Exploring Crustacean Health: Effect of *Profilicollis botulus* Infection on the Behaviour of *Carcinus maenas* and Antilipopolysaccharide Factor Phylogeny in Decapoda

by

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Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
December 2021

Dalhousie University is located in Mi'kma'ki,
the ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

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Abstract

Learning more about crustacean health and immunity is important to protect them from infections and disease outbreaks. Little is known about the evolution of antilipopolysaccharide factors (ALFs), so this study examined the phylogeny of 291 ALF sequences of decapods using unrooted Maximum Likelihood trees. Due to the heterogeneity of the trees, it is likely that ALFs evolved over a series of successive gene duplications followed by neofunctionalization. The acanthocephalan *Profilicollis botulu*s has the intermediate host *Carcinus maenas*. The goal of this project was to identify whether infected crabs displayed different behaviours compared to uninfected ones. Three behaviour trials were conducted on crabs (n = 37) from a Nova Scotia population (mirror approach test, open field test, and background preference test). No significant differences were detected between infected and uninfected crabs. This has interesting implications for our knowledge of acanthocephalans, as host manipulation is predicted to be their ancestral trait.

List of Abbreviations Used

ALF	Antilipopolysaccharide factor
AMP	Antimicrobial peptide
BGBP	β-glucan-binding protein
DAMP	Damage-associated molecular pattern
DF	Degrees of freedom
DSCAM	Down syndrome cell adhesion molecule
LBD	Lipopolysaccharide-binding domain
LGBP	Lipoplysaccharide-binding protein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
ML	Maximum likelihood
MSA	Multiple sequence alignment
OTU	Operational taxonomic unit
PAMP	Pathogen-associated molecular pattern
PIPA	Parasite-induced phenotypic alteration
ProPO	Prophenoloxidase
PRR	Pathogen recognition receptor
RNAi	Anti-viral RNA interference
SNP	Single-nucleotide polymorphism
THC	Total haemocyte count

Acknowledgements

Thank you to my supervisor and committee members for all their help.

Thank you to my friends and family that supported me throughout these tough years.

Thank you to my Juniper for keeping me company while writing.

Thank you to my Panchito and especially my Domino for keeping my legs warm.

Thank you my dear Tovah for being my best friend, I hope you are eating all the parsley you can find in rabbit heaven.

Chapter 1: Introduction

1.1 Introduction

Decapoda is an order of crustaceans that includes crabs, lobsters and shrimp. These animals are an important source of food and economic security in many countries across the world (FAO, 2020b). The crustacean fisheries in Atlantic Canada specifically are responsible for over $2 billion Canadian dollars in revenue (DFO, 2019). Thus, learning more about crustacean health and immunity is important to protect the wild populations and aquaculture-produced stocks from potential infections and disease outbreaks. This thesis will examine two main topics: the diversity of antilipopolysaccharide factors in decapod crustaceans, and the impact of the acanthocephalan parasite *Profilicollis botulus* on the behaviour of the green crab (*Carcinus maenas*).

Antimicrobial peptides (AMPs) are a particular type of immune effector that are key to the defense of crustacean hosts against pathogens. One particular family, the antilipopolysaccharide factors (ALFs), have significant sequence diversity and can display broad-spectrum antimicrobial activity against bacteria, fungi and viruses (Hou et al., 2017; Li and Li, 2020). Most ALF studies focus on the discovery and expression patterns of the peptides. Different ALF types can be expressed simultaneously (Rosa et al., 2013), at different times (Antony et al., 2011), at different levels (Lv et al., 2018), and in a specific tissue (Li et al., 2019) or several tissues (Lv et al., 2018).

Although there are some studies that look at ALFs in other crustaceans, most studies focus on penaeid shrimp, whose ALFs have been well-studied and classified into several distinct types unique to this taxon (Matos et al., 2018). There is therefore a lack of information about ALFs in crustaceans of other infraorders, and it is challenging to obtain

the bigger picture of the functional and mechanistic specificity of AMPs due to their incredible diversity in both structure and function.

This study will examine the phylogeny of ALFs with the goal of identifying similarities of these peptides in decapod crustaceans. This is significant because to date, very few studies have comprehensively explored the phylogeny of these molecules in crustaceans. Increasing our knowledge of this AMP family will guide us towards its many potential applications as antimicrobial compounds.

The green crab is native to the coast of Atlantic Europe, but it has invaded other temperate regions like Atlantic North America (Darling et al., 2008). In an attempt to reduce the population size of this invasive species, the government of Canada has issued licences that allow the capture of these animals in order to sell them as bait for the commercial fishing of other crustacean species (Bojko et al., 2018). In Atlantic Canada, green crabs are currently being used as a bait source in the American lobster (*Homarus americanus*) fishery, which is an industry of great economic importance for this region (DFO, 2019). However, there is potential for parasite and disease transfers between the two crustaceans. Accordingly, it is important to assess the risks posed by invasive hosts and their parasites on native populations in order to gain the necessary knowledge for maintaining their health (Bojko et al., 2018).

Some parasites that have complex life cycles can manipulate the behaviour and/or physiology of their intermediate host to increase the contact rate of their intermediate and final hosts. This is the case for many animals in the phylum Acanthocephala, which is composed of parasitic worms that have been reported to cause alterations in their intermediate crustacean hosts that ultimately enhance their own transmission to their final

hosts (Maynard et al., 1996; Rojas and Ojeda, 2005; Tain et al., 2006). One acanthocephalan, *P. botulus*, has the green crab as its intermediate host.

This study will examine what happens to the behaviour of the green crab during *P. botulus* infection. The goal is to determine if infection results in behavioural alterations in the crab that promote its predation. It would be beneficial for this parasite to create a more vulnerable behavioural and physiological state in the crab since their ultimate goal is to increase their own transmission to their definitive host, the common eider duck (*Somateria mollissima*) (Thompson, 1985b; Rojas and Ojeda, 2005). This is significant because no study to date has determined the impact of *P. botulus* on the green crab. It would be important to know if it does have an effect because of the potential risk of green crab parasites, like *P. botulus*, being unintentionally transferred to other commercially important crustaceans like the American lobster. Ultimately, studying this specific parasite-host interaction will help broaden our knowledge about parasitic manipulation and its potential impacts on marine ecosystems.

1.2 Literature Review

This literature review has six key sections: the importance of crustacean fisheries and aquaculture, basic crustacean immunology, crustacean AMPs, the green crab, the phylum Acanthocephala (including *P. botulus*), and the interactions between *P. botulus* and its hosts. Crustaceans are globally important both economically and as a food source; therefore, knowledge of their health and diseases is crucial to the fishery and aquaculture industries. A brief review of the major components of crustacean immunology is also included. Antimicrobial peptides have received a lot of attention in recent years due to their potential applications in aquaculture animal husbandry, as well as

in the medical field (Huang et al., 2015; Hou et al., 2017). It is thus important to increase our understanding of crustacean immunology to develop disease treatments in this age of antibiotic resistance and climate change (Ahmad et al., 2021). As an invasive species with a history of multiple invasions in Nova Scotia, the green crab has many effects on marine ecosystems and impacts other native crustaceans. *Profilicollis botulus* is one of many acanthocephalan species that exist all over the world, and animals in this phylum are notorious parasitic manipulators. Therefore, there is merit in investigating host-parasite interactions between *P. botulus* and its hosts.

1.3 Importance of Crustaceans

1.3.1 Global Crustacean Production

Crustaceans are a diverse arthropod subphylum, with a specific order, the Decapoda, that is important in the fishery and aquaculture industries in many countries around the world (Behringer and Duermit-Moreau, 2020). Decapods include crabs, shrimp, and lobsters. According to the Food and Agriculture Organization of the United Nations, the global marine capture production of crustaceans reached almost 6 million tonnes in 2018 (FAO, 2020b). In 2018, global aquaculture production of crustaceans reached 9.4 million tonnes, with marine and coastal crustacean aquaculture reaching roughly 5.7 million tonnes (heavily comprised of marine shrimp species), and inland crustacean aquaculture produced around 3.6 million tonnes (FAO, 2020b). For total global inland water catches, catches of freshwater crustaceans and freshwater molluscs combined were 0.45 million tonnes in 2018 (FAO, 2020b). Crustaceans are an important food source, but also an important source of jobs. Approximately 60 million people worked in the fishing

and aquaculture sectors in 2018, with a high number of jobs created in rural and coastal communities (FAO, 2020b).

1.3.2 Crustaceans in Atlantic Canada

Crustacean fisheries are important to the economy of Atlantic Canada. The value of Atlantic coast commercial landings of crustacean decapods was over $2.6 billion Canadian in 2019 (DFO, 2019). The major crustaceans fished commercially in Atlantic Canada, in order of decreasing value, are the American lobster, shrimp collectively (includes species such as the shrimp *Pandalus borealis* and *Pandalus montagui*), the snow or queen crab (*Chionoecetes opilio*), and other crab species collectively (including the Jonah crab *Cancer borealis* and Atlantic rock crab *Cancer irroratus*) (DFO, 2019). The lobster industry alone brought in over $1.5 billion in 2019 (DFO, 2019).

1.3.3 One Health

Crustaceans are important both ecologically and to the economic and nutritional health of humans around the globe (Behringer and Duermit-Moreau, 2020). Indeed, crustaceans are a significant source of dietary protein and income, especially for coastal communities (Behringer and Duermit-Moreau, 2020). The One Health concept refers to the relationships between human health, animal health, and environmental health, and is applicable to crustaceans (Behringer and Duermit-Moreau, 2020). Although crustaceans only transmit a relatively small number of zoonotic parasites that usually cause foodborne illness (most commonly gastrointestinal illnesses caused by bacterial contamination), global changes and changing climates are expected to increase the transmission of

foodborne and contact illnesses to humans (Behringer and Duermit-Moreau, 2020). In addition, as human-mediated pathogen spread from invasive to native species is understudied, it is important to predict future pathogen spread and parasite host-switching (Roy et al., 2017; Behringer and Duermit-Moreau, 2020). Another aspect of crustacean One Health to consider is the strong dependence of coastal communities on the health of their crustacean fishery resources (Behringer and Duermit-Moreau, 2020). Declines in crustacean fisheries could have abrupt and/or gradual consequences on these communities in terms of employment and mental health (Behringer and Duermit-Moreau, 2020).

1.4 Crustacean Immunity

1.4.1 The Innate Immune System

Crustaceans possess an innate immune system. The innate immune system is based on germline-encoded receptors and effectors that recognize and kill pathogens, while the acquired immune system present in vertebrates is based on receptors produced by somatic gene rearrangement that recognize highly specific antigens, allowing for immune memory to take place (Fearon, 1997). Invertebrates do not have adaptive immunity; however, some crustaceans have evolved specificity to the level of being able to discriminate between closely related parasites (Armitage et al., 2015). Innate immunity does have the capacity to distinguish self from non-self and was initially believed to be non-specific, but all eukaryotes can recognize non-self particles (Jiravanichpaisal et al., 2006). Specificity is required for immune memory as some characteristics of the pathogen must be remembered so that there is increased host protection at the next encounter (Armitage et al., 2015). A crustacean's immune response could be specific for larger clades of bacteria, but non-

specific at the strain level, or the receptors may not be specific at the whole parasite level, instead binding to conserved pathogenic molecules (Armitage et al., 2015).

The innate immune system is further divided into humoral and cellular defence responses. In crustaceans, cellular immunity includes all reactions directly mediated by haemocytes, such as phagocytosis and encapsulation (Kulkarni et al., 2021). In comparison, humoral responses occur in the haemolymph and involve the production and secretion of a multitude of immune proteins and effectors like AMPs, proteinases and their inhibitors and agglutinins, which are associated with many signalling and proteolytic cascades like the clotting and melanisation cascades (Jearaphunt et al., 2015; Kulkarni et al., 2021). There is overlap between humoral and cellular responses because a lot of humoral factors affect the function of haemocytes, a major source of humoral factors (Jiravanichpaisal et al., 2006). The following is a brief review of the crustacean immune system, starting with the exoskeleton barrier and ending with specific endogenous molecules.

1.4.2 The Exoskeleton as a Barrier

There are several innate immune defences in crustaceans, but the first barrier to pathogens is the exoskeleton, which is made of chitin with some calcified regions giving it more mechanical strength (Roer et al., 2015). The exoskeleton is the primary external barrier, preventing the entry of eukaryotic parasites and infection by microorganisms (Moret and Moreau, 2012). Even the moulting of the cuticle has an immunological function, as it helps to reduce the crustacean's surface pathogen load by replacing the damaged shell and removing epibionts or parasites from the old shell (Moret and Moreau, 2012). If a pathogen penetrates through the exoskeleton or peritrophic membrane, the last line of

defense is the haemocoelic internal defenses, which includes several signal transduction pathways, receptors that recognize pathogens, and effectors that inactivate and eliminate them (Moret and Moreau, 2012; Kulkarni et al., 2021).

1.4.3 Immune Organs

Several crustacean organs have immune functions. There is the peritrophic membrane of the midgut, which is a secreted acellular layer that separates ingested material from the gut epithelium, thereby providing a tract barrier to pathogens (Martin et al., 2006). The peritrophic membrane is thought to protect the midgut from abrasive food particles and pathogens (Wang et al., 2012). In addition, the hepatopancreas has specialized fixed phagocytic cells that can also trap and retain particles with a layer of granular material on their surface (Johnson, 1987). This organ also produces immune proteins such as clotting proteins and haemocyanin (Burnett and Burnett, 2015), β-1,3-glucan binding protein, AMPs and lectins (Li et al., 2013a). The heart, gills, antennal glands, and Y-organs are also minor sites of haemolymph particle removal (Burnett and Burnett, 2015). Finally, the lymphoid organ specific to penaeid shrimp can accumulate foreign particles and express various AMPs (Burnett and Burnett, 2015; Sun et al., 2021).

1.4.4 Major Immune Pathways

Pathogen recognition works in several ways in crustaceans. Pathogen recognition receptors (PRRs) represent a variety of germline-encoded proteins that can recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs); the latter are danger signals associated with cellular stress (Chen et al.,

2014). Endogenous molecules containing DAMPs trigger immune responses when lipopolysaccharide (LPS), β-glucan or peptidoglycan-induced necrosis occurs in host cells and tissues (Chen et al., 2014). Pathogen recognition receptors can target specific conserved motifs of a pathogen's surface, triggering the activation of immune effector molecules, phagocytosis, encapsulation, and AMP production depending on the pathogen load and type, as well as the route of entry (Burnett and Burnett, 2015; Kulkarni et al., 2021). Pathogen recognition receptors in crustaceans include lipopolysaccharide-binding proteins (LGBP) and β-glucan-binding proteins (BGBP), lectin-like proteins, Gram-negative binding proteins and Down Syndrome Cell Adhesion Molecules (DSCAMs) (Burnett and Burnett, 2015).

There are several major immune pathways that occur within crustaceans. Innate immune responses are regulated by signal transduction pathways where PRRs get activated when they bind with PAMPs. Activated PRRs can trigger many cellular or humoral responses, directly or indirectly, such as clotting, phagocytosis, AMP production and the activation of the NF-kB-homologues Dorsal and Relish (Kulkarni et al., 2021). The Toll and Dorsal/Relish signaling pathways are present in all invertebrates, and these proteolytic cascades can activate shrimp AMP production (Wang et al., 2014b). The proteolytic cascades of coagulation, encapsulation, and melanisation are also crucial innate immune responses. Exoskeleton damage and/or the presence of pathogens triggers coagulation in the haemolymph: haemocytes will release calcium-dependent transglutaminase (TGase) that directly promotes the plasmatic clotting protein, which is then polymerized (Perdomo-Morales et al., 2019). However, crustacean coagulation research has only focused on a few species of crayfish and shrimp. These species seem to have similar coagulation

mechanisms, but the haemocyte type carrying TGase is variable depending on crustacean species, which could result in differences in how TGase is released and thus how clotting is initiated (Perdomo-Morales et al., 2019). Clotting is therefore activated by PRRs, such as LGBP or BGBP, and/or Toll receptors. These PRRs recognize a pathogen and cause the exocytosis of cytoplasmic granules containing TGase (Hoeger and Schenk, 2020). Encapsulation occurs when invading pathogens are too large for individual haemocytes to phagocytose (Burnett and Burnett, 2015). It is a process in which multiple haemocytes gather to form a cellular barrier around the pathogen, forming a capsular space where toxic immune products may be released (Burnett and Burnett, 2015). The coagulation and encapsulation responses are succeeded by melanin deposition, which is the end result of the prophenoloxidase cascade (proPO cascade). The activation of the cascade is often in conjunction with the production and release of AMPs from haemocytes, at least in shrimp (Jearaphunt et al., 2015). This proteolytic cascade is activated when PRRs recognize PAMPs, and then many serine proteinases and serine proteinase homologs are involved in controlling the proteolysis steps that lead to the activation of the enzyme phenoloxidase (Jearaphunt et al., 2015). The proPO cascade's melanin production inhibits the growth and spread of pathogens, and the intermediary metabolite quinones are cytotoxic for pathogens (Kulkarni et al., 2021).

1.4.5 Major Immune Proteins

Many immune effector proteins exist in crustaceans, and so only a select few will be briefly described below: lectins, DSCAMs, haemocyanin, and lipoproteins. Crustacean lectins have a carbohydrate recognition domain that promotes phagocytosis of bacteria

through opsonization (Kulkarni et al., 2021). C-type lectins specifically have multiple roles but have important immune functions involved in bacterial agglutination, opsonization, encapsulation, promoting oxidative burst, activating the proPO cascade, and participating in microbicidal and antiviral activities in penaeid shrimp (Wang and Wang, 2013).

Down syndrome cell adhesion molecules are present in crustaceans and these cell surface proteins are a part of an immunoglobulin superfamily (Ng and Kurtz, 2020). The genes encoding DSCAMs are unique in the sense that through alternative splicing, the resulting proteins are extremely diverse especially in the cytoplasmic tail region, and there is a high gene copy number normally observed in crustaceans (Ng and Kurtz, 2020). As hypervariable immune molecules, DSCAMs have a variety of roles (Ng and Kurtz, 2020). In freshwater crayfish for example, specific DSCAM proteins are produced in response to bacteria, and will bind with specificity to these pathogens (Cerenius and Söderhäll, 2013). The exact functions of DSCAMs have not yet been fully established in arthropods (Ng and Kurtz, 2020).

Haemocyanin is the main protein of the crustacean haemolymph, representing 95% of its total protein component (Coates and Nairn, 2014). It is a respiratory protein that also contributes to lipid transport (Hoeger and Schenk, 2020). This protein is secreted by the crustacean hepatopancreas, and though its general function is transporting oxygen, it can also activate the proPO cascade through phenoloxidase activity and release AMPs following proteolysis (Coates and Nairn, 2014).

Lipoproteins are also present in relatively large amounts of many mg/mL (Hoeger and Schenk, 2020). There is a PRR family called β-1,3-glucanase related proteins that includes LGBPs, BGBPs and Gram-negative bacteria binding proteins (Chai et al., 2018).

Both the BGBPs, which are pattern recognition proteins also involved in lipid transport, and the LGBPs, which bind glucans, are part of the innate immune system and act as opsonins for microbial compounds (Hoeger and Schenk, 2020). Another immune function of BGBPs is that they can enhance the proPO cascade's activation when bound to β-1,3-glucans, as this complex's formation causes the exocytic release of haemocyte-derived proPO components (Duvic and Söderhäll, 1990).

1.4.6 Haemocytes

Along with their soluble products, haemocytes are the main mediators of the crustacean immune response and use several PRRs, effector functions, and signaling pathways (Burnett and Burnett, 2015). The cardiovascular system of crustaceans is the facilitator for transporting haemocytes and soluble immune factors (Burnett and Burnett, 2015). Haemocytes are believed to be derived from undifferentiated haematopoietic cells present in the haematopoietic tissue. They then differentiate based on various microenvironmental factors and enter the circulation through mechanisms that are still unknown in most invertebrates (Jiravanichpaisal et al., 2006). The process of haemocyte maturation and entrance into haemolymph circulation is called haematopoiesis, and mature haemocytes are not believed to be able to divide (Jiravanichpaisal et al., 2006). The haematopoietic tissue of decapods forms a sheet located on the foregut (Johansson et al., 2000).

The number of circulating haemocytes in a healthy decapod is quite high and varies with several biotic and abiotic factors such as species, temperature, and infection status. For example, in a healthy state the total haemocyte count (THC) of *Litopenaeus vannamei*

is roughly 32.0×10^9/L at 27°C (Hsieh et al., 2013). In comparison, the THC of healthy *H. americanus* at 2°C is on average $8.1\text{-}22.4\times10^9$/L, but the THC of *Aerococcus viridans* var. *homari*-infected animals is $5.2\text{-}31.3\times10^9$/L (Battison et al., 2013).

Crustacean haemocytes play a variety of roles in innate immunity, including pathogen recognition, phagocytosis, secretion of immune effectors, melanisation, cytotoxicity and cell-to-cell communication (Johansson et al., 2000; Jiravanichpaisal et al., 2006). Crustaceans can heal wounds by the cell adhesion of haemocytes (Kulkarni et al., 2021) and the formation of extracellular traps (Robb et al., 2014). Extracellular traps are the controlled release of chromatin from cells that trap and kill microorganisms outside of the cells, which have been documented in *L. vannamei* and *C. maenas* (Robb et al., 2014).

Haemocyte types are differentiated by their morphology and functionality (Jiravanichpaisal et al., 2006). In decapods, haemocytes are divided into three types based on their cytoplasmic granule content: hyaline cells, semigranular cells, and granular cells (Johansson et al., 2000). The functions performed by each type vary between species. In crayfish for example, hyaline cells mainly have a phagocytosis function, semigranular cells participate in encapsulation, and granular cells are involved in the activation of the proPO cascade and cytotoxicity (Johansson et al., 2000).

1.4.7 RNA Interference

Effector molecules are not exclusively proteins and their effectors. Crustaceans can also perform antiviral RNA interference (RNAi). This mechanism can recognize, process, and destroy viral double-stranded RNA and DNA through cleavage (Wang et al., 2014a). This post-transcriptional gene silencing is mediated by small noncoding RNA sequences

(around 21 to 30 nucleotides), either siRNA or miRNA (Huang and Zhang, 2013; Wang et al., 2014a).

1.5 Antimicrobial Peptides

1.5.1 Definition and Diversity of Antimicrobial Peptides

Antimicrobial peptides are one component of the innate immune system that has been studied intensely during the last decades in terms of their synthesis, mechanism of action, and medical implications (Antony et al., 2011). Antimicrobial peptides are an extremely diverse group of molecules (Lai and Gallo, 2009). They are short-chain peptides present in a variety of organisms, including archaea, bacteria, plants, fungi, invertebrates, and vertebrates (Kumar et al., 2018a; Olatunde et al., 2020). These molecules vary when it comes to their sequence, structure and source, but in general they have a net positive charge and display some level of hydrophobicity and amphipathicity (Kumar et al., 2018a).

There are several families within AMPs. For example, the penaeid shrimp has four known families of AMPs: antilipopolysaccharide factors (ALFs), lysozymes, crustins, and penaeidins, that are stored in and secreted by haemocytes (Kulkarni et al., 2021). These protein families all have different roles in the innate immunity of a crustacean. A study examined the differential expression of AMP genes in the haemocytes of *Penaeus monodon* in response to a WSSV infection and found that ALF, crustin, and penaeidin genes were differentially expressed in the haemocytes pre- and post-challenge (Antony et al., 2011). More specifically, the ALF and penaeidins were up-regulated during the early hours of the challenge, and both ALF and crustin-3 were up-regulated during the late hours of the

challenge while the other AMPs studied were down-regulated (Antony et al., 2011). In addition, the ALF gene was also up-regulated post-challenge (Antony et al., 2011).

1.5.2 Discovery of Antilipopolysaccharide Factors

Antilipopolysaccharide factors are an important family of AMPs. Lipopolysaccharides are amphiphilic molecules on the outer leaflet of Gram-negative bacteria that consist of a hydrophilic polysaccharide component covalently bound to a hydrophobic lipid component called lipid A (Chaby, 2004). Lipid A is a conserved hydrophobic region of LPS molecules and is the bioactive center that is toxic to many organisms (Somboonwiwat et al., 2005). Antilipopolysaccharide factors were initially isolated in the horseshoe crabs *Limulus polyphemus* (ALF-L) and *Tachypleus tridentatus* (ALF-T) (Tanaka et al.,1982; Ohashi et al., 1984). When haemocytes from these species were exposed to LPS, an intracellular clotting system of several protein components was activated (Morita et al., 1985). When these clotting factors were separated and purified, an anticoagulant was found and named anti-LPS factor as it inhibited the activation of the LPS-induced plasma clotting cascade (Morita et al., 1985; Liu et al., 2006). Indeed, ALFs inhibited the activation of the B and C coagulation factors of horseshoe crabs' granular haemocytes through LPS-binding (Tanaka et al., 1982; Morita et al., 1985). It was subsequently observed that this novel factor had an antibacterial effect on the growth of Gram-negative *Salmonella minnesota*, but not on the Gram-positive *Staphylococcus aureus* (Morita et al., 1985). These ALFs bind to lipid A, thereby inhibiting bacterial growth (Liu et al., 2006). The crystal structure of ALF-L was established and consists of 101 amino acids with a single domain made up of three α-helices packed against a four-

stranded β-sheet, with an extended amphipathic loop at residues 31-52 functioning as the LPS binding site (Hoess et al., 1993; Chaby, 2004). The fact that ALF-L reduced the mortality of animal models when injected before or after a bacterial challenge (Hoess et al., 1993) was one indication of possible future applications for animal and human health. Apart from inhibiting Gram-negative bacteria, ALFs have many roles in innate immunity, such as reducing bacterial growth and decreasing viral replication (Liu et al., 2006).

1.5.3 Structure of ALFs

After being isolated in horseshoe crabs, ALFs were identified in the penaeid shrimp species *L. vannamei* and *L. setiferus* (Gross et al., 2001). They have since been identified in many crustaceans and contain a large sequence diversity (Li and Li, 2020). The structure of very few ALFs has been resolved at the molecular level because AMPs have been predominantly studied for their response to microbes (Antony et al., 2011). However, the tertiary structures of ALF-L and ALFPm3 of *P. monodon* are both similar, consisting of three α-helices against a four-stranded β-sheet (Li and Li, 2020). The central β-hairpin is delimited by two conserved cysteines that form an intramolecular disulfide bridge (Rosa et al., 2013). The β-hairpin of ALF-L has positively charged residues, stabilized by the disulfide bridge (Mora et al., 2008), that recognize the lipid A moiety of LPS (Hoess et al., 1993). Similarly, a recombinant ALFPm3 will bind to lipid A, and the predicted LPS-binding site consists of six positively charged and one negatively charged residue located in the β-hairpin and the two closest β-strands (Yang et al., 2009). This recombinant ALF could also bind to the lipoteichoic acid (LTA) of Gram-positive bacteria through an unknown binding site (Somboonwiwat et al., 2008), but most ALFs bind to LTA through

their LPS-binding site (Li and Li, 2020). Therefore, the clustering of positive charges mainly within the disulfide loop is usually defined as the putative LPS-binding domain, or LBD (Somboonwiwat et al., 2005). Interestingly, positively charged residues near the LBD region can also contribute to the binding and antimicrobial activity. For example, the variant SpALF6-V of the ALF SpALF6 from *Scylla paramamosain* has amino acid mutations due to single nucleotide polymorphisms (SNPs) and the mutation of H46 to R46 near the LBD resulted in stronger antimicrobial and cell-binding activities compared to SpALF6 (Hou et al., 2017).

1.5.4 Functions of ALFs

Antilipopolysaccharide factors display broad-spectrum antimicrobial activity against bacteria, fungi, and viruses (Li and Li, 2020). The last decade has seen several studies reporting broad-spectrum antimicrobial activity of ALFs in various crustacean species, often with distinct ALFs within one species exhibiting specific activity against different microbial pathogens (Li and Li, 2020).

As discussed above, ALF molecules are structured in a way that confers antibacterial activity. Many studies have suggested that the disulfide loop and the basic amino acids in the LBD have a major function in ALF antibacterial activity (Li and Li, 2020). There is also a relationship between the predicted isoelectric point of the LBD and its activity. Highly cationic ALFs like ALFPm3 exhibit strong, broad-spectrum activities against bacteria, fungi, and viruses, while lesser cationic ALFs such as EsALF2 from *Eriocheir sinensis* exhibit a lower strength antibacterial activity, sometimes only against Gram-negative bacteria (Tassanakajon et al. 2015). Meanwhile, highly anionic ALFs have

an impaired LBD and thus do not display any antimicrobial activity (Rosa et al., 2013). This is likely because of the lack of positively charged residues in the LBD, corroborating the suggestion that basic amino acids in the LBD have an important role for antibacterial activity (Li and Li, 2020).

In contrast to their antibacterial activity, the antiviral activity of ALFs seems to be based on the role of specific lysine residues in the LBD region. The anti-WSSV activity of a synthetic peptide corresponding to the LBD of an ALF from the Chinese shrimp *Fenneropenaeus chinensis* (FcALF-LBDc) is inhibited when lysine residues are replaced by other residues (Guo et al., 2014). The LBD directly interacts with several structural proteins of the WSSV envelope (Suraprasit et al., 2014), but the specific mechanism of action is still not fully understood (Li and Li, 2020). Similarly, differential gene expression in the freshwater crayfish *Pacifastacus leniusculus* in response to a WSSV challenge found that an ALF was up-regulated upon infection and that ALF RNAi reduced viral propagation (Liu et al., 2006). This was the first crustacean gene product identified with the capacity to interfere with WSSV replication (Liu et al., 2006). The study was not able to confirm if the ALF interacted with the virus extracellularly or intracellularly but suggested that the presence of a signal sequence meant that the protein acted outside of the cell (Liu et al., 2006).

Other immune system roles of ALFs include the regulation of the crustacean's endemic microbiota (Li and Li, 2020). Several AMP gene expression-knockdown studies have shown that these molecules help maintain the host-microbiota homeostasis by preventing the proliferation of the haemolymph's endemic bacteria, reviewed in Wang and Wang (2015). Antilipopolysaccharide factors in particular have a protective function, and

their strong antimicrobial activity against Gram-negative bacteria, which dominate the haemolymph microfauna, could be why (Wang and Wang, 2015). Two ALFs (ALF4 and ALF6) from kuruma shrimp (*Marsupenaeus japonicus*) are ubiquitously expressed by haemocytes, and their expression can be affected by a C-type lectin (MjHeCL) (Wang et al., 2014b). When MjHeCL expression was suppressed via RNAi, the haemolymph bacterial count quickly increased from approximately 5×10^2/mL to 6×10^3/mL, resulting in high animal mortality (Wang et al., 2014b). Suppression of endemic bacterial growth is not the only other role of ALFs. In *Macrobrachium rosenbergii*, an ALF has been suggested to have a role as an opsonin since the phagocytic activity of haemocytes was enhanced after they were treated with it, compared to haemocytes treated with a sterile medium (Liu et al., 2014). Lv et al. (2017) found that silencing an ALF in apparently healthy *Exopalaemon carinicauda* caused hepatopancreas lesions ultimately causing death, possibly due to increased bacterial growth of mainly *Vibrio* spp. (Lv et al., 2017).

1.5.5 ALFs of Crustacean Species Studied So Far

Many studies have focused on the identification of novel ALF isoforms and their specific functions. Recombinant molecules of ALFPm3 (rALFPm3) expressed in the yeast *Pichia pastoris* revealed that it inhibited most of the tested Gram-positive and Gram-negative bacteria, as well as filamentous fungi species (*Fusarium oxysporum*, *Botrytis cinerea,* and *Penicillium crustosum*) (Somboonwiwat et al., 2005). The ALFPm3 peptide also has other activities than just directly antimicrobial: it is an effector of the *P. monodon* immune system and could use its LPS-binding property to facilitate many reactions such as inhibition of inflammatory reaction or anticoagulant activity (Somboonwiwat et al.,

2005). Penaeid shrimp seem are the most studied decapods when it comes to ALFs (eg. Somboonwiwat et al., 2008; Jiang et al., 2015; Sun et al., 2021), but there is a growing number of studies that involve other decapods (eg. Afsal et al., 2012; Hou et al., 2017; Sruthy and Philip, 2021). These studies focus on identifying and characterizing the antimicrobial activity of new molecules. For example, in the crayfish *Procambarus clarkii*, recombinant PcALF1 could bind to all tested Gram-positive bacteria, Gram-negative bacteria and fungi as well as LPS, LTA, and β-glucan (Sun et al., 2011). Similarly, the mud crab *S. paramamosain*'s recombinant ALFs (rSp-ALF1 and rSp-ALF2) had a strong inhibitory effect against most tested Gram-positive and Gram-negative bacteria and WSSV (Liu et al., 2012). Although the focus on decapod crustaceans was initially on penaeid shrimp, studies of ALFs in other decapod species are growing in numbers today.

1.5.6 ALF Expression Patterns

All ALF types can be simultaneously expressed in one shrimp (Rosa et al., 2013). Transcripts of ALFs can be differentially expressed in significant ways: some ALFs are tissue-specific, such as FcALF8 of *F. chinensis* only being expressed in the lymphoid organ (Li et al., 2019), while others are expressed in a variety of tissues at different levels, such as EcALF2 of *E. carinicauda* (Lv et al., 2018). However, many ALFs are mainly expressed in haemocytes (Li and Li, 2020). These different expression patterns could be related to the transcription regulation of ALFs through the IMD, JAK/STAT, and especially Toll pathways, which have all been proven to regulate ALFs through gene knockdown studies (Li and Li, 2020). In *Cherax quadricarinatus,* expression of CqALF was inhibited after the gene silencing of a specific Toll receptor (CqToll) in haematopoietic tissue cell cultures

(Li et al., 2017). The regulation of ALF expression is still not fully understood as we still need to observe direct interactions between transcription factors and ALF gene promoter regions (Li and Li, 2020).

1.5.7 ALF Classifications

The classification of ALFs has only been established on genes derived from penaeid shrimp (Li and Li, 2020). A recent study of penaeid shrimp ALFs found that they cluster into seven groups, A to G, that represent seven unique genomic sequences identified in *P. monodon* (Matos et al., 2018). These genes also have the same coding region structure of three exons separated by two introns, with the second exon coding for the four stranded β-sheets and the central β-hairpin, which is delimited by the two conserved cysteines (Matos et al., 2018). The ALF classes are based on the full-length sequences, as well as the highly conserved LBD sequences (Tassanakajon et al., 2018). The positively charged LBD groups include groups A, B, C, and E, while group D has an impaired LBD (Tassanakajon et al., 2018). Groups B and G have broad antimicrobial activity while groups A, C and E have more limited activity, and groups D and F have almost no antimicrobial activity (Matos et al., 2018). Matos et al. (2018) describes the amino acid signatures and biochemical properties of the seven shrimp ALF groups in detail.

Even if classifications are currently based on penaeid shrimp genes, they are not applicable to other crustacean species, and are not completely consistent even within shrimp (Li and Li, 2020). One sequence of *M. japonicus*, MjALF-E1, was initially classified as a type E ALF in Jiang et al. (2015), but a more recent study could not fit this sequence into any ALF class (Matos et al., 2018). The combination of shrimp, crab, and

lobster sequences increases the classification complexity. The *P. monodon* ALFPm3 sequence was initially placed in class B, and ALFPm6 was initially placed in class C; however, they were both classified into the E type in one study (Li et al., 2019). There are many papers that cover a large selection of crustacean species that are not penaeid shrimp (see Afsal et al., 2012; Wang et al., 2015; Lai and Aboobaker, 2017), and these studies are unable to classify most of their sequences into the current classification paradigm. Combining ALF evolution among crustacean species with a more comprehensive bioinformatic analysis is needed (Li and Li, 2020).

1.5.8 Potential Future ALF Uses

There are many potential uses of ALFs that have been suggested or explored. They could help fight bacterial antibiotic resistance, which is pushing science to discover novel AMPs as sources or templates for designing new antibiotics (Somboonwiwat et al., 2005). Antilipopolysaccharide factors have been suggested as alternatives to antibiotics for crustaceans themselves, such as a potential use in shrimp larviculture as therapeutic agents (Somboonwiwat et al., 2005). Once ALFs and their variety of immune functions were discovered, there was a strong interest in applying them as anti-disease compounds in shrimp aquaculture, but early efforts were limited by the high preparation costs of these proteins (Li and Li, 2020). Recent efforts have focused on the LBD as this well-described functional domain consists of only 22 amino acids and more efficient and cost-effective technology now allows for lower production costs (Li and Li, 2020). Pathogenic diseases are the major threat to the global crustacean aquaculture industry, so the applicability of ALF genes in disease resistant breeding is of big interest due to their variety of

antimicrobial activities and sequence diversity (Li and Li, 2020). Some SNPs in ALF genes have been identified as important target genes for marker selection for disease resistance, such as 16 SNPs detected in seven ALF isoforms of *Portunus trituberculatus* associated with resistance to *Vibrio alginolyticus* (Li et al., 2013b).

Antilipopolysaccharide factors and similar LPS-binding molecules and derivatives have been of interest to human and overall veterinary health, as they could potentially help as therapeutic, and/or prophylactic, agents for treating septic shock (Somboonwiwat et al., 2005). In mammals, LPS can induce disseminated intravascular coagulation (Chaby, 2004). Any molecule that can bind to LPS and neutralize its effects, or enhance its clearance, could have potential clinical applications as this component is the main mediator of septic shock (Hoess et al., 1993). Recent studies have demonstrated how ALFs have a role in anti-tumor growth due to their immune regulation functions. For example, a study on mice with bladder-associated tumors found that a synthetic LBD peptide from *P. monodon*, in conjunction with inactivated murine bladder carcinoma cell lysate, could enhance antitumor immunity for this tumor type in mice (Huang et al., 2015). Another example includes a synthetic peptide derived from a *P. trituberculatus* ALF that was encapsulated in raw milk-derived extracellular vesicles that were successfully delivered to human monocytes (Lee et al., 2019). The peptide increased the levels of reactive oxygen species, superoxide anion production, phagocytosis and several cytokines, demonstrating its potential to be developed into an immune stimulator (Lee et al., 2019). The potential for ALF use in future aquaculture husbandry and in medical applications is huge.

1.6 The Green Crab

1.6.1 General Biology of the Green Crab

Green crabs are usually found in sheltered marine and estuarine habitats on various substrates, although they tend to aggregate under rocks or similar debris at low tide during daylight hours (Orlosk et al., 2011; Blewett et al., 2017). These crabs have both circadian and circatidal rhythms, with activity peaks during hours of darkness and high tides (Orlosk et al., 2011). The colouration of adult green crabs ranges from green to red, and it is known that red individuals are delaying molting in order to allocate more energy into reproduction (Lee et al., 2005). Green to yellow individuals are more physiologically tolerant to environmental stressors than orange to red individuals (Lee et al., 2005). Previous research has found that the red colouration only occurs in larger size classes, where crabs are in prolonged intermoult and have a thicker carapace than comparable crabs with a green colouration (McGaw et al., 1992). Green crabs consume a variety of prey with a preference for molluscs including soft shell clams, quahogs, mussels and oysters (McNiven et al., 2013; Tan and Beal, 2015). They also have several predators along the Atlantic coast of North America, including other crab species, shrimp, fish, birds and seals (Cohen et al., 1994). Even though green crabs have many predators, they are known for being a very successful invasive species across the world.

1.6.2 The History of the Green Crab's Global Invasion Events

The green crab originated on the coasts of Atlantic Europe and possibly northwest Africa, but has established itself in six other temperate regions, namely in Atlantic and Pacific North America, South America, Australia, South Africa, and Japan (Carlton and

Cohen, 2003; Darling et al., 2008). This marine invasive species has invaded many areas throughout the world through natural and anthropogenic dispersal (Darling et al., 2008) such as being present in ballast water, solid ballast and contaminated packing material of commercial seafood (see Carlton and Cohen, 2003). Three major human-mediated dispersion events helped successfully disperse this crab globally in roughly 1800, in the 1850s-70s, and in the 1980s-90s (Carlton and Cohen, 2003). The first documented invasive population was established on the Atlantic coast of the United States in the early 1900s (Darling et al., 2008). The established population in Nova Scotia possesses levels of genetic diversity comparable to those observed in the species' native range, which has been proven to be due to multiple introductions to eastern North America (Darling et al., 2008). The range of this species in both native and introduced regions is likely regulated by temperature parameters, as no tropical populations have been established so far (Carlton and Cohen, 2003).

1.6.3 The Green Crab as an Invasive Species

The green crab is listed as an invasive aquatic species in Canada (DFO, 2019). This species is also considered an ecosystem engineer as it can create extensive ecosystem alterations through predator-prey interactions, interspecific competition and ecological disturbances (Cohen et al., 1994). The green crab modifies marine ecosystems by destroying eelgrass (*Zostera marina*) beds, consuming native species and displacing other crab populations (Matheson et al., 2016). This crab can also cause general sediment disturbance by digging through several centimeters to search for prey (Cohen et al., 1994). Green crabs can even decimate small stocks of commercially valuable soft-shell clams

(*Mya arenaria*) (Floyd and Williams, 2004; Williams et al., 2006). Due to all of these negative environmental impacts, the Canadian government issues nuisance permits to fishermen that allow the destruction of any green crabs they catch (DFO, 2019). Culling efforts have thus been attempted to help reduce green crab numbers, but none have been successful in eradicating populations so far (McNiven et al., 2013). This is likely because it is hard to remove all the life stages of this animal (McNiven et al., 2013). The successful control of green crab populations would require intensive trapping for several years over large geographical distances which is not economically feasible at the moment (McNiven et al., 2013). Finding a use for this species would help lessen the costs of trapping it. Although this species is ubiquitous along Nova Scotian coasts, it is still a host to a diverse group of parasites (Bojko et al., 2018).

1.6.4 Parasitism in Green Crabs

Green crabs harbour many metazoan parasites, namely nematodes, an acanthocephalan (*P. botulus*), a barnacle (*Sacculina carcini*), trematodes, and ectoparasitic crustaceans (Bojko et al., 2018). In addition, many microbial eukaryotes, bacteria, and viruses parasitize this animal as well (Bojko et al., 2018). As the North American East Coast is relatively geographically close to the European shoreline, this allowed for a relative ease of parasite transport from the crab's original population (Blakeslee et al., 2009). The most common green crab parasite of green crab populations along the North American East Coast is the trematode *Microphallus similis*, with an overall prevalence of roughly 40% (Blakeslee et al., 2009). Non-native species tend to lose parasites upon introduction into novel regions (Torchin et al., 2001). Indeed, the absence of parasitic

castrators like *S. carcini* along the North American East Coast has had physiological and ecological benefits to green crab populations (Torchin et al., 2001). Crab body size and biomass are inversely proportional to parasitic castrator prevalence, with crabs in introduced regions being larger and having greater biomass (Torchin et al., 2001). The North American East Coast green crab populations have thus shown a significant loss in parasite species richness and prevalence upon introduction compared to the native European population (Blakeslee et al., 2009).

1.7 The Phylum Acanthocephala

1.7.1 General Description of Acanthocephalans

Acanthocephala is an independent and monophyletic phylum with around 1300 described species that all have complex life cycles, and always have an intermediate arthropod host that must be trophically transmitted to a final vertebrate host (Moore, 1984; García-Varela et al., 2000; Rojas and Ojeda, 2005; García-Varela and Pérez-Ponce de León, 2015). Acanthocephalans are endoparasitic worms of arthropods and vertebrates (García-Varela et al., 2000). Adults undergo sexual reproduction in the intestine of the definitive host and their eggs are released through the feces of this host (Moore, 1984). The egg is the only life stage that can survive outside of a host (Moore, 1984). The intermediate host then inadvertently ingests an egg, which penetrates the invertebrate's gut wall and develops exclusively in the host's body cavity until the cystacanth stage, where it is capable of infecting the definitive host if it ingests the parasitized intermediate host (Moore, 1984; Rojas and Ojeda, 2005; Lafferty and Shaw, 2013). The cystacanths can enter a state of quiescence within the intermediate host until this host is either eaten or dies (Thompson,

1985a). Many acanthocephalans use crustacean species as their intermediate hosts (Latham and Poulin, 2001).

1.7.2 Acanthocephalans and their Crustacean Intermediate Hosts

Many studies performed on amphipods have revealed that some acanthocephalans alter intermediate host phenotypes as a strategy for parasite transmission by increasing the risk of predation by the final host (Guler and Ford, 2010). Some acanthocephalans can reverse predator avoidance behaviours in gammarids. For example, *Gammarus pulex* individuals infected by the acanthocephalan *Pomphorhynchus laevis* are significantly less photophobic than uninfected ones while *Polymorphus paradoxus* causes a dramatic change in the evasive response of *Gammarus lacustris* (Bethel and Holmes, 1973).

There are relatively few studies that have examined the effects of acanthocephalan infections on crabs (Haye and Ojeda, 1998), and no studies on the green crab specifically. Most studies involving acanthocephalans have focused on the burrowing behaviour of shore crabs like the sand crab (*Emerita analoga*) or the mud crab (*Macrophthalmus hirtipes*), with many acanthocephalan species causing altered burrowing behaviour that leaves infected crabs more exposed and thus more vulnerable to predation by seabirds, the definitive hosts (Latham and Poulin, 2001, 2002a, 2002b; Kolluru et al., 2011). A study of *Hemigrapsus crenulatus* infected with *Profilicollis antarcticus* cystacanths found a higher metabolic rate (oxygen consumption) and more excitatory activities in infected crabs (Haye and Ojeda, 1998). This increase in excitatory behaviour may increase the vulnerability of these crabs

to predators as they are more visually explicit in the environment (Haye and Ojeda, 1998). Conversely, sand crabs infected with *Profilicollis altmani* have a lower metabolic rate (oxygen consumption) compared to uninfected ones, although the reason for this is unknown (Figueroa et al., 2019). It is likely, however, that a lower metabolic rate decreases the ability of the crabs to escape predators, which would be beneficial to the parasite as well (Figueroa et al., 2019). Acanthocephalan manipulation can vary according to the species of intermediate crustacean host, but it is certain that parasitic manipulation is worth looking at more closely, especially in species not studied in this way before.

1.7.3 Profilicollis botulus

The genus *Profilicollis* is composed of nine acanthocephalan species characterized by a long neck and fully ovoid proboscis that use decapods as intermediate hosts and seabirds as final hosts (Ching, 1989). More specifically, *P. botulus* has multiple species of shore crabs as intermediate hosts, including the genera *Carcinus*, *Hyas*, and *Hemigrapsus* (Ching, 1989; McDermott, 2011; Skirnisson, 2015). *Profilicollis botulus* cystacanths have also been recovered from American lobsters (Brattey and Campbell, 1986). Many diving duck species can become definitive hosts of *P. botulus*, including the common eider (Ching, 1989).

The complex life cycle of this parasite is as follows: adult *P. botulus* worms are dioecious and live in the intestines of the duck, where eggs are passed with the feces into the surrounding environment (Skirnisson, 2015; Thompson, 1985a). A green crab then ingests the eggs, which are immediately infective to the crab and develop in the midgut from the acanthella into the cystacanth stage (Thompson, 1985a). The cystacanth is the

parasite's long-living infective stage, as it likely stays infective throughout the life of the green crab or until it gets eaten by a duck (Thompson, 1985a). Once a duck consumes an infected crab, the worm's life cycle can be completed, with adult *P. botulus* living for at least two months within the duck's intestine (Thompson, 1985a).

1.7.4 Background on Manipulative Parasite-Intermediate Host Interactions

There are many possible explanations for how and why parasitic manipulation exists. The survival of parasites with complex life cycles depends on their ability to contact appropriate hosts, so in theory, natural selection favours parasite-induced changes in the intermediate host's phenotype that increase the likelihood of encountering the parasite's definitive host (Kolluru et al., 2011). Host alterations may simply be due to a decrease in general vigour, and not necessarily adaptive parasitic manipulation (Kolluru et al., 2011). However, a number of studies have demonstrated that some parasites manipulate the behaviour and physiology of their intermediate hosts in order to ultimately enhance their own transmission to their final hosts (Moore, 1984; Tain et al., 2006; Lafferty and Shaw, 2013). Since acanthocephalans are trophically transmitted, phenotypic alterations such as increased conspicuousness, increased overall activity and alteration of microhabitat choice in intermediate hosts would increase the contact rates between intermediate and final hosts (Kolluru et al., 2011; Lafferty and Shaw, 2013). Since the larval stage of acanthocephalans in their intermediate hosts is essential for their trophic transmission, it seems unlikely that these helminths only accumulate randomly and rest passively in the intermediate hosts (Poulin et al., 2003). There is direct empirical evidence that some acanthocephalans can change the phenotype of their intermediate hosts by changing their appearance, influencing

their microhabitat choice and/or changing other aspects of their behaviour and physiology (Cézilly et al., 2000; Tain et al., 2006). These induced changes could increase the likelihood of ingestion by the final host if infected animals are proportionally more predated upon than non-infected ones (Lagrue et al., 2007). Although there are many theories on the mechanisms of parasitic manipulation, many scientists are still trying to determine the proximate causes of this phenomenon.

1.7.5 Proximate Causes of Parasitic Manipulation

Many proximate mechanisms of parasitic manipulation have been suggested. Parasites can manipulate host behaviour through energetic drain and/or the damaging of key organ systems, but more complex methods can be used as well when the physiological systems target behaviour (Lafferty and Shaw, 2013). This includes the neural, endocrine, neuromodulatory and immunomodulatory systems, which have many overlaps that make the task of isolating the specific mechanisms of host manipulation quite difficult (Lafferty and Shaw, 2013). The physiological pathways manipulated by parasites in order to alter host behaviour are still poorly understood, but the most recent evidence suggests that parasites can actively modulate the neurochemistry of their hosts (Poulin et al., 2003). In most infected crustaceans, the acanthocephalans live freely in the haemocoel (Rojas and Ojeda, 2005), such as in the parasitized gammarid *G. lacustris*, where *P. paradoxus* floats without causing apparent mechanical damage (Maynard et al., 1996). This suggests some kind of biochemical interference by the acanthocephalan with the neuroendocrine system of these intermediate hosts as there is no direct contact with the host's central nervous system (Rojas and Ojeda, 2005). In *G. lacustris* infected by *P. paradoxus*, the serotonergic

neurons of the gammarid's central nervous system change, with a greater number of localized points of serotonin storage, and these gammarids exhibit altered predator avoidance behaviour (Maynard et al., 1996). This provides evidence that serotonin is a key player in the behavioural alteration observed, and that *P. paradoxus* is capable of influencing the central nervous system of this amphipod (Maynard et al., 1996). Another example includes how *P. antarticus* causes a dramatic increase in the haemolymph dopamine levels of parasitized hairy-handed crabs (*H. crenulatus*) through a biochemical process that has yet to be identified (Rojas and Ojeda, 2005). Overall, the proximate causes of parasitic manipulation are encompassed by a variety of traits of the neuromodulatory, endocrine and immune systems that are altered simultaneously (Perrot-Minnot and Cézilly, 2013). Although many possible explanations for parasitic manipulation exist, there is still much work to be done to determine which theories are most accurate.

1.8 Host-Parasite Interactions Involving *P. botulus*

1.8.1 Green Crabs and P. botulus

Relatively little is known about the interactions between *P. botulus* and the green crab. The parasite is located on the midgut of infected green crabs and infection can result in an enlarged gut (Bojko et al., 2018). This parasite can elicit a melanisation response in the crab if the infection breaks through the gut epithelium (Bojko et al., 2018). These pathological effects are the only documented effects of this worm on the crab. A 2018 study reported an average *P. botulus* infection prevalence of 2.0 ± 4.4% at two Nova Scotia sites (Pubnico and River Port), with infections being more common in male specimens (Bojko

et al., 2018). There is clearly a need for a more careful observation of *P. botulus* effects on this crustacean.

1.8.2 Eider Ducks and P. botulus

There are two subspecies of eider ducks that could be impacted by the green crab in Nova Scotia: *S. m. borealis*, which winters in eastern North America, and *S. m. dresseri*, which has an overlap of breeding and wintering populations in eastern North America (Vestbo et al., 2019). *Profilicollis botulus* is ingested by eiders through the consumption of infected green crabs (Thieltges et al., 2006). Historically, *P. botulus* infection prevalence in eiders has always been relatively high, with an annual cycle connected to seasonal peaks in crab consumption (Thompson, 1985b). In mass mortality events of eiders, the high prevalence and intensity of *P. botulus* infections is often identified as a secondary cause of death (Camphuysen et al., 2002; Thieltges et al., 2006). Although the life cycle of *P. botulus* has been established, there is still a research gap when it comes to the potential effects of the worm on its intermediate host to achieve transmission to the eider.

1.9 Summary and Conclusions

Globally, most crustaceans harvested from wild fisheries and aquaculture are from the order Decapoda, which includes shrimp, lobsters, and crabs (DFO, 2019; FAO, 2020a). Crustaceans are important nutritionally and economically to the global human population, and therefore provide food security in both producing and exporting countries (Bondad-Reantaso et al., 2012). The One Health concept should be applied to crustaceans for these reasons as climate change and human-mediated pathogen spread from invasive to native

species both increase; it is crucial to increase our understanding of infections and diseases pertaining to these species (Behringer and Duermit-Moreau, 2020).

Even though crustaceans only possess an innate immune system without true adaptive immunity, their immune system is still quite complex and consists of several cellular and humoral components that are often interlinked (Kulkarni et al., 2021). Disease susceptibility of the important decapods is a major concern due to global anthropogenic changes. Host-pathogen interactions for example are responding to global environmental changes and it is vital to understand how these changes impact disease risks and dynamics function (Bernardo-Cravo et al., 2020).

Antimicrobial peptides are important immune effector proteins, especially in crustaceans. These peptides exhibit high diversity in terms of structure and function, and ALFs are an AMP family that is gaining more attention from comparative immunological human and veterinary health perspectives (Hou et al., 2017). Although many studies have incorporated phylogenetic trees of decapod ALFs, no study to date has attempted to comprehensively evaluate their phylogeny. In fact, many ALF papers focus on the identification, genomic/transcriptomic characterization and functions of newly discovered peptides. Thus, thoroughly determining the sequence-level similarity of ALFs in decapod crustaceans is vital to our understanding of these molecules, especially in light of their possible medical applications.

Although the above section examines the effects of infection at the molecular level, this section examines the effects of infection at the level of the whole animal. To summarize the present research gaps in the *P. botulus*-green crab relationship, there are few studies about acanthocephalans that involve intermediate crab hosts, and none performed on the

green crab specifically. Furthermore, although the life cycle of *P. botulus* has been described already, no study has ever looked at the impact of this specific acanthocephalan on the green crab. Understanding this parasite-host interaction is key in order to determine the potential effects of *P. botulus* on its ecosystem.

Furthermore, no study to date has empirically determined the impact of *P. botulus* on the green crab. There is currently an approaching imbalance between theoretical and empirical articles when it comes to parasitic host manipulation, with a rapid rise in theoretical papers that published empirical studies have trouble keeping up with (Poulin and Maure, 2015). There is a trade-off to be made between extensive knowledge of a few model systems and broader knowledge of a wider range of organisms (Poulin and Maure, 2015). Even if the green crab is not commercially important in North America, it is still considered an important species for academic studies, especially in neurotoxicity studies involving water contaminants (Blewett et al., 2017) and pharmaceutical drug exposure (Mesquita et al., 2011; Hamilton et al., 2016). Therefore, there is merit in investigating the interaction between *P. botulus* cystacanths and green crabs for the sake of broadening our scientific knowledge of parasitic manipulation.

In addition, pathogens are more likely to have an effect at the ecosystem level when they influence host behaviour and/or make hosts more vulnerable to predators (Hudson et al., 2006). Parasites can be a good proxy for estimating ecosystem health because they integrate biodiversity over time (Hudson et al., 2006). The transmission of pathogens to native species from an invasive host has proven to be quite destructive on those native populations in the past (Bojko et al., 2018). If infected invasive hosts get established in a new location and their parasites become established as well, the invasive parasites may

impact native species if they can infect novel hosts (Torchin et al., 2002). On the Atlantic coast of North America, a legal fishery has been developed where green crabs are caught and sold mainly as bait to trap more commercially important crustaceans, such as the American lobster (Bojko et al., 2018). There is little knowledge about the symbionts of the introduced green crab populations and the potential risk of these symbionts being unintentionally transferred to native populations (Bojko et al., 2018). To summarize, determining the effects of *P. botulus* on the green crab will be essential in order to expand our empirical scientific knowledge about parasitic manipulation.

Chapter 2: Phylogeny of Antilipopolysaccharide Factors in Decapoda

2.1 Introduction

Antimicrobial peptides (AMPs) are evolutionarily conserved in all life forms, and they have retained their antimicrobial activity over hundreds of millions of years (Tassanakajon et al., 2015). As crustaceans lack an acquired immune system, AMPs are important immune effectors that defend the host by inhibiting the growth of microorganisms (Tassanakajon et al., 2018). These peptides usually quickly inactivate microbes and can inhibit antibiotic-resistant microbes, while also displaying antiviral and anticancer activities (Kumar et al., 2018a; Olatunde et al., 2020). Antimicrobial peptides use several different mechanisms of action that are broadly divided into direct killing and immune modulation (Kumar et al., 2018a).

Antilipopolysaccharide factors (ALFs) are a diverse family of AMPs exclusive to marine chelicerates and crustaceans (Matos et al., 2018). Initially discovered in the horseshoe crabs *Limulus polyphemus* and *Tachypleus tridentatus* (Tanaka et al., 1982; Ohashi et al. 1984), ALFs have since been reported in various crustaceans such as shrimp, crabs, and the American lobster *Homarus americanus* (Polinski et al., 2021; Sruthy and Philip, 2021; Sun et al., 2021). Horseshoe crab ALFs were first observed to inhibit the activation of coagulation factors through lipopolysaccharide (LPS)-binding and were therefore classified as anticoagulant or anti-LPS factors (Tanaka et al., 1982; Morita et al., 1985). It was subsequently observed that this novel factor had an antibacterial effect on the growth of Gram-negative bacteria (Morita et al., 1985), and the number of antimicrobial functions of this AMP family has continued to increase since then.

The tertiary structures of the *Limulus* ALF, as well as ALFPm3 from the penaeid shrimp *Penaeus monodon*, have been resolved and revealed that ALFs consist of three α-helices against a four-stranded β-sheet with a characteristic central β-hairpin delimited by two conserved cysteines forming an intramolecular disulfide bridge (Hoess et al., 1993; Yang et al., 2009; Rosa et al., 2013). This central β-hairpin that often exhibits a clustering of positive charges is usually defined as the putative LPS-binding domain, or LBD (Somboonwiwat et al., 2005; Matos et al., 2018). Despite sharing a conserved tertiary structure and a length of roughly 100 amino acids, ALFs display tremendous sequence diversity and their evolutionary mechanisms have yet to be extensively studied (Hou et al., 2017). Many ALFs are cationic molecules that have broad-spectrum antimicrobial activity against bacteria, fungi and viruses (Li and Li, 2020). There are some exceptions however, with the smaller number of anionic ALFs discovered displaying low or no antimicrobial activity (Tassanakajon et al., 2015). The disulfide loop and the basic amino acids in the LBD are thought to play a major role in ALF antibacterial activity (Li and Li, 2020), and antiviral activity seems to be related to certain lysine residues in the LBD region (Guo et al., 2014).

These molecules are expressed in various tissues and organs, often with different isoforms being expressed simultaneously (Rosa et al., 2013; Lv et al., 2018). Specific ALF differential expression patterns and functions are determined via *in vivo* pathogen challenges (Liu et al., 2006; Li et al., 2013b; Jiang et al., 2015) and *in vitro* studies, which usually implement inhibition zone tests, minimal inhibitory concentration assays, and other types of assays to detect the antimicrobial activities of synthetic peptides based on ALFs (Li et al., 2014; Liu et al., 2014; Lee et al., 2019). Antilipopolysaccharide factors are

therefore key to maintaining host-microbiota homeostasis by inhibiting endemic bacterial growth in the haemolymph (Wang and Wang, 2015).

Many proteins that participate in crustacean innate immunity have been characterized at the protein and molecular levels, but ALFs and AMPs in general have been mostly studied for their response to microbes (Antony et al., 2011). Many studies have briefly explored the phylogeny of crustacean ALFs, with phylogenetic trees often being produced in papers that primarily identify and then test the antimicrobial activity of novel ALFs (Li et al., 2014; Sruthy et al., 2015; Sruthy and Philip, 2021). As ALF classifications have only been established in penaeid shrimp, a number of studies have exclusively looked at the ALF phylogeny of these shrimp species (Rosa et al., 2013; Jiang et al., 2015; Gu et al., 2018). To our knowledge only two studies so far have attempted to comprehensively examine ALF phylogeny (Lai and Aboobaker, 2017; Matos et al., 2018). The goal of this study is therefore to examine ALF phylogeny in decapod crustaceans to understand the similarities and patterns of these proteins.

2.2 Methodology

2.2.1 Sequence Sources

Sequences were obtained from the public database GenBank of NCBI, the papers of Matos et al. (2018) and Polinski et al. (2021), as well as unpublished Clark laboratory transcriptomes. Only mature peptide amino acid sequences were used to build phylogenetic trees. Descriptions of the sequences used (abbreviations, species name, GenBank accession numbers, mature peptide sequence) are provided in Table 4 of the Appendix.

2.2.2 Steps to Acquiring Mature Peptide Sequences

To acquire sequences through GenBank, the query sequences used to mine the protein database were the mature peptides of all *Litopenaeus vannamei* ALFs from Matos et al. (2018). Each query sequence was run through the non-redundant protein sequences (nr) database using the blastx BLAST algorithm and all relevant decapod ALF hits were retrieved. Duplicate sequences (i.e. identical amino acids) were removed, and the remaining amino acid sequences were put through SignalP 5.0 (Almagro Armenteros et al., 2019) to detect the presence of a signal peptide. Sequences with a signal peptide had the signal peptide removed to obtain just the mature peptide sequence, while sequences with no signal peptide detected were removed from the sample because as ALFs are secretory proteins, the signal peptide is a necessary component for secretion out of haemocytes (Short, 2017). Similarly to the GenBank sequences obtained, the ALF sequences from Polinski et al. (2021) were put through SignalP 5.0 and the same process was followed. The sequences from Matos et al. (2018) were already separated into signal and mature peptide sequences. For the Clark lab transcriptomes, the Galaxy Europe server version 20.05 (Afgan et al., 2018) was used to obtain nucleotide sequences from the unpublished Clark lab transcriptomes. The same *L. vannamei* query sequences from Matos et al. (2018) were used to mine a BLAST nucleotide database generated for each transcriptome with the tblastx tool (Camacho et al., 2009; Cock et al., 2015). Retrieved sequences were translated to protein sequences using two methods to maximize the amount of potential sequences retrieved: transeq (Galaxy Version 5.0.0) followed by the CD-HIT PROTEIN tool (Galaxy Version 1.3) with a similarity threshold of 0.99 within the Galaxy Europe server, and the Translate tool of Expasy (Duvaud et al., 2021). Duplicate sequences were removed. Only

full-length coding sequences that contained the characteristic two cysteines separated by 20 amino acids of the LBD, that did not have other cysteines within or proximate to the LBD, and had a signal peptide that was removed via SignalP 5.0 were selected. In species where more than one published sequence was available, the lab sequences were removed if they did not contain the conserved residues (obtained through multiple sequence alignments, described below) of the published sequences. The predicted tertiary structure of the resulting lab sequences was compared to the structure of PenmonALF3 in the SWISS-MODEL software of Expasy using the template alignment function (Bienert et al., 2017; Waterhouse et al., 2018) and if the lab sequence did not have the characteristic three alpha helices against four beta strands it was removed. Once the mature peptides of all sources used were obtained, the Blast2GO feature of OmicsBox (Götz et al., 2008; OmicsBox, 2019) was used to functionally annotate all protein sequences to confirm that they were described as ALFs. Any sequence that did not have ALF in its description, or that was described as any ALF from an order other than Decapoda, was removed.

2.2.3 Multiple Sequence Alignments

Multiple sequence alignments (MSAs) were performed in MEGA X version 10.2.6 (Kumar et al., 2018b) using the MUSCLE algorithm with default parameters. An alignment was built using all combined mature peptide sequences, and then an alignment was built for each of these specific infraorders present in the sample: Achelata, Anomura, Astacidea, Brachyura, Caridea, and Penaeidae (although Penaeidae is considered a family, for the purposes of this study it will be referred to as an infraorder). No manual sequence deletion or trimming was required for any of the alignments. The software Seq2Logo 2.0 (Thomsen

and Nielsen, 2012) was used with default parameters to generate sequence logos of the LBD alignments specifically, but any conserved residues outside of the LBD region were also included. The images generated display the numerical position in the alignment on the x-axis, large letters signify frequently observed residues, big letter stacks signify highly conserved residues and small stacks signify variable residues (Thomsen and Nielsen, 2012). The y-axis represents the information in bits (Thomsen and Nielsen, 2012).

2.2.4 Phylogeny

Bootstrap consensus trees (set at 50% bootstrap cutoff) were built using the MEGA X software. The phylogeny method used was Maximum Likelihood (ML) with the following parameters: 1000 bootstraps, five discrete gamma categories, the gaps/missing data treatment of partial deletion with a site coverage cut-off of 95%, nearest neighbor-joining interchange as the ML Heuristic method, the initial tree being made automatically and no branch swap filter. For each individual alignment, the optimal ML model was chosen based on the best DNA/protein model prediction tool in MEGA X using the following parameters: Automatic (Neighbor-joining tree) for tree to use, ML for statistical method, amino acid for substitution type, the gaps/missing data treatment of partial deletion with a site coverage cut-off of 95 and no branch swap filter. An unrooted tree with all sequences (291) was built, followed by unrooted trees for Achelata (16 sequences), Anomura (15 sequences), Astacidea (43 sequences), Brachyura (128 sequences), Caridea (42 sequences), and Penaeidae (47 sequences). The bootstrap values for each branch were included to give an idea of branch support, with the exception of the all sequences and Brachyura trees due to their high number of sequences. Unpublished laboratory sequences are indicated with the "*" symbol at the end of the sequence abbreviation. The trees for all

sequences and Brachyura were edited in iTOL (Interactive Tree of Life) version 6.3.1 (Letunic and Bork, 2021), while all other trees were edited in Microsoft Word. For all trees generated, the main patterns that were examined were homogeneity in regards to several types of operational taxonomic units (OTUs): species, genus, family, superfamily and/or infraorder. All sequences were arbitrarily assigned an abbreviation consisting of the first few letters of the genus name followed by the first few letters of the species name, similar to the penaeid shrimp nomenclature proposed by Rosa et al. (2013), in an effort to standardize ALF terminology. In addition, since penaeid shrimp ALFs have been classified into seven types, A to G (Matos et al., 2018), this notation was also implemented.

2.2.5 Terminology

Wilkinson et al. (2007) argued that the terms used to describe rooted phylogenetic trees do not apply to unrooted trees. Therefore, in this study "adjacent group" will be used instead of the term "sister group" used for rooted phylogenies and "clan" instead of "clade" (Wilkinson et al., 2007). The new terminology is needed because in unrooted trees, we cannot pick which nodes are ancestral and which ones are derived (Lapointe et al., 2010). In order to make evolutionary claims, rooted phylogeny is necessary because the phylogenetic tree topology must be globally polarized in time, and a root is needed to make the distinction between ancestral and derived nodes to infer genealogical relationships (Lapointe et al., 2010). In our case, the rationale for producing unrooted instead of rooted trees was the possibility of several ALF isoforms resulting from different evolutionary pressures based on environmental pathogens and not speciation. Adjacent groups do not necessarily derive from the same common ancestor since there can be up to three adjacent

groups for any clan (Lapointe et al., 2010). Clans are defined by a bipartition of OTUs, while slices are defined by pairs of splits (bipartitions) that form tripartitions (Lapointe et al., 2010). Clans are homogenous when they are composed of OTUs of a single type and heterogenous when this is not the case, with the OTUs of a different type compared to the dominant type defined as intruders (Lapointe et al., 2010). Homogenous clans containing all the OTUs of a type are called perfect clans (Lapointe et al., 2010).

2.3 Results

2.3.1 Multiple Sequence Alignments

The conservation of amino acid residues in the LBD was the first area of ALFs to be examined in this study. When looking at the MSA of all sequences combined, only the two cysteines of the LBD were conserved (Fig. 1). Most MSAs of the other infraorders also exhibited additional conserved amino acids. The Achelata MSA had one in the LBD, P7 (Fig. 2) and three near the LBD (a P, G and W immediately after the carboxyl-terminal end of the LBD). The Anomura MSA had one conserved residue in the LBD, P7 (Fig. 3) and three near the LBD carboxyl-terminal end (a P, W, and G). While the Astacidea MSA only displayed the two cysteines as LBD conserved residues (Fig. 4), this infraorder also had one other conserved residue near the LBD, a P positioned immediately after the LBD. The MSA of Brachyura (Fig. 5) only exhibited the two conserved cysteines. The Caridea MSA also only had the cysteines as conserved LBD residues (Fig. 6) with the addition of two other conserved residues near the LBD (a G and W toward the carboxyl-terminal end). The Penaeidae MSA had one other conserved residue in the LBD, P7 (Fig. 7) as well as three other conserved residues (a P, G and A) toward the carboxyl-terminal end.

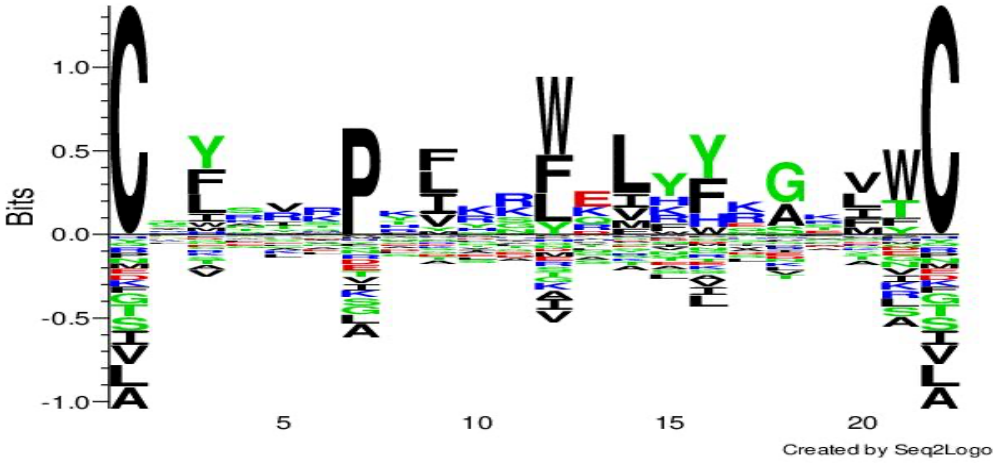

Figure 1. Sequence logo of the LBD alignment of 291 decapod ALF sequences.

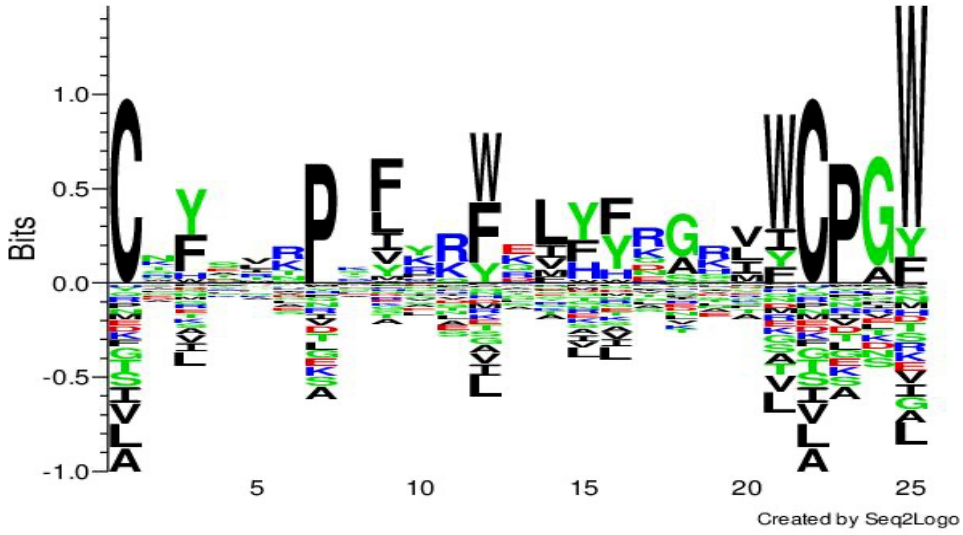

Figure 2. Sequence logo of the LBD and part of the carboxyl-terminal end alignment of 16 Achelata sequences.

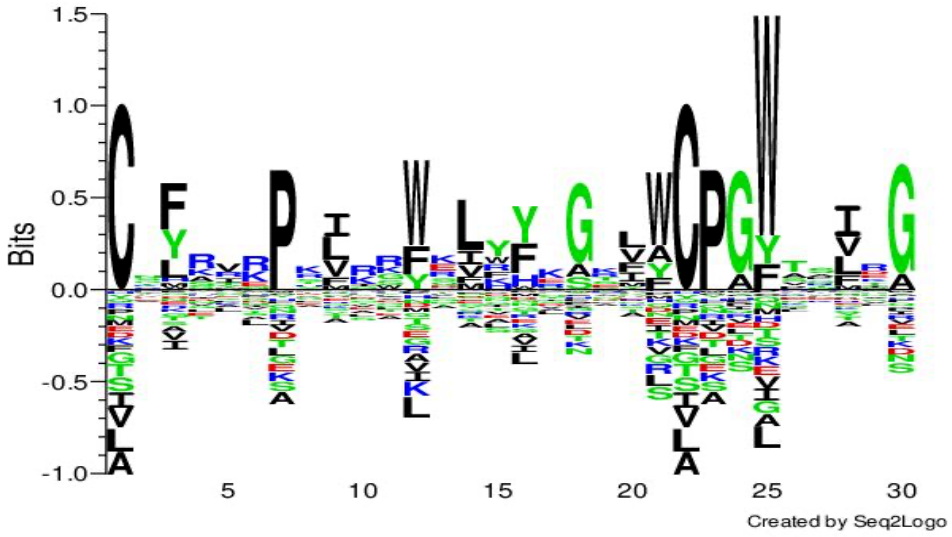

Figure 3. Sequence logo of the LBD and part of the carboxyl-terminal end alignment of 15 Anomura sequences.

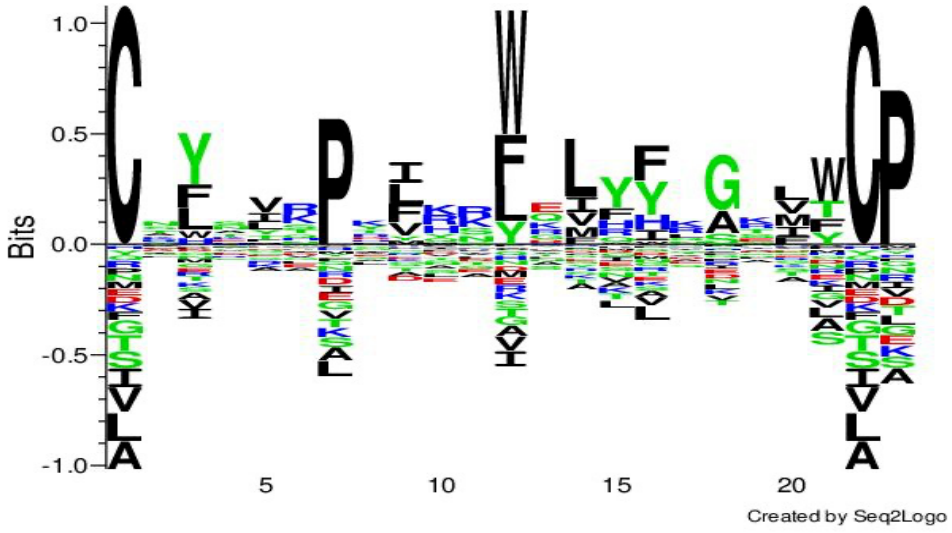

Figure 4. Sequence logo of the LBD and part of the carboxyl-terminal end alignment of 43 Astacidea sequences.

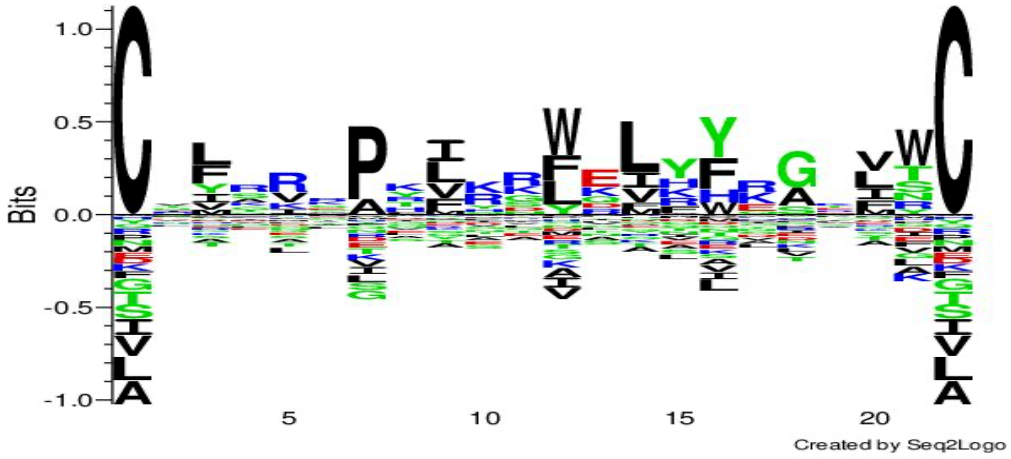

Figure 5. Sequence logo of the LBD alignment of 128 Brachyura sequences.

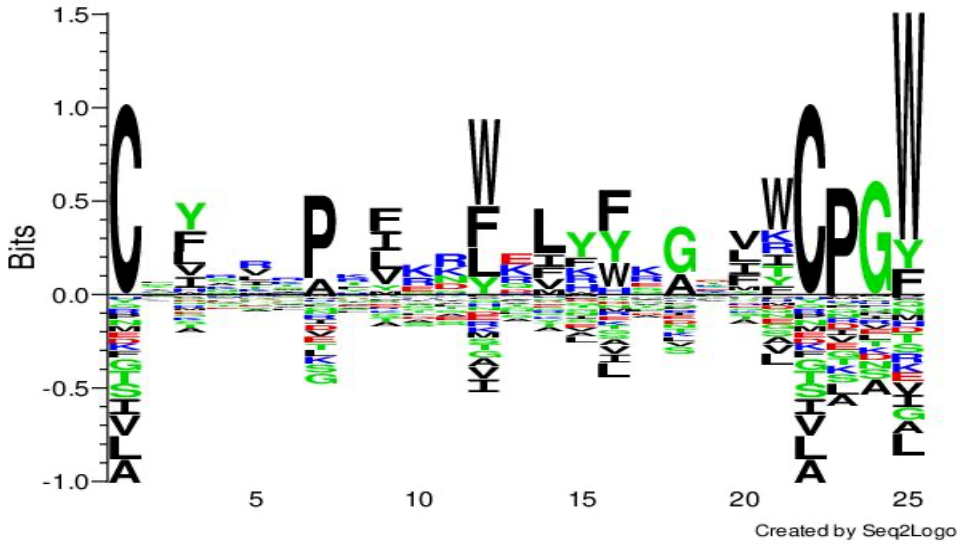

Figure 6. Sequence logo of the LBD and part of the carboxyl-terminal end alignment of 42 Caridea sequences.

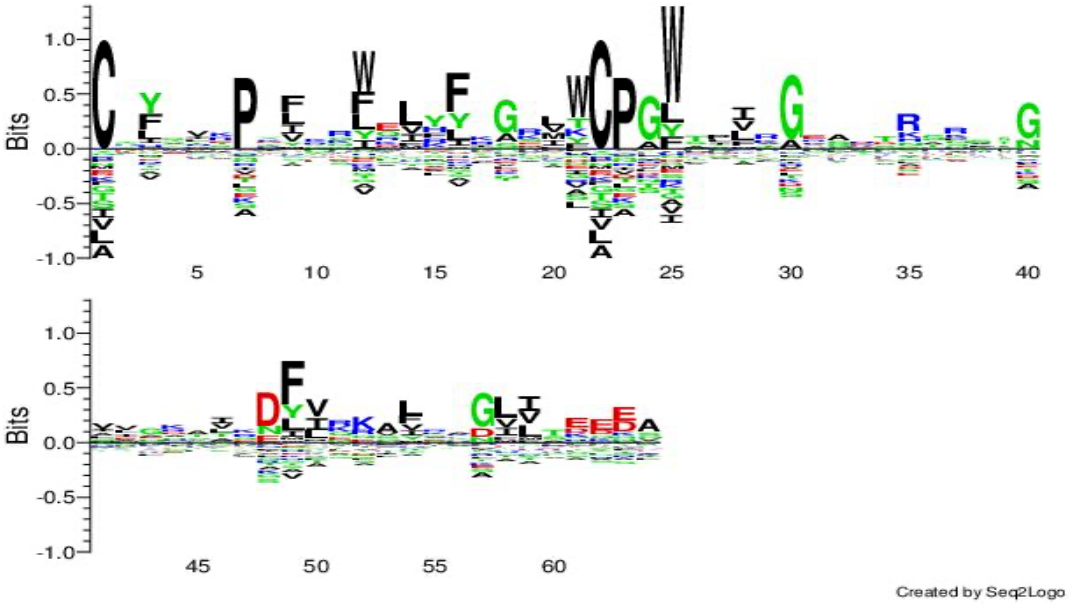

Figure 7. Sequence logo of the LBD and part of the carboxyl-terminal end alignment of 47 Penaeidae sequences.

2.3.2 All ALF Sequences

The next step was to examine the entire mature sequence of ALFs. It is apparent that when all sequences were aligned, their phylogeny produced a complex tree (Fig. 8). Sequences seem quite scattered without highly discernible patterns, but clans tend to be homogenous for infraorder. However, the clans of each specific infraorder are scattered around the tree and are not forming adjacent groups.

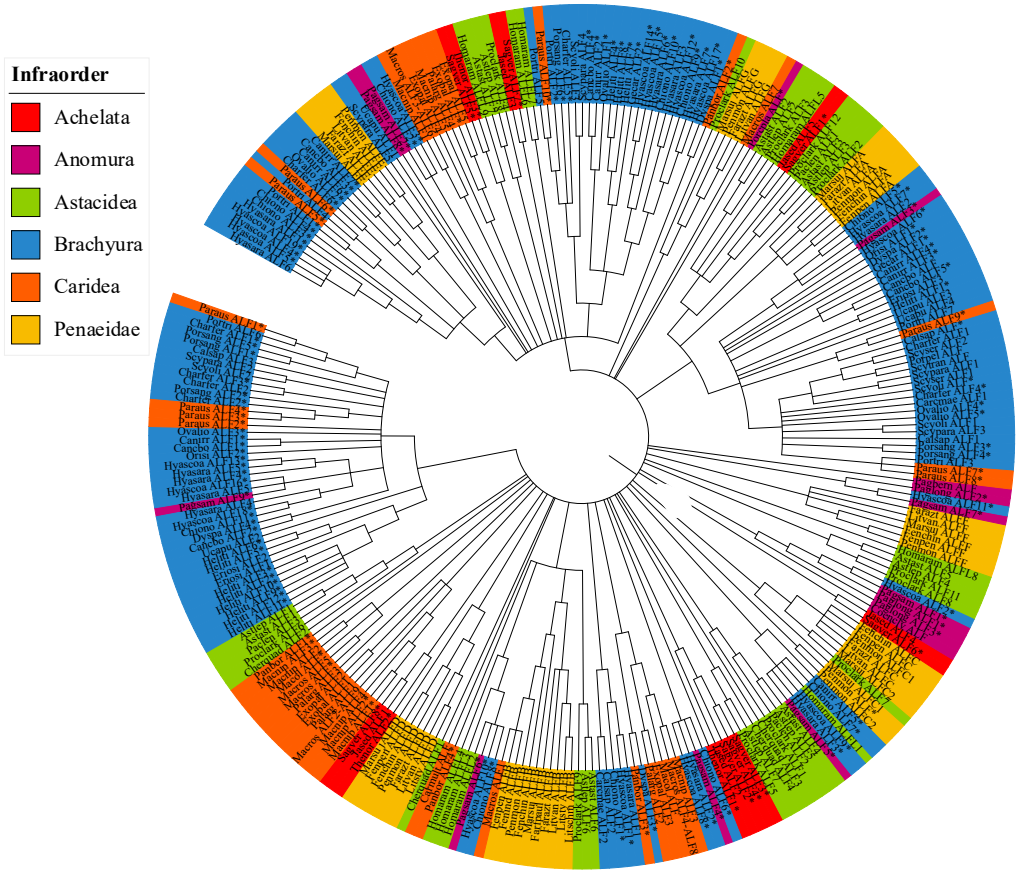

Figure 8. Unrooted phylogenetic tree of 291 decapod ALF sequences using Maximum Likelihood method with 1000 bootstraps. Each infraorder was assigned a specific colour and unpublished laboratory sequences are indicated with the "*" symbol. A list with descriptions of all sequences (abbreviations, species name, GenBank accession numbers, mature peptide sequence) is provided in Table 4.

2.3.3 Achelata Sequences

Almost all bipartitions and the resulting Achelata clans are heterogenous for species, with the exception of one clan of three *Sagmariasus verreauxi* sequences (Fig. 9).

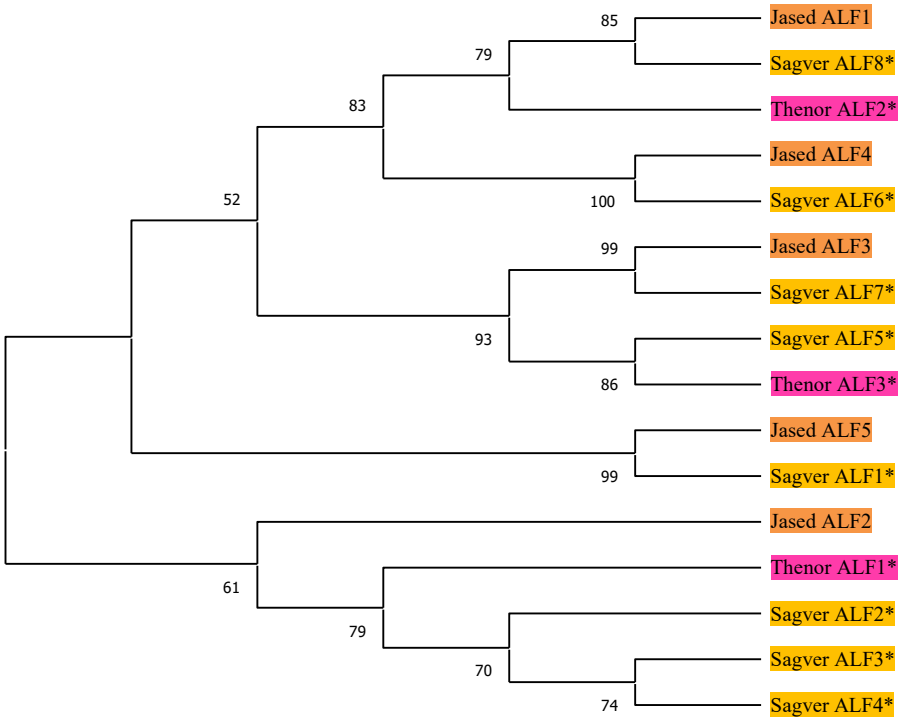

Figure 9. Unrooted phylogenetic tree of 16 Achelata ALF sequences using Maximum Likelihood method with 1000 bootstraps. Each genus was assigned a specific colour and unpublished laboratory sequences are indicated with the "*" symbol.

2.3.4 Anomura Sequences

Most Anomura OTUs did not form branches with other OTUs, and out of the three clans formed, only one is homogenous for species (two *Pagurus samuelis* sequences) (Fig. 10).

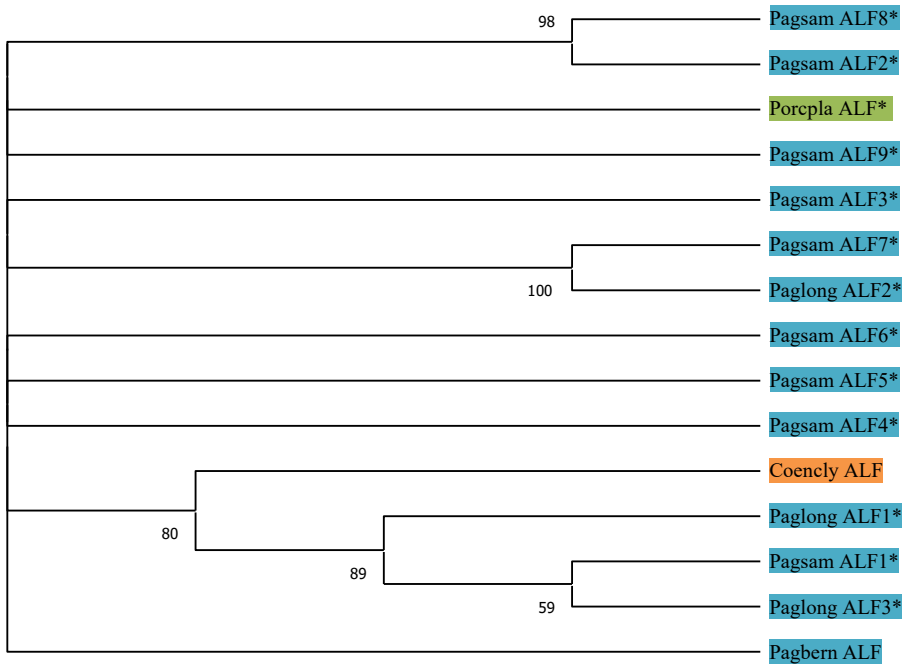

Figure 10. Unrooted phylogenetic tree of 15 Anomura ALF sequences using Maximum Likelihood method with 1000 bootstraps. Each genus was assigned a specific colour and unpublished laboratory sequences are indicated with the "*" symbol.

2.3.5 Astacidea Sequences

Almost all Astacidea clans are heterogenous for species except for two *Homarus americanus* clans and two *Procambarus clarkii* sequences (Fig. 11). In addition, five OTUs did not form branches with other OTUs.

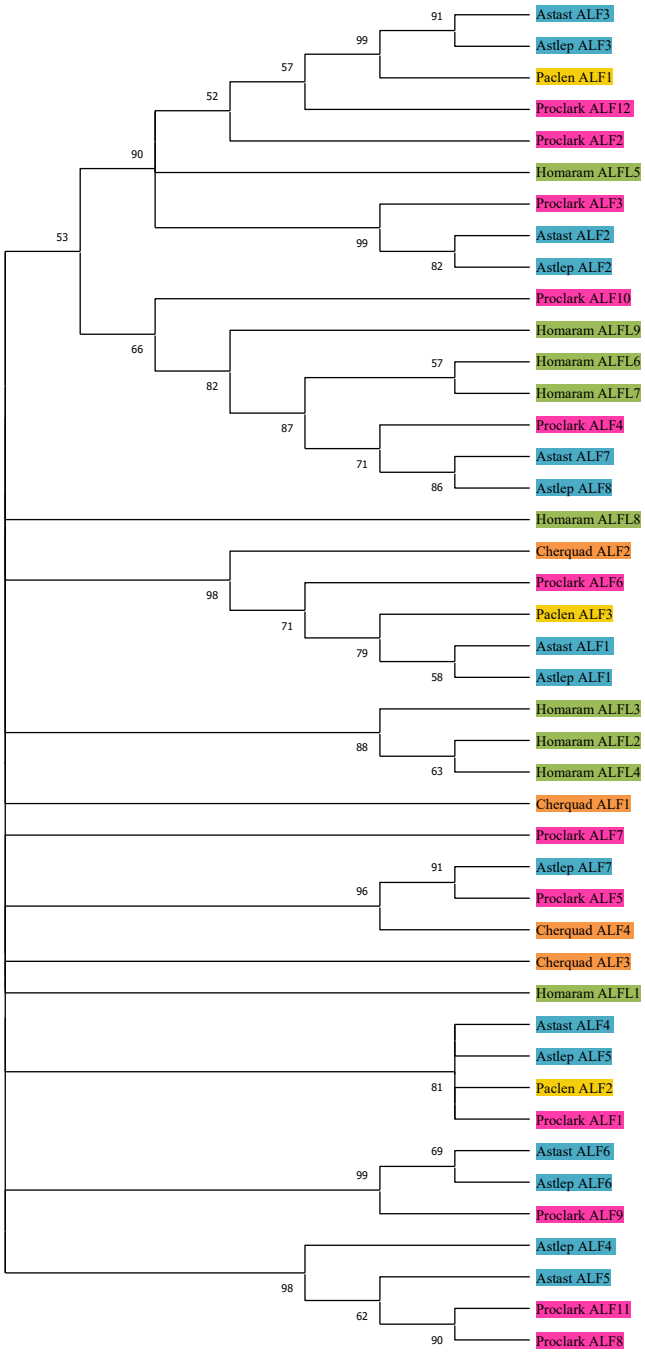

Figure 11. Unrooted phylogenetic tree of 43 Astacidea ALF sequences using Maximum Likelihood method with 1000 bootstraps. Each genus was assigned a specific colour and unpublished laboratory sequences are indicated with the "*" symbol.

2.3.6 Brachyura Sequences

Many Brachyura clans are homogenous for family and/or superfamily and many are homogenous for species or genus (Fig. 12). Quite a few OTUs did not form branches and family and superfamily clans are not often adjacent.

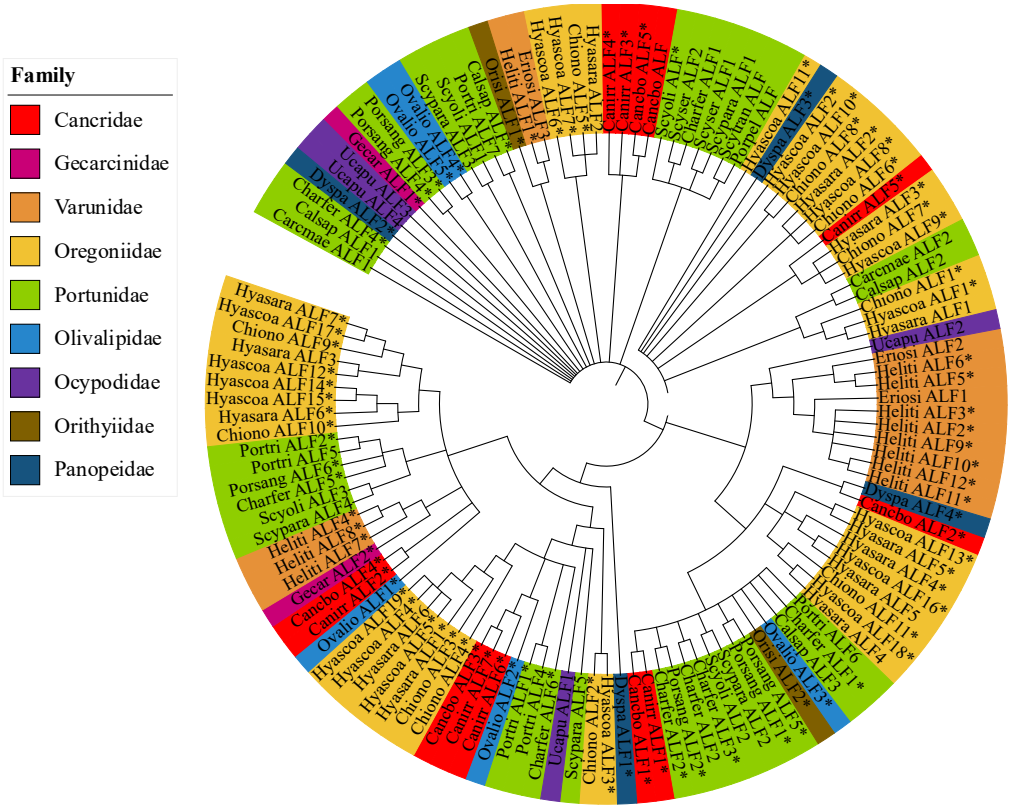

Figure 12. Unrooted phylogenetic tree of 128 Brachyura ALF sequences using Maximum Likelihood method with 1000 bootstraps. Each family was assigned a specific colour and unpublished laboratory sequences are indicated with the "*" symbol.

2.3.7 Caridea Sequences

Most Caridea clans are homogenous for species or genus (Fig. 13). All *Paratya australiensis* sequences formed homogenous clans.

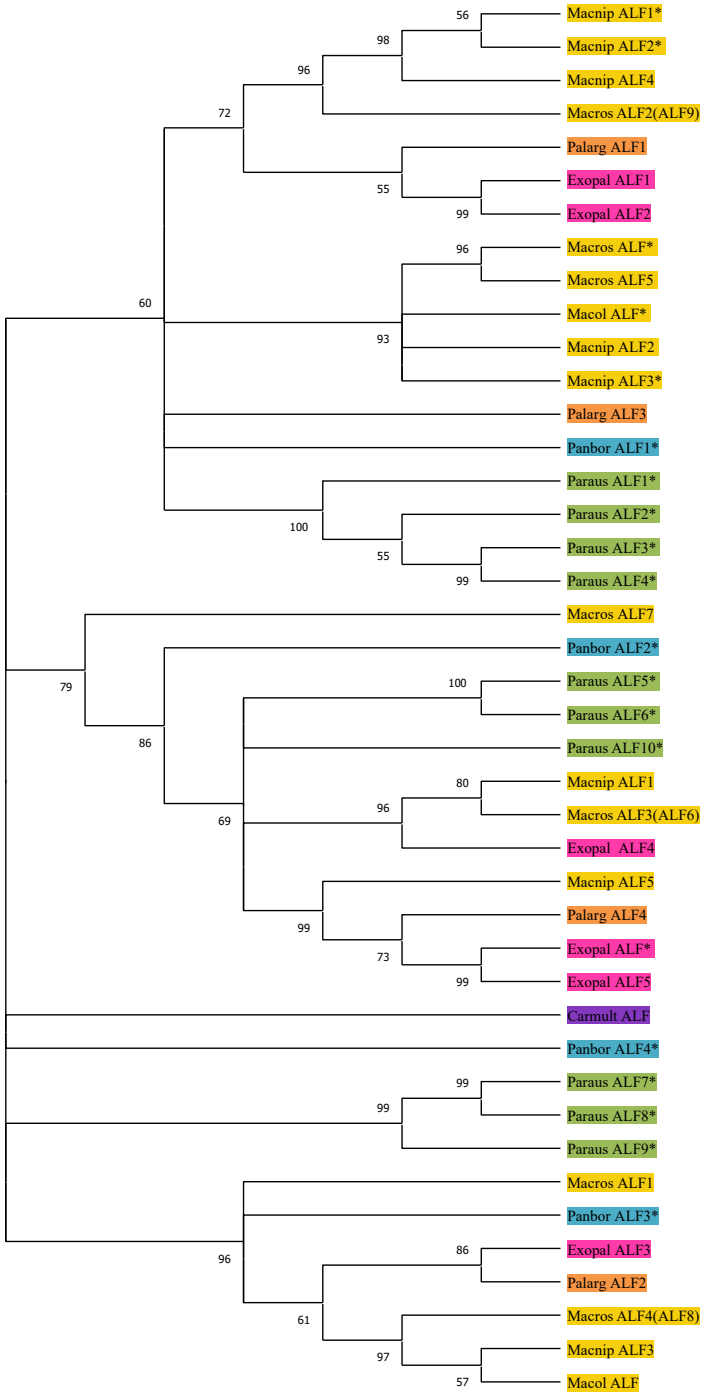

Figure 13. Unrooted phylogenetic tree of 42 Caridea ALF sequences using Maximum Likelihood method with 1000 bootstraps. Each genus was assigned a specific colour and unpublished laboratory sequences are indicated with the "*" symbol.

2.3.8 Penaeidae Sequences

All Penaeidae clans are perfect and homogenous for ALF type, but all subclans are heterogenous for species except for one branch of two *P. monodon* sequences (Fig. 14). A few clans are homogenous for genus. The ALF C type sequences Penmon_ALF* and Penmon_ALFC2 seem more closely related to the other classes than the rest of the C-type sequences. The anionic groups (A, D, E, and G) are all adjacent to each other, as are the cationic groups (B, C, and F).

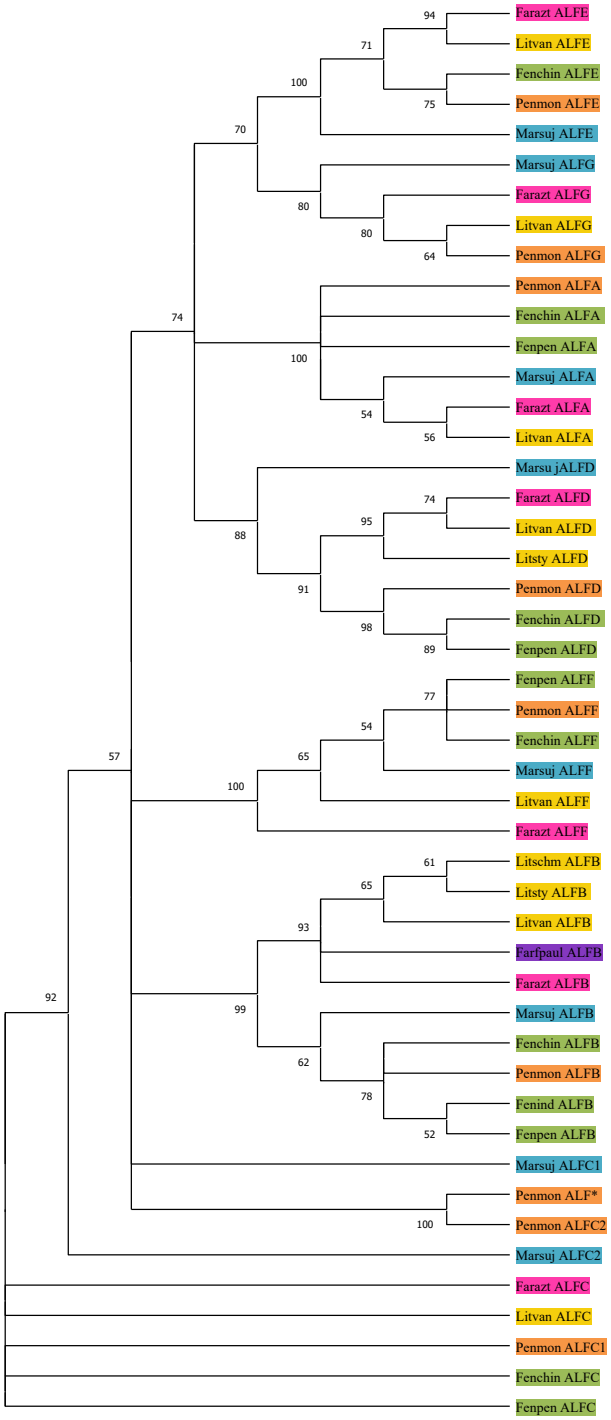

Figure 14. Unrooted phylogenetic tree of 47 Penaeidae ALF sequences using Maximum Likelihood method with 1000 bootstraps. Each genus was assigned a specific colour and unpublished laboratory sequences are indicated with the "*" symbol.

2.4 Discussion

2.4.1 Multiple Sequence Alignments Comparisons

As seen in all MSAs generated, the two cysteines enclosing the LBD have been highly conserved throughout the evolution of decapods. A variety of other amino acids have been conserved throughout the evolution of ALFs, and infraorders often have unique conserved residues compared to other infraorders, suggesting lineage-specific trends in ALF evolution. In terms of Penaeidae specifically, the MSA had one less conserved residue compared to the results of Matos et al. (2018), which reported a conserved lysine residue. In this study, this residue was replaced by an isoleucine in the sequences Marsuj_ALFD and Fenchin_ALFF.

Only one other study was found that incorporated an LBD sequence logo. Ren et al. (2012) created an MSA of the LBD of 31 penaeid shrimp, freshwater crayfish, caridean shrimp, crab, and *H. americanus* sequences. Their sequence logo also revealed that the two cysteines were the only fully conserved residues while other residues were relatively conserved (Ren et al., 2012). Similarly, the 7th residue strongly tended to be a P (Ren et al., 2012), which was consistent with the findings in this study. However, Ren et al. (2012) reported a strong tendency for a conserved G and W at positions 18 and 21 respectively, which was not observed in this study. This discrepancy could be due to the larger sample of sequences used here, which likely increased the variability in sequence similarity.

2.4.2 Patterns in Phylogenetic Trees

The tree with all sequences combined exhibited some patterns that were reported in previous studies. Based on recent mitogenomic research, the suborder Dendrobranchiata is

considered a sister group to the suborder Pleocyemata, and the infraorders Anomura and Brachyura are sister taxa (Tan et al., 2019). That Anomura and Brachyura are considered sister taxa was also apparent in this particular tree, as the comparatively fewer Anomura sequences tended to be intruders in Brachyura clans. A 2018 study found that penaeid shrimp ALFs are paralogous and evolved before the speciation of the Dendrobranchiata suborder, as ALF diversity extends to species of the suborder Pleocyemata, which includes the rest of the crustaceans in this study (Matos et al., 2018). This trend was also present in this study, as penaeid shrimp sequences did not form a perfect clan in the tree with all sequences. This study therefore corroborates the hypothesis that ALFs evolved before the speciation of the only two decapod suborders, and so it will be key to examine ALF phylogeny in studies that incorporate other species of the crustacean class Malacostraca.

However, the biggest overall pattern obtained in each of the trees of this study was that clans overwhelmingly tended to be heterogenous, often for more than one OTU category. It is interesting that very few homogenous clans were found, even within infraorder-specific trees. As the LBD MSA sequence logos have shown, even within the LBD region the amino acid residues are quite variable, so it is unlikely that generating ML trees based on the LBD exclusively would produce results that are that much more enlightening. Most clans were composed of species of various, often non-overlapping, habitat ranges, so the lack of taxonomical or environmental homogeneity makes it difficult to interpret these trees. Perhaps the reason why there are no clear-cut splits of homogenous OTUs is because many of the ALFs used in this study, even some of the published sequences, are actually non-functional, as many ALFs are simply predicted and their antimicrobial activity has not yet been tested or confirmed. In addition, most of the

sequences retrieved were obtained from unpublished transcriptome data. Careful interpretation of the results in this study is needed as ALF sequence databases are currently incomplete, some sequences may have had errors in their assembly, and there are always inter-individual differences in a species such as SNPs that may not have been caught. The number of ALFs retrieved per species was highly variable. The difficulty in interpreting a lack of ALFs in a specific species is that this could be due to missing data in public databases or a gene loss event, making experimental evidence of antimicrobial activity necessary for future studies. Nevertheless, one previously described explanation for the heterogenous trees that is generally suggested is that ALFs have gone through extensive gene duplication followed by neofunctionalization and/or subfunctionalization (Rosa et al., 2013; Matos et al., 2018), which is explained below.

2.4.3 Sequence Diversity Explanations

Sequence diversity of ALFs has been explained by different genome loci (Matos et al., 2018), alternative splicing (Tharndata et al., 2007), and single nucleotide polymorphisms (SNPs) (Li et al., 2013b). A 2018 study showed how penaeid shrimp ALFs are encoded by at least seven genes that came from duplication events followed by mutations in the form of nucleotide substitutions and insertion/deletions (Matos et al., 2018). In *P. monodon*, alignment of genomic sequences revealed that ALF transcripts were obtained through the alternative RNA splicing of pre-mRNA transcripts (Tharndata et al., 2007). Another study of ALFs of the mud crab *Scylla paramamosain* found that positive selection resulting from pathogen pressure likely created two SNPs in SpALF6, with the other called SpALF6-V, which has more potent anti-microbial activity compared to

SpALF6 (Hou et al., 2017). Therefore, there are many mutation types at play in the evolution of ALFs.

The apparent gene expansion of ALFs is likely due to positive selection pressure for gene duplication and mutation, as was mentioned by Matos et al. (2018). They suggested that penaeid shrimp ALF genes specifically originated in duplication events followed by nucleotide substitutions and insertion/deletions, which resulted in neo- and/or subfunctionalization and retention of ALF genes in penaeid shrimp (Matos et al., 2018). Duplicated genes, or paralogs, can go through three processes. Nonfunctionalization is where silencing or null mutation of a paralog occurs: deleterious mutations in a coding sequence of a gene cause a dysfunctional protein or the absence of one (Duarte et al., 2006; Karanth et al., 2009). Neofunctionalization is where novel functions are gained in a paralog: mutations in a coding sequence result in novel functions of the protein of one of the duplicates (Duarte et al., 2006; Karanth et al., 2009). Subfunctionalization is where functional modules are partitioned and the complement of both copies retains the functional capacity: in other words, the function of the ancestral gene is sub-divided between two sister duplicate genes (Duarte et al., 2006; Karanth et al., 2009). The tertiary ALF structure is similar across penaeid shrimp classes in spite of differences in primary structure and biochemical characteristics, but LPS binding residues in Penaeidae are not strongly conserved across the seven ALF classes, which has likely caused neofunctionalization (Matos et al., 2018). This makes sense because it is known that AMPs like ALFs are multifunctional peptides with several defense functions (Lai and Gallo, 2009). Also, the extreme sequence diversity of ALFs in general suggests that many of the duplicated ALF genes in decapods are unlikely to be partially or totally functionally redundant. Duplicated

genes can have different or overlapping effects, especially when they have differing expression patterns (Sun et al., 2021), a trend exhibited in ALF isoforms of the same species (Ren et al., 2012; Jiang et al., 2015; Srisapoome et al., 2018). It is important to note that to date, direct evidence of ALF gene duplication has not been proven. Chromosomal organization studies are used to determine if gene duplications, deletions, inversions, and translocations have occurred (eg. Yamamoto et al., 2014). Knowledge of the ancestral expression pattern before duplication is needed in order to identify specific shifts in expression due to sub- and neofunctionalization (Duarte et al., 2006). Therefore, discovering the chromosomal gene locations and distributions of ALFs will be crucial to detect the timing of duplication events.

2.4.4 Comparison to Other ALF Phylogeny Studies

Although rooted crustacean ALF phylogeny cannot be directly compared to this study, rooted trees also tend to recover clans heterogenous for infraorder, containing penaeid shrimp, freshwater crayfish, crabs, etc. (Ren et al., 2012; Wang et al., 2015; Matos et al., 2018). Gu et al. (2018) reported clear splits based on infraorder, specifically penaeids and brachyuran crabs, but the crabs were considered the outgroup. When looking at unrooted crustacean ALF trees, a common theme of trees that include decapods other than penaeid shrimp is that three groupings end up being formed: only shrimp (usually penaeid), all crustaceans, and only crabs (Afsal et al., 2012; Sruthy et al., 2015; Sruthy and Philip, 2021). However, other studies have not found these broad groupings and all infraorders or orders are scattered around the tree (Lai and Aboobaker, 2017; Lv et al., 2018). Most of the phylogeny trees in the ALF literature are Neighbor-Joining trees with 1000 bootstraps

done using the MEGA software (Afsal et al., 2012; Li et al., 2014; Sruthy et al., 2015; Lv et al., 2018). Even though a different phylogeny model was used in this experiment, the typical lack of homogeneity was obtained here as well.

In terms of penaeid shrimp specifically, a rooted tree showed that the seven ALF groups clustered into two clades, the first containing type A ALFs and the second containing all other groups (Matos et al., 2018). The second clade was further clustered into all cationic groups (B, C, and F), anionic groups (E and G), and the third branch comprised group D (Matos et al., 2018). In this study, most clans of the unrooted tree were heterogenous for species and all clans were homogenous for ALF class (A through G). When comparing this to the literature, Jiang et al. (2015) produced an unrooted penaeid shrimp ALF tree with only the A to E classes, and all classes were grouped as clans, but the clans are in different positions than our tree and the anionic and cationic classes are not clearly separate as they are in this study (Jiang et al., 2015). A similar study with an unrooted penaeid ALF tree using the classes A to G found that all classes were grouped as clans but that the class E clan clearly diverged from the other types (Srisapoome et al., 2018), while in our tree it is the class B clan that is clearly divergent. Similarly, the anionic and cationic sequences are not clearly separate in the study conducted by Srisapoome et al. (2018). The first paper built a neighbor-joining tree with an unspecified number of bootstrap replications (Jiang et al., 2015), while the second built an unspecified tree with 1000 bootstraps (Srisapoome et al., 2018), making it difficult to compare these trees in more detail. However, it is likely that different phylogeny models and parameters are what resulted in different outcomes.

2.4.5 In-Depth Unrooted Trees

Unrooted trees are not equivalent to rooted trees, which poses some difficulties when it comes to interpretation of results. A root implies a temporal relationship of the internal nodes in terms of the divergence of OTUs in time, something that is not possible to do with unrooted trees where all nodes are equivalent (Lapointe et al., 2010). In other words, the branch lengths in this study do not reflect the degree of relatedness of the OTUs. As it is ambiguous to say that adjacent groups are more closely related to each other than to other groups, characteristics of path-length distances could be used to determine the closest adjacent groups (Lapointe et al., 2010). One way to determine if groups of OTUs of a similar divergence have topological proximity was proposed to be the clip concept: a clip is a group of OTUs where all pairwise path-length distances are smaller than the given threshold value, s (Lapointe et al., 2010). In other words, clips define sets of OTUs that are exposed to similar selective pressures (Lapointe et al., 2010). In addition, several dispersion measures to examine OTU distribution in unrooted trees have been suggested, such as the Shannon diversity index and the Equitability index, which can be calculated with various mathematical formulas (Lapointe et al., 2010). These indices often have online calculators available and as none of these measures were applied in this study, it would be interesting to incorporate them to better understand the OTU divergence patterns.

2.4.6 Next Steps

Several future directions are necessary to further our understanding of ALFs. There are still many crustacean species that have not been studied in terms of ALFs. In Decapoda specifically, there are 11 infraorders composed of almost 15,000 extant species in total (De

Grave et al., 2009). Additionally, to date only one study has examined an ALF in a deep-sea crustacean (*Rimicaris sp*.), with other studies focusing on neritic and freshwater species (Gu et al., 2018). Exploring ALFs in less studied crustaceans would thus be beneficial. The tertiary structure and two conserved cysteine residues of ALFs are well-conserved in malacostracan crustaceans specifically, not just Decapoda (Lai and Aboobaker, 2017). In addition, more in-depth analysis of ALF phylogeny would be interesting, such as looking at motifs and gene location and distribution. Examples include chromosomal organization studies (Yamamoto et al., 2014), quantifying clan diversity (Schliep et al., 2010), testing for signatures of natural selection by comparing ratios of nonsynonymous and synonymous mutations (Nielsen, 2005), and taking into account biochemical properties of the sequences such as the theoretical isoelectric point. Also, additional studies about ALF antimicrobial functions are needed to confirm that the large number of predicted ALFs are indeed composed of functional peptides. Examples include gene-silencing technology like RNA interference to determine expression patterns (Matos et al., 2018), evaluating the consequences of amino acid changes outside of the LBD on antimicrobial activity (Hou et al., 2017), and examining how different isoforms respond to the same immune challenge (Smith and Dyrynda, 2015).

2.5 Conclusions

To conclude, the phylogeny of several ALF sequences of decapods was examined through a tree with all sequences combined and with trees based on individual infraorders. All trees had a substantial amount of heterogenous clans when examining many OTU categories, such as species, genus and family. Based on this study, the evolution of ALFs

was likely comprised of a series of successive gene duplications followed by the neofunctionalization of novel peptides. Indeed, positive selection in the form of gene duplication followed by nucleotide changes could have allowed genes to encode ALFs with novel, advantageous functions that were then selected for. Recommendations for future studies include building unrooted trees that quantify divergence and diversity, expanding on the range of species studied, increasing the depth of phylogeny analysis, and collecting evidence of antimicrobial activity in putative ALFs. The search for antimicrobial compounds is increasing as solutions are needed to control pathogenic and spoilage microorganisms (Olatunde et al., 2020), and ALFs are a promising avenue for research in this regard that need to be examined properly.

Chapter 3: Effect of *Profilicollis botulus* on Behaviour of *Carcinus maenas*

3.1 Introduction

Evidence that some parasites can manipulate their hosts is becoming more and more clear. Host manipulation in the form of physiological, morphological, and behavioural alterations has evolved in all of the main parasite clades studied so far and its adaptive significance has been established in some well-studied parasite species (Bhattarai et al., 2021). Adaptive host phenotypic alterations may make intermediate hosts more susceptible to definitive hosts by interfering with predator avoidance behaviors like refuge use, microhabitat choice and concealment (Kolluru et al., 2011). It is important to note that the specific criteria that define adaptive host manipulation are complex and still subject to debate (Poulin, 1995; Poulin and Maure, 2015; Bhattarai et al., 2021).

One parasite taxon that is known for intermediate host manipulation is Acanthocephala. This phylum is composed of parasitic helminth worms that must be trophically transmitted from an intermediate arthropod host to a final vertebrate host (Moore, 1984). Intermediate host manipulation is currently predicted to be an ancestral trait in this taxon (Fayard et al., 2020). In general, acanthocephalans induce low to moderate phenotypic trait changes that favour their own trophic transmission (Fayard et al., 2020). This is in terms of signed effect size estimates where meta-analytic r values of 0.1 and 0.3 were respectively interpreted as low and moderate effects (Fayard et al., 2020). The host trait subcategories that exhibit the largest alterations are taxis/phobia, responses to stimuli, vulnerability to predation, reproductive traits, and immunosuppression (Fayard et al., 2020). These worms seem to increase their transmission by modifying host microhabitat

choice and anti-predation behaviour, as well as increasing the activity of host energy-saving mechanisms (Fayard et al., 2020). Most acanthocephalan host manipulation studies have explored the crustacean intermediate host-parasite relationship with amphipods, in particular the gammarid *Gammarus pulex* (Bethel and Holmes, 1973; Maynard et al., 1996; Médoc et al., 2006; Tain et al., 2006; Kaldonski et al., 2007; Cornet et al., 2009; Rauque and Semenas, 2009). However, there are also studies that have looked at host manipulation in various shore crab species, including: *Macrophthalmus hirtipes*, *Hemigrapsus edwardsi*, *H. crenulatus*, and *Emerita analoga* (Haye and Ojeda, 1998; Latham and Poulin, 2001, 2002a, 2002b; Rojas and Ojeda, 2005; Kolluru et al., 2011). The behaviour these studies have focused on is the burrowing behaviour of these crabs, and it has been found that acanthocephalan infection tends to cause animals to burrow more slowly. However, no other crab behaviour category has been studied so far. Therefore, there is a large imbalance when it comes to the acanthocephalan intermediate crustacean host species studied, with much less information known about how these worms affect decapod hosts such as brachyuran crabs. In addition, there is a lack of information about how other shore crab behaviours are affected by infection.

The *Profilicollis* genus belongs to the acanthocephalan class Palaeacanthocephala. Birds are the definitive hosts of *Profilicollis* species, specifically diving ducks and gulls, while crustaceans are the intermediate hosts (Kolluru et al., 2011; McDermott, 2011). One of these species, *P. botulus*, has a lifecycle that has been described in detail with the green crab (*Carcinus maenas*) as the intermediate host and the common eider duck (*Somateria mollissima*) as the putative main definitive host. Adult *P. botulus* are found in the intestines of eider ducks, where they reproduce and are estimated to live two to three months

(Thompson, 1985b). Heavy infection levels are often reported from apparently healthy eiders (Liat and Pike, 1980; Thompson, 1985b; Skirnisson, 2015). Their eggs, immediately infective to intermediate hosts, pass in the feces individually or through expelled gravid worms into the external environment and are ingested by green crabs (Thompson, 1985b; Skirnisson, 2015). Once worms have developed into the cystacanth stage, they are able to infect their final vertebrate hosts (Rojas and Ojeda, 2005; Tain et al., 2006). Cystacanths are long-lived and are thought to remain infective over the course of the green crab's life (Thompson, 1985a). Although the ecology of the *P. botulus*-green crab-eider duck relationship is well-known, there is a lack of knowledge about the parasite's effects on its intermediate host.

The green crab easily invades new areas because it tolerates a wide range of water temperatures and salinities (McNiven et al., 2013), is a rapid learner (Orlosk et al., 2011), and is able to live in various marine habitats (McNiven et al., 2013). This animal has been present on the North American East Coast for over two centuries and is a threat to native fauna because it can introduce novel pathogens and/or become a reservoir for native pathogens (Blakeslee et al., 2009; Bojko et al., 2018). There is concern about parasite spillover from the green crab to the economically important American lobster, *Homarus americanus* (Behringer and Duermit-Moreau, 2020). It is thus important to obtain knowledge of green crab pathogens in order to determine potential impacts to native crustacean species. Therefore, the purpose of this study is to examine the effect of *P. botulus* infection on various behaviours of the green crab.

Manipulation of the intermediate host can phenotypically make it more vulnerable to predation by the definitive host, such as by increasing the intermediate host's

conspicuousness or by changing its behaviour to make it more easily captured (Fenton and Rands, 2006). In order to determine increased vulnerability to definitive host predation, it would be reasonable to observe more visible parasitized hosts that spend more time without cover, more time on contrasting substrate cover compared to body colour and more time in motion (Poulin, 1995). Therefore, the experiments in this study were designed to measure whether parasitized crabs are more vulnerable to predation due to behavioural alterations. The objective was to determine if infected crabs behave significantly differently than uninfected ones.

3.2 Methodology

3.2.1 Subjects

Green crabs were captured in East Pubnico, Nova Scotia using mackerel (*Scomber scombrus*)-baited traps under a Section 52 scientific license from the Department of Fisheries and Oceans. They were then transported to the Aquaculture Centre of Dalhousie University Faculty of Agriculture. Two separate batches of crabs were obtained on October 29th, 2020 (n = 26) and November 14th, 2020 (n = 11). Both batches were allowed to acclimate to the aquaculture center for a few weeks before testing. Animals were fed biweekly on a diet of mackerel pieces and were fasted on the weeks of the trials. The crabs were housed individually in trays in a recirculating system with cold saltwater (roughly 32 ppt and a temperature of 5-6^{o}C). A small sticker with a unique number was glued to the carapace of each crab for identification. Several biological characteristics of the animals were noted initially: batch number (1 or 2), sex (male or female), colour morph (red or green) and carapace width (mm). It is important to note that the infection status of

the crabs can only be confirmed through necropsy, and so following all experimental trials the crabs were euthanized. Therefore, the following response variables were noted after necropsies were performed: worm presence (yes or no) and worm intensity (number of worms).

3.2.2 Determining Test Lengths

In order to determine the average length of the tests, the activity of green crabs was plotted against time in Minitab 18®. This was done to ensure that the tests were not too short and lasted long enough until there was a clear decrease in crab activity. To accomplish this, the behaviour tracking software (described below) ToxTrac version 2.91, lastly updated on September 15th, 2020 (Rodriguez et al., 2017, 2018) was used to calculate the distance (mm) traveled by individual crabs per one-minute intervals. Five crabs of various sizes, both sexes and colour morphs were put into one of the experimental tanks (either a half black and half white tank or a white tank) and these were filmed from overhead for about an hour each. As seen in Fig. 15, the average decrease in crab activity (average of the third time point where distance travelled was at or below 10 mm) occurred around 30 to 40 min, and so all tests were 35 min in length.

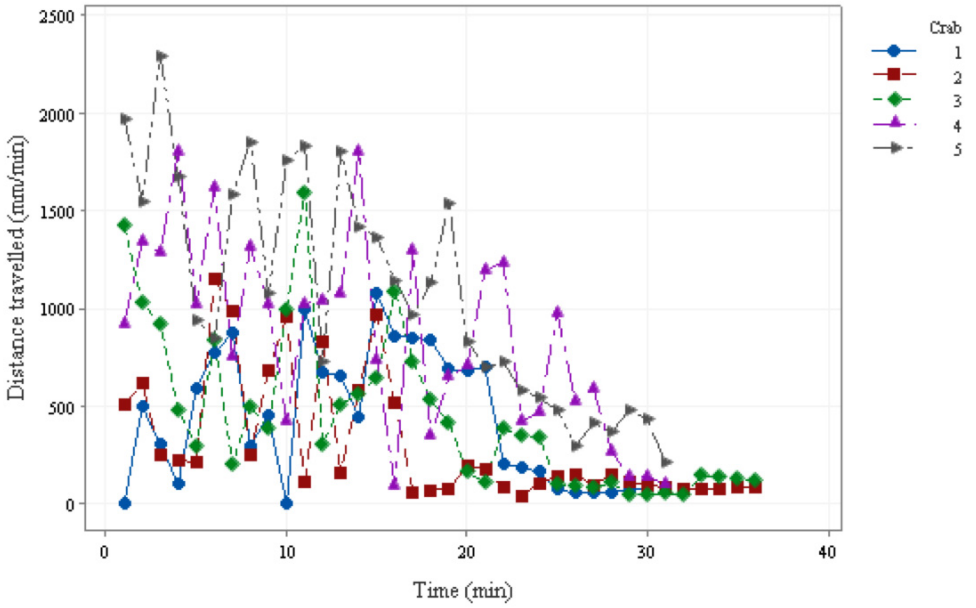

Figure 15. Scatterplot of five green crab individuals' distance traveled (mm/min) in one-minute increments for around 35 min.

3.2.3 ToxTrac Organism Tracking Software

Five behaviour response variables for two behaviour tests (the mirror approach and open field tests) were obtained via ToxTrac. This is a free open-source organism tracking software that detects and tracks animals in rectangular arenas of an image (Rodriguez et al., 2017). For the software to work optimally, the arena must be uniformly brightly lit with high contrast (Rodriguez et al., 2017). Static objects can be removed with a background subtraction technique, while moving objects, reflections, and shadows interfere with the tracking of the animals (Rodriguez et al., 2017).

The ToxTrac response variables chosen were the following: mobility rate, the rate of instant speed above a preset value; exploration rate, the number of arena areas divided by the number of explored areas; number of frozen events, the count of the frozen events; total time frozen, the total time the animal is in the frozen state; and time spent in a specific

zone of the arena, which is the time spent inside the zone divided by the total duration of the video. For this last variable, a rectangular "zone of interest" was manually selected, and the software automatically calculated how long the crab stayed inside this zone. It was decided to focus more on "rate" variables due to the slight changes between video angles/zone sizes that could affect the variability of the results.

Instantaneous speed is automatically calculated in mm/s (Rodriguez et al., 2017). In this experiment, the default sampling distance of two frames was used. For exploration, the arena is automatically divided in non-overlapping squares, and the use of each square zone is calculated by the software. In this experiment, the software divided the experimental tanks into 50 squares. A frozen event is a moment when the animal is still for a certain amount of time. In this experiment, the defaults were used: a frozen event was detected when the crab moved less than 5 mm in 3 s.

To determine the reliability of measures given by the software, correlation tests were performed on various responses obtained from two outputs of the same crab video. The Pearson correlations calculated with Minitab 18® were all above 0.90 with many close to 1 for several measures. Two quality control measures were implemented as well to increase the reliability of the results: one of the software outputs is "visibility", which is the number of visible video frames divided by the sum of the visible and invisible frames. If this percentage was lower than 98%, the video was run through ToxTrac again until a result above 98% was achieved. The only setting changed was the animal detection parameter "threshold", which selects the minimum intensity level value (1 to 255) for the detection of objects (Rodriguez et al., 2017). In addition, another output is a video of the software's tracking path of the crab. For each crab, the first minute of tracking was viewed

to determine if the algorithm correctly identified the crab - i.e. if the object being tracked was something other than the crab (such as a water ripple) or there was no object being tracked, the video was run through ToxTrac again (with the only change in settings being the detection threshold parameter) until the crab was correctly identified during that first minute.

3.2.4 Experimental Set-Up

The experimental tanks were placed on a table in the holding system room (Fig. 16). Four Logitech® cameras (model HD Webcam C525) were used to record the tanks from a position one meter overhead of the tanks using the software OBS Studio Windows version 26.1.1 by Open Broadcaster Software® (Open Broadcaster Software, 2021). Three behaviour tests were performed on each crab: the open field test, mirror approach test, and background preference test. The same crab transport method was used for each test. Right before recording would start, crabs were removed from their respective tray positions in tank A and put into a plastic container to transport them to the table. Then, each crab was initially placed at the center of its respective arena and the recording started. Once the video recording was done, crabs were placed back into the plastic container to transport them to the "recovery" tray in tank B to avoid chemical communication between crabs that had been tested and those that had yet to be tested. All experimental tanks were rinsed with cold saltwater between each tested crab to avoid chemical communication between crabs. When all recordings for the day were done, all crabs were transferred from the tank B tray into their original positions in the tank A tray.

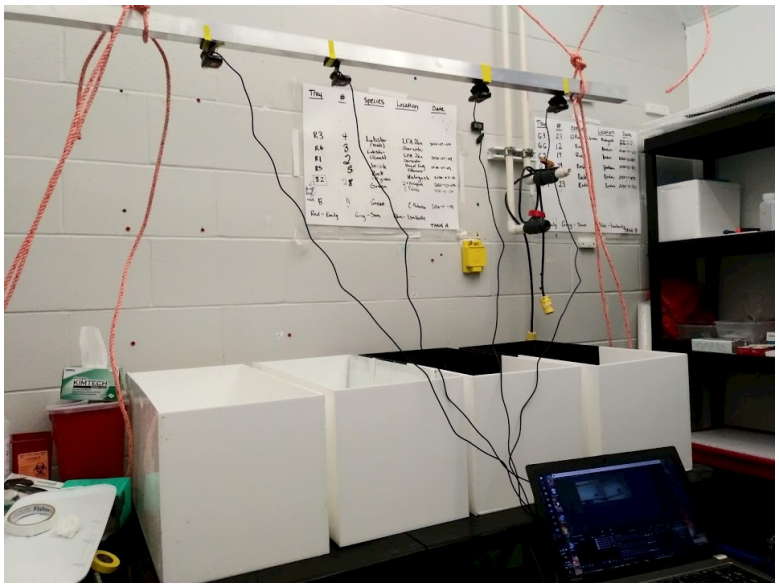
Figure 16. Picture of experimental set-up viewed from the side.

For the open field test, the experimental arena was a white acrylic tank with the dimensions of 60 cm x 30 cm x 30 cm (l x w x h) filled to a ~4 cm depth of ~4°C saltwater (Fig. 17a). ToxTrac computed the mobility rate, exploration rate, number of frozen events, total time frozen, and total % of time spent in the "center zone". The "outer zone" is defined as the outer area of all sides of the arena, for which there is a width of at least one body length. As the maximum carapace width of the crabs in this sample was roughly 6 cm, the outer zone was 6 cm in width. Since the area of the arena was 60 cm x 30 cm, the center zone of the tank measured 18 cm x 48 cm and was manually selected in ToxTrac as a zone of interest.

For the mirror approach test, the experimental arena was the same tank as the open field test with a removable lengthwise mirror panel filled to a ~4 cm depth of 4°C saltwater (Fig. 17b). ToxTrac computed the mobility rate, exploration rate, number of frozen events, total time frozen, and total % of time spent in the "mirror zone". The mirror zone is defined

as the area of one body length directly against the side of the mirror panel. As the maximum carapace width of the crabs in this sample was roughly 6 cm, this zone was 6 cm in width and was manually selected in ToxTrac as a zone of interest.

For the background preference test, the experimental arena was a half white and half black acrylic tank with 60 cmx 30 cm x 30 cm (l x w x h) filled to a ~4 cm depth of 4ºC salt water (Fig. 17c). The following response variables were manually computed: the percentage of time spent on the black side of the tank, and the number of times the crab moved to the white side of the tank, or the number of events to the white side. These were calculated by hand since as mentioned above, ToxTrac is quite sensitive to lighting and the crabs were not being tracked when they were in the black side of the tank.

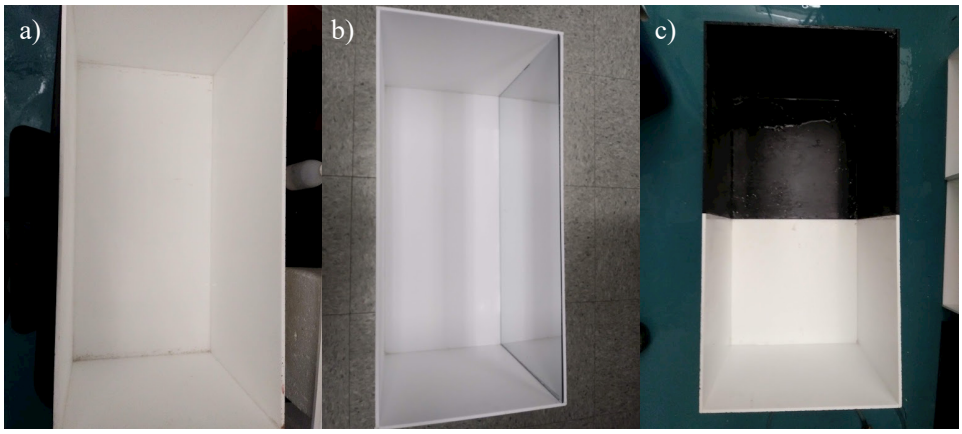

Figure 17. Overhead view of the experimental tanks used for: a) open field test, b) mirror approach test, and c) background preference test.

3.2.5 Statistical Procedures

Several measures were implemented to ensure randomization. Each individual crab performed each test, and so all animals were randomly assigned an order of the three tests to avoid order effects. Each crab performed one test per day of testing, always with two to

three days in between testing days to allow for stress recovery. Each batch was tested individually, with batch 1 crabs being tested on November 18th, 22nd, and 25th (2020) and batch 2 crabs being tested on November 30th, December 2nd, and December 4th (2020). For the background preference test, the orientation of the black side of the tank (front or back of the table) was noted and alternated between crabs. Similarly, for the mirror approach test the mirror orientation (left or right side of the tank) was noted and alternated between crabs.

To simplify the statistical analysis, three factors were combined into eight possible treatments: green crab colour morph (red or green), sex (female or male), and worm presence (yes or no). The batch (1 or 2) was considered a blocking factor. Two types of statistical tests were performed which looked at the significance of treatment and alpha was set at 0.05. Both tests have the same assumptions: normality and constant variance, and this was checked and confirmed for each response variable in Minitab 18® using the following methods. Normality was assessed using the Anderson-Darling test (Anderson and Darling, 1952) and constant variance was assessed by plotting residuals against fits to obtain a scatterplot. If there was a horizontal band impression, the variance was considered constant. The time spent in center zone response for the open field test had to undergo a square root transformation to achieve normality and constant variance. All numerical variables were checked for significant outliers via the Grubbs's test (Grubbs, 1950) in Minitab 18®. For the first test type, since the mirror and open field tests shared four response variables, a mixed model with repeated measures was done in SAS ® version 15.1 for Windows (SAS Institute Inc., 2020). The batch was considered a blocking factor and the day of testing ("1" for open field test and "2" for mirror approach test were arbitrarily

chosen) was included as a fixed effect to see if the testing day had an impact on the results. The interaction between the treatment and testing day was also included in the model. The second test type was performed for all other responses: A randomized incomplete blocking design with batch as the block (ANOVA) was done using Minitab 18®. This design was chosen as there was an uneven number of replicates per treatment. Additionally, a power analysis test was done to determine the minimum sample size of parasitized and non-parasitized animals to detect significant differences between the two groups. The procedure was performed on a few of the largest effect sizes (partial eta squared) of response variables using an online calculator (Brant, 2021) for comparing the means of two independent samples with the default desired power of 80%. A Spearman correlation was also performed in Minitab 18® to determine if crab carapace size affected infection intensity.

3.3 Results

The repeated measures design for the four common responses of the mirror approach and open field tests (mobility rate, exploration rate, number of frozen events and total time spent frozen) revealed that the day, treatment, and day*treatment effects were all non-significant (Table 1). Similarly, the zone response variables of those two behaviour tests (time spent in mirror approach zone and time spent in center zone) showed that the treatment effect was non-significant (Table 2). Additionally, the two background preference test variables (time spent in the black side and number of events going to the white side) also had non-significant treatment effects (Table 3).

Table 1. Results of mixed model with repeated measures for four response variables common to the mirror approach and open field tests (α = 0.05) of *Carcinus maenas* (n = 37).

Response	Source	Numerator DF	Denominator DF	F-value	P-value
Mobility rate (%)	Batch	1	56	3.63	0.0620
	Day	1	56	1.11	0.2963
	Treatment	7	56	0.03	1.0000
	Day*treatment	7	56	0.25	0.9698
Exploration rate (%)	Batch	1	55	0.42	0.5179
	Day	1	55	0.02	0.8755
	Treatment	7	55	1.70	0.1277
	Day*treatment	7	55	1.26	0.2876
Number of frozen events	Batch	1	56	3.63	0.0620
	Day	1	56	1.11	0.2963
	Treatment	7	56	0.03	1.0000
	Day*treatment	7	56	0.25	0.9698
Total time frozen (min)	Batch	1	55	0.42	0.5179
	Day	1	55	0.02	0.8755
	Treatment	7	55	1.70	0.1277
	Day*treatment	7	55	1.26	0.2876

Table 2. Results of randomized incomplete block design ANOVA for time spent in zones (%) for mirror approach test (mirror zone) and open field test (center zone) (α = 0.05) of *Carcinus maenas* (n = 37).

Response	Source	DF	SS	MS	F-value	P-value
Time in mirror zone (%)	Batch	1	60.56	60.56	0.20	0.655
	Treatment	7	971.43	138.78	0.47	0.849
	Error	28	8302.94	296.53		
Time in open center zone (%) (λ = 0.5)	Batch	1	0.8087	0.8087	0.32	0.578
	Treatment	7	13.4048	1.9150	0.75	0.633
	Error	27	68.9697	2.5544		

Table 3. Results of randomized incomplete block design ANOVA for background preference test response variables (α = 0.05) of *Carcinus maenas* (n = 37).

Response	Source	DF	SS	MS	F-value	P-value
Time in black side (%)	Batch	1	1.59	1.586	0.01	0.927
	Treatment	7	1302.04	186.097	1.00	0.449
	Error	28	5182.71	185.097		
Number of white side events	Batch	1	39.83	39.83	0.63	0.433
	Treatment	7	193.64	27.66	0.44	0.868
	Error	28	1759.88	62.85		

Regarding the sample size, the rule of thumb for these tests is that the error degrees of freedom (DF), or denominator DF, must be greater than or equal to five in order to detect significant differences; all error DF in the tests above are over five, signifying that the sample size was not too small to violate the test. In addition, power analysis of several large effect sizes assuming 80% power revealed that the minimum sample size to detect significant differences between parasitized and non-parasitized crabs was 16. As there were 20 infected crabs and 17 uninfected crabs in this study, this condition was also satisfied.

In addition, carapace width had a non-significant correlation to infection intensity ($r = -0.033$; P-value = 0.891).

No significant differences were detected between infected and non-infected crabs at the five percent level for all response variables measured. In this experiment, infected green crabs sampled from an East Pubnico population did not behave in any significantly different manner compared to non-infected crabs from this population.

3.4 Discussion

3.4.1 Comparisons of P. botulus Infection Intensity and Prevalence

A thorough study about green crab symbionts found that out of 15 UK sites, only one (Norfolk) had *P. botulus* present, which was prevalent in 3.3% of the population (Bojko et al., 2018). The five Faroe Islands sites all had the presence of the worm, with average prevalence being 0.5 ± 8.4% (Bojko et al., 2018). The parasite was only detected in 2 out of 7 Nova Scotia sites, Pubnico and Riverport, with the average prevalence of 2.0 ± 4.4%, while Pubnico alone had a prevalence of 11.7% (Bojko et al., 2018). In this study, infection prevalence was 54.1% in the 37 crabs sampled. Bojko et al (2018) reported that across

several green crab populations, *P. botulus* infection was more commonly associated with male green crabs. This could not be verified in this study due to the small number of females in our sample.

3.4.2 Exploring the Lack of Colour Morph, Carapace Width and Sex Effects

Several crab traits did not affect behaviour in these experiments. The general colour morph, red or green, had no effect and this is interesting as it has already been shown that red morphs have a different physiology compared to green morphs (Lee et al., 2005); thus, we can tentatively say that the cystacanths did not affect this facet of the species' physiological ecology. However, there is a large spectrum of colours in green crabs both on the carapace and the abdomen, and more specific categories have been suggested by others (Lee et al., 2005; Stevens et al., 2014). There are even more detailed measurements of colour that could have been done, such as looking for individual differences in brightness or hue. Green crabs have been proven to change their camouflage by adjusting their brightness over a period of hours to influence the detection probability by their predators (Stevens et al., 2014). It would be interesting to compare the more detailed colour categories and aspects in terms of worm effect.

The crab carapace width also had no effect on the behaviours measured in this study and did not affect infection status as well. This is contrary to an older study that reported that *P. botulus* cystacanth counts increased with green crab carapace width, with the largest size class of 60-64 mm being infected with a mean of three cystacanths per individual (Thompson, 1985a). Similarly, a study in Scotland reported that depending on size class, there were significant differences in green crab *P. botulus* infection rates and intensity (Liat

and Pike, 1980). The size classes used were small (0.5-2.0 cm carapace width), medium (2.1-4.0 cm) and large (4.7-7.0 cm) (Liat and Pike, 1980). Overall, the infection rate and intensity increased with size without significant sex differences or temporal differences (Liat and Pike, 1980). This trend is also observed in other crab species such as the mud crab *M. hirtipes*, which has relatively high numbers of *Profilicollis* species cystacanths that are positively correlated with carapace width (Latham and Poulin, 2001). However, the green crab size range in this study was relatively limited, with a range of approximately 3-6 cm, which could explain the lack of size effect on infection.

No sex differences were observed, despite previous studies having avoided using female crabs in acanthocephalan studies to avoid behaviour differences due to sex (Latham and Poulin, 2001). Conversely, a sex difference in host behavioural manipulation has been observed in another crab species. A study done on the sand crab *E. analoga* infected with *Profilicollis altmani* found that only females, especially heavily infected ones, had significantly slower sand burrowing times compared to uninfected females (Kolluru et al., 2011). This trend was not observed in males, but this could be due to the small male to female ratio in their sample.

3.4.3 Background on Behaviour Trials Used

The open field test has been performed in crustacean studies that have based their measurements on rodent and zebrafish studies (Mesquita et al., 2011; Hamilton et al., 2016; Blewett et al., 2017). This test has been used in previous green crab studies to examine locomotion and thigmotaxis, which is considered a proxy for anxiety-like behaviour (Hamilton et al., 2016; Blewett et al., 2017). Locomotion is a behaviour required for many

complex behaviours, such as predator avoidance, and so impaired locomotion caused by *P. botulus* could potentially affect these behaviours in the green crab (Mesquita et al., 2011). Alteration of locomotion could have severe energy costs in terms of muscular activity or may cause shifts in energy metabolism (Guler and Ford, 2010), hence the importance of examining this behaviour category.

Some studies have used mirror approach tests in crustaceans, commonly crayfish, as a proxy for measuring boldness or intraspecific aggression (Drozdz et al., 2006; May and Mercier, 2006, 2007; Hamilton et al., 2016). Green crabs have compound eyes that can detect motion in their environment (Horseman et al., 2011) and it was hypothesized that they would be able to respond to their reflection in a mirror. If *P. botulus* infection increases the likelihood of agonistic interactions, since a pair of crabs fighting is more visible to avian predators this could make them more vulnerable to predation (Latham and Poulin, 2001).

Light/dark preference testing is based on rodent paradigms and is convenient for large-scale screening in behavioural studies (Blaser and Peñalosa, 2011). Green crabs have a preference for dark shelters compared to brightly lit areas, as in the wild they tend to hide under rocks at low tide and during daylight in order to avoid their predators (Barr and Elwood, 2011; Orlosk et al., 2011). It has been hypothesized that individuals of this species therefore have a strong motivation to avoid light (Barr and Elwood, 2011). This is suggestive of predator avoidance behaviour since avian predation relies on vision, so foraging on green crabs takes place during the daytime (Orlosk et al., 2011). In theory, if infected crabs displayed a reversed background preference, this would make them more

vulnerable to predation. This type of background preference testing has been done on the green crab before, but in a toxicologic context (Hamilton et al., 2016).

3.4.4 The Behaviour Tests in Other Crustacean Studies

Open field tests were not significantly different between infected and uninfected crabs which could have been due to the tank size as mentioned above. Only a few toxicology studies have performed open field tests on green crabs. A study on the effect of cadmium exposure performed an open field test in a 69 cm diameter circular experimental tank and found no significant differences between control and exposed crabs (Blewett et al., 2017). Another study on the effect of fluoxetine used a 60 cm diameter circular experimental tank to perform the open field test and found a significant relationship with fluoxetine concentration and time spent moving as well as number of arena segments crossed (Mesquita et al., 2011). As both studies employed circular tanks for their open field trials, it would be interesting to see if a circular tank enabled us to detect any differences in terms of green crab acanthocephalan infection status.

Mirror approach was not significantly different in parasitized green crabs. Only one other crab study that used the same test was found. A study on exposure to fluoxetine in the striped shore crab *Pachygrapsus crassipes* that used the same mirror response test in a tank of nearly identical size to our study also reported no significant difference in time spent in the mirror approach zone compared to control animals (Hamilton et al., 2016). With their compound eyes, crabs have poor spatial acuity and colour vision but can detect ultraviolet light and are sensitive to the plane of polarization of light (Horseman et al., 2011). Crab eyes are able to detect form and movement (Horseman et al., 2011), so it is reasonable to hypothesize that the crabs in this study could detect their reflection in the

mirror. Even if mirror use has a long history of being used to study aggression or agonistic behaviours in animals, behavioural responses are not always correlated to responses to live conspecifics and may not be associated with the same physiological responses (Li et al., 2018), which could have occurred in this case. Agonistic behaviour between pairs of male green crabs has already been described in detail (see Sneddon et al., 1997). Male green crabs will display abdomen to abdomen agonistic behaviours, specifically when fighting for access to receptive females, such as displaying claws to the opponent, pinching with the claws and more (Sneddon et al., 1997). The fact that fights quickly get intense at the beginning suggests that green crabs do not visually assess their opponent and must use physical contact to gauge their opponent (Sneddon et al., 1997), which could explain the lack of agonistic behaviours observed. However, associations between acanthocephalan infection and crab agonistic behaviours have been studied in another host-parasite relationship: The likelihood of a male *M. hirtipes* winning a ritualized fight against a size-matched conspecific in the field was related to *Profilicollis* species infection level, but this was predicted to only be a pathological consequence rather than adaptive manipulation (Latham and Poulin, 2001). Indeed, a pair of crabs fighting is more visible to avian predators compared to single resting crabs but the parasite affecting the outcome of the fight is unlikely to benefit the parasite if it simply wants to attract birds; thus, it was proposed that *Profilicollis* infection may simply deplete the energy reserve of male *M. hirtipes* (Latham and Poulin, 2001).

A few studies have examined how crabs behave in background preference and shelter use tests. In a study with a dark tank divided into a dark side with no lighting and a shelter and a light side with a bright light, control green crabs moved to the dark shelter

and avoided the bright light (Barr and Elwood, 2011). Another older study reported that in a laboratory setting, green crabs were very photonegative during daytime when given microhabitat choices comprised of a green-coloured tank filled with a 3 cm layer of sand and dark green plastic shelters (McPhee and Wilkens, 1989). The control animals were hiding under rocks or buried in the sand 76% of the time in that study (McPhee and Wilkens, 1989). Additionally, a study on exposure to fluoxetine in *P. crassipes* used the same black/white preference tank and found that 25 mg/L fluoxetine significantly reduced time in the dark zone compared to control crabs, which they suggested indicated reduced "anxiety" (Hamilton et al., 2016). It is interesting that in our study, the proportion of time spent in the dark side of the tank was only around 60% in both parasitized and non-parasitized crabs. There are many other factors that could be implemented in future trials to elucidate if this lack of difference persists across different contexts, such as tank substrate, illumination, or colour.

3.4.5 Possible Reasons for Not Observing Significant Differences

The captive environment is inevitably different from the actual environment of the crabs. Captivity definitely disrupted their biological rhythms since the green crab has both circadian and circatidal rhythms, with activity peaks during hours of darkness and high tides (Naylor, 1958; Reid and Naylor, 1989; Rewitz et al., 2004). In daylight hours, green crabs exhibit a strong negative phototaxis and spend most of their time hidden, either under rocks or buried in sand (McPhee and Wilkens, 1989). In contrast, the housing of the crabs in this study was always dark unless it was time to feed the animals, which was only done biweekly. With the additional absence of tides, these artificial housing conditions may have

caused the crabs to become stressed, which in theory should have led to the usual display of strong behavioural variability under stressful conditions (Parsons, 1988). It may have been the case that the tests used here simply had little to no relevance to contexts that occur in the wild. Perhaps the experimental tanks were too restrictive to measure biologically relevant changes in locomotion, as adult green crabs generally undergo daily migrations, moving inshore and offshore with the tides to forage in the intertidal zone during high tide and retreating to the subtidal zone at low tide (Klassen and Locke, 2007; Waser et al., 2018). This is especially relevant to our sampled population, which was captured in an estuary and therefore tidal migration was vital in obtaining these individuals. The highly restrictive nature of their captive environment could have impeded the detection of behavioural differences. However, another study done on a shore crab in similarly sized tanks was capable of detecting behavioural changes, albeit in the context of chemical exposure and not parasite infection (Hamilton et al., 2016). In addition, no study thus far on crab behaviour has used ToxTrac. Crustacean studies use a diversity of organism tracking software, such as Ethovision XT (Bossus et al., 2014; Hamilton et al., 2016; Blewett et al., 2017), EthoWatcher (Fürtbauer, 2015), idTracker (Fürtbauer and Fry, 2018), or Image J (Mesquita et al., 2011). This complicates direct comparisons of locomotion behaviours, and it is possible that ToxTrac had systematic errors when it came to tracking green crabs that obscured the true locomotion of the animals. This is unlikely however as the authors of the software have reported an average detection rate of 99.2% using various animal species and the detection algorithm does not require a specific animal shape to work (Rodriguez et al., 2018).

Another complicating factor for comparisons to previous studies is the test time used. Overall, relevant crab studies seem to use much shorter test times for all three tests we performed. For example, the study on *P. crassipes* used similar background preference and mirror approach tanks but both trials were 5 min in duration, justified by being based on common anxiety testing paradigms in zebrafish and rats (Hamilton et al., 2016). Another previous study on the green crab also performed the open field test, and the test was 2 min long justified by the fact that 2 to 5 min for an open field test is generally considered an acceptable measure of exploratory locomotion in a new environment for mice (Mesquita et al., 2011). Some of these studies could detect significant differences in behaviour in a toxicology context for that short amount of time, so perhaps shortening the trial time of our study could have had an impact on our results. It would be worth investigating if significant differences occurred during the first 5 min of our trials, which ToxTrac could easily perform. The large differences in testing times between the literature and our experiment makes direct comparisons difficult, but in this study the trial time was based on the time that crabs were active in the experimental tanks and so was more biologically relevant for the species.

Different intermediate host species can be differentially affected by acanthocephalan manipulation. This has been shown in other crab species: A previous study looked at the effect of *Profilicollis* species as a whole on the low tide hiding behaviour of the crabs *M. hirtipes* and *H. crenulatus* in New Zealand (Latham and Poulin, 2002a). These crab species are in different families but are still closely related; even so, the two species seem to have different susceptibility to cystacanth manipulation (Poulin et al., 2003). *M. hirtipes* individuals that were exposed above the sand had significantly higher

mean infection levels compared to hidden individuals, while this observation was not reported in *H. crenulatus*, for which the mean cystacanth numbers were significantly lower compared to *M. hirtipes* (Latham and Poulin, 2002a). Crabs exposed at low tide are suspected to be more vulnerable to the bird definitive hosts (Latham and Poulin, 2002a). In addition, carapace width did not affect infection level in *M. hirtipes*, but there was a significant correlation between infection level and carapace width in *H. crenulatus* (Latham and Poulin, 2002a). This is evidence that *Profilicollis* species cystacanths may affect their various intermediate hosts differentially, as *M. hirtipes* is ubiquitous at the study site while the *H. crenulatus* population is less dense (Latham and Poulin, 2002a). If *M. hirtipes* is the competitively dominant crab and is preferentially manipulated by *Profilicollis* cystacanths over the other intermediate species present, this process could be contributing to the diversity of this ecosystem by affecting crab population dynamics (Latham and Poulin, 2002a).

The green crab is clearly not the only *P. botulus* host. Intermediate hosts of *P. botulus* include the brachyurans *H. oregonensis*, *H. sanguineus*, *C. maenas*, *Hyas araneus*, and *Cancer irroratus*, the American lobster, as well as the anomurans *Paralithodes camtschaticus* and *Pagurus pubescens* (Brattey and Campbell, 1986; Christiansen et al., 2009; McDermott et al., 2010; McDermott, 2011; Bojko et al., 2018). Prevalence of the worm varies by species and location. For example, the prevalence of *P. botulus* cystacanths in *Hemigrapsus oregonensis* was 9% of 692 crabs at two Vancouver (British Columbia) locations in 1976, and a prevalence of 62% of 42 crabs in another Vancouver site in 1987 (Ching, 1989). Studies performed in the Barents Sea reported an almost 100% presence of *P. botulus* cystacanths in the crab *H. Araneus*, while a prevalence of 12% was found in the

hermit crab *P. pubescens* (Uspenskaya, 1960; Ching, 1989). Whether all of these species are true intermediate hosts or paratenic hosts is unknown at this stage, but with the number of possible intermediate hosts for *P. botulus*, it would make sense that its cystacanths differentially affect different host species. There is some evidence that parasites who use more than one intermediate host species show a regional variation in host preference (Westram et al., 2011). If a parasite has the ability to infect multiple host species, differences in infection success between hosts are commonly present, which may occur when the parasite gains a higher fitness advantage through local adaptation to the most common host in a region (Westram et al., 2011). Additionally, differences in infection rates could also be caused by differences in parasite encounter rates due to microhabitat choice (Westram et al., 2011). It would be interesting to see if evidence of adaptive host manipulation could be found in the other *P. botulus* intermediate host species.

In this experiment, the acanthellae of *P. botulus*, the parasite's developmental stage preceding the cystacanth, were not accounted for. Larval acanthocephalans in the acanthella stage can induce the opposite host alterations in roughly the same magnitude as the cystacanth stage alterations (Fayard et al., 2020). For example, *G. pulex* infected with *Pomphorhynchus laevis* have stronger anti-predator behaviour when the worm is at the acanthella stage compared to when the mature cystacanth stage is reached, as at this point the anti-predator behaviour decreased (Dianne et al., 2012). The concept of the switcher-paradigm has been suggested as the answer to why manipulative parasites that enhance the predation of their intermediate hosts do not lead to instability in bottom-heavy predator-prey ratios: some parasites engage in predation suppression sequentially followed by enhancement, which could increase the persistence of the parasite and oppose the effects

of intense enhancement, meaning that bottom-heavy ratios would persist (Iritani and Sato, 2018). Evidence of the switcher-paradigm in acanthocephalans exists, though this possibility is tentative. Host predation suppression in acanthocephalans should be carefully interpreted as only a small number of studies have examined the effect of these worms at the acanthella stage on their hosts (Fayard et al., 2020). Detailed analysis of predation suppression succeeded by predation enhancement has not been performed due to the lack of available data, but at this point it is tentatively predicted that the acanthella stage can induce changes that decrease intermediate host vulnerability to predation (Fayard et al., 2020). In terms of this study, it is possible that the crabs in the sample also harboured *P. botulus* acanthellae. As observed in an older study, green crabs of larger size classes can get infected with acanthellae, meaning that they are still susceptible to infection (Thompson, 1985a). Additionally, the eider subspecies in the Pubnico area, *S. m. dresseri* and *S. m. borealis*, overwinter along the Atlantic coast (Mendall, 1986; Vestbo et al., 2019) and individuals were present at the time of crab collection, likely harbouring adult *P. botulus*. Therefore, we cannot assume that the crabs in this study were not also harbouring eggs and acanthellae at the same time as cystacanths. A study on gammarids infected by an acanthocephalan suggested that delayed host manipulation in their experiment could be due to hosts harbouring multiple parasite stages at once (Labaude et al., 2021). Perhaps the crabs in our case harboured enough acanthellae to suppress the manipulative effects of the cystacanths, but this would need to be confirmed through histology and microscopy in future studies.

Parasitic host manipulation is considered the expression of a parasite's extended phenotype, as they usually alter several host phenotypic traits (Fayard et al., 2020). There

is a lack of studies that examine this multidimensionality of host manipulation in terms of its magnitude, extent, and adaptive significance (Fayard et al., 2020). Most acanthocephalan studies only consider one manipulated trait at a time even though most manipulative parasites tend to affect several phenotypic traits in their hosts, either simultaneously or successively (Thomas et al., 2010; Fayard et al., 2020). For example, a study on *G. pulex* infected with *Pomphorhynchus tereticollis* revealed that decreased photophobic behaviour itself in the amphipod did not increase vulnerability to fish predation (Perrot-Minot et al., 2012). This is because even at two light intensities, infected individuals were not predated on more than uninfected ones, and a blend of serotonin and fluoxetine injected into uninfected gammarids that mimicked the decreased photophobia did not increase fish predation bias compared to sham-inoculated animals (Perrot-Minot et al., 2012). This multidimensionality of host alteration could be considered adaptive if it permits the parasites to complete their lifecycle in a multitude of ecological situations (Thomas et al., 2010). There are a few theories on how multidimensionality may have evolved, such as the successive addition of independently manipulated traits or a major physiological mechanism disruption in the host (Cézilly and Perrot-Minnot, 2010; Fayard et al., 2020). The exact impact of manipulative parasites on their ecosystems' dynamics is difficult to assess as their manipulative abilities may be influenced by several environmental factors that could interact with the abilities or have additive effects, leading to variations of host manipulation magnitude within and between hosts (Fayard et al., 2020). For example, temperature can affect the level of acanthocephalan manipulation of gammarid phototaxis, with the effect of infection increasing with increased temperature (Labaude et al., 2017). Therefore, since only behaviour was considered in this study, future

studies could examine the potential multidimensionality of *P. botulus* phenotypic alterations in the green crab.

3.4.6 Next Steps

There is a large variety of potential research avenues that could help determine if *P. botulus* exhibits adaptive green crab manipulation or not. These include comparing natural and experimental infections, the latter of which can confirm that host alterations are caused by infection itself and prior phenotypic differences did not cause infection (Poulin and Maure, 2015). Whether personality is a factor in potential *P. botulus* alterations is also worth exploring, as green crabs do seem to possess a boldness-shyness continuum (Fürtbauer, 2015, Fürtbauer and Fry, 2018). While this study focused on possible behaviour changes, acanthocephalans can affect a number of other phenotypic traits (Fayard et al., 2020), meaning that there is a range of possible alterations that *P. botulus* could cause in the green crab. Looking at potential proximate mechanisms of host alteration is always needed, as this aspect of parasitic manipulation is still not fully understood and studies demonstrating molecular changes caused by parasitic infection are rare (Herbison, 2017; Fayard et al., 2020). Predation trials that demonstrate how eiders preferentially prey on infected green crabs would ultimately confirm the adaptive nature of any alteration caused by *P. botulus*, as the ultimate goal of adaptive intermediate host manipulation is to increase trophic transmission to the definitive host (Auld and Tinsley, 2015). Finally, manipulative parasites, including acanthocephalans, tend to modify multiple traits at once, and so it may be that the combination of multiple host alterations through different pathways is what

results in increased trophic transmission (Herbison, 2017; Fayard et al., 2020). There are many possibilities to consider before ruling out green crab manipulation by *P. botulus*.

3.5 Conclusions

In this study, *P. botulus* did not significantly alter green crab behaviour in any of the responses measured. This could be explained by a number of factors, including unnatural captive environment, particular tests used, differential host manipulation, lack of parasite acanthella stage measurements, and the multidimensionality of host manipulation. The lack of behavioural impact on the green crab is a tentatively promising result for knowledge of the worm's impact. A recent meta-analysis of acanthocephalan-intermediate host studies revealed a large study effect, meaning that differences in study results about the type and magnitude of parasite-induced phenotypic alterations can occur due to differences in experimental design (Fayard et al., 2020). Therefore, testing additional experimental designs will be essential before the lack of overall green crab manipulation is confirmed. Indeed, there are several future research avenues when it comes to acanthocephalan manipulation. It is important to report negative evidence of parasite host manipulation and it will be crucial to measure the effects of *P. botulus* in more diverse green crab phenotypic traits, as well as in its many other intermediate host species.

Chapter 4: Conclusion

4.1 Phylogeny of Antilipopolysaccharide Factors in Decapoda

4.1.1 Summary and Conclusions

Antimicrobial peptides (AMPs) are an important component of crustacean innate immunity. One AMP family, the antilipoplysaccharide factors (ALFs), was initially found in horseshoe crabs (Tanaka et al.,1982; Ohashi et al., 1984) and ALF genes were subsequently discovered in several crustacean species, including shrimp, lobsters and crabs (Rosa et al., 2013; Hou et al., 2017; Polinski et al., 2021). These small peptides have attracted significant scientific interest due to their broad-spectrum antimicrobial activity against several types of pathogens (Liu et al., 2006; Rosa et al., 2013; Tassanakajon et al., 2015). Though many studies that focus on ALF expression and functions include small phylogenetic trees, the detailed phylogeny of many sequences from several decapod crustaceans has only been attempted a few times (Lai and Aboobaker, 2017; Matos et al., 2018).

Thus, the goal of this study was to comprehensively examine the phylogeny of 291 ALF sequences of various decapod species to find trends and patterns in unrooted Maximum Likelihood trees. The overall pattern obtained in all trees was general heterogeneity of the tree groupings in terms of several operational taxonomic unit (OTU) categories, from species to infraorder. This finding was consistent to those from several other studies that constructed both rooted trees (Ren et al., 2012; Wang et al., 2015; Matos et al., 2018) and unrooted ones (Lai and Aboobaker, 2017; Lv et al., 2018). The lack of homogeneity has been suggested to be due to gene duplication events that occurred at least

before the speciation of the Dendrobranchiata and Pleocyemata suborders (Matos et al., 2018).

4.1.2 Future Directions

Many possible research avenues are available to further decipher the evolution of ALFs, with a non-exhaustive list described below.

A relatively small portion of extant crustacean species have been studied when it comes to ALFs, with most studies concentrating on Decapoda members. However, ALFs are well-conserved in all of Malacostraca (Lai and Aboobaker, 2017), meaning that there is a wealth of potential ALFs to be discovered and categorized. Adding ALFs of different crustacean orders into phylogenetic analyses of ALFs could therefore provide more clues towards their evolution patterns.

The unrooted trees in this study could have revealed more quantitative information about ALF evolution through the implementation of divergence and diversity measures. The clips of unrooted trees can give information about the evolution rates of groups of OTUs that are related in terms of path-length distances (Lapointe et al., 2010). More specifically, a clip is defined as a group of OTUs where all pairwise path-length distances are smaller than the given threshold value, s (Lapointe et al., 2010). There are also different dispersion measures to account for the distribution of OTUs of different categories in clans. For example, with the Shannon diversity index a diversity of 0 indicates that all OTUs of a type are in the same clan, while positive values mean that OTUs from the same type are dispersed in different clans (Shannon, 1948). Additionally, the Equitability index can be used to compare trees of various sizes (Lapointe et al., 2010). Implementing these indices in future ALF unrooted trees could reveal more useful information.

More in-depth analysis of ALF phylogeny will also be key, such as looking for motifs, gene location and distribution, and more. Chromosomal organization studies (eg. Yamamoto et al., 2014) can look at the chromosome locations of specific genes to infer more specific gene mutations. More detailed measurements of clan diversity could also be implemented, such as placing OTUs in lifestyle and/or phenotype categories in order to detect more distribution patterns (Schliep et al., 2010). There are also ways to test for natural selection signatures. Testing for signatures of natural selection by comparing ratios of nonsynonymous (dN) and synonymous (dS) mutations is common as positive and negative selection leave molecular signatures that can be detected through statistical tests (Nielsen, 2005). The dN/dS ratio signifies the ratio of nonsynonymous mutations/nonsynonymous site to synonymous mutations/nonsynonymous site where if synonymous and nonsynonymous substitutions occur at the same time, $dN/dS = 1$, if there is negative selection, $dN/dS < 1$, and if there is positive selection, $dN/dS > 1$ (Nielsen, 2005). Therefore, several methods can be used to dive deeper into the evolution of this AMP family.

Confirming the antimicrobial function of predicted ALF sequences is essential to determine if any of these sequences are non-functional. Studies implement a variety of tests to get evidence of ALF functionality across several situations. This can include administering pathogen challenges to live animals (Sun et al., 2011; Liu et al., 2013; Sun et al., 2021) and several *in vitro* techniques such as developing and testing synthetic LBD peptides against a variety of pathogens through measuring agglutination activity (Hou et al., 2017; Sun et al., 2021) or liquid bacterial culture inhibition assays (Jiang et al., 2015).

The more ALFs that have confirmed antimicrobial activities or the lack thereof, the more accurate the phylogenetic analysis will be.

4.2 Effect of *Profilicollis botulus* on Behaviour of *Carcinus maenas*

4.2.1 Summary and Conclusions

Complex lifecycle parasites are parasites that must consecutively infect more than one host during their lifecycle (Auld and Tinsley, 2015). One group that employs this lifestyle is the Acanthocephala taxon. These parasitic worms must infect an intermediate arthropod host and undergo development before being trophically transmitted to their vertebrate definitive host, where they can reproduce (Moore, 1984). There is evidence that these parasites can manipulate a variety of phenotypic traits in their crustacean intermediate hosts to increase their own trophic transmission. Most acanthocephalan-crustacean studies focus on gammarids and small amphipods (Helluy and Holmes, 1990; Kaldonski et al., 2007; Lagrue et al., 2007; Perrot-Minnot et al., 2016). The few studies done on the crabs infected by acanthocephalans focus on physiological changes as well as the burrowing behaviour of the shore crabs *Macrophthalmus hirtipes*, *Hemigrapsus edwardsi*, *H. crenulatus*, and *Emerita analoga* (Latham and Poulin, 2001, 2002; Kolluru et al., 2011). Thus, there is a research gap when it comes to the effects of acanthocephalans on more varied crab behaviours.

In this study, the effect of *Profilicollis botulus* on several behaviours of the green crab, *Carcinus maenas*, was measured. As an invasive species in Atlantic Canada, the green crab is a suitable model for studying the movement and dispersal of symbionts associated with marine invasions (Torchin et al., 2003) and the use of green crab as bait for American lobster (*Homarus americanus*) fisheries may facilitate disease and parasite transmission to

the commercially important crustacean (Bojko et al., 2018). Thus, three tests were performed on a green crab population located in Nova Scotia: an open field test, a mirror approach test, and a background preference test. Although several responses were measured, there were no significant differences reported between parasitized and non-parasitized crabs. This was suggested to be due to several reasons including a captive environment and tests that were perhaps not biologically relevant, differential host manipulation as *P. botulus* can infect a number of other crustacean hosts (Uspenskaya, 1960; Ching, 1989; McDermott, 2011), the lack of parasite acanthella stage measurements, and the multidimensionality of host manipulation.

4.2.2 Future Directions

Host manipulation is incredibly complex and to find if *P. botulus* does manipulate the green crab in some way, there are several possibilities for future research. To expand on the conclusions of Chapter 3, there are several ways to further study this interaction. Experimental infections can help us determine if an altered behaviour is a cause of infection rather than a consequence of the behaviour that predisposed the host to infection (Poulin and Maure, 2015). Intermediate hosts can be experimentally infected by exposing them to acanthocephalan eggs (Labaude et al., 2021). A recent meta-analysis revealed that the type of infection, experimental or natural, did not affect the overall intensity of host manipulation, meaning that the results of experimental infection studies can be reliably compared to natural infection studies (Fayard et al., 2020).

There is evidence of personality in green crabs. There are consistent individual differences in haemolymph density and the crabs' response to a novel environment, with individuals with a higher density spending more time near shelter (Fürtbauer, 2015).

Individuals also display repeatability in levels of activity, immobility and hiding (Fürtbauer and Fry, 2018). This link between consistent physiology and behaviour signifies the potential presence of personality in this invertebrate (Fürtbauer, 2015). Many factors influence personality, such as population density, temperature, and more (Decker and Griffen, 2012), so it would be interesting to examine the role of parasite infection in intraindividual behaviour variability.

Parasite-induced phenotypic alterations (PIPAs) have been extensively studied, but most studies thus far have concentrated on changes in host behaviour (Fayard et al., 2020). However, acanthocephalans can alter several phenotypic traits. Although behaviour does tend to be the most significantly influenced, other host trait categories such as life history, morphology, and physiology for example can be altered by cystacanths (Fayard et al., 2020). Even within behaviour, changes in behavioural variability in infected hosts is another concept that merits attention (Fayard et al., 2020). Previous crab studies have explored some non-behavioural traits. A study found weak evidence that *Profilicollis* cystacanths can manipulate the carapace colour of *M. hirtipes* (Latham and Poulin, 2001), and *H. crenulatus* crabs infected with *P. antarcticus* cystacanths exhibit a higher metabolic rate in terms of oxygen consumption compared to uninfected crabs (Haye and Ojeda, 1998). In addition, traits that are not directly related to predator-prey interactions have seldom been studied in acanthocephalan-intermediate host systems, so it is important to look at these to see if the adaptiveness of modified traits also applies to parasite stages that are not infective to the final host (Cézilly and Perrot-Minnot, 2010).

It is quite rare for studies to investigate molecular manipulation in parasite-host systems, never mind demonstrating molecular changes in a host that are either directly

caused by active parasite manipulation, or that the changes do increase parasite fitness (Herbison, 2017). There are four systems of the host that parasites can target for manipulation: neural, endocrine, neuromodulatory, and immunomodulatory (Lafferty and Shaw, 2013). Parasites can communicate with these systems via many chemicals (such as neurotransmitters, neuromodulatory chemicals, hormones, proteins, enzymes, etc.), genetic or epigenetic mechanisms, parasite gene expression, or interactions of parasite and host chemicals in response to each other (Bhattarai et al., 2021). We still need to decipher the links between behavioural, life-history, and physiological traits in these host-parasite relationships, and this could be achieved with studies that manipulate infected hosts' immune responses and measure effects on their immuno- and neuromodulatory systems (Fayard et al., 2020). Host manipulation mechanisms can be broadly divided into the categories of immunological, genomic/proteomic, neuropharmacological, and symbiont-mediated (Herbison, 2017). Many studies have provided evidence that parasite-induced behavioural changes are linked to altered levels of neuromodulators or neuropeptides or changes in genetic expression related to biogenic amine metabolism in the brain (Perrot-Minnot and Cézilly, 2013). A lot of studies on the proximate causes of host manipulation have concentrated on the role of neuromodulators so far, and various parasite-host interactions have evidence of altered levels of specific neuromodulators in the nervous system of the host (Perrot-Minnot et al., 2016).

Predation trials would be important to conduct to determine if host manipulation truly leads to increased trophic transmission to the definitive host. Behaviour changes due to infection are not necessarily adaptive and may increase the rate that infected intermediate hosts are consumed by predators other than the definitive host, where the parasite cannot

complete their lifecycle (Auld and Tinsley, 2015). Many studies report PIPAs, but as of 2020 only 5.4% of acanthocephalan cystacanth studies quantified trophic transmission to the final host, and even fewer looked at the contributions of specific altered traits to increased transmission (Fayard et al., 2020). Even simulated predator exposure would be beneficial. For example, a previous field study simulated the approach of a bird predator by passing a cardboard model of a gull in flight passing over the crab and recorded the behavioural state of the crab (Latham and Poulin, 2001).

An important quality of manipulative parasites is that they tend to combine the use of multiple host pathways to enact manipulation, but most PIPA studies will focus on one manipulation pathway at a time instead of a more holistic approach (Herbison, 2017). A certain specific behavioural alteration may not result in increased final host predation compared to uninfected hosts, at least not on its own (Fayard et al., 2020). In the case of amphipod intermediate hosts, the cystacanth has a bright orange colouration visible on the cuticle of the host and induces behaviour alterations like decreased photophobia (Fayard et al., 2020). More recent studies manipulated one phenotypic trait at a time and found that the worm colour or altered host behaviour alone did not increase gammarid vulnerability fish predation, suggesting that multiple phenotypic changes could be acting in concert to enhance parasite transmission (Perrot-Minnot et al., 2012; Fayard et al., 2020).

The green crab also has a large range of genetic diversity across the globe. The Nova Scotia populations in particular have a high proportion of mitochondrial DNA haplotypes common in the native range (Darling et al., 2008). Phylogenetic analysis of microsatellite data revealed the formation of three clusters: one with native populations and Nova Scotia, one with all North American populations except Nova Scotia, and one with

populations from Australia, Tasmania, and Argentina (Darling et al., 2008). Overall, the genetic diversity in introduced populations is significantly lower compared to the native range, but diversity is quite varied depending on the particular invasive population (Darling et al., 2008). Due to the large genetic and geographic ranges of the green crab, it would be interesting to see if green crabs from the native population or a different introduced population exhibit similar responses to *P. botulus* infection.

It is important to note that all of the possibilities for future directions described above are not exhaustive; there are many more experimental designs and measurements that could potentially be done to solve the question of if *P. botulus* can adaptively manipulate the green crab to increase its trophic transmission.

4.3 Conclusion

Both components of this project demonstrate the incredible complexity of crustacean health, both at the molecular and phenotypic level. As demonstrated by the topology of all ALF trees constructed, this AMP family exhibits tremendous diversity in terms of decapod phylogenetic groupings. This lack of clear patterns is likely due to several gene duplication events. Ultimately, to get a clearer picture of the evolution of ALFs we will need to study the chromosomal organization and function of ALFs in several crustacean species. Additionally, although no evidence of behavioural manipulation was obtained in the relationship between *P. botulus* and *C. maenas*, this does not necessarily rule out overall manipulation by the worm. There are many research avenues to confirm the nature of this host-parasite interaction, but a lack of evidence for adaptive manipulation would be a significant result as well.

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Appendix

Table 4. Supplementary information of 291 ALF sequences used in this study.

Suborder	Infraorder	Superfamily	Family	Abbreviation	Species	Source	NCBI identification of nucleotide sequence	NCBI identification of amino acid sequence	Mature peptide sequence
Dendrobranchiata	N/A	Penaeoidea	Penaeidae	Farazt_ALFA	*Farfantepenaeus aztecus*	Matos et al., 2018	GEUA01066862	N/A	QGVQDLIPSLVDRIVGLWHSDEVEFMGHSCRYSQRPSFYRWELYFNGRMWCPGWAPFTGRSRTRSPSGAVEHATRDFVQKALQSNLITEDDARIWLEH
				Farazt_ALFB	*Farfantepenaeus aztecus*	Matos et al., 2018	GEUA01068264	N/A	QGWEAVAAAVASKIVGLWRNEETELLGHKCRFTVKPYIKRLQLYYKGKMWCPGWTPIRGEARTRSHSGVAGRTARDFVQKAFRDGLISEQDAKRWLNS
				Farazt_ALFC	*Farfantepenaeus aztecus*	Matos et al., 2018	GEUA01066953	N/A	SGWEALVPAIANKLTGLWESGELELLGHYCSFNVTPKFKRWQLYFRGRMWCPGWTAITGQAETRSRSGVVGRTTQDFVRKAFSAGLITESEARVWLNS
				Farazt_ALFD	*Farfantepenaeus aztecus*	Matos et al., 2018	GEUA01067287	N/A	FSLKDLFVPVIKEQVSDLWKTGDIDLVGHSCTYNVKPDIDGFELYFIGSVTCPGWTTLRGESNTRSKSGVVNAAVKDFIQKALKQGLVTEEEAKPHLV
				Farazt_ALFE	*Farfantepenaeus aztecus*	Matos et al., 2018	GEUA01004511	N/A	RPQLGDILGSVVTTFLENAVKTTEITILDNYCYVSRSPYLKKFEVHYRAEVKCPGWTIIVGRGRDHTNPTNSELAAIKDFVNQAVEKGLMTDQEATEYL
				Farazt_ALFF	*Farfantepenaeus aztecus*	Matos et al., 2018	GEUA01070429	N/A	QGWEALIPLITQQVVGLWKTGERELFGHECTYFVTPRVKRIQLYFRGRMTCPTLSNVRGEALTRSRSGVEAKTVEDYVKKAVAKGVITEEDAKAWLNK
				Farazt_ALFG	*Farfantepenaeus aztecus*	Matos et al., 2018	GEUA01069818	N/A	QEMEDQKNHVSDILSNIFNSLVKDGEIELLGHYCSYSTRPYFIGWRLKFKSKVWCPGWTLVYGSSGNNASASNSIQNAIVNFIQKAYQEGVITEEDAKPWLQGNH
				Farfpaul_ALFB	*Farfantepenaeus paulensis*	NCBI	EF601051	ABQ96193.1	QGWQAVAAAVASKIVGLWRNEETELLGHKCRFTVKPYIKRIQLYYRGKMWCPGWTPIRGEASTRSHSGVAGRTARDFVQKAFRDGLISEQDAKRWLNS
				Fenchin_ALFA	*Fenneropenaeus chinensis*	NCBI	JX853777	AFU61127.1	QGVQDLLPALVDRIVGLWHSDEVEFMGHSCRYSQRPSFYRWELYFNGRMWCPGWAPFTGRSRTRSPAGAVEHATRDFVQKALQSDLISEEDARIWLEH
				Fenchin_ALFB	*Fenneropenaeus chinensis*	NCBI	AY859500	AAX63831.1	QGWEAVAAAVAVKIVGLWRNEKTELLGHECKFTVKPYIKRFQLYYKGRMWCPGWTAIRGEAKTRSRSGVAGRTAKDFVRKAFQQGLISQQQANQWLNS
				Fenchin_ALFC	*Fenneropenaeus chinensis*	NCBI	JX853775	AFU61125.1	SGWEALVPAIADKLTGLWESGELELLGHYCSFNVTPKFKRWQLYFRGRMWCPGWTTIGGQAETRSRSGVVGRTTQDFVRKAFRAGLITESEAQAWLNN
				Fenchin_ALFD	*Fenneropenaeus chinensis*	NCBI	JX853779	AFU61129.1	FGLKDLLLPAVTDQVKDLWRNGDVDLVDHSCSYSVKPDIQGFELYFIGSVTCPGWTTIRGESNTRSKSGVLNAAIKDFIQKALKAGLVTDEEAKPYLV
				Fenchin_ALFE	*Fenneropenaeus chinensis*	NCBI	JX853778	AFU61128.1	RPQLGDILGSLVTTFVENAVKTTETTILDNYCLLSRSPYLKKLEVHYRAELKCPGWTTIVGKGRDHTNPTNSELDAIKDFIRQALEKGLVTDEEAAQYL
				Fenchin_ALFF	*Fenneropenaeus chinensis*	NCBI	JX853774	AFU61124.1	QVWEALVPLITQQVVGLWKNGEREFFGHQCTYSVTPTVKSFELYFKGRMSCPSLSSVRGEALTRSRSGVEVKTVEDYVKKVLAQGVITEEEAKAWITK
				Fenind_ALFB	*Fenneropenaeus indicus*	NCBI	GU727863	ADE27980.1	QGWEAVAAAVASKIVGLWRNEKTELLGHECKFTVKPYIKRFQLYYKGRMWCPGWTAIRGEARTRSHSGVAGRTAQDFVRKAFQKGLISQQEANQWLNS
				Fenpen_ALFA	*Fenneropenaeus penicillatus*	Matos et al., 2018	GFRT01025471	N/A	QGVQDLLPALVERIVGLWHSDEVEFMGHSCRYSQRPSFYRWELYFNGRMWCPGWAPFTGRSRTRSPSGAVEHATRDFVQKALQRGLISEEDARIWLEH
				Fenpen_ALFB	*Fenneropenaeus penicillatus*	NCBI	GFRT01025680	QCQ82547.1	QGWEAVAAAVASKIVGLWRNEKTELLGHECKFTVKPYIKRFQLNYKGRMWCPGWTAIRGEARTRSHSGVAGRTAQDFVRKAFQQGLISQQEANQWLNS
				Fenpen_ALFC	*Fenneropenaeus penicillatus*	Matos et al., 2018	GFRT01017039	N/A	SGWEALVPAIADKLTGLWESGELELLGHYCSFNVTPKFKRWQLYFRGRMWCPGWTTIRGQAETRSRSGVVGRTTQDFVRKAFRAGLITESEARVWLNN
				Fenpen_ALFD	*Fenneropenaeus penicillatus*	Matos et al., 2018	GFRT01004925	N/A	FGLKDLILPAVTTQVKDLWRNGDVDLVDHSCSYSVKPDIQGFELFFIGSVTCPGWTTIRGESNTRSKSGVLNAAIKDFIQKALKAGLVTDEEAKPYLV
				Fenpen_ALFF	*Fenneropenaeus penicillatus*	Matos et al., 2018	GFRT01004448	N/A	QIWETLVPLITQQVVGLWKNGEREFFGHQCTYSVTPKVKSLELHFKGRMTCPTLSSVRGEALTRSRSGVEAKTVEDYVKKVLAQGVITEEEAKAWITN
				Litschm_ALFB	*Litopenaeus schmitti*	NCBI	DQ991357	ABJ90465.1	QGWQAVAAAVASKIVGLWRNEETELLGHKCRFTVKPYIKRLQLHYKGKMWCPGWTPITGEARTRSHSGVAGRTARDFVQKAFERGLISEQDAKRWLSS
				Litsty_ALFB	*Litopenaeus stylirostris*	NCBI	KC346373	AGH32549.1	QGWQAVAAAVASKIVGLWRNEETELLGHKCRFTVKPYIKRIQLHYKGKMWCPGWTPITGEARTRSHSGVAGRTARDFVEKAFRKGLISEQDAKRWLSS
				Litsty_ALFD	*Litopenaeus stylirostris*	NCBI	DQ010421	AAY33769.1	FSLKDLFVPVIKDQVSDLWRTGDIDLVGHSCTYNVKPDIQGFELYFIGSVTCPGWTTLRGESNTRSKSGVVNSAVKDFIQKALKAGLVTEEEAKPHLV
				Litvan_ALFA	*Litopenaeus vannamei*	NCBI	GETD01024664	AVP74301.1	QGVQDLIPSLVQKIVGLWHSDEVEFMGHSCRYSQRPSFYRWELYFNGRMWCPGWAPFTGRSRTRSPSGAVEHATRDFVQKALQSNLITEDDARIWLEH
				Litvan_ALFB	*Litopenaeus vannamei*	Matos et al., 2018	GQ227486	N/A	QGWQAVAAAVASKIVGLWRNEETELLGHKCRFTVKPYIKRLQLNYKGKMWCPGWTTIKGEARTRSHSGVAGRTARDFVEKAFRDGLISEQDAKRWLN
				Litvan_ALFC	*Litopenaeus vannamei*	Matos et al., 2018	GETD01033447	N/A	SGWEALVPAIANKLTGLWESGELELLGHYCNFSVTPKFKRWQLYFRGRMWCPGWTAIRGQAETRSRSGVVGRTTQDFVRKAFGAGLITESEAQVWLNS
				Litvan_ALFD	*Litopenaeus vannamei*	NCBI	FE115964	AVP74303.1	FSLKDLFVPVIKDQVSDLWRTGDIDLVGHSCTYNVKPDIDGFELYFIGSVTCPGWTTLRGESNTRSKSGVVNAAVKDFIQKALKAGLVTEEEAKPHLV
				Litvan_ALFE	*Litopenaeus vannamei*	NCBI	FE069658	AVP74304.1	RPQLGDVLGSVVETFLKNAVKTSELTILDNYCYVNRSPYLKKFEVHYRADVKCPGWTIIVGRGSDHTNPNNSELDAIKDFVKQAVIKGIMTDVEAAEYL
				Litvan_ALFF	*Litopenaeus vannamei*	NCBI	KJ000049	AHG99284.1	QVWETLIPLITQQVVGLWKTGEREMFGHECTYFVTPKVKSFELYFKGRMTCPTLSNVRGEALTRSRSGVEAKTVEDYVRKVVEKGVITEEEAKAWLNK
				Litvan_ALFG	*Litopenaeus vannamei*	NCBI	GETZ01049665	AVP74305.1	QEMEDQDNYASDIFSNIFNSLVKDGEIELLGHYCSYSTRPYFLRWRLKFKSKVWCPGWTLVYGSASESSSVSNSIQNAIINFIQKAYQEGVITEEDAKPWLQGNH
				Marsuj_ALFA	*Marsupenaeus japonicus*	NCBI	AB453738	BAH22585.1	QGVQDLIPALVQRIVGLWHSDEVEFMGHSCRYSQRPTFYRWELYFRGSMWCPGWAPFTGRSMTRSPSGAVEHATRDFVEKALQRNLITEDDARIWLED
				Marsuj_ALFB	*Marsupenaeus japonicus*	NCBI	KY627759	ASR74829.1	QGWEAVAAAVASKVVGLWRNEKTELLGHECNFTVKPYIKKFQLYYKGRMWCPGWTPIRGEASTRSQSGVAGRTARDFVEKAFRGGLISEKEANQWLHG
				Marsuj_ALFC1	*Marsupenaeus japonicus*	NCBI	AB210110	ANA91279.1	QGWEALVPAIAEKLTGLWENGELELLGHYCNFYVEPKFRNWQLRFKGRMWCPGWTIIKGEADTRSRSGVVGKTIQDFVKKAFSQGLITEEEARAWLSQ
				Marsuj_ALFC2	*Marsupenaeus japonicus*	NCBI	KU160498	AME17862.1	QGWEALVPAIAEKLTGLWQTGELELLGHYCNFSVTPKFKRWQLYFRGRMWCPGWTPIRGEAETRSRSGVVGRTTQDFVRKAFRSGLISESEAQAWLNQ
				Marsuj_ALFD	*Marsupenaeus japonicus*	Matos et al., 2018	N/A	N/A	QGLKDFLLPVISQQIAGLWRTGTVDLLDHLCTYNVKPDLQRFELYFLGTVTCPSWTVIKGESETRSQSGVVNDAVKDFVKKALKAGLVTEEEAKPYIV
				Marsuj_ALFE	*Marsupenaeus japonicus*	NCBI	KY627761	ASR74831.1	RPQFNIGGILGSVVETFVEEAYETREITLFDNYCVLTRSPYVKRLEVHYRADVKCPGWTPIVGKGRDHTNPTNSEMDAIKDFVRQALGKGLVTADEAAEWL
				Marsuj_ALFF	*Marsupenaeus japonicus*	NCBI	KU160499	AME17863.1	QIWETLIPLITKEVVSLWKNGEREMFGHRCTYSVTPKIKSFELYFKGRMTCPSLSNERGEAFTRSRSGVEAKTVEDYVRKIVAKGVITEEEAKAWLNK
				Marsuj_ALFG	*Marsupenaeus japonicus*	Matos et al., 2018	N/A	N/A	QEVESRDEILPEILGKFFDVLVRDGEIELLGHYCSYGTRPHLIRWRLKFKSKLWCPGWTLLYGTSSDDSSASNSLQNAIVNFVNKAVQAGLVSAEDAMPWLQ
				Penmon_ALFA	*Penaeus monodon*	NCBI	EF523561	ABP73290.1	QGVQDLLPALVEKIAGLWHSDEVEFLGHSCRYSQRPSFYRWELYFNGRMWCPGWAPFTGRSRTRSPSGAIEHATRDFVQKALQSNLITEEDARIWLEH
				Penmon_ALFB	*Penaeus monodon*	NCBI	EF523562	ABP73289.1	QGWEAVAAAVASKIVGLWRNEKTELLGHECKFTVKPYLKRFQVYYKGRMWCPGWTAIRGEASTRSQSGVAGKTAKDFVRKAFQKGLISQQEANQWLSS
				Penmon_ALFC1	*Penaeus monodon*	NCBI	JN562340	ADM21460.1	SGWEALVPAIANKLTSLWESGEFELLGHYCSFNVTPKFKRWQLYFRGRMWCPGWTTIRGQAETRSRSGVVGRTTQDFVRKAFRAGIITESEAQTWLNN
				Penmon_ALFC2	*Penaeus monodon*	NCBI	KX431031	ANP92039.2	AGWGAFMPSIATRLTGLWETGELELLGRYCTYSVKPTFQQWQLYFIGSMWCPGWTPIRGVAETRSRSGVVGKMTQDFVRKALRADLLSKEEAETWLSH
				Penmon_ALFD	*Penaeus monodon*	NCBI	GEME01010116	BCB17104.1	FGLKDLFLPAITDQVKDLWRNGDVDLVDHSCSYSVKPDIQGIELYFIGSVTCPGWTTIRGESNTRSKSGVLNAAIKDFIQKALKAGLVTEEEAKPYLV
				Penmon_ALFE	*Penaeus monodon*	NCBI	GEEP01059175	BCB92165.2	RPQLGDILGSLVETFVENAIKTTEITILDNYCLLSRSPYLKKFEVHYRAELKCPGWTTIVGKGRDHTNPTNSELAAIKDFIGQALKKGLVTDEEAAQYL
				Penmon_ALFF	*Penaeus monodon*	NCBI	GEME01077326	BCB17106.1	QIWETLVPLITQQVVGLWKNGEREFFGHQCTYSVTPKIKSLELHFKGRMSCPSLSSVRGEALTRSRSGVEGKTVEDYVRKVLAQGVITEEEAKAWLTK
				Penmon_ALFG	*Penaeus monodon*	Matos et al., 2018	JR205808	N/A	QEMEEQENHVSDIVSKIYNFLVRNGEIELLGHYCSYSTRPYFLRWQLKFKTKIWCPGWTLVYGSAKGNSSVSSSLQDAIVNFVQKAYQEDVISEEDAKPWLQGRK
				PenmonALF*	*Penaeus monodon*	Clark lab	N/A	N/A	AGWGAFMPSIATRLTGLWETGELELLGHYCTYSVKPTFQQWQLYFIGSMWCPGWTPIRGVAETRSRSGVVGKMTQDFVRKALRADLLSKEEAETWLSH
Pleocymata	Achelata	N/A	Palinuridae	Jased_ALF1	*Jasus edwardsii*	Matos et al., 2018	GGHM01074860	N/A	RGFEAFLPVLADQLGGLWRDGDMELMGRYCNYSVNPTFKKFELFFQGSVWCPGWTLITGSALTTSKSGVVGKTTKDFVKKVYEAGLVTKEEAGEWLV
				Jased_ALF2	*Jasus edwardsii*	Matos et al., 2018	GGHM01063785	N/A	QGWEAVAGAVATKIIGLFREGEVELMGHYCNYSVKPKIKRWQLYYKGRMWCPGWTTIRGEAETRSRSGVVGKTTRDFVNKAFKAGVIHQQEAQQWLNA
				Jased_ALF3	*Jasus edwardsii*	Matos et al., 2018	GGHM01137719	N/A	FDFSGLLSTVADNVVDRLYNEGEMELLGHYCRYERRPYFYRWELHYKAQVSCPGWTVIIGKAQGYVNPTSAEREATRDFVRKAVDRELVTEEEASEWL
				Jased_ALF4	*Jasus edwardsii*	Matos et al., 2018	GGHM01068293	N/A	QSLKDILPGLLKQVSEALFDNSEFELLGYYCNFSMTPSIKSFQLYYNGRVWCPGWTDVRGEASTRSRSGVVGKAIKDFLQKAAAKGIVTDAEVQEYLGS
				Jased_ALF5	*Jasus edwardsii*	Matos et al., 2018	GGHM01060172	N/A	QVWEQLVGLIVTKLANLWHDGEMDFMGHPCRYSRRPTIYRWQLHYKGKMWCPGWAPF
				Sagver_ALF1*	*Sagmariasus verreauxi*	Clark lab	N/A	N/A	QPTPCEGQSWGQLVGLIVTKLAGLWHDGEMDFMGHACRYSHRPSIYRWKLFYKGEMWCPGWAPFSGSSKTKSRSGAIEHAVRDFVQKAFEKGLITGEEASAWINQ
				Sagver_ALF2*	*Sagmariasus verreauxi*	Clark lab	N/A	N/A	QGWEAVAAAVASKLVGLWRQEEVELLGHYCQYSVRPKIKRWQLYFRGKMWCPGWTTIRGEAETRSRSGVVGKTTRDFVNKAFNAGLIHQQEAEQWLSA
				Sagver_ALF3*	*Sagmariasus verreauxi*	Clark lab	N/A	N/A	QGWEAVAAAVASKLVGLWRQEEVELLGHYCQYSVNPKIKRWQLYFRGKMWCPGWTTIRGEAETRSRSGVVGKTTRDFVNKAFNAGLIHQQEAEQWLNA
				Sagver_ALF4*	*Sagmariasus verreauxi*	Clark lab	N/A	N/A	QGWEAVAAAVASKLVGLWRQEEVELLGHYCQYSVNPKIKRWQLYFRGKMWCPGWTTIRGEAETRSRSGVVGKTTRDFVNKAFNAGLIHQQEAEQWLSA
				Sagver_ALF5*	*Sagmariasus verreauxi*	Clark lab	N/A	N/A	FDFEQILSQVVTGVASRAYQEGELELLDHYCWYSTKPQFYRWRLYYRSRVWCPGWTPIIGRDSRARNPLASRTEATKDFVRKIVNTGLVTREEAQQWL
				Sagver_ALF6*	*Sagmariasus verreauxi*	Clark lab	N/A	N/A	QSLKDILPGLLQQISSVLFENTEFELLGRYCNFSMTPSFKRFQLYYDGRVWCPGWTDIRGEASSRGRSGVIGKTIRDFLKKAEARGVVTHDQVVEYMGTE
				Sagver_ALF7*	*Sagmariasus verreauxi*	Clark lab	N/A	N/A	FDFSRLLSTVADNVIDRLYKEGEMELLGHYCTYERRPYFYKWELHYRAHVICPGWTTVIGKANGYLNPTSAEREATRDFVRKAVNQGILTAEEASEWL
				Sagver_ALF8*	*Sagmariasus verreauxi*	Clark lab	N/A	N/A	RGLEAFLPIISEQLGGLWRDGDMELLGRFCNYSVKPTFKRFELFFQGSVWCPGWTHIRGSALTTSKSGVVGKTTKDFVQKVFKAGLVTKEEAGEWLS
			Scyllaridae	Thenor_ALF1*	*Thenus orientalis*	Clark lab	N/A	N/A	QGWEAVAAAVVNKLTGLWSHEEVELLGHICKYSVRPKFRRFKLYFRGRMWCPGWTIIRGEAQTSSRSGVVGKTTQDFVRKAFRSGLISQEEAEQWLNS
				Thenor_ALF2*	*Thenus orientalis*	Clark lab	N/A	N/A	GGLEALLPIITDQISGLWRDGEMELLGHYCNFHVKPTFMRFELYFQGSVTCPGWTVIRGEALTRSKSGVLGKTTKNFIQKALAAGLITKEDATPWLN
				Thenor_ALF3*	*Thenus orientalis*	Clark lab	N/A	N/A	LDFKQILAHFATGVVGQVFQEGEIELLDHYCSFTTKPHFYRWKMYHRAKVWCPGWTPIVGEAKGYRSSVTARIEATKDFVRKIVDSGLVTREEAQPWL
	Anomura	Paguroidea	Coenobitidae	Coencly_ALF	*Coenobita clypeatus*	Matos et al., 2018	HABY01000044	N/A	QWQAVVPVLVNKIKGLWRDGEFELMGHICNYSVTPKIKRWQLYYKGRMWCPGWTTIRGEAETRSQSGVVGKTVSDFVEKAHNAGFITEQQARQWLAGQ
			Paguridae	Pagbern_ALF	*Pagurus bernhardus*	Matos et al., 2018	HABX01000001	N/A	QGWQSLVPALAGKIIGLWRGGNLNILGHQCSYRVRPKIIRWRLWYKGTLACPGWTSITGSDKARNPNHVVTYTVTDFVKKAFEAGLIKESDAQRWLQ
				Paglong_ALF1*	*Pagurus longicarpus*	Clark lab	N/A	N/A	NWQAIVPVLVSKVAGLWQNGSFEFMDTTCNYSVKPKIYRWELHYEGRMWCPSWPTIRGKARTRSQSGVVGKTVKDFVEQAHRAGKITEAQAQNWLNTH
				Paglong_ALF2*	*Pagurus longicarpus*	Clark lab	N/A	N/A	QGWETLIAAAVKQVAGLWTKGNHMILGNTCYYKTRPTFRKWKLKFKGKFWCPGWTSVQGQVVTSSRSKVLQRTVEDFVRKAVSAGVIKKSDLQKLQ
				Paglong_ALF3*	*Pagurus longicarpus*	Clark lab	N/A	N/A	NFKDIVPALVSKLTGLWQNDSFDLLDQTCNYSVKPMIQSWKLYYVGRVWCPGWPGIRGEAX
				Pagsam_ALF1*	*Pagurus samuelis*	Clark lab	N/A	N/A	NWQAIVPVLVSKISGLWQNGNFNLLDETCSYSVKPKIRRWQLYYDGRAWCPGWPGIRGEARTRSQSGVVGKTVTDFVQKAHNAGKITEADAQRIINSK
				Pagsam_ALF2*	*Pagurus samuelis*	Clark lab	N/A	N/A	KPFKIGDIIGALADVAKDTFQQGEIELFGHYCIYRREPHIKSLELYYRSEVRCPGWTSIIGRSDDRQNPTHSESEAEKDFVIKAVAKGLVTKEEAAAYF
				Pagsam_ALF3*	*Pagurus samuelis*	Clark lab	N/A	N/A	QWEALVAPVVAKIQGLWHSDHVEFMGHNCQFRRRPKIRKWKLYHEGKFWCPGWAPFDGRSRTKSRSGSSSEATKDFVRKAFANGLVTQEDAALWLKNHR
				Pagsam_ALF4*	*Pagurus samuelis*	Clark lab	N/A	N/A	QGWAAVAAAVASKVVGLWQNGHVDLLDHPCRFSVKPTVRRFQLYFKGRMWCPGWTSIRGEAKTRSRSGVVGKTTRDFVNKAFQAGLITERDAQQWLSH
				Pagsam_ALF5*	*Pagurus samuelis*	Clark lab	N/A	N/A	QGWETLVAGVSSQLVSLWHQGELELMGHYCNFQVKPKIRRWQLYFVGSMWCPGWTNIRGSAQTRSRSGVVGKTTTDFVRKAFRAGLITQQDAQEWLDN
				Pagsam_ALF6*	*Pagurus samuelis*	Clark lab	N/A	N/A	QGWEALLPAFVDKLSGLWRNGEVELLGRHYCSFAVKPKIKSWELYFLGTVSCPGWTRIRGHSETRSYSNVVNKATEDFLRNALKAGIITEEDLNTWLNN
				Pagsam_ALF7*	*Pagurus samuelis*	Clark lab	N/A	N/A	QGWETLIGVAVKQIAGLWTKGNHVFLGHTCQYKTKPTFRRWKLKFKGKFWCPGWTSITGKVTTSSRSKVLRRTVEDFVRKAVSAGVIKKTDLQSLQ
				Pagsam_ALF8*	*Pagurus samuelis*	Clark lab	N/A	N/A	IDLSSIISTIAGTAADQLYKSEEILLFDHYCTLTRRPYVKRWELHYKSEVYCPGWTSIIGKADNHRNPIKSDRSATQDFVTQALARDVVTQEQADLWLKE
				Pagsam_ALF9*	*Pagurus samuelis*	Clark lab	N/A	N/A	GFLDRLFDKVIDTLVEEGTIEVLDNICNFRVLPRLRGKSLSYKGSLWCPGWTSIRGESLTRSRTRVVNKAIEDFARKALEAGLILEADAQTLYNA
		Galatheoidea	Porcellanidae	Porcpla_ALF*	*Porcellana platycheles*	Clark lab	N/A	N/A	EREVTRHREHDLKHLELREYQQIDDDDDDDDAEVRTDEEPERALKEAMQLVASLYSSGPVDIAGHSCQFRYRPSLSWFKLRYKGKVWCPGWTSIEGRSKTKSRSGSVKEAIKDFVNKAVANGKLTQQEASTVLQS
	Astacidea	Astacoidea	Astacidae	Astast_ALF1	*Astacus astacus*	Matos et al., 2018	GEDF01041873	N/A	QLDLLGPLLGDSVKGLFQQGEVLLLDHYCNYSVKPKIKKWELHFDGSFWCPGWTNIKGEAITRSRSSIVNKATEDFIRKAQAKGLITEEEAKGWFGSH
				Astast_ALF2	*Astacus astacus*	Matos et al., 2018	GEDF01017751	N/A	QDWDATVTLIINKLAGLWHEGEAKFMGHTCHYSYNPTFYRWKMYYKGKMWCPGWVPFSGNSRTESRAGAVEHATRDFVKQALEKNLFTAEEVSAWINN
				Astast_ALF3	*Astacus astacus*	Matos et al., 2018	GEDF01038173	N/A	QVSQEVVNLIISKLVSLWSDGQVDFMGHTCNYSYSPTISRLQLYYKGKMWCPGWAPFSGNSRTKSRAGSIEHATRDFVTKAIEQKLITAKEASVWTKN
				Astast_ALF4	*Astacus astacus*	Matos et al., 2018	GEDF01001283	N/A	QILEGLAAAVANKLVGLWQNGEVELMGHFCNYSVTPKIRRWQLYFKGRMWCPGWTSIRGEAMTRSRSGVVGDTSRDFVNKAFSAGLISEQDAQAWLNS
				Astast_ALF5	*Astacus astacus*	Matos et al., 2018	GEDF01042255	N/A	QGIRESLVGAVTQQLLGLWSNGEVELLGHYCQYSVTPKIKKFELYYKGRMTCPGWTPIRGEAETRSRTGILVDTTADFVGKAFQAGLITEEEARQWLS
				Astast_ALF6	*Astacus astacus*	Matos et al., 2018	GEDF01005126	N/A	QGWEAVAALVAQKVVSLFRNDEVEFMGRVCKISVKPYIKKFQLYYKGRMTCPGWTHIRGEANTRSQSGVATKTTEDFVRKAFASGLIQEADAQQWLKT
				Astast_ALF7	*Astacus astacus*	Matos et al., 2018	GEDF01012076	N/A	KPFDFSHIISTVADNVITRLYRDGEIELLGHYCTWNRRPYFHRWELNYRASTTCPGWSSAVGKAEGFVNPMTAETEATKDLVRKLVQRGLATTDEASAWL
				Astlep_ALF1	*Astacus leptodactylus*	Matos et al., 2018	GBEI01002126	N/A	QLDLLGPLIGDSVKGLFQEGEVLLLDHYCNYSVKPKIKRWELHFDGTFWCPGWTNIKGEALSRSRSSIVNRATEDFIRKAQAKGLITEEEAQGWFGSN
				Astlep_ALF2	*Astacus leptodactylus*	Matos et al., 2018	GAFY01006393	N/A	QDWDATVTLIINKLAGLWHEGEAKFMGHSCRYSYNPTFYRWKMYYKGKMWCPGWVPFSGNSRTESRAGAVEHATRDFVKQALEKNLFTAEEVSAWINN
				Astlep_ALF3	*Astacus leptodactylus*	Matos et al., 2018	GAFY01000962	N/A	QVSQEVANLIISKVVSLWSDGQVDFMGHTCNYSYSPTISRLQLYYKGKMWCPGWAPFSGNSKTKSRAGSIEHATRDFVTKAIAQKLITAEEASVWIKN
				Astlep_ALF4	*Astacus leptodactylus*	Matos et al., 2018	GAFS01013149	N/A	QGISESLIGAVAQQLQGLWSNGEVELLGHYCQYSVTPKIKKFELYYKGRMTCPGWTPIRGEAETRSRTGVLVDTTADFVRKAFQAGLITEEDARQWLS
				Astlep_ALF5	*Astacus leptodactylus*	Matos et al., 2018	GAFY01001088	N/A	QILEGLAAVASKLVGLWQNGEVELMGHYCNYSVTPKMRRWQLYFKGRMWCPGWTSIRGEAMTRSRSGVVGDTSRDFVNKAFSAGIISEQDAQAWLNS
				Astlep_ALF6	*Astacus leptodactylus*	Matos et al., 2018	GAFY01012902	N/A	QGWEAVAGLVAQKVVSLFRNDEVEFMGHVCKISVKPYIRKFQLYYKGRMTCPGWTHIRGEANTRSQSGVAAKTTEDFVRKAFASGLIQEADAQQWLKT
				Astlep_ALF7	*Astacus leptodactylus*	Matos et al., 2018	GAFS01008651	N/A	QVWEPLAVAIAEKLVDLWQHGEFQLLNHQCNYSVSPKIRSWELYFKGTMWCPGWTPIKGEALTRSRSGVVGQTTRDFVRKAFNAGLITEQEGKDWLAL
				Astlep_ALF8	*Astacus leptodactylus*	Matos et al., 2018	GAFY01007701	N/A	KPFDFSYIVSTVADNVITRLYRDGEIELLGHYCTWNIRPYFHRWELKHRASTTCPGWSPAVGRAKGYLSPRSAQTEATKDLVRKLVQRGLVTTDEASAWL
				Paclen_ALF1	*Pacifastacus leniusculus*	NCBI	EF523760	ABQ12866.1	QVPPEVVSLIISKLVNLWSDGQVEFMGHTCNYSYSPTISKFQLYYKGKMWCPGWAPFSGNSKTKSRAGSIEHATRDFVTKAIDQKLITAEQASAWIKN
				Paclen_ALF2	*Pacifastacus leniusculus*	Matos et al., 2018	GBYW01031117	N/A	QILEGLAAAVANKLVGLWQNGEVELMGHYCNYSVKPTIRRWQMYFRGRMWCPGWTSIRGEAMTRSRSGVVGDTSRDFVNKAFSAGLISEQDAQTWLNA
				Paclen_ALF3	*Pacifastacus leniusculus*	Matos et al., 2018	GBYW01032814	N/A	QLDLLGPLLGESVKGLFNEGEVVLLDRYCNYLVRPKIKKWELYFDGSFWCPGWTNIKGEALSRSRSSIVNKATEDFIRKAQAKGLITEEEAKSWFGSN
			Cambaridae	Proclark_ALF1	*Procambarus clarkii*	NCBI	HM005306	ADX60063.1	QVLEGLAAAVTGKLAGLWRNGEVELLGHYCSYSVTPTIRRWQLYFRGRMWCPGWTSIRGEAMTRSNSGVQGDTTRDFVTKALNAGLISQQEAQAWLDG
				Proclark_ALF2	*Procambarus clarkii*	NCBI	KU680793	AOG75595.1	EESKGLFGSIIDHFSNFWKNGEMEFMGQICRYSYSPKFQNWGLTYSGKMWCPGWAPFAGNSRTLSRAGAIENATKDFVRKAVDHGLITAEEASVWLQ
				Proclark_ALF3	*Procambarus clarkii*	NCBI	KU680794	AOG75596.1	QDWDDTITLIINKLAGLWHEGEAKFMGHSCRYSYKPTFYRWKMYYKGKMWCPGWVPFSGHSRTESRSGAIEHATRDFVKQALEKNLFTAEEASAWIKN
				Proclark_ALF4	*Procambarus clarkii*	NCBI	KU680795	AOG75597.1	KPFDFSQIISTVADNVITRLYQEGEIELLGHYCNYSRRPYFHRWELNYRATTSCPGWTTAVGKAQGYLNPTTAEIEATKDLVRKLVQAGLVTREEASAWL

Table 4 Continued. Supplementary information of 291 ALF sequences used in this study.

Suborder	Infraorder	Superfamily	Family	Abbreviation	Species	Source	NCBI identification of nucleotide sequence	NCBI identification of amino acid sequence	Mature peptide sequence
Pleocyemata	Astacidea	Astacoidea	Cambaridae	Procfalt_ALF3	*Procambarus clarkii*	NCBI	KU-680796	AOG75596.1	QAWETLAVAIA IK LVELWQHGEVRLLNHQCN YSVTPKVRSWELYYQGSMWC PGWTPIRGEALTRSRSGVVGKTTQDFVRKA FTA GLITQEGKDWLV S
				Procfalt_ALF6	*Procambarus clarkii*	NCBI	KU-680797	AOG75598.1	QLDLAPLLGDQVKG UFIQAIAVLLDHV CN YSVKPK FRKWELYYDGSFWCPGWTNIKGEALTRQSSVVNKATEDFVKQAAGLISDEEAKSWFGAN
				Procfalt_ALF7	*Procambarus clarkii*	Matos et al., 2018	KU-680798	N/A	QGWEALVPAIA IK LTGLWQTGELELLGHYC NFSVTPKFKRWQLYFRGRMWC PGWTPIRGEALTRSRSGVVGRTQDFVRKAFRSG LISEEEAQASWLNQ
				Procfalt_ALF8	*Procambarus clarkii*	NCBI	KU-680799	AOG75601.1	QVLEGLAAAVTGKLAGLW RNGEVELLGH YCQYSVSPKFK SFILY FRGRMTCPGWTPIRGEAESSSTGILAA TTA DFVNKAFQAGLITDDAKRWLN
				Procfalt_ALF9	*Procambarus clarkii*	NCBI	KU-680800	AOG75602.1	QGWEAVAAAVAQKLVGLFDSVIEVELLGHKC KLTVKPVTKRFQLYHKG TMSCPGWTHIKGEIDTRSASGVANKTQDFVRKAFASGVANKTQDFVRKAFASGVEGEAQFLWLKS
				Procfalt_ALF10	*Procambarus clarkii*	Matos et al., 2018	KU-680801	N/A	RPLHWDSITVMEIDRVTHRMHVGNEFELLNH QCAYHTRPLLMWVSMHELAKVW CPGWVN LSG TATDRIPKSEFATRDFVMKALQAGVVSQEDAKRWLSNLP
				Procfalt_ALF11	*Procambarus clarkii*	NCBI	KU-680802	AOG75604.1	QSLQGLLGAVTQGLVGLWNGEVELLGHYC QYSVSPKFKSFILYFRGRMTC PGWTPIRGEAESSSTGILAA TATDFVNKAFQAGLITDDAKRWLN
				Procfalt_ALF12	*Procambarus clarkii*	Matos et al., 2018	GA.RH0110403-41	N/A	QVSPELINLISN KLGGLWKDGEVFPMGHACNY SYSPKISWRLVYKGKMWC PGWAPFSQDSQTKSRA GAIEHATRDFVRKA IRNKLITAEEAAA WLKQ
		Parastacoidea	Parastacidae	Chrquad_ALF1	*Cherax quadricarinatus*	NCBI	KX-083340	AOE23899.1	QITEALVTAFAGKVA GLWDNGQELLGNYC NYVNVRPTIKKFQLYFNGSMYC PGWTTIRGEASTRSRSGVVGETTKDFIRKAMAAG LITQQQATDFLKT
				Chrquad_ALF2	*Cherax quadricarinatus*	Matos et al., 2018	HACK01047976	N/A	QSDILTSLLGDKIGL WKEIWEVLLMDHV CN YSVKPTFKKFELYFAGNFWCPGWTNI
				Chrquad_ALF3	*Cherax quadricarinatus*	Matos et al., 2018	HACK01046235	N/A	QLWEAIAGAVSNKVRLWDQGEVELMGHYC NYRVVPKPRRWQLYFNGRMWC PGWTTIRGEATSRSRSGVKGTTRDFVRKAFEAGIITEQSAQQWLNS
				Chrquad_ALF4	*Cherax quadricarinatus*	Matos et al., 2018	HACD02010080	N/A	QAWEQLAVAVAEKLEDLWQLGDFQLLGH KCNYSVTPK IRRLELYFQGSMWC PGWTLIRGEALTRSRSGVGDTTQDFVRKAFTAGLITDFQEAQAWLTT
				Hommam_ALF1.1	*Homarus americanus*	Polinski et al., 2021	N/A	N/A	QGWETLVAGVSSQLVSLWHQGELELMGHYC NYSPQYKPLRRWQLYFGSRGVPGWTNIRGAEQTSRSRSGVVGKTTDFVRKAFRAGLITQDRAQWLEN
			Nephropidae	Hommam_ALF1.2	*Homarus americanus*	Polinski et al., 2021	N/A	N/A	QGWEALLPAFADKLSGLWRNGEVELLGHYC NFAVKPKIRSWKLYYLGSTCPGWTSIKGEYQSRSSSKV INQTKDFIN KALAAGLIDEQQLN DWLKTS
				Hommam_ALF1.3	*Homarus americanus*	Polinski et al., 2021	N/A	N/A	QVWEALLPAFADKLSGLWRNGEVELLGHYC NYSVTVPHLRSLELYYKGKMCPGWTAIGEAETRSRSKVLNKTTEDFLRKALAAGITTEDLNTWLSH
				Hommam_ALF1.4	*Homarus americanus*	Polinski et al., 2021	N/A	N/A	QGWELLPAFVDKLSGLWRNGEVELLGRHYCN VAVKPKIRSWKLYYLGSTCPGWTSIKGEYQSRSSSKV INQTKDFIN KALAAGLIDEQQLN DWLKTS
				Hommam_ALF1.5	*Homarus americanus*	Polinski et al., 2021	N/A	N/A	QNTESIVETVLQKLGGLWRNGEVDPMGHSCMYNYRPTESRWKLYYKGKMWC PGWAPFSGRSRKKSSRGAEHATRDFVQKALDSGLITKEDASAWEQ
				Hommam_ALF1.6	*Homarus americanus*	Polinski et al., 2021	N/A	N/A	IPFD LSKVPETLADN VEWLLYNKGHMNLLGHYC TYKRQPFYHRWELEYRAQATCPGWSTSVGKSUNHRPNSAIERATRNLVQQIV AEGLVAKEAAEWL
				Hommam_ALF1.7	*Homarus americanus*	Polinski et al., 2021	N/A	N/A	TPDLTSVBSLLADNVISDRLFSEGQDLTLGHRC TYKKQPRIYSWQLQYQ AEVTPCGWTSIN GKSGRYLSSNTA LREATKNLMEQAADKGLVTKEEAQEWL
				Hommam_ALF1.8	*Homarus americanus*	Polinski et al., 2021	N/A	N/A	KDWDELVPDVVKK LIRAWQQKGEVNLLGHYC QYSVSPKFKSFILYFDSHK RIQATTFC PTLSDQGRATSRNKFGVPGQVHDYRKAKRLKEVTEVKVDIWLTS
				Hommam_ALF1.9	*Homarus americanus*	Polinski et al., 2021	N/A	N/A	PFD ISEFFAQMTHV ADKSFAIDGELDLLDHFCSYNRPYFHKWRLLYYRQ VWCPGWSPIKTADRIITNSVHAK MAATRSIPVHQAVDRGLVRKEEAV NWL
				Cancho_ALF	*Cancer borealis*	Matos et al., 2018	GEFH01001087	N/A	QWEALVGPLLTKLSGLWHSDSFDFMGHTCNFRRPSVRRFKLYHNGKFWCPG WASFEGRSKTK SRSGSARDATQDFVRQAFQKGLITQED ASAWLKN
				Cancho_ALF1*	*Cancer borealis*	Clark lab	N/A	N/A	FLK DLLFDKAKDALLEDGNTELDKCNSFRVTPQLKKWELLYFRGDV WCPGWTTIRGDSFTRSRTKVLKAVADFAEKAVKAGGLFQEDADPLFE
				Cancho_ALF2*	*Cancer borealis*	Clark lab	N/A	N/A	FLK GLLSKATDALLESGTREVLGHVCNYRTPRVKSWELYFRGDVWCPGWSIKGESLTRSRTSVVNKAIDFARKAVSAGV VLQEDAEPLLH
				Cancho_ALF3*	*Cancer borealis*	Clark lab	N/A	N/A	KPFNKSILGSVAGSLIKGAIKTKDDLLDHVCVLAKTPVGKSWELHYKAGVTCPGWTTIRGRGSGHTNPNSENAALRDFMEKAVKGGLVTKAEA SPWLDN
				Cancho_ALF4*	*Cancer borealis*	Clark lab	N/A	N/A	AFDFSALBNVADTAIKKLLYVEKEITLLDHYCVMNRSPHFYRWQLKWKTNVTCPGWTPIVGRASGFASPTTSENEATRQFVKKAVATGLVTREEATAWL
				Cancho_ALF5*	*Cancer borealis*	Clark lab	N/A	N/A	QWEALVGPLLTKLSGLWVSDSFDFMGHTCNFRRPSVRRFKLYHNGKFWCPG WASFEGRSKTK SRSGSARDATQDFVRQAFQKGLITQED ASAWLKN
				Cantir_ALF1*	*Cancer irroratus*	Clark lab	N/A	N/A	FLK EILFDKAKDALLEDGTTELDKCNSFRVTPRLKSWELYFRGDV WCPGWTSTGESFTRSRTRVVLKAVDFAEKAVKGGLLTQEEAQPLFE
				Cantir_ALF2*	*Cancer irroratus*	Clark lab	N/A	N/A	PFD FSALBNVADTAIKKLLYVEKEITLLDHYCVMNRSPHFYRWQLKWKTNVACPGWTPIVGRASGFASPTTSENEATRQFVKKAVATGLV IREEAAAWL
				Cantir_ALF3*	*Cancer irroratus*	Clark lab	N/A	N/A	QWEALVGPLLTKLSGLWHSDSFDFMGHTCNFRRPSVRRFKLYHNGKFWCPGWTSVGESFTRSRTRVVLKAVDFAEKAVKGGLFTQEEAQPLFE
				Cantir_ALF4*	*Cancer irroratus*	Clark lab	N/A	N/A	QWEALVGPVLTKLSGLWNSDSFDFMGHTCHFRRKPTVRRFKLYHKGKFWCPGWAPFDQRSRTKSRSGSARDATQDFVRQAFQKGLITQEDASDWLKN
				Cantir_ALF5*	*Cancer irroratus*	Clark lab	N/A	N/A	QGWETLVAGVSSQLVSLWHQGELELMGHYC NFQVKPKIRRWQLYFVGSMWCPGWTNIKGASQTSSRSGVVGKTTFDFVRKAFRAGLITQDRAQWLEN
				Cantir_ALF6*	*Cancer irroratus*	Clark lab	N/A	N/A	KPFNKSILGSVAGSLIKGAIKTKDDLLDHVCVLAKTPVGKSWELHYKAGVTCPGWTTIRGRGSGHTNPNSENAALRDFMEKAVKGGLVTKAEA SPWLDN
				Cantir_ALF7*	*Cancer irroratus*	Clark lab	N/A	N/A	KPFSKSILGSVAGSLIKEA TK TQIELLDHYC VLKRTPVGK SWELHYRA DVSCPGWTTIRGRSGHTNPNSENAALRDFMEKAVKGLV TRAEASPWLDN
		Geocarcinidae	*Gecarcoidea natalis*	Cantir_ALF1*	*Gecarcoidea natalis*	Clark lab	N/A	N/A	QWEALVGPLGKLTG LWHDDVDFMGHVCKFRRPTFKRWELYHGKFWCPGWIA PFSGRSRTKSRSGVINATQDFVRQAFQKGLITEDDANLWLKG
				Gnar_ALF2*	*Gecarcoidea natalis*	Clark lab	N/A	N/A	LD ISNILSSVADTVIERMY VDRITI TIM DHYC TLRRPHFYRWELKWRAAVTCPGW TPSGTAKNYAN IPVSAEREATRDFVKKAVER
	Grapsoidea	Varunoidae	*Helice tientsinensis*	Eriosi_ALF1	*Eriocheir sinensis*	NCBI	DQ793214	ABG82027.1	GWLDRIGTAVDSVA IFG TIN IVDQICNTRVMPTIKKFELYFRGRV WCPGWTTIQGESLTRSRTRV VNKAVEDFARKAVAAGLM IQQEADNPLLNA
				Eriosi_ALF2	*Eriocheir sinensis*	NCBI	AC Y25186.1	ACY25186.1	NFD DDFGKV TETLV DFGTTIDAVGNPCNYRLSPILIKFELYFVGLV WCPGWTTIQGESLTRSRTRV VNKAVEDFARKAVAAGIMTQEDADNPLLNA
				Eriosi_ALF3	*Eriocheir sinensis*	NCBI	HQ850572	ADZ46233.1	QWQALVGPLIKVS SGLWHNDTVDFMGRICKFRRSPKIKRWELYHDGKFWCPGWAPFSGN ISK RSRSGSLEEATRDFVRQAFEKGLITEADAKLWKLQ
				Heltri_ALF1*	*Helice tientsinensis*	Clark lab	N/A	N/A	QWQALVGPEIEKVSGLWHADTVDFMGRICKFRRPTVKRWQLYHDGKFWCPGWA AFSGTSKKSRSRGALEHATRDFVRKAFENGLINEDADKLWIKN
				Helti_ALF2*	*Helice tientsinensis*	Clark lab	N/A	N/A	GWLDRKIPAVSSAVFGSHITLDTCNTRVMPTIKKFEVYFKGRVWCPGWTSIQGESLTRSRSVVNKAVEDFARKAVTAGLMTQEEASNPLLN
				Helti_ALF3*	*Helice tientsinensis*	Clark lab	N/A	N/A	GWLDRIGTAVNSAVIFG SHTLDTCNTRVMPTIKRFEVYFKGRVWCPGWTSIQGESLTRSRSRVVNKAVEDFAKKAVAAGLMTQEEA
				Helti_ALF4*	*Helice tientsinensis*	Clark lab	N/A	N/A	IDFSALSSVADSAINRLYVDRITI VDHYC TLSRSPHFYRWELKWRVTVTCPGWTPIKGTAKNFTASPSTAEREATRDFVRKAVDRGLIEREDATEWL
				Helti_ALF5*	*Helice tientsinensis*	Clark lab	N/A	N/A	NFD DVFGKVTETLVDFG TTIDVPNCNYRVSPRLIKFELYFVGVVWCPGWTTIQGESLTRSRSRVVNKAVEDFARKAVTAGLMTQEEANPLLNA
				Helti_ALF6*	*Helice tientsinensis*	Clark lab	N/A	N/A	NFD DVFGKVTETLVDFG TTIDVPNCNYRVSPRLIKFELYFVGVVWCPGWTTIQGESLTRSRSRVVNKAVEDFARKAVTAGLMTQEEADPLLNA
				Helti_ALF7*	*Helice tientsinensis*	Clark lab	N/A	N/A	IDFSALSSVADSAINRLYVDRITI VDHYC TLSRSPHFYRWELKWRVTVTCPGW TPIKGTAKNFYSPVTA ERATRDFVRKAVDRGLIERDDATEWL
				Helti_ALF8*	*Helice tientsinensis*	Clark lab	N/A	N/A	IDFSALSSVADSAINRLYVDRITI VDHYC TLSRSPHFYRWELKWRVTVTCPGW TPIKGTAKNFANPSTA ERATRDFVRKAVDRGLIERDDATEWL
				Helti_ALF9*	*Helice tientsinensis*	Clark lab	N/A	N/A	GWLDRIGTAVNSAVIFG SHTLDTCNTRVMPTIKKFEVYFKGRVWCPGWTSIQGESLTRSRSRVVNKAVEDFARKAVTAGLMTQEEANPLLN
				Helti_ALF10*	*Helice tientsinensis*	Clark lab	N/A	N/A	GWLDRIGTAVNSAVIFG SHTLDRTCNTRVMPTIKKFQVYFKGRVWCPGWTSIHGESLTRSRSRVVNKAVEDFAKKAVAAGLMTQE
				Helti_ALF11*	*Helice tientsinensis*	Clark lab	N/A	N/A	GWLDRIGPAVSSAVFGSHITLDRTCKTRVMPTIKKFQVYFKGKVWCPGWTSIQGESLTRSRSRVVNKAVEDFARKAVTAGLMTQE
				Helti_ALF12*	*Helice tientsinensis*	Clark lab	N/A	N/A	GWLDRMGTAVSSAVFGSHITLDRTCDTRVMPTIKKFQVYFKGKVWCPGWTSIQGESLTRSRSRVVNKAVEDFARKAVTAGLMTQE
Brachyura		Majoidea	Oregoniidae	Chiono_ALF1*	*Chionoecetes opilio*	Clark lab	N/A	N/A	QVPYAALAALLGIEKLIG LWKSQETFLDHTCH LTVKPKIRWELYYIG RMWCPGLTPIGEALTRSQSGVAGKTVQDFARKALESGVITEED AKIWK T
				Chiono_ALF2*	*Chionoecetes opilio*	Clark lab	N/A	N/A	KPFKGDIGALADVAKDTFQQGEIELLGHYC TYRREPHRKSLELYY RSVWCPGWTSIKGRSDRQNPTHESEAKDFVRKAVAKGLVTKEEAAAYF
				Chiono_ALF3*	*Chionoecetes opilio*	Clark lab	N/A	N/A	APFDKGILTGVLGTLVQD AFKTQEITLLDRYCTLSRRAYISRAELHYKA VVSCPGWTIRKGESGTHNPLNSEKTALKDFMTQAVA RDLVTREDAAPWLD
				Chiono_ALF4*	*Chionoecetes opilio*	Clark lab	N/A	N/A	APSSKGDLTGVLGTIENAFKTQEITLLDRYCTLGERA YLHKVELRYK AEVTCPGWTTIKGKGSGIESNA IN SENDALKDFMTQAVA RDLVTREDAAPWLDH
				Chiono_ALF5*	*Chionoecetes opilio*	Clark lab	N/A	N/A	QWEALVAPVVAKIQGLWHSDHVEFMGHNCQFRRPPKIRKWELYHEGKFWCPGWTPIQGRSRTKSRSGIRSHATKDFVRKAFANGLVTQEDAALWLKNNR
				Chiono_ALF6*	*Chionoecetes opilio*	Clark lab	N/A	N/A	QGWAAVAAA YASKVVGLWQNGHVDLLDHPCRFSVKPTVRRFQLYFKGRMWCPGWTSIRGEAK TKSRSGVVGKTTRDFVNKAFQAGLITERDAQQWLSH
				Chiono_ALF7*	*Chionoecetes opilio*	Clark lab	N/A	N/A	QGWETLVAGVSSQLVSLWHQGELELMGHYCNFQVKPKIRRWQLYFVGSMWCPGWTNIRGAQTRSRSGVVGKTTFDFVRKAFRAGLITQDRAQWLEN
				Chiono_ALF8*	*Chionoecetes opilio*	Clark lab	N/A	N/A	QGWEALPAFVDKLSGLWNGEVELLGRHYCSFAVKPKIRSWELYFKGSMWCPGWTNIRGSAQTRSRSGVVGETTDFVRKAFEAGLITQDRAQEWLDN
				Chiono_ALF9*	*Chionoecetes opilio*	Clark lab	N/A	N/A	GFDLSSILSSVAGDAVNRLYVDREINLMDRYCVMSRRPHFHRWELKWRSTVTCPGWTPKGRA SGYSSPTTSERE ATKDFVTQAVATGLIQEEADTEWL
				Chiono_ALF10*	*Chionoecetes opilio*	Clark lab	N/A	N/A	GFDLSQVLSSVAGSVVDRLYVDREITLMDRYCVMSRQPGFHWELKWRTTVTCPGWTPKGRASGYANPTSEQEA TKDFVKQA VERGLVPKEDA TEWL
				Chiono_ALF11*	*Chionoecetes opilio*	Clark lab	N/A	N/A	GWADFIFDKVKDVLVEIGTTEVLDKVCNFRVLPRLKGLELSFGDLWCPGWTSIRGDSLTRSRTRVVNKAEDFAKKALEAGLIL
				Hyasaran_ALF1	*Hyas araneus*	Matos et al., 2018	HACG01101 0733	N/A	QADYAALAVLLGKKJVGLWRSQETFLDHKCHLTVKPKIRWQLYYIGRSWCPGLTPIGEALTRSQSGVAGKTVQDFARKALESGVITEEDAKIWY RT
				Hyasara_ALF2	*Hyas araneus*	Matos et al., 2018	HAA.B01 003816	N/A	QWEALVAPVVAKIQGLWHSDHVEFMGHNCQFRRPPKIRKWELYHEGKFWCPGWAPFEGRSRTKSRSGSSSEATKDFVRKAFANGLVTQEDAALWLKNNR
				Hyasara_ALF3	*Hyas araneus*	Matos et al., 2018	HAA.B01 002604	N/A	GFDLSSILSSVAGNAVDRLYVDQEITLMDKYCVMSRRPHFYRWELKWRTTVTCPGWTPIKGRASGYSSPTSERE ATKDFVKQAVARGLITKEDATEWL
				Hyasara_ALF4	*Hyas araneus*	Matos et al., 2018	HAA.B01 017501	N/A	GLFDFIFDKVQDVLVIEG TTEVLDKVCNFRVLPRLKGDLWCPGWTSIRGDSLTRSRTRVVNKAEDFAKKALAEGALILEVDAQTLVNA
				Hyasara_ALF5	*Hyas araneus*	Matos et al., 2018	HAA.B01 005456	N/A	GFFDRLFDKVIDTLVEEGTVEVLDNICNFRVLPRLKGKLSLSYKGALWCPGWTSIRGTESLTSSRTRVVNKAIEDFAKKALKAGLILIA DAQTLVNA
				Hyasara_ALF6	*Hyas araneus*	Matos et al., 2018	HACG01102 9590	N/A	APGLGDLLTGVLGTLIKDV IK TQEITLLDRY CTLSRRGYISRAELHYRAVVSCPGWTTIRGKGSGHTNPLNSENAALKDFMTQAVARDLVTPEDAAPWLRR
				Hyasara_ALF7*	*Hyas araneus*	Clark lab	N/A	N/A	APGLGDLLTGVLGTLIKDV IK TQEITLLDRY CTLSRRAYISRAELHYRAVVSCPGWTTIRGKGSGHTNPLNSENAALKDFMTQAVARDLVTPEDAAPWLRR
				Hyasara_ALF2*	*Hyas araneus*	Clark lab	N/A	N/A	QGWAAVAAA YASKVVGLWQNGHVDLLDHPCRFSVKPTVRRFQLYFKGRMWCPGWTSIRGEAK TKSRSGVVGKTTRDFVNKAFQAGLITERDAQQWLSH
				Hyasara_ALF3*	*Hyas araneus*	Clark lab	N/A	N/A	AQGWETLVAGVSSQLVSLWHQGELELMGHYCNFQVKPKIRRWQLYFVGSMWCPGWTNIRGAQTRSRSGVVGKTTFDFVRKAFRAGLITQDRAQWLEN
				Hyasara_ALF4*	*Hyas araneus*	Clark lab	N/A	N/A	GFLDRLFDKVIDTLVEEGTIEVLDNICNFRVLPRLKGDLSLSYKGALWCPGWTSIRGESLTSSRTRVVNKAIEDFAKKALKAGLILIVDAQTLVNA
				Hyasara_ALF5*	*Hyas araneus*	Clark lab	N/A	N/A	GWGDFIFKKVKDALIEDGTTEVLDKVCNFRVLPRLKGKLELFYRGDLWCPGWTSIRGDSLTRSRTRVVNKAIEDF
				Hyasara_ALF6*	*Hyas araneus*	Clark lab	N/A	N/A	APGLGDLLTGVLGTLIKDV IK TQEITLLDRY CTLSRRGVYSRAELHYRAVVSCPGWTTIRGKGSGHTNPLNSENAALKDFMTQAVARDLVTPEDAAPWLRR
				Hyasara_ALF7*	*Hyas araneus*	Clark lab	N/A	N/A	GFDLSNILSSVAGDAVDRLYVDREITLDRYCVMSRRPHFYRWELKWRTTVTCPGWTPIKGRASGYASPVTSERE ATKDFVKQAVERGLITKEDAAEWL
				Hyasara_ALF8*	*Hyas araneus*	Clark lab	N/A	N/A	GFDLSNILSSVAASVVDRLYVDREITLVDRYCVMSRQPHFYRWELKWRTTVTCPGWTPIKGRASGYANPVTSERE ATRDFVKQAVERGLITKEDAAIWL
				Hyasara_ALF9*	*Hyas araneus*	Clark lab	N/A	N/A	GFLDRLFDKVIDTLVEEGTIEVLDNICNFRVLPRLKGKLSLSYKGALWCPGWTSIRGESLTSSRTRVVNKAIEDFAKKALKAGLILIVDAQTLVNA
				Hyasara_ALF10*	*Hyas araneus*	Clark lab	N/A	N/A	GFDLSNILSSVAGDAVDRLYVDREITLDRYCVMSRRPHFYRWELKWRTTVTCPGWTPIKGRASGYSSPTTSERE ATKDFVKQAVARGLIPKEDATEWL
				Hyasara_ALF11*	*Hyas araneus*	Clark lab	N/A	N/A	GWGDFIFKKVKDVLVEIGTTEVLDKVCNFRVLPRLKGKLELFYRGDLWCPGWTSIRGDSLTRSRTRVVNKAIEDF
				Hyasara_ALF12*	*Hyas araneus*	Clark lab	N/A	N/A	APGLGDLLTGVLGTLIKDV IK TQEITLLDRY CTLSRRGVYSRAELHYRAVVSCPGWTTIRGKGSGHTNPLNSENAALKDFMTQAVARDLVTPEDAAPWLRR
		Portunoidea	Portunidae	Calsap_ALF1	*Callinectes sapidus*	Matos et al., 2018	CV467249	N/A	QYEALVASRLGKLTGLWHNNTVTFMGHTCHFRRPIKPRKWKLVHEGKFWCPGWAPTIGRSRTKSRSGSAREATKDFVRKALRNGLISQNDASVWLRN
				Callap_ALF2	*Callinectes sapidus*	Matos et al., 2018	GH1D01 006178	N/A	QDWESLAVSKGRLVGLWRNEITFLGRICQLTVKPKIKQLYHEGRMWCPGWTPIQEALTRSFSGVAGNTVKDFVRKAHDKGLVTEDAKVWLEA
				Callap_ALF3	*Callinectes sapidus*	Matos et al., 2018	GH1D01 102301	N/A	ELK DLLFKAKNALIEDGRTELDHVCNYRVMPSRSWELYFKGDVWCPGWTSLIRSRTRVVNKAIDFAQKALAGGLITQEDADPLLE
				Callap_ALF4	*Callinectes sapidus*	Clark lab	N/A	N/A	QYEALATVMQKLAGLWHNDTLDFLGHTCHSRKPTVRRLKLYWKGKFWCPG WVPFSGSSRNK SRSGSTRGATEDFVKQALQRGLVTESEVAAWLKG
				Carmae_ALF1	*Carcinus maenas*	Matos et al., 2018	DV340354	N/A	QYEALASVLGKLTGLWHNNSVDFLGHTCQFRPKLKRWKLYHEGKFWCPGWAPEIRSRTKSRSGAAEDATKDFVQKAFKQGLVSEEEASWLIS
				Carmae_ALF2	*Carcinus maenas*	Matos et al., 2018	GFX.P0134028	N/A	QDWESLAVSKGRLVGLWRNQETEVMGHKCTLTVKPKIKSFQLYHEGRMWCPGWTPI

Table 4 Continued. Supplementary information of 291 ALF sequences used in this study.

Suborder	Infraorder	Superfamily	Family	Abbreviation	Species	Source	NCBI identification of nucleotide sequence	NCBI identification of amino acid sequence	Mature peptide sequence
Pleocyemata	Brachyura	Portunoidea	Portunidae	Charfer_ALF1	*Charybdis feriatus*	NCBI	KP688577	AKF40834.1	QYEALVASILGKLSGLWHSDTVDFMGHTCHIRRKPKFRKFKLYHEGKFWCPGWTHLIGNSRTKSRSGSTREATKDFVHKALQNKLITKNSADAWLKG
Charfer_ALF2	*Charybdis feriatus*	Matos et al., 2018	GGFD01017303	N/A	FLKDLIFGEVKKALVEDGTTEILDRVCNFRVMPRLRRFELYFRGDVWCPGWTIKGESLTRSRTRVVNKAIDFAKKALAQQLITQEDAQPLLE
Charfer_ALF1*	*Charybdis feriatus*	Clark lab	N/A	N/A	FLKDLIFGEAKKALLDEGTTEILDHVCNFRVMPRIRSWELYFRGDVWCPGWTVIKGESLTRSRTRVVNKAVADFAQKALAQGLITQEDAQPLLE
Charfer_ALF2*	*Charybdis feriatus*	Clark lab	N/A	N/A	FVKELLFKVKDATLHEDGTTEILDRVCNYRVVPGLENLEVYFKGDVWCPGWTVIKGESLTRSRTKVVDKAIDFAKKALAQGLVTQEDYQPLLQ
Charfer_ALF3*	*Charybdis feriatus*	Clark lab	N/A	N/A	FLKDLIFGEVKKALVEDGTTEILDRVCNFRVMPRLRSFELYFRGDVWCPGWTIKGESLTRSRTRVVNKAIDFAKKALA
Charfer_ALF4*	*Charybdis feriatus*	Clark lab	N/A	N/A	QYDALIASILGKLTGLWHNNSVDFMGHTCHFRRRLEIRRLKLYHKGKFWCPGWVPFEGTSRTSRSRGSVREATKDFVRKALQNGVISQQDATLWLTN
Charfer_ALF5*	*Charybdis feriatus*	Clark lab	N/A	N/A	FDTFSFISTAADHVIKRLVYDRETILFDRYLVLSRSPHIYKWELKWEATVTCPGWTPEKGKVRGYSNPLSAIREATRDFVQRIVKRGLVTREEARRWV
Charfer_ALF6*	*Charybdis feriatus*	Clark lab	N/A	N/A	APFGFKDILGSIKPASDAKQTTELTILDHYCRMTRRPVIKRLELHYRAEVSCPEWTTLKGRGSNHTNATNSENDALKDFMNKALKAGLVTREEAKPWENP
Pospel_ALF	*Portunus pelagicus*	NCBI	JQ899452	AFM77968.1	QYEALVASILGKLSGLWHSDTVDFMGHTCHFFRKPKFRKFKLYHEGKFWCLAGHLLFNSRSKSRSGSTREATKDFVHKALQNKLITKNSADVWLKG
Posnag_ALF1*	*Portunus sanguinolentus*	Clark lab	N/A	N/A	FLKDLIFGEAKKALLEDGTTEILDHVCNFRVMPRIRWELYFKGDVWCPGWTVIKGESLTRSRTRVVNKAVADFAQKALAQGLITQEDAQPLLE
Posnag_ALF2*	*Portunus sanguinolentus*	Clark lab	N/A	N/A	HSLVKEKIFKEVKDALHEDGTTEILDRVCNYRVVPRLESLELYFKGDVWCPGWTVIKGESLTRSRTMVVNKAIADFAKKAVTQQLITQEDAQPLL
Posnag_ALF3*	*Portunus sanguinolentus*	Clark lab	N/A	N/A	QYEALVASILGKLTGLWHNNTVDFMGHTCHFRRRPKFRKFKLYHEGKFWCPGWAPFDGRSRTKSRSGSARDATKDFVRKALQNGLVTQEDASLWLNS
Posnag_ALF4*	*Portunus sanguinolentus*	Clark lab	N/A	N/A	QYEALVASILGKLTGLWHNNTIVDFMGHTCHFRRRPKFRKFKLYHEGKFWCPGWAPFDGRSRTKSRSGSARDATKDFVRKALQNGLVTQEDASLWLNN
Posnag_ALF5*	*Portunus sanguinolentus*	Clark lab	N/A	N/A	FLKDLIFGEAKKALLEDGTTEILDHVCNFRVMPRIRRWELYFKGDVWCPGWTVIKGESLTRSRTRVVNKAVADFAQKALARGLITQEDAQPLLE
Posnag_ALF6*	*Portunus sanguinolentus*	Clark lab	N/A	N/A	FDTFSFISTAGNTVIKRLYLDQEINLFDHYCLMSRSPHISNWELKWEASVNCIGWTPEKGKVRGYSNPMSAETEATREFVKKIVQKGLVTREEASEWL
Potstj_ALF1*	*Portunus trituberculatus*	Clark lab	N/A	N/A	GILLDIVKSIVVPAAREITKTQDITLLDHYCTLSRSPYIKRFELHYRADVTCPGWTSKGRGNSHTNPTNSEKDALKDFMTQAVAAGLVTKEEAAPWLNHR
Potstj_ALF2*	*Portunus trituberculatus*	Clark lab	N/A	N/A	FDSSSFISTADTVIKRLVYDQEINLFDHYCLSRSPHISRWELKWQATVTCPGWTPYKGKVRGVSNPLSAIREATRDFVQRIVQRGLVTRDEASEWL
Potstj_ALF3	*Portunus trituberculatus*	NCBI	GQ165621	ADU23042.1	QYEALVTSILGKLTGLWHNDSVDFMGHTCYFRRRPKIRRFKLYHEGKFWCPGWAPFEGRSRTKSRSGSREATKDFVRKALQNGLVTQQDASLWLNN
Potstj_ALF4	*Portunus trituberculatus*	NCBI	JF736050	AFA42332.1	GWLDIVKAIVVPAAREITKTQDITLLDHYCTLSRSPYIKSLELHYRAEVTCPGWTIRKGRGSNHRNPTNSGKDALKDFMTQAVAAGLVTKEEAAPWLNHR
Potstj_ALF5	*Portunus trituberculatus*	NCBI	JF736051	AFA42333.1	ASFDISSFISTADTVIKRLVYDRETILFDHYCLMSRSPHISRWELKWQATVTCPGWTPYKGKVRGYSNPLSAIREATRDFVQRGLVTRDEASEWL
Potstj_ALF6	*Portunus trituberculatus*	NCBI	JF736052	AFA42334.1	FLKDLLFGEAKTALLEDGTTEILDHYCNFRVMPRLRSWELYFRGDVWCPGWTVIKGESLTRSRTRVVNKAVADFAQKALAQGLITQEDAQPLLE
Potstj_ALF7	*Portunus trituberculatus*	NCBI	JF736057	AFA42335.1	QYEALTAILTKLSKMWHSDTLNFLGHTCHVSRTPTVKRFKLYWKGKFWCPGWAPFSGTSRTSRSGSARETADFVQKIVRRGLVTREEASEWL
Scyol_ALF1	*Scylla olivacea*	Matos et al., 2018	GDRM01072213	N/A	QYEALVASVLGKLTGLWHNNSVNFMGHTCHFRRRPKVRKFKLYHEGKFWCPGWAPFEGRSRTKSRSGSAREATKDFVRKALQNGLITQQDATVWLNN
Scyol_ALF2	*Scylla olivacea*	Matos et al., 2018	GDRM01007616	N/A	FLKDLLFGEAKKALLDEGNTIELDHVCNYRVMPRFKNWELYFRGDVWCPGWTVIKGESLTRSRTRVVNKAVADFARKAVQGLITQEDAQPLLE
Scyol_ALF3	*Scylla olivacea*	Matos et al., 2018	GDRM01065957	N/A	GSFDFSSLISSVANTAIKRLVYERIEILFDHYCVLSRSPHIYRWELKFEATVTCPGWTPEKGRVRGYSSPTSAIREEATRDFVQKIVRRGLVTREEASEWL
Scymon_ALF1	*Scylla olivacea*	Clark lab	N/A	N/A	QYEALVASILGKLSGLWHSDTVDFMGHTCHIRRKPKFRKFKLYHEGKFWCPGWTHLIGNSRTKSRSGSTREATKDFVRKALQNGLITQQDATVWLNN
Scypam_ALF1	*Scylla paramamosain*	NCBI	EF207786	ABP96981.1	QYEALVASILGKLSGLWHSDTVDFMGHTCHIRRRPKFRKFKLYHEGKFWCPGWTHLIGNSRTKSRSGSARDATKDFVYKALQNKLITNSNAAAWLKG
Scypam_ALF2	*Scylla paramamosain*	NCBI	HM345950	ADT71676.1	FLKDLLFGEAKKALLDEGNTIELDHVCNYRVMPRFKDWELYFRGDVWCPGWTSKGESLTRSRTRVVNKAVADFARKAVAQGLITQEDAQPLLE
Scypam_ALF3	*Scylla paramamosain*	NCBI	JQ069030	AFH37796.1	QYETLIASVLGKLTGLWHNNSVDFMGHTCHFRRRPKVRKFKLYHEGKFWCPGWAPFEGRSRTKSRSGSSEEAKDFVRKALQNGLITQQDATVWVNN
Scypam_ALF4	*Scylla paramamosain*	NCBI	KF484747	AIB62419.1	GSFDFSSLISSVANTAIKRLFVFERIEILFDHYCVLSRSPHIYSWELKFEATVTCPGWTPEKGRVRGYSSPTSAEREATKDFVQKIVRRGLVTREEASEWL
Scysur_ALF5	*Scylla paramamosain*	NCBI	HF952161	CCW43202.1	GIGDIFQDVAISLISEIFKVKEITLLDHYCVLSRSYBLRGLKLLYRATLTCPEWSTLKGRGDESSAVNAENNABNYMEQAVANGLVTKEEAQPWLH
Scysur_ALF1	*Scylla serrata*	Matos et al., 2018	HQ638024	N/A	QYEALVASILGKLSGLWHSDTVDFMGHTCHIRRKPKFRKFKLYHEGKFWCPGWTHLIGNSRTKSRSGSARDATKDFVYKALQNKLITENSNAAAWLKG
Scysur_ALF2	*Scylla serrata*	NCBI	HF952161	B5TTX7.1	QYEALVASILGKLSGLWHSDTVDFMGHTCHIRRKPKFRKFKLYHEGKFWCPGWTHLIGNSRTKSRSGSTREATKDFVHKALQNKLITKNSADAWLKG
Scytun_ALF	*Scylla tranquebarica*	NCBI	JQ899453	AFM77969.1	QYEALVASILGKLSGLWHSDTVDFMGHTCHFFRKPKFRKFKLYHEGKFWCPGWTHLIGNSRSKSRSGSTREATKDFVHKALQNKLITKNSADVWLKG
Ovoalo_ALF1*	*Ovalipes ocellatus*	Clark lab	N/A	N/A	GFDFSSLISSVADNVIERLVYDRETILDHYCVMSRTPHFYRWELKWESTVTCPGWTPVKGRASGFASPTSREATKDFVRQIVARGLVTREEARJWL
Ovoalo_ALF2*	*Ovalipes ocellatus*	Clark lab	N/A	N/A	KPFSKIDILKGAAGSLIKEAIQSREINLLDHYCVMERSPVLKRLELHYRAEVSCPGWTTIGRGSNHTNATNSENDALVDFMQKAVKAGLVTRKEASPWLDH
Ovoalo_ALF3*	*Ovalipes ocellatus*	Clark lab	N/A	N/A	FLKDLLIGEVKDALLDDGTTEILDHLCNYRVLPRLRRWEIYFRGDVWCPGWTSIKGESFTRSRTRVVTKAVADFAQKAVAQGLITQAEAEPLLE
Ovoalo_ALF4*	*Ovalipes ocellatus*	Clark lab	N/A	N/A	QYEALIASVLGKLTGLWHNNTIVDFMGHTCHFRRRPKVRKFKLYHEGKFWCPGWAPFDGRSRTLSRSGAASEATKDFVRKALQNGLITQQDATLWLNS
Ovoalo_ALF5*	*Ovalipes ocellatus*	Clark lab	N/A	N/A	QYEALIASVLGKLTGLWHNNTVDFMGHTCHFRRRPKVRKFKLYHEGKFWCPGWAPFDGRSKTKSRSGSAREATKDFVRKALQRGLIKQEDASRWINS
Ocypodoidea	Ocypodidae	Ocypodidae	Uospu_ALF1	*Uca pugilator*	Matos et al., 2018	JO476301	N/A	QGGVFDFLEFTIVKDAIKVTEIDLLGHYCTLRRQPYVKRFELYYRAWLNCPGWTTI
			Uospu_ALF2	*Uca pugilator*	Matos et al., 2018	JO477865	N/A	SWTDKLTDTLVNSLVDTGTTEILDYVCNYNYRPTKRWELYFGHVWCPGWTSIRGESETSRSRSSVVNKAVEDFIRKAVAGLVTEEDAQPFLNA
			Uospu_ALF3	*Uca pugilator*	Matos et al., 2018	JO476724	N/A	QYEALVGAVLTKISGLWHDNTVDFMGHTCRFRRRPTIKRFQLYHEGKFWCPGWAPFSGRSKTRSRSGSVEQATRDFVRQAFQNRLISEEDARLWLKG
			Uospu_ALF4	*Uca pugilator*	Matos et al., 2018	JO476440	N/A	QYEALVGALFQKIAGLWHDDTVDFMGHTCRFSRSPTIYRWEDHYKGKFWCPGWSPSGNSRTKSRSGSVEQATKDFVRQAFENGLINEEDARIWLQN
	Orithyioidea	Orithyiidae	Orivi_ALF1*	*Orithyia sinica*	Clark lab	N/A	N/A	N/A	QYEALVGPLIAKISGLWHNDTVDFMGHQCQFRRRPKISRWKLYHEGKFWCPGWAPFSGNSRTKSRSGSVEQATKDFVRQAFANGLITQEDASLWLNN
			Orivi_ALF2*	*Orithyia sinica*	Clark lab	N/A	N/A	N/A	FLKDLLIDKAKDALFEDGTTEVLDRKCNFRVMPRLRRLELYFRGDVWCPGWTTIKGESLTRSRTRVVNKAIGDFAKKALAAGLITEEAARPLIE
	Xanthoidea	Panopeidae	Dyspsp_ALF1*	*Dyspanopeus sayi*	Clark lab	N/A	N/A	N/A	KPIDLTSILSSATDQLKVFIFDQNEMELLGHYCLYATWATFKDWKLRYKCGVKCPGWTPIVGTAEDADKPTAAERGATRNFVEQALESKLITEEAARSWLV
			Dyspsp_ALF2*	*Dyspanopeus sayi*	Clark lab	N/A	N/A	N/A	QWEALVGPVIKKISGLWHSDSVDFMGHTCNFRRRPKIRKFKLYHEGKFWCPGWATFEGRSRTKSRSGSVREATKDFVNQAFSEQDAKLWLNKHG
			Dyspsp_ALF3*	*Dyspanopeus sayi*	Clark lab	N/A	N/A	N/A	QGWEVLALAVGEKLVDLWKSDHLIFMGHQCNYSVNPKVKRFQLYFRGRMWCPGWTTIRGEAETRSRSGVVGKTTQDFARKAFIKGLITEQAARDWLNA
			Dyspsp_ALF4*	*Dyspanopeus sayi*	Clark lab	N/A	N/A	N/A	FWKELLGGPIKDALIEEGTRDVKGHVCNYRVMPRLRDWELYFRGDVWCSGWTSIKGESFTRSRTSVVNKAFADFARKAVAAGVMVQEDADYLLH
	Cotmedi_ALF	Cotmedidae	Cotmedi_ALF1	*Cardiosoma armatum*	JABX01113740	Matos et al., 2018	N/A	N/A	DIWEGLVPTLVGAIGQLWSIGEMIDFMGHYVCFYNVRPTIRRKWELYFNGSVWCPGWTIRGGSSTRSRSGSVVNAATEDFVRKAIQSGLITEEDADWLTRG
Aytoidea	Aytoidae	Aytoidae	Patsus_ALF1*	*Paratya australiensis*	Clark lab	N/A	N/A	N/A	FLKDLLPGEAKKALLDEGTTEILDHVCNFRVMPRLRSWELYFRGDVWCPGWTVIKGESLTRSRTRVVNKAVDFAQKALAQGLITQEDAQPLLE
			Patsus_ALF2*	*Paratya australiensis*	Clark lab	N/A	N/A	N/A	SQDYLKEELFNEVKDALFDFGATEILDRVCNYRVMPRFENWRFYFKGDVWCPGWTFIKGESLTRSRTRVVNKAIADFAQKALAQGLITQEDAQPLLE
			Patsus_ALF3*	*Paratya australiensis*	Clark lab	N/A	N/A	N/A	FVKELFKEVKDTLHDEGTTEILDRVCNYRVVPRLISELLYFKGDVWCPGWTVIKGESLTRSRTRVVNKAIADFAQKALAQGLITQEDAQPLLE
			Patsus_ALF4*	*Paratya australiensis*	Clark lab	N/A	N/A	N/A	FVKELLPKEVKDTLHDEGTTEILDRVCNYRVVPRLISELLYFKGDVWCPGWTVIKGESLTRSRTRVVNKAIADFAKKAVTQQLITQEDAQPLLE
			Patsus_ALF5*	*Paratya australiensis*	Clark lab	N/A	N/A	N/A	GWLDIVKAIVVPAAREITKTQDITLLDHYCTLSRSPVIKSLELHYRADVTCPGWTSIKGRGSNHTNPTNSEKDALKDFMTQAVAAGLVTKEEAAPWLNHR
			Patsus_ALF6*	*Paratya australiensis*	Clark lab	N/A	N/A	N/A	GWLDIVKAIVVPAAREITKTQDITLLDHYCTLSRSPVIKSLELHYRADVTCPGWTSIKGRGSNHRNPTNSEKDALKDFMTQAVAAGLVTKEEAAPWLNHR
			Patsus_ALF7*	*Paratya australiensis*	Clark lab	N/A	N/A	N/A	QYEALVTSILGKLTGLWHNDSVDFMGHKCYFRRRPKIRRFKLYHEGKFWCPGWAPFEGRSRTKSRSGSREATKDFVRKALQNGLVTQQDASLWLNN
			Patsus_ALF8*	*Paratya australiensis*	Clark lab	N/A	N/A	N/A	QYEALTAILTKLSKMWHSDTLNFLGHTCHVSRTPTVKRFKLYWKGKFWCPGWAPFSGTSRTSRSGSARETADFVQKIVRRGLVTREEASEWL
			Patsus_ALF9*	*Paratya australiensis*	Clark lab	N/A	N/A	N/A	QYEALTAILTKLSKMWHSDTLNFLGHTCHVSRTPTVKRFKLYWKGKFWCPGWAPFSGTSRTSRSGSARETADFVQKIVRRGLVTREEASEWL
			Patsus_ALF10*	*Paratya australiensis*	Clark lab	N/A	N/A	N/A	FDTFSFISTADTVIKRLVYDRETILFDHYCTLSRSPHISRWELKWQATVTCPGWTPEKGKVRGYSNPLSAIREATRDFVQRGLVTREEASEWL
Caridea	Palaemonoidea	Palaemonidae	Exspal_ALF*	*Exopalaemon car incanda*	Matos et al., 2018	N/A	N/A	N/A	KPFDIGSILIDTLPAIVDGLYSEDTIELFGRACQYKRIPYIKRLELHYRAEVRCLGWTPIVGKARDIHRNPTNAEQAIKDFARQALAQGLITEESKPWLSR
			Exspal_ALF1	*Exopalaemon car incanda*	Matos et al., 2018	JX123494	N/A	N/A	QDLSVAGLLGGLTGNELVEATVIKKLVGLWKTGDTVLMDRVCSYQVKPTRKFELYFKGTWCPGWTPIRGKSLTRSNSNAVNDAVRDFVQQAMAKGVITEEAAREWLSQIRK
			Exspal_ALF2	*Exopalaemon car incanda*	Matos et al., 2018	N/A	N/A	N/A	QDLSVAGLLGGLTGNELVEATVTKKLVGLWKTGDTVLMDRVCSYQVKPTRKFELYFKGTWCPGWTPIRGKSLTRSNSNAVNDAVRDFVQQAMAKGVITEEAAREWLSQIRK
			Exspal_ALF3	*Exopalaemon car incanda*	Matos et al., 2018	N/A	N/A	N/A	QAWQAVAAAVATIKIAGLWVNDEMELLGHTCNYRVDPKIKRFQLYFKGRMWCPGWTPIRGEAETRSRSGVVGKTTSDFVTKAFKSGLISEDDARAWLNSKK
			Exspal_ALF4	*Exopalaemon car incanda*	Matos et al., 2018	N/A	N/A	N/A	LDWSDLKSTLDFTRIQMYVEGDMELLDQYCVYKRIGYFYKWELNYKAEVRCPGWTPIIGRAKNYVSPSSAESEATKDFVRQAIAAGLITREEAQRWLD
			Exspal_ALF5	*Exopalaemon car incanda*	Matos et al., 2018	N/A	N/A	N/A	KPFDIGSLLITLPAIVDGLYSEDTIELFGRACQYKRIPYIKRLELHYRAEVRCLGWTPIVGKARDIHRNPTNAEQAIKDFARQALAQGLITEESKPWLSR
			Macmip_ALF1*	*Macrobrachium nipponense*	Clark lab	N/A	N/A	N/A	QGVSDLVGGLTGSGLVEALTSKIVGLWRSGDIVFLDHVCSFQVKPKLRKFQLYFEGKFWCPGWTPIKGEALTRSNSSVVNKAVADFLQKALANGLITEDEARAWLQQ
			Macmip_ALF2*	*Macrobrachium nipponense*	Clark lab	N/A	N/A	N/A	QGVSDLVGGLTGSGLVEALTSKIVGLWRSGDIVFLDHVCSFQVTPKIRKFQLYFEGKFWCPGWTSIKGEALTRSNSSVVNKAVADFLQKALANGLITEDEARAWLQQ
			Macmip_ALF3*	*Macrobrachium nipponense*	Clark lab	N/A	N/A	N/A	QGLADLLGGSTRDILVQSLTSQLVGLWETGDLIFLDHVCSFQVKPKLRKFKLYWKGKFWCPGWTPIGTSETRSRTSSVVNEAVADFVQKALSSGLVTEEARNWLNHH
			Macmip_ALF4	*Macrobrachium nipponense*	NCBI	KF696305	AJO05394.1	N/A	KPLDWTELTPIPGLARSALYTEGDMELLDHYCVYKRTGYFYKWELIYKAEVRCPGWTKIGKAKNHLNPTSAERDATEDFVRKVVAAGLVTEEEALPWLM
			Macmip_ALF2	*Macrobrachium nipponense*	NCBI	KR013242	ALF02818.1	N/A	VGRGPKGVADLLGGSTRDELVQSLTSQLVGLWETGDLIFLDHVCSFQVKPKIKRWELYFRGTMWCPGWTPIRGTSETRSRSSVVNRAVADFVQKALTSGLVTEEARNWLNHQ
			Macmip_ALF3	*Macrobrachium nipponense*	NCBI	KF696306	AJO05395.1	N/A	QGWEVVAAAVAEKIAGLWVDDHMTFLGHTCQYSVNPKVKRFQLYFRGRMWCPGWTSIRGEAETRSRSGVVTKTTQDFVRKAFESGLVTEEQAEAWLNNK
			Macmip_ALF4	*Macrobrachium nipponense*	NCBI	KR013241	ALF02817.1	N/A	QGVSDLVGGLTGSGLVEALTSKIVGLWRSGDIVFLDHVCSFQVKPKLRKFQLYFEGKFWCPGWTPIKGEALTRSNSSVVNKAVADFLQKALANGLITEDEARAWLQQ
			Macmip_ALF5	*Macrobrachium nipponense*	NCBI	KR013245	ALF02821.1	N/A	KPLDILSLVTGVVPKIVDGVYDEGDIDLFGKPCQYKRIPYIKRLELHYRAEIRCLGWTPIVGEARDIHRNPTIAEQAAKDFVRQAMAQALVTEEQAKPWLTD
			Macrob_ALF*	*Macrobrachium olfersii*	Clark lab	N/A	N/A	N/A	EAIGQQLDDLLGGVGQLSVSALTSQLVGLWETGDLIFLDHVCSFQVKPKIKRWELYFRGTMWCPGWTPIRGTSETRSRTSVVNKAVTDFVQKALRSGLVTEEARNWLSHQ
			Macrol_ALF	*Macrobrachium olfersii*	NCBI	EU289220	ABY20736.1	N/A	QGWEAVAAAVAEKIAGLWVNDIMVFLGHTCQYSVTPRIKRLIELWFKGRMWCPGWTAIRGEADTRSRSGVVTKTTQDFVRKAFESGIVTEAQAKAWLNSK
			Macros_ALF*	*Macrobrachium rosenbergii*	Clark lab	N/A	N/A	N/A	EAIGQDLGDLLGGLTGDGLVQALTSQLVGLWESGDLEFLDHVCSFQVKPKIKRWELYFRGTMWCPGWTPIRGTSETRSRSGVVNRAVADFVQKALASGLVTEEARNWLSHQ
			Macros_ALF1	*Macrobrachium rosenbergii*	Matos et al., 2018	JN572544	N/A	N/A	QGWEAVAAAVASKIVGLWRNEKTELGHHCQFTKPVRKRFQLYFKGRMWCPGWTAIRGEASTRSHSGVAGRTAQDFVRKAFQKGLISQQDAWQWLNS
			Macros_ALF2(ALF9)	*Macrobrachium rosenbergii*	Matos et al., 2018	KR013241	N/A	N/A	QGVADLVGGLTGSGLVEALTSRIVGLWRTGDIVFLDHVCSFQVKPKLRKLQLYFDGTFWCPGWTPIKGEALTRSNSSVVNKAVQDFLQKALASGLITSEDARAWLKH
			Macros_ALF3(ALF6)	*Macrobrachium rosenbergii*	NCBI	GQ377840	ADB80708.1	N/A	KPIDWSDILKSTFEFTRSQLYVQGDMELLDHYCIYKRTGYFYKWELHYKAEVRCPGWTNIGTAKNHKNPTTAEREATVDFVRKVIAAGHVTEEAALPWFV
			Macros_ALF4(ALF8)	*Macrobrachium rosenbergii*	NCBI	ACC60660	ACC60660.2	N/A	QGWEVVAAAVAEKLAGLWVDDQMVFLGHTCQYSVNPKVKRFQLYFKGRMWCPGWTTIRGEAETRSRSGVVTKTTQDFARKAFEKGLITEQQARDWLNA
			Macros_ALF5	*Macrobrachium rosenbergii*	NCBI	JQ412135	AFW04104.1	N/A	EAIGQDLGDLLGGLTGDGLVQALTSQLVGLWESGDLEFLDHVCSFQVKPRIKRWELYFRGTMWCPGWTPIRGTSETRSRSGVVNRAVADFVQKALASGLVTEEARNWLSHQ
			Macros_ALF7	*Macrobrachium rosenbergii*	NCBI	JQ412137	AFW04106.1	N/A	TSFLDLILPTLTTHFVKLWRQGDLELLGKYCTYNMRPFKNWKLYYSASVICPGWTQIRGSAKASSMTAVLNQAIEDFLTKALKAGVVKREEARTYYGK
		Palaemonidae	Palarg_ALF1	*Palaemon argentinus*	Matos et al., 2018	GJFP01027500	N/A	N/A	QDLSDLHSLTGKILWGSLASKVAIEWRTGEVVFMDRVCSYQVKPDLKKFQLYFGSFWCPGWTSI
			Palarg_ALF2	*Palaemon argentinus*	Matos et al., 2018	GJFP01012563	N/A	N/A	QSWEAVAVAVAEKIAGLWVNDEMELLGHTCKYRVQPTIKRFQLYFKGRMWCPGWTPIRGEAETRSRSGVVQKTTQDFVKKAFQSGISEEARIWLNSRK
			Palarg_ALF3	*Palaemon argentinus*	Matos et al., 2018	GJFP01020690	N/A	N/A	QNLVDLLGGLSGNSLGEAVGSQLAGLWGSGELLFLDHVCNFQVKPKFRKWQLKFVATVYCPGWTTRGTSDTSSRTNAVNLAAADFSRKAIVEGLITEEQATEWLRYN
			Palarg_ALF4	*Palaemon argentinus*	Matos et al., 2018	GJFP01021225	N/A	N/A	KPINDWSLLTPSIPKLIDGLYYYDDIDLFGRVCQYKRIPYIKRLELHYRAEVRCLGWTPIVGKACGYNPTNAEQAIKDFARQALGQGLISEEEAKPWLSR
		Pandalidae	Panbar_ALF1*	*Pandalus borealis*	Clark lab	N/A	N/A	N/A	QGLGGLVTGCLSGGLSGDSLVDALTQQLVGLWQTDEVLLDHYCNFQVKPKFRSWELYFQGTMWCPGWTPIRQQLSTRSRTGVVNKAIQDFVKKAFAGGLINEQADANAWLNA
			Panbar_ALF2*	*Pandalus borealis*	Clark lab	N/A	N/A	N/A	KPIDLTSILSSATDQLKVFIFDQNEMELLGHYCLYATWATFKDWKLRYKCGVKCPGWTPIVGTAEDADKPTAAERGATRNFVEQALESKLITEEARSWLV
			Panbar_ALF3*	*Pandalus borealis*	Clark lab	N/A	N/A	N/A	QGWEVVALAVGEKLVDLWKSDHLIFMGHQCNYSVNPKVKRFQLYFRGRMWCPGWTTIRGEAETRSRSGVVGKTTQDFARKAFIKGLITEQAARDWLNA
			Panbar_ALF4*	*Pandalus borealis*	Clark lab	N/A	N/A	N/A	DNWSDALLPALTNQLTSLWDQGEMELLGHYCSYTVRPKIRSWELYFVGNLWCPGWTPIRGDGLTRSRSGILINTTKDFIRKTLAAGITEEEARPWLI