston & Horvitz, 1977; Sulston, Schierenberg, White, & Thomson, 1983). Classically, nematodes were thought to be determinate as a result of *C. elegans* and *Ascaris suum* research (Boveri, 1899; Müller, 1903; Sulston et al., 1983). While this holds true for these two nematodes, over time it became clear that determinate development was not ubiquitous among nematodes (Malakhov, 1994; Schierenberg, 2005; Schulze & Schierenberg, 2008, 2011). While all derived nematode lineages studied display determinate development (Schulze, Houthoofd, Uenk, Vangestel, & Schierenberg, 2012), research shows many basal nematodes are indeterminate, to the point where it is now an accepted concept that the ancestral state of nematodes was indeterminate development (Schulze & Schierenberg, 2011). *Plectus*, a nematode evolutionarily balanced between the basal and derived groups, was found to have a style of embryogenesis that is transitional between the indeterminate ancestors and the determinate descendants (Schulze et al., 2012). In this way, not only has embryogenesis shed light on the evolution of nematodes, but it also has revealed another instance where reliance on *C. elegans* generalizations undermined the advancement of our understanding.

1.3 THE PROGRESSION AND FAILINGS OF NEMATODE PHYLOGENY

Comprehension of nematode phylogeny is a critical part of relating nervous system concepts to evolution. As such, a reliable phylogeny is essential in order to infer accurate relationships. The following section endeavors to provide a history of nematode and outgroup phylogenies as well as their limitations. The concepts presented provide a premise upon which evolutionary interpretations of the data can be made.

Thinking broadly, nematodes are protostomes, more specifically they fall into the superphylum Ecdysozoa, which most notably contains arthropods. Ecdysozoa was established molecularly by Aguinaldo et al. (1997). Today, this superphylum is widely recognized as monophyletic, however a lot of morphological evolution questions still remain (Ivanova-Kazas, 2015). Most regard the unifying synapomorphy of the taxon to be ecdysis, or cuticle/exoskeleton molting via ecdysone (Schmidt-Rhaesa, Bartolomaeus, Lemburg, Ehlers, & Garey, 1998), but other potential synapomorphies postulated include a three-layered cuticle, a trilaminate epicuticle, and α-chitin within the inner cuticle (Harvey, Dong, & Donoghue, 2010). While nematodes do undergo ecdysis and have a trilaminate epicuticle, the cuticle layers vary taxonomically and chitin has only been observed in eggshells and esophagi (Schmidt-Rhaesa, 2014). Currently, nematodes' positioning as an accepted Ecdysozoan taxon is not in question but it has long been a point of contention elucidating the relationships within Ecdysozoa.

More recently, Nematoda has been placed within Cycloneuralia with four other taxa: Nematomorpha, Priapulida, Kinorhyncha, Loricifera (Nielsen, 1995). The autapomorphy for this grouping are a cycloneuralian brain that is circumpharyngeal (Teuchert, 1977). Curiously, the exception to this is Nematomorpha, which lacks a cycloneuralian brain. The only hypothesis put forth to justify this is their entirely parasitical nature (Schmidt-Rhaesa, 2014), but it is important

to note that parasites within the other cycloneuralian taxa retain their distinctive cephalized structure. The cycloneuralian brain itself does not have a known origin as a result of no known morphologically similar structures (Schmidt-Rhaesa, Harzsch, & Purschke, 2016). An argument has been made that it is an adaptation to compensate for the terminal mouth in Cycloneuralia so structures are situated around a central axis rather than other animals which are organized around a ventral mouth (Schmidt-Rhaesa & Rothe, 2014).

It is essential to note, however, that no matter what taxonomic groupings have been made based on morphological data, no one has yet to produce convincing molecular evidence to support monophyly (Schmidt-Rhaesa et al., 2016). For many years, proponents of Nematomorpha as the sister clade to Nematoda have pushed this agenda, but phylogeneticists consistently fail to provide proof (Schmidt-Rhaesa et al., 1998; Schmidt-Rhaesa & Rothe, 2014; Zrzavý, 2001). More recent molecular studies even include Tardigrada when attempting to resolve the relationships, perhaps as a potential outgroup to Cycloneuralia, but these analyses fail as well (Holterman et al., 2006; Smythe, Holovachov, & Kocot, 2019; van Megen et al., 2009). It has been hypothesized that there may be a lost ancestor to nematodes that hinders tree reconstruction, but this argument shouldn't affect recovering monophyly.

Thinking more narrowly to the relationships within Nematoda, while we are currently reliant on molecular investigations, DNA was not a tool casually available when a great deal of animal phylogenies were initially constructed. Nematode morphological classification was dominated by a few nematologists, no doubt due to the difficult nature of distinguishing distinctive characteristics in animals that are superficially so morphologically similar. Developing a nematode phylogeny has been attempted for nearly a century, starting with the creation of designations Aphasmidia and Phasmidia (Chitwood, 1937), which eventually

morphed into the long utilized Adenophorea and Secernentea, respectively (Chitwood, 1958). Secernenteans were characterized by possession of sensory phasmids while Adenophoreans lacked them. Through time, many argued that Adenophorea was not monophyletic and that the strict presence or absence of phasmids was not diagnostic, but Adenophorea and Secernentea remained the recognized classes as no major phylogenies overturned them. An important shift in thinking occurred in the 1980s, when a paper suggested the use of a clade system (Lorenzen, 1981). This laid the groundwork for the future cladistic phylogenies.

Nematode systematics is a relatively young field. The first attempt at a molecular phylogeny was by Blaxter et al. with a small sample of only 53 species (1998). They used 18S rDNA, which followed as the gold standard for nematode systematics and barcoding. In their study, they determined there were five nematode clades, and they used the now debunked nematode classes of Secernentea and Adenophorea. De Ley & Blaxter (2002) improved on the 1998 phylogeny, with the inclusion of more taxa but it was centered around economically important nematodes. They overturned the use of Secernetea and Adenophorea as the nematode classes and instead replaced them with Chromadorea and Enoplea.

A major development occurred when a massive phylogenetic collaboration was published by Holterman et al. (2006). They took a more comprehensive look at the phylum by using 339 SSU rDNA sequences. Their phylogeny split Nematoda into 12 clades (Figure 1.1), which was further corroborated in a follow-up paper that expanded the sample using 1215 specimens (van Megen et al., 2009), though they only addressed 15 of the 19 orders as defined by Holterman et al. (2006). Both found paraphyletic groupings, such as the distinctive Plectida, based off the included species and called for revisions to be made. Within the already established taxonomic framework, Clades 1 and 2 belong to Enoplea and the rest of the clades (3-12) are accepted under

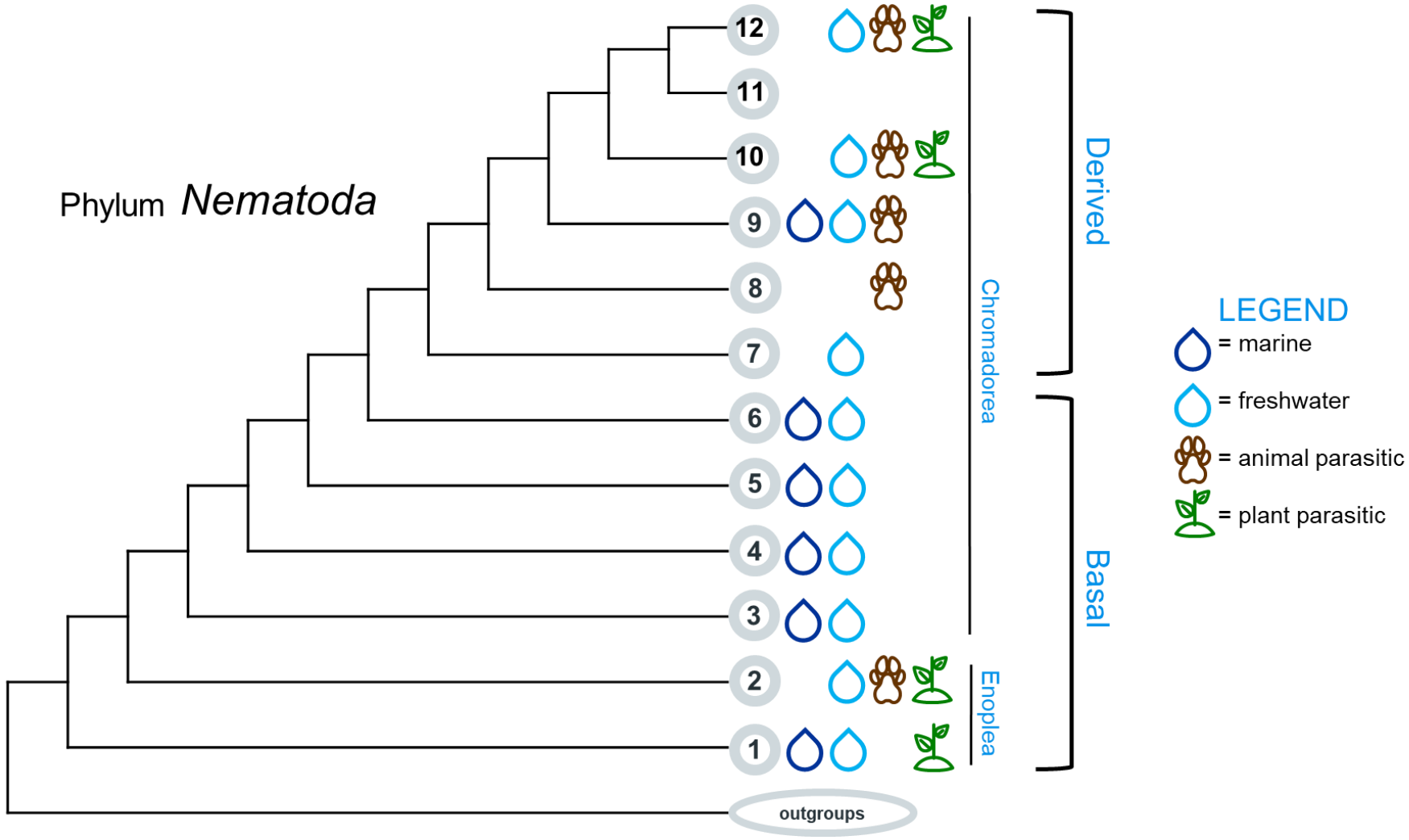

Figure 1.1. A representation of the modern, cladistic nematode phylogeny adapted from Holterman et al. (2006) and van Megen et al. (2009). Basal clades contain Clades 1-6 while derived clades are 8-12. All clades contain terrestrial representatives.

CLADE	CLADE TAXA	NOTABLE INFORMATION	DISTINGUISHED SPECIES
1	Enoplia	Largely marine; unique sensory structures	*Tobrilus* spp.
2	Dorylaimia	Common in soils and includes many parasites	*Mononchus* spp.
3	Chromadorida	Mostly aquatic	
4	Desmodorida	Issues with monophyly	
5	Monhysterida and Araeolaimida	Mostly aquatic	
6	Plectida	Issues with monophyly	*Camacolaimus* spp.
7	Teratocephalidae	Contains a single genus and a transitional clade	
8	Spirurina	Mostly animal parasitic	*Ascaris* spp.
9	Myolaimina and Rhabditina	Widely investigated	*C. elegans*
10	Aphelenchoidea and Panagrolaimomorpha	Contains many parasites	*Steinernema* spp.
11	Cephalobomorpha	Mostly free-living	
12	Tylenchida and Aphelenchidae	Contains devastating plant parasites	*Meloidogyne* spp.

Table 1.1. An expansion of Figure 1.1 including both taxa and special properties contained within the twelve nematode clades.

Chromadorea, although there have since been concerns of polyphyly within Chromadorea (Schmidt-Rhaesa, 2014).

All clades contain terrestrial representatives but further lifestyles are also prevalent among nematodes. Special areas of interest are highlighted here but an overview of the clades is given in Table 1.1. Clade 1is largely marine and freshwater nematodes. It has two orders, Enoplida, with the true marine nematodes, and Triplonchida, with largely freshwater worms. This clade also tends to possess unique sensory structures that could have nervous system implications (Malakhov, 1994). Clade 4 is entirely composed of Desmodorida but there have been concerns regarding the monophyly of this grouping. Conventionally, nematologists tend to establish Epsilonematidae and Draconematidae as their own families due to their exceptional morphologies but the molecular evidence is very messy for their order and Draconematidae was

not even included in the two comprehensive nematode studies (Holterman et al., 2006; van Megen et al., 2009). Clade 6 is Plectida, which today is still not regarded as a monophylum, likely due to the numerous orphaned morphologies that were cast aside in this order throughout the years that made attempts at monophyletic analyses lamentable (Holovachov, 2013). Clade 7 features morphological characteristics of both the Secernenteans and Adenophoreans, causing taxonomists to believe it was a transitional grouping. There are not as many issues regarding monophyletic relationships with the derived clades, likely due to the plethora of sequences available compared to the basal clades, leading to greater skewed input into tree analyses.

Rapid speciation towards the base of the nematode tree does not aid in the recognition of a nematode ancestor or construction of a backbone as poor resolution in genes is common in rapidly evolving groups. For instance, even with an extensive number of sequences, the family relationships within Dorylaimia are a difficult to resolve and appear paraphyletic. A potential reason for this is the belief that Dorylaimia underwent rapid radiation due to the conserved nature of the 18S sequences (Holterman et al., 2006). For better resolution, a phylogeny was built using 28S rDNA of 72 Dorylaimida species (Holterman et al., 2008). They found that the LSU rDNA did provide better resolution but resulted in a large basal polytomy, likely due to the small representative sample for a rapidly speciating subclass. This clearly helps to establish that the overreliance on the 18S gene prevalent in nematode systematics is a shortcoming that hinders effective tree reconstruction for the more difficult questions. A failing that needs to be acknowledged as well is that single gene phylogeny studies have become the norm in nematodes, not by intention but through neglect of multi-gene basal clade sequencing (Blaxter et al., 1998; Holterman et al., 2008; Holterman, Schratzberger, & Helder, 2019; Holterman et al., 2006; van

Megen et al., 2009). Recently, Smythe et al. used transcriptome data to construct a nematode phylogeny; going forward, hopefully this indicates a shift away from single gene studies (2019).

In 2012, a review was written regarding nematode genome sequencing efforts (Kumar, Koutsovoulos, Kaur, & Blaxter, 2012). At the time, only ten genomes had been sequenced. This number has now grown to 153 nematode genomes available through GenBank and WormBase (Harris et al., 2010). While this expansion is certainly an accomplishment, it must also be examined with a critical eye. Of those 153 sequences, 136 of them are from the derived clades (9 through 12). Of the 17 representatives from the basal clades, 16 of them are from Clade 2 and they are all animal parasites belonging to the same two genera. Sixty-six percent of all the genomes are animal or plant parasitic. Among the free-living genomes, 55% are *Caenorhabditis* spp. Hence, while nematology should celebrate its genomic accomplishments, we also need to acknowledge the limitations often seen in research that are reflected here. There is simply less representation of the basal clades across the board. Even when there is representation, it is biased towards parasitic nematodes. Seeing as there are no current hypotheses of a parasitic nematode ancestor, it is unfortunate there is not as much research available regarding free-living basal nematodes which could help to explain nematode evolution.

If we are to examine evolution, it would be beneficial to have a recognized nematode ancestor. Just as with outgroups, the ancestral clade is uncertain. However, in terms of the nematode ancestor, there is a lot less speculation involved. There have been two camps seriously floated and they will both be discussed below. The longer-running, tentatively-accepted as truth hypothesis is that the most similar extant relative to the nematode ancestor are Enoplians (Poinar, 1983). The reasons for the wider acceptance of this premise are multi-faceted: they possess ancestral animal traits such as indeterminate development (Schierenberg, 2005; Schulze &

Schierenberg, 2011) as well as a persistent nuclear envelope in spermatozoa (Justine, 2002) and they are largely marine, which is a common evolutionary birthplace for animal phyla (Maggenti, 1963; Malakhov, 1994). Keep in mind that Enoplia is not entirely marine however, so the acceptance of this hypothesis does not necessitate a definitive marine ancestor. The other camp suggests that Dorylaimia was possibly the starting point for nematode radiation with a terrestrial or freshwater ancestor (De Ley & Blaxter, 2004). The evidence for this hypothesis is equal to the Enoplian support but it is not favored in discussion. However, it cannot be ruled out due to the nature of nematode SSU data, which has failed to reveal an ancestor throughout many analyses. There have been targeted Enoplian phylogenetic studies, and they are not capable of revealing the nematode backbone without other genes included. Either way, nematologists strongly advocate for a Clade 1 or 2 ancestor which does establish Enoplea as basal to the rest of the tree. Recently, a study found strong support for the Enoplia hypothesis while Dorylaimia was not rooted as basal in their analyses (Smythe et al., 2019). Given the morphological support and the phylogenetic support, it does not seem farfetched to accept this hypothesis until contradicting data comes along. It is difficult to conclusively elucidate an ancestor when the nematode backbone has still not been resolved. One large cause of this is the lack of Enoplian and Dorylaimian representatives in phylogenetic analyses as compared to Chromadorian. Efforts have been made in recent years to remedy this gap.

1.4 CRITICAL EVOLUTIONARY JUNCTURES IN INVERTEBRATE NERVOUS SYSTEMS

Complex organ systems do not spontaneously generate; through evolution, the groundwork for their morphology is laid and requires higher level structures to evolve over time, which is not a guarantee. By understanding morphology in simpler models, we can apply the

information to more complex systems. This is why it is so vital to thoroughly investigate model nervous systems, especially in light of the increasing elderly population and prevalence of neurodegenerative diseases. There are an innumerable amount of *C. elegans* strains painstakingly created that are used to demonstrate the effects of certain neurodegenerative diseases, novel drugs, or genetic knockouts. Interpreting the results of these disease studies and genetic screenings hinges on scientists' knowledge of morphological and functional differences between nematodes and humans.

We need to shift our thinking regarding what constitutes a nervous system or the origin of one. For instance, Porifera lacks neurons but they have homologs to genes that are required for neurons (Smith et al., 2014). This indicates that the role of a neuron can be carried out by non-neuronal cells and, more importantly, that the underpinnings of a nervous system took time to develop the complex organization and diversity we are familiar with today. While there is no adamant scientific debate that sponges have nervous systems, it is more difficult to dismiss the fact that they possess rudimentary precursors to the bilaterian central nervous system (CNS).

While a long-lasting source of argument, evidence has mounted in support for a shared origin of the central nervous system (Lowe et al., 2003; Nomaksteinsky et al., 2009; Pani et al., 2012; Telford, 2007). Bilaterian nervous systems have similarities between their two superphyla, protostomes and deuterostomes. The urbilaterian is essentially a Last Universal Common Ancestor (LUCA) equivalent for bilaterians, a Last Universal Bilaterian Ancestor, if you will. Some evidence is controversial in support or against of an urbilaterian having a nervous system that could then be further adapted by protostomes and deuterostomes. Examining supporting evidence, a tripartite bilaterian brain is corroborated by gene expression patterns in specific nervous tissues of the *D. melanogaster* brain bearing homology to vertebrate brain tissues (Hirth

et al., 2003). In addition, genes responsible for the patterning that maintains the dorso/ventral orientation are conserved within bilaterians, but missing within close relatives such as the sister clade cnidarians (Mieko Mizutani & Bier, 2008). This indicates that they were exclusively adapted to their role within bilaterians themselves. Beyond *D. melanogaster*, annelids feature mushroom bodies in their brains which also show gene homology to the vertebrate brain, specifically localized to the pallium (Denes et al., 2007; Tomer, Denes, Tessmar-Raible, & Arendt, 2010). In the spirit of maximum parsimony, it would be unlikely for the similarity observed between protostome and deuterostome central nervous systems to be entirely due to analogous structures that arose through convergent evolution.

If we accept the fact that there is a common ancestry to the CNS, then we also have to acknowledge that at some point the long-debated dorso/ventral (D/V) inversion must have occurred (Dohrn, 1875; Gerhart, 2000; Nomaksteinsky et al., 2009). The D/V inversion is the phenomenon whereby, at some point, the ventral nerve cord in protostomes must have migrated to the dorsal side in deuterostomes or vice versa. Currently, there are three hypotheses regarding the mechanics of the inversion. The oldest, which has been rejected and renewed since its inception, postulates that the shared ancestor of protostomes and deuterostomes was annelid-like; from this, protostomes maintained the structure we are familiar with but a chordate precursor eventually underwent the inversion (Dohrn, 1875). The second hypothesis states that an ancient deuterostome similar to a hemichordate possessed both nerve cords and a nerve net (Gerhart, 2000; Lowe et al., 2003; Pani et al., 2012). They argue that the nerve net was adapted by chordates into a CNS with a ventral cord. An offshoot of this argument is that the CNS evolved directly from the ancient deuterostome's dorsal nerve cord itself (Nomaksteinsky et al., 2009). The final hypothesis—which isn't widely discussed—suggests that the urbilaterian had more

than one nerve cord, which eventually resulted in the protostome and deuterostome nervous systems arrangements (Gerhart, 2000). Either way, no matter which hypothesis holds true, they all represent the rigid belief that there is a common origin of both the invertebrate and vertebrate nervous systems. In this way, their similarities allow them to be compared and allows for invertebrate models to be simpler mirrors to understand the vertebrate nervous system.

It is also important to acknowledge that evolution is not strictly linear. While it may seem appropriate and logical that complexity is an always increasing phenomenon, evolution is not a sentient mechanism working towards a specific goal. There are instances of reversions to simplicity in the nervous system. A controversial example of this is seen in comb jellies, which possess neurons. Their genome indicated that they are ancestral to sponges, which lays out two scenarios: neurons do not have a shared origin or sponges could have lost neurons (Moroz et al., 2014). Some scientists argue the fact that comb jellies are not reactive to most standard neurotransmitters as proof that there must have been two evolutionary neuronal events. On the other hand, opponents maintain that the comb jelly genome is inaccurate or that accelerated evolution accounts for the differences seen in neurotransmitters (Gavilán, Perea-Atienza, & Martínez, 2016; Jékely, Paps, & Nielsen, 2015). Sponges do have neuronal homologs so neither argument is farfetched. They both show that complexity is a relative concept when you are dealing with all these lineages off shooting from an event; either neurons evolved once and eventually comb jellies altered them significantly from bilaterians or neurons were lost in a close relative. Maintaining an open mind when evaluating evolutionary events is crucial because supporting end-goal complexity in itself is a bias.

1.5 THE NEMATODE NERVOUS SYSTEM AND THE VENTRAL NERVE CORD

Nematode nervous systems are among the most extensively studied of any metazoan. The complete *C. elegans* connectome—a map of the entire nervous system and its connections—has been charted to the cellular level (White et al., 1986). Outside of nematodes, the only other animals that can boast such a feat are *Ciona intestinalis* (Ryan, Lu, & Meinertzhagen, 2016) and *Drosophila melanogaster* (Zheng et al., 2018).

As this review acts as an argument for nematode nervous systems as models for more complex organisms, it is necessary to discuss the differences between nematode and human nervous systems. As mentioned previously, they have a simplified nervous system with fewer neurons compared to vertebrates (White et al., 1986). One of the most stark examples of this is the lack of myelination on their axons; given the short distance nematode axons tend to travel, myelin is likely not necessary for quick impulse generation like in larger vertebrates (Oikonomou & Shaham, 2011). It is unclear how larger nematodes, such as *A. suum*, deal with current loss through their longer processes. Contrary to the reliance on action potentials in vertebrates, nematodes tend towards non-spiking graded potentials (Lockery & Goodman, 2009). Another major difference is that nematode neurons do not require glial trophic support to survive, at least in determinate *C. elegans* (Oikonomou & Shaham, 2011). This is possible due to the fact that determinate lineages do not produce excess neurons when developing like many other animals and therefore do not require glial signals to prune cells; however, it has not been investigated whether this property extends beyond *C. elegans*. Examining proteins, nematodes are notable for having calcium gated ion channels instead of sodium, more diverse ion channel families in their neuronal genome, as well as more GPCRs documented in *C. elegans* (Schafer, 2016). While humans have over 100 known neuropeptides (Russo, 2017), nematodes have more

than 250; likewise, humans have only 5 neprilysin proteases responsible for degrading neuropeptides while *C. elegans* has 27 (Schafer, 2016). These examples demonstrate that while nematodes may be different and simpler in some ways, nervous systems are still incredibly complex creations that are flexible through evolution.

In actuality, there are six nerve cords—ventral, dorsal, and four sublateral cords—but the ventral nerve cord contains the majority of nerve cord cells and is responsible for coordination with the other nerve cords (Schafer, 2016). The cords themselves are not in direct contact with local muscles, instead they are separated through a layer of basal lamina. All the cords are made up of dense tracks of longitudinal neuron processes that lack myelination (Oikonomou & Shaham, 2011). Commissures are circumferential highways of processes responsible for connecting the cords, in particular connecting the ventral cord to others. The ventral cord originates at the nerve ring's retrovesicular ganglion and extends to the preanal ganglion (White, Southgate, Thomson, & Brenner, 1976). It is largely made of motor neuron axons and interneurons, allowing it to direct movement. The dorsal cord lacks the interneuron component.

Nematodes have been a model for neuroanatomy for 200 years (Otto, 1816), with *A. suum* and eventually *C. elegans* at center stage. Initially, in the early 1900s, evaluations of the nervous systems of a few nematodes—all of which were in clades 8 and 9, save for one clade 2 representative led Chitwood & Chitwood to believe in a simplicity and conservation of the nematode nervous system (1950). Later on, *C. elegans* was determined to have 302 neurons in an adult hermaphrodite (White et al., 1986), 57 of which are ventral nerve cord neurons (Sulston, 1976; White et al., 1976). When the retrovesicular ganglion and preanal ganglion are included, there are a total of 75 motor neurons within the VNC. *Ascaris suum* is a large intestinal parasite of humans and has tens of thousands of cells more than *C. elegans* (Schafer, 2016). In addition to

their size, they also have vastly different lifestyles, *C. elegans* being bacterial-feeding and *A. suum* being parasitic. In spite of their differences, *A. suum* has 298 neurons that control its body that can be up to 40,000 times the length of *C. elegans* and *A. suum* has a diminutive 55 VNC neurons and 72 motor neurons in the VNC (Schafer, 2016; Stretton et al., 1978; Stretton & Maule, 2013). This striking resemblance of the ventral nerve cord acted as a microcosm for general nervous system complexity, so it was assumed from this data, and the earlier nervous system descriptions of mostly derived nematodes, that the nematode nervous system is highly conserved. This concept was perpetuated without further research beyond mainly clades 8 and 9 until recently (Angstadt, Donmoyer, & Stretton, 1989; Burr & Robinson, 2004; Hallem & Sternberg, 2008; Kimber & Fleming, 2005; Martin, Purcell, Robertson, & Valkanov, 2002; Srinivasan, Durak, & Sternberg, 2008).

Limited research has looked at nematode ventral nerve cord counts, and fewer still studies have completed comprehensive examinations. Malakhov (1994) argued that Enoplida, which falls in clade 1, features the most complex of the nematode nervous systems, with a few thousand neurons overall in observed specimens. He found that in small Rhabditids, their neurons can amount to forty percent of all cells and sizeable parasites such as *A. suum* likely evolved from these small Rhabditids with a preservation of nerve cell number. This ancestry would certainly explain why *A. suum* has a starkly similar nervous system to *C. elegans*. Sulston & Horvitz (1977) found that three species in clade 10 exhibit similar ventral nerve cord development and number to *C. elegans* (clade 9), however one species had an increased number from the rest. In addition, a clade 2 nematode had over one thousand neurons near to its adulthood. The only other paper to briefly mention this topic discusses a clade 2 nematode and how they estimated its VNC neurons must exceed a thousand based on the number of

circumferential commissures (Gans & Burr, 1994). Overall, these papers tend to mention instances of deviation from the *C. elegans* archetype nervous system, but these findings are always brief remarks. These examples all have observations from clade 1 and 2 in common, and that their neuron numbers are far in excess of anything else described from the derived clades. This brings into question two issues. First, is there a marked difference between the nervous systems of basal and derived clades that is represented by VNC neurons? Second, as the only basal evidence is from Enoplean clades (1 and 2), are the basal Chromadorean clades (3 through 6) in agreement with these high counts or is this strictly a nervous system difference between the two nematode classes?

The only paper to do an in-depth comparative analysis on ventral nerve cord enumeration is Han, Boas, & Schroeder (2016). They found that even within clades there were gains and losses of ventral nerve cord neurons, which could indicate it is a much more common event than given credit for. At the genus level, however, ventral nerve counts were observed to be stable. Despite the established knowledge that there is a difference in VNC counts between *C. elegans* hermaphrodites and males (White et al., 1976), there was no significant difference found between sexes in this study. They also evaluated food habit and found no connection between it and ventral nerve cord counts. Intriguingly, they choose to use dye filling, a technique that allows dyes to infiltrate the anterior sensory neurons and be visualized and enumerated in *C. elegans* (Tong & Bürglin, 2010). While the cause of this is unknown, it can be effective for comparing sensory structures. They found that life stage influenced the dye pattern, revealing that sensory structures are altered and sensitive to development. Most notably, they found differences in staining patterns between and even within clades, indicating these patterns are not phylogenetically robust. Given the unique sensory structures of some of the basal nematodes, it

is uncertain if this method would be effective on them. One difficulty of working with nematodes lies in their many life stages; this paper had to use different stages depending on the species. At the time of this research, there was no reason to suspect that the ventral nerve cord development would differ from *C. elegans*, whose neurons are in place by J2 (Sulston, 1976; Sulston & Horvitz, 1977). The variable developmental timeline of the ventral nerve cord is something this paper brought to light. An additional area for improvement would certainly be addressing clades 1 through 7 and determining whether they characteristically possess much more intricate nervous systems than clades 8 through 12. If this is true, it would indicate a simplification of the nematode nervous system from the nematode ancestor.

While the question of final complements of VNC neurons is illuminating, the concept of differences in rate and timing of their development is also a consideration. *C. elegans* possesses 302 neurons in an adult hermaphrodite. Their generation occurs in two phases. Most are created during embryogenesis with the rest developing at the end of J1. In terms of the VNC itself, upon hatch the juveniles have 15 VNC neurons. The full 57 cells is reached by J2, before which precursor cells migrate and divide in the VNC (Sulston, 1976). Han et al. decided to evaluate differences between nematodes that hatch as J1—*C. elegans*—and nematodes that hatch as J2 (2016). Both *Pristionchus pacificus* (Clade 9) and *Aphelenchus avenae* (Clade 12) hatched as J2s without a full complement of neurons. A third species, *Pratylenchus penetrans* (Clade 12), did hatch as J2s with a full complement of neurons. Not only did the two Clade 9 species reach VNC maturity at different times, but so too did the two Clade 12 species, indicating that neuronal heterochrony is largely nematode specific and prone to adaptation.

To examine precisely when development occurred in these J2 hatch aberrations, Han et al. followed *A. avenae* post-hatch and counted the VNC neurons (2016). The counts remained

largely consistent through J2 until a rapid addition of neurons preceding the J3 molt that ended in a full complement entering J3. Unfortunately, save for *C. elegans*, there is no other VNC count data to compare this to. All that can be established is that ventral nerve cord development, and possibly nervous system development itself, is not as highly conserved as others might suggest it to be.

1.6 NEMATICIDES AND THEIR CONNECTION TO THE NERVOUS SYSTEM

As a Crop Sciences graduate student, I would be remiss if I did not discuss the agricultural ramifications of the nervous system research application. Historically, the nematode nervous system was a substantial target for discovered nematicides. Nematodes are estimated to be responsible for 12.3% of global yield losses (S. Singh, Singh, & Singh, 2015). Three crops—corn, soybeans, and sugarcane—total 90% of all nematicide applications (Ferraz & Brown, 2016). Despite these intriguing numbers, the nematicide market is extremely limited compared to other pests (Mordor Intelligence, 2020). Pesticides are becoming more costly to make with increasingly strict regulation and registration procedures (Desaeger, Wram, & Zasada, 2020). Considering there is no guarantee that a successful nematicide will be created following years of development and adding in the relatively small profit to be made compared to other pesticides, it comes as no surprise that industry shied away from nematicide generation for decades until recently.

The first large-scale use of nematicides followed World War I, when surplus chloropicrin (tear gas) was repurposed as an agricultural fumigant (Ravichandra, 2018). It resulted in phenomenal yields via soil sterilization that drew interest into the field of nematology, despite its devastating environmental and human effects. Following Cold War research into nerve agents,

derivatives such as organophosphates and carbamates began taking stage as nematicides (Oka, 2020). In terms of the nervous system, the organophosphates and carbamates are of particular importance. Their mode of action is as acetylcholinesterase inhibitors, preventing the breakdown of acetylcholine and leading to paralysis and potentially death (Opperman & Chang, 1990). At field concentrations, the levels are often low enough that an endless paralysis results until the nematicide dissipates and the nematode can resume normal function; nematicides that induce this condition are termed nematostatic (McGarvey, Potter, & Chiba, 1984).

Broad-spectrum nematicides have fallen out of favor; industry is moving towards target specificity, which is possible through modern screening methods (Oka, 2020). *Meloidogyne* spp. are unanimously considered the most devastating of plant-parasitic nematodes. It makes sense then that in screening it is used frequently as a model, often alongside *C. elegans* (Desaeger et al., 2020). There has been thorough documentation that not all nematodes are affected by nematicides to the same degree, with sedentary parasites like *Meloidogyne* spp. being most susceptible, other plant-parasites being affected, and free-living nematodes like *C. elegans* being tolerant (Gourd, Schmitt, & Barker, 1993; Wada & Toyota, 2008). This spectrum is overwhelmingly beneficial to understand, especially in light of the fact that fumigants were non-discriminatory.

The new generation nematicides are called the 3F nematicides (fluensulfone, fluopyram, fluazaindolizine) due to their trifluoro group (Desaeger et al., 2020). These are the most likely nematicides that will dominate the market in the coming years. They are much safer for the environment and applicators and their current known modes of actions do not exclusively involve the nervous system.

Of course, as with any attempt at chemical control of an organism, resistance is always a consideration. Oddly, there has been no documentation of nematicide resistance in plant-parasitic nematodes, which is even more of a curiosity when you consider that there has been documented anthelminthic resistance within animal-parasitic nematodes (Moens & Hendrickx, 1998; Wolstenholme, Fairweather, Prichard, Von Samson-Himmelstjerna, & Sangster, 2004). The exception is fluopyram in particular, resistance has been floated as a concern as a result of its long soil half-life and mode-of-action against fungi (Desaeger et al., 2020). These factors, in contrast to previous nematicides, could result in selection pressure towards resistance. Enhanced biodegradation has been flagged as a concern, in which nematicides lose effectiveness with additional applications due to adapted microbial activity (J. P. E. Anderson, Nevermann, & Haidt, 1998; Smelt, Van De Peppel-Groen, Van Der Pas, & Dijksterhuis, 1996). This effect has even been noted if different nematicides of the same chemical group are applied (Karpouzas, Fotopoulou, Menkissoglu-Spiroudi, & Singh, 2005; B. K. Singh, Walker, & Wright, 2005). While not mentioned in the literature, it seems concerning that the 3F nematicides all possess a trifluoromethyl group. If microorganisms have a preference for metabolizing this group, it could render the new-generation of nematicides ineffective.

1.7 OVERVIEW

Previous work on the ventral nerve cord established that there is conservation of the nematode nervous system throughout the phylum. While not overturned, smatterings of evidence suggested that not all nematodes fell into the documented pattern seen in other worms. Further thorough investigation revealed that there are measurable differences within the derived clades, all the way down to genus level.

This thesis, while on its path to test the validity of a simplification of the nematode ventral nerve cord, also addresses the lack of concrete information associated with VNC data. It attempts to add broader basal nerve cord information to the greater body of knowledge as well as to make clade and subphylum comparisons that have not truly been made before. Prior to this investigation, only derived data had been thoroughly presented, with some tangential observation peppered in the literature. Rarely were any conclusions made of this data in terms of the nervous system, save for assumptions of conservation based on the previous information. Examining the established research, I hypothesize that there is a simplification of the VNC from the basal to the derived clades, indicating that there are significant differences among the VNC neuron numbers, upending the concept of nervous system conservation; furthermore, there may well be measured differences in neuron numbers between the basal clades and derived clades.

CHAPTER 2: A PHYLOGENETIC EVALUATION OF THE NEMATODE VENTRAL NERVE CORD

2.1 ABSTRACT

Comparative analyses of the nematode ventral nerve cord have been limited to essentially only the derived clades. These investigations found that in spite of the long-running belief that the nematode nervous system is highly conserved, there was variety within the derived clade ventral nerve cords. This study endeavors to expand upon the derived clade data by including the basal clades and drawing comparisons between not only the two groupings but also between classes Enoplea and Chromadorea. In addition, I investigate *Mononchus laminatus* ventral nerve cord development to compare it against previous reports. The findings indicate that there was a significant depression of ventral nerve cord neurons from Enoplea to Chromadorea as well as from basal to derived clades. *M. laminatus* also features rapid neurogenesis during ventral nerve cord development, which is consistent with derived species experiencing a spike in neurons prior to a molt, though the timing varied between species. Together, these results represent that the nematode nervous system is not as highly conserved as previously argued; in fact, neuron numbers and neuron developmental timing are subject to change.

2.2 INTRODUCTION

While there is a staggering understanding of the *C. elegans* nervous system, nematology still has limited knowledge of nervous system evolution in nematodes. Nematoda itself is divided into twelve clades, with Clades 1 through 6 designated here as basal and Clades 7 through 12 considered derived (Holterman et al., 2006; van Megen et al., 2009). Furthermore, there are two nematode classes: Enoplea, which contains Clades 1 and 2, and Chromadorea, which contains

Clades 3 through 12. There are multiple pieces of evidence that support the true nematode ancestor being most similar to an Enoplean.

C. elegans has been documented to have 57 VNC neurons while *A. suum* has 55 (Stretton et al., 1978; Sulston, 1976; White et al., 1976). This contributed to the belief that the nematode nervous system is highly conserved. Previous observations have revealed that a Clade 1 nematode has thousands of VNC neurons (Malakhov, 1994), and two different genera in Clade 2 have over a thousand neurons in their VNC (Gans & Burr, 1994; Sulston & Horvitz, 1977). Beyond these observations, only one paper has done a comprehensive analysis of ventral nerve cord neuron enumeration (Han et al., 2016). They found that within the derived clades there was variation in VNC neuron numbers, all the way down to the genus level. This conclusion is in direct opposition to the upheld belief of nervous system conservation. In addition, they monitored VNC neuron development and found neuronal heterochrony between multiple species as well as corroborated the rapid neurogenesis seen in *C. elegans* within *A. avenae* (Sulston, 1976).

This chapter addresses the gap of basal clade VNC enumeration data and draws comparisons between the clades and classes. Moreover, the VNC development of *M. laminatus* is analyzed to assess if the differences seen within derived nematodes hold up in basal and indeterminate members. I hypothesize that there is a simplification of the nematode ventral nerve cord from the basal to the derived clades as well as from Enoplea to Chromadorea that will further upend the notion of nematode nervous system conservation. Additionally, I postulate that there may be concerted differences in VNC development of a basal nematode as compared to a derived model.

2.3 MATERIALS AND METHODS

Sample Collection

Shallow aquatic and intermediate terrestrial sediment samples were collected from aquatic or terrestrial sources into jars stored at 4°C until further processing, preferably immediate. For aquatic samples, a liquid layer was always included to simulate the natural environment up to the time of processing.

Sample Location	*Locality*	*GPS Coordinates*	*Biome*	*Time of Sampling*
Dana Colbert Park Pond	Savoy, IL	40°03'02.1"N; 88°14'58.9"W	Freshwater	12/2019; 6/2020
Prairie Meadows Subdivision Pond	Savoy, IL	40°02'58.5"N; 88°14'47.4"W	Freshwater	6/2020
Lake Park Residential Pond	Savoy, IL	40°03'53.2"N; 88°14'21.1"W	Freshwater	6/2020
Moorman Sewage Retention Pond, Large (Lake Nalbandov)	Urbana, IL	40°05'22.2"N; 88°14'08.4"W	Freshwater	6/2020
Moorman Sewage Retention Pond, Small (Lake Nalbandov)	Urbana, IL	40°05'16.5"N; 88°14'11.3"W	Freshwater	6/2020
UIUC Japan House Main Pond	Urbana, IL	40°05'34.0"N; 88°12'59.2"W	Freshwater	6/2020; 10/2020
UIUC Japan House Minor Pond	Urbana, IL	40°05'37.4"N; 88°12'55.8"W	Freshwater	10/2020
Boneyard Creek	Champaign, IL	40°06'40.4"N; 88°13'40.4"W	Freshwater	12/2019
Private Residence Pond	Urbana, IL	40°05'49.4"N; 88°12'12.4"W	Freshwater	11/2019; 6/2020
Salt Fork River, Mulberry Tree	Homer, IL	40°05'39.1"N; 87°99'65.4"W	Terrestrial	10/2019
Homer Lake Forest Preserve Collins Pond	Homer, IL	40°05'39.1"N; 87°99'65.4"W	Freshwater	10/2019
Homer Lake Forest Preserve, White Oak	Homer, IL	40°05'39.1"N; 87°99'65.4"W	Terrestrial	6/2020
*Indian Grass (*Sorghastrum nutans*) Prairie*	Homer, IL	40°05'39.1"N; 87°99'65.4"W	Terrestrial	6/2020
Northwestern Illinois Agricultural Research and Demonstration Center	Monmouth, IL	40°93'64.6"N; 90°72'06.5"W	Terrestrial	2020
Agricultural Field	Belleville, IL	38°31'59.5"N; 89°53'40.2"W	Terrestrial	2020
Carpinteria Salt Marsh, Muddy Site	El Estero, CA	34°24'01.7"N; 119°32'21.6"W	Marine	9/2019
Carpinteria Salt Marsh, Sandy Site	El Estero, CA	34°23'55.6"N; 119°32'19.3"W	Marine	9/2019
Carpinteria State Beach, Intertidal Site 1	Carpinteria, CA	34°23'35.4"N; 119°31'30.4"W	Marine	9/2019
Carpinteria State Beach, Intertidal Site 2	Carpinteria, CA	34°23'35.4"N; 119°31'30.4"W	Marine	9/2019

Table 2.1. A list of sampling locations for the nematodes used for DAPI staining and immunohistochemistry within this thesis.

Most samples were collected local to Champaign-Urbana and surrounding areas, with exceptions in Illinois being Monmouth and Belleville (Table 2.1). In order to obtain some marine samples to capture the more exclusive clades, sediment samples were taken from the Carpinteria State Beach and Salt Marsh in California, courtesy of Dr. Tiago José Pereira and Dr. Holly Bik, formerly of the University of California, Riverside. A small subset of terrestrial samples had

previously been collected and DAPI stained by Jaeyeong Han, a fellow doctoral candidate of Dr. Nathan Schroeder.

C. elegans strain N2 were grown standardly on NGM with OP50 at RT (Brenner, 1974).

Sample Extraction

Sieve Extraction

Extraction was accomplished through the Baermann funnel method with sugar centrifugation (MacGuidwin & Bender, 2012). Nematodes were separated from sediment using 250 and 38 µm pore size sieves. Centrifugation at 3000 rpm for 3 minutes preceded sugar centrifugation with 45% sucrose. The supernatant was applied to a 25µm pore size sieve for rinsing and collection.

Since the marine samples were sensitive to tap water, rinses were carried out with Instant Ocean®—prepared as per manufacturer's instructions—to prevent osmotic imbalance.

Baermann Funnel Extraction

The Baermann funnel method was utilized to target nematodes not freely living in the sediment (i.e. within roots) or perhaps too large to transfer sieves. Sediment left behind on the 250µm pore size sieve was transferred to a Baermann funnel apparatus (MacGuidwin & Bender, 2012). After 48 hours, a second clamp was added higher on the tubing. The lower clamp was removed and the sample was drained for collection.

As with the sieve extraction steps, marine samples were steeped in Instant Ocean® mixture to ensure cellular preservation.

Sample Processing

Fixation

The 15 mL Falcon tubes were centrifuged at 4000 rpm for 4 minutes. The liquid was gently pipetted off until approximately 1 mL was left. For aquatic nematodes, 50 µl of .1M Levamisole was added to relax them since their natural positions are often coils which are not conducive to ventral nerve cord visualizing. They were left with the sedative for 30 minutes then assessed for movement. Once adequately still, all liquid except a thin layer was pipetted off and 1 mL of DESS (dimethyl sulfoxide, disodium EDTA, and saturated NaCl) was added (Yoder et al., 2006). For terrestrial samples, no sedative was added and all liquid except a thin layer was immediately pipetted off so that 1 mL of saturated DESS could be added. Samples were stored at 2°C.

Belleville and Monmouth samples utilized the fixation method described under Mononchidae Developmental Analysis.

DAPI Staining

Depending on the amount of sediment in each sample, 2-8 µg/mL DAPI was added. The sample was kept in the dark at RT overnight then returned to 2°C until imaging.

Fluorescent Microscopy

Slide Preparation

To prepare an agar pad, 0.1g of agarose was added to 5 mL of an appropriate buffer. For *C. elegans*, this was M9 while for environmental worms this was typically .1M PBS. It was microwaved on low power until molten then quickly applied and flattened on a slide. Nematodes were added to the pad and a small amount of appropriate buffer was added prior to placing the cover slip. After imaging, the prepared slides were disposed of.

Imaging

Nematodes were imaged using a Zeiss® Axio Imager M2 fluorescent microscope equipped with an Axiocam 506 mono camera and an X-Cite® Series 120Q fluorescent lamp on Zen 2012 software.

Samples were screened looking for adults with consistent staining. Z-stacks were taken with slices at .35-.5 µm intervals, collecting both the DIC and DAPI channels for future reference. The entire worm was imaged, with sufficient overlap for later stitching.

Identification

Morphological Identification

Given the three distinct biomes that specimens were drawn from, there were also different methods of identification. For terrestrial nematodes, the following sources were used as main reference material: Bongers (1988); Mai, Mullin, Lyon, & Loeffler, (1996); and Peña-Santiago (2013). Freshwater identification relied heavily on Bongers (1988) and Eyualem-Abebe, Andrássy, & Traunspurger (2006). For the marine nematodes, there were no central resources to rely on but instead a patchwork of materials. A general reference overall for the different orders and genus descriptions was found within Schmidt-Rhaesa (2014). All sources referenced are included in the table below along with their taxonomic specialty (Table 2.2).

Molecular Identification

A range of aquatic nematodes, with *C. elegans* and Tobrilidae acting as controls, were evaluated through 18S SSU rDNA molecular barcoding. Nematodes were applied to lysis buffer, as per Williams, Schrank, Huynh, Shownkeen, & Waterston (1992). A thermocycler program removed their cuticle (60°C for 4 hours, 95°C for 15 minutes, then 12°C). A master mix of 12.5 µL GoTaq Green Master Mix (2x), .625µL of 100µM primers, 3 µL nematode lysate, and 8.25

µL water was added to PCR tubes and run. Primers from both Foucher & Wilson (2002) and Holterman et al. (2006) were tested. Nem1/nem2 primers were chosen with the following PCR profile: 94°C for 5 min, 35x (94 °C, 30 s; 50 °C, 30 s; 72 °C, 90 s), 72°C for 5 min. The PCR product was purified then gel extracted. Three PCR reactions for a single worm would them be run using the gel purified product. The PCR product of the three reactions was pooled and gel purified again prior to being sent to the UIUC Core Sequencing Facility for sequencing.

Reference	***Biome***	***Reference Specialty***	***Additional Notes***
Bongers (1988)	Terrestrial and Freshwater	Nematodes of the Netherlands	Primary reference
Mai et al. (1996)	Terrestrial	Plant-parasitic nematodes	Primary reference
Peña-Santiago (2013)	Terrestrial	Dorylaimida	Primary reference
Goodey & Goodey (1963)	Terrestrial	Soil and freshwater nematodes	Supplementary reference
Jairajpuri & Ahmad (1992)	Terrestrial	Dorylaimida	Supplementary reference
Qing & Bert (2019)	Terrestrial	Tylenchidae	Supplementary reference
Eyualem-Abebe et al. (2006)	Freshwater and Marine	Freshwater nematodes	Primary reference
Ahmad & Jairajpuri (2010)	Freshwater	Mononchida	Supplementary reference
Holovachov (2016)	Freshwater and Marine	Plectina	Supplementary reference
Tarjan et al. (1977)	Freshwater	Freshwater nematodes	Supplementary reference
Hope & Tchesunov (1999)	Marine	Camacolaiminae	Supplementary reference
Keppner & Tarjan (1989)	Marine	Enoplida	Supplementary reference
Keppner & Tarjan (1991)	Marine	Araeolaimida	Supplementary reference
Keppner & Tarjan (1994)	Marine	Microlaimoidea and Desmodoroidea	Supplementary reference
Tarjan & Keppner (1999)	Marine	Chromadoroides (exclusive of Chromadoridae)	Supplementary reference
Platt & Warwick (1983)	Marine	Marine nematodes	Primary reference
Schmidt-Rhaesa,(2014)	Terrestrial, Freshwater, and Marine	All orders	Universal reference

Table 2.2. A collection of pertinent references used for morphological identification of nematodes in different biomes.

Ventral Nerve Cord Enumeration

Merging

Micrographs were opened in Fiji ImageJ and DAPI channels were isolated and cropped (Rueden et al., 2017; Schindelin et al., 2012). Images were stitched together using linear blending and 5 peaks (Preibisch, Saalfeld, & Tomancak, 2009). The stitched image was assessed for continuity. For images that were more difficult to stitch, the settings were changed to up to 50 peaks, subpixel accuracy, and zero values were ignored. The entire nematode was stitched from head to tail.

Counting

Using the stitched DAPI image, the VNC neurons could then be counted. Cells were considered VNC neurons based on morphology, size, position, and relative intensity of stain, often in accordance with previous work (Han et al., 2016; Sulston, 1976; Sulston & Horvitz, 1977; White et al., 1976). VNC neurons tend to be small, punctate, aligned, and brighter than surrounding cells. Counts began at the preanal ganglion immediately anterior to the anus and proceeded towards the retrovesicular ganglion—among the concentrated neurons behind the nerve ring—using the multipoint tool. Figure 2.1 provides insight into VNC length and VNC neuron distinction.

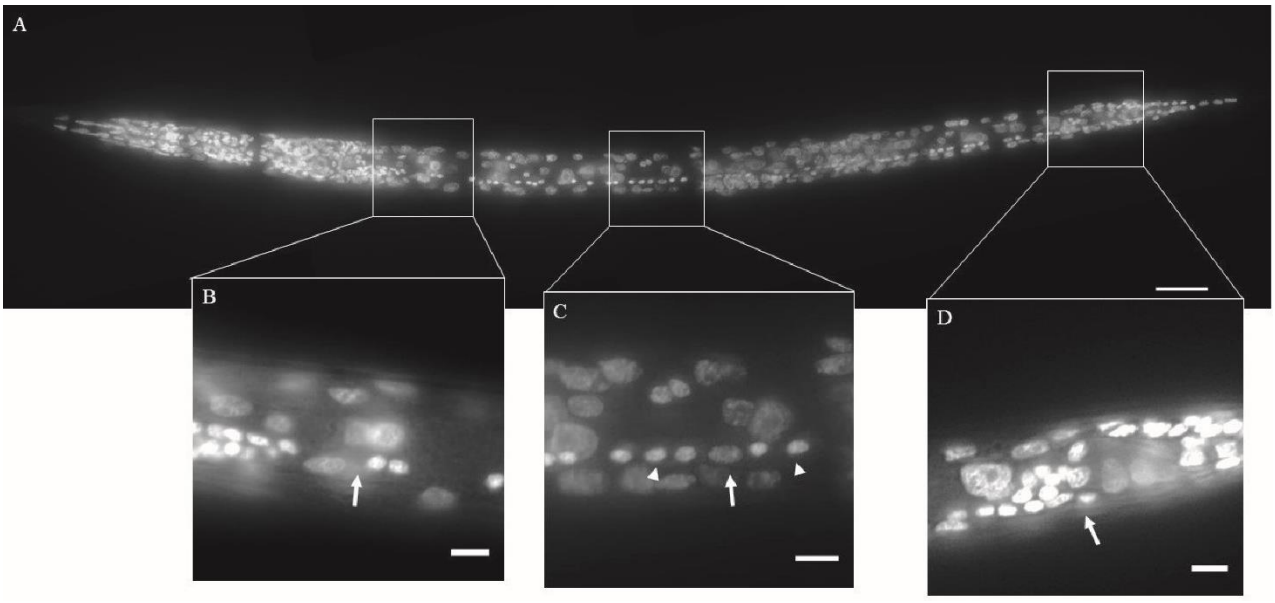

Figure 2.1. A micrograph of a DAPI-stained *C. elegans* dauer taken with permission from Han et al. (2016). Neurons can be seen proceeding longitudinally through the worm in the VNC. The VNC begins posterior to the nerve ring where the intense localization of cells—the retrovesicular ganglion—clears up. Just prior to the anus, the VNC visibly terminates at a cluster of neurons that is the preanal ganglion.

Density

Stitched images were scaled using 4.3712 pixels/μm. A line was drawn down the center of the worm from the nerve ring to the retrovesicular ganglion to determine the VNC length. To calculate density, the VNC neuron count was multiplied by 100 then divided by the VNC length to obtain a neurons/100 μm measurement.

$$Density\ (neurons/100\mu m) = \frac{(VNC\ Neurons) \times 100}{VNC\ Length}$$

Data Analysis

VNC Neuron Counts and Densities

Data was inputted into GraphPad Prism 9. To test for normality, Anderson-Darling, Shaprio-Wilk and D'Agostino & Pearson tests were compared. To test for distribution of variances, the Kruskal-Wallis test was used. Dunn's multiple comparisons test was run to both

compare individual families to other families as well as to compare entire clades against each other. In both cases, individual species from previous published data were treated as their own individual family or clade to compare novel data against (Han et al., 2016).[1] The previous report of 55 VNC neurons for *A. suum* was also used to draw comparisons against (Stretton et al., 1978). Data was also combined into basal (1-6) and derived (8-12) clades in order to evaluate direct group differences. In addition, the two classes Enoplea (Clades 1-2) and Chromadorea (3-12) were analyzed against each other. The non-parametric nature of the chosen tests was due to the lack of normality, unequal variances, and low sample sizes in the data that transformation could not compensate for.

When comparing clades, across all three normality tests sample size was an issue for Clade 8, which only had the single accepted count from *A. suum*. The only other clade that was unable to be assessed due to sample size was Clade 4, which failed both the Anderson-Darling and D'Agostino & Pearson tests. The clades that passed normality using all tests were Clades 5 and 11. Clades 3, 6, 9, and 12 failed to be normal across all three tests. Clades 1, 2, and 10 passed normality under D'Agostino & Pearson but failed under the other two tests. While Clade 4 had too small a sample size for two tests, it passed normality using the Shapiro-Wilk test. The Kruskal-Wallis test determined the distributions differed ($p < .0001$).

When comparing families, normality was lacking at the family level due to the sample sizes. The families that failed all three normality tests due to sample size are as follows: Diphtherophoridae, Prismatolaimidae, Aporcelaimidae, Ethmolaimidae, Epsilonematidae, Metateratocephalidae, Plectidae, Ascarididae, and Rhabditidae. The families that passed all three

[1] Genera were adapted for the purposes of this study to their family names: *Ascaris* to Ascarididae, *Caenorhabditis* to Rhabditidae, *Heterorhabditis* to Heterorhabditidae, *Pristionchus* to Diplogastridae, *Steinernema* to Steinernematidae, *Acrobeles* to Cephalobidae, *Aphelenchus* to Aphelenchidae, *Heterodera* to Heteroderidae, *Meloidogyne* to Meloidogynidae, and *Pratylenchus* to Pratylenchidae.

tests included Aphanolaimidae, Diplogastridae, Cephalobidae, Aphelenchidae, Heteroderidae, and Meloidogynidae. Seven families—Tobrilidae, Longidoridae, Mylonchulidae, Desmodoridae, Monhysteridae, Xyalidae, and Camacolaimidae—had too small of an n value for the D'Agostino & Pearson and Anderson-Darling tests but were calculated to be normal through Shapiro-Wilk. Mononchidae and Achromadoridae failed to be normal in all three tests. Finally, Heterorhabditidae, Steinernematidae, and Pratylenchidae all failed the Anderson-Darling and Shapiro-Wilk tests but passed the D'Agostino & Pearson test. The family distributions were also determined to be different according to the Kruskal-Wallis test ($p < .0001$).

Focusing on the neuron density data, the D'Agostino & Pearson and the Anderson-Darling normality tests were inapplicable to a majority of families due to the small n values. The exceptions to this were Mononchidae, Achromadoridae, Aphanolaimidae, and *C. elegans*. Aphanolaimidae and *C. elegans* both passed the two normality tests. Mononchidae and Achromadoridae both only passed only the Anderson-Darling test. The Shapiro-Wilk normality test was much more forgiving; only 7 families had n values too small to evaluate (Diphtherophoridae, Prismatolaimidae, Aporcelaimidae, Ethmolaimidae, Epsilonematidae, Metateratocephalidae, and Plectidae). Of the remaining families, Tobrilidae, Longidoridae, Mylonchulidae, Achromadoridae, Desmodoridae, Xyalidae, Aphanolaimidae, Camacolaimidae, and *C. elegans* all passed the Shapiro-Wilk test. In addition, a Kruskal-Wallis analysis found that the distributions differed between families ($p < .0001$).

Mononchidae Developmental Analysis

Culturing

Mononchid-type nematodes were isolated and cultured using a Soil Extract Agar (SEA) method adapted from Salinas & Kotcon (2005) and Springett (1964). Mononchids were applied

to a 1% SEA plate with a sterilized protective soil layer on top. The prey species, *Aphelenchus avenae,* was grown on half to full strength Potato Dextrose Agar inoculated with *Botrytis. A. avenae* was introduced via chunking and was successful as the prey species, which is consistent with *Mylonchulus sigmaturus* preference (Koohkan & Shokoohi, 2014). A single culture was viable for over half a year and adults were transferred to new plates when *A. avenae* levels were low. One particular line sourced from an agricultural field at the Northwestern Illinois Agricultural Research and Demonstration Center was identified as *Mononchus laminatus* based on morphometric parameters and taxonomic descriptions (Ahmad & Jairajpuri, 2010; Zullini, Loof, & Bongers, 2002). It was quite successful at colonization and featured only parthenogenic females, resulting in an isogenic culture.

Fixation and Mounting

Due to the slow-growing nature of Mononchids compared to more common lab nematodes, months-old cultures were ideal to source nematodes from. Individuals of various sizes throughout development were selected. They were picked into .1M PBS for rinsing then onto a slide with a thin layer of .1M PBS. The slide was transferred to a hot plate between 65°C and 70°C. Until the nematodes had fully relaxed and ceased movement, small amounts of .1M PBS were added to prevent desiccation. Once ready, as much PBS was siphoned off as possible with a Kimwipe and heated 4% formaldehyde was applied in its place. The nematodes were transferred to a watchglass and sealed with more 4% formaldehyde to allow them to harden straight overnight at 2°C. Post-fixation, they were rinsed with .1M PBS buffer.

DAPI staining and microscopy were consistent with previous reporting. For permanent mounting, exceptions were made as glycerol was used as the mounting medium in place of a

buffer and following microscopy the slide was sealed three times with nail polish and transferred to long term storage at -20°C.

Data Analysis

The VNC neuron counts and densities of adults were incorporated and analyzed with the family data. Separately, graphs of the counts through developmental time, with VNC length acting as the proxy for time, were made. Adult females versus juveniles were distinguished in the data. Linear regression analyses were applied to the neuron counts and density data of adults and juveniles separately.

Immunohistochemistry

Adult *C. elegans* strain N2 and environmentally sourced Tobrilidae were utilized for testing this procedure. Tobrilidae were collected from local drainage ponds. The intention was to tag tubulin and GABA. This procedure was adapted from Henne, Sombke, & Schmidt-Rhaesa (2017).

Fixation

Fixation was identical to that used for Mononchidae Developmental Analysis with the exception of the choice of buffer was adapted to the nematode of consequence; *C. elegans* used M9 as its standard buffer while Tobrilidae/aquatic worms used .1M PBS, pH 7.3.

Permeabilization

Specimens were centrifuged for a minute at 6000rpm. The supernatant was discarded and replaced with β-mercaptoethanol solution (5% β-mercaptoethanol, 1% Triton X-100, .1M TRIS buffer [pH 7.4]) for 24 hours at 37°C. They were rinsed six times in the centrifuge with .1M TRIS buffer then with collagenase buffer (1 mM calcium chloride dihydrate, .1M TRIS buffer). Upon transferring to collagenase solution (1000U/ml collagenase, 1% Triton X-100, 1 mM

calcium chloride dihydrate, .1M TRIS buffer), they were gently shaken for 24 to 36 hours at 37°C. The vessel recommended for this would be concave glass, not plastic, to prevent sticking and desiccating on the sides. To cease enzymatic action, they were rinsed with cold collagenase buffer twice then again with .1M PBS twice. The samples were left overnight in preincubation buffer (1% DMSO, .5% bovine serum albumin, 6% goat serum, 2% Triton X-100, .05% sodium azide, .1M PBS) at 37°C.

Hybridization

Primary antibody (1:500 in preincubation buffer) incubation lasted for 72-96 hours at 37°C. Following several wash steps in .1M PBS, secondary antibody (1:200 in preincubation buffer) incubation was 24 hours at 37°C. The secondary antibody incubation and following steps all took care to be done in minimal lighting.

Staining and Mounting

For nuclear staining, 4 µg/mL DAPI was added and sat at RT for an hour. The samples were then washed several times with .1M PBS. If they could not be immediately visualized, they were stored at 2°C. They were mounted just as the Mononchid specimens were, to allow for long term storage. Microscopy followed the standard protocols previously described, excepting for the use of z-stacks utilizing FITC and Texas Red filter cubes in addition to DAPI and DIC.

2.4 RESULTS

Demonstrable Differences in VNC Neurons and Density Both Between and Within Clades

Clade Differences

Combining the clade data, whether basal versus derived or Enoplea versus Chromadorea, produced significant differences between their ventral nerve cord numbers. Tackling the basal

versus derived question, data from Clades 1-6 and 8-12 were combined and analyzed. Both the Mann-Whitney and Kolmogorov-Smirnov p-values were less than .0001. This indicates there is a statistically significant increase in neurons within the basal clades as compared to the derived. However, this information does not provide as much evolutionary insight so the clade data was divided into the two classes, Enoplea (Clades 1 and 2) and Chromadorea (Clades 3-12). The p-values for their tests were also less than .0001, revealing Clades 1 and 2 specifically have a significant increase in neurons from the rest of the data and are likely skewing the basal data upwards.

An analysis of individual clades against others resulted in mostly insignificant comparisons (Table 2.3). There were 55 comparisons per clade within the mean separation test. Exceptions to this include Clades 1 vs 9; 2 vs 5; 2 vs 9; 2 vs 10; 2 vs 11; 2 vs 12; 3 vs 9; 4 vs 9; 6 vs 9; 6 vs 11; 6 vs 12; 9 vs 10; 10 vs 11; and 10 vs 12. In summary, a lot of basal clades differed from Clade 9 and some differed from 11 and 12 (Figure 2.3). There were limited differences within the basal clades but there were multiple clade differences between the derived clades. This corroborates the Kolmogorov-Smirnov and Mann-Whitney p-values above stating that collectively there are basal versus derived differences.

Clades	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
1		ns	ns	ns	ns	ns	ns	ns	**	ns	ns	ns
2	ns		ns	ns	***	ns	ns	ns	****	*	****	****
3	ns	ns		ns	ns	ns	ns	ns	***	ns	ns	ns
4	ns	ns	ns		ns	ns	ns	ns	*	ns	ns	ns
5	ns	***	ns	ns		ns	ns	ns	ns	ns	ns	ns
6	ns	ns	ns	ns	ns		ns	ns	****	ns	****	****
7	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns
8	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns
9	**	****	**	*	ns	****	ns	ns		****	ns	ns
10	ns	*	ns	ns	ns	ns	ns	ns	***		***	**
11	ns	****	ns	ns	ns	****	ns	ns	ns	*		ns
12	ns	****	ns	ns	ns	****	ns	ns	ns	**	ns	

Table 2.3. A Dunn's comparison test of VNC neuron counts between clades. ns indicates no significant difference, * indicates $p \leq .05$, ** indicates $p \leq .01$, *** indicates $p \leq .001$, **** indicates $p \leq .0001$

Family Differences

Given the number of families involved, the comparison data was chaotic to interpret (Table 2.4); however, a pattern emerged where most significant differences were between basal and derived families, though there were also a decent number of differences between the derived families, which is in agreement with the assessment of that data by Han et al. (2016) (Figure 2.2). There were 351 comparisons per family under the Dunn's multiple comparisons test. The significant differences can be divided into three groupings: within basal clades, within derived clades, and between basal and derived clades. The significant family differences within the basal clades were between Prismatolaimidae vs Longidoridae; Prismatolaimidae vs Mononchidae; and Prismatolaimidae vs Camacolaimidae. The families with significant differences in the derived clades were Heterorhabditidae vs Steinernematidae; Diplogastridae vs Steinernematidae; Diplogastridae vs Heteroderidae; Steinernematidae vs Cephalobidae; and Steinernematidae vs Pratylenchidae. Interestingly, Diplogastridae vs Heteroderidae is the only derived family significant difference where both families belong to the same clade. Lastly, there were 26 significant differences between basal and derived families. They are recorded in the table below.

FAMILIES	*Diphtherophoridae*	*Prismatolaimidae*	*Tobrilidae*	*Aporcelaimidae*	*Longidoridae*	*Mononchidae*	*Mylonchulidae*	*Achromadoridae*	*Ethmolaimidae*	*Desmodoridae*	*Epsilonematidae*	*Monhysteridae*	*Xyalidae*	*Aphanolaimidae*	*Camacolaimidae*	*Metateratocephalidae*	*Plectidae*	*Ascaridiidae*	*Rhabditidae*	*Heterorhabditidae*	*Diplogastridae*	*Steinernematidae*	*Cephalobidae*	*Aphelenchidae*	*Heteroderidae*	*Meloidogynidae*	*Pratylenchidae*
Diphtherophoridae		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Prismatolaimidae	ns		ns	ns	*	*	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Tobrilidae	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	****	ns	*	ns	ns	ns	**
Aporcelaimidae	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Longidoridae	ns	*	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	****	****	ns	****	ns	ns	ns	****
Mononchidae	ns	*	ns	ns	ns		ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	****	****	ns	****	*	**	**	****
Mylonchulidae	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Achromadoridae	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	****	ns	ns	ns	ns	ns	ns
Ethmolaimidae	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Desmodoridae	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Epsilonematidae	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Monhysteridae	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Xyalidae	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Aphanolaimidae	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	****	ns	ns	ns	ns	ns	****
Camacolaimidae	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	****	****	ns	****	ns	ns	ns	****
Metateratocephalidae	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Plectidae	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Ascaridiidae	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns
Rhabditidae	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns
Heterorhabditidae	ns	ns	*	ns	****	****	ns	ns	ns	ns	ns	ns	ns	ns	****	ns	ns	ns	ns		ns	***	ns	ns	ns	ns	ns
Diplogastridae	ns	ns	****	ns	****	****	ns	****	ns	ns	ns	ns	ns	****	****	ns	ns	ns	ns	ns		****	ns	ns	*	ns	ns
Steinernematidae	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	****		**	ns	ns	ns	****
Cephalobidae	ns	ns	*	ns	****	****	ns	ns	ns	ns	ns	ns	ns	ns	****	ns	ns	ns	ns	ns	ns	**		ns	ns	ns	ns
Aphelenchidae	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns
Heteroderidae	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns		ns	ns
Meloidogynidae	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns
Pratylenchidae	ns	ns	**	ns	****	****	ns	ns	ns	ns	ns	ns	ns	****	****	ns	ns	ns	ns	ns	ns	****	ns	ns	ns	ns	

Table 2.4. A Dunn's comparison test analysis of VNC count across numerous families. Italics indicate the use of published data from Han et al. (2016). ns indicates no significant difference, * indicates $p \leq .05$, ** indicates $p \leq .01$, *** indicates $p \leq .001$, **** indicates $p \leq .0001$

Neuron Density

A majority of density comparisons under Dunn's test were insignificant. The exceptions were Longidoridae vs Aphanolaimidae; Longidoridae vs *C. elegans*; Mononchidae vs *C. elegans*; Aphanolaimidae vs Camacolaimidae; and Camacolaimidae vs *C. elegans*. While neuron density is an interesting parameter, it does not appear to be enlightening to infer evolutionary relationships on a family or clade scale for nematodes given its perplexing variability (Figure 2.4).

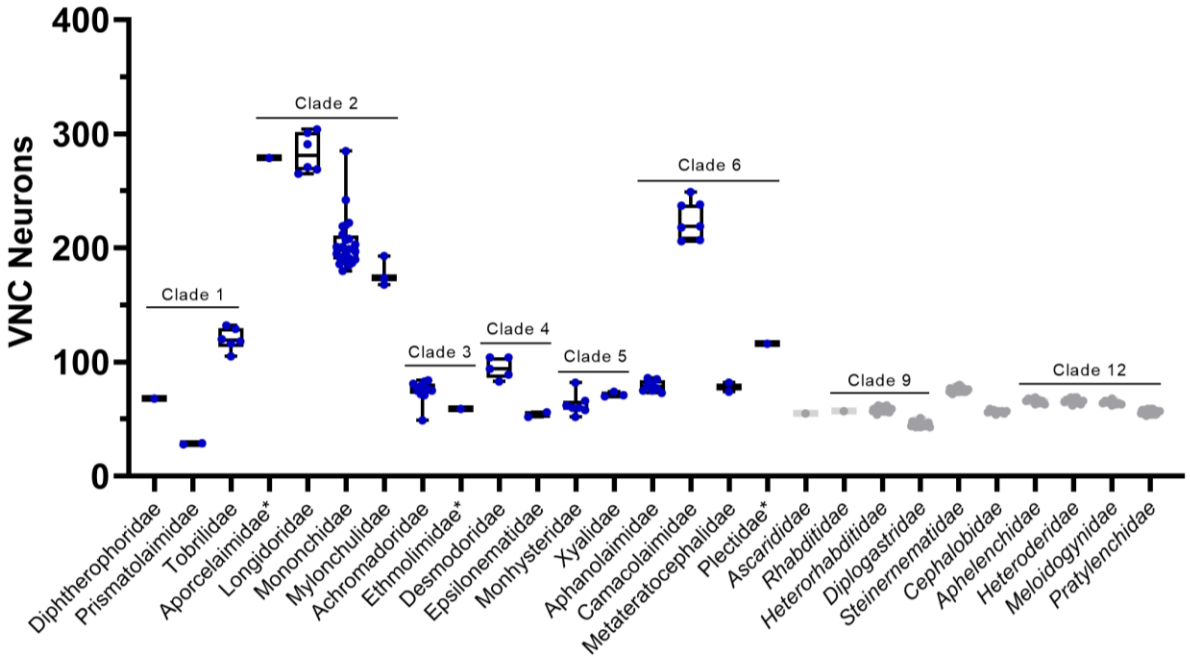

Figure 2.2. A family representation of VNC neuron counts across all Nematoda. Grey and italicized data indicates prior juvenile-dominant reporting from Han et al. (2016). All blue points indicate single female adults, with the exception of Camacolaimidae and Epsilonematidae which included males. Boxes extend between the 25th and 75th percentiles; whiskers indicate minimum and maximum values.

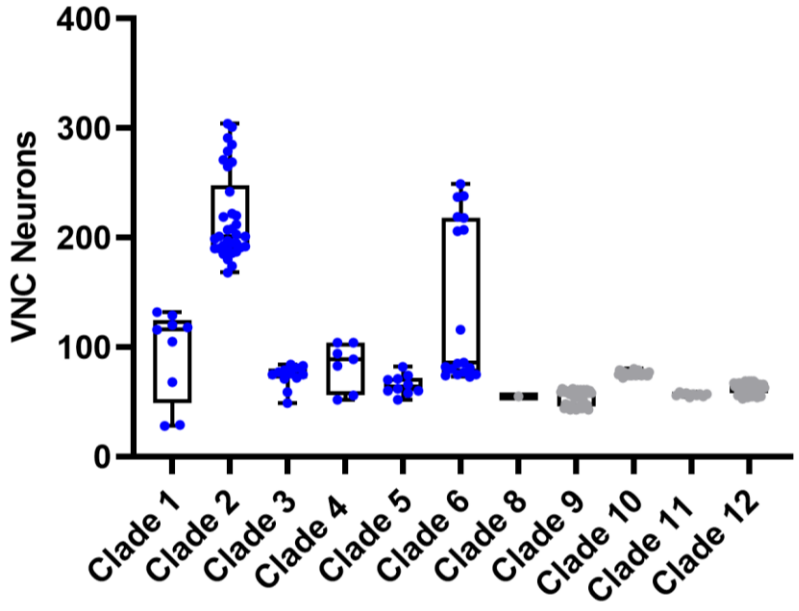

Figure 2.3. A clade analysis of VNC counts across the nematode phylogeny. Grey and italicized data indicates prior derived juvenile-dominant reporting from Han et al. (2016). All blue points indicate single female adults, with the exception of Camacolaimidae and Epsilonematidae which included males. Boxes extend between the 25th and 75th percentiles; whiskers indicate minimum and maximum values.

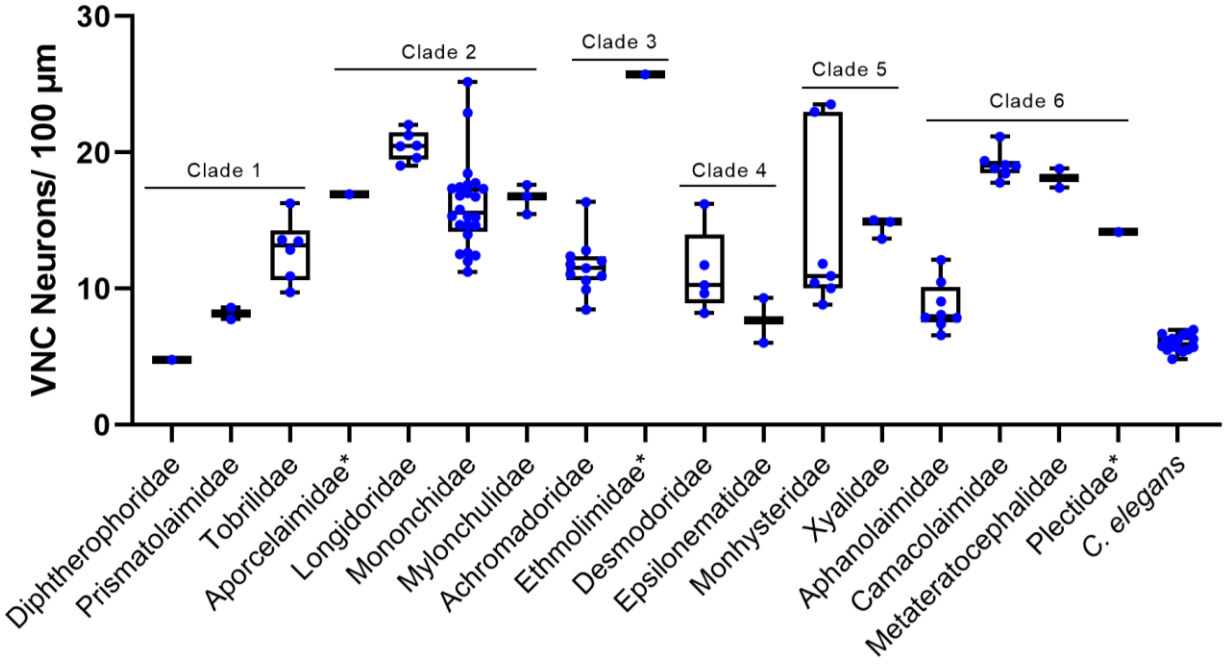

Figure 2.4. Average VNC neurons per 100 µm of nerve cord by family. Asterisks indicate families with only one representative. Boxes extend between the 25th and 75th percentiles; whiskers indicate minimum and maximum values.

Mononchus laminatus VNC Development Reveals Determinate Development Consistencies and Parallels to *A. avenae*

Raw Counts and Density

M. laminatus ranged from 324 to 1672 µm. Early in development (under 500 µm), juveniles have a small complement of 54-83 VNC neurons. Molts were not tracked so determining exact life stages is not possible; however, given that another *Mononchus* sp. was documented as J1 upon hatch, it is reasonable to assume the smallest juveniles are J1s (Grootaert & Maertens, 1976). Once they approach approximately 600 µm, there seems to be an explosion of neural generation and counts abruptly jump to the 170s (Figure 2.5). No nematodes were found in this swift developmental range suggesting that it is a sudden part in the life cycle. At this point, the nematodes are still juveniles and quickly level off their counts by reaching a full complement of on average 197 VNC neurons in adulthood. The adult counts do not appear to increase with length, which is further investigated with linear regression analyses.

Examining density, the results mirror the VNC counts (Figure 2.6). In that initial period around 500 µm, densities are decreasing until they hit that rapid neural development timepoint, at which time densities reach their highest possible point before the nematodes begin to grow and densities eventually fall to below that of the early juveniles in the longer adults. It is important to note that the there is a substantial number of adults that hover around 15 neurons/100µm; this is also the density that the early juveniles.

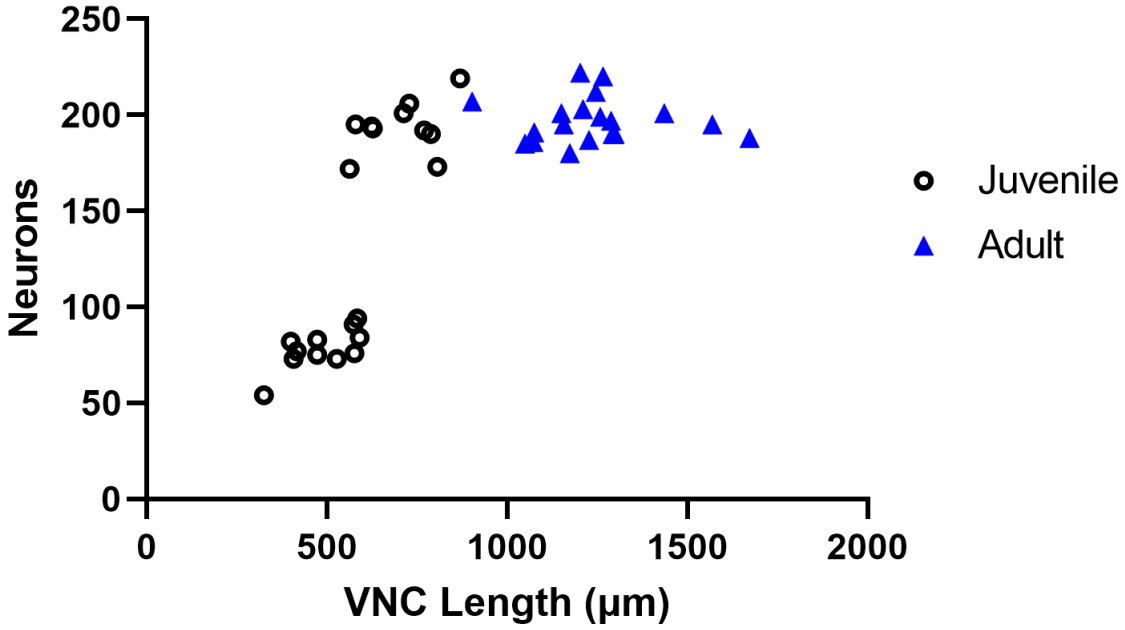

Figure 2.5. VNC neuron enumeration through the development of cultured *M. laminatus*. There is a sharp increase in neuronal development once the juvenile reaches approximately 600µm.

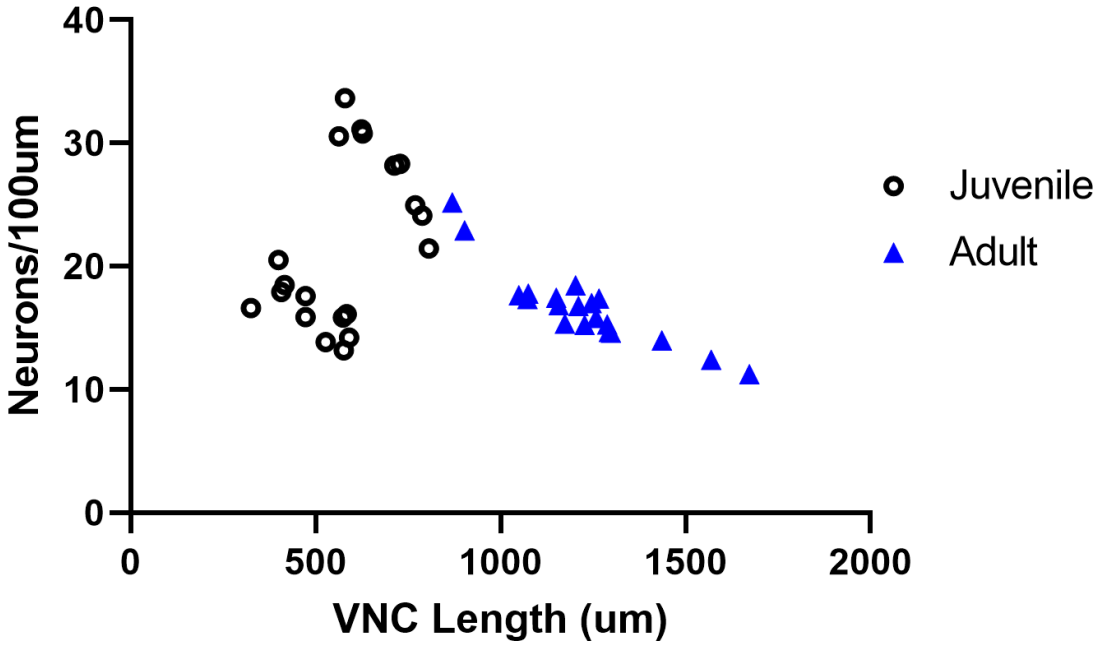

Figure 2.6. VNC neurons per 100µm of nerve cord throughout development of cultured *M. laminatus*. The lack of an increase in density as an adult suggests determinate development.

Linear Regression

The linear regression for adult neurons was of particular interest as it found adult counts were not statistically significant from zero ($y = -.01385x + 215.3$, $r^2 = .04682$) as length increased (Figure 2.8). A stagnant nerve cord is consistent with determinate development, which is not documented in Enoplea. The juvenile neuron counts had a strong positive correlation ($y = .3383x – 66.33$, $r^2 = .6276$), which aligns with the concept of a developing nerve cord (Figure 2.7).

Following up on the determinate evidence, the adults had a strong negative correlation as they grew and became less dense ($y = -.01476x + 34.69$, $r^2 = .8130$) (Figure 2.10). A more in-depth explanation for these determinate observations will occur in the discussion. The juveniles had a weak and insignificant positive correlation ($y = .02176x – 9.108$, $r^2 = .1966$), likely affected by the sudden increase in neurons (Figure 2.10).

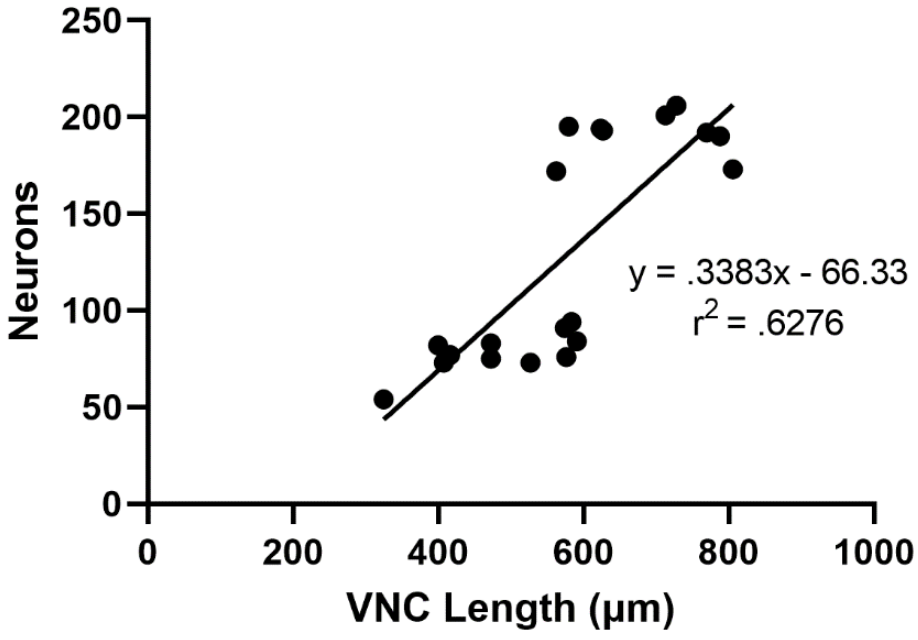

Figure 2.7. A linear regression of the VNC neuron counts of juvenile *M. laminatus* as they reach adulthood. There is a positive association of added neurons and growth. $p < .0001$

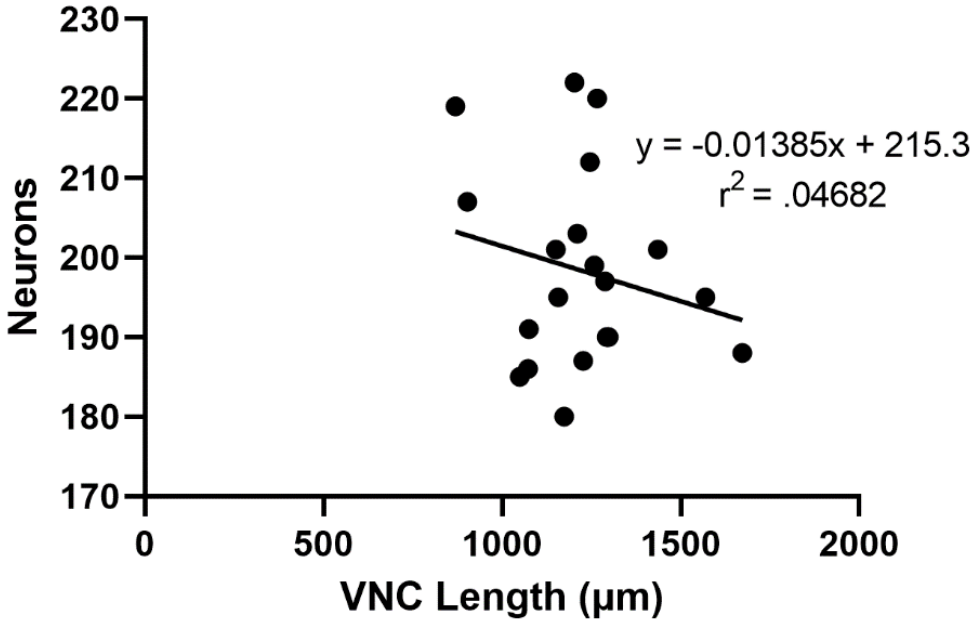

Figure 2.8. A linear regression of the VNC neuron counts of adult *M. laminatus*. There is no association which suggests the neuron counts stagnate even as the adult continues growing, consistent with determinate development. $p < .3595$

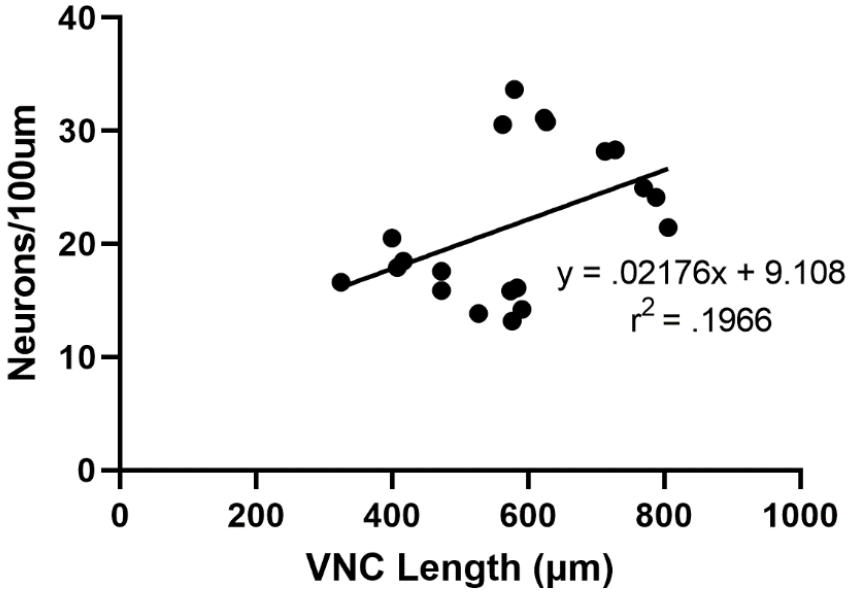

Figure 2.9. A linear regression of the VNC neuron densities of juvenile *M. laminatus* as they reach adulthood. There is a negligible positive association of density and growth. The abrupt increase in VNC neurons likely does not aid in a linear analysis. $p < .0502$

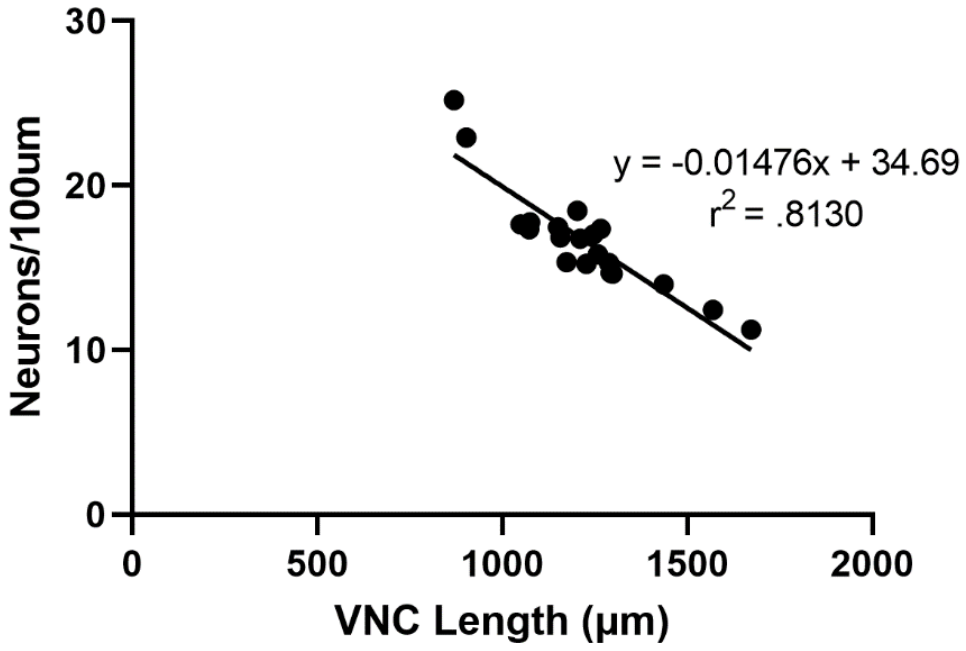

Figure 2.10. A linear regression of the VNC neuron counts densities of adult *M. laminatus*. There is a strong negative association of density and growth. This is very suggestive of determinate development. $p < .0001$

Immunohistochemistry of Clade 1 Tobrilidae

Attempts of antibody staining a basal nematode were partway successful. Visualization was better for Tobrilidae than *C. elegans*, which did not respond to the treatment as well, likely due to cuticular differences. GABA featured more cell body localization while tubulin was often seen within long cords or processes. Commissures in Tobrilidae were visualized more frequently towards the tail and often easier to distinguish in males (Figure 2.11a). Tubulin localized to the ventral nerve cord itself as seen when comparing the DAPI stain to tubulin (Figure 2.11b, b'). There was some crossover in GABA and tubulin staining as evidenced by Figure 2.12a and a'.

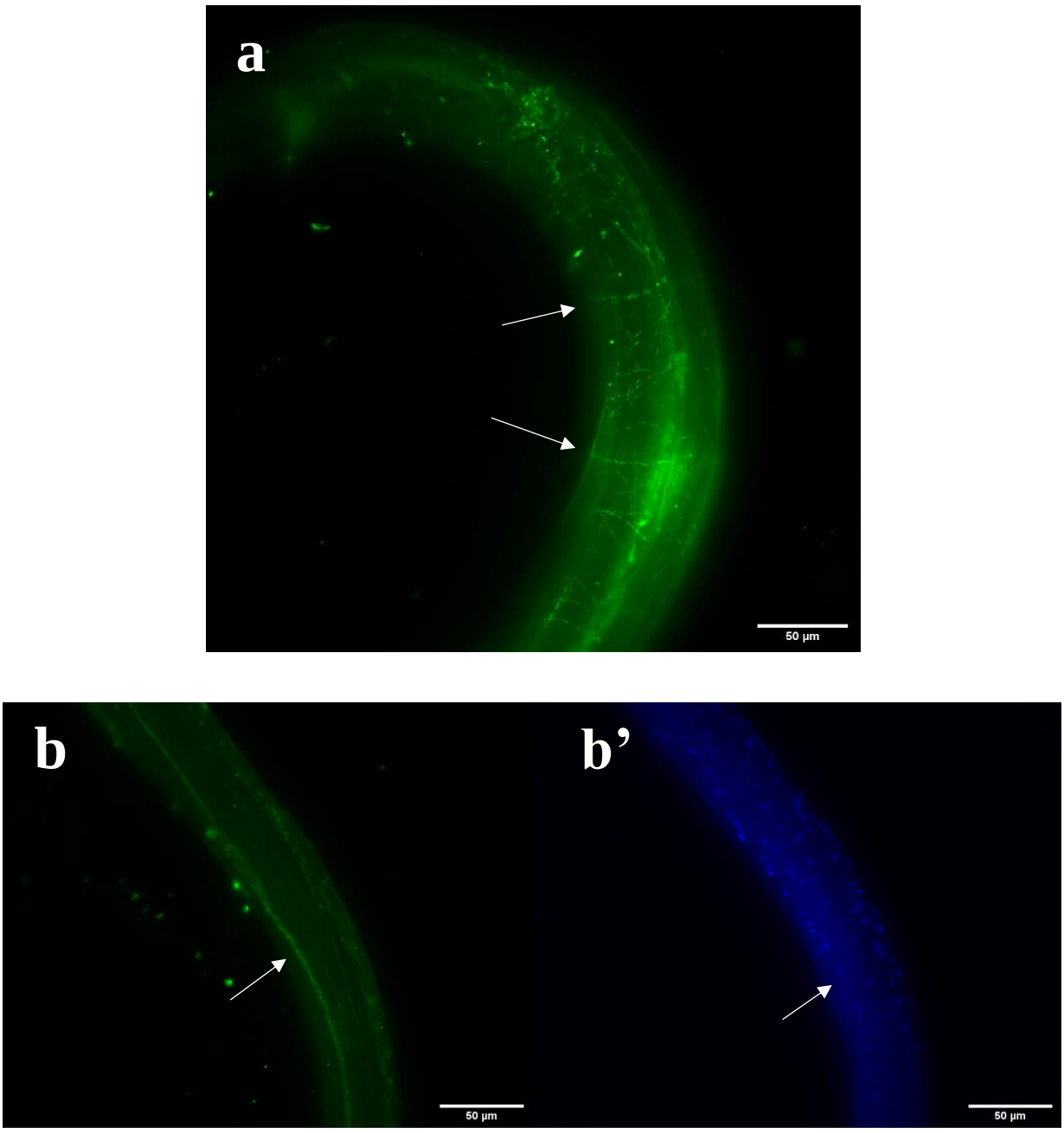

Figure 2.11. Fluorescent micrographs of immunohistochemistry featuring anti-tubulin within a Tobrilidae target. (a) Anterior tail region of male Tobrilidae. Tubulin staining of commissures indicated by arrows. (b) Mid-body image of tubulin staining of the ventral nerve cord as indicated by arrow. (b') DAPI channel of the same slice as depicted in (b) with the arrow still indicating the ventral nerve cord. Scale bars, 50 µm.

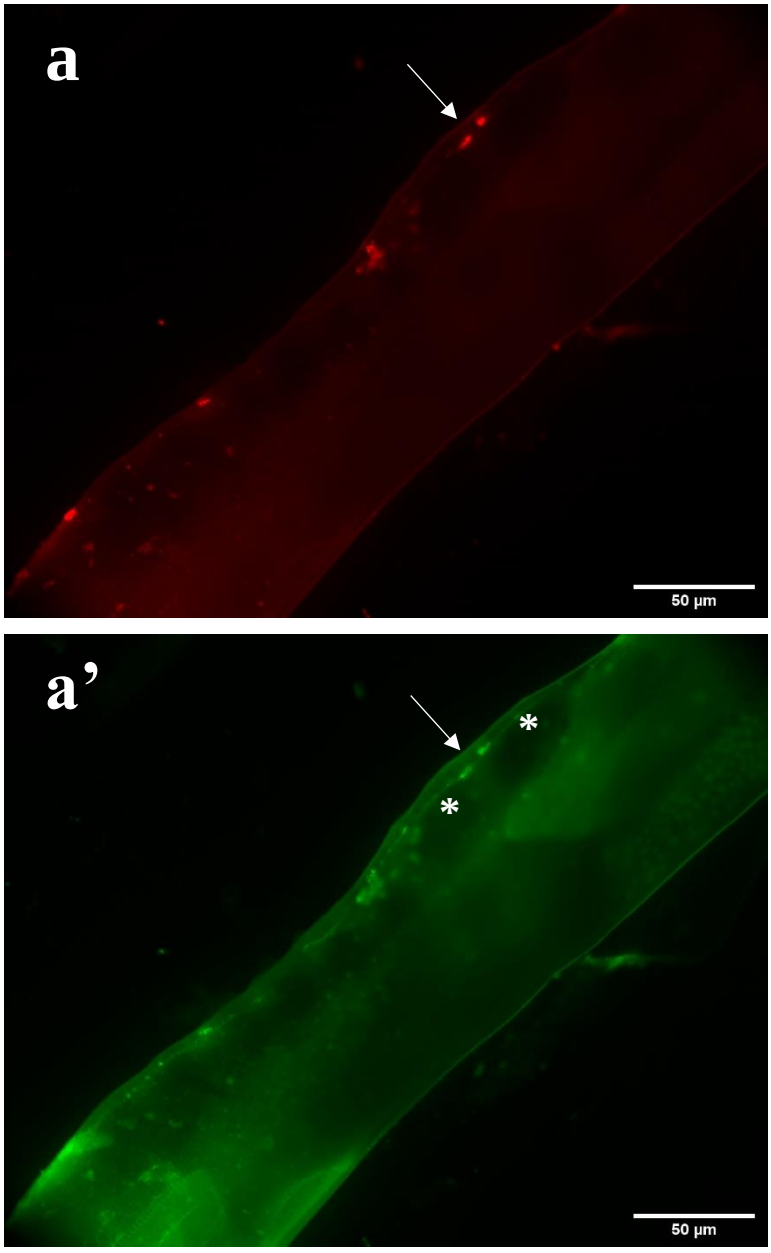

Figure 2.12. Fluorescent micrographs of immunohistochemistry featuring anti-tubulin and anti-GABA within a *C. elegans* target. (a) Mid-body image of a pregnant hermaphrodite. GABA staining of cell localization indicated by arrow. (a') Tubulin staining of the same slice as depicted in (a). Tubulin localizes to the same cells (arrow) as well as to thin processes extending laterally (asterisks). Scale bars, 50 µm.

2.5 DISCUSSION

The intent of this thesis was to provide evidence on a phylum-wide scale that the nematode nervous system is not as conserved as previously suggested. Through the inclusion of the basal clades, I also hoped to detect evolutionary relationships between classes Enoplea and Chromadorea or basal and derived clades. I found that there was a simplification of the ventral nerve cord between the Enoplean and Chromadorean classes as well as the basal and derived clades. In addition, *Mononchus laminatus* contributes to previous data that neuronal heterochrony is present in the ventral nerve cord. The data was effective at providing evidence that the nematode nervous system is more varied and complex than given credit for and that there are some relationships indicative by the nerve cord.

Interpretation and Limitations of Results

Beyond Clade 1 and 2, there seems to be a near global depression of the nematode ventral nerve cord neurons. Exceptions to this include Triplonchida and Camacolaimidae. Of the three Triplonchida families, Diphtherophoridae and Prismatolaimidae have counts similar to the derived clades. Tobrilidae has a nerve cord with more neurons but certainly not on the level of thousands as reported in Enoplida. The other exception is Camacolaimidae, which falls firmly into the territory of the depressed nerve cord numbers. While another Clade 6 representative is elevated from the derived VNC numbers, Camacolaimidae is very distinct and set apart from the rest of the surrounding families.

The density data itself is not illuminating. While each species seems to have a contained range of neuron density within their VNC, the variation within clade and beyond makes density a poor parameter to assess nervous system evolution and change. Likely, density is not

evolutionarily imperative and therefore, unlike with the raw neuron numbers, I did not observe stability within density calculations as this variable may be prone to fluctuation.

In addition, it must be noted that conclusions are made almost entirely from DAPI data alone. There is no unique fluorescent distinction for VNC neurons besides the characteristics listed in the methods. This means there is reasonable expectation of limited human error in regards to miscounts of neuronal-like cells that have migrated into the VNC as well as potential false counts of neurons on the cusp of the restrovesicular and pre-anal ganglion. Given that there is established precedence of using DAPI for VNC enumeration and comparison, the utilization of DAPI within this thesis is not unreasonable (Han et al., 2016).

Due to the nature of erratic environmental sampling, the overall sample sizes of individual families was poor. For this reason, nonparametric tests needed to be used. For families with lower sample sizes and given the low statistical power of Dunn's test, there appeared to be some questionable lack of significant differences for families with lower sample sizes. Many of the significant differences that were apparent originated from families that passed normality tests. It is understandable that clade normality tests failed when we consider that a clade is made of multiple families, each with its own clustered distribution; this clustering is far from a standard bell-curve.

Examining neuron development as *M. laminatus* grew, there appeared to be a drastic addition of neurons all at once in juveniles. Regrettably, as molts were not tracked, it is uncertain what juvenile stage this neuronal explosion occurred. The general trend though is similar to reports from both *C. elegans* and *A. avenae*. It seems consistent throughout the phylum that there is a rapid addition of neurons at some point during the juvenile stages. It is noteworthy that a nematode from the basal clades has neuronal development consistent with reports from derived

clades. It appears there is not some remarkable difference in VNC development, no matter determinate or indeterminate development. In accordance with previous data, it would not be surprising for the timing of Clade 2 VNC development to differ from derived worms given the documentation of neuronal heterochrony within the derived clades.

In regards to antibody staining, the method was originally created for *Plectus* spp. but appeared to be effective on Tobrilidae (Henne et al., 2017). *C. elegans* also stained with the treatment; however, the conditions were harsh on the cuticle and the staining was poor in many cases. Exposed openings such as the stoma and sliced sections tended to over fluoresce. GABA staining was often diffuse and difficult to interpret. Repeatedly, the anterior region featured incredibly diffuse staining of both GABA and tubulin so little information could be retrieved regarding the nerve ring or retrovesicular ganglion.

The Highly Conserved Debate: What was Right and What was Wrong

There are clearly underlying differences between taxa. While the organization is highly conserved, there appears to be some form of genetic underpinning influencing lower nerve cord numbers. In addition, based on the excessive numbers of neurons featured in clades 1, 2, and 6, their connectomes may widely differ from the standards established by *C. elegans* and derived company. The concept of nervous system divergence should not come as a surprise to the field of nematology—particularly within the basal clades—given the occurrence of structures such as double nerve rings or shifts in ganglia locations and numbers that have previously been documented (R. V. Anderson, 1966; J. B. Goodey & Hooper, 1963).

Given the documented uniqueness of Enoplean nervous structures (Malakhov, 1994), to assume that a *C. elegans* type connectome is directly relevant to Enopleans would be foolhardy. They have sensory structures that are unseen in other clades and how these structures

are assimilated into the established circuitry is a mystery. While trying to address differences in connections like these at a mammalian level would be unpalatable, consider that nematodes are the best research vessel to question this dilemma. Given their status as simple models, it is presumed that divergences in evolution would be clearer, and the implications of the variety of potential connectome differences within nematodes is important to understanding nervous system evolution. In addition, it also begs the question of why were these structures lost and the nervous system downsized? We always tend to assume nervous system evolution is a one way street where increasing complexity is the destination. The loss of sensory structures and ventral nerve cord neurons is in opposition to that view point.

The fact that the VNC neurons in *M. laminatus* were not in flux as adults could be an indicator that nervous system development is highly conserved, but perhaps not in the all-encompassing way the statement was first intended. While the range of neuron numbers appears to vary, the overall organization of the nervous system itself appears to be conserved. When large-scale variation is observed, it appears to occur more often in sensory structures and not central structures like the nerve cord (Malakhov, 1994). The extent of VNC change documented is in instances like distinct clustering of neurons in certain species. In addition, the development of the nerve cord is similar between species; multiple sources document a rapid spike in neurogenesis during a juvenile stage, often directly prior to a molt (Han et al., 2016; Sulston, 1976; Sulston & Horvitz, 1977). The only noted major difference in this developmental process is the timing at which these neurons are added. It appears that while the numbers of neurons may shift due to some outside, likely genetic, pressure, the overall nervous system morphology and development are less susceptible to change, with changes being to extraneous modifications like VNC neuron clustering or developmental timing shifts. This lack of diversity in the nematode

nervous systems can also be extrapolated as an argument against using it as a target for modern nematicides which are now made with specificity in mind.

Questions of Environmental Pressures on Nervous System Evolution

Curiously, Plectidae and Camacolaimidae both fall under Plectida, but Camacolaimidae has a distinct increase in its VNC neurons, even when compared to the related Plectidae representative. Perhaps whatever pressure responsible for the downsizing of the nerve cord in Chromadorea was somehow lost in Camacolaimidae specifically. While there are a myriad of taxonomic descriptions of the Camacolaimidae species, there is little research into them and no morphological documentation to explain potential causes of such a stark nervous system difference from other Chromadoreans. Superficially, the Camacolaimidae family is entirely marine while Plectidae is often encountered terrestrially or in freshwater (Eyualem-Abebe et al., 2006). Marine Enoplids are suggested to have the most complex nervous systems and possess high VNC counts so there is a question of habitat influence (Malakhov, 1994). Unfortunately, without a larger sample of families, this is very difficult to assess. Of course, in direct contrast to that explanation, marine representatives from Desmodoridae and Epsilonematidae are in alignment with their derived cousins.

While nematodes are not planktonic in nature (Fenchel, 1978; Jacobs, 1984), there is thorough documentation of their prevalence in the water column (Boeckner, Sharma, & Proctor, 2009; Jensen, 1981). It used to be assumed their presence indicated passive distribution but it is now understood that there is some manner of active swimming and sediment choice for suspended nematodes (Palmer, 1984; Ullberg & Ólafsson, 2003). However, it appears larger nematodes are not as capable of this active distribution due to three factors. First, sinusoidal movement in large nematodes often cannot overcome water viscosity (Crofton, 1966). Second,

larger nematodes need to produce enough propulsive power to overcome gravity (Ullberg & Ólafsson, 2003). Third, active upwards swimming in the water column requires high wave frequencies of the body, which are also more difficult in larger worms (Wallace & Doncaster, 1964). All these factors combine and support observations made that smaller nematodes are more likely to navigate the water column and remain in it longer (Crofton, 1966; Ullberg & Ólafsson, 2003). This appears to even be corroborated by sediment layer preference; smaller nematodes like Monhysterids prefer upper sediment where chances of suspension are high while larger worms like Enoplids prefer deeper sediment and are adapted against suspension (Bergtold, 2001; Eder, 1983; Schratzberger, Whomersley, Warr, Bolam, & Rees, 2004). Furthering the acknowledgement of size influence on water column navigability, juveniles of species that prefer deeper sediment migrate to shallower sediment (Traunspurger & Drews, 1996). While the larger worms sacrifice water column navigation, they are better suited to sediment colonization (Gallucci, Moens, Vanreusel, & Fonseca, 2008; Schratzberger et al., 2004). This begs the question of body movement differences between nematodes capable of active migration and sediment dwellers. There is limited information that analyzes the effects of habitat on movement of nematodes in conjunction with their nervous system. While most nematodes use a sinusoidal movement, there can be differences in that movement that are hard for the human eye to measure. Software such as WormLab can allow for the analysis of criteria like speed, body wavelength, body/head bending angles, and omega bends that might reveal clear habitat differences in marine nematode movements such as Enoplids and Camacolaimidae. If there are concerted differences, it is not implausible to suggest they might also be reflected in innervations or the connectome. The marine data reflects this as large Camacolaimidae, juvenile

Oncholaimidae, and outside observations have more numerous neurons in the VNC but smaller marine worms like Epsilonematidae do not.

One issue with this habitat-size hypothesis lies in why large freshwater nematodes visualized in this study do not have as stark VNC differences. Firstly, Prismatolaimidae is a small worm so it may conform to other derived clade VNC pressures since it can navigate the water column. Secondly, Tobrilidae is a larger worm with a slightly elevated neuron number that upsets this argument. The pressures associated with freshwater though are different than marine in some cases. For example, a freshwater species of *Chromadorita* was capable of swimming while a morphologically similar benthic *Chromadorita* species would not swim (Jensen, 1981). Freshwater nematodes are also commonly found extended into surrounding terrestrial habitats, which presents the need to be adapted to multiple biomes (Niemann, Arens, Koczwara, & Sturhan, 1996). The lower biomass and productivity of freshwater nematodes could indicate less competition and reduced need to colonize (Eyualem-Abebe et al., 2006). The influence of freshwater depth and velocity also need to be considered in comparison to marine habitats (Filipjev, 1930; Hirschmann, 1952). Unfortunately, these is not as much interest in the assessment of freshwater factors on nematode communities as compared to marine but the above reasons may help to open a discussion regarding this issue.

In nematodes closely related to the marine Enoplids, we do still observe vestiges of the elevated counts within the Triplonchids; they are just not on the level of Malakhov's observations (1994). This also brings into question why Dorylaimians also have a plethora of VNC neurons. This could be an argument that there is a genetic component to the counts that resulted in high numbers within Enoplea. If the true nematode ancestor was a marine worm that could explain the Enoplean nerve cord complexity, with a long marine nematode like

Camacolaimidae establishing that the ancestors cord was due to marine adaptations specifically. In direct contrast to this argument, Camacolaimidae belongs to a molecularly frustrating order that classically confused morphologists; it could simply be an isolated aberration in terms of evolutionary changes in the nematode VNC (Holovachov, 2013).

Implications of Determinate Development Indicators in *Mononchus laminatus*

While determinate development was accepted as a convention of nematodes, over time this belief waned as indeterminate members like the Enoplians and Dorylaimians were investigated. The scope of determinate development among nematodes has not been thoroughly examined but it appears to be more of an exception than the evolutionary norm. The data presented here indicates an invariant number of neurons in a Clade 2 nematode; this contradicts the idea of indeterminate development that was hypothesized (Schulze & Schierenberg, 2008, 2011). Previous data is rather insistent that a Clade 2 nematode is subsumed under an indeterminate model so deeper analysis is required to explain this aberration. First, determinate and indeterminate development are intended to refer to the gastrulation process. While an observation of increasing neurons in adults would be indicative of indeterminate development, the lack of change in neurons is not an immediate assumption of determinate development. *Enoplus brevis* was documented to have variable cells during embryogenesis but as a first stage juvenile these cells became invariant, which is a property often associated with determinate development (Voronov & Panchin, 1998). Second, *M. laminatus* continued to grow in length while the neurons were stagnant so perhaps outside of the nervous system cell fates were variant. Examining adults seems to be counterproductive to determine developmental process if they are not followed through from the embryo; however, based on previous research, it would be highly consistent and agreeable to say that *M. laminatus* is indeterminate. In addition, that is one of the

pieces of evidence for Enoplians as the nematode ancestor that can also be definitively applied to Dorylaimia, if molecular data didn't suggest that Dorylaimia is probably not the source of nematode ancestry, that is (Smythe et al., 2019).

Future Research: Taxa of Interest

In terms of representation, there are certainly areas of improvement for this study. For example, Clade 7 was the only clade not included. Its single genus has been described as having morphology that bridges former classes Adenophorea and Secernentea (Zhang & Baldwin, 2001). Given the research presented here, it is not farfetched to predict that Clade 7 will follow the patterns laid out by the derived clades.

Another lack of representation falls to Enoplida. Malakhov described Enoplids as having the most complex nervous systems of nematodes (1994). Having more evidence to support this idea, especially within a comparative analysis like this, would improve conclusions. While the Enoplids and Dorylaims found in this study did not have thousands of neurons, there is previous documentation of this that adds a further piece of evidence distinguishing Enoplea from Chromadorea; along with the commonalities between Enoplea and other Ecdysozoan phyla, this nervous system data can further establish Enoplea as basal to the nematode tree.

A final group that presents interesting questions is Plectida. The two Plectid families included here had very different nerve cords. While the SSU rDNA of Plectida was not as messy as Dorylaimida, a higher resolution analysis of the group could not hurt to solidify family relationships, especially given its history as a polyphyletic order (Holovachov, 2013). Knowing if further families within the order also have elevated nerve cords like Camacolaimidae could make this an order of interest to research gene discovery in order to understand the underpinnings that differ between nematodes with higher neuron counts versus lower. Plectida

presents such a unique opportunity because not as much change will occur on the level of an order. The problem could still be tackled using *C. elegans* and Enoplean nematodes, for instance, but given the evolutionary distance between them there will be significant genetic changes to sift through. As such, I suggest the Plectid model for investigation into the genetic underpinnings of nematode nervous system simplification. Of course, the genes responsible for an increase in VNC neurons may differ between a Clade 1 and a Clade 6 nematode.

Unfortunately, this research did not have the opportunity to analyze a marine Enoplid as stated above. Assessing the differences between a marine nematode from Enoplida versus a freshwater nematode from its sister order Triplonchida would provide illuminating evidence for or against habitat pressures on the nervous system. Given the question of habitat-size-VNC relationships, a multivariate analysis of the VNC would be beneficial to understand if there is environmental pressure for nervous systems. From there, understanding the specialization of movement across habitats and its effect on innervations or the connectome would be enlightening if there are noted habitat differences.

In addition, Schafer suggests the diversity seen in nematode proteins compared to more complex organisms is a result of their simpler nervous systems offsetting their restricted morphology (2016). It would be engaging to corroborate this idea through a comparison of Enoplid and Rhabditid proteomes given the data we have regarding higher Enoplean nervous system complexity.

2.6 CONCLUSIONS

This thesis set out to expand on previous work that established nematode ventral nerve cords are not conserved across the phylum as was previously thought, contribute to the lack of

basal clade nervous system data, as well as to examine comparative nerve cord development in the basal clades for the first time. Using the ventral nerve cord as a microcosm for nervous system complexity, the data gathered here confirms that nervous system evolution across nematodes is more complex than given credit and suggests that the Enoplean clades, long thought to be the closest representatives to nematode ancestors, have more neurons and no doubt more complex connectomes. This indicates there was a simplification of the nematode nervous system, potentially due to environmental pressures that need to be explored further. In addition, basal clade indeterminate adults also represent the evolutionary predecessors and it was discovered their neuron proliferation appears to be consistent with invariant individuals, suspending the notion that cell proliferation continues in indeterminate adults. Going forward, innervations in basal clades should be investigated to understand the evolutionary precursor to nervous system downsizing as well as the genetic influence responsible. Furthermore, additional examination of neuronal heterochrony in indeterminate individuals is called for given their looser developmental cascade than the *C. elegans* archetype. The research represented here and in the future is pivotal to our understanding of nervous systems as the study of nervous system evolution enables us to better discern the underpinnings of the nervous system in its totality and thereby apply that knowledge to modern neuroscientific challenges.

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