BEHAVIOURAL, ANATOMICAL AND PHYSIOLOGICAL EXAMINATIONS OF CRAB HOST-PARASITE INTERACTIONS

by

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Dedicated to my parents, Brian, Kimberly and Craig
& my grandparents, Margaret and Merlin
For their loving support and inspiring me to work hard

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ABSTRACT

The main objective of this thesis was to study crab host-parasite interactions though the examination of changes in behaviour, anatomy and physiological processes. Atlantic rock crabs (*Cancer irroratus*) infected with *Nectonema agile* and green crabs (*Carcinus maenas*) infected with *Profilicollis botulus* were subjected to predator evasion and aggression tests. Additionally, green crabs were tested for their righting response. Size (carapace width), body weight, testes weight and hepatopancreatic lumen areas were measured in rock crabs. Hepatopancreatic glycogen reserves were measured in green crabs. Parasitized rock crabs increased their speed in the aggression test and had significantly smaller testes than unparasitized counterparts. As *P. botulus* transitioned from acanthellae to cystacanths, they caused green crabs to increase time spent at the midline and decreased their speed, distance travelled and glycogen reserves. During the later cystacanth stage, these crabs righted themselves faster and had larger glycogen reserves than unparasitized crabs. These results provide important insight into host health and parasitic lifecycles.

LIST OF ABBREVIATIONS AND SYMBOLS USED

Abbreviation/Symbol	Definition
5-HT	5-hydroxytryptamine, serotonin
ANCOVA	Analysis of covariance
B-cell	Blister cell
BLAST®	Basic local alignment search tool
CAD	Canadian dollars
DFO	Fisheries & Oceans Canada
DNA	Deoxyribonucleic acid
EPA	Environmental Protection Agency
FAO	Food & Agriculture Organization of the United Nations
MUSCLE	Multiple sequence comparison by log-expectation
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
sp./spp.	Species/Multiple species
USD	United States dollars
ºC	Degrees in Celsius
~	Approximately
#	Number

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CHAPTER 1: INTRODUCTION

1.1 Introduction

The global crustacean industry includes the fishing and aquaculture of many species of prawn, shrimp, lobster and crab, totaling over $69.5 billion USD in 2017 (FAO 2020; Fisheries & Aquaculture Department 2020). The Canadian crustacean industry relies completely on wild fishery landings and was worth over $1.6 billion CAD in 2020 (DFO 2021). Atlantic Canadian fish and seafood, including decapod shellfish species, are a large export, with products being sold to the United States, China and the European Union (DFO 2022). Top production from this region includes the American lobster, snow crab and shrimp (DFO 2021). The North American fishing industry is threatened by disease that has already caused massive mortalities and losses, especially in the US (Shields 2011; Shields 2019). This is expected to increase as global air and seawater temperatures rise over the next several decades (Poulin & Mouritsen 2006). In the Long Island Sound estuary, the average bottom temperature has increased by 0.4 ºC to 1.6 ºC per decade (Groner et al. 2018). This warmer water, along with eutrophication and exposure to environmental toxins, has caused American lobsters within this area to develop newly emerging diseases and secondary infections responsible for a greater than 90% decline in the population (Shields 2011; Shields 2019). The snow crab fishery has experienced two major outbreaks of bitter crab disease along the coast of Newfoundland (Shields 2019). This disease, caused by the parasite *Hematodinium*, overwhelms the crab's cardiovascular and immune system through destruction of its haemocytes (Lee & Frischer 2004). Infected crabs become bitter in flavour and have significantly high mortalities, resulting in losses to the fishery (Shields et al. 2019). As water temperatures increase, the crab's molting activity is enhanced and the parasite becomes

more metabolically active, presumably making the crab more susceptible to infection (Lee &
Frischer 2004; Shields 2019).

The emergence of parasite-driven diseases is expected to continue to be detrimental to
crustacean industries. For this reason, studies investigating the interactions between these
organisms and their hosts are essential. This research project will examine host-parasite
interactions through behavioural, anatomical and physiological studies of parasitized crabs. The
main objectives of these studies are to gain a better understanding of the impact that parasites
have on the behaviour, anatomy and physiology of the Atlantic rock (*Cancer irroratus*) and
green crabs (*Carcinus maenas*) as indicators of the relationship between a decapod host and its
parasitic pathogens.

1.2 Atlantic rock crab

1.2.1 General characteristics and fishery

The Atlantic rock crab is found along the Atlantic coast of North America, from
Labrador, Canada to South Carolina, USA (DFO 2013). A smaller established population also
exists in Icelandic waters (Gíslason et al. 2014). Rock crabs inhabit the intertidal zone and
mesopelagic zone, to a depth of 575 m (DFO 2013). They live in many different substrates, with
a preference for sandy bottoms (DFO 2013). These crabs have a broad, oval carapace with nine
smooth teeth-like ridges on either side of their eyes (DFO 2000). Rock crabs grow by molting
their shells and are sexually dimorphic; with the males reaching sexual maturity at a carapace
width of 65-75 mm and the females reaching maturity at a width of 50-57 mm (DFO 2000; DFO
2013). Rock crabs mate in late summer and fall after the female has molted (DFO 2013). After
mating, the female releases 125,000 – 500,000 eggs and carries them beneath her abdomen for
about 10 months (DFO 2000; DFO 2013). These eggs hatch in mid-June and the planktonic

larvae finally settle to the bottom in mid-September (DFO 2000; DFO 2013). Rock crabs are mostly carnivorous and cannibalistic; consuming crustaceans, fish, mollusks and polychaetes (DFO 2013). They are very generalist eaters and will consume other prey such as mussels, starfish and sea urchins depending on availability and habitat (Scarratt & Lowe 1972; DFO 2013). Rock crabs are essential prey for the American lobster and are important for the lobster's molting, growth, condition and ovary development (Gendron et al. 2001; DFO 2013).

The rock crab fishery is a male-only fishery restricted to crabs that have a carapace width ≥ 102 mm. It takes approximately 5-6 years for rock crabs to reach the minimum commercial size (DFO 2000; DFO 2013). This fishery has existed largely as bycatch in the American lobster industry (DFO 2000; Bradt 2015); however, directed fisheries exist today (Bradt 2015). Fisherman do not receive a high price for rock crab, but the fishery is economically viable due to low operating costs and high catch rates (DFO 2000).

1.2.2 Behaviour

The agonistic behaviours toward conspecifics and heterospecifics is well described in rock crabs (Hobbs 2016). Rock crabs have shorter fights than lady crabs (*Ovalipes ocellatus*) and are more aggressive than the spider crab, *Libinia emarginata*, when fighting amongst themselves (Hobbs 2016). Rock crabs are often displaced out of their preferred substrate by grapsid crabs and remove their conspecifics from this substrate (Hobbs 2016). Large, intact rock crabs initiate contests with green crabs over scarce resources more frequently than smaller rock crabs in warmer water and win their contests much more than smaller rock crabs in any water temperature (Matheson & Gagnon 2012a). These larger rock crabs are more aggressive, resulting in more frequent contests between rock and green crabs. In laboratory experiments, small and medium sized rock crabs spend significantly more time buried in sediments or within sheltered

areas than large crabs when in competition with green crab (Matheson & Gagnon 2012a; Matheson & Gagnon 2012b). Juvenile rock crabs frequently shelter in spaces between rocks and underneath seaweed in their natural environment (McVean & Findlay 1979; Breen & Metaxas 2009).

1.2.3 Parasites of rock crabs

A few parasitic worm species have been found in rock crabs off the Atlantic coast of Canada (Leslie *et al*. 1981; Brattey et al. 1985). The most prevalent of these parasitic worms is a trematode *Microphallus* sp. (Brattey et al. 1985). Two other helminths, a *Nectonema* sp. and *Profilicollis* sp. infect very few crabs (Brattey *et al*. 1985). Bratty et al. (1985) did not observe any pathology associated with *Microphallus* sp. infection. The *Profilicollis* sp., likely *Profilicollis botulus*, however, embedded into and perforated crab intestines (Brattey et al. 1985). Leslie et al. (1981) observed gonadal deterioration in a few *Nectonema*-parasitized male crabs.

The parasites documented thus far in rock crabs collected along the Atlantic Coast of the United States are different from those along the coast of Atlantic Canada. Helminths of the genus *Profilicollis* have also been found in rock crabs in Maine, but these infections are with another *Profilicollis* species, *P. major* (Schmidt & MacLean 1978). This worm is associated with the proliferation of host cells in the connective tissue of the midgut and the infiltration of eosinophilic granulocytes. In addition, MacLean and Ruddell (1978) found the parasitic dinoflagellate *Hematodinium perezi* in a small number of rock crabs collected off of New York Bight. Infection with this parasite was associated with pink colouration in its hosts' tissues. This parasite is present in many hosts across numerous continents and causes fatal disease with no treatments (Small 2012). Crabs become lethargic as disease progresses through their hemolymph and tissues (Small 2012).

1.3 Nematomorpha (horsehair worms)

1.3.1 Phylum characteristics

Little is known about phylum Nematomorpha (Hanelt et al. 2005). The phylum is exclusively parasitic and is divided into two main groups: the gordiids and the nectonematids (Hanelt et al. 2005). The gordiids infect millipeds and insects such as mantids, beetles and crickets (Thomas et al. 2002; Chiu et al. 2015; Ernst et al. 2016; Chiu et al. 2020), while the nectonematids parasitize isopods and decapod crustaceans such as crabs, lobsters and shrimp (Ward 1892; Nielsen 1969; Leslie et al. 1981; Skaling & MacKinnon 1988; Schmidt-Rhaesa et al. 2013; Kakui et al. 2021). Nematomorphs are only parasitic during the juvenile stage and gordiids move from one host to the next via paratenesis (Hanelt et al. 2005). Juveniles develop in their host's hemocoel, absorbing nutrients through their body walls rather than through direct feeding (McDermott et al. 2010). Once mature, these worms leave their host to become aquatic and free-living (Hanelt et al. 2005; McDermott et al. 2010). After mating, the embryos are released from females and develop into infective larvae (McDermott et al. 2010).

1.3.2 Gordiids

Gordiids, or freshwater horsehair worms, are known for their ability to alter the behaviour of their host to benefit their lifecycle (Thomas et al. 2002; Ponton et al. 2011). The worm must manipulate its terrestrial host to move toward, or within, water so it can emerge from the animal and become a free-living adult (Hanelt et al. 2005). The cricket, *Nemobrius sylestris*, has been shown to be more likely to jump into water in response to infection (Thomas et al. 2002). This behaviour may be due to the hairworm inducing directed responses to light; parasitized crickets direct their walk to a light stimulus (Ponton et al. 2011). When a similar test is done on horsehair worm-parasitized mantids, they are shown to be specifically attracted to the

horizontally polarized light associated with water (Obayashi et al. 2021). Studies have revealed that gordiids produce mimetic molecules that act directly on their host to induce the differential expression of proteins related to neurogenesis, visual processes, signalling and neurotransmitter activities; these may be involved in behavioural manipulation (Biron & Loxdale 2013).

The freshwater horsehair worm impairs the reproductive ability of its host and reduces its survival (Biron et al. 2005; Barquin et al. 2015). Male crickets infected with gordiids undergo parasitic castration (Biron et al. 2005) and spend less time mate-calling during later stages of infection (Barquin et al. 2015). Female crickets, on the other hand, form ovaries and produce eggs (Biron et al. 2005). Male mantids become partially feminized by their worm parasite (Chiu et al. 2015). The testes of highly infected males disappear or shrink in size.

1.3.3 Nectonematids

Very little is known about the lifecycle of the marine horsehair worm. Only one genus, *Nectonema*, has been identified, despite being widespread throughout the world (Nielsen 1969; Leslie et al. 1981; Oku et al. 1983; Poinar Jr. & Brockerhoff 2001). Ward (1892) observed that *N. agile* only had one host in its entire lifecycle, unlike that of freshwater hairworms. Nielsen (1969) described a species in Sweden, *N. munidae*, and found that at the end of its parasitic juvenile stage, its gonads matured, and natatory bristles formed under its final juvenile cuticle. The cuticle is then shed, and the adult penetrates the thin dorsal membrane between the posterior margin and abdomen of its squat lobster host to emerge as a free-living adult. It is assumed that all members of the genus follow a similar process of development. An intestine of a *Nectonema* sp. infecting the shrimp, *Pandalus montagui*, was investigated and found to have absorptive and secretory cells (Skaling & MacKinnon 1988). These, along with acid and alkaline phosphatases, as well as non-specific esterases found within the body wall are believed to be involved in the

absorption of nutrient substances. No paratenic hosts are known to exist for *Nectonema* spp. (Hanelt et al. 2005).

The marine horsehair worm has been associated with gonadal degeneration in some decapod hosts (Leslie et al. 1981; Born 1967). Some male rock crabs from Nova Scotia had deteriorated testes when infected by a *Nectonema* sp. (Leslie et al. 1981). The shrimp, *Palaemonetes vulgaris*, is a host for *N. agile* in Massachusetts and has been shown to have atrophied, oocyte-absent, opaque ovaries, when parasitized (Born 1967). In comparison to this, a juvenile female American lobster infected with the same species in New Brunswick was found to have normal, developing ovaries and no signs of internal damage (Schmidt-Rhaesa et al. 2013).

1.4 Green crabs

1.4.1 General characteristics

The green crab is a prolific and successful invasive species native to Atlantic Europe with non-indigenous populations in the northwestern Atlantic, northwestern Pacific, Australia, Argentina, and South Africa (Young & Elliot 2020). Their success can be attributed to their wide temperature and salinity tolerance, as well as their generalist omnivorous diet (Young & Elliot 2020). Despite this, they are limited to temperate coastlines for breeding and larval development and are commonly found in wave-protected sheltered bays, harbours, and estuaries (Young & Elliot 2020). These crabs live 5-7 years in their native and western Atlantic range and reach sexual maturity at the age of 2-3 years, or a carapace width of 22-40 mm for females and 21.33-34 mm for males in the northwestern Atlantic (US EPA 2017; Young & Elliot 2020). Both juvenile and adult crabs are found in the intertidal zone; adults are found in the lower intertidal and subtidal zones, often at a depth of 6-7 m (Young & Elliot 2020). Their diet consists of clams, mussels, cockles, periwinkles, common brown shrimp, juvenile lobsters, polychaetes,

amphipods, and other crabs, including conspecifics (Young & Elliot 2020). Green crabs mate in July-October in both their northwestern and native ranges once the female has recently molted (Klassen & Locke 2007; Young & Elliot 2020). Once fertilized, a female can store spermatophores for 4.5 – 12 months and releases larvae from August to December (Klassen & Locke 2007; Young & Elliot 2020). One female can produce 1-2 clutches per year, each consisting of between 4,781-185,000 eggs, carried on swimmerets for up to several months (Klassen & Locke 2007; Young & Elliot 2020).

1.4.2 Behaviour and impact

Green crabs are quite aggressive, likely leading to frequent agonistic associations with conspecifics (Young & Elliot 2020). These crabs also compete with grapsid crabs (*Hemigrapsus* spp.) over both food and shelter (Jensen et al. 2002). The green crab dominates one grapsid species over the other when it comes to food but is consistently outcompeted when it comes to shelter. Like rock crabs, juvenile green crabs tend to avoid agonistic interactions and are found in spaces between rocks and underneath seaweed, moving inside of shelters when disturbed (McVean & Findlay 1979; Breen & Metaxas 2009). Green crabs have caused considerable damage to blue mussel, American oyster, Atlantic rock crab and American lobster populations along the northwestern coast and have destroyed valuable eelgrass beds (US EPA 2017; Young & Elliot 2020). Garbary et al. (2014) determined that green crab activity had led to the extensive decline of an eelgrass bed in Nova Scotia, related to their foraging behaviour. When foraging, the crab uproots sediments in intertidal mudflats, cutting and tearing eelgrass shoots' sheath bundles (US EPA 2017; Garbary et al. 2014). Sixty percent of commercially fished marine finfish species use eel grass beds as nurseries for their juvenile stages, so the impact of eel grass destruction extends beyond intertidal invertebrate populations.

1.4.3 Parasites of green crabs

Green crabs serve as hosts to a wide array of parasites. However, the number of species that infect these crabs and the prevalence of infection is lower in introduced populations of crabs when compared to those found in their native range (Blakeslee et al. 2009; Zetlmeisl et al. 2011; Bojko et al. 2018). This is a well described phenomena in invasive populations since not all pathogens in a population get transported to the region of introduction. Helminth parasites infecting green crabs in their native range include *Profillicolis botulus, Maritrema portucalensis, Microphallus* spp., cestodes and nemerteans (Coe 1902; Stentiford & Feist 2005; Blakeslee et al. 2009; Pina et al. 2011; Bojko et al. 2018). Only 3 species of helminth have been reported to infect crabs in introduced regions: *P. botulus, Microphallus similis* and an unindentified larval nematode (Brattey et al. 1985; Blakeslee et al. 2009; Blakeslee et al. 2015; Bojko et al. 2018). *Profilicollis botulus* elicits a melanization response when infection breaches gut epithelium in crab intestines (Bojko et al. 2018). No observable immune response has been found in other helminth infections in green crabs (Coe 1902; Brattey et al. 1985; Stentiford & Feist 2005; Pina et al. 2011; Blakeslee et al. 2015; Bojko et al. 2018).

Non-helminth parasites infecting green crabs in their native range include gregarines, the microsporidian *Ameson pulvis*, *Haplosporidium* spp., *Hematodinium* spp., *Sacculina carcini* and isopods (Stentiford & Feist 2005; Bojko et al. 2018; Davies et al. 2019; Davies et al. 2020). Only 2 non-helminths have been reported in the crab's introduced range: the microsporidian *Parahepatospora carcini* and an unidentified amoeba (Bojko et al. 2017; Bojko et al. 2018). *Ameson pulvis*, a *Hematodinium* sp. and *Sacculina carcini* elicit observable host immune responses (Stentiford & Feist 2005; Bojko et al. 2018). *Hematodinium* sp. infecting crabs of the Faroe Islands colonize the hemolymph and turn it opaque and white (Bojko et al. 2018). *Ameson*

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pulvis and *S. carcini* are both associated with melanization (Stentiford & Feist 2005; Bojko et al. 2018). *Ameson pulvis* spores are phagocytized and melanized by their host, and *S. carcini* rootlets are encapsulated and melanized (Stentiford & Feist 2005; Bojko et al. 2018). Despite the observable immune response to *S. carcini*, the parasite does eventually overcome its host's defenses; Goddard et al. (2005) reported that *S. carcini*-infected crabs died at twice the rate of uninfected crabs.

1.5 Acanthocephala

1.5.1 Phylum characteristics

Acanthocephalans attach to the intestinal walls of their intermediate and definitive hosts via an eversible hooked proboscis (McDermott et al. 2010). They absorb predigested nutrients from their host across their body wall (McDermott et al. 2010; Richardson 2013). They mate and reproduce in their definitive vertebrate host, releasing their eggs in the host's feces (McDermott et al. 2010). These eggs contain the acanthor stage, which are then consumed by the intermediate arthropod hosts (McDermott et al. 2010; Richardson 2013). Once ingested, the acanthor penetrates the intestine, where it transitions from a non-infective acanthella into the infective stage, a cystacanth (McDermott et al. 2010; Richardson 2013; Schmidt & Nickol 1985). At this stage, it is transmitted to the definitive host when the intermediate host is consumed (McDermott et al. 2010; Richardson 2013). Like freshwater nematomorphs, acanthocephalans may make use of paratenic hosts in their lifecycle (Richardson 2013). When a cystacanth is ingested by a paratenic host it penetrates the intestinal wall and re-encysts until a definitive host ingests the infected paratenic host (Richardson 2013).

Host pathology is often due to mechanical trauma from attachment and penetration of the proboscis into the intestine (Richardson 2013). Chronic inflammation at the site of damage

results in local fibrosis and nodule formation (Richardson 2013). Acanthocephalans may cause a humoral immune response in their vertebrate host; however, this response does not appear to serve any protection against the parasites (Richardson 2013).

1.5.2 *Profilicollis* spp. and *P.botulus*

Profilicollis spp. frequently use brachyurans as their intermediate hosts. Research on these species has focussed on their manipulation of crab behaviour from a neurotransmitter and conspicuous standpoint (Latham & Poulin 2001; Latham & Poulin 2002; Poulin et al. 2003; Rojas & Ojeda 2005; Kolluru et al. 2011; Loh 2017). Dopamine has been implicated in parasitism of *Hemigrapsus crenulaus*, while serotonin is associated with parasitism in *Macrophthalmus hirtipes* (Poulin et al. 2003; Rojas & Ojeda 2005). Heavy infections of *Profilicollis* spp. are linked to increased burrowing time and increased exposure to predators in the *Emerita analoga* and *M. hirtipes*, respectively (Latham & Poulin 2002; Kolluru et al. 2011).

One of the most important and widespread *Profilicollis* spp. is *P. botulus*. This acanthocephalan parasitizes common eider ducks (*Somateria mollissima*) from the eastern and western Atlantic, to the northeastern Pacific and the Arctic Oceans (McDermott et al. 2010). The common eider is its primary definitive host (McDermott et al. 2010). *Profilicollis botulus* parasitizes this eider at high incidences (76.9-92%) and causes observable, yet non-lethal damage to host intestines in the form of small nodules (Bishop & Threlfall 1971; Liat & Pike 1980; Borgsteede et al. 2005). Several intermediate hosts exist for *P. botulus*, including the green crab, Atlantic rock crab, velvet swimming crab, great spider crab and the grapsid crabs *Hemigrapsus oregonensis*, *H. sanguineus* and *H. takanoi* (Brattey et al. 1985; Ching 1989; Christiansen et al. 2009; McDermott et al. 2010; Goedknegt et al. 2017). Other hosts include the American lobster and the anomurans *Pagurus pubescens* and *Paralithodes camtshaticus* (Brattey & Campbell

1986; McDermott et al. 2010). The green crab and great spider crab are the main intermediate

hosts of *P. botulus* in Northern Europe and the Arctic region (McDermott et al. 2010).

1.6 Parasite manipulation of behaviour

1.6.1 Evolutionary significance

Parasites with complex lifecycles are believed to have the ability to control the behaviour

of their hosts to aid in the transmission from one organism to the next (Moore 1995; Hughes &

Libersat 2019). Parasitism is a form of feeding that has evolved more frequently across history

than carnivory or herbivory, with as many as half of all known species being parasitic (Hughes &

Libersat 2019). Most parasites are easily transmitted from one host to another without

behavioural manipulation (Hughes & Libersat 2019). There are a few cases, however, when

manipulation increases the likelihood of parasite transmission to ensure the propagation of the

parasite's genes and continued existence of the species (Hughes & Libersat 2019).

1.6.2 Amphipod behavioural manipulation

1.6.2.1 Neuromodulation

The main theory behind behavioural manipulation is the alteration of neuromodulators

(Adamo 2002). Parasites may not only suppress their host's immune system but also induce it to

release neuromodulatory compounds (Adamo 2002). One of the most investigated crustacean-

parasite relationships in this field of study are acanthocephalan-infected amphipods (Perrot-

Minnot & Cezilly 2013). The neuromodulatory systems underlying such manipulation in

acanthocephalan-parasitized crustaceans are hypothesized to involve serotonin (Perrot-Minnot &

Cezilly 2013). Injection of serotonin (5-HT) in the gammarid *Gammarus lacustris* matches the

clinging behaviour seen in acanthocephalan-infected individuals (Helluy & Holmes 1990).

Ventral nerve cords of *G. lacustris* also show a greater amount of serotonin storage when infected with *Polymorphus paradoxus* (Maynard et al. 1996). When serotonin is injected into the gammarids *G. pulex*, *G. roeseli*, and *G. fossarum*, natural photophobic behaviour is reversed, mimicking acanthocephalan-influenced modifications that are expected to increase the hosts' susceptibility to predation (Tain et al. 2006; Tain et al. 2007; Perrot-Minnot et al. 2014).

1.6.2.2 Behavioural manipulation

Many studies on parasitized amphipods investigate behavioural manipulation by acanthocephalan parasites, with the vast majority focussing on *G. pulex*. This manipulation involves changes in the anti-predator responses of the gammarid (Kaldonski et al. 2007; Dianne et al. 2011; Durieux et al. 2012; Baldauf et al. 2007; Bailly et al. 2018). *Pomphorhynchus laevis*-infected *G. pulex* are more susceptible to predation by bullhead fish, even when a refuge is available (Kaldonski et al. 2007). A large proportion of infected *G. pulex* will remain outside of the refuge, despite the presence of their predator in the same environment (Kaldonki et al. 2007). A clear difference in geotactic behaviour is seen in *G. pulex* when infected by different developmental stages of *Polymorphus minutus* (Bailly et al. 2018). When the parasite is in its non-infective stage (acanthella), it induces a stronger positive geotaxis, making it less susceptible to predation (Bailly et al. 2018). In comparison, when the parasite is in its infective stage (cystacanth), it causes its host to display negative geotaxis, exposing the gammarid to predators (Bailly et al. 2018). The acanthella stage of *P. laevis* also is associated with less predation upon its host, as well as a decrease in activity and food intake (Dianne et al. 2011; Dianne et al. 2014). Acanthella -infected *G. pulex* hide in refuges much more than uninfected individuals and are less predated upon by trout (Dianne et al. 2011). This behaviour changes when the parasite enters the cystacanth stage and anti-predator behaviour is reversed (Dianne et al. 2011). In the same

parasite-host system, parasitism is associated with a decrease in attraction to conspecifics and a

reversal of natural photophobia in the presence of a predator cue (Durieux et al. 2012). The faster

the growth of a cystacanth, the more quickly it can induce phototaxis reversal in its *G. pulex* host

(Franceschi et al. 2010).

Other acanthocephalan-parasitized amphipods include *Hyalella azteca, Echinogammarus*

stammeri, Gammarus fossarum, G. roeseli and *G. pseudolimnaeus* (Maynard et al. 1998; Bauer

et al. 2005; Lewis et al. 2012; Perrot-Minnot et al. 2014; Stone & Moore 2014; Labaude et al.

2017a; Labaude et al. 2017b). When *Leptorhynchiodes thecatus*-infected *H. azteca* are presented

with both alarm pheromones from conspecifics and kairomones from a predator, they are more

active and spent less time on their refuge (Stone & Moore 2014). A reversal of anti-predator

behaviour manifests in *P.laevis*- infected *E. stammeri* as an increase in preference for lighted

environments and increase in activity levels (Maynard et al. 1998). When *G. fossarum* is infected

by *Pomphorhychus tereticollis*, its parasite-induced photophila increases with increasing

temperature (Labaude et al. 2017a). Temperature, however, does not affect manipulated refuge

use or geotactic behaviour (Labaude et al. 2017a). Like *G. pulex, P.minutus*-infected *G. roeseli*

display negative geotaxis, swimming to the top of the water column, but do so at a lower

magnitude (Bauer et al. 2005). When infected by *Corynosoma* sp., *G. pseudolimnaeus* avoids

aggregating with conspecifics; this may increase predation upon individual infected gammarids

(Lewis et al. 2012).

1.6.3 Crab behavioural manipulation

1.6.3.1 Neuromodulation

Studies on neuromodulation of acanthocephalan-infected crabs have investigated the crab

species *Macrophthalmus hirtipes*, *Uca spinicarpa* and *Hemigrapsus crenulatus* (Poulin et al.

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2003; Rojas & Ojeda 2005; Pérez-Campos et al. 2012). *Macrophthalmus hirtipes* co-infected with both the acanthocephalan *Profilicollis* spp. and the trematode *Maritrema* sp. have an increase of serotonin content in their brain (Poulin et al. 2003). The fiddler crab, *U. spinicarpa*, also exhibits higher hemolymph serotonin levels when infected by *Hexaglandula corynosoma*; this association increases with the intensity of infection (Pérez-Campos et al. 2012). Dopamine levels do not change between infected and uninfected crabs (Pérez-Campos et al. 2012). In contrast, *H. crenulatus* infected with *Profilicollis antarcticus* shows an increase in hemolymph dopamine content (Rojas & Ojeda 2005).

1.6.3.2 Behavioural manipulation

As with amphipod-parasite relationships, investigated crab-parasite relationships also largely involve acanthocephalans. Like with amphipods, predator evasion is the commonly modified behaviour in acanthocephalan-infected crabs. *Macrophthalmus hirtipes* serves as an intermediate host to *Profilicollis* spp. and higher infection levels are found in those crabs exposed to predators (Latham & Poulin 2001; Latham & Poulin 2002). Despite this, infection intensity in *M. hirtipes* does not influence its response to simulated predator approach (Latham & Poulin 2001). *Hexaglandula corynosoma*-infected *Uca spinicarpa* show a similar exposure behaviour to *M. hirtipes* (Pérez-Campos et al. 2012). These crabs are found outside their burrows more often than uninfected crabs (Pérez-Campos et al. 2012). As previously mentioned, *Emerita analoga* burrows more slowly into its environment when infected by *Profilicollis altmani*, making it potentially more vulnerable to predators (Kolluru et al. 2011). *Profilicollis antarcticus*-infected *Hemigrapsus crenulatus* crabs exhibit higher metabolic rates and corresponding increases in activity and excitability (Haye & Ojeda 1998). It is unclear whether this has an influence on predator evasion.

Investigations of the parasite-crab relationships have also involved the Atlantic mole crab, *Emerita talpoida*, co-infected by a *Profilicollis* sp. and a trematode *Microphallus* sp. and found some behavioural modifications associated with trematode infection intensity (Loh 2017). Crabs exhibiting rhythmic behaviour have higher trematode infection intensities than arrhythmic crabs but no difference in this behaviour is seen in acanthocephalan infection intensities (Loh 2017). In one study, long-term *Microphallus similis* infection in green crabs increases their feeding time; this change in behaviour increases with infection intensity (Blakeslee et al. 2015). In earlier stages of infection, a short-term increase in righting response time occurs in these crabs (Blakeslee et al. 2015). A later study looking into this parasite-host relationship found no influence of infection on feeding behaviour or righting response (Ro et al. 2022).

1.7 Effects of parasitism on anatomy and physiology

1.7.1 Amphipod-parasite interactions

Pomphorhynchus laevis-infected gravid female *Gammarus pulex* have lower lipid contents than uninfected gravid females (Plaistow et al. 2001). This relationship is not seen in non-gravid or male *G. pulex* (Plaistow et al. 2001). However, both parasitized males and females (gravid and non-gravid) do display increases in glycogen content (Plaistow et al. 2001). When experimentally fed a protein deficient diet, infected *G. pulex* have a lower metabolic rate (Labaude et al. 2015). In comparison, both sexes of *G. roeseli* infected with *Polymorphus minutus* have decreased lipid and protein contents and increased glycogen content (Gismondi et al. 2012). Antioxidant defense may be lower in the host due to lower glutathione and γ-glutamylcysteine ligase activity (Gismondi et al. 2012). When infected by the acanthocephalan, *Corynosoma sp.*, an elevated concentration of estrogen and decreased concentrations of testosterone are seen in male *G.pseudolimnaeus* (Lewis et al. 2016). This could be an example of

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male feminization normally seen in parasitized crab species (Okada & Miyashita 1935; Yang et al. 2014; Fazhan et al. 2020).

1.7.2 Crab-parasite interactions

1.7.2.1 Metabolic and immune effects

Metabolic effects have been investigated in *Profilicollis* and *Sacculina*-parasitized crabs. *Emerita analoga* infected by *Profilicollis altmani* have a lower metabolic rate (Figueroa et al. 2019). In contrast, *P. antarticus* causes its host, *Hemigrapsus crenulatus*, to have a higher metabolic rate (Haye & Ojeda 1998). This is proposed to be due to a difference in the life stage or parasite infection intensity of the crabs investigated (Figueroa et al. 2019). The crabs investigated by Figueroa et al. (2019) were juveniles with low infection intensities, while those of Haye & Ojeda (1998) were adults with higher intensities. Green crabs parasitized by *Sacculina carcini* have decreased numbers of reserve inclusion bodies (Stentiford & Feist 2005). Metabolic rate has not been looked at in this association, but it would be expected to be higher due to evidence of increased energy store usage.

Immune effects have been investigated in trematode-crab associations. Soon after parasitism is established *Microphallus similis*, green crabs exhibit a short-lived, but marked drop in circulating haemocytes (Blakeslee et al. 2015). Green crabs infected by *M. primas* do not elicit an acute immune response to this parasite but are believed to have stunted haemocyte movement within their gill lamellae (Stentiford & Feist 2005). Another trematode, *Maritrema novazealandensis*, increases the haemocyte count of its *Macrophthalamus hirtipes* host (Koehler & Poulin 2012).

1.7.2.2 Reproductive effects

One of the most well-studied crab-parasite interactions are those involving rhizocephalan barnacle parasitism. Green crabs infected by *S. carcini* have lower testes weight than uninfected crabs in their native range (Zetlmeisl et al. 2011). Infected female crabs display atresia and arrest of oocyte development in the pre-vitellogenic phase, as well as a noticeable immune response against the parasitic invasive rootlets (Stentiford & Feist 2005). Invasive green crabs in California appear to have unhindered reproduction and can produce reproductive sacs; infected native crabs, *Hemigrapsus oregonensis, H. nudus, Pachygrapsus crassipes and Cancer magister*, are unable to produce this structure (Goddard et al. 2005). *Sacculina beauforti* infection in *Scylla olivacea* effects both sexes, arresting spermatogenesis in testes and severely damaging the structure of the ovaries (Fazhan et al. 2020). Male rhizocephala-parasitized *Eriocheir* spp. experience an intersex condition, resulting in a reduction in size, broadening of the abdomen and reduction of testes and copulatory styles (Okada & Miyashita 1935; Tang et al. 2005; Li et al. 2011). In the blue crab, *Callinectes sapidus*, rhizocephalan parasitism results in smaller gonad size, and the broadening of the abdomen in both males and some females (Reinhard 1950).

The bopyrid isopod, *Allokepon hendersoni*, has been reported as decreasing the oocyte count in its host, *Charybdis bimaculata* and, unlike with rhizocephalan infection, causes a decrease in the abdominal flap size of its male host (Corral et al. 2021). Also, in contrast to rhizocephalans, the parasites *Microphallus nicolli* and *P. atlmani* do not affect egg production in female *Emerita analoga* (Constancio 2011; Sandhu 2017; Bhaduri 2020). As previously mentioned, *N. agile* may cause gonad degeneration in male Atlantic rock crabs, however this occurrence appears to be relatively rare (Leslie et al. 1981).

1.8 Project Rationale

As the average global temperature rises, the prevalence and incidence of parasitic diseases of shellfish is expected to increase. Due to the commercial and cultural importance of shellfish fisheries and aquaculture production, it has become important to investigate the potentially harmful relationships that decapod species have with other organisms, from a behavioural, anatomical and physiological standpoint. The main goal of this project is to build upon previous parasitic research.

1.9 Objectives

1. To determine whether *N. agile* and *P. botulus* influence the behaviour of their Atlantic rock and green crab hosts.

2. To quantify hepatopancreatic glycogen reserves and lumen area/to total tubule area ratios in *P. botulus* and *N. agile*-parasitized and non-parasitized green and rock crabs, respectively.

3. To determine if *N. agile* infection alters the hepatopancreas and body size, body weight and testes weight/to carapace width ratio of their rock crab host.

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CHAPTER 2: THE IMPACT OF *NECTONEMA AGILE* INFECTION IN THE ATLANTIC ROCK CRAB, *CANCER IRRORATUS*

2.1 Introduction

The Atlantic rock crab (*Cancer irroratus*) is native to the Atlantic coast of North America; from Labrador, Canada to South Carolina, USA (DFO 2013). This crab inhabits many different substrates within the intertidal and mesopelagic zones down to a depth of 570 m (DFO 2013). Rock crabs are generalist feeders, consuming many different prey species depending on availability and habitat (Scarratt & Lowe 1972; DFO 2013). Their prey includes crustaceans, fish, mollusks, polychaetes and echinoderms (Scarratt & Lowe 1972; DFO 2013). The American lobster (*Homarus americanus*) feeds on rock crabs, gaining essential nutrients from it for molting, growth, condition and ovary development (Gendron et al. 2001; DFO 2013). Most parasites affecting rock crabs are helminth species. The most prevalent of these helminths in Eastern Canada is a trematode, *Microphallus* sp. (Brattey et al. 1985). *Profilicollis* sp. and *Nectonema* sp. also infect rock crabs at a low prevalence in this area (Brattey et al. 1985).

Nectonema spp. are members of the phylum Nematomorpha, or horsehair worms (Hanelt et al. 2005). These worms consist of two main groups: the freshwater gordiids and the marine nectonematids (Hanelt et al. 2005). *Nectonema* spp. belong to the latter group (Hanelt et al. 2005). The gordiids infect insects and millipeds (Thomas et al. 2002; Chiu et al. 2015; Ernst et al. 2016; Chiu et al. 2020). In contrast, the nectonematids parasitize decapod and isopod crustaceans (Ward 1892b; Nielsen 1969; Leslie et al. 1981; Skaling & MacKinnon 1988; Schmidt-Rhaesa et al. 2013; Kakui et al. 2021). Nematomorphs are parasitic as juveniles and gordiids often move from one host to the next via paratenesis (Hanelt et al. 2005). Juvenile worms develop within their host's haemocoel and absorb host nutrients through their body wall

(McDermott et al. 2010). Once mature, they leave the host to live as a free-living, aquatic adult (Hanelt et al. 2005; McDermott et al. 2010). These adults then mate, and embryos are released from females to develop into infective larvae (McDermott et al. 2010).

Gordiids can alter their host's behaviour to benefit their lifecycle (Thomas et al. 2002; Ponton et al. 2011). This worm manipulates its terrestrial host to move toward, or within, freshwater when transitioning from its juvenile stage to its adult stage (Hanelt et al. 2005). Both infected crickets (*Nemobius sylvestris*) and infected arboreal mantids (*Hierodula patellifera*) are attracted to water (Thomas et al. 2002; Obayashi et al. 2021). This behaviour appears to be due to the parasite inducing directed responses to light (Ponton et al. 2011; Obayashi et al. 2021). In infected arboreal mantids, this is related to an attraction to horizontally polarized light reflected by the water surface (Obayashi et al. 2021). In terms of anatomical and physiological impairments, gordiids impair the reproductive ability of their host and reduce their survival (Biron et al. 2005; Barquin et al. 2015). Male *N. sylvestris* crickets undergo parasitic castration during horsehair worm infection, while infected male *Acheta domesticus* crickets spend less time mate-calling in later stages of infection (Biron et al. 2005; Barquin et al. 2015). In contrast, female *N. sylvestris* can still form ovaries and eggs (Biron et al. 2005). Horsehair worms cause male mantid *Hierodula formosana* to become partially feminized and the testes of the most infected males disappear or shrink in size (Chiu et al. 2015).

Less information is known about nectonematids. This group consists of one genus, *Nectonema,* and is widespread throughout the world (Nielsen 1969; Leslie et al. 1981; Oku et al. 1983; Poinar Jr. & Brockerhoff 2001). Unlike gordiids, no paratenic hosts are known to exist for *Nectonema* spp. and Ward (1892b) noted that *N. agile* has only one known host in its entire lifecycle. While no manipulation in host behaviour has been reported by *Nectonema* spp., they

have been associated with gonadal degeneration in some decapods (Pérez 1927; Nouvel & Nouvel 1934; Born 1967; Leslie et al. 1981). Very few *Nectonema* sp.-parasitized rock crabs (4.35 % of total parasitized) from Nova Scotia have been found thus far with deteriorated testes (Leslie et al. 1981). Infection by *Nectonema agile* in female *Palaemonetes vulgaris*, *Palaemon serratus* and *Anapagurus hyndmanni* has generally caused ovarian atrophy and stunted oocyte development (Pérez 1927; Nouvel & Nouvel 1934; Born 1967).

Behavioural manipulation is used by some species of parasites to either increase host survival, increase the chance that the parasite will be transmitted from one host to the next, or both (Hafer 2016). Some parasites decrease the chance of intermediate host or vector predation during development of non-infective parasite life stages (Hafer 2016). While this predation suppression has not been explored in Nematomorpha, it has been demonstrated by other helminth species, including members of Nematoda and Acanthocephala. Copepods containing *Camallanus lacustris* not yet in their infective stage are less likely to be predated upon by sticklebacks than non-parasite containing conspecifics (Weinreich et al. 2013). Acanthella-stage *Polymorphus minutus* and *Pomphorhynchus laevis* cause the amphipod, *Gammarus pulex*, to sink lower in the water column and retreat more frequently into refuges, decreasing exposure to predators (Dianne et al. 2011; Bailly et al. 2018).

Many decapod crustaceans, including crabs, are cannibalistic (Romano & Zeng 2017). Cannibalism often occurs during aggressive encounters and can be an issue in aquaculture (Romano & Zeng 2017). While cannibalism is not problematic for freshwater horsehair worms, who use paratenic hosts, it may be problematic to the marine horsehair worm, who only has one host throughout its lifecycle (Hanelt et al. 2005). Therefore, it would be beneficial for *Nectonema* to manipulate not only its host's predator evasion behaviour but also its level of

aggression. Changes in aggression have not been thoroughly researched in Nematomorpha-host

relationships but have been investigated in Nematoda-host associations. Horned passalus beetles

(*Odontotaenius disjunctus*) infected with *Chondronema passali* show a modest reduction in

fighting ability and *Myrmeconema neotropicum*-infected tropical ants (*Cephalotes atratus*) with

higher parasite infection intensities do not bite or produce detectable alarm pheromones

(Yanoviak et al. 2008; Vasquez et al. 2015).

2.2 Objectives

Despite the discovery of a *Nectonema* sp. in Atlantic rock crabs in Eastern Canada, the

parasite has yet to be identified to the species level. The first objective of this study was to

identify the species of *Nectonema* parasitizing rock crabs collected from the Northumberland

Strait via DNA sequencing. The second objective of this study was to determine whether this

Nectonema sp. alters predator evasion behaviour and aggression in its crab host. In earlier

studies, decapods have shown anatomical alterations when infected by parasites of this genus.

The third objective of this study was to determine if parasitism by *Nectonema* sp. impacts body

size, body weight, testes weight/to carapace width ratio, hepatopancreas size and hepatopancreas

lumen area/to total tubule area ratio in rock crabs collected from the Northumberland Strait.

2.3 Hypotheses

1. The *Nectonema* sp. in this study will be identified as *Nectonema agile* based on previous

studies done along the coast of eastern North America.

2. This *Nectonema* sp. will increase predator evasion behaviour and decrease aggression in its

rock crab host to protect its host as well as itself.

3. The *Nectonema* sp. will decrease the body size, body weight, testes weight/to carapace width ratio and hepatopancreas size and increase the hepatopancreas lumen area/to total tubule area ratio of infected rock crabs.

2.4 Materials and Methods

2.4.1 *Nectonema* sp. identification

   DNA was extracted from 25 mg of a 100% ethanol-preserved specimen of *Nectonema* sp. from a Borden-Carleton, Prince Edward Island, rock crab using a Qiagen DNeasy® Blood & Tissue Kit. After extraction, the quality and quantity of DNA were determined using a Nanodrop ND-1000 Spectrophotometer. The DNA was then amplified using CAS2 and CASIS primers (5' – ACG-GGC-GGT-GTG-TAC-AAA-GG – 3'& 5'- GGA-ATT-GAC-GGA-AGG-GCA-CC – 3', respectively; Le Roux et al. 1999) and a Taq-polymerase chain reaction (PCR). A 10 µl reaction mixture consisting of 5 µl iTaq™ Universal SYBR® Green Supermix, 0.2 µl each primer, 2 µl DNA template, and 2.6 µl molecular water was used. The reaction conditions were: 92º C for 3 min, 35 cycles of 94º C for 1 min, 50º C for 1 min and 72º C for 1 min, and 72º C for 5 min. The PCR products were then electrophoresed on a 2 % agarose gel at 70 V for 45 min. PCR product was removed from the gel and purified using Omega Bio-Tek's MicroElute® Gel Extraction Kit before being sent to Génome Québec for Sanger sequencing. Once sequenced, a 750 bp section of high quality linear 18S rRNA was used for species identification through a standard nucleotide BLAST® (https://blast.ncbi.nlm.nih.gov/Blast). A positive identification was made if percent identity was ≥ 99 %.

2.4.2 Phylogenetic analysis

Once the *Nectonema* sp. was identified, the corresponding 18S rRNA sequence from the National Center for Biotechnology Information (NCBI's) GenBank was aligned with MUSCLE in Mega-X (Kumar et al. 2018) with that of a *Nectonema* sp. that parasitizes isopods (GenBank: LC605988.1), 23 gordiid species and eight Nematoda species. The Best-Fit DNA Model for phylogenetic analysis was determined and a maximum-likelihood tree was constructed using these sequences. The tree was made using 1000 bootstrap replications with partial deletion and a site coverage cut-off of 95%. The eight Nematoda species were used as an outgroup to root the tree and the bootstrap cut-off value was set to 50%.

2.4.3 Animal collection and husbandry

One hundred three rock crabs were caught using Promar collapsible crayfish trays and Russel green crab traps in Borden-Carleton in Prince, Edward Island, Canada (46.24910786374199, -63.702706905230926); an area known for higher *Nectonema* sp. prevalence (Baker 2019; personal observations). These crabs were then transported and housed at Dalhousie University's Agricultural Campus (Bible Hill, Nova Scotia) in a drip-tray system with cooled and recirculated natural seawater (12 ± 1 ºC, pH 7 ± 1) and a 12:12 light/dark cycle. They were fed small pieces of mackerel (*Scomber scombrus*) every two weeks over the study period.

2.4.4 Behaviour tests

A mix of male and female crabs were measured (carapace width), weighed (g) and tested for predator evasion behaviour and aggression. On a testing day, crabs were taken out of their recirculated system and kept in their tray until tested. Tested crabs were then placed in a separate

dry drip tray from untested crabs. At the end of each testing day, all crabs were placed in their original tray and left for 30 min before being placed back into the system.

2.4.4.1 Predator evasion

This test was conducted over a four-day period on 89 crabs (49 ♂, 40 ♀) using a modified version of the test from Hamilton et al. (2016). An acrylic tank (60 cm x 30 cm x 30 cm, l x w x h) divided in half by a dark zone (black) and a light zone (white) was filled with 8 ± 1 cm of new natural seawater (4 ± 1°C). Crabs were individually placed along the line dividing the dark and light zones and immediately recorded for 5 min using a Logitech HD camera. Time spent in the dark zone was measured as predator evasion behaviour and time in the light zone was measured as exploratory (non-evasive) behaviour. Water was changed between each crab to avoid residual chemical signals being passed from one crab to the next.

2.4.4.2 Aggression

Aggression was tested on 82 crabs (45 ♂, 37 ♀) over a ten-day period after the predation evasion test using a protocol modified from Hamilton et al. (2016). An all-white acrylic tank (60 cm x 30 cm x 30 cm, l x w x h) with a panel covering a mirror on one side (approach zone) was filled with the same amount of new natural seawater as the predator evasion test. Crabs were individually placed into the tank and left for 30 min to habituate. Once the panel was removed, the crab was moved to the center of the wall across from the mirror (avoidance zone), showing the crab their reflection. The crab's behaviour was recorded for 5 min using the Logitech HD camera. The time spent in the approach zone was measured as aggressive behaviour. The time spent in the avoidance zone was measured as submissive behaviour. Water was again changed between each crab to avoid residual chemical signals from affecting the behaviour of subsequent

crabs. Crab movement, measured as mobile average speed (mm/s) and total distance travelled (mm) was measured via ToxTrac™ (Rodriguez et al. 2017, 2018).

2.4.5 Necropsy, dissection, slide preparation and lumen area /to total tubule area ratio

Crabs that died before the end of the study period were necropsied upon discovery. Those still alive at the end of the study period, both tested and untested, were humanely euthanized and necropsied after the aggression test. Infection status (parasitized or unparasitized), crab sex (male or female), parasite length (mm) and parasite sex (male or female) were noted for all crabs at the time of necropsy. Testes of male crabs were removed and weighed (g) to determine testes weight/to carapace width ratio. Gross pathology was only noted in full for euthanized crabs as tissues were partially deteriorated in crabs that died prematurely.

Hepatopancreatic tissues were fixed in Davidson's fixative for 48 h and then stored in 70% ethanol, before being routinely processed and embedded in paraffin wax, sectioned at 5 µm, mounted onto glass slides and stained with hematoxylin and eosin (Hopwood 1996). Histologic slides were then viewed under a Motic BA310E™ light microscope at 100 x total magnification using Omax ToupView™ 3.7. Pictures were taken using an Omax A3550U3™ 5.1 MP USB camera attached to the microscope and images were analyzed using ImagePro Plus 10™. The total tubule area and lumen area (μm^2) were calculated to determine lumen area/to total tubule area ratio for 3 random tubules per crab, 13 crabs per group (infection status). Total tubule area was calculated using the formula for an ellipse or series of ellipses, depending on the overall shape of the tubule. Lumen area was calculated based on the formula for a combination of shapes, including rectangles, triangles and ellipses, to determine the total area of the lumen.

2.4.6 Statistical analysis

A multiple regression analysis was performed to determine if crab sex, crab carapace width and worm sex affected worm length. Behaviour, anatomical and physiological data were checked for normality using the Anderson Darling test in Minitab 19™. Large outliers were removed from parametric data and a Johnson transformation was used to normalize the data. These data were then analyzed using a two-way analysis of covariance (ANCOVA). Parametric data unable to be transformed were analyzed in SPSS IBM SPSS Statistics 27™ using a Quade's two-way ANCOVA. All data, except for testes weight/to carapace width ratios, were compared for the effects of infection status (parasitized/unparasitized) and sex (male/female) on the response variable. Only the effects of infection status were compared for testes weight/to carapace width ratio. Worm length was tested as a covariate in these ANCOVAs. Those crabs containing brown, worm-shaped structures or *Profilicollis botulus* cystacanths were removed from statistical analysis. A result was deemed significant when a p-value was < 0.10.

2.5 Results

2.5.1 *Nectonema* sp.

2.5.1.1 Identification and phylogenetic analysis

The *Nectonema* sp. sequence extracted from Atlantic rock crabs had 99.8% similarity, 100% query cover and an e-value of 0 with *Nectonema agile* 18S rRNA (GenBank: AF421767.1). The Tamura-Nei model with gamma distributed rates was determined to be the best-fit DNA model for maximum-likelihood phylogenetic analysis. *Nectonema agile* and *Nectonema* sp. (GenBank: LC605988.1) grouped together in the tree with a bootstrap value of 100% (Figure 1). Members of Gordiida were sorted into two large separate groups; one consisting of eight *Gordius* spp. and the other members of the family Chordodidae and three

Gordius spp. Chordodidae consisted of *Paragordius* spp., *Gordionus* spp., *Paragordionus* spp.,

Chordodes spp., a *Spinochordodes* sp., and a *Neochordodes* sp. Two *Chordodes* spp. grouped

separately from the third. The group consisting of the eight *Gordius* spp. branched off before the

larger group consisting of the other members of Gordiida and Nectonematida.
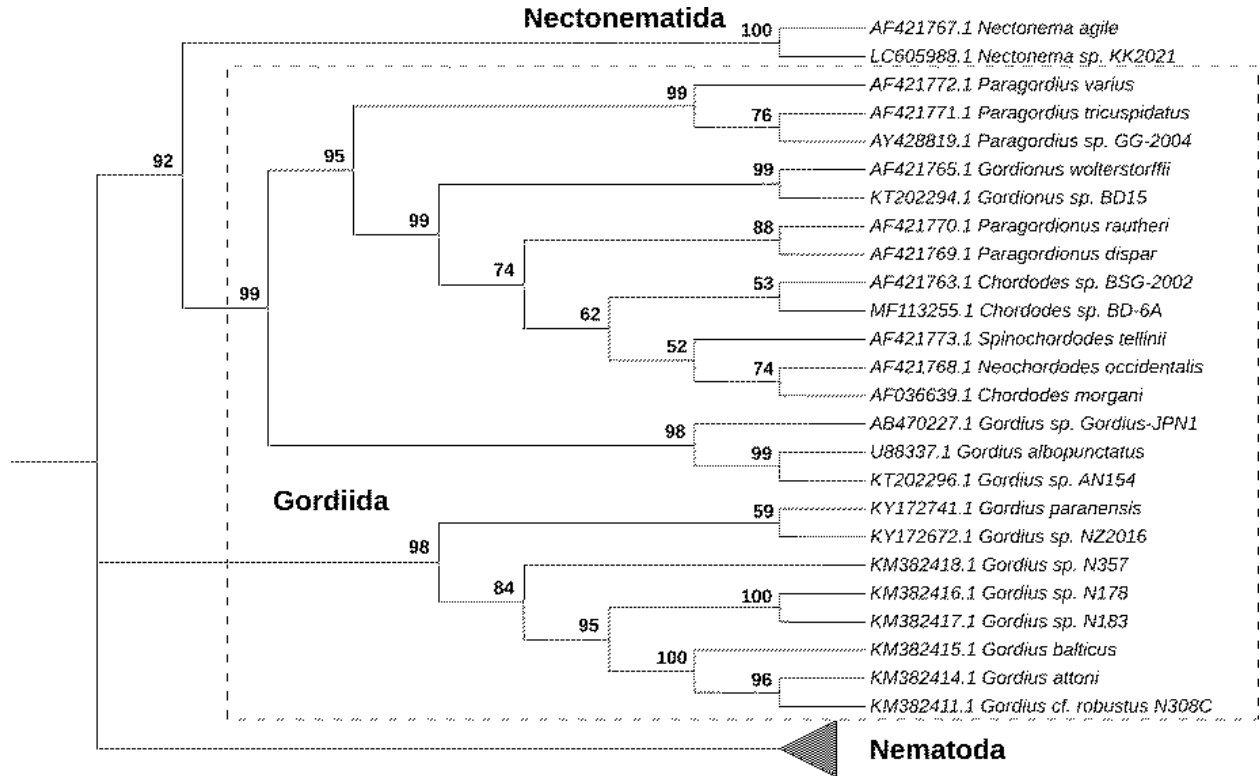
Figure 1. Maximum likelihood tree (Tamura-Nei model) showing placement of *Nectonema agile*
and other nematomorphs. The tree is rooted with Nematoda as the outgroup. Members of
Gordiida are contained within the dotted rectangle. Values at nodes represent bootstrap values.

2.5.1.2 Location, behaviour and physical characteristics

Live *Nectonema agile* were found within the haemocoel of 16 rock crabs (Figure 2).

These juvenile worms were generally immobile, except for two females, that continuously

moved in a wave-like fashion once their host was euthanized. Worm length was 567 ± 146 mm.

Multiple regression analysis revealed that crab size (carapace width) and sex had no influence on

worm length. Female worms were significantly longer than males and were opaque and white, while males were transparent (873.0 ± 209 mm vs.174.6 ± 27.5 mm; Figure 3) (p = 0.028). One of the mobile females was the longest worm found, at 2207 mm; the shortest worm was a male at 71 mm. The shorter mobile female (411mm) was removed from the host and placed into a small container with cold seawater (4 ± 1 ℃) where it tied itself into gordian knots (Figure 4). When placed into a larger aquarium, the worm continuously swam in wave-like motion, probing the water ahead of itself (Figure 5). The worm died ~ two hours of removal and seawater exposure.
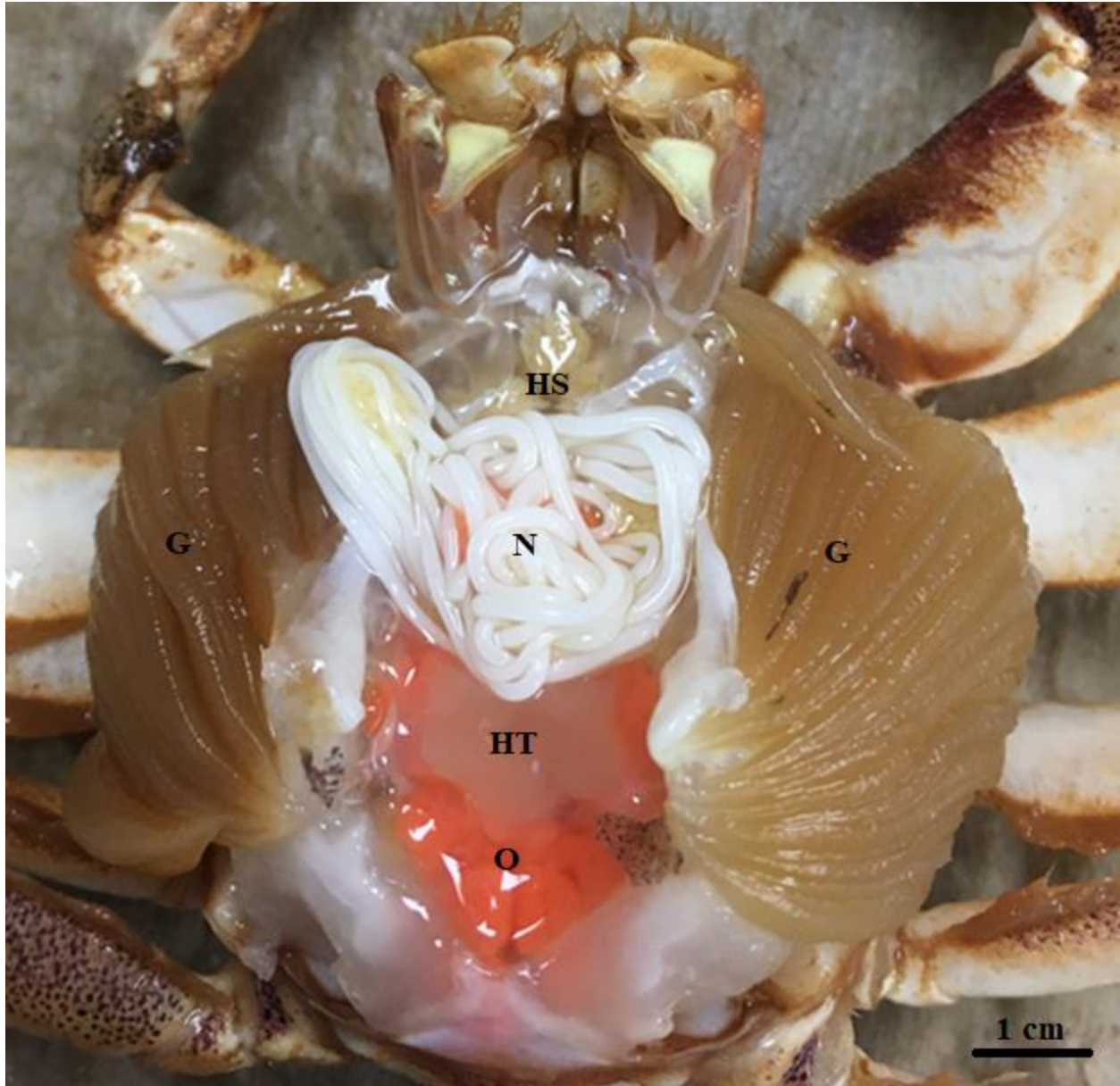
Figure 2. Juvenile female *Nectonema agile* (**N**) found within haemocoel of euthanized female rock crab. The worm was located anteriorly to the heart (**HT**) and ovaries (**O**), dorsal to the hepatopancreas (**HS**) and medial to the gills (**G**).
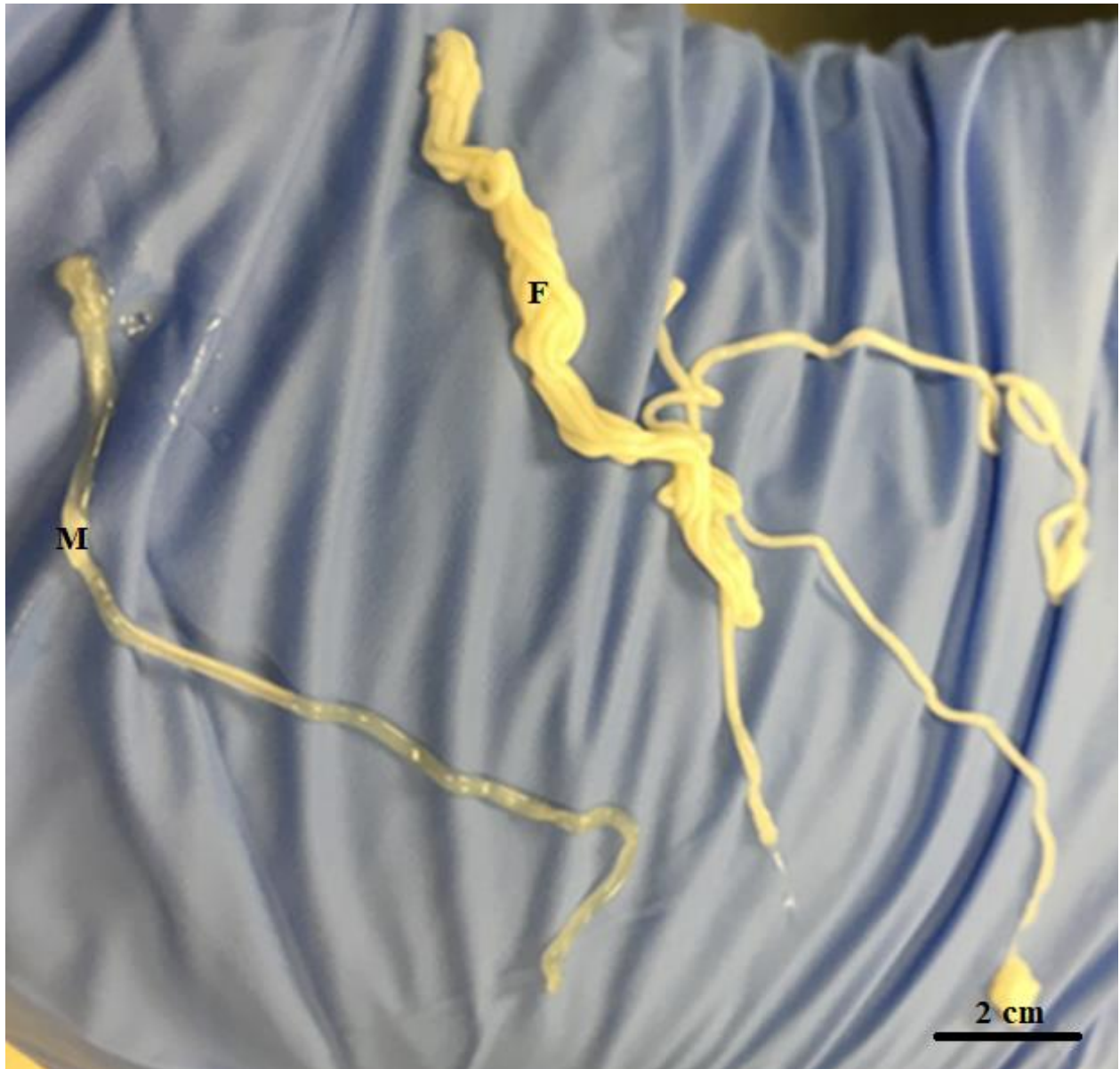
Figure 3. Juvenile male (**M**) and female (**F**) *Nectonema agile* removed from their host.
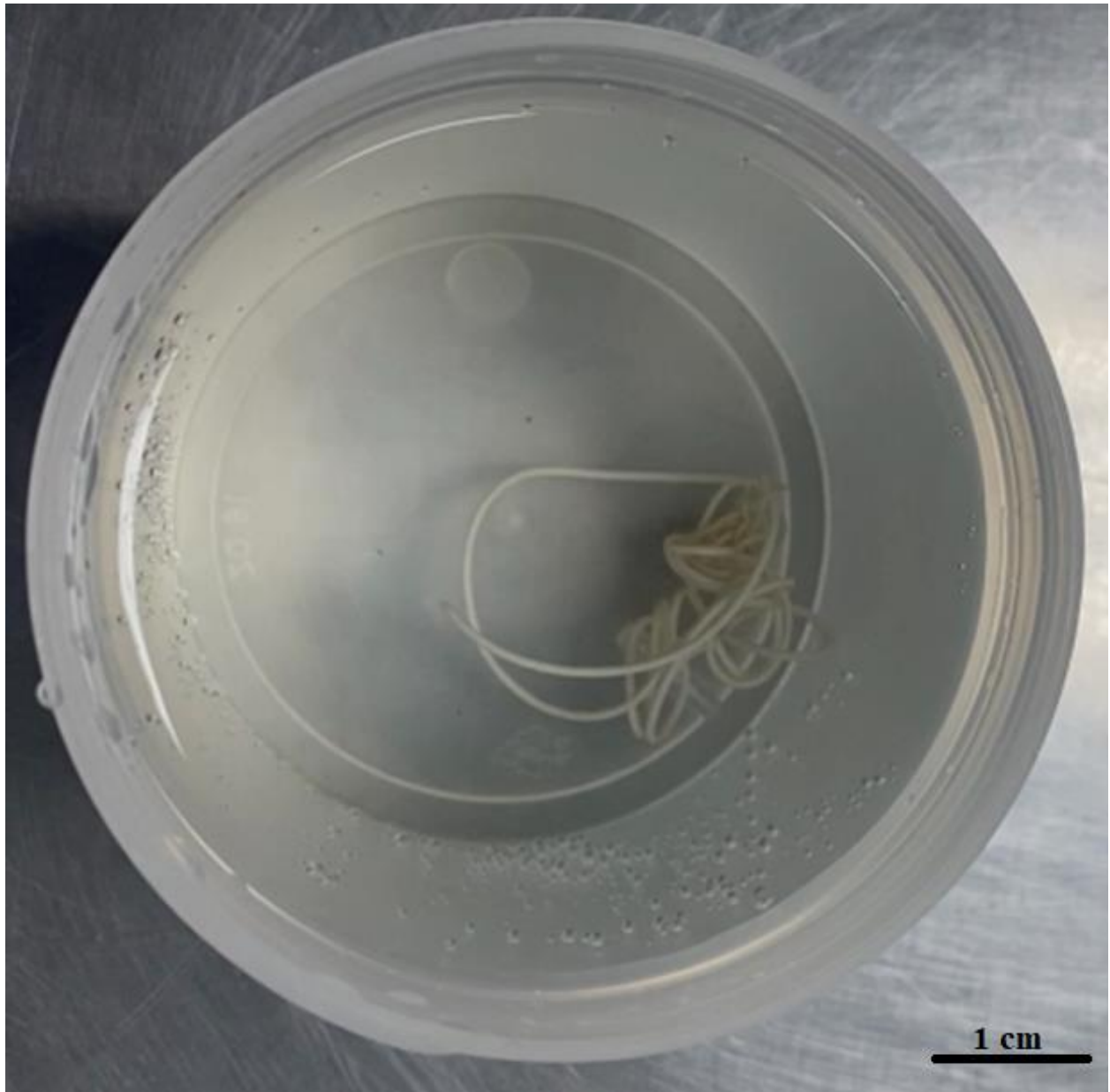
Figure 4. Juvenile female *Nectonema agile* in seawater (4 ± 1 ºC) tying itself up into gordian knots after removal from its host.

Figure 5. Juvenile female *Nectonema agile* swimming in seawater (4 ± 1 ºC) after removal from its host.

2.5.2 Behaviour tests

2.5.2.1 Predator evasion

These data failed the normality test and were unable to be transformed, therefore a Quade's ANCOVA was used for analysis. Eleven parasitized and 72 unparasitized crabs were tested. The time spent in the white zone, at the midline or in the black zone was not statistically significant between males and females or parasitized and unparasitized individuals ($p > 0.10$; Figure 6). No interaction effects occurred between sex and infection status ($p > 0.10$). Quade's ANCOVA indicated that worm length and time spent in the black zone were correlated ($p = 0.022$). However, linear regression analysis on unstandardized residuals revealed that worm length explained only a small amount of the variance seen in time spent in this zone by parasitized crabs ($R^2_{adj} = 27.50\%$; $p = 0.056$).
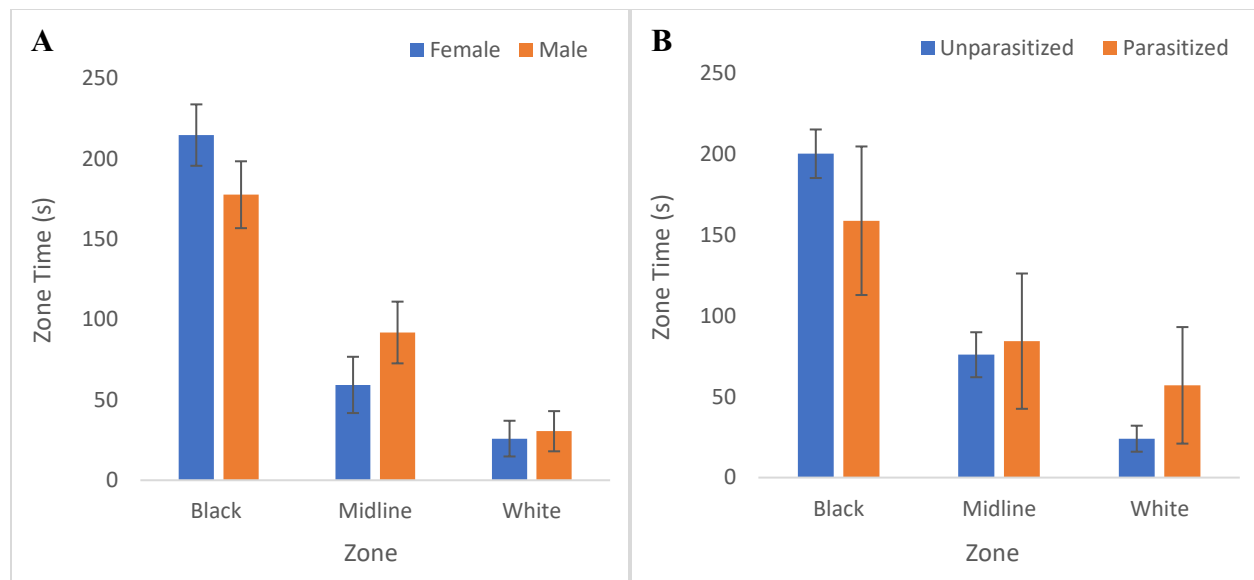
Figure 6. Time spent in the black and white zones and at the midline (s) (mean ± standard error) for rock crabs. **A.** Female and male. **B**. Unparasitized and *Nectonema agile*-parasitized.

2.5.2.2 Aggression

The aggression data also failed the normality test and were unable to be transformed so a Quade's ANCOVA was used for analysis. Eleven parasitized crabs and 65 unparasitized crabs were tested. Time spent in the approach and avoidance zone was significantly different between male and female rock crabs ($p = 0.051$; Figure 7A). Females spent more time in the approach zone and males spent more time in the avoidance zone. There was no significant difference between parasitized and unparasitized crabs ($p > 0.10$; Figure 7B). No interaction effects occurred between sex and infection status ($p > 0.10$). Worm length was not associated with, or explained variances in, time spent in either of the zones ($p > 0.10$).
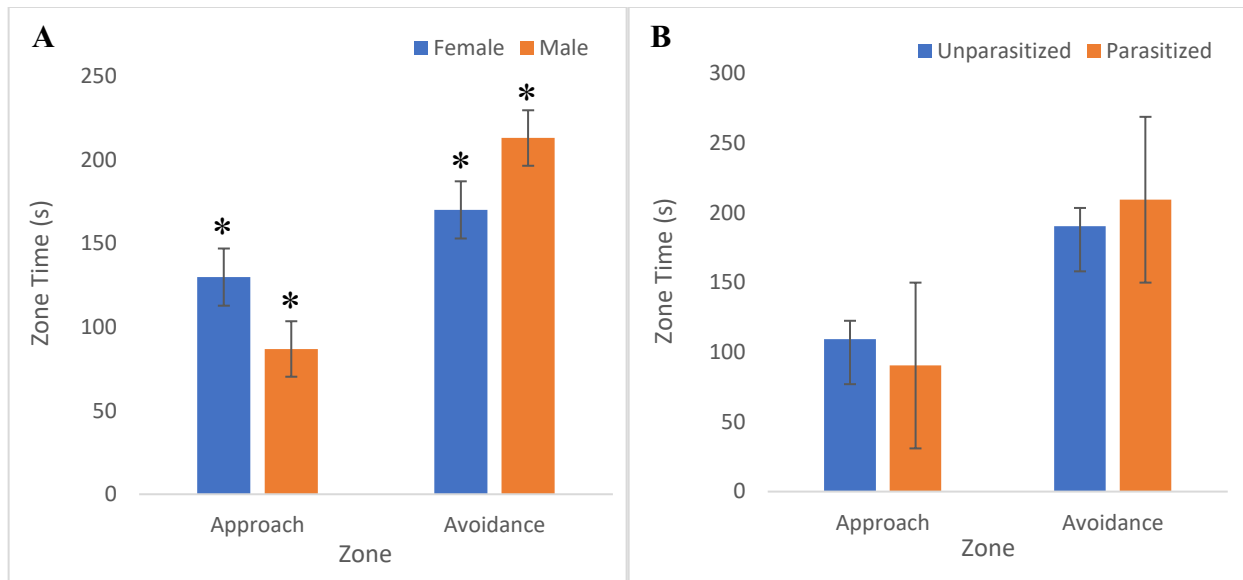
Figure 7. Time spent in the approach and avoidance zones (s) (mean ± standard error) for rock crabs. **A.** Female and male. **B**. Unparasitized and *Nectonema agile*-parasitized. Asterisks indicate a significant difference between groups (p = 0.051).

2.5.2.3 Movement

These data failed the normality test and were normalized through transformation. An ANCOVA found a significant difference in mobile average speed between sexes and infection statuses (Figure 8). Females were faster than males and parasitized crabs were faster than unparasitized crabs for mobile average speed (p = 0.059, p = 0.013). There was no significant difference between infection statuses and sexes for total distance travelled (p > 0.10; Figure 9). No interaction effects occurred between sex and infection status (p > 0.10). Worm length was not associated with or explained variances seen in movement (p > 0.10).
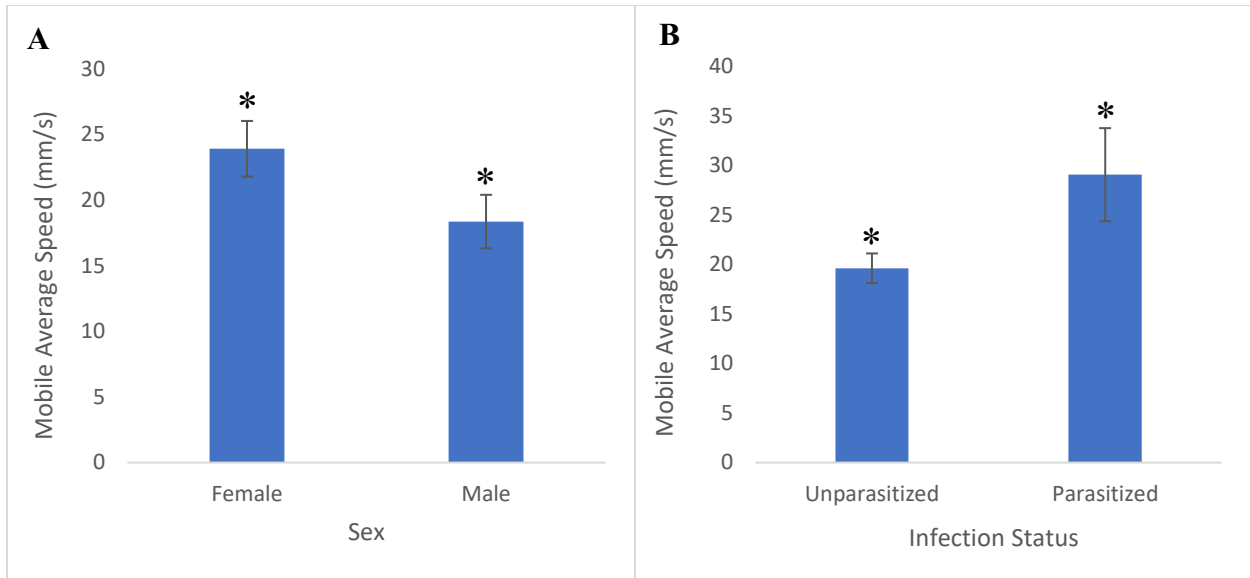
Figure 8. Mobile average speed (mm/s) (mean ± standard error) for rock crabs. **A.** Female and male. **B**. Unparasitized and *Nectonema agile*-parasitized. Asterisks indicate a significant difference between groups (p = 0.059, p = 0.013).
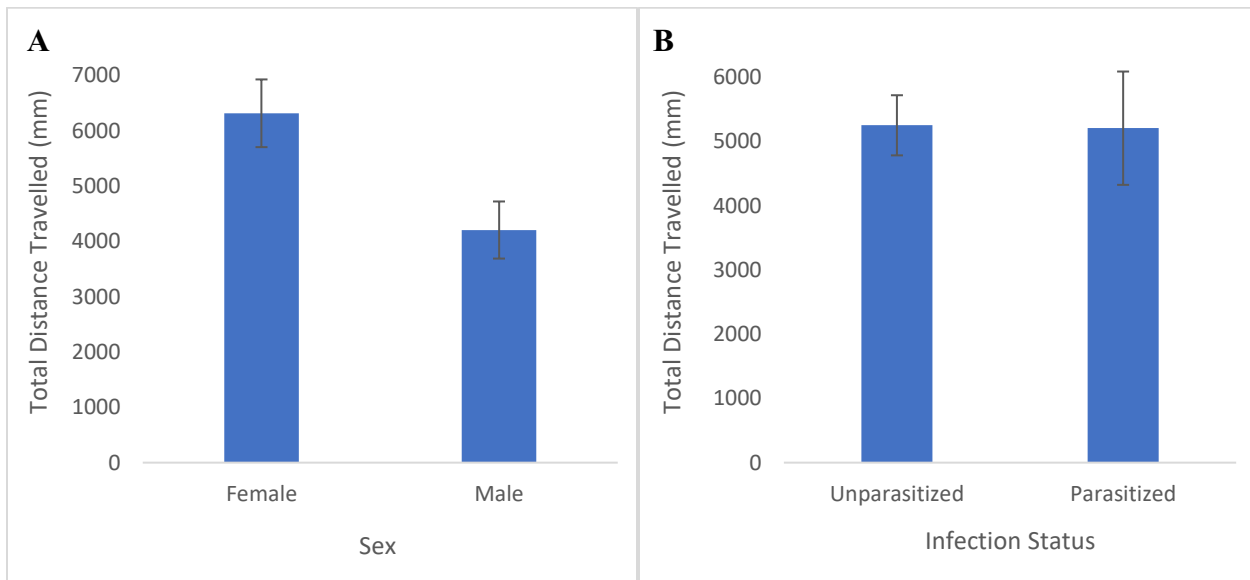
Figure 9. Total distance travelled (mm) (mean ± standard error) for rock crabs. **A**. Female and male. **B**. Unparasitized and *Nectonema agile*-parasitized.

2.5.3 Anatomical and physiological alterations

2.5.3.1 Gross and microscopic examination

Upon necropsy, four crabs contained darker brown worm-shaped structures that looked like melanized worms or remnants of shed worm cuticle (Figure 10). Two live female *N. agile* had a partially melanized body. Total live horsehair worm prevalence, including crabs that died during the study and were euthanized at the end of the study, was 15.5% (16/103). Male crabs were only slightly more parasitized than female crabs at 17.0% (10/59) vs. 13.6% (6/44), however a chi-square test revealed that this difference was not significant (p > 0.10). Thirteen of the crabs found with live worms survived until the time of necropsy; two of those that prematurely died were tested when alive for predator evasion and aggressive behaviour. Ten out of the 13 (76.9%) parasitized crabs euthanized at the end of the study had a dark brown hepatopancreas. The hepatopancreas of the female crab containing the largest live worm was dark brown and appeared to be smaller than expected. This crab was one of the smallest females in the study. Two out of four (50.0%) of those crabs with brown worm-shaped structures had a pale yellow, almost white hepatopancreas. Only 12/62 (19.4%) unparasitized crabs had a dark brown hepatopancreas. The same percentage of unparasitized crabs had a pale yellow, almost white hepatopancreas. The remaining unparasitized crabs had dark yellow, lighter brown or green hepatopancreases with varying shades and hues.

Both parasitized and unparasitized hepatopancreases contained no glycogen reserve inclusion bodies and appeared to have increased B-cell (blister cell) and tubule lumen size (Figure 11). This was in comparison to previously analyzed hepatopancreas tissue from rock crabs collected from the same area (Baker 2019; personal observations). Hepatopancreas lumen area/to total tubule area ratio data failed the normality test and were transformed. An ANCOVA

indicated that ratios were not significantly different between sexes or parasitized and unparasitized crabs (p > 0.10; Figure 12). No interaction effect occurred between sex and infection status and worm length was not associated with, or explained variances seen in, the data (p > 0.10). All other organs and tissues from both parasitized and unparasitized crabs were either slightly melanized or appeared normal.
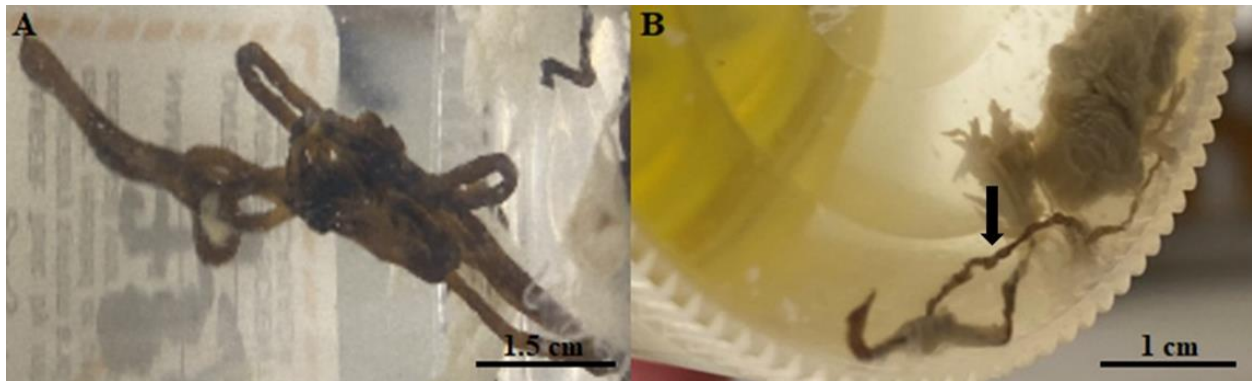
Figure 10. Preserved brown worm-shaped structures from crabs (70 % ethanol). **A**. Large example **B**. Small example (arrow).
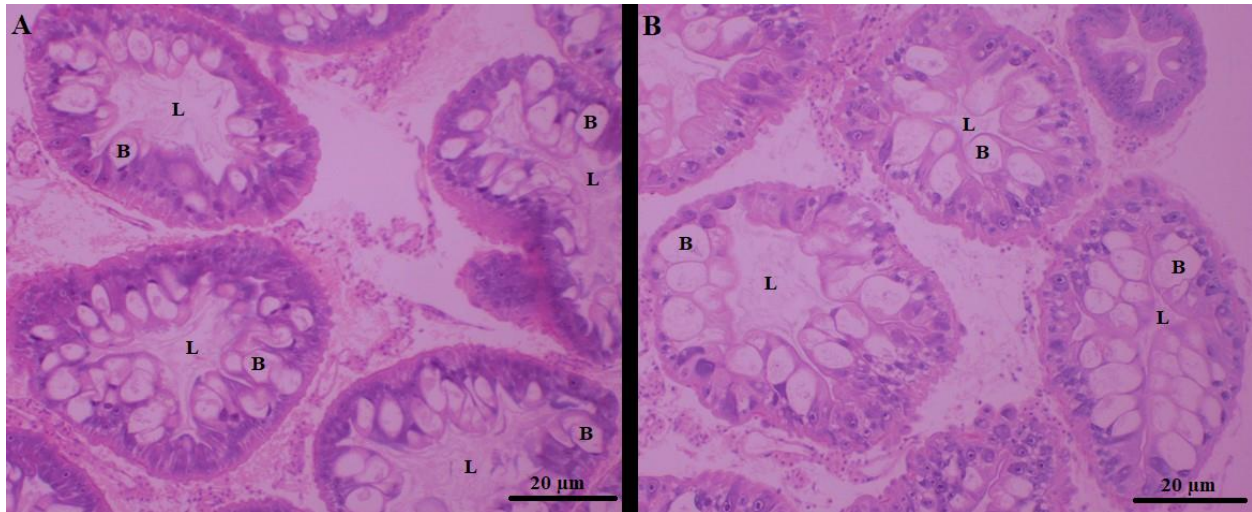
Figure 11. Hepatopancreatic tubules from rock crabs showing increased lumen (**L**) and B-cell size (**B**) at 100 x total magnification. **A**. Parasitized. **B**. Unparasitized.
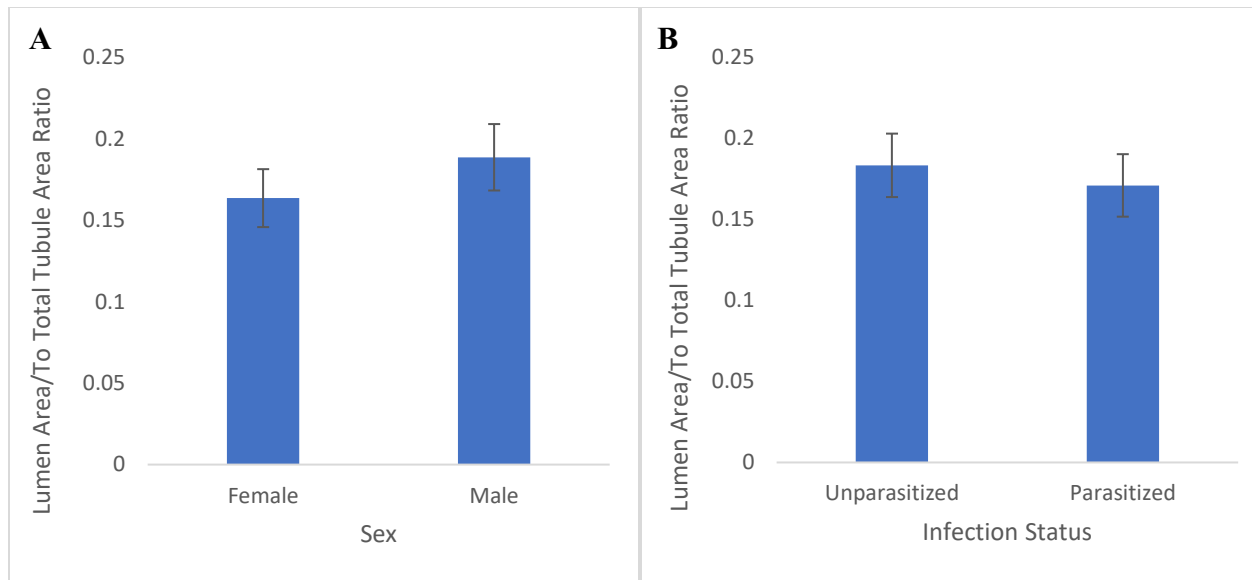
Figure 12. Lumen area/to total tubule area ratios (mean ± standard error) for rock crabs. **A**. Female and male. **B**. Unparasitized and *Nectonema agile*- parasitized.

2.5.3.2 Carapace width, body weight and testes weight/to carapace width ratio

Carapace width and body weight failed the normality test and were transformed. An ANCOVA indicated that sex significantly affected these measurements (p = 0.000; Figure 13A, 14A). As expected, males were larger and weighed more than females. There was no significant difference in carapace width and body weight between parasitized and unparasitized crabs (p > 0.10; Figure 13B, 14B). An interaction effect occurred between sex and infection status for carapace width and body weight (p = 0.019, 0.015). Both parasitized and unparasitized males were significantly larger and weighed more than parasitized, as well as unparasitized females. Despite the ANCOVA indicating that worm length was correlated with carapace width and body weight (p = 0.045, 0.015), linear regression analysis revealed that worm length did not explain any of the variance seen in carapace width and body weight, indicating a causative relationship did not exist (p > 0.10).

Testes weight/to carapace width ratio data were parametric. An ANCOVA found a significant difference in testes weight/to carapace width ratio measurements between infection statuses (p = 0.089; Figure 15). Parasitized male crabs had a significantly smaller testes weight/to carapace width ratio than unparasitized male crabs. Worm length was not associated with, or explained variances seen in testes weight/to carapace width ratio of rock crabs (p > 0.10).
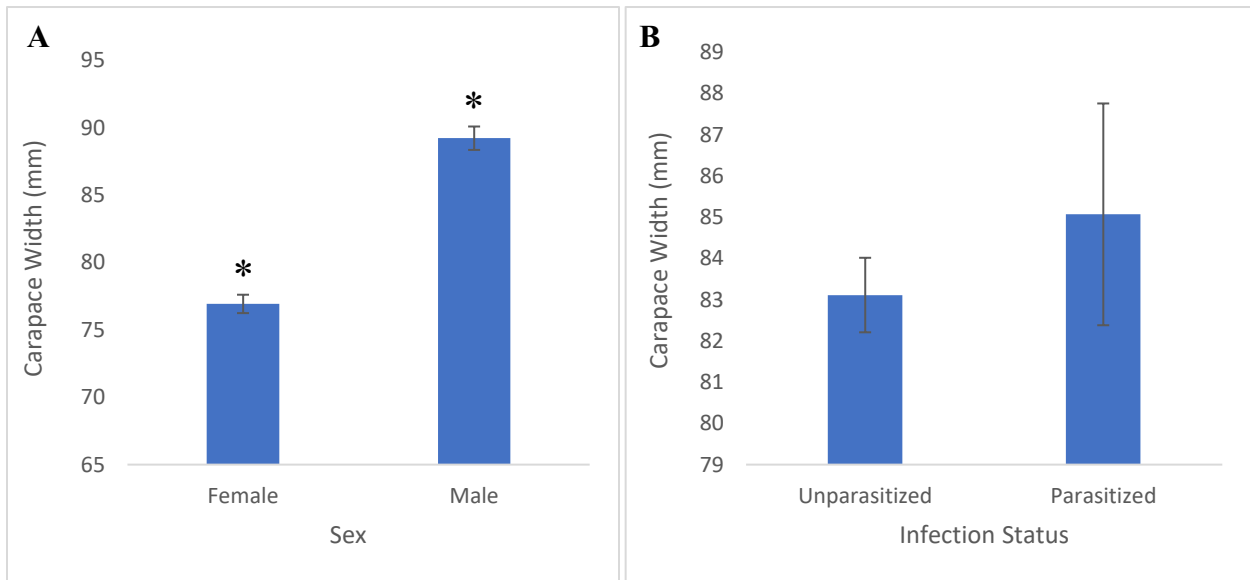
Figure 13. Carapace width (mm) (mean ± standard error) for rock crabs. **A.** Male and female. **B.** Unparasitized and *Nectonema agile*-parasitized. Asterisks indicate a significant difference between groups (p = 0.000).
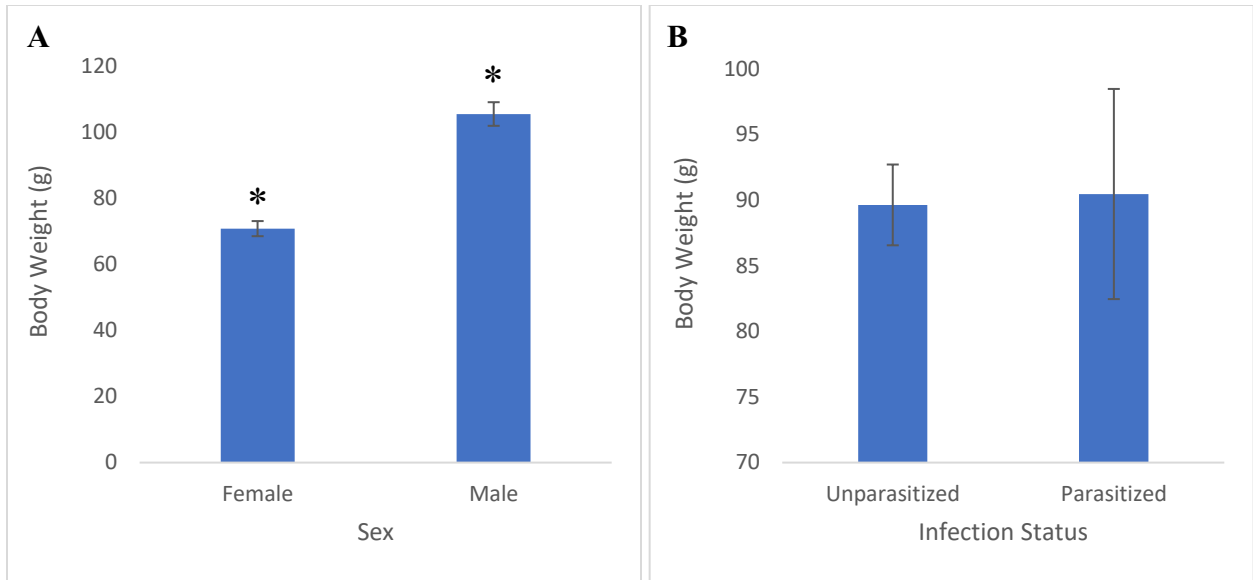
Figure 14. Body weight (g) (mean ± standard error) for rock crabs. **A**. Male and female. **B**. Unparasitized and *Nectonema agile*-parasitized. Asterisks indicate a significant difference between groups (p = 0.000).
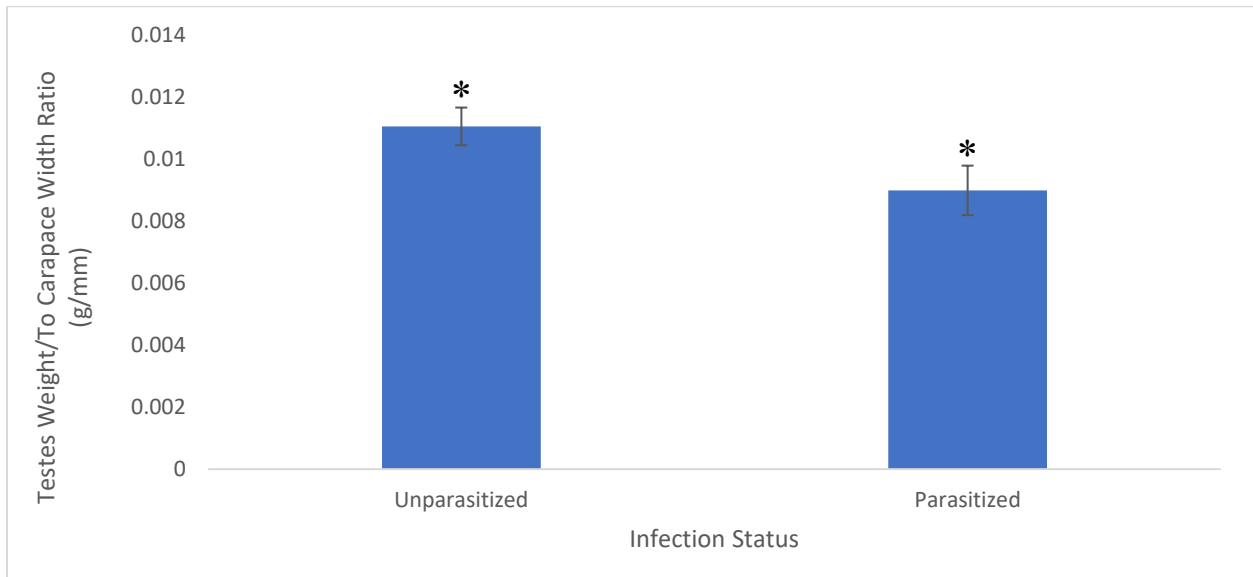
Figure 15. Testes weight/to carapace width ratios (g/mm) (mean ± standard error) for unparasitized and *Nectonema agile*-parasitized rock crabs. Asterisks indicate a significant difference between infection statuses (p = 0.089).

2.6 Discussion

2.6.1 *Nectonema agile*

2.6.1.1 Phylogeny

The 18S rRNA extracted from the *Nectonema* sp. in this study was a 99.8% match to a previously identified *N. agile* sequence extracted from worms of Passamaquoddy Bay, New Brunswick (GenBank: AF421767.1; Bleidorn et al. 2005). Query cover was 100% and the e-value was 0. The higher the percent identity and query cover and the lower the e-value, the more significant the BLAST® match is (NCBI 2022; Tufts University 2022). Due to the high percent identity, complete query cover and e-value of zero, it can be confidently concluded that the horsehair worm parasitizing rock crabs in the Northumberland Strait is *Nectonema agile*.

When Bleidorn et al. (2005) constructed a Nematomorpha phylogeny via maximum-likelihood, they found that *N. agile* (and therefore Nectonematida) acted as a sister group to all analyzed freshwater horsehair worms. In the present study, Nectonematida acted as a sister group to most freshwater horsehair worms, except for eight *Gordius* spp. The other three *Gordius* spp. grouped together with members of Chordodidae, suggesting that *Gordius* spp. may be a paraphyletic group. Kakui et al. (2021) also found evidence of *Gordius spp.* paraphylogeny in their maximum-likelihood analysis. Both Bleidorn et al. (2005) and Kakui et al. (2021) found *Chordodes* spp. to be paraphyletic, corroborating the observation made in the current study. Neither found *Gordius* spp. to branch off before the other members of Gordiida and Nectonematida. The placement of the eight *Gordius* spp. outside and before these two groups seems to suggest that the whole of Gordiida is paraphyletic and that *Gordius* spp. may possess some ancestral trait found in Nematomorphs. Further analysis of *Gordius* spp. should be done to

determine whether this may be the case. Differences found between previous studies and the present study may be due to the use of different models for maximum-likelihood analysis.

2.6.1.2 Behaviour and physical characteristics

In the current study, *N. agile* juveniles were generally immobile, except for two females who exhibited rapidly undulating movements. Nielsen (1969) reported that juvenile *Nectonema munidae* were often immobile within their squat lobster (*Munida tenuimana*) host. One juvenile worm showed rapid movements when encountering seawater. Those that moved slowly and continuously were adults ready to exit their host. Free-living *N. agile* adults swim in rapid, undulatory motions (Ward 1892a). It is possible that the two mobile worms from the current study were exposed to droplets of seawater upon host euthanization. However, it is more likely that their movements are related to damage to the host or getting ready for transition to a free-living adult. When one of the rapidly moving female *Nectonema* worms was placed in cold seawater, she showed proficient swimming ability and continued her rapidly undulating patterns of behaviour, further suggesting that she was close to being ready for free-living. Her death within two hours after removal from the host, however, suggests that she either was not physiologically prepared for free-living or the experimental environmental conditions were not optimal for an adult worm. The gordian knot behaviour exhibited by this same female when in a small environment is also seen in freshwater horsehair worms. Knotting behaviour in *Paragordius tricuspidatus* consists of multiple male worms and is believed to be associated with a decreased water level (Daoust et al. 2012). The length of the female *N. agile* in this study clearly required knotting up in the small container to stay submerged in water. It can be assumed that exposure to air would desiccate the worm and increase the chances of death.

Crab size and crab sex did not affect the size of *N. agile* in the present study. Nielsen (1969) found a similar trend in *N. mundidae*; there is no correlation between parasite size and host size for *N. mundidae* and its squat lobster host (*M. tenuimana*). While no correlation between parasite size and host size was noted for a *Nectonema* sp. surveyed in rock crabs from the Bay of Fundy, the size of individual worms was related to host species (Leslie et al. 1981). The average size of this *Nectonema* sp. was smaller in the hermit crab *Pagurus acadianus* than in rock crabs. *Nectonema agile* found in rock crabs in the current study were larger, on average, than recorded in earlier studies. These studies recorded *N. agile* juveniles from marsh grass shrimp (*Palaemonetes vulgaris*) to be 50-100 mm in length and free-living adults to be 50-200 mm in length (Ward 1892; Born 1967). A female *N. agile* found in an immature female American lobster (*Homarus americanus*) in New Brunswick was found to be 590 mm (Schmidt-Rhaesa et al. 2013). The *Nectonema* sp. (likely *N. agile*) from rock crabs in the Bay of Fundy were described in such a way to suggest that they were all female and were 300-650 mm long (Leslie et al. 1981). The difference in size of *N. agile* between past studies and the current study is likely due to the presence of one particularly large female (above 2000 mm in length) and the small sample size of parasites.

2.6.2 Behaviour tests

2.6.2.1 Predator evasion and movement

In the current study, *N. agile*-infected rock crabs were expected to have increased photophobia, if worm infection induced predator avoidance. This, however, did not occur. Parasite-induced host photophilia has been documented in previous helminth-amphipod studies and is believed to be related to the parasitic lifecycle (Brown & Thompson 1986; Durieux et al. 2012; Casalins et al. 2015). Light also has a strong affect on the behaviour of freshwater

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horsehair worm-parasitized insects (Ponton et al. 2011; Obayashi et al. 2021). Both infected

crickets (*Nemobius sylvestris*) and arboreal mantids (*Hierodula patellifera*) have directed

responses to light and walk more than uninfected insects (Ponton et al. 2011; Obayashi et al.

2021). Parasitized mantids are specifically attracted to horizontally polarized light associated

with water and parasitized crickets walk faster than uninfected (Ponton et al. 2011; Obayashi et

al. 2021). The increased mobility seen in infected mantids is believed to increase the encounter

rate between infected hosts and water. While parasitized rock crabs did not change their

attraction to light, they did significantly increase their speed in the presence of light, in

comparison to uninfected crabs. Unlike hairworm-infected insects, parasitized crabs did not

travel increased distances. This may be due to the confined space in which they were held or due

to the smaller sample size of the parasitized group.

Despite there being no increased photophobia or photophilia in *N. agile*-infected rock

crabs, the increased speed in these crabs when encountering light may indicate a method of

predation suppression via movement. A parasitized crab moving more quickly than conspecifics

may decrease its chances of being predated upon. This could be one reason why freshwater

horsehair worms increase mobility in their definitive host when they are close to emerging as a

free-living adult. Directing their host to water as quickly as possible increases not only host

survivability, but also parasite survivability and reproduction. An increase in speed of infected

rock crabs may improve their chances of encountering other parasitized individuals in water and

increase *N. agile* reproduction by being in proximity of another soon-to-emerge worm. An

example of such behaviour is seen in the cricket *N. sylvestris* infected by the horsehair worm *P.*

tricuspidatus (Sanchez et al. 2008). *Paragordius tricuspidatus* will first induce erratic behaviour

(go to an abnormal environment) in its host before becoming fully mature and then direct its host

toward water. This behaviour ensures mating; the fecundity of worms is better in crickets that exhibit this suicidal behaviour. In future studies, *N. agile*-parasitized rock crabs should be tested for attraction to horizontally polarized light and then placed outside of a body of water to gauge their attraction to it, as performed in Obayashi et al. (2021). A second experiment should measure rates of predator capture of parasitized versus unparasitized crabs to determine if the increased speed found in parasitized individuals decreases predation upon them.

2.6.2.2 Aggression

In the current study, *N. agile* was expected to reduce host aggression toward conspecifics in its rock crab host to escape cannibalism. Despite this prediction, no significant differences in aggression between infection statuses were observed. Host predator evasion behaviour has frequently been studied in helminth-crustacean relationships. Crustacean host aggression, on the other hand, has not been thoroughly investigated in these associations. An annelid ecto-symbiont (*Xironogiton victoriensis*) has been found to reduce aggression in its signal crayfish host, *Pacifastacus leniusculus* (James et al. 2015). As previously mentioned, nematode parasites also reduce aggression in their insect hosts. *Chondronema passali* reduces fighting ability in its beetle host and *Myrmeconema neotropicum* stops its ant host from biting (Yanoviak et al. 2008; Vasquez et al. 2015). The only such study involving a nematomorph found a similar trend of decreased aggression in *Paragordius varius*-infected crickets (*Acheta domesticus*), but this result was insignificant (Keck et al. 2017). The results obtained in the current study, in combination with those obtained by Keck et al. (2017), suggest that horsehair worms do not alter aggression in their host. Aggression may therefore not significantly affect the survivability of the host, nor the parasite. Further horsehair worm studies should be conducted to confirm this. Nematoda is the sister phylum to Nematomorpha, sharing many characteristics with it. It is possible that

decreased aggression also occurs in nematomorph hosts, but the change in behaviour was simply not seen in Keck et al. (2017) or the current study. In the future, it may be beneficial to expose crabs to conspecifics and measure rates of direct aggression, rather than simply using a mirror test.

2.6.3 Anatomical and physiological alterations

2.6.3.1 Necropsy and hepatopancreatic examination

In the present study, small brown worm-shaped structures were found in some rock crabs. Leslie et al. (1981) reported that some rock crabs surveyed from the Bay of Fundy contained brittle brown tubes and believed them to be shed *Nectonema* sp. cuticle. A later study on grapsid crabs (*Hemigrapsus edwardsi*) infected by *N. zealandica* found that most worms were melanized by their hosts and in various stages of encapsulation (Poinar Jr. & Brockerhoff 2001). Poinar Jr. and Brokerhoff (2001) suggested that the brown tubes observed by Leslie et al. (1981) were probably melanized worms, rather than shed cuticles. It is unclear whether the small brown structures found in the current study are small melanized worms or shrunken shed cuticles. A thorough microscopic examination of these structures will need to be done to conclude their identity. If they are melanized worms, then this shows that some *N. agile* cannot escape the immune response of their host and succumb to toxic melanisation.

The total parasite prevalence of *N. agile* infecting crabs in the current study was slightly lower than that reported by Leslie et al. (1981) (15.5% vs. 21.4%, respectively). This could be due to the sampling area being a different body of water than that investigated by Leslie et al. (1981) (Bay of Fundy vs. Northumberland Strait), a difference in overall sample size or due to convenience sampling, which would have reduced the chance of randomly sampling the

population. Based on personal observations, parasite prevalence was expected to be closer to 20%. Leslie et al. (1981) also found no difference in parasite prevalence between crab sexes. Two other studies reported that *N. agile* favours males of its hermit crab (*Pagurus bernhardus*) and prawn (*Palaemonetes vulgaris*) hosts (Mouchet 1931; Born 1967). The current study corroborated the lack of preference for one host sex over another found by Leslie et al. (1981).

A higher percentage of dark brown hepatopancreases were found in parasitized crabs euthanized at the end of this study than in unparasitized crabs. Decapod hepatopancreases tissue can be a variety of colours: brown, green, yellow, tan, red or blue (Ceccaldi 1989). The colouration depends mainly on stored reserves, such as carotenoids, that are obtained from the animals' diet (Ceccaldi 1989). The dark brown hepatopancreases seen in parasitized individuals may suggest that a possible change in carotenoid concentration occurred, brought on by *N. agile*. Amphipods (*Hyalella patagonica*) parasitized by the acanthocephalan *Corynosoma* sp. exhibit lower carotenoid concentrations than uninfected conspecifics (Rauque & Semenas 2009). Either direct or indirect impacts of *N. agile* parasitism on host metabolism could explain the darker colour seen in parasitized hepatopancreases. It is possible that as *N. agile* feeds from within its host, it draws upon hepatopancreatic carotenoids as one of its nutrient sources. Another explanation is that *N. agile* parasitism decreases host feeding. To test these theories, host feeding behaviour should be monitored between parasitized and unparasitized crabs. Once testing is complete, a spectrophotometric analysis should be done on crab hepatopancreas tissue to compare carotenoid concentrations between groups and determine if an association between *N. agile* parasitism, host feeding behaviour and hepatopancreatic carotenoid concentration exists.

Other than possible carotenoid alteration, hepatopancreas tissues of parasitized crabs were generally unchanged in the current study. Only one hepatopancreas appeared reduced in

size due to parasitism; this belonged to a small host containing an above average-sized female *N. agile*. A similar occurrence was discovered in *N. munidae*-infected squat lobster; heavy infestation occasionally resulted in smaller hepatopancreases in comparison to uninfected hosts of similar size (Nielsen 1969). Microscopic examination of hepatopancreas tissue revealed that both parasitized and unparasitized rock crabs lacked glycogen reserve inclusion bodies and had apparent increases in B-cell and tubule lumen size. These observations were possibly due to diet and/or infrequent feeding. Hepatopancreatic glycogen reserve inclusion bodies have previously been used to compare the effects of parasitism on crab host energy reserves (Stentiford & Feist 2005; Wheeler et al. 2007). However, due to the lack of glycogen reserve inclusion bodies in the current study, a lumen area/to total tubule area ratio comparison was made between infection statuses. Hepatopancreatic lumens increase in size in Chinese mitten crabs (*Eriocheir sinensis*) under long-term starvation (Huang et al. 2020). When subjected to this condition, this crab depletes hepatopancreatic energy stores and consumes its hepatopancreatic cells to maintain energy metabolism. An increase in B-cell size can be seen in both these and in long-term starved lobsters (*Homarus gammarus*) (Albalat et al. 2019). It was expected that the combination of starvation, higher holding temperature and parasitism of rock crabs in this study would result in significantly larger lumen area/to total tubule area ratios in infected hosts than uninfected conspecifics. This was not the case. These results indicate that hepatopancreas tissue is largely undamaged by *N. agile* parasitism and no additional damage is observed in starved crabs when parasitized.

2.6.3.2 Body size, body weight and testes weight/to carapace width ratio

In the current study, the only factor that influenced body size and body weight was crab sex; parasitism did not change these parameters. Male rock crab testes size, however, was

decreased by *N. agile* parasitism. Body and gonad modifications are common in parasite and

crustacean host relationships (Mouritsen & Jensen 2006; Sargent et al. 2014; Romero-Rodríguez

et al. 2016; Bailly et al. 2018; Corral et al. 2021). Hosts infected by freshwater horsehair worms

grow significantly less and have a lower fecundity (Villalobos et al. 1999; Anaya & Bolek 2021).

Crabs infected by *N. zealandica* and another *Nectonema* sp. exhibit no pathological damage from

their parasites (Oku et al. 1983; Poinar Jr. & Brockerhoff 2001). Nielsen et al. (1969) reported no

effects of *N. munidae* parasitism on *M. tenuimana* growth or molting, but did find smaller

ovaries in severely infected female hosts. Gonads of male hosts, however, were normal. A few

studies also note that *N. agile* parasitism is associated with only female gonad deterioration

(Pérez 1927; Nouvel & Nouvel 1934; Born 1967). While Leslie et al. (1981) found some male

crabs with degenerated gonads, this percentage was very small (4.35 % of total parasitized) and

was not found in rock crabs examined by Brattey et al. (1985). Degeneration of rock crab testes

in the current study was directly measured through weight. Leslie et al. (1981) did not weigh

rock crab testes and therefore may have missed many crabs affected by *N. agile* parasitism. The

lack of alteration of body size and body weight, but decrease in testes weight, of parasitized rock

crabs suggests that when the parasite impacts host metabolism, the testes are some of the organs

that are affected negatively. Ovaries were not compared between infection statuses in the current

study. In the future, ovaries need to be compared to determine if there is a sex difference in the

effects of *N. agile* parasitism on rock crab gonads.

2.7 Conclusion

Despite the discovery of marine horsehair worms decades ago, little is known about the

anatomical and physiological effects on their host and, until now, nothing was known about their

effects on host behaviour. The objectives of the current study were to determine the exact species

of *Nectonema* infecting the Atlantic rock crab in the Northumberland Strait (Borden-Carleton) and to determine its influence on host behaviour (predator evasion & aggression), anatomy (body size, body weight, testes weight/to carapace width ratio, hepatopancreas size) and physiology (hepatopancreas lumen area/to total tubule area ratio). In contrast to what was predicted, parasitized crabs did not spend less time in a lighted area when given the choice between a dark and light zone. They did, however, show increased speed when exposed to a completely lighted environment. Also, unlike what was expected, these crabs did not spend any less time showing aggression than their unparasitized conspecifics. Regarding anatomy and physiology, there were no changes in body size or hepatopancreas size, body weight or hepatopancreatic lumen area/to total tubule area ratio in parasitized crabs. However, there was a decrease in testes weight/to carapace width ratio in parasitized males and a darkening of the hepatopancreas in many parasitized crabs. This is the first study investigating how *Nectonema agile* manipulates the behaviour of its host and the first to look at internal structures of the parasitized decapod hepatopancreas. The exact reasoning behind increased speed, changes in hepatopancreas colour and decreased testes weight in parasitized individuals is unknown but possible explanations were presented in this study.

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CHAPTER 3: DEVELOPMENTAL STAGES OF *PROFILICOLLIS BOTULUS* AND ITS IMPACT ON THE INVASIVE GREEN CRAB (*CARCINUS MAENAS*) BEHAVIOUR AND PHYSIOLOGY

3.1 Introduction

The green crab (*Carcinus maenas*) is native to the Atlantic coast of Europe and is invasive to the Northwestern Atlantic, Northeastern Pacific, Argentinian, South African and Australian Oceans (Young & Elliot 2020). Its widespread success is likely due to its broad salinity and temperature tolerance and generalist omnivorous diet (Young & Elliot 2020). Green crabs have caused considerable damage to multiple aquatic species, including the blue mussel (*Mytilus edulis*), American oyster (*Crassostrea virginica*), American lobster (*Homarus americanus*), Atlantic rock crab (*Cancer irroratus*) and eelgrass (*Zostrea marina*) habitat (US EPA 2017; Young & Elliot 2020).

Helminths are one of the major groups of parasites that infect the green crab. Helminths that infect green crabs within their native range include *Profilicollis botulus*, *Maritrema portucalensis*, and *Microphallus* spp. (Stentiford & Feist 2005; Blakeslee et al. 2009; Pina et al. 2011; Bojko et al. 2018). Very few parasite species infections have been reported in green crabs in their introduced regions, with *P. botulus* the only helminth infection found thus far in green crabs in Atlantic Canada (Brattey et al. 1985; Blakeslee et al. 2009; Blakeslee et al. 2015; Bojko et al. 2018).

Profilicollis botulus parasitizes ducks on both sides of the Atlantic, Northeastern Pacific and Arctic oceans (McDermott et al. 2010). Its most common definitive host is the common eider (*Somateria mollissima*) (McDermott et al. 2010). While several intermediate hosts exist for *P. botulus*, its main hosts in the Northern Europe and Arctic region are the green crab and great spider crab (*Hyas araneus*) (McDermott et al. 2010). *Profilicollis* spp., like all acanthocephalans,

attach to the intestinal walls of their host, absorbing predigested nutrients across their body wall (McDermott et al. 2010; Richardson 2013). These worms mate and reproduce within their definitive vertebrate host and release their eggs in host feces (McDermott et al. 2010). The arthropod intermediate hosts consume the released eggs, which then develop into an infective stage known as the cystacanth (McDermott et al. 2010; Richardson 2013). Once the definitive host consumes the intermediate host infected with a cystacanth, the acanthocephalan completes its development into an adult capable of sexual reproduction in the definitive host's intestine and the life cycle starts again (McDermott et al. 2010; Richardson 2013).

Research on *Profilicollis* spp. infecting brachyurans largely focusses on their manipulation of intermediate host behaviour (Latham & Poulin 2001; Latham & Poulin 2002; Poulin et al. 2003; Rojas & Ojeda 2005; Kolluru et al. 2011; Loh 2017). Parasites with complex life cycles such as *Profilicollis* spp. are believed to control the behaviour of their intermediate host to aid in their transmission to their definitive hosts (Moore 1995; Hughes & Libersat 2019). Both serotonin and dopamine have been implicated as key neurotransmitters involved in this process (Poulin et al. 2003; Rojas & Ojeda 2005). Serotonin is associated with both predator evasion and aggression in crustaceans (Helluy & Holmes 1990; Huber et al. 1997; Tierney & Mangiamole 2001; Pedetta et al. 2010; Fossat et al. 2014). *Profilicollis* spp. infections are linked to alteration or reversal of predator evasion behaviour in *Emerita analoga* and *Macrophthalamus hirtipes*, respectively (Latham & Poulin 2002; Kolluru et al. 2011). Crab aggression has not been significantly investigated in *Profilicollis* spp. infections. Latham and Poulin (2001) investigated male-male fights in infected *M. hirtipes* and determined that infection intensity was correlated with who won in these confrontations; winners had more parasites than losers during the mating

season and less after the mating season. The researchers noted that this finding was likely due to pathology, rather than parasite manipulation.

The physiological impacts of acanthocephalan parasitism have been investigated in both gammarid and decapod crustaceans. Gammarid studies have focussed on the parasites' effects on energy reserves, while decapod research has targeted their effects on metabolic rates (Haye & Ojeda 1998; Plaistow et al. 2001; Gismondi et al. 2012; Figueroa et al. 2019). Acanthocephalan infection results in lower lipid and protein reserves and increased glycogen reserves in gammarids (Plaistow et al. 2001; Gismondi et al. 2012). *Profilicollis* spp. infection in decapods may result in either an increase or decrease in metabolic rates, depending on the life stage of the host and infection intensity (Haye & Ojeda 1998; Figueroa et al. 2019).

The behavioural and physiological effects of *P. botulus* have yet to be investigated in the green crab. The physiological effects of the rhizocephalan *Sacculina carcini* and both the behavioural and physiological effects of the trematode *Micropahllus similis* on the green crab have been studied (Stentiford & Feist 2005; Blakeslee et al. 2015; Ro et al. 2022). *Sacculina carcini* infection is associated with a decrease in numbers of reserve inclusion cells (Stentiford & Feist 2005). While metabolic rate has not been directly examined, it is expected to be higher due to increased depletion of energy stores. *Microphallus similis* infection results in a short-lived drop in circulating haemocytes (Blakeslee et al. 2015). Two studies have investigated behavioural manipulation by *M. similis* in green crabs. In one study, early stages of infection were associated with an increase in righting response time and long-term infection led to an increase in feeding time (Blakeslee et al. 2015). In the second study, no differences in behaviour were seen between the parasitized and unparasitized crabs (Ro et al. 2022).

3.2 Objectives

Acanthocephalan behavioural manipulation has been extensively studied in gammarid species. However, behavioral modification induced by *P. botulus* infection of its intermediate host has yet to be examined. The first objective of this study was to determine whether *P. botulus* manipulates the behaviour of its host to aid its transmission from intermediate host to definitive host, and if so, at what stage of development does it elicit this effect. As the parasite develops in the intestine of its intermediate host, it would need to draw on energy resources from within the host to fuel its development. The second objective of this study was to determine if, like other acanthocephalans, *P. botulus* alters the energy reserves of its crustacean host.

3.3 Hypotheses

1. Parasitized crabs will have increased predator evasion response and show decreased aggression during the non-infective stage (acanthella) of the parasite compared to unparasitized crabs and those parasitized by the infective stage (cystacanth) of *P. botulus*.

2. Cystacanth-parasitized crabs will have decreased predator evasion response and show increased aggression in comparison to unparasitized crabs and those infected with the acanthella stage of *P. botulus*.

3. The hepatopancreatic glycogen reserve inclusion body area of parasitized crabs will decrease in size during times of significant parasitic growth.

3.4 Materials and Methods

3.4.1 Animal collection, husbandry, and experimental infection

One hundred green crabs were caught using Promar collapsible crayfish trays and Russel green crab traps at Malagash, Nova Scotia, Canada, an area known to be low in *P. botulus* prevalence (Dr. Fraser Clark, personal communication). These were then transported and housed at Dalhousie University's Agricultural Campus (Bible Hill, Nova Scotia) in adjoining tanks with cooled and recirculated natural seawater (16 ± 2 ºC, pH 7 ± 1) and a 12:12 shaded light/dark cycle. *Profilicollis botulus* adults were removed from two dissected common eider intestines and minced to release the parasite's eggs (Figure 16). Fifty crabs were placed in individual plastic dishes twice and fed these eggs (parasite-exposed). Both parasite-exposed and unexposed crabs were fed one frozen shrimp (*Pandalus borealis*) each, once a week for the entire experiment.

Figure 16. Dissected small intestine from common eider showing attached *Profilicollis botulus* adults (black arrows).

3.4.2 Behaviour Tests

    A mix of male and female crabs were randomly assigned to different groups (parasite-exposed versus unexposed). Behavioural analysis was initiated one month after parasite exposure and was repeated every 4 weeks for 16 weeks, on subsets of each group. Crabs were tested for their predator evasion, aggression and righting response behaviour. Exposed and unexposed crabs were kept in separate trays until tested. Tested crabs were then placed in a separate dry drip tray from untested crabs, again separating the exposed and unexposed groups. Once all crabs were tested, they were placed in a glass aquarium filled with aerated natural seawater (4 ± 1 ºC) until the next testing day (one per group). Water was replaced at the end of each day in these aquaria.

3.4.2.1 Predator evasion

    This test was conducted over a one-day period using a protocol modified from Hamilton et al. (2016). Twenty crabs each month, for the first 3 months, were tested (exposed = 6 ♂, 4 ♀; unexposed = 5 ♂, 5 ♀). Twenty-four crabs (exposed = 8 ♂, 4 ♀; unexposed = 8 ♂, 4 ♀) were tested in the final month. An acrylic tank (60 cm x 30 cm x 30 cm, l x w x h) divided in half by a dark zone (black) and a light zone (white) was filled with 5 ± 1 cm of new natural seawater (4 ± 1 ºC). Crabs were individually placed along the line dividing the dark and light zones and immediately recorded for 5 min using a Logitech HD camera. Time spent in the dark zone was measured as predator evasion behaviour and time in the light zone was measured as exploratory (non-evasive) behaviour. Water was changed between each crab to avoid residual chemical signals from affecting subsequent experimental trials.

3.4.2.2 Aggression

This test was performed on the same crabs over a period of two days after the predation

evasion test using a protocol modified from Hamilton et al. (2016). An all-white acrylic tank (60

cm x 30 cm x 30 cm, l x w x h) with a panel covering a mirror on one side (approach zone) was

filled with the same amount of fresh natural seawater as the predator evasion test. Crabs were

individually placed into the tank and left for 35 min to habituate. Once the panel was removed

the crab was moved to the center of the wall across from the mirror (avoidance zone), showing

the crab their reflection. The crab's behaviour was recorded for 5 min using the Logitech HD

camera. The time spent in the approach zone was measured as aggressive behaviour. The time

spent in the avoidance zone was measured as submissive behaviour. Water was again changed

between each crab to avoid potential effects of residual chemical signals from previous

experimental trials. Crab movement, measured as mobile average speed (mm/s) and total

distance travelled (mm) was measured via ToxTrac™ (Rodriguez et al. 2017, 2018).

3.4.2.3 Righting response

This test was performed a couple of days after the aggression test on most of the crabs

previously tested, over a 2 h period ($n_{4th\ week}$ = 9 exposed: 6 ♂, 3 ♀, 10 unexposed: 5♂, 5 ♀; n_{8th}

$_{week}$ = 10 exposed: 6 ♂, 4 ♀, 9 unexposed: 5 ♂, 4 ♀; $n_{12th\ week}$ = 10 exposed: 6♂, 4♀, 10

unexposed: 5♂, 5♀; $n_{16th\ week}$ = 11 exposed: 7♂, 4♀, 12 unexposed: 8 ♂, 4 ♀). Crabs were placed

dorsal side down in a dry tank and the time taken to flip back over or right themselves was

measured. This test was performed three times on each crab, as described by Blakeslee et al.

(2015). The crabs were timed for 5 min in total before being placed in a dry drip tray for humane

euthanasia and necropsy. Crabs with less than 4 walking legs were not included in the righting

response test and those that did not right themselves after 5 min were removed from analysis.

3.4.3 Necropsy and dissection

Tested crabs were necropsied each month after righting responses were performed to determine infection status (parasitized or unparasitized), infection intensity (# of acanthellae/cystacanths) and to look for any gross signs of pathology. Some hepatopancreas tissue and the intestine of each crab were removed and placed in Davidson's Seawater Fixative for two days (Hopwood 1996). These were then removed and placed into 70% ethanol for tissue storage until they were trimmed into histology cassettes for slide preparation.

3.4.4 Slide preparation, parasite identification and hepatopancreas analysis

Tissues were embedded in paraffin wax, sections trimmed to 5 µm and mounted onto glass slides and stained with hematoxylin and eosin. These slides were then viewed under a Motic BA310E™ light microscope. Any parasites not identified in the initial necropsy were noted and counted via histologic parasite profiles to determine infection intensity. Hepatopancreas tissue was viewed at 100 x total magnification using Omax ToupView™ 3.7 and pictures were taken using an Omax A3550U3™ 5.1 MP USB camera attached to the microscope. Images were analyzed using ImagePro Plus 10™'s segmentation software, targeting the red color associated with glycogen reserve inclusion bodies. The total area of these bodies was determined for 3 random fields of view per crab. Glycogen body presence was also scored on a scale of 0 to 4 (0 = no glycogen bodies, 4 = > 50 % interstitial space cover) to determine the effectiveness of the software (Stentiford & Feist 2005).

3.4.5 Statistical analysis

Those crabs in the exposed group that were not confirmed to be parasitized were removed from statistical analysis. Infection intensity, behaviour and glycogen body data were checked for

normality using the Anderson Darling test in Minitab 19™. When determining the effects of infection status (parasitized/unparasitized) on behaviour and glycogen body data, normally distributed data were analyzed using a 2-sample t-test and those that failed the normality test were analyzed using a Mann-Whitney test. Mann-Whitney tests were also used to determine whether infection intensity, behaviour and glycogen body data changed over time. Regression analysis was performed to determine the effect of carapace width on infection intensity and the effect of infection intensity on tested behaviours and glycogen body area. A result was deemed significant when a p-value was < 0.10.

3.5 Results

3.5.1 Infection status and infection intensity

Twenty percent (2/10: 2 ♂) of the crabs were confirmed to be parasitized in the exposed group when tissues were analyzed during the 4th week of infection. The number of infected crabs was higher in the second month, at 60% (6/10: 2 ♂, 4 ♀). Seventy percent and 58.3% of crabs were parasitized at the 12th (7/10: 5 ♂, 2 ♀) and 16th (7/12: 3 ♂, 4 ♀) weeks of infection, respectively. A significant difference in infection intensity only occurred between the 4th week and 8th and 16th weeks; infection intensity was higher in the 4th week (p = 0.065, p = 0.056; Figure 17). A strong, positive correlation was seen between carapace width and infection intensity at the 4th and 8th week (r = 1.00, r = 0.864). This trend did not continue in weeks 12 and 16 of this study. Regression analysis (x = carapace width, y = infection intensity) could only be determined for the 8th – 16th week due to only 2 crabs being parasitized in the 4th week. Carapace width explained the majority of variance seen in infection intensity for the 8th week only (R^2 = 68.41 %, p = 0.026).
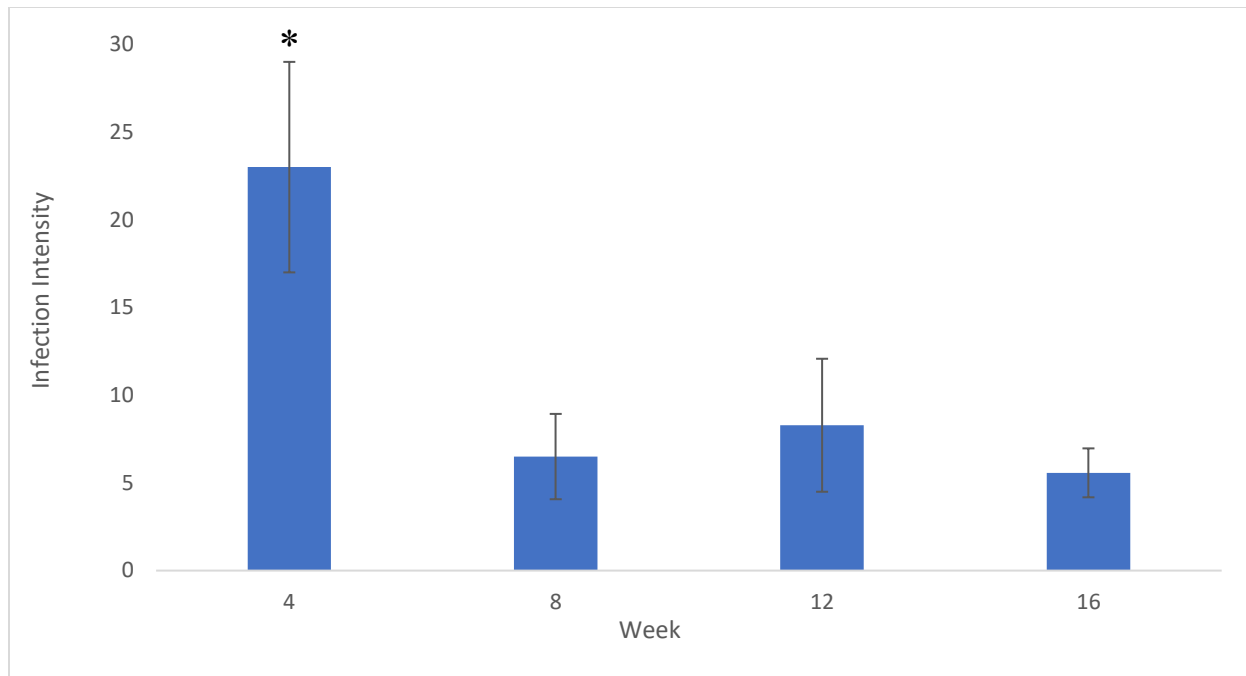
Figure 17. Infection intensity of green crabs (mean ± standard error) exposed to *Profilicollis botulus* eggs over 4 months (16 weeks). Asterisk represents week with highest infection intensity (vs. 8th & 16th week; p = 0.065, p = 0.056).

3.5.2 *Profilicollis botulus* development

Young acanthellae were only seen microscopically in the intestinal tissue of parasitized crabs during the 4th week of infection. It was only during the later acanthella and cystacanth stage (8th week +) that developing *P. botulus* could be viewed macroscopically (Figure 18). In the first month of infection, acanthellae were initially ovoid or elliptical in shape (Figure 19A). These acanthellae contained giant nuclei; elliptical shaped acanthellae had a clearly defined inner cell mass and proboscis anlage. These acanthellae ranged in size from ~ 10-17 µm in length. Upon the second month, they elongated and began to develop a proboscis and cement glands, growing to ~ 50-170 µm (Figure 19B). The rate of proboscis development varied among acanthellae, with some more defined that others. Acanthellae transitioned into cystacanths between 8 and 12 weeks, remaining similar in size (~ 50-160 µm). Cystacanths were identified

by their thorny invaginated proboscis and encapsulated tegument at 12 weeks (Figure 19C). Thorns on the proboscis appeared to enlarge in some cystacanths over the course of the next month (Figure 19D). No observable haemocytic immune reaction could be seen around developing *P. botulus*.
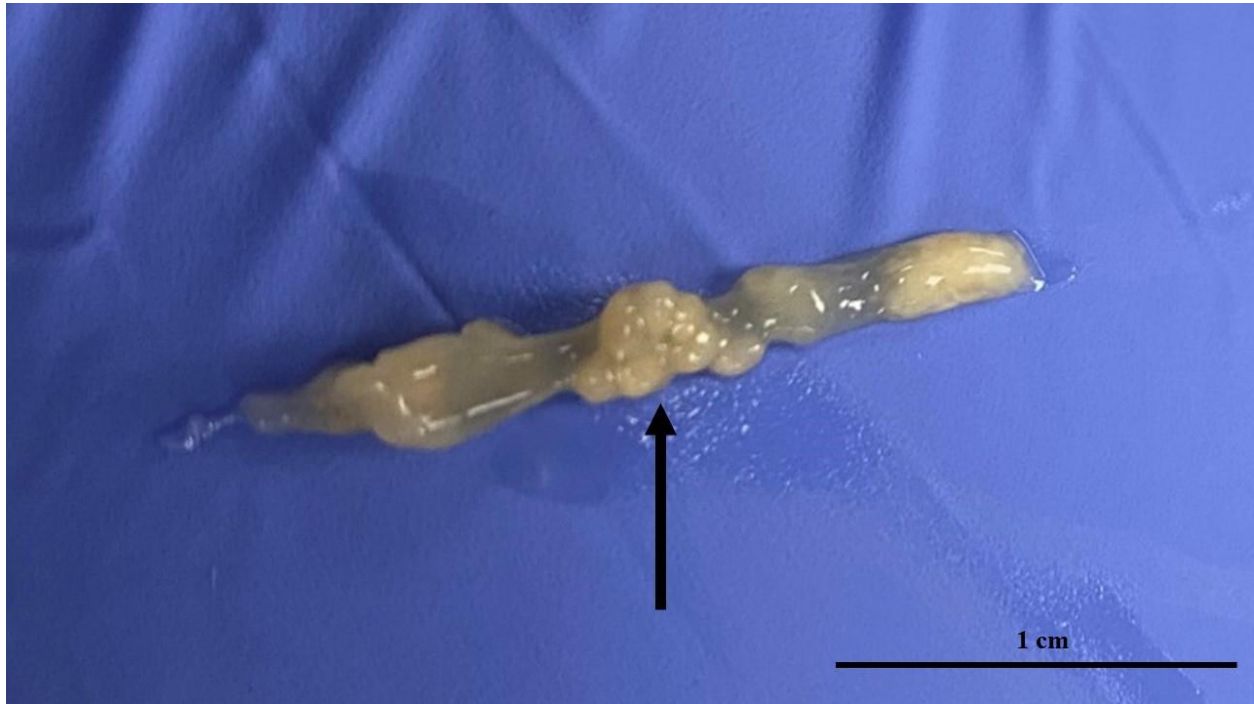
Figure 18. Dissected green crab intestine showing a cluster of *Profilicollis botulus* cystacanths developing along the tissue wall (black arrow).
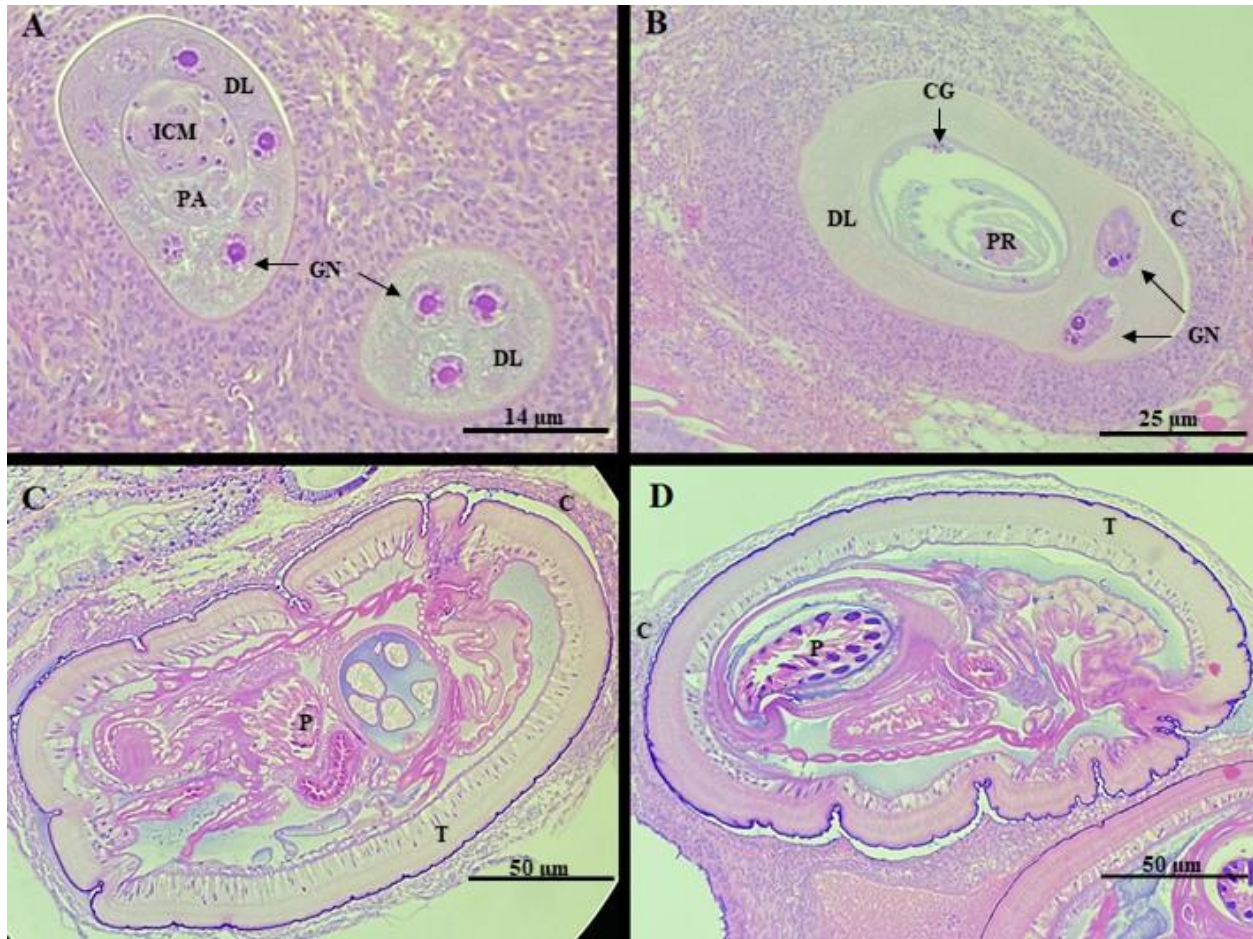
Figure 19. Development of *Profilicollis botulus* from 4th - 16th weeks (100 x total magnification). **A.** Acanthellae consisting of a dermal layer (**DM**), an inner cellular mass (**ICM**), proboscis anlage (**PA**) and giant nuclei (**GN**) developing within the intestinal wall of green crab host (4 weeks post-exposure). **B.** Elongated acanthella developing proboscis region (**PR**) and cement glands (**CG**) (8 weeks post-exposure). **C.** Twelve-week-old cystacanth with noticeable, invaginated proboscis (**P**), tegument (**T**) and capsule (**C**). **D.** Sixteen-week-old cystacanth with fully thorned proboscis.

3.5.3 Behaviour tests

3.5.3.1 Predator evasion

All data, except for time spent in the black zone during the 12th week, failed the

normality test. A significant difference in predator evasion behaviour for parasitized crabs only

occurred between weeks 8 and 12; crabs spent more time at the midline on the 12th week (p =

0.008; Figure 20). Unparasitized crabs, however, showed a more widespread change in

behaviour. They spent significantly more time in the black zone at the 4th and 8th week in comparison to the 12th and 16th week tested (4 vs. 12: p = 0.064; 4 vs. 16: p = 0.044, 8 vs. 12: p = 0.028; 8 vs. 16: p = 0.008). These crabs also spent significantly more time in the white zone during the later half of the study, in comparison to the first eight weeks (4 vs. 12: p = 0.093; 4 vs. 16: p = 0.098; 8 vs. 12: p = 0.046; 8 vs. 16: p = 0.072). They spent significantly more time at the midline in the first eight weeks in comparison to the 16th week (4 vs. 16: p = 0.037; 8 vs. 16: p = 0.009). Despite this, parasitized and unparasitized green crabs showed no significant difference between groups in the time they spent within the black zone and white zone during the entire study period (p > 0.10). There was only a significant difference in the time spent at the midline at 12 weeks (p = 0.028). Parasitized crabs spent ~ 2.2 times more time at the midline than unparasitized crabs during this time. There was no association between infection intensity and predator evasion behaviour at any point in the study period (p > 0.10).
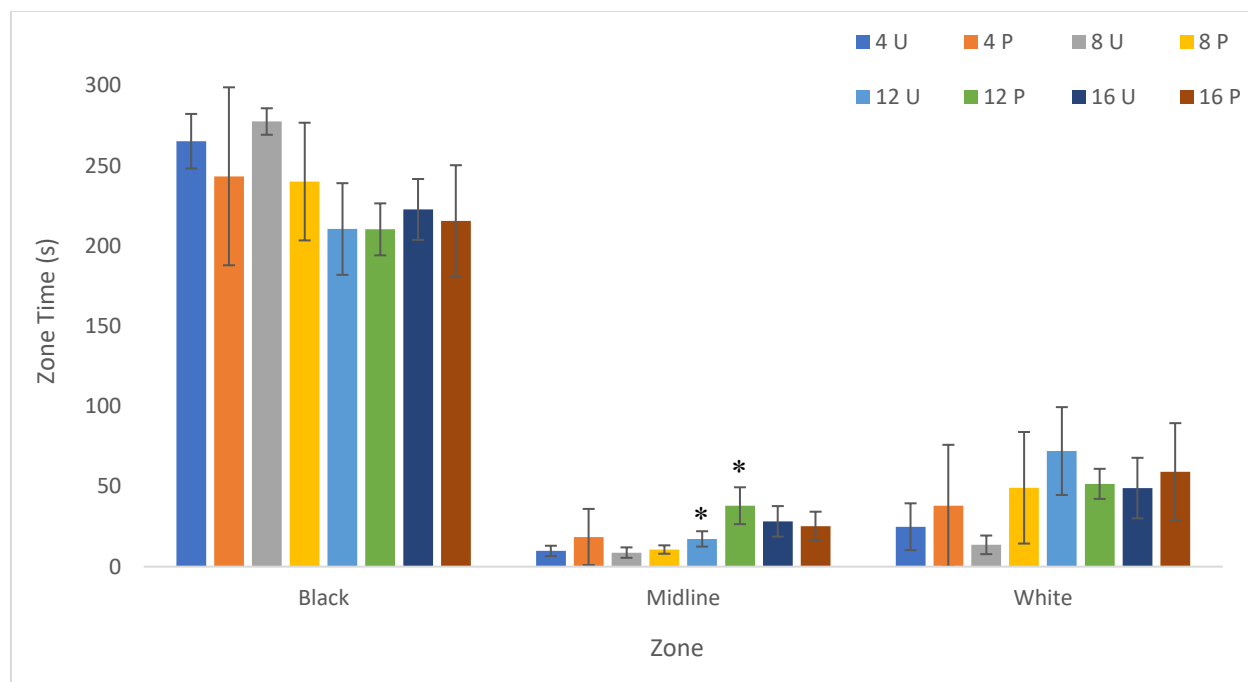
Figure 20. Time spent in the black zone, at the midline and in the white zone (s) (mean ± standard error) for *Profilicollis botulus*-parasitized (**P**) and unparasitized (**U**) green crabs. Numbers (**4**, **8**, **12** & **16**) represent the week tested. Asterisks represent significance between infection statuses (p = 0.028).

3.5.3.2 Aggression

Most of the data, except for the 4th week, failed the normality test. Parasitized crabs spent significantly more time in the avoidance zone than the approach zone during the 12th and 16th week of study (p = 0.009, p = 0.001; Figure 21). Unparasitized crabs spent significantly more time in the avoidance zone than the approach zone during the 4th and 16th week (p = 0.063, p = 0.013). When looking at each zone separately over time, unparasitized crabs spent more time in the avoidance zone during the 8th week versus the 16th (p = 0.072). No such trend existed for the parasitized crabs (p > 0.10). Also, there was no significant difference in the time spent in the avoidance or approach zones during the duration of the study period between parasitized and unparasitized green crabs (p > 0.10). Infection intensity had no influence on avoidance or approach behaviour at any time during the study (p > 0.10). It is important to note that the

majority of crabs, both parasitized and unparasitized, showed no physical signs of aggression toward the mirror during the test, such as raising the body or extending claws.
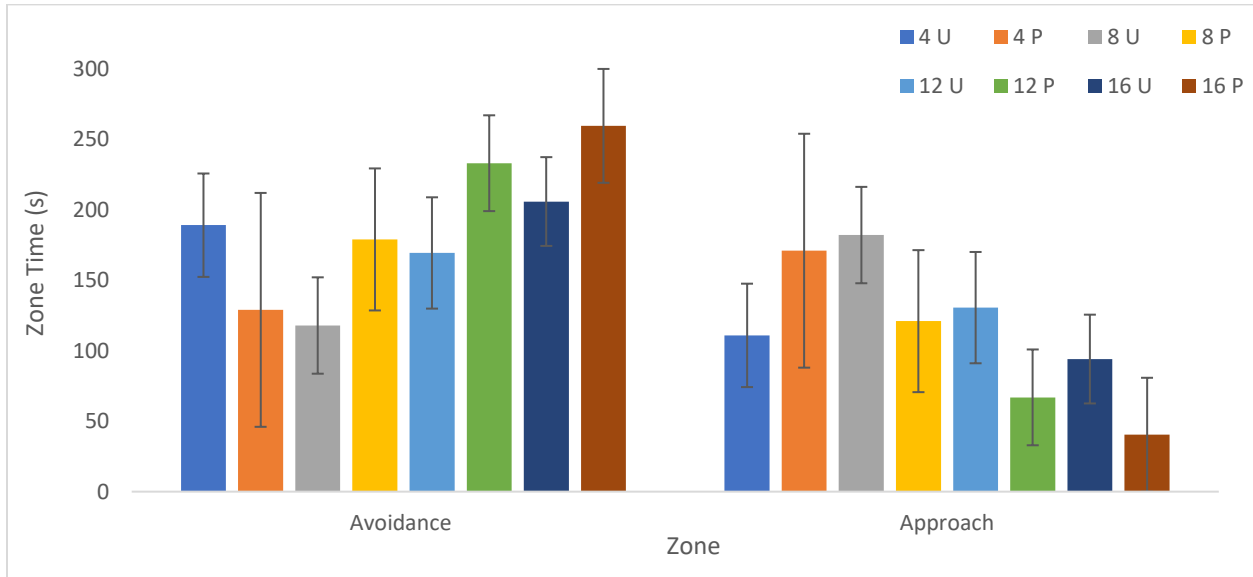
Figure 21. Time spent in the avoidance and approach zones (s) (mean ± standard error) for *Profilicollis botulus*-parasitized (**P**) and unparasitized (**U**) green crabs. Numbers (**4**, **8**, **12** & **16**) represent the week tested.

3.5.3.3 Movement

Total distance travelled data failed the normality test and mobile average speed data had a normal distribution during the 4th week. This was the opposite during the 8th week. All data were normally distributed for the 12th week and failed the normality test for the 16th week. Parasitized crabs significantly decreased mobile average speed and distance travelled between the first eight weeks and 16th week (4 vs. 16: $p = 0.057$, $p = 0.057$; 8 vs. 16: $p = 0.008$, $p = 0.005$; Figure 22; Figure 23). Unparasitized crabs only significantly decreased their speed and distance travelled between the 8th and 12th week ($p = 0.054$, $p = 0.064$). Parasitized and unparasitized crabs had no statistically significant difference in their mobile average speed and total distance travelled during the first 12 weeks of the study ($p > 0.10$). However, there was a significant difference

between crabs at week 16 (p = 0.008, 0.004). Parasitized crabs were ~ 3.1 times slower and

travelled ~ 3.4 times less distance at this time. Infection intensity had no effect on these

behaviours over the 16 weeks (p > 0.10).
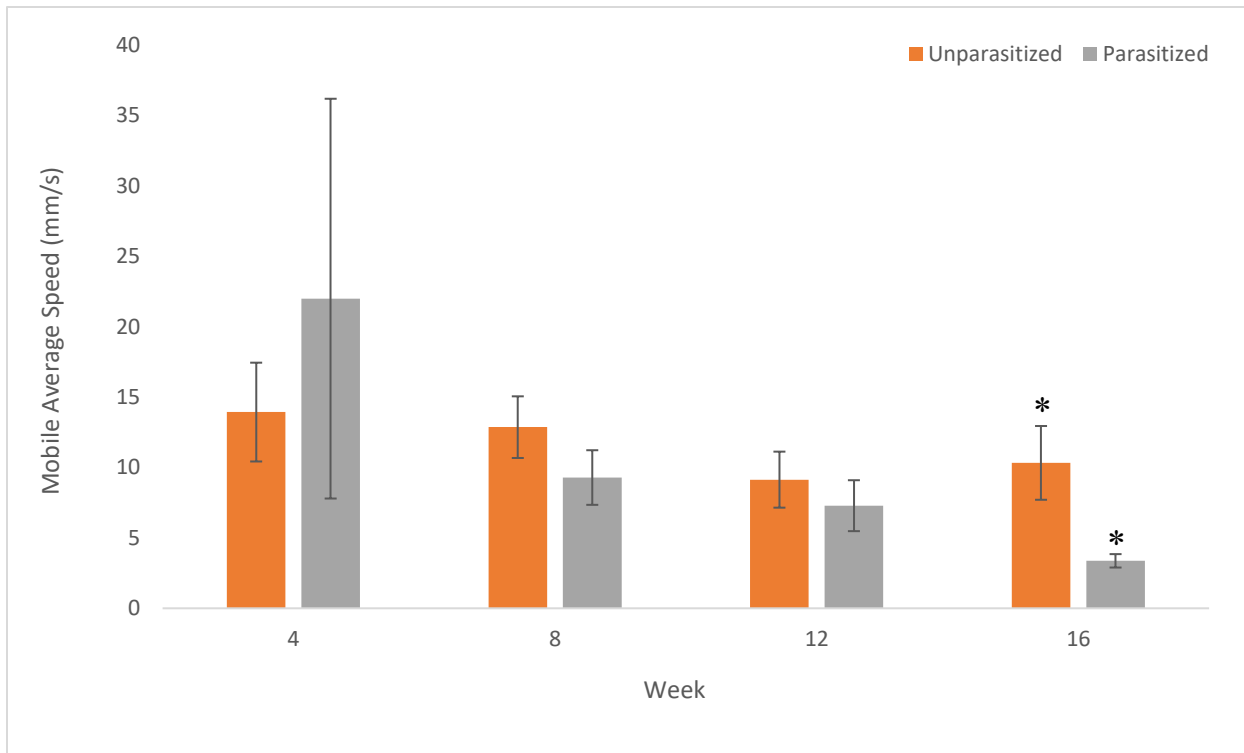
Figure 22. Mobile average speed (mm/s) (mean ± standard error) for *Profilicollis botulus*-parasitized and unparasitized green crabs. Asterisks represent significance between infection statuses (p = 0.008).
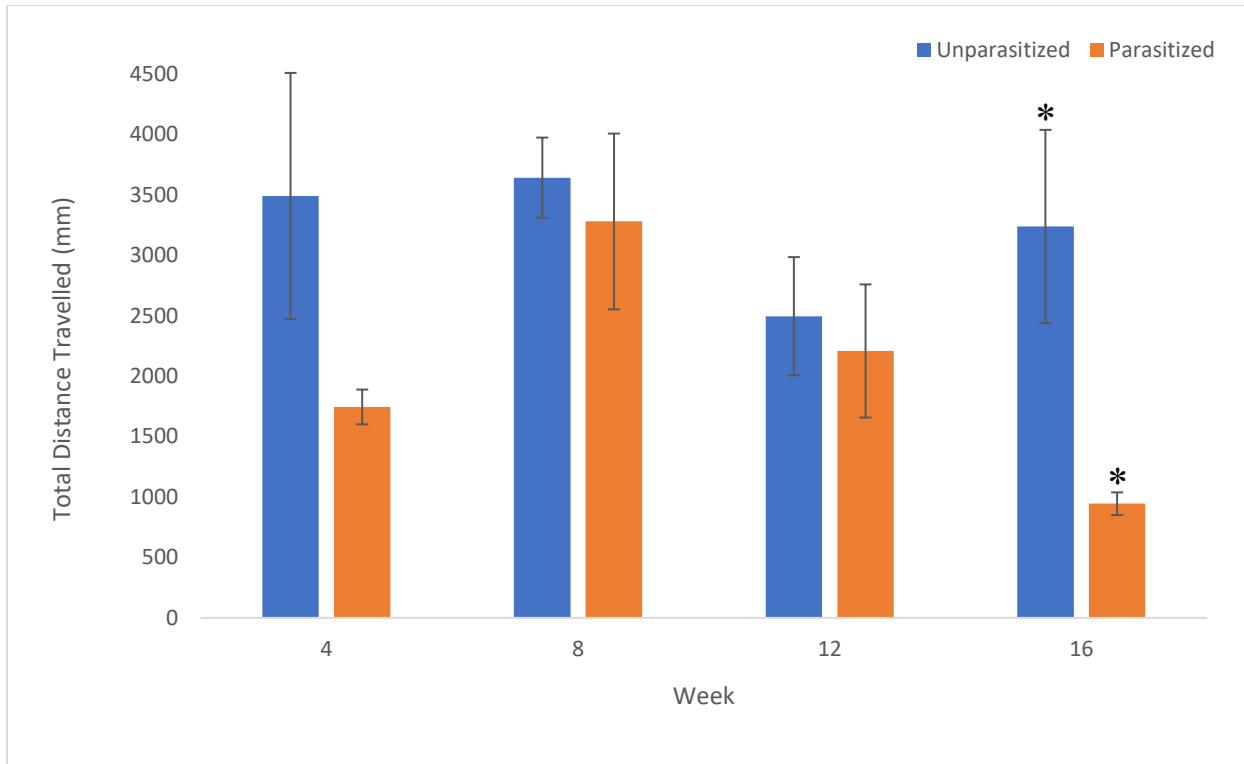
Figure 23. Total distance travelled (mm) (mean ± standard error) of *Profilicollis botulus*-parasitized and unparasitized green crabs. Asterisks represents significance between infection statuses (p = 0.004).

3.5.3.4 Righting Response

All data failed the normality test. No significant change in righting response time occurred over time for parasitized crabs (p > 0.10; Figure 24). Unparasitized crabs took significantly longer to right themselves during the 16th week of testing compared to the first eight weeks (4 vs. 16: p = 0.017; 8 vs. 16: p = 0.016). No significant difference was found in the righting response time between parasitized and unparasitized crabs for the first 12 weeks of the study (p > 0.10). A significant difference in righting response between parasitized and unparasitized crabs was found at week 16 (p = 0.012). Parasitized crabs righted themselves ~ 3.4 times faster than unparasitized crabs at this time. Infection intensity had no influence on righting response throughout the duration of the study (p > 0.10).
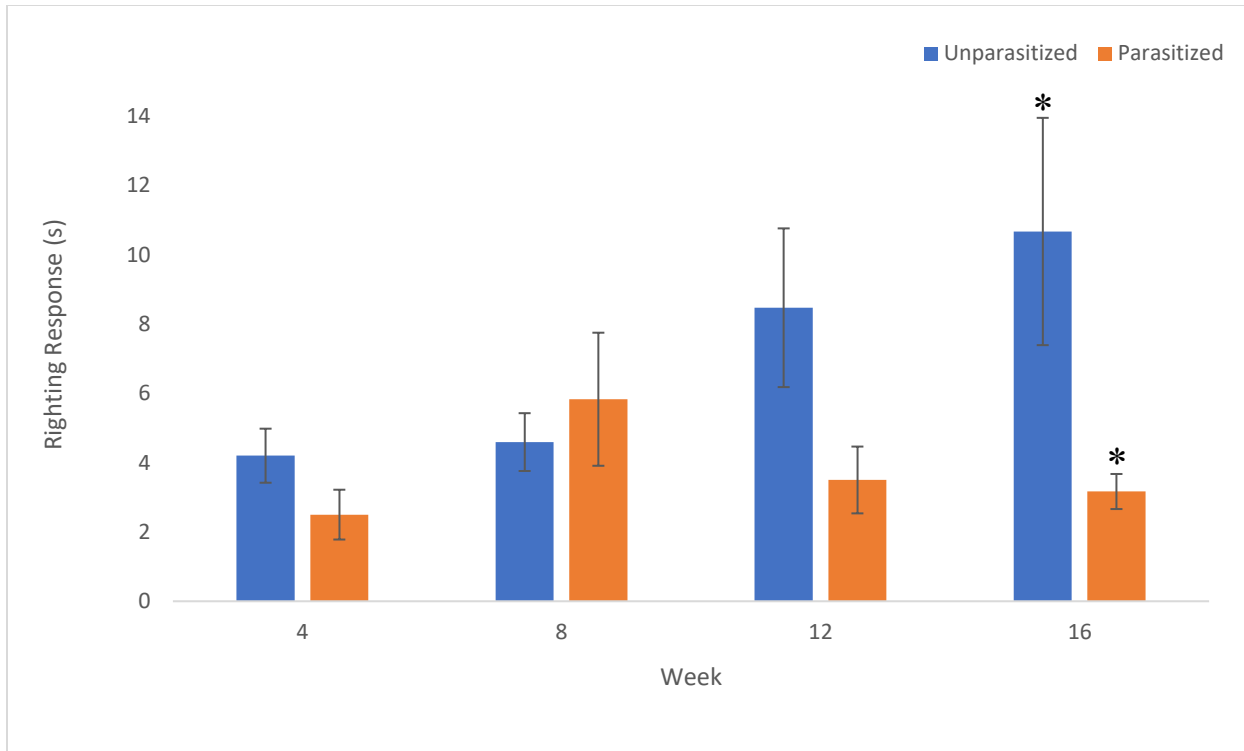
Figure 24. Time spent to right (s) (mean ± standard error) for *Profilicollis botulus*-parasitized and unparasitized green crabs. Asterisks represents significance between infection statuses (p = 0.012).

3.5.4. Glycogen reserve inclusion body area

All data failed the normality test. Parasitized crabs had a significant decrease in glycogen reserves during the 12th week, in comparison to the previous eight weeks (4 vs. 12: p = 0.013; 8 vs. 12: p = 0.003). This was followed by a significant increase between the 12th and 16th week (p = 0.000; Figure 25). Scoring also indicated that reserves were significantly higher in the 16th week, in comparison to the 4th week (p = 0.021). Unparasitized crabs had significantly more glycogen reserves in the first eight weeks of the study, in comparison to the 16th week. (4 vs. 16: p = 0.002; 8 vs. 16: p = 0.008). Additionally, scoring indicated a significant decrease between the 4th and 12th week (p = 0.098). There was a significant difference in glycogen reserve inclusion body area between parasitized and unparasitized crabs at 12 and 16 weeks (p = 0.021, p = 0.067;

Figure 26). About 6.2 times less glycogen reserves were present in parasitized crabs during the 12th week. The difference between infection statuses was less intense at week 16; parasitized crabs only had about 1.4 times more glycogen reserves than unparasitized crabs. Scoring also revealed significant differences between infection statuses during the first 8 weeks of the study. Unparasitized crabs had higher glycogen reserve scores during the 4th and 8th week, in comparison to parasitized crabs (p = 0.008, p = 0.088). Infection intensity explained very little of the variance seen in glycogen body area during the 4th and 12th weeks of the study period (R^2 = 5.84%, p = 0.084; R^2 = 5.70%, p = 0.051).
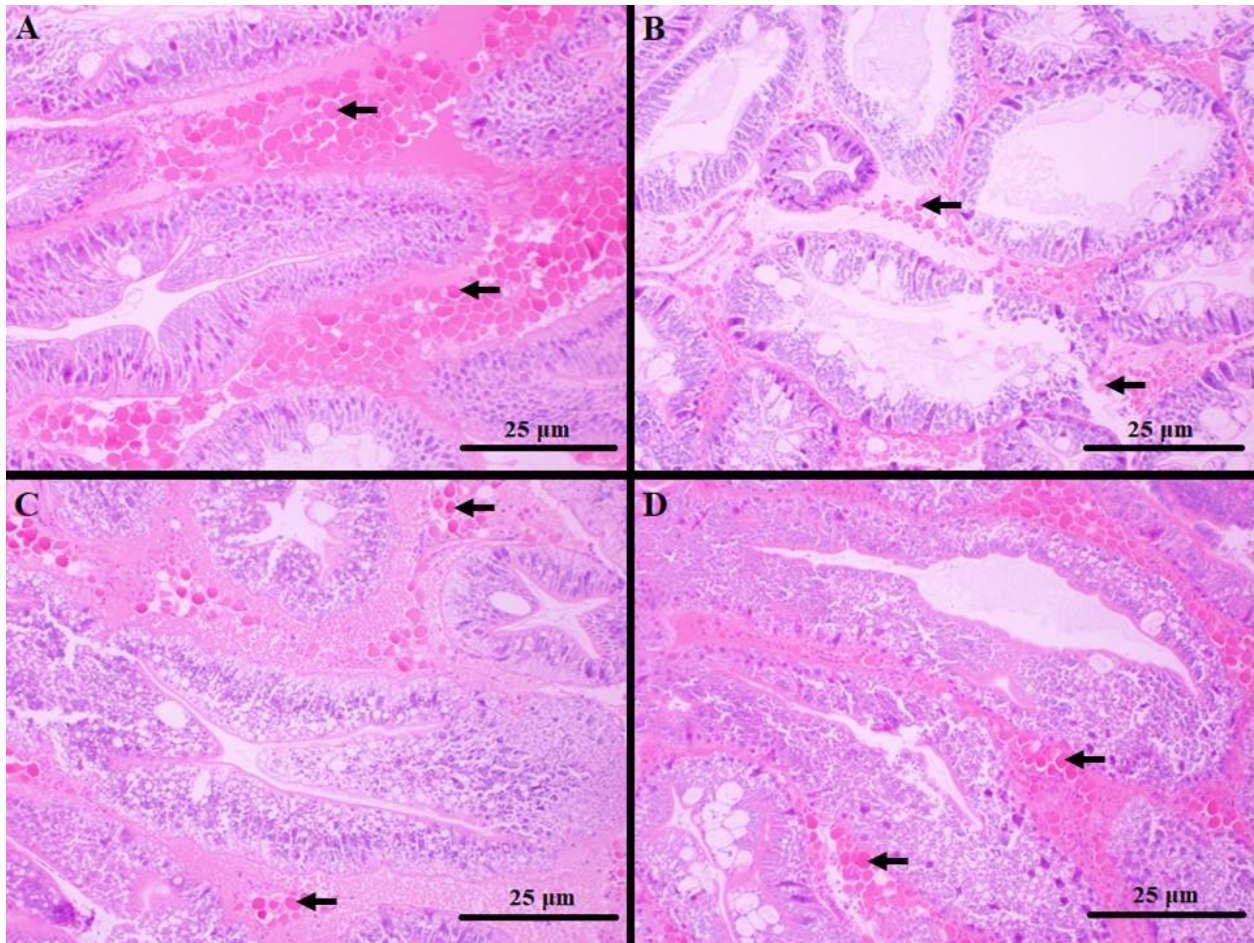
Figure 25. Hepatopancreatic glycogen reserve inclusion bodies in *Profilicollis botulus*-parasitized and unparasitized green crabs at 100 x total magnification (black arrows). **A**. Twelve-week unparasitized. **B**. Twelve-week parasitized. **C**. Sixteen-week unparasitized. **D**. Sixteen-week parasitized.
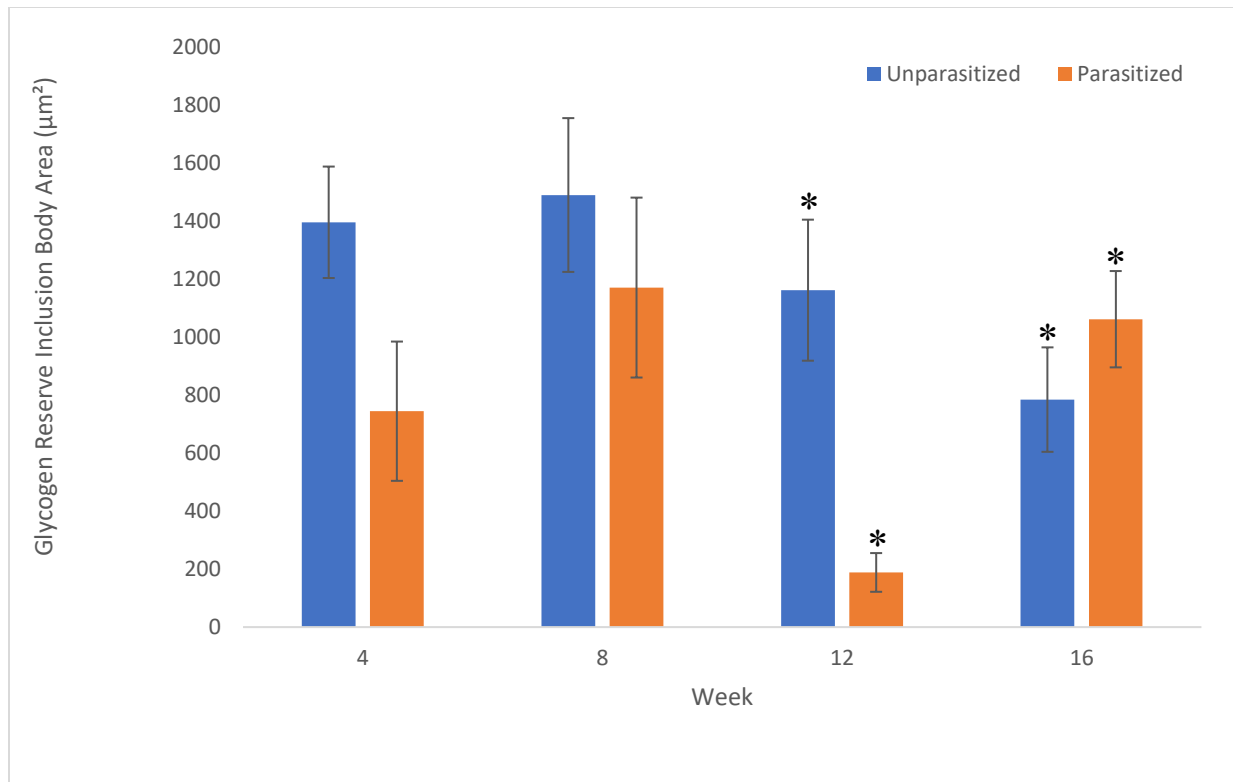
Figure 26. Glycogen reserve inclusion body area (μm^2) (mean ± standard error) for *Profilicollis botulus*-parasitized and unparasitized green crabs. Asterisks represents significance between infection statuses (p = 0.021, p = 0.067).

3.6 Discussion

3.6.1 Infection status and infection intensity

In the first four weeks of the experiment, a very low number of crabs exposed to *P. botulus* eggs were confirmed to be parasitized. There are several reasons this may occur, including variations in acanthellae growth, the small sample size of crabs tested or the way the intestinal tissue was sliced during slide preparation. The number of parasitized crabs detected at eight weeks increased, but a large apparent reduction in infection intensity was observed. This may be due to intraspecific competition between the acanthellae. Intraspecific competition has been noted to cause a decrease in cystacanth size for the acanthocephalans *Profilicollis* spp., *Pomphorhynchus laevis* and *Acanthocephalus tumescens* (Dezfuli et al. 2001; Poulin et al. 2003;

Rauque & Semenas 2011). As conspecifics compete for limited resources, there is a strain on the growth of individual acanthocephalans. It may be that as *P. botulus* acanthellae were growing in their green crab host, some acanthellae outcompeted others, decreasing and eventually stunting the growth of weaker ones. The surviving acanthellae went on to develop into cystacanths and unlike previous studies, showed little evidence of competition at this stage. It is important to note that the previous studies looked at cystacanth volume, which was not calculated in this study. It is possible that evidence of intraspecific competition among cystacanths may have been seen if *P. botulus* size was compared among parasitized individuals, rather than simply noting an overall range in size.

Another explanation for the large difference in infection intensity between the 4th and 8th week may be due to the method of counting. *Profilicollis botulus* individuals were counted using histologic parasite profiles. If some of the worms were coiled and sliced through during slide preparation, then two pieces of the same worm may have appeared as two separate worms, resulting in an overestimation of infection intensity.

In this study, infection intensity was highest in the largest parasitized crabs only for the first 8 weeks. This contrasts with what was found in other *Profilicollis* spp. infections (Liat & Pike 1980; Thompson 1985; Latham & Poulin 2001). Typically, a positive relationship exists between crab carapace width and cystacanth infection intensity; larger crabs harbour more cystacanths. Two of these studies looked at *P. botulus*-infected green crabs so it is odd that this pattern was not found with cystacanth infection in the current study (Liat & Pike 1980; Thompson 1985). It is possible that this trend may have been observed in this trial if larger sample sizes were used during each testing week, increasing the probability of finding the expected relationship via a wider range of carapace widths. However, it is important to note that

these studies were done four decades ago and on green crabs in their native range. Perhaps warming ocean temperatures altered the host-parasite association between *P. botulus* and green crabs. Increased water temperatures can enhance molting frequency in decapod species, including the snow crab (*Chionoecetes opilio*) and the green mud crab (*Scylla paramamosain*) (Shields 2019; Nur Syafaat et al. 2020). This enhancement in molting may make these animals more susceptible to parasitic infections (Shields 2019). It is possible that green crabs are molting more frequently with increasing water temperatures and thus becoming more susceptible to infections at all sizes. In the case of the parasite, *Hematodinium perezi*, increasing temperatures stimulate the development of its presumptive infectious stage and enhance transmission to susceptible juvenile hosts (Shields 2019). *Profilicollis botulus* may be following a similar trend; as water temperatures rise more juvenile green crabs are being infected and thus the previous positive carapace width-infection intensity relationship no longer exists. Alternatively, invasive green crabs infected with *P. botulus* may not follow the positive carapace width-infection intensity relationship. Blakeslee et al. (2009) compared parasite infection prevalence between Asian shore (*Hemigrapsus sanguineus*) and green crabs in their native and invasive ranges and found that the invasive conspecifics were often able to escape infection. While *P. botulus* is found on both sides of the North Atlantic, it is possible that invasive green crabs in Eastern Canada have escaped higher levels of infection, affecting the previous reported relationship.

3.6.2 *Profilicollis botulus* development

In the present study, acanthellae could be seen in parasitized crabs at 4 weeks, and some variation in acanthellae stages could be seen at 8 weeks (56 days). No cystacanths were detected via histologic examination at 8 weeks. *Profilicollis botulus* development was only tracked every 4 weeks; however, based on observations, it was expected that cystacanth development started

between 60 and 90 days (8th-12th week). A similar trend of development has been noted by previous studies. Thompson (1985) noted that once *P. botulus* have infected their green crab host, they should take ~70 days to reach their cystacanth stage at 16 ºC. Rayski & Garden (1961) conducted feeding trials on green crabs and tracked significant life stages of *P. botulus*. They found that acanthors had emerged by day 2, acanthellae had began to develop by 3-5 weeks and many acanthellae stages could be seen at 50 days. By day 62, some crabs had began developing cystacanths, while others contained large numbers of acanthellae.

3.6.3 Behaviour tests

3.6.3.1 Predator evasion

During the acanthella (non-infective) stage (4th & 8th weeks), there was no significant difference in predator evasion behaviour between unparasitized and *P. botulus*-parasitized green crabs. Studies investigating predator evasion in acanthocephalan-infected gammarids have found a difference in host behaviour associated with varying parasite developmental stages. When *Gammarus pulex* is infected by the acanthella stage of *Polymorphus minutus* or *Pomphorhynchus laevis* it exhibits behaviour that makes it less susceptible to predation (Dianne et al. 2011; Bailly et al. 2018). When infected by *P. minutus* acanthellae, *G. pulex* shows a stronger positive geotaxis; they are found lower in the water column (Bailly et al. 2018). Acanthellae of *P. laevis* cause *G. pulex* to hide in refuges more frequently and decrease their predation by trout (Dianne et al. 2011). The findings from these gammarid studies suggest that the acanthocephalan parasite induces behavioural changes that protect its host from predation when it is in the acanthella stage. Despite what these studies have shown*, P. botulus* does not appear to affect the predator evasion behaviour of its green crab intermediate host in a light/dark test during the acanthella stage. While it is not exactly clear why this is the case, a larger sample size could have resulted

in different results and yielded altered behaviour in the acanthellae-infected crabs. It is also possible that, due to the high infectivity of *P. botulus*, it is not worthwhile for the parasite to protect its host from predation during the acanthella stage as one adult has many opportunities to propagate its genes through many crabs and have them reach the definitive host.

As *P. botulus* acanthellae transitioned into cystacanths (infective stage), a significant increase in time spent at the midline was seen in parasitized green crabs (8th vs 12th week). Also, early *P. botulus* cystacanth stage crabs (12th week) spent more time at the midline than unparasitized crabs. Previous studies report that acanthocephalan infection alters intermediate host behaviours in such a way as to promote predation once the parasite reaches the cystacanth stage. The predator evasion host behaviours promoted by *P. minutus* and *P. laevis* acanthella stages are reversed once the parasites reach their cystacanth stage; thereby exposing their hosts to predation (Dianne et al. 2011; Bailly et al. 2018). Cystacanths of *P. laevis* within *G. pulex* cause a decrease in host attraction to conspecifics, and a reversal of natural photophobia in the presence of a predator cue (Durieux et al. 2012). Cystacanth parasitism by *Profilicollis* spp. and *Hexaglandula corynosoma* are associated with a lower chance of the host being burrowed, and thus more exposure to light and predators (Latham & Poulin 2001; Pérez-Campos et al. 2012). In the present study, it was not clear whether predation was suppressed or promoted by *P. botulus* cystacanths, as the only significant difference in behaviour was time spent at the midline. While being at the midline does expose these crabs to more light, and thus more predators, it also provides a quick escape into the simulated sheltered or black zone. Therefore, it is more likely that the increase in time spent at the midline by these crabs was due to their slower movement, rather than changes in predator evasion behaviour. Unfortunately, the crab tracking software was

unable to calculate crab speed on the black background due to problems separating the green crab from its dark surroundings so a direct comparison of speeds in each zone could not be made.

It is not completely clear why unparasitized crabs spent more time in the black zone during the first half of the study and then decreased this time in the later half. One possible explanation is that human contact reversed the phototactic behaviour naturally seen in unparasitized crabs, as repetitive human contact often leads to habituation (Geffroy et al. 2020). Crabs typically avoid humans and flee to a sheltered (or dark) area when encountered (Hemmi 2005b, Oliva et al. 2007; Hemmi & Tomsic 2012; Bateman & Fleming 2015; Belgrad & Griffen 2016). As these crabs spent more time interacting with humans through normal weekly husbandry, this may have led to a decrease in this predator evasion behaviour. This trend, however, was not seen in parasitized crabs, who received the same care. They maintained natural phototactic behaviour through most of the study; they did not significantly alter their time spent in the black or white zones over time. This could suggest that the parasite is somehow affecting the habituation of its crab host, or the slower movement induced by infection is changing the amount of time the parasitized crab spends in each zone. It can be deduced from this study that it is difficult to interpret the predator evasion response of green crabs using a light/dark test. It may be beneficial to instead subject these crabs to simulated predation events in which they are provided with sediments, seaweed and/or rocky shelters, as a direct reflection of their natural habitat (McVean & Findlay 1979; Breen & Metaxas 2009; Young & Elliot 2020). Such experiments have been quite successful when subjecting fiddler crabs to looming stimuli representing bird predators (Hemmi 2005 a,b).

3.6.3.2 Aggression

Uninfected and *P. botulus*-infected green crabs showed a general trend of spending more time in the avoidance zone than the approach zone during each testing period. When comparing infection statuses, there was no significant difference in the time spent within avoidance or approach zones between parasitized and unparasitized crabs over the entire duration of the study, no matter the life stage of *P. botulus*. Also, most of the crabs from both infection statuses did not display any physical signs of aggression in response to a mirror. Increases in hemolymph serotonin have been shown to induce behavioural changes like those seen in cystacanth-infected amphipods, suggesting serotonin is a modulator of these behaviours in acanthocephalan-crustacean relationships (Perrot-Minnot et al. 2014). Since increases in serotonin have also been associated with increased aggression in crustaceans, including green crabs, it was predicted that *P. botulus*-infected green crabs would display less aggression during the acanthella stage, when hemolymph serotonin concentrations are expected to be lower, and increased aggression in their cystacanth stage, when these concentrations are higher (Huber et al. 1997; Sneddon et al. 2000; Pedetta et al. 2010). Despite evidence of serotonin being altered in acanthocephalan parasitism, a change in aggressive behaviour does not appear to be present in parasitized green crabs. Green crabs, no matter the infection status, appear to prefer avoiding confrontation when presented with what they perceive as an opponent of similar size. To see if this is in fact the case, a follow-up study should measure direct aggression displays between two green crabs, instead of the use of a mirror to simulate an opponent, like the method used by Sneddon et al. (2000). It is possible that the lack of olfactory communication signals from conspecifics, such as pheromones, resulted in the lack of aggressive displays and relatively small amount of time spent in the approach zone (Wang & Anderson 2010). Chemical signalling is a very important form of communication for

crustaceans (Frommen 2019). Lobsters and crayfish communicate through urinary signals; staging fights in seawater would be beneficial for proper dissemination of these chemicals (Frommen 2019).

3.6.3.3 Movement

In the present study, green crabs parasitized by *P. botulus* had a general trend of decreasing their speed and distance travelled over time. Mobile average speed and distance travelled significantly decreased between the acanthella stages and later cystacanth stage. Unparasitized crabs only significantly decreased their speed and distance travelled between the 8th and 12th week. Parasitized crabs were significantly slower and travelled a shorter distance than unparasitized during the later cystacanth stage only. Changes in host activity level have been previously investigated in other *Profilicollis* spp. cystacanth-crab relationships. *Profilicollis altmani* infection in *Emerita analoga* results in its host having a lower metabolic rate and burrowing more slowly into its environment (Kolluru et al. 2011; Figueroa et al. 2019). In contrast, *P. antarcticus*-infected *Hemigrapsus crenulatus* exhibit a higher metabolic rate and are more active and excitable (Haye & Ojeda 1998). The exact reasoning behind the difference in host metabolic activity between these two studies is unclear, however, it is important to note that higher dopamine levels are found in *P. antarticus*-parasitized *H. crenulatus* (Rojas & Ojeda 2005). While no neurotransmitter was investigated in *P. altmani*-parasitized *E. analoga*, low doses of serotonin have been shown to cause slow, awkward walking in green crabs, suggesting that manipulated serotonin levels may be at play in both *P. botulus* and *P. altmani* parasitism (McPhee & Wilkens 1989). *Microphallus similis*, like *Profillicollis* spp., also uses a bird as its definite host and may slow down green crab host activity, resulting in increases in feeding time (Blakeslee et al. 2015). Metabolic rate was not investigated in the current study, however, the

decrease in movement seen in *P. botulus*-parasitized crabs was very likely due to a decreased metabolic rate induced by the later cystacanth stage of the parasite. The decrease in movement efficiency could result in more exposure to the definitive host and impair the infected crabs' chances at getting away in the presence of a predator. This could also be the case in *P. altmani*-parasitized *E. analoga* and *M. similis*-parasitized green crabs; the parasites need to move from their intermediate crab host to their definitive host to complete their lifecycle. An experiment in which the parasites' definitive hosts are exposed to these slow-moving crabs would need to be conducted to determine whether such crabs are indeed caught more than their quick, unparasitized conspecifics.

It is unclear why there is a significant decrease in speed and distance travelled in unparasitized crabs between the 8th and 12th week. While not significant, the speed and distance travelled do increase between the 12th and 16th week. It is possible that the general decrease in glycogen reserves seen in unparasitized over time may influence this, but an increase would be expected to concur with the increase in movement between the 12th and 16th weeks. A transcriptomic study would help to determine what specific metabolic pathways may be at play that influence this finding.

3.6.3.4 Righting response

Profilicollis botulus-parasitized green crabs had no significant change in righting response over time. In comparison, unparasitized crabs took longer to right themselves during the last week of the study, versus the first eight weeks. Surprisingly, *P. botulus*-infected green crabs righted themselves significantly faster than the unparasitized individuals at the later cystacanth stage. Righting responses have been used to test both the negative effects of chemicals on crab response time and the effects of parasites on their host's behaviour (Weis &

Perlmutter 1987; McPhee & Wilkens 1989; Burger et al. 1991; Blakeslee et al. 2015; Correia & Smee 2018). Petrochemical-based oil and tributyltin have opposite effects on fiddler crab righting response time (Burger et al. 1991; Weis & Perlmutter 1987). Immediately after an oil spill, exposed crabs take longer to right, with many unable to right at all (Burger et al. 1991). Repeated exposure to tributyltin results in a hyperactivity in crabs, decreasing righting response time (Weis & Perlmutter 1987). In blue crabs, the organophosphate pesticide, malathion, acts more like petrochemical-based oil in fiddler crabs, increasing righting response time (Correia & Smee 2018). When injected with serotonin, green crabs also follow this trend and display impaired righting responses (McPhee & Wilkens 1989). Blakeslee et al. (2015) found that the trematode, *M. similis*, exerted a similar effect to serotonin injection on its green crab host and increased righting response time in earlier stages of infection. This result was not found in the subsequent study (Ro et al. 2022). The faster righting response time in parasitized crabs during the later cystacanth stage, in comparison to unparasitized crabs, suggests hyperactivity in the host, rather than the decreased activity levels suggested by their speed and distance travelled. It is unclear why these behaviours contrast. It may be useful to measure metabolic rate in *P. botulus*-parasitized crabs in a follow-up study and repeat the righting response test to determine whether it more closely relates to a decrease or increase in crab activity. Another explanation for these contrasting behaviours may be due to the apparent decrease in predator evasion over time in unparasitized crabs seen in the light/dark test. Unparasitized crabs not only decreased the time they spent in the black zone over time but also slowed the time they took to right between the first half and the last week of the study. Repetitive exposure to humans during feeding and cleaning would result in habitation, which was not seen in parasitized crabs.

3.6.4 Glycogen reserve inclusion body area

Parasitized green crabs in the present study showed a significant decrease in hepatopancreatic glycogen reserves during the 12th week, in comparison to the first 8 weeks. A significant increase was then seen between the 12th and 16th week. Unparasitized crabs had significantly more reserves during the first eight weeks than the 16th week. These crabs had significantly more of these reserves during the 12th week than parasitized crabs, but significantly less than them during the 16th week. The decrease in parasitized crab glycogen content occurred during the first month in which cystacanths were observed, meaning this decrease likely resulted as a consequence of great parasitic growth between the parasite's acanthella and cystacanth stages. The transition between these developmental stages may have resulted in transient damage to the intestinal wall of the green crab host. Such damage could reduce feeding behaviour or the feed conversion ratio, resulting in a reliance on glycogen reserves as a source of energy. It could also result in depletion of glycogen reserves due to the energy source being used for wound repair and healing. If such an experiment were repeated, it may be useful to observe crab feeding behaviour and calculate feed conversion ratio between infection statuses to corroborate changes in glycogen reserves. Feed conversion ratios have been successfully investigated in the mud crab (*Scylla serrata*) and Chinese mitten crab (*Eriocheir sinensis*) (Ali et al. 2011; Yu et al. 2019).

Acanthocephalan infections have been frequently associated with increased glycogen reserves in their crustacean hosts (Plaistow et al. 2001; Gismondi et al. 2012; Chen et al. 2015; Korkofigas et al. 2016). In *P. laevis*-infected *G. pulex*, this effect appears to be independent of sex and reproductive status (Plaistow et al. 2001). A few theories have been proposed as to why this occurs. An increase in glycogen reserves may be due to increasing energetic demands exhibited by parasitized hosts, or quick mobilizations of energy storage during parasite growth

(Plaistow et al. 2001; Chen et al. 2015). Alternatively, the parasite may be manipulating its host's energy reserves to directly cause a change in behaviour, or to provide energy to fuel a modified behaviour (Plaistow et al. 2001). It certainly appears as though *P. botulus* elicits a high energetic demand in its green crab host as it transitions from the acanthella to the cystacanth stage, possibly leading to increased feeding behaviour in the crab after significant parasite growth has ceased. Increased feeding would promote anabolism of hepatopancreatic glycogen stores, evident in the later cystacanth stage. Unparasitized crabs lacked this effect and therefore had a general decrease in reserves over time. Since parasitized crabs had a significant decrease in speed and distance travelled during the later cystacanth stage, it is unlikely that the increase in energy reserves at this stage affected their movement.

A few differences between statistically significant values were found when comparing ImagePro™'s glycogen inclusion body area values and the reserve inclusion scoring used by Stentiford & Feist (2005). Significant differences in glycogen reserves between infection statuses in the first 8 weeks were not found by the software. Also, the increase in reserves between the 4th and 16th week in parasitized and decrease in reserves between the 4th and 12th week in unparasitized crabs indicated by scoring were not found. While many important trends in glycogen reserves were found with ImagePro™, fewer were revealed than with scoring. This result may indicate one of two things: either detection of glycogen reserve inclusion bodies is not sensitive enough in the program, or scoring is too subjective to use as a valid measure of energy reserves. Regardless, the program appears to be a feasible tool to use when studying hepatopancreatic glycogen reserves in parasitized crustaceans and could be implemented in future studies.

3.7 Conclusion

The exact method by which parasites modify their host's behaviour is still relatively unknown, despite the vast number of studies investigating this field. The objectives of the current study were to determine whether *P. botulus* manipulates the behaviour of its host, when this occurs and whether it alters the energy reserves of its host. Parasitized crabs did not spend less time in the light (white) zone and more time in the dark (black) zone during the parasite's acanthella stage nor did they spend more time in the light zone and less time in the dark zone during its cystacanth stage. However, these crabs did spend more time at the midline between light and dark than unparasitized individuals in the earlier cystacanth stage; this was likely because of slower movement in parasitized crabs. Unlike what was expected, parasitized crabs did not spend less time displaying aggression during the parasite's acanthella stage and did not spend more time displaying aggression during its cystacanth stage; crabs spent more time in the avoidance zone than the approach throughout parasitic growth. There was no significant change in the time they took to right themselves throughout the study period. Unparasitized crabs did take significantly longer to right themselves during the last study week, in comparison to parasitized crabs. It was theorized that this may be due to a decrease in anti-predator behaviour in unparasitized crabs over time. Also, when tested in a lighted environment, parasitized crabs slowed in their speed and travelled shorter distances during the parasite's later cystacanth stage, in comparison to the acanthella stages. When *P. botulus* transitioned from an acanthella to cystacanth, the parasite depleted glycogen energy stores, either directly or indirectly, showing that it elicited significant metabolic effects on its host during this crucial growth period. The glycogen reserves rebounded once the parasite had attained its later cystacanth stage. This is the first study investigating how *P. botulus* manipulates both the behaviour and physiology of its

intermediate host, the green crab. While the exact mechanism by which behaviour manipulation occurs remains a mystery, some possible ways in which the parasite manipulates its host were illustrated in this study. The physiological impact *P. botulus* has on crucial energy reserves has also been shown, providing a key link to parasitism and energy mobilization. Earlier studies have only investigated *P. botulus* development, prevalence and intensity; while this study was also able to report the first photographic evidence of its transitional developmental stages.

3.8 References

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CHAPTER 4: CONCLUSIONS

4.1 THE IMPACT OF *NECTONEMA AGILE* INFECTION IN THE ATLANTIC ROCK CRAB, *CANCER IRRORATUS*

4.1.1 Summary and conclusions

      The Atlantic rock crab is native to the Atlantic coast of North America, being found from Labrador, Canada, to South Carolina, USA (DFO 2013). Most of the parasites affecting this species are helminths. *Nectonema* sp., a marine horsehair worm, is one of these species, parasitizing at a low prevalence in Eastern Canada (Brattey et al. 1985). Despite its discovery in rock crabs in this region decades ago, little is known about the effects of this parasite on its host (Leslie et al. 1981). The first objective of this study was to identify the species of *Nectonema* parasitizing rock crabs in the Northumberland Strait (Borden-Carleton). This was achieved through DNA extraction, amplification and sequencing from ethanol-preserved parasite tissue. The second objective of this study was to determine whether the *Nectonema* species identified altered predator evasion and aggressive behaviour in its host. Two tests were used to deduce this: a light/dark test and a mirror test, respectively. Tanks were divided into zones and the time spent in each zone was recorded for parasitized and unparasitized crabs. Additionally, crab speed and distance travelled was recorded and compared between infection statuses. The last objective of this study was to determine if body size, body weight, testes weight/to carapace width ratio, hepatopanceas size and hepatopancreas lumen area/to total tubule area ratio in these crabs were modified by *Nectonema* sp. parasitism. Crabs were weighed and measured, and necropsies were performed to note testes weight and gross pathology. Slides were prepared of hepatopancreas tissue to calculate lumen area/to total tubule area ratio.

The marine horsehair worm infecting the Atlantic rock crab was identified as *Nectonema agile*. Maximum likelihood phylogenetic analysis revealed it to have a 100% bootstrap value with a relatively newly identified *Nectonema* sp. identified in isopods (Kakui et al. 2021). When a rock crab host was euthanized, *N. agile* were generally immobile, however two active females were observed. One of these females showed a continuously undulating swimming pattern when placed in cold seawater (4 ± 1°C). Crab sex and crab size had no significant effect on the worm's size, only worm sex did: females *Nectonema* worms were larger than male worms. Similar sexual dimorphism was noted for *N. munidae* infecting squat lobsters (Nielsen 1969).

Predator evasion behaviour was not significantly different between infection statuses. Helminth-parasitized amphipods display increased photophilia in infective stages, possibly allowing definitive host predators to capture parasitized prey more easily, completing the parasite's lifecycle (Brown & Thompson 1986; Durieux et al. 2012; Casalins et al. 2015). Since *N. agile* completes its lifecycle in one host, it was expected that it would manipulate its rock crab host to seek shelter (dark) in predator situations to protect its host (Ward 1892). This expected behaviour was not observed in this study. The behaviour of parasitized rock crabs in a fully lighted environment (mirror test tank) closely resembled that of freshwater horsehair worm hosts; increased mobility (Ponton et al. 2011; Obayashi et al. 2021). Parasitized rock crabs significantly increased their speed in this environment, in comparison to unparasitized individuals. This increased speed would not only provide a method of predation suppression, but also increase the likelihood of parasitized hosts encountering each other, allowing worms to quickly emerge and mate.

No significant difference in aggressive behaviour between infection statuses was found in rock crabs. A previous study on freshwater horsehair worm-parasitized crickets made a similar

finding (Keck et al. 2017). It was expected that parasitized rock crabs would have significantly decreased aggression than their unparasitized counterparts, as decreased aggression would reduce the likelihood of cannibalism (Romano & Zeng 2017). Aggression may therefore not be a significant factor in this host-parasite relationship.

Rock crab body size, crab weight, hepatopancreas size and lumen area/to total tubule area ratio were not significantly different between infection statuses. Hepatopancreas tissue varied among crabs, with many parasitized crabs having a dark brown hepatopancreas. This dark brown hepatopancreatic colouration may be due to altered carotenoid concentrations, such as those found in acanthocephalan-infected amphipods (Rauque & Semenas 2009). This suggests that carotenoids may be a nutrient source by *N. agile* during their growth. Testes weight/to carapace width ratio was significant between parasitized and unparasitized individuals. Leslie et al (1981) only found degenerated testes in a small percentage of male rock crabs from the Bay of Fundy. This finding was not noted in rock crabs studied by Brattey et al. (1985). This suggests that when rock crabs in the Northumberland Strait are parasitized by *N. agile*, one of the tissues that are sacrificed upon nutrient depletion are the gonads.

4.1.2 Future directions

In the current study, *N. agile*-parasitized rock crabs were tested for predator evasion behaviour using a light/dark test consisting of distinct black and white zones. Differences in predator evasion behaviour between infection statuses were not found in this test, but rather the mirror test, which consisted of an all-white or light environment. In both tests crabs were immersed in water during the entire testing period. Parasitized rock crabs did not show increased attraction to light, but did increase their speed in the all-white, or completely light environment. Freshwater horsehair worm-infected mantids and crickets show an increased attraction to water;

this reflects horizontally polarized light (Ponton et al. 2011; Obayashi et al. 2021). In a follow-up experiment investigating the effects of *N. agile* on rock crab behavior, crabs should be placed outside a body of water, to gauge the attraction they have for it, as performed in Obayashi et al. (2021). If *Nectonema*-parasitized rock crabs show an increased attraction to light, like gordiid-infected hosts, then they should not only increase their speed, as shown in the current study, but also have a clear path of trajectory toward the light source. A second experiment should measure rates of predator capture between parasitized and unparasitized crabs to determine if increased speed is indeed a method of predation suppression influenced by parasitism. Unfortunately, to do this, the infection status of each crab would need to be known. Infection trials would need to be set up, similarly to what was done with green crabs. The freshwater horsehair worm, *Gordius robustus,* has been successfully reared in laboratory conditions using this method (Hanelt & Janovy Jr. 1999). Horsehair worm larvae were fed to intermediate hosts, which were in turn fed to definitive hosts for successful infection. Since *N. agile* appears to only have one host, its definitive host, larvae would just need to be fed to one host for infection. If larvae penetrate the midgut, encyst and then later complete their development in the hemocoel, a predator capture experiment will be feasible.

Some nematode parasites reduce aggression in their insect hosts (Yanoviak et al. 2008; Vasquez et al. 2015). Since Nematoda is a sister phylum to Nematomorpha and shares similar parasitic life-cycle traits to it, *N. agile*-parasitized rock crabs were expected to show decreased aggression as well. However, this did not occur. In the current study, a mirror test was used to test aggressive behaviour in rock crabs. The mirror reflected the crab's image back to it, acting as a proxy for another crab. Some previous studies investigating crayfish and hermit crab aggression used direct fighting between conspecifics to measure aggressive behavior (Huber et

al. 1997; Sneddon et al. 2000; Pedetta et al. 2010). Without the presence of an actual conspecific in an aggression test, there is a lack of olfactory communication, and therefore a lack of pheromones that may be associated with this behaviour (Wang & Anderson 2010). Crustaceans commonly use chemical signalling when communicating with each other, through urinary signals (Frommen 2019). In a future experiment, rock crabs should be exposed to a conspecific in a seawater tank environment to allow dissemination of these chemical signals.

Upon necropsy, external hepatopancreas colouration was noted in this study. While an observable difference in hepatopancreas colour was seen between crab infection statuses, further analysis into what caused this change was not conducted. Spectrophotometric analysis has been used to determine hepatopancreatic carotenoid concentration in acanthocephalan-parasitized amphipods (Rauque & Semenas 2009). It would therefore be possible to use this method in future studies to determine whether the change in hepatopancreas colour is due to parasitic influence. In the current study, rock crabs were kept at high temperatures, fed relatively infrequently and not given a diet rich in nutrients. High temperatures would have sped up crab metabolism and, with little nutrient availability, it caused the hepatopancreatic glycogen stores to be completely broken down and utilized. These stores provide important insight into the effects of parasites on nutrient stores in their hosts (Stentiford & Feist 2005; Wheeler et al. 2007). Without them, a comparison was made in lumen area/to total tubule area ratio between infection statuses. No significant difference was found between parasitized and unparasitized individuals. Had glycogen reserves been present, it is possible that a more accurate depiction of parasite-induced nutrient strain on the host body may have been observed. In future studies, water holding temperature should be kept low (4 ± 1 ºC) and diet should be rich and adequate to

prevent metabolic strain on the host species and allow proper comparisons to be made in energy storage between infection statuses.

One measurement that was not made in the current study was ovary weight in female rock crabs. In past studies, *Nectonema* sp. parasitism has been associated with smaller and/or deteriorated ovaries in host species (Pérez 1927; Nouvel & Nouvel 1934; Born 1967; Nielsen et al. 1969). *Nectonema agile*, in particular, has been found to be associated with ovary deterioration in prawns and hermit crabs (Pérez 1927; Nouvel & Nouvel 1934; Born 1967). In contrast, Leslie et al. (1981) and Brattey et al. (1985) did not note any decreased size or deterioration of ovarian tissues in the Atlantic rock crab in Eastern Canada. To determine if the reproductive capabilities of the female rock crabs in the Northumberland Strait are affected by *N. agile* parasitism, ovary weights should be taken and compared between infection statuses.

4.2 DEVELOPMENTAL STAGES OF *PROFILICOLLIS BOTULUS* AND ITS IMPACT ON THE INVASIVE GREEN CRAB (*CARCINUS MAENAS*) BEHAVIOUR AND PHYSIOLOGY

4.2.1 Summary and conclusions

The green crab is native to Atlantic Europe, but is invasive almost worldwide, with established populations in the Northwestern Atlantic, Northeastern Pacific, Argentina, South Africa and Australia (Young & Elliot 2020). Like the Atlantic rock crab, helminths are some of the most common parasites found in the green crab. Despite its escape from many of these parasitic species in its Atlantic Canadian region, the green crab remains vulnerable to *Profilicollis botulus* (Brattey et al. 1985; Blakeslee et al. 2009; Bojko et al. 2018). Studies identifying parasites in green crabs collected from Atlantic Canada have yet to thoroughly investigate the behavioural and physiological effects of *P. botulus* on this host. The first

objective of this study was to determine whether *P. botulus* manipulates the behaviour of the green crab in such a way as to aid its transmission from this intermediate host to a definitive host, and what time in its development this effect is elicited. This was investigated by feeding *P. botulus* eggs to unparasitized green crabs and subjecting them to two tests: a light/dark test and a mirror test, testing for predator evasion and aggressive behaviour, respectively. Using the same set up as the Atlantic rock crab study, testing tanks were divided into zones and the time spent in each zone was recorded for experimentally infected and uninfected crabs. Crab speed and distance travelled were also recorded and compared between infection statuses during the aggression test. Additionally, righting responses were conducted and compared between infection statuses. These tests were repeated every four weeks. The second objective of this study was to determine if the *P. botulus* infecting green crabs alter the energy reserves of their hosts. Once behaviour tests were concluded each testing week, crabs were necropsied and hepatopancreas tissue was removed. Slides were prepared from this tissue to calculate glycogen reserve inclusion body area.

Profilicollis botulus development was divided into 4 distinct stages over the study period: the early acanthella (4th week), the late acanthella (8th week), the early cystacanth (12th week) and the late cystacanth (16th week) stages. Previous studies have reported a similar trend of development, with acanthellae appearing in the first month and the cystacanth stage being reached between 62 and 70 days (Rayski & Garden 1961; Thompson 1985). No significant difference in predator evasion behaviour was seen between green crab infection statuses during the acanthella stages. Previous studies have reported that acanthocephalan-infected gammarids exhibit behaviours that make them less susceptible to predation during their acanthella stages (Dianne et al. 2011; Bailly et al. 2018). It was expected that *P. botulus* would increase the time

its green crab host spent in a sheltered (dark) zone in predation situations to protect its host during the acanthella stages. The contrast between these findings and those of previous studies may come down to the high infectivity of *P. botulus*; it may not be worthwhile for the parasite to protect its intermediate host during this time if one adult has many opportunities to propagate its genes through many larvae-infected crabs. If many crabs are infected, it is almost guaranteed that some acanthellae will grow to cystacanths; it is only during this point in time that it would be important to increase the chances of predation by the definitive host. When *P. botulus* transitioned into the early cystacanth stage, parasitized crabs significantly increased the time they spent at the midline; early cystacanth stage-parasitized crabs spent more time at the midline than unparasitized crabs during this time. Previous research notes that acanthocephalans alter their gammarid hosts' behaviour to promote their predation once the cystacanth stage is reached (Dianne et al. 2011; Bailly et al. 2018). Unfortunately, in the present study it was not clear whether *P. botulus* cystacanths supressed or promoted predation of their host. Being at the midline both exposes crabs to more light and provides a quick escape into a simulated shelter (dark zone). Therefore, an increase in the time spent at the midline in the early cystacanth stage was likely due to slower crab movement, rather than direct changes in predator evasion behaviour.

There was no significant difference in aggression between infection statuses over the entire duration of the study, no matter the life stage of *P. botulus*. Crabs spent more time in the avoidance zone of the mirror test and most did not display physical signs of aggression in response to the mirror. Increases in serotonin, a key neurotransmitter identified as being involved in crustacean aggression, have been shown to induce similar behavioural changes to those seen in cystacanth-infected amphipods (Huber et al. 1997; Sneddon et al. 2000; Pedetta et al. 2010;

Perrot-Minnot et al. 2014). It would not be much of a stretch to suggest that serotonin is one modulator of behaviours in acanthocephalan-crustacean relationships. Despite the link between serotonin and aggression, no change in aggressive behaviour was seen in *P. botulus*-parasitized green crabs. These crabs, no matter the infection status, appear to avoid confrontation when presented with what they would perceive as a similar-sized opponent.

In the present study, parasitized crabs significantly decreased their mobile average speed and distance travelled between the acanthella stages and the later cystacanth stage. Parasitized crabs were significantly slower and travelled less than unparasitized crabs during the later cystacanth stage. Mole crabs parasitized by *Profilicollis altmani* are reported to have similar decreases in activity; they exhibit a lower metabolic rate and burrow more slowly into their environment (Kolluru et al. 2011; Figueroa et al. 2019). Low doses of serotonin also cause slow, awkward movement in green crabs, further supporting the role of serotonin in behavioural manipulation (McPhee & Wilkens 1989). If *P. botulus* elicits a lower metabolic rate and/or decreases movement efficiency, the crab will be less likely to escape and thus more prone to predation by the definitive host.

Parasitized crabs showed no significant change in righting response over time. However, when compared to unparasitized crabs, they righted themselves significantly faster during the later cystacanth stage of *P. botulus*. This result was surprising, as green crabs injected with serotonin display impaired righting responses; parasitized crabs were expected to do the same (McPhee & Wilkens 1989). A possible explanation for this discrepancy may be due to the apparent decrease in predator evasion behaviour of unparasitized crabs during the light/dark test. These crabs not only decreased the time they spent in shelter (dark zone), but also slowed the time they took to right themselves between the first half and last week of the study. Repeated

exposure to humans during animal husbandry likely resulted in habituation of these crabs. The same habituation was not seen in parasitized crabs, suggesting that *P. botulus* infection somehow altered the natural habituation seen in these animals.

A significant decrease in hepatopancreatic glycogen reserves was seen in parasitized crabs between the acanthella stages and the early cystacanth stage. This was followed by a significant increase between the early and later cystacanth stages. These crabs had significantly less glycogen reserves during the early cystacanth stage than their unparasitized counterparts, but significantly more than them during the later cystacanth stage. The significant decrease in glycogen reserves during the early cystacanth stage likely occurred as a result of great parasitic growth as *P. botulus* was transitioning from an acanthella to a cystacanth and/or indirect effects on crab hosts due to acanthella life stage emergence from the intestinal wall. This transition would have been energetically costly and required the parasite to draw more nutrients from its host and/or reduce host food consumption or food conversion rates. Increased glycogen reserves have frequently been seen in acanthocephalan-infected amphipods (Plaistow et al. 2001; Gismondi et al. 2012; Chen et al. 2015; Korkofigas et al. 2016). Once the *P. botulus* reached the later cystacanth stage, it no longer needed to draw as many nutrients from its host, and those anabolic processes may have been hyper-promoted, resulting in an increase in glycogen stores once again. An increased host appetite could also have occurred after great parasitic growth subsided and/or parasite-induced intestinal wall damage healed, allowing glycogen reserves to replenish.

4.2.2 Future directions

In the predator evasion test, early cystacanth-parasitized crabs spent more time at the midline than unparasitized crabs during that time. However, it is difficult to interpret whether

being in this location would promote or supress predation and the slower movement of parasitized crabs was likely the reason that they spent more time here. Therefore, a better test would need to be conducted to determine the predator evasion behaviour of *P. botulus*-parasitized green crabs. Subjecting crabs to simulated predation events in which they are provided with sediments, seaweed and/or rocky shelters would provide a more accurate depiction of this behaviour. In their natural environment, they would use these as sources of refuge in the presence of a predator (McVean & Findlay 1979; Breen & Metaxas 2009; Young & Elliot 2020). Predator evasion tests have also been successful when subjecting crabs to a looming stimulus representing bird predators (Hemmi 2005 a,b). Since the common eider is the most common definitive host of *P. botulus* and a green crab predator, it would be safe to assume that this test would accurately reflect how green crabs would react during predation events (McDermott et al. 2010).

Metabolic rate was not investigated in the current study, however it may be useful to investigate the link between metabolic rate and *P. botulus* infection, especially in regard to behaviour. *Profilicollis altmani* decreases the metabolic rate of its mole crab host and shows a clear, and associated decrease in movement (Kolluru et al. 2011; Figueroa et al. 2019). Therefore, it was expected that the decrease in movement seen in parasitized green crabs may be a result of cystacanth-induced decreases in the metabolic rate. The best way to conduct this experiment would be to set up a respiratory chamber, submerging parasitized and unparasitized crabs in seawater and measuring the decreasing rate of oxygen (Dr. Fraser Clark, personal communication). Also, an additional experiment should be done to determine if common eiders capture slow-moving *P. botulus*-parasitized green crabs more frequently than their unparasitized

counterparts. This would provide further evidence of parasitic manipulation and provide a clear evolutionary advantage to it.

Host feeding behaviour was not monitored in the current study. Trematode infection (*Microphallus* spp.) has been shown to affect feeding behaviour in rusty crayfish (*Orconectes rusticus*) and green crab hosts (Sargent et al. 2014; Blakeslee et al. 2015). The parasite reduces foraging behaviour in its crayfish host and has shown to increase mussel handling time in its green crab host (Sargent et al. 2014; Blakeslee et al. 2015). Since trematodes, like acanthocephalans, also have crustacean intermediate hosts and bird definitive hosts, it is highly probable that a similar effect on feeding behaviour would occur in *P. botulus*-infected green crabs. Therefore, it would be beneficial in future studies to monitor feeding behaviour, especially to determine its relationship with glycogen body reserves in parasitized crabs. Feed conversion ratios may also be impacted by *P. botulus* parasitism. These also should be calculated in subsequent investigations to determine if intestinal damage caused by the growing parasite influences absorption and usage of nutrients.

4.3 Conclusion

The crustacean industry contributes billions of dollars to economies worldwide (Fisheries & Aquaculture Department 2020). Currently, the North American industry is threatened by mass-casualty disease (Shields 2011; Shields 2019). This problem is only expected to increase as global air and sea temperatures rise in the next several decades (Poulin & Mouritsen 2006). It is for this reason that research investigating the interactions between parasitic pathogens and their hosts is essential. The main objective of the current studies was to gain a better understanding of decapod health in these relationships by using helminth-parasitized Atlantic rock and green crabs

as models. Specifically, the behavioural, anatomical and physiological impacts of helminth parasitism on these crab species were investigated. Parasite behavioural manipulation of hosts can be used to either increase host survival or increase the chance that the parasite will be transmitted from one host to the next, sometimes at the expense of the parasite's host (Hafer 2016). Many parasites use manipulation for both reasons (Hafer 2016). Findings made on *Nectonema agile* parasitism in rock crabs showed that the host increased its speed in a lighted environment, suggesting that this would decrease predation by making the host harder to catch. This would protect the parasite and increase the chances of encountering other parasites for mating. *Profilicollis botulus*, on the other hand, showed no evidence of suppressing predation on its green crab host and instead changed its behaviour in such a way as to promote predation during the parasite's later cystacanth stage. Parasitized crabs decreased their speed and distance travelled during this time. These crabs righted themselves significantly faster than their unparasitized conspecifics during the later cystacanth stage of development, however, this significance was likely due to natural habituation in unparasitized crabs. Body, gonad and energy reserve modifications commonly occur in parasite and crustacean host relationships (Plaistow et al. 2001; Mouritsen & Jensen 2006; Gismondi et al. 2012; Sargent et al. 2014; Chen et al. 2015; Korkofigas et al. 2016; Romero-Rodríguez et al. 2016; Bailly et al. 2018; Corral et al. 2021). While body size and body weight changes were not seen in parasitized rock crabs, male parasitized crabs had significantly smaller testes than unparasitized crabs and the majority of parasitized crabs had a darker brown hepatopancreas. These findings suggest that *N. agile* parasitism impacts host metabolism, resulting in a sacrifice of testicular tissue and a possible change in hepatopancreatic carotenoid concentration. In green crabs, *P. botulus* parasitism caused a significant decrease in hepatopancreatic glycogen reserves as parasites transitioned

from acantellae to cystacanths. Once cystacanths stopped growing, glycogen reserves increased

once again. This suggests that *P. botulus* draws a significant amount of energy from within its

host, alters host feeding behaviour and/or feed conversion ratio, or causes host energy reserves to

be redirected to intestinal wound healing as it is transitioning from its non-infective stage to its

infective stage. These studies gave valuable insight into the relationship between the Atlantic

rock and green crabs and their respective helminth parasites. They illustrate the importance of

researching crustacean health in an ever-changing global environment.

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