Examination of crustacean immune responses through dsRNA-nanoparticle injection and phylogenetic analysis

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
November 2021

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Abstract

Crustaceans are a group of economically and culturally valuable species in Canada both as wild-caught and cultured. Increasing the knowledge of the crustacean innate immune system will provide valuable information that can be utilized in policy decisions impacting crustacean aquaculture and fisheries. This study examined several features of the crustacean immune system. A novel nanoparticle-synthetic dsRNA analogue complex was tested for use as a dsRNA delivery system on *Faxinious propinquus*. The experiments resulted in the discovery of 404 differentially expressed transcripts, 41 associated with the synthetic dsRNA analogue alone and 382 associated with the nanoparticle-synthetic dsRNA analogue complex. Immune-related differentially expressed transcripts were found only with the nanoparticle complex. A crustacean antimicrobial peptide, crustin, was examined across the order Decapoda. A phylogeny of the crustin sequences suggests a long period of time between gene duplication events in the common ancestor.

List of Abbreviations Used

3D	three-dimensional
Ago2	Argonaute RISC catalytic component 2
AHPND	acute hepatopancreatic necrosis disease
ALF	antilipopolysaccharide factor
AMP	antimicrobial peptide
BGBP	β-1,3-glucan binding protein
BGRP	β-1,3-glucanase-related protein
bp	base pairs
CND	Canadian dollars
CRD	carbohydrate recognition domain
DET	differentially expressed transcripts
DNase	deoxyribonuclease
ds	double stranded
DSCAM	down syndrome cell adhesion molecule
EMS	early mortality syndrome
ERK	extra-cellular signal-regulated kinase
FREP	fibrinogen-related proteins
GFF	general feature format
GNBP	Gram-negative bacteria binding protein
HMW	high molecular weight
HN	high molecular weight nanoparticle
IFN	interferon
IHHNV	infectious hypodermal and haematopoietic necrosis virus
Imd	immune deficiency
IMNV	infectious myonecrosis virus
JAK/STAT	Janus kinase-Signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
KAAS	KEGG automatic annotation server
KEGG	Kyoto Encyclopedia of Genes and Genomes

KO	KEGG orthology
LGBP	lipopolysaccharide and β-1,3-glucan binding protein
LMW	low molecular weight
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAFFT	multiple alignment using fast Fourier transform
MAPK	mitogen-activated protein kinase
MBL	mannose-binding lectins
MEGA	Molecular Evolutionary Genetics Analysis
MEM	minimal essential media
mRNA	messenger RNA
MSGS	monodon slow growth syndrome
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHP	necrotizing hepatopancreatitis
nr	non-redundant
nt	nucleotides
ORF	open reading frame
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PGN	peptidoglycan
PO	phenoloxidase
Poly (I:C)	polyinosinic:polycytidylic acid
PPAE	prophenoloxidase-activating enzyme
proPO	prophenoloxidase
PRR	pattern recognition receptor
RAS	recirculating aquaculture system
RdRP	RNA-dependent RNA polymerase
redox	reduction-oxidation
RISC	RNA-induced silencing complex
RLR	RIG-like receptors

RNAi	RNA interference
ROS	reactive oxygen species
siRNA	small interfering RNA
ss	single stranded
TEP	thioester-containing protein
THC	total haemocyte count
TLR	toll-like receptor
TMM	trimmed Mean of M values
TNFR	tumor necrosis factor receptor
TR	thioredoxin reductase
TRX	thioredoxin
TSV	Taura syndrome virus
USD	United States dollar
WAP	whey acidic protein
WSSV	white spot syndrome virus
YHD	yellow head disease
~	approximately

Acknowledgements

I would like to thank my graduate supervisor Dr. Fraser Clark for all of his experience and guidance that he provided during my Master's journey. I would like to thank my supervisory committee, Dr. Stephanie DeWitte-Orr, Dr. Sarah Stewart-Clark and Dr. Young Chang, for their feedback and insights that helped to shape my projects.

I would like to thank the Clark lab members for their unconditional support as well as the DeWitte-Orr lab members for their assistance and collaboration during the projects, especially Dr. Tamiru Alkie and Emma Monod. I would also like to thank the collaborations of Wilfred Laurier University, McMaster University, University of Fraser Valley, Fisheries and Oceans Canada and Glysantis™.

I would like to acknowledge the staff at the Dalhousie University Agricultural Campus aquaculture centre for their support and assistance with the crustacean holding areas used to conduct these experiments. A special acknowledgement to the fishermen who assisted in these projects, Ken Snow, Bill Bond, Alan Newell and their crews, who provided crustaceans as well as knowledge and unconditional support for our research.

I would like to personally thank my friends and family for all their support. I would like to send a special thank you to my parents, Wayne and Nancy, for their willingness to listen to my crustacean facts, care packages and encouragement, as well as to my mother for reading and re-reading my thesis. I would also like to give a special thank you to my partner, Elliott, for his unconditional support during this process.

I would like to thank Alexandra Elbakyan for her contributions to science.

Finally, I would like to thank NSERC and Dalhousie University for funding my research.

Chapter 1: Introduction

1.1 Introduction

Crustaceans are a valuable group of species in Canada through fisheries, importation and aquaculture. An understanding of their immune capabilities is essential to preserve the stocks, and to be able to predict how infections from viruses, parasites, bacteria or other pathogens may affect their populations. The following chapter is a comprehensive literature review of the value of crustaceans, disease impacting crustaceans and crustacean immunity in context to the research objectives of this thesis.

1.2 Canadian Crustacean Fisheries

Commercial fisheries are important industries for both the East and West coasts of Canada. In 2019, Canadian fisheries landings were valued at over $3.6 billion CND. Crustaceans accounted for 72% of Canada's total fishery landings, with 69% of the total crustacean value coming from fisheries in Atlantic Canada (Boone, 1931; Government of Canada, 2021). The American lobster (*Homarus americanus*) fishery brought in over 100,000 metric tons of live weight at a landed value of over $1.5 billion CND for 2019 (Government of Canada, 2021). The Atlantic fishery for snow crab brought in over 74,000 metric tons of live weight for a total landed value of over $700 million CND (Government of Canada, 2021). Various other crab species fished in Atlantic Canada include rock crab (*Cancer irroratus*) and Jonah crab (*Cancer borealis*), with 3,500 metric tons of live weight landed in 2019, for a total landed value of over $8 million CND (Boone, 1931; Government of Canada, 2021). The Atlantic fishery for shrimp, targeted to northern shrimp (*Pandalus borealis*), brought in over 58,000 metric tons of live weight at a landed value of over $200 million CND in 2019 (Government of Canada, 2021). Along with the high value of the landings, Canadian fisheries

provide a large number of employment opportunities. In 2018, the Canadian fisheries resulted in jobs for over 143,000 people directly and over 88,000 indirectly (Ganter et al., 2021).

1.3 Global Crustacean Aquaculture and Hatcheries

Crustacean aquaculture is an important industry worldwide as a sustainable seafood source. Global crustacean aquaculture includes several species of shrimp, crab and lobster (Cai et al., 2019). These species are cultured for consumption, although many other species are also cultured as ornamental crustaceans (Calado et al., 2003b, 2003a).

Shrimp aquaculture accounts for 50% of the global shrimp consumed by human, with 5 million tonnes of live weight produced in 2019, for a value of $5-$22 USD/kg (Food and Agriculture Organization of the United Nations, 2021). Majority of this live weight is from the Pacific whiteleg shrimp (*Litopenaeus vannamei*) (70%) and giant tiger prawn (*Penaeus monodon*) (21%) (Food and Agriculture Organization of the United Nations, 2021). Shrimp aquaculture also includes the culture of the genera: *Acetes, Metapenaeus, Palaemon* and other species in the *Litopenaeus* and *Penaeus* genera (Food and Agriculture Organization of the United Nations, 2021).

Lobsters have been cultured as both grow out operations from broodstock to adult and as hatcheries for restocking. Although lobster culture is not happening at a large commercial scale, culture and hatcheries for lobster do still exist (Carere et al., 2015; Jones et al., 2019; Nicosia and Lavalli, 1999). Current species cultured include: the European lobster (*Homarus gammarus*), bamboo lobster (*Panulirus versicolor*), adik-adik lobster (*Panulirus edulis*), tiger lobster (*Panulirus ornatus*) and rock lobster (*Panulirus interruptus*) (Burton, 2001; Jones, 2009; Nicosia and Lavalli, 1999; Waddy and Aiken, 1995).

Culturing of crabs largely focuses on the Chinese mitten crab (*Eriocheir sinensis*) and mud crab (*Scylla serrata*) (Cheng et al., 2018; Paterson and Mann, 2011). Chinese mitten crabs are a very high-value species with prices as high as $50.00 USD/crab, depending on size, grade and sex (Cheng et al., 2018). Mud crab are a high-value species with average prices ranging from $10.00-19.42 USD/crab, depending on size, grade and sex (Bhuiyan et al., 2021). Although *S. serrata* is the most cultured species, other cultured species include *Scylla paramamosain*, *Scylla olivacea* and *Scylla tranquebarica* (Paterson and Mann, 2011). In North America, the blue crab, *Callinectes sapidus*, has been cultured for both the stock enhancement in the Chesapeake Bay and for the controlled production of soft-shelled crab (Perry et al., 2011; Zmora et al., 2005).

1.4 Crustacean Aquaculture in Canada

Although shrimp are fished in Canadian waters, the majority of shrimp sold to consumers in Canada are imported. Shrimp importation to Canada in 2019 was valued at over $400 million CND, with shrimp coming from countries in Central America, South America, Asia, Africa, Europe, the Middle East and other parts of North America (Canadian Importers Database, 2021; Government of Canada, 2021). With the high value of these shrimp importations, cultured shrimp produced in Canada would be highly beneficial to the Canadian economy.

Shrimp aquaculture has existed in Canada, however it has not existed at a commercial scale. Canadian farms still in operation include: Good4UShrimp (Sudbury, Ontario), RAS Technologies formerly Planet Shrimp (Aylmer, Ontario), Rocky Mountain Shrimp Farm (Calgary, Alberta), Waterford Farms (Strathmore, Alberta) and Shrimp Canada (Guelph, Ontario) (Good4UShrimp, 2019; Planet Shrimp, 2019; RAS Technologies, 2019; Rocky Mountain Shrimp Farm Inc, 2018; Shrimp Canada, 2019; Waterford Farms, 2020). These farms all cultured the Pacific whiteleg shrimp (*L. vannamei*) and import stock of 10-day old shrimp from facilities in Texas and Florida,

United States, which are then grown to adult size (Beer, 2020; Good4UShrimp, 2019; Lynch, 2019; Planet Shrimp, 2019; RAS Technologies, 2019; Rocky Mountain Shrimp Farm Inc, 2018; Shrimp Canada, 2019; Waterford Farms, 2020).

Canadian shrimp farms rely on recirculating aquaculture systems (RAS), which allow these farms to exist on land (Lucas and Southgate, 2012; Alang, 2021). Shrimp aquaculture requires large ponds with shallow water in order to farm the shrimp; a requirement that can be met by converting pig farms to shrimp production facilities (Lynch, 2019; MacNaughton and News, 2015; Nuttall-Smith, 2015; Small, 2019). Simple RAS systems on these converted farms can create large profit for a full grow out system. Waterford Farms in Alberta predicted 2000 lbs of harvested live weight per week, and Planet Shrimp in Ontario was producing 300,000 lbs of live weight per year (Jones, 2019; Small, 2019).

Although profits are possible, many of these farms have since shut down for various reasons. First Ontario Shrimp (Campbellford, Ontario) and Berezan Shrimp Farm (Langley, British Columbia) were previously in operation until their closure in 2019 (Beer, 2020; Lynch, 2019). In September 2019, Berezan shrimp farm shut down after 22 months of operation due to the inability to afford the farm costs (Mayer, 2019). The farm was placed under quarantine two months prior to the shut down due to an outbreak of infectious hypodermal and haematopoietic necrosis virus (IHHNV) detected in the brood stock imported from Texas; a location that also supplies the other Canadian whiteleg shrimp farms (Lynch, 2019). First Ontario Shrimp has also closed down due to a variety of factors including difficulty obtaining stock due to weather impacting shipments of stock from the United States, as well as growth issues in the shrimp (Lynch, 2019). Although some farms are still active, the stock supply has been the largest of the challenges to the success of the farms.

1.5 Effect of Disease in Canadian Crustacean Aquaculture

Pathogens, once on the farms, can infect the entire stock resulting either in the death of stock, or shrimp that are not acceptable for market (Lucas and Southgate, 2012). Whiteleg shrimp is the most common broodstock imported into Canada (Beer, 2020; Good4UShrimp, 2019; Lynch, 2019; Planet Shrimp, 2019; RAS Technologies, 2019; Rocky Mountain Shrimp Farm Inc, 2018; Shrimp Canada, 2019; Waterford Farms, 2020). In 2019, the Canadian Food Inspection Agency shut down all importation of the whiteleg shrimp due to the most recent outbreak of IHHNV (Government of Canada C.F.I.A, 2019). A confirmed diagnosis of IHHNV results in the halt of all importation of whiteleg shrimp for culture, research, diagnostic testing, aquariums and outdoor holding units, to protect the Canadian crustacean populations. The inability to obtain broodstock has resulted in the halt of most shrimp aquaculture currently in Canada (Government of Canada C.F.I.A, 2019).

1.6 Crustacean Immunity

Crustaceans effectively use their innate immune system for protection against pathogenic and opportunistic microorganisms such as bacteria, parasites, fungi and viruses, since they lack an adaptive immune system (Hauton, 2012; Roth and Kurtz, 2009; Rowley, 2016; Sánchez-Salgado et al., 2021; Vazquez et al., 2009). The first line of defence in crustacean immunity is the cuticle and epithelia; crustaceans have strong exoskeleton and cuticle tissues that cover areas of articulation (Rowley and Powell, 2007). The cuticle covers the entire outer surface of the animal and creates a protective barrier. These barriers protect the host from most pathogenic and opportunistic microorganisms, which could enter through the cuticle or epithelia via opportunistic cracks, or by pathogen-secreted proteases (Rowley, 2016). The cellular and humoral responses become vital in protecting the host in cases where the microbes are able to enter (Rowley and Powell, 2007).

The crustacean innate immune system relies on several responses to protect the host, which are often divided into two categories: cellular responses and humoral responses. These responses can work separately or collaboratively and rely on the host's ability to differentiate its own tissues from those that are foreign (self vs non-self), known as allorecognition (Vazquez et al., 2009). The cellular responses are completed primarily by the circulating haemocytes and some fixed haemocytes in the hepatopancreas and gills (Ellender et al., 1992; Johansson et al., 2000). Humoral responses work alongside the cellular responses to help destroy pathogenic non-self material once the immune response is initiated (Leclerc, 1996; Wootton et al., 2006). These responses require the host to effectively recognize foreign cells. Pattern recognition receptors, including lectins, play a large role in allorecognition and pathogen recognition (Chorney and Cheng, 1980; Ji et al., 2009; Marques and Barracco, 2000; Sánchez-Salgado et al., 2021).

1.6.1 Haemocytes

The cellular immune responses of the crustacean innate immune system are initiated by circulating haemocytes, which comprise the majority of the cells found in the open circulatory system of crustaceans. Haemocytes utilize a variety of responses to protect the host, which depend on the type of pathogen and on the type of haemocyte responding (Jiravanichpaisal et al., 2006).

There are three main classifications of haemocytes that can be distinguished based on morphology and function, differing in the presence, number and size of cytoplasmic granules. These granules facilitate the distinct functions of each type of haemocyte (Johansson et al., 2000; Johansson, 1995; Rowley, 2016). The haemocyte classifications that are currently used are: hyaline, semi-granular and granular cells. Hyaline cells act through phagocytosis and encapsulation as well as producing cytotoxic molecules and antimicrobial peptides (AMP) (Rowley, 2016; Söderhäll and Smith, 1983; Thörnqvist et al., 1994). Semi-granular cells act as

phagocytes and have functional roles in encapsulation, nodule formation and cytotoxicity, as well as storage and release of prophenoloxidase (proPO) (Kobayashi et al., 1990; Persson et al., 1987; Rowley, 2016; Söderhäll and Smith, 1983; Thörnqvist et al., 1994). Granular cells act through nodule formation, encapsulation and have the ability to release cytotoxic molecules, AMP, as well as store and release proPO (Johansson and Söderhäll, 1989; Rowley, 2016).

Haemocytes are produced in a specialized haematopoietic tissue that covers the dorsal and dorsolateral sides of the stomach (Figure 1). The total number and type of circulating haemocytes can vary greatly at the intra- and interspecies level as well as at different physiological time points; thus, a single time point evaluation of the number of circulating haemocytes is not a reliable measure of the physiological state of an animal (Huang et al., 2020). Variation in the total and differential circulating haemocyte counts can be caused by factors including but not limited to infection, environmental stress, moult stage and reproduction (Johansson et al., 2000). During an infection, total haemocyte count (THC) initially increases and in some cases is followed by a rapid decrease as the animal becomes moribund (Johansson et al., 2000; Vazquez et al., 2009). This has been observed in a variety of crustacean species, including *L. vannamei* and the Danube crayfish (*Astacus leptodactylus*), in the presence of pathogen associated molecular patterns (PAMPs) such as laminarin, lipopolysaccharide (LPS) and Poly (I:C) (Ji et al., 2009; Safari et al., 2015).

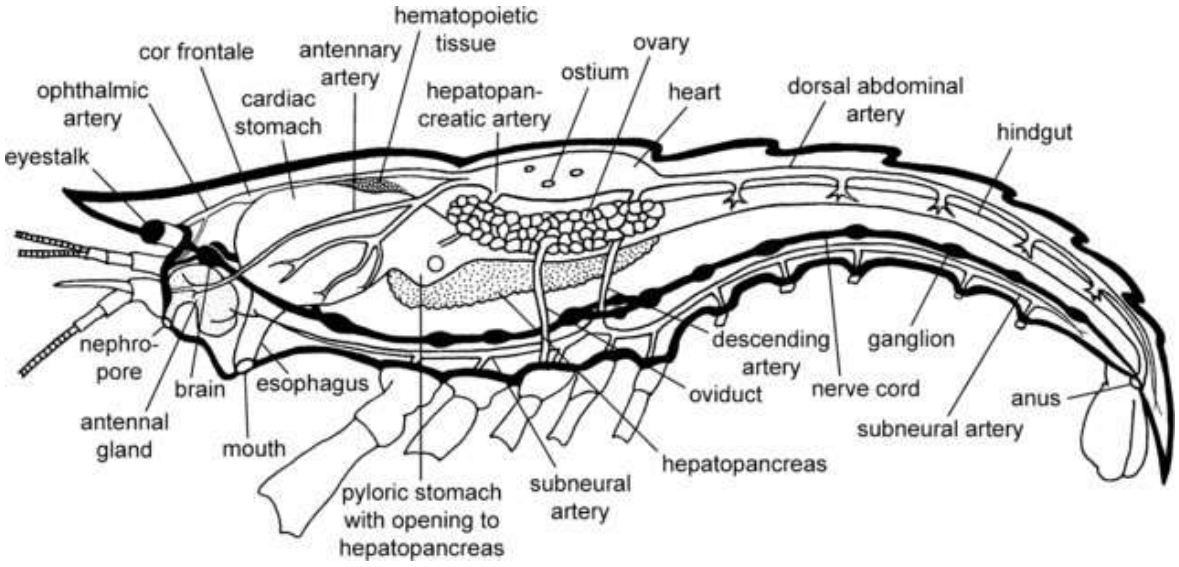


Figure 1 An illustration of the internal anatomy of a crayfish showing the location the haematopoietic tissue and hepatopancreas (Gherardi et al., 2010).

Fixed haemocytes in the hepatopancreas play an important role in crustacean immunity (Factor et al., 2009; Roth and Kurtz, 2009). The hepatopancreas plays an important role in metabolism, detoxification and immune defence (Figure 1) (Factor, 1995). Hepatopancreas fixed haemocytes are found in the outer walls of the terminal hepatic arterioles and are present in the haemal spaces, where they can have access to haemolymph. As the haemolymph circulates through the hepatopancreas, these fixed haemocytes are able to remove foreign particles including bacteria and pathogens (Factor et al., 2009). Fixed phagocytes of *H. americanus* have been observed to remove *Aerococcus viridans* var. *homari* from the haemolymph of infected lobsters (Johnson et al., 1981). In Caribbean spiny lobster (*Panulirus argus*) injected with haemolymph from another *P. argus* infected with an unknown virus, the fixed hepatopancreas phagocytes were observed filtering out the virions (Shields and Behringer, 2004).

1.6.2 Pattern Recognition Receptors

Pattern recognition receptors (PRRs) in the crustacean immune system are able to recognize molecular patterns that are shared among many pathogens (Clark and Greenwood, 2016; Kurtz, 2005). Pathogen associated molecular patterns, such as LPS, peptidoglycan (PGN) and β-glucan, are detected by PRRs on the surface of haemocytes (Battistella et al, 1996). Many compounds can act as PRRs, including lectins, β-1,3-glucanase-related protein (BGRP), Toll-like receptors (TLRs), scavenger receptors, down-syndrome cell adhesion molecules (DSCAMs) and thioester-containing proteins (TEPs) (Fujita et al., 2004; Sánchez-Salgado et al., 2021). Each of the PRRs have a variety of intra- and extra-cellular signaling pathways that they activate, such as the TLR, Imd, JAK/STAT, and JNK pathway, which will be discussed further in 1.6.3. Activation results in the upregulated expression of effector genes and immune pathways, which aim to protect the host from the pathogen (Table 1).

Table 1 Several crustacean pattern recognition receptors and their associated signaling pathways, immune genes and immune functions (Modified from Sánchez-Salgado et al. 2021).

PRR	Signaling Pathways	Immune Genes	Immune Functions
Lectins	TLR Pathway Imd Pathway JAK/STAT Pathway	ALFs Crustins Lysozymes Penaeidins MBL proPO Genes Histin	AMPs Expression Phagocytosis proPO System Encapsulation
β-1,3-Glucanase-Related Protein (BGRP)	No Documented Pathways	ALFs Crustins	proPO System AMPs Expression Phagocytosis
Toll-Like Receptor (TLR)	TLR Pathway	ALFs Crustins Lysozymes Penaeidins Histin Arasin Hyastatin	AMPs Expression
Scavenger Receptor	TLR Pathway Caspase 3 Activity	ALFs Crustins Lysosomes TLR Genes Phagocytosis Genes	Phagocytosis AMPs Expression Apoptosis
Down-Syndrome Cell Adhesion Molecules (DSCAM)	ERK Pathway Dorsal	ALFs Crustins Lysozymes	Phagocytosis AMPs Expression
Thioester-Containing Proteins (TEP)	NF-κB Pathway Homology TLR Pathway JNK Pathway	ALFs Crustins TLR Genes Relish	Clotting System proPO System Phagocytosis

1.6.2.1 Lectins

Lectins are a type of PRR in crustaceans and other animals that have the ability to recognize and bind carbohydrates on the surface of microorganisms (Marques and Barracco, 2000). Lectins are proteins or glycoproteins that are synthesized in haemocytes and are present as transmembrane receptors or soluble proteins (Jin et al., 2013). They bind non-covalently to the PAMPs found on pathogenic microorganism cell surfaces (Söderhäll, 2010; Wang and Wang, 2013). Lectins are characterized by having a carbohydrate recognition domain (CRD), which binds to specific

carbohydrates on the cell surface of the pathogenic microorganisms. The variations in CRDs allow for different domains to recognize and bind different types of sugars, such as LPS, lipoteichoic acid (LTA), fungal chitin, PGN and maltose (Chen et al., 2018; Sun et al., 2008; Utarabhand et al., 2017; Wang et al., 2009; Zhang et al., 2009). This variation in binding allows for some specificity within the lectins.

There are several different types of lectins, but C-type lectins, or calcium dependent lectins, are the most diverse and most well studied lectin family in invertebrate immunity (Söderhäll, 2010; Wang and Wang, 2013). C-type lectins are found throughout the haemolymph and are easily extracted from the hepatopancreas. C-type lectins have been found to be upregulated in the presence of pathogenic microorganisms, such as viruses and bacteria (Hauton, 2012). Specifically, C-type lectins have been found to be upregulated of *P. monodon* and *L. vannamei* due to WSSV infection, and in *P. monodon* and the kuruma prawn (*Marsupenaeus japonicus*) during Gram-negative bacterial infection, in both the hepatopancreas and haemolymph (Luo et al., 2006, 2003; Ma et al., 2007; Yang et al., 2007)

Pathogen neutralization is one of the many functions C-type lectins have in crustacean immunity (Söderhäll, 2010). This neutralization includes the role that C-type lectins play as opsonins to encourage phagocytosis, encapsulation and nodule formation. These cellular responses are some of the most important in crustacean immunity. The responses disable the pathogenic material once it has been detected in the host, and through interaction with humoral factors, are able to kill the pathogenic microorganisms. Crustaceans have open circulatory systems, therefore pathogenic microorganisms can easily spread around the host (Factor, 1995). Because haemocytes are present in both the haemocoel and the tissues, fast recognition by C-type lectins allow for prevention of damage by microorganisms that are pathogenic.

Mannose-binding lectins (MBLs) are a form of C-type lectin that play a crucial role in the innate immune system (Arockiaraj et al., 2015). Mannose-binding lectins are involved in proPO system activation and some studies have suggested that MBLs can act as scavenger receptors for LPSs in certain stages of the molting cycle. Further research needs to be conducted to better understand the mechanisms that are associated with the MBLs (Arockiaraj et al., 2015; Sánchez-Salgado et al., 2021).

Fibrinogen-related proteins (FREP) are carbohydrate binding proteins that are related to the crustacean immune response (Hanington and Zhang, 2010). Ficolins are proteins that belong to the FREP family and can agglutinate a broad range of bacteria. Binding of FREPs to their ligand can promote phagocytosis and the stimulation of AMP production. These immune functions have been found in several species to agglutinate various pathogens, participate in bacterial clearance and antiviral response (Hanington and Zhang, 2010; Sánchez-Salgado et al., 2021).

L-type lectins are widely distributed within Crustacea and activate a variety of immune functions through their interaction with N-glycans from glycoproteins involved in cellular trafficking (Fujita et al., 2004; Marques and Barracco, 2000; Wang and Wang, 2013). L-type lectins are known to participate in bacterial and pathogenic clearance and promote phagocytic activity. The mechanisms of these lectins require further investigation (Sánchez-Salgado et al., 2021).

Galectins, formerly S-type lectins, recognize endogenous “self” glucans and are able to mediate processes including cell differentiation, tissue organization and immune homeostasis (Marques and Barracco, 2000; Wang and Wang, 2013). Galectins can also recognize and bind glycans on pathogenic microorganisms. Galectins agglutinate and trigger phagocytosis to

eliminate bacteria and have been involved in haemolymph bacterial clearance (Marques and Barracco, 2000; Sánchez-Salgado et al., 2021; Wang and Wang, 2013).

Several crustacean lectins have been functionally characterised as having important roles in the host immune response. The lectins FC-L and LVL, from the Chinese white shrimp (*Fenneropenaeus chinensis*) and *L. vannamei*, respectively, play a role in agglutination (Sun et al., 2008, 2007). Agglutination is vital for humoral defense against pathogens because it triggers phagocytosis and the production of reactive oxygen species (Sun et al., 2008, 2007). Lectins in the giant freshwater prawn (*Macrobrachium rosenbergii*) and the red claw crayfish (*Cherax quadricarinatus*) also have roles in agglutination and in the production of oxidative bursts (Sánchez-Salgado et al., 2014). In *L. vannamei*, lectins LvCTLD and LvGal have been found to activate the proPO pathway and phagocytosis of bacteria, respectively; while the red swamp crawfish (*Procambarus clarkii*) lectin Pc-Lec1 has been found to enhance haemocyte encapsulation (Hou et al., 2015; Zhang et al., 2011). These are just some examples of the roles of lectins in crustaceans.

1.6.2.2 β-1,3-Glucanase-Related Protein

Members of the β-1,3-glucanase-related protein (BGRP) family include lipopolysaccharide and β-1,3-glucan binding protein (LGBP), β-1,3-glucan binding protein (BGBP) and Gram-negative bacteria binding protein (GNBP) (Chai et al., 2018). The family has been widely identified in arthropods. In crustaceans, BGRPs are involved in immunity through activation of the proPO system, proPO gene expression and expression of antimicrobial peptides (AMPs) including anti-lipopolysaccharide factors (ALFs) and crustins (Chai et al., 2018; Sánchez-Salgado et al., 2021). The BGRPs were examined in *P. clarkii* and were found to be expressed in both the haemocytes and hepatopancreas (Chai et al., 2018). The *P. clarkii* BGRP (PcBGRP) was induced

by carbohydrates and bacterial stimulants, especially LPS and β-1,3-glucan. *Procambarus clarkii* had a more noticeable response from the BGRP during infection by Gram-negative *Aeromonas hydrophila* than Gram-positive bacteria (Chai et al., 2018). Evaluation of BGRP in *E. sinensis* found that LGBP is able to activate the proPO cascade and trigger melanization in cases of *Vibrio parahaemolyticus* infection (Zhang et al., 2016). Findings from the signal crayfish (*Pacifastacus leniusculus*) demonstrated that the use of an anti-LGBP antibody would inhibit the proPO cascade when different PAMPs were introduced (Lee et al., 2000). These studies show the importance of BGRPs in crustacean immunology.

1.6.2.3 Toll-Like Receptors

Toll-like receptors (TLR) are evolutionarily conserved in both vertebrates and invertebrates, although their mechanisms of action differ. Toll-like receptors respond to the presence of PAMPs allowing for the recognition of a variety of pathogens (Deepika et al., 2014; Habib and Zhang, 2020; Sánchez-Paz and Muhlia-Almazán, 2020). In vertebrates, TLRs bind directly to pathogens; however, in crustaceans, TLRs bind to Spätzle, a cytokine-like ligand. The binding of TLR to their ligands results in the activation of an intracellular signalling cascade that initiates the expression of several immune genes. Toll-like receptors have been found to stimulate the expression of AMPs including: ALFs, crustins, penaeidins, histins, arasins and hyastatin (Deepika et al., 2014; Habib and Zhang, 2020; Sánchez-Paz and Muhlia-Almazán, 2020). Research on TLRs has been centred mostly around crabs, shrimps and crayfishes of the genera *Litopenaeus, Procambarus, Penaeus, Fenneropenaeus, Macrobrachium, Marsupenaeus, Scylla, Eriocheir* and *Portunus* (Habib and Zhang, 2020).

1.6.2.4 Scavenger Receptors

Scavenger receptors recognize a large range of PAMPs and function in response to immune and homeostasis changes. The scavenger receptors bind low-density lipoproteins, specifically oxidized low-density lipoprotein and acetylated low-density lipoprotein (Pearson, 1996). Scavenger receptors have been found to bind LTA, Gram-positive bacteria and apoptotic cells (Pearson, 1996; Sánchez-Salgado et al., 2021). Depending on the lipoprotein that is bound, scavenger receptors have been known to initiate phagocytosis pathways, as well as the synthesis of AMPs.

1.6.2.5 Down-Syndrome Cell Adhesion Molecules

Down-syndrome cell adhesion molecules (DSCAMs) have been proposed to participate in the crustacean immune system as hypervariable PRRs (Li et al., 2018; Ng and Kurtz, 2020). As well as being a cell surface receptor able to initiate phagocytosis, a hypothesis has emerged that DSCAMs are also able to act as an opsonin (Ng and Kurtz, 2020; Watson et al., 2005). Down-syndrome cell adhesion molecules can be generated through the alternative splicing of variable exons from a single-locus gene. Originally, DSCAM was first discovered in humans and found to be involved in axon guidance. It has since been discovered in invertebrates where it is involved in the innate immune system as a hypervariable immune molecule due to the high versatility of the gene and the presence of numerous immunoglobulin domains (Ng and Kurtz, 2020). Both membrane-bound and soluble DSCAMs have been discovered in crustaceans and have been found to bind directly to pathogens to promote their elimination (Li et al., 2018).

Current methods of DSCAM-mediated pathogen elimination are known to be through phagocytosis; however, the production of AMPs have also been investigated. The alternative splicing of DSCAMs is reported to result in 38,016 isoforms in the common fruit fly (*Drosophila*

melanogaster), 30,600 isoforms in *E. sinensis* and at least 13,000 isoforms in the water flea, *Daphnia pulex arenata* (Brites et al., 2008). The full extent of the hypervariability found in DSCAMs and the functional immune roles that they play are not well understood (Brites et al., 2008; Li et al., 2018; Sánchez-Salgado et al., 2021).

Within the crustacean innate immune response, the role of DSCAMs is still being investigated. One study found that the Imd and TLR pathways in the mosquito *Anopheles malaria* modulates the transcriptional regulation of the splicing factors that are able to generate DSCAMs specific to *Plasmodium* infections (Dong et al., 2012). This pathogen-specific DSCAM creation has been found after pathogen exposure and leads to binding of the pathogens with a degree of specificity to promote clearance (Ng and Kurtz, 2020). *Eriocheir sinensis* DSCAMs were examined for their role in phagocytic receptors and opsonins. The study determined that after exposure to *Vibrio parahaemolyticus, Staphylococcus aureus, Bacillus subtilis* and *Aeromonas hydrophila*, DSCAMs found in the hemolymph bound specifically to the eliciting bacteria, triggering phagocytosis (Li et al., 2018). A similar study on *C. quadricarinatus* found this same DSCAM specificity for WSSV (Ng et al., 2014). This specificity demonstrates the value and diversity of DSCAMs as a PRRs.

1.6.2.6 Thioester-Containing Protein

Thioester-containing proteins (TEPs) are found in several arthropods, including crustaceans, and are known to be humoral receptors (Ning et al., 2019; Vaseeharan et al., 2007). These proteins are mediated by the thioester bond, which in turn mediate the binding of the PRR to its ligand. Thioester-containing proteins are able to bind directly to bacterial cell walls and are associated with the expression of genes that result in phagocytosis and AMP production through the TLR and NF-κB pathways. The involvement in these pathways suggest that TEPs may be

involved in other immune mechanisms as well (Sánchez-Salgado et al., 2021). A novel TEP was discovered in *S. serrata* that is similar to a TEP in *P. japonicus* and *H. americanus*. In all cases, the presence of the TEP was upregulated significantly after injection of LPSs (Vaseeharan et al., 2007). In gazami crab (*Portunus trituberculatus*), a TEP was discovered that was induced by both bacteria and fungi, causing activation of the proPO cascade (Ning et al., 2019). The TEPs in *P. leniusculus* gills and intestines were examined after infection with *Pseudomonas libanensis,* which found that when these TEPs were inactivated, mortality rates increased suggesting the importance of TEPs in intestinal immune defense.

1.6.3 Responses to Activated Haemocytes

Recognition and binding of PAMPs results in a variety of immune responses from haemocytes. The binding of the PRRs to PAMPs triggers intracellular signaling cascades, which determine the immune response(s) that will be activated. Pathways involved in the crustacean innate immune responses include nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway homologies, known as Dorsal and Relish, c-Jun N-terminal kinase (JNK) pathway and Janus kinase-Signal transducer and activator of transcription (JAK/STAT) pathway. Many of these pathways also interact with one another (Clark and Greenwood, 2016). Other pathways exist that are extracellular pathways, such as the proPO pathway. Understanding these pathways is important in the management of cultured crustaceans for regulation of immune response.

1.6.3.1 Dorsal/Relish Pathways

The Dorsal/Relish pathways have homology to the NF-κB pathways in vertebrates and are involved in the defense against viruses (Li et al., 2019; Wang et al., 2013). The two NF-κB homology pathways in crustaceans are the TLR pathway and the immune deficiency (Imd)

pathway. These pathways are the major regulators of shrimp immune responses through the control of AMP expression. Bacterial infections activate these pathways by activating two NF-κB homology transcription factors, Dorsal and Relish, that cause an increase in the expression of AMPs. The Imd and TLR pathways often work together and are responsible for controlling the expression of the majority of immune genes (Clark and Greenwood, 2016).

The TLR pathway utilizes PRRs to initiate the expression of immune genes through nuclear transcription factors. The TLR pathway is involved in defense against fungi, Gram-positive bacteria and viruses. This pathway has also been observed to respond to Gram-negative bacteria in shrimp species including: *L. vannamei*, *P. monodon, F. chinensis, M. japonius, M. rosenbergii* and *P. clarkii* (Kulkarni et al., 2021; Li et al., 2019). The activation of the TLR pathway results in an activated regulatory protein, Cactus, that releases the transcription factor Dorsal, which is then able to translocate into the nucleus and activate gene expression (Clark and Greenwood, 2016; Li et al., 2019; Tanji et al., 2010; Vaniksampanna et al., 2019). The TLR pathways are responsible for controlling the expression of microbial immune response genes including AMPs and genes involved in clotting and melanization (Clark and Greenwood, 2016).

The Imd pathway plays an important role in the response to Gram-negative bacteria and viruses. This pathway is triggered by a type of PGN, y meso-diaminopimelic acid-type PGN, which is found in the cell walls of most Gram-negative bacteria (Li et al., 2019). This pathway has also been observed to be activated by Gram-positive bacteria in shrimp such as: *L. vannamei*, *P. monodon, F. chinensis, M. japonius, M. rosenbergii, Macrobrachium nipponense* (oriental river prawn) and *P. clarkii*, and is similar to the tumor necrosis factor receptor (TNFR) pathways in vertebrates that can trigger the expression of antibacterial genes (Clark and Greenwood, 2016; Li et al., 2019; Li et al., 2018). The Imd pathways are responsible for controlling the expression of

microbial immune response genes including AMPs and genes involved in clotting and melanization (Clark and Greenwood, 2016; Li et al., 2018; Valenzuela-Muñoz and Gallardo-Escárate, 2014). The Imd signaling pathways contain a death-domain-containing protein that has a similar function to the receptor-interacting protein from the TNF-R pathway in vertebrates. Similar to the TLR pathway, a NF-κB transcription factor homolog, known as Relish, becomes activated, translocates into the nucleus and induces the production of immune-relevant transcripts (Li and Xiang, 2013; Li et al., 2018; Valenzuela-Muñoz and Gallardo-Escárate, 2014).

1.6.3.2 JNK Pathway

The c-Jun N-terminus kinase (JNK) pathway is a member of the mitogen-activated protein kinase (MAPK) superfamily. The JNK pathway is reported to be present in arthropods. Although the specific role of this pathway has not yet been determined, the JNK pathway has been found to play an important role in cell proliferation, differentiation and apoptosis in crustaceans (Wei et al., 2020). The JNK pathway also has involvement in a variety of immune responses in aphids, including: regulation of phenoloxidase (PO) activity, hydrogen peroxide concentration and phagocytosis of bacteria (Ma et al., 2020).

1.6.3.3 JAK/STAT Pathway

The Janus kinase-Signal transducer and activator of transcription (JAK/STAT) pathway is involved in antiviral defense in insects and in some crustaceans (Clark and Greenwood, 2016; Kulkarni et al., 2021). The JAK/STAT pathway has been well studied in *Drosophila*, where it has a role in hematopoiesis, cellular immunity, viral response and gut immunity (Pascual, 2019). The JAK/STAT pathway is activated by Gram-negative bacteria and viruses, leading to the production of AMPs and possibly interferons (IFN) (Kulkarni et al., 2021). A possible IFN system-like antiviral regulatory mechanism has been suggested in shrimp (Kulkarni et al., 2021). This possible

antiviral mechanism is suggested to regulate the expression of the *vago* gene, an antiviral cytokine. *Vago* is then suggested to activate the JAK/STAT pathway to limit viral infection (Kulkarni et al., 2021). This proposed mechanism is not well defined in crustaceans; however, it is gaining traction as a research topic especially in cultured shrimp (Kulkarni et al., 2021; Li et al., 2015; Silveira et al., 2018).

1.6.4 Immune Responses in Crustaceans

Although immune responses in crustaceans are often divided into cellular and humoral responses, the collaboration between both responses is very important for proper host immune response. Here, the immune responses in general terms will be discussed without the categorization of cellular and humoral responses.

1.6.4.1 Restricted Movement of Pathogens

Restricting the movement of pathogens is important to control infection. This is accomplished by haemocytes through phagocytosis, encapsulation or nodulation (Ji et al., 2009; Kulkarni et al., 2021; Vazquez et al., 2009). Restriction of the movement of these pathogens can be promoted by opsonin substances that are released by granular haemocytes (Vazquez et al., 2009). Opsonization is the process whereby opsonins, such as lectins, attach to the surface of the microbe to mark it for phagocytosis (Abbas et al., 2018). This use of opsonization has been found in *F. chinensis* and *M. japonicus*, where C-type lectins bind and promote phagocytosis of *Vibrio anguillarum* (Wang et al., 2014).

One role of haemocytes is phagocytosis, the ability to recognize and ingest pathogenic microbes in order to restrict their movement. Phagocytosis is an endocytic process that involves the recognition and uptake of pathogens into a phagosome, in this case a haemocyte (Liu et al., 2020). The process of phagocytosis is mediated by PRRs and a variety of signalling cascades.

Following the isolation of the pathogen in the haemocyte, various methods of destruction can be used to further reduce the possible effects that the pathogen may have on the host (Liu et al., 2020); these methods of destruction will be discussed further in 1.6.4.2. Phagocytosis is the most common cellular event that occurs in crustaceans in the presence of a pathogen (Söderhäll and Cerenius, 1992).

Phagocytosis can be carried out on spores, bacteria and dead or virus-infected host cells. Depending on the crustacean species, different selectivity exists in what is phagocytosed. In *M. japonicus*, phagocytosis was observed from circulating haemocytes encountering *Vibrio parahaemolyticus* (Zong et al., 2008). In *P. scaber*, phagocytosis from circulating haemocytes was observed in the presence of *Bacillus thuringiensis* and *Escherichia coli* (Roth and Kurtz, 2009). A notable finding by Roth and Kurtz (2009) was the specificity of the phagocytes; the response time of the phagocytes was decreased and more phagocytes participated in the phagocytosis in cases where the bacteria was encountered for a second time. This study found a high degree of selectivity that enabled the differentiation between bacterial strains (Roth and Kurtz, 2009). Phagocytosis may not be enough to stop the foreign particle when the particles are too large, greater than 10 μm, such as helminths, protists, fungal spores or when the small foreign particles are too numerous (Battistella et al., 1996; Söderhäll, 2010; Vazquez et al., 2009). Because of this limitation of size, other methods can be utilized by the haemocytes to control the foreign particles, such as encapsulation and nodule formation (Battistella et al., 1996; Söderhäll, 2010; Vazquez et al., 2009).

Encapsulation is the term used when dealing with the particles too large (> 10 μm) for phagocytosis, while nodule formation is the term used for dealing with particles too numerous for phagocytosis. In both situations, haemocytes surround the particles in several layers causing the

particles to be trapped and rendering them unable to move (Wang and Yao, 2007). This will stop the foreign particles from venturing further into the haemocoel.

Recently, extracellular traps have been discovered in crustaceans as a way to restrict the movement of pathogens including bacteria, fungi, viruses and other parasites (Sánchez-Salgado et al., 2021). These extracellular traps are known to be produced by hyaline haemocytes; however, other types of haemocytes have not been investigated individually (Ng et al., 2013; Robb et al., 2014). Extracellular traps are utilized by crustaceans and have specifically been studied in *L. vannamei* and *C. maenas* (Ng et al., 2013; Robb et al., 2014). Although the mechanisms behind invertebrate extracellular traps are still being investigated, initial studies suggest they are very similar to extracellular traps in vertebrates, where a signaling cascade will result in a type of cell-death program known as ETosis, a distinct type of cell death from apoptosis or necrosis. ETosis results in the removal of the nuclear membrane and membrane surrounding cytoplasmic granules, which causes the contents of the nucleus and granular antimicrobial peptides to come in contact. With the disruption of the cellular membrane, chromatin fibres and granular proteins are released into the extracellular region entrapping the pathogen (Ng et al., 2013; Robb et al., 2014)

1.6.4.2 Destruction of Pathogens

Once the movement of a pathogen has been restricted, haemocytes destroy the pathogen to ensure it will cause no further harm to the host. A variety of methods can be utilized for this including production of AMPs, production of reactive oxygen species (ROS) and melanization.

Antimicrobial peptides are small peptides with a wide range of inhibitory effects against bacteria, fungi, parasite and viruses. These peptides are small, cationic, amphipathic molecules (<10 kDa, 15-100 amino acids) that are gene encoded (Rosa and Barracco, 2010). Antimicrobial peptides have been well studied in arthropods and have started to be examined further in

crustaceans (Battistella et al., 1996; Vazquez et al., 2009). These peptides have been identified in the granules of circulating haemocytes with variation observed among crustacean species and their associated function. Once the pathogen is trapped, the haemocytes can degranulate and release AMPs into the layers of haemocytes surrounding the pathogen, resulting in elimination. Antimicrobial peptides are able to act on pathogens by disrupting the cells membrane integrity (Rosa and Barraco, 2010). The cationic portion of the peptide attracts to the negatively charged bacterial or fungal cell walls and membranes, where the electrostatic interaction allows the peptide to insert into the pathogen wall or membrane through the hydrophobic portion of the peptide. The resulting membrane destabilization and/or pore formation results in the destruction of the pathogen (Rose and Barracco, 2010). Along with this function, AMPs generally aim to inhibit bacterial growth and can be found in many other immune responses (Destoumieux-Garzón et al., 2016; Johansson, 1995; Rowley, 2016).

Production of ROS is another way that haemocytes can destroy the pathogens. Haemocyte mitochondria contain a variety of enzymes including NADPH-oxidase, which are activated upon recognition of the PAMP (Gopalakrishnan et al., 2011). This in turn increases the oxygen consumption of the haemocyte, known as a "respiratory burst". The enzyme complex, NADPH-oxidase, facilitates electron transfer from cytosolic NADPH to intracellular oxygen. This transfer of electrons produces reactive oxygen species, which include superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2), that can kill foreign material in the cell directly or in combination with other enzymes. Reactive oxygen species are toxic to both the foreign material and the host cells, not discriminating between self and non-self. If regulation does not occur on the reactive oxygen species, they can cause deleterious effects against the host cells when an imbalance in the ROS (oxidants) and anti-oxidants occurs (Gopalakrishnan et al., 2011). A variety of antioxidants can be

deployed in the innate immune system that are able to prevent this damage to the host, including superoxide dismutase, catalase, glutathione peroxidase and thioredoxin. In the freshwater crab *Sinopotamon henanense*, cadmium exposure resulted in immune responses including increases in total haemocyte counts and production of ROS (Zhou et al., 2016). Along with these immune responses, the production of antioxidants were upregulated for superoxide dismutase, catalase and glutathione peroxidase within the haemocytes. In *S. paramamosain*, LPSs were injected to examine the immune parameters that changed, including the production of ROS. As the ROS was found to increase after injection, the antioxidant production was observed to be positively correlated (Gopalakrishnan et al., 2011). In *H. americanus* challenged with WSSV, thioredoxin expression increased (Clark et al., 2013d). Thioredoxin (TRX) is a reduction-oxidation (redox) protein, which can neutralize ROS. The TRX system incorporates the redox protein TRX and the enzyme thioredoxin reductase (TR) where TR catalyzes the reduction of TRX with electrons from NADH (Aispuro-Hernandez et al., 2008). Without these antioxidants, the ROS that are produced in an immune response could cause severe damage and possible death to the host.

Prophenoloxidase eliminates the threat from a pathogen by creating protective barriers around the pathogen, where these AMPs and ROS can function without harming the host (Battistella et al., 1996; Söderhäll et al., 2009; Vazquez et al., 2009). The proPO cascade results in the melanization of the layered haemocytes surrounding the pathogen, to help in creating a protective layer and making it impermeable to the trapped pathogenic particle (Battistella et al., 1996; Cerenius et al., 2008; Söderhäll, 2010; Vazquez et al., 2009). Prophenoloxidase is the inactive precursor of PO; an enzyme responsible for activating the melanization cascade in crustaceans (Rodríguez and Le Moullac, 2000). After proPO is activated by bacterial LPS, bacterial lysine-PGN, diaminopimelic acid-PGN, or fungal β-1,3-glucan through a PRR, the PO

cascade initiates, resulting in the production of melanin (Battistella et al., 1996; Park et al., 2006; Vazquez et al., 2009). Melanin is a brown-black pigment that has beneficial properties in the immune response including inhibition of fungal and bacterial enzyme activity. In crustaceans, melanin is produced in the cuticle in response to a wound or parasitic attack. Melanin is also produced during nodule and encapsulation responses. Once the PRR and PAMP bind, the triggered release of prophenoloxidase proteins from haemocytes occurs where the pro-form of proPO-activating enzyme (PPAE) is released into the hemolymph and is converted to the active form of PPAE by a cascade of proteinases (Amparyup et al., 2013; Cerenius et al., 2008; Söderhäll et al., 2009). Once activated, PPAE is able to cleave proPO into PO, resulting in the production of melanin (Amparyup et al., 2013; Cerenius et al., 2008; Söderhäll et al., 2009). As well as the production of melanin, quinones may be present that inhibit the growth of the pathogenic particle and result in its death (Marques and Barracco, 2000; Munoz et al., 2000; Sloan et al., 1975).

1.7 Management of Crustacean Disease

A variety of methods, both direct and indirect, have been proposed for disease management within crustacean populations, both wild and cultured. The methods used for management must be determined on a case-by-case basis due to the variety of factors that influence each disease outbreak, including pathogen, location, species affected, environmental conditions and metabolic stress (Flegel et al., 2008; Hauton, 2012; Karunasagar and Ababouch, 2012).

The first step in the creation of these management strategies is understanding the disease needing to be managed. Challenges occur with crustacean disease research due to the lack of continuous cell lines for crustacean organs and tissues. The lack of these continuous cell lines has encouraged histopathological, molecular and phylogenetic approaches to provide insight into the pathogen taxonomy (Stentiford, 2012). Many more challenges occur that are directly associated

with wild or cultured species. Wild crustacean populations lack information on diseases due to the open nature of the crustacean fisheries. A lack of effort is directed to the studies of wild crustacean populations, whereas cultured population studies are approached with greater effort due to the control that can be obtained with cultured stocks compared to wild populations. The majority of crustacean pathogen studies that have occurred in wild crustacean populations have been completed on life-stages above a legal landing requirement, which leaves a knowledge gap on those life-stages below this requirement, or in species that are not commercially valuable. In many of the cultured crustacean studies, access to all life stages is possible. Both wild and cultured crustacean studies provide valuable information; however, there is research bias towards the studies that examine cultured crustacean species (Stentiford, 2012).

1.7.1 Management of Disease in Wild Crustaceans

Wild populations of crustaceans are not directly treated for disease as treatment of disease would not be possible on a mass scale and could put other species at risk. Instead of direct treatments, the aim for the management of disease in wild crustacean populations is to mitigate the negative impact of the disease (Flegel et al., 2008; Hauton, 2012; Karunasagar and Ababouch, 2012). Many approaches implemented include the destruction of any landed diseased animals, overfishing of the affected regions, overfishing of targeted sexes, limitations on importation of diseased animals and protocols for land-based culture operations (Flegel et al., 2008; Hauton, 2012; Karunasagar and Ababouch, 2012).

One method to limit the spread of crustacean disease to healthy populations, cultured or wild, is the destruction of diseased animals. Any landed crustaceans that are diseased will be destroyed to prevent the risk of transfer of the disease to healthy populations (Bower, 2017; Flegel et al., 2008; Stentiford, 2012). Results of this destruction method are similar to results with

limitations on the importation of diseased animals. By limiting the introduction of diseases to wild populations, the risk for further management needed in the future is decreased (Stentiford, 2012).

As well as the management methods that aim to decrease the introduction of the diseases to wild populations, methods of targeted fishing have been used to help decrease the prevalence of disease in wild populations (Bower, 2017; Flegel et al., 2008.; Stentiford, 2012). Overfishing in regions with high prevalence of a disease or overfishing a sex that has a higher prevalence of the disease has been found to greatly decrease the disease spread within the species population or to other species populations (Flegel et al., 2008). This is a mitigation method that employs the already active fishery and allows for data collection from landings. Management through decreasing the introduction of the diseases and decreasing the spread of the disease in wild populations have both been utilized for different diseases. For wild populations, an understanding of the diseases that are present and how to manage them is the most important management technique (Bower, 2017; Flegel et al., 2008; Stentiford, 2012).

1.7.2 Management of Disease in Cultured Crustaceans

Disease in cultured crustaceans is often more easily managed due to the use of many different disease management methods. Most commonly, the management of disease in cultured crustaceans focuses on warm water shrimp species, which are cultured worldwide. The first serious outbreaks of disease in cultivated shrimp occurred from 1987-1992 (Flegel et al., 2008). These outbreaks started the management strategies and resulted in better industry practices, including the use of probiotics, immunostimulants, quorum sensing, phage therapy, breeding and selections and most recently, a surge in RNA interference (RNAi) (Flegel et al., 2008).

Industry practices have been used in all types of aquaculture to mitigate the risks of disease and disease transmission (Lucas and Southgate, 2012). These practices include, but are not limited

to: sterilization of equipment after use, testing of brood stock before introduction to the facility, destruction and disposal of diseased animals, treatment of incoming water, lower stocking densities and stress reduction. These practices are readily used in all types of aquaculture including shrimp culture (Flegel et al., 2008; Lucas and Southgate, 2012).

Another commonly used industry practice in shrimp culture is the addition of probiotics to the culture water. Probiotics are preparations of living bacterial cells that are added to the water to improve water quality and prevent disease (Flegel et al., 2008). There are no large-scale studies supporting the use of probiotics for disease reduction or improved water quality; however, it is still a common practice in land-based operations. Probiotics have been utilized most commonly in the cultured shrimp species, *P. monodon*, against *Leiminus virbios* and *Vibrio harveyi* with a *Bacillus* probiotic (Meunpol et al., 2003; Moriarty, 1999; Rengpipat et al., 2003; Vaseeharan and Ramasamy, 2003). Along with showing increased resistance to the bacteria, the probiotics have also found other beneficial effects in culture including increased growth rates (Meunpol et al., 2003; Moriarty, 1999; Rengpipat et al., 2003; Vaseeharan and Ramasamy, 2003).

Immunostimulants are another common practice that still require large-scale studies to determine their effectiveness. Immunostimulants are preparations of whole or dead microbial cells, or semi-purified products from plants, microbes or chemicals, that are used to cause an immune response in the shrimp (Flegel et al., 2008). If the shrimp immune system stays in an active phase due to the presence of the immunostimulants, the immune system will already be active when a pathogen occurs and may better prepare shrimp to fight off the infection. A variety of immunostimulants have been explored in shrimp aquaculture of *P. monodon* and *L. vannamei* with a variety of different outcomes, all which are positive for the shrimp aquaculture industry (Apines-Amar and Amar, 2015). These immunostimulants include: vitamins such as vitamin C and vitamin

E, specific elements such as copper (Cu) and zinc (Zn), chitin and chitosan, sodium alginate, β-1,3-glucan, bacterial LPS and PGN (Chang et al., 2003, 2000; Cheng et al., 2005, 2004; Felix, 2005; Lee and Shiau, 2004, 2003, 2002a, 2002b; Shariff et al., 1995; Sivakumar et al., 2019). From these immunostimulants, a variety of results have been seen including: an increase in THC, proPO activity, respiratory burst and superoxide anion production, as well as an increased resistance to bacterial and viral infections (Chang et al., 2003, 2000; Cheng et al., 2005, 2004; Felix, 2005; Lee and Shiau, 2004, 2003, 2002a, 2002b; Shariff et al., 1995; Sivakumar et al., 2019).

Selective breeding of shrimp for disease tolerance to pathogens has been implemented to accelerate genetic improvement of specific traits of commercial production benefit. Producing specific disease tolerant lines of shrimp is the first step in long-term genetic improvement, which will promote the sustainable development of the shrimp industry (Trang et al., 2019). This selective breeding has occurred to increase resistance of *L. vannamei* to WSSV, IHHNV and TSV (Lucas and Southgate, 2012; Lillehammer et al., 2020; Moss et al., 2005; Trang et al., 2019; Zhang et al., 2019). Although the individuals may become infected, they do not become diseased.

RNA interference (RNAi) is a method of gene silencing through degradation of gene-specific dsRNA (Nguyen et al., 2018). RNA interference utilizes an RNA-induced silencing complex (RISC) by complementary RNA fragments introduced into the cell (Ufaz et al., 2018). RNA interference is used in aquaculture for combating viral diseases in shrimp. In *L. vannamei*, scientists are utilizing the RNAi pathway to reduce the effects of WSSV (Rijiravanich et al., 2008; Ufaz et al., 2018). Due to this success, RNAi is a common technology in WSSV management in aquaculture (Rijiravanich et al., 2008; Ufaz et al., 2018). RNA interference has also been used in *L. vannamei* for protection against IMNV and *Vibrio harveyi* with some success (Feijó et al., 2015; Wang et al., 2010). Researchers have shown promise with *P. monodon*, another cultured species

of shrimp, with RNAi being used as protection method against YHV in Thailand (Tirasophon et al., 2005). RNA interference has been shown to have promising results in combating viruses within shrimp aquaculture; however, further investigation is needed to understand the full extent of the benefits from the utilization of RNAi. RNA interference will be discussed further in 2.1.4.

1.8 Purpose of Research

This research aims to 1) investigate the response of crustacean cells (*Faxonius propinquus*) to a nanoparticle complexed to synthetic dsRNA analogue, and 2) examine the phylogeny of the AMP, crustin, in multiple crustacean species. As crustacean immunology is a more recent field of study, research conducted in this project may help to fill gaps in knowledge that may exist in our understanding of the crustacean innate immune system. Crustacean fisheries and aquaculture industries make a significant contribution to the economy and more research is needed to prevent economic loss that can results from disease. Some mechanisms of the crustacean immune system are described in previous research; however, gaps in knowledge still exist. Little can be done to alter the immune systems of wild crustacean populations, but knowledge of crustacean immune capabilities can be used in crustacean aquaculture systems to understand how they may respond to different types of infections and would greatly benefit the growing crustacean aquaculture industry.

Chapter 2: Examination of the Crustacean Immune Responses to a dsRNA-Nanoparticle Injection

2.1 Introduction

New methods are being developed to decrease the high mortality rates, up to 100%, caused by viral pathogens. The first serious outbreaks of disease in cultivated shrimp occurred from 1987-1992, starting with monodon baculovirus in Taiwan, followed by yellow head disease in Thailand and infectious hypodermal and hematopoietic necrosis virus and Taura syndrome virus in the Americas (Flegel et al., 2008). These outbreaks started the desire for management strategies to combat these viral pathogens, including: better industry and husbandry practices, the use of probiotics, immunostimulants, quorum sensing, phage therapy, selective breeding and most recently, a surge in RNA interference (RNAi) (Flegel et al., 2008). RNA interference (RNAi) is being utilized as a viral management strategy through dsRNA injections. This study examines the injection of synthetic dsRNA analogue, in the form of Poly (I:C) complexed to a phytoglycogen nanoparticle, into a crustacean to determine the impact that this dsRNA-nanoparticle complex will have on the immune system of the crustaceans.

2.1.1 Viral Mimetic: Poly (I:C)

Polyinosinic:polycytidylic acid (Poly (I:C)) is a synthetic analog of double-stranded RNA (Ding et al., 2018; InvivoGen, 2016). Poly (I:C) is comprised of long strands of inosine poly(I) homopolymer annealed to strands of cytidine poly(C) homopolymer. This composition allows for Poly (I:C) to have a structure similar to that of the dsRNA present in some viruses. This makes it an ideal model as a viral mimetic for many immunological studies (Caipang et al., 2011; Dai et al., 2017; Ding et al., 2018). Poly (I:C) is available in both high molecular weight (HMW) and low molecular weight (LMW). The HMW Poly (I:C) ranges from 1.5 kb to 8 kb, while the LMW Poly

(I:C) ranges from 0.2 kb to 1 kb. This study used the HMW Poly (I:C) because it is a stronger immunostimulant (InvivoGen, 2016).

2.1.2 Use of Poly (I:C) in Crustacean Research

Poly (I:C) is a promising immunostimulant in crustacean aquaculture and many immunological studies have been performed on both saltwater and freshwater crustaceans (Apines-Amar and Amar, 2015; Bricknell and Dalmo, 2005; Caipang et al., 2011; Inada et al., 2012; Ji et al., 2009). Generally, the use of dsRNA has caused an increase in the expression of immune related genes, and the production of superoxide anions when injected into either marine or freshwater crustaceans (Caipang et al., 2011; Inada et al., 2012; Ji et al., 2009).

Poly (I:C) injection into *L. vannamei* and *P. borealis* resulted in an initial spike in total circulating haemocyte counts, followed by a large decrease in the first hours post-injection (Caipang et al., 2011; Ji et al., 2009). Following Poly (I:C) injection, there were differences in the activity of haemolymph phenoloxidase between the two species of shrimp. Haemolymph phenoloxidase decreased in *L. vannamei* but increased in *P. borealis*. Post-injection, total antioxidant capacity and total glucose concentration of the haemolymph increased in *P. borealis*, while lysozyme and anti-protease levels decreased (Caipang et al., 2011; Ji et al., 2009). Intramuscular injections of Poly (I:C) in *M. japonicus* and *L. vannamei* increased expression of genes coding for NADPH oxidases, which produce free radicals such as reactive oxygen species (ROS) (Inada et al., 2012; Ji et al., 2009).

Freshwater *P. clarkii* and *M. rosenbergii*, injected intramuscularly with Poly (I:C), had increased expression of many immune genes including: anti-lipopolysaccharide factor (ALF), spätzle, heat shock protein 70, serine/threonine protein kinase, cactus, lectin, toll-like receptors (TLR), farnesyl diphosphate synthesis, peptidoglycan (PGN) recognition protein, gamma-

interferon-inducible lysosomal thiol reductase, glycogen synthase kinase-3 and C-type lectin (Dai et al., 2017; Liu et al., 2020; Wu et al., 2017). Some other notable transcripts observed include peroxiredoxin 5 and cathepsin C. Peroxiredoxin 5 is from the family of peroxiredoxins that play a critical role in host defense against oxidative stress. They can also be involved in immune responses following microbial infections (Wu et al., 2017). Cathepsin C is a lysosomal cysteine protease, which degrades peptides and proteins that have been brought into the cell through autophagy or endocytosis (Liu et al., 2020).

2.1.3 Use of Nanoparticles in RNA Interference

Injected RNA is at risk for enzymatic degradation when it is outside of cells (Schroeder et al., 2010; Ufaz et al., 2018). A molecular template is used to help reduce this degradation and facilitate the entry of the RNA into a cell. A variety of cationic vectors have been used as molecular templates to help transport the RNA into the cells, including lipids (lipoplexes), dendrimers (dendriplexes) and polymers (polyplexes) (Krishnan et al., 2009). The lipids and polymers can induce gene expression changes in biological systems that might have siRNA activity. Therefore, it is critical to screen delivery systems prior to adding dsRNA to reduce the induction of off-target effects (Krishnan et al., 2009; Nguyen et al., 2018).

Some experiments have tried to inject dsRNA without the use of a conjugate; however, conjugates are highly beneficial in reducing the degradation of the dsRNA, which ensures the crustacean receives intact RNA (Ufaz et al., 2018). Crustacean dsRNA injections have tested a variety of nanoparticle conjugates to improve the injection of the dsRNA. One conjugate that has been tested is viral particles. In *M. rosenbergii*, nodavirus-like particles were used as a conjugate for WSSV dsRNA and were found to improve protection to the crayfish against WSSV (Jariyapong et al., 2015). Another conjugate that has been utilized with WSSV in *L. vannamei* is

chitosan; a sugar that is obtained from the exoskeletons of crustaceans (Ufaz et al., 2019, 2018). Polymer complexes such as chitosan are excellent candidates for conjugates due to the protection they offer both DNA and RNA from degradation (La Fauce and Owens, 2012). Polymer complexes can be synthetically derived or, like chitosan, can be naturally derived (La Fauce and Owens, 2012).

2.1.4 RNA Interference

RNA interference is a process where RNA is involved in sequence-specific suppression of gene expression resulting in gene silencing (Sagi et al., 2013). Viruses contain genetic material made of DNA or RNA that encode replication information and structural proteins critical for viral genome packaging and virion assembly. RNA interference has been investigated as a mechanism for combating viruses in crustacean aquaculture due to the significant economic impact associated with severe viral outbreaks (Sagi et al., 2013).

RNA interference starts with the introduction of target specific dsRNA that corresponds to the viral RNA. In this situation, dsRNA is used as a viral PAMP, which is capable of inducing immune responses in haemocytes (Caipang et al., 2011; Desmet and Ishii, 2012; Ding et al., 2018; Inada et al., 2012; Ji et al., 2009; Poynter and DeWitte-Orr, 2018; Saray et al., 2018). Once the dsRNA enters the cytoplasm, the multidomain ribonuclease II enzyme Dicer2 cleaves the dsRNA into small (20-30 nt) RNA fragments. These fragments are known as small interfering RNAs (siRNAs), which have a characteristic 3'hydroxide, 5'phosphate and 3'dinucleotide overhang (Gong and Zhang, 2021; Krishnan et al., 2009; Li, 2014; Nguyen et al., 2018; Ufaz et al., 2018). The dsRNA forms a precursor RNA-induced silencing complex (pre-RISC) by binding with Argonaute-2 (Ago2) protein (Hannon, 2002; Maralit et al., 2015; Xu et al., 2007). The Ago2 protein is a catalytic component of RISC, which unwinds the dsRNA resulting in two strands of

ssRNA: the sense and antisense strands. The sense strand is known as the passenger strand and is either released into the cytoplasm with the possibility of triggering further dsRNA synthesis by RNA-dependent RNA polymerase (RdRP), or is degraded and used as RISC complex substrate (Hannon, 2002; Maralit et al., 2015; Xu et al., 2007). The antisense strand of the siRNA is known as the guide strand and remains bound to the RISC complex to act as a targeting sequence for the enzyme complex. RNA-induced silencing complexes use this ssRNA guide to bind to complementary target mRNA (Hannon, 2002; Maralit et al., 2015; Xu et al., 2007). After binding, the nuclease activity from RISC results in cleavage of the target mRNA. The resulting damaged mRNA is then degraded or causes sequence specific, post-transcriptional gene silencing (Hannon, 2002; Maralit et al., 2015; Xu et al., 2007). Once Ago2 unwinds the dsRNA, the mature RISC is formed and is able to target the complementary viral mRNA. The RISC complex is a multienzyme complex that has the ability to bind the siRNAs and unwind them (Hannon, 2002; Maralit et al., 2015; Xu et al., 2007).

2.1.5 NanoDendrix™

Nanoparticles have become increasingly popular in a variety of applications including cosmetics and pharmaceuticals. Often, biopolymers are used as nanoparticles that can include a highly branched, water soluble polymer of glucose formed by plants, known as phytoglycogen. Phytoglycogen is chemically simple, but has a distinct dendrimeric structure, which allows for a compact, monodispersed nanoparticle, with many applications (Dutcher et al., 2017).

Phytoglycogen from sweet corn known by the trade name NanoDendrix™ (Mirexus Inc. (Glysantis™), Guelph, Ontario, Canada) has been proposed as a potential nanoparticle for dsRNA injections in shrimp. NanoDendrix™ is a plant-based glycogen with distinct dendrimers with dendrimer fingers that can carry up to its own weight in nucleic acids (MW ~5 MDa; Diameter

~45 nm). The NanoDendrix™ is 0.38 type catatonic nanoparticle that has a neutral charge when bound to dsRNA. These nanoparticles are biodegradable, hydrophilic, stable with temperature and pressure, and easily chemically modifiable (Glysantis, 2020).

2.1.6 Pacific Whiteleg Shrimp (*Litopenaeus vannamei*)

Pacific whiteleg shrimp (*Litopenaeus vannamei*) are decapod crustaceans with ten walking legs. They can range in weight up to 45 g, where the females are typically larger than the males (Boone, 1931). Their carapace colouration is a blueish grey and they are found in tropical marine habitats in their adult stage, but are found in brackish waters of mangroves, lagoons and coastal estuaries in their sub-adult stage. They are found naturally in eastern Pacific waters from Mexico to Peru, where they are commercially harvested (Boone, 1931).

Pacific whiteleg shrimp are the most cultured shrimp species worldwide, accounting for 82% of all cultured shrimps in 2019 equalling 24 million tons of live weight (Fisheries and Oceans Canada, 2021). Aquaculture practices have introduced new viral disease challenges due to the high stocking density of *L. vannamei*. One challenge of viral outbreaks is the speed that the virus spreads, leading to up to 100% mortality in 2-7 days from the first clinical signs of infection (Loy et al., 2012). Shrimp are highly susceptible to a number of viral diseases, resulting in losses of over $1 billion USD annually (Loy et al., 2012).

2.1.7 Northern Clearwater Crayfish (*Faxonius propinquus*)

Current restrictions for the importation of *L. vannamei* resulted in a necessary substitution of the northern clearwater crayfish (*Faxonius propinquus*) for this study (Government of Canada C.F.I.A, 2019). *Faxonius propinquus* made an ideal substitution based on the readily available source of the crayfish; however, there are some notable differences in the two species. *Litopenaeus vannamei* is a saltwater crustacean that can survive in fresh water, compared to *F. propinquus* that

is a freshwater species. This is a difficult comparison as no studies exist that compare the immune responses between freshwater and saltwater crustaceans. *Litopenaeus vannamei* is able to survive in fresh water and is commonly cultured in fresh water farms, so the fresh water nature of the experimental animals should not be confounding (Seibert and Pinto, 2012). Phylogenetically, the species are in the same order: Decapoda (Tan et al., 2019).

The northern clearwater crayfish has a natural habitat range of the north-eastern United States (Illinois, Indiana, Iowa, Massachusetts, Michigan, Minnesota, New York, Ohio, Pennsylvania, Vermont and Wisconsin) and southern Ontario and Quebec. They inhabit streams, rivers, ponds and lakes. The highest concentrations of the crayfish are found in the Great Lakes of North America (Crandall and De Grave, 2017; Rosenthal et al.,2006). They have a maximum carapace length of 35 mm and weight ranging from 8.0-11.0 g at maturity (Simon et al., 2015). Carapace colouration is typically a brownish green with a notable feature of a broad dark dorsal band running the length of the abdomen (Figure 2). The chelae are s-shaped with orange or red tips. Other notable features include blade-like ridges (carina) on the rostrum (Crocker and Barr, 2008).

Figure 2 Northern clearwater crayfish (*Faxonius propinquus*) caught in the Conestogo River, St. Jacobs, Ontario, Canada in October 2020. Photo provided by Emma Monod.

2.2 Objective

The increase in shrimp aquaculture means that new methods are needed to combat viruses causing mass mortalities in the global crustacean aquaculture trade. The use of a synthetic dsRNA analogue-NanoDendrix™ complex is being explored as an option to mitigate these concerns. Therefore, the objective of this study is to examine the transcriptomic response of a crustacean immune system to the injection of a synthetic dsRNA analogue-NanoDendrix™ complex into the northern clearwater crayfish *F. propinquus*.

2.3 Hypothesis

There were two hypotheses for this study: (1) the injection of Poly (I:C) would show an upregulation of immune associated differentially expressed transcripts in the hepatopancreas, and (2) the injection of a Poly (I:C)-NanoDendrix™ complex would show an upregulation of RNA interference associated transcripts in the hepatopancreas, great than that of Poly (I:C) alone.

2.4 Materials and Methods

2.4.1 Collection and Husbandry of Crayfish

Northern clearwater crayfish (*F. propinquus*) were collected from the Conestogo River (St. Jacobs, Ontario, Canada) and held based on standard protocols from the DeWitte-Orr lab. A total of 19 crayfish were used for this study and weighed 5.22 g +/- 1.68 g (mean +/- SD). The sex and pathogen status of these crayfish were unknown. Crayfish were held in 208 L tanks (maximum 16 individuals/tank) in reverse-osmosis freshwater (19 °C) with continuous circulation and oxygenation with an air stone to maintain an oxygenation level of 80%. Water changes of 75% occurred every 3 days. Feeding occurred daily with Omega One shrimp feed with each crayfish receiving ~0.16 g of feed (Omega Sea LLC, Painesville, Ohio, United States). The crayfish were allowed to acclimate for 3 weeks prior to the injections.

2.4.2 Complexing Poly (I:C) to NanoDendrix™

Following standard protocols from the DeWitte-Orr lab, the Poly (I:C)-NanoDendrix™ complex was produced. NanoDendrix™ (0.38 type) (Mirexus Inc. (Glysantis™), Guelph, Ontario, Canada) was dissolved in RNAse and DNase free molecular biology grade water (VWR International, Mississauga, Ontario, Canada) at 1 mg/mL. The solution was vortexed to remove any large particle aggregates before being aliquoted in 500 µL aliquots and stored at -20 ºC. Prior to use, an aliquot of NanoDendrix™ solution and an aliquot of high molecular weight Poly (I:C) (1 mg/mL) were thawed at room temperature. Both the NanoDendrix™ solution and Poly (I:C) (Millipore Sigma, Mississauga, Ontario, Canada) solution were placed in a heating block at 55 ºC for a 40 min incubation period. After 10 min in the heating block, the NanoDendrix™ solution was vortexed and the Poly (I:C) was mixed by pipette, before being returned to the heating block for the remaining 10 min. During the last 20 min, periodic vortexing was completed. The solutions were left for 30 min at room temperature.

To complex the Poly (I:C) to the NanoDendrix™, methods were modified from Alkie et al. (2019). To start the complex, 10 µL of the 1 mg/mL NanoDendrix™ solution was mixed with 15 µL of phosphate buffered saline (PBS) . Separately, 10 µL of the Poly (I:C) solution was mixed with 15 µL of PBS. To make the Poly (I:C)-NanoDendrix™ complex, 25 µL of the NanoDendrix™ PBS solution and 25 µL of the Poly (I:C) PBS solution were mixed together by pipetting up and down 10 times. This final Poly (I:C)-NanoDendrix™ complex was incubated for 30 min at room temperature.

2.4.3 Injection of Poly (I:C)-NanoDendrix™ Complex

Northern clearwater crayfish were injected in the research laboratory of Dr. Stephanie DeWitte-Orr at Wilfrid Laurier University (Ontario, Canada) following standard protocols from

the DeWitte-Orr lab. One of three intramuscular injections was given to each of the crayfish at random: PBS (control), Poly (I:C) and Poly (I:C)-NanoDendrix™ complex. The PBS injection consisted of 25 µL of 0.6 M PBS. The Poly (I:C) injection consisted of a 0.6 M PBS and 0.4 mg/mL Poly (I:C), and the Poly (I:C)-NanoDendrix™ complex consisted of 0.6 M PBS, 0.4 mg/mL Poly (I:C) and 0.04 mg/mL ($2x10^{-19}$ M) NanoDendrix™ as outlined in 2.4.2.

The injections were given using a 6 mm 31-gauge insulin syringe. The intramuscular injections were completed between the 3rd and 4th or 4th and 5th tergum depending on crayfish size. The crayfish were returned to the holding tanks and hepatopancreas samples were taken after 6 h (Rise et al., 2010). No anesthesia was used for the dissection. The hepatopancreas samples were placed into TRIzol® (ThermoFisher Sceintific, Mississauga, Ontario, Canada) and stored at -20 °C. The samples were shipped on dry ice to Dalhousie University (Truro, Nova Scotia, Canada).

Total RNA was extracted from Trizol® preserved samples of the hepatopancreas. RNA isolation was completed using methods based on phenol-chloroform RNA extraction (Chomczynski and Sacchi, 1987) and followed by an RNA clean-up using a Qiagen RNAeasy mini kit with an on-column deoxyribonuclease (DNaseI) treatment (Qiagen, Toronto, Ontario, Canada) according to the manufacturer's instructions (Clark et al., 2013a). RNA was quantified using a NanoDrop® 1000 ND-UV-Vis Spectrophotometer (ThermoFisher Scientific, Mississauga, Ontario, Canada) and the quality was assessed using an Agilent Bioanalyzer (Agilent, Mississauga, Ontario, Canada) prior to sending the samples to Novogene Co., Ltd (Beijing, China) for Illumina NovaSeq 6000 sequencing with 30 samples per lane (Clark et al., 2013b, 2013c, 2013d). Some samples were concentrated with a Savant™ SpeedVac™ High-Capacity Concentrator (ThermoFisher Scientific, Canada) due to low RNA concentration prior to shipping.

2.4.4 Differential Expression Analysis

Raw RNA sequence reads were uploaded into Galaxy Europe (Freiburg Galaxy Team, 2021) and the quality of the reads were assessed by FastQC (Babraham Bioinformatics, 2019a). Poor sequence reads, minimum Phred score of 37 (Wang et al., 2012), were filtered out through Trim Galore! (Babraham Bioinformatics, 2019b) before once again being assessed again for quality by FastQC. The samples were then concatenated with Concatenate Datasets Head to Tail (Grüning, 2021). The Trinity (Grabherr et al., 2011; Haas et al., 2013) pipeline was run on the paired-end samples to generate a *de novo F. propinquus* transcriptome. Fasta Statistics (Freiburg Galaxy Team, 2021) were run to determine the descriptive statistics for the *de novo* transcriptome.

The filtered sequence read files were aligned to the new transcriptome using HISAT2 (Daehwan et al., 2015). The number of transcripts in each sample from the HISAT2 output was then determined by running StringTie with no general feature format (GFF) (Freiburg Galaxy Team, 2021). The quantified transcript files from the StringTie outputs were then merged with StringTie merge to create a new consensus annotation of the transcriptome (Freiburg Galaxy Team, 2021). Counts of the sample transcripts were generated using featureCounts (Liao et al., 2014) run on the HISAT2 output.

To determine the differentially expressed transcripts (DETs), both DESeq2 and edgeR pipelines were used (Liu et al., 2015; Love et al., 2014; Robinson et al., 2010). The featureCounts output was loaded into both DESeq2 and edgeR with the comparisons of the control to the Poly (I:C) (PBS vs. HMW) and control to the Poly (I:C)-NanoDendrix™ complex (PBS vs. HN) (Li and Dewey, 2011; McCarthy et al., 2012; Robinson et al., 2010).

Significant differentially expressed transcripts (FDR < 0.05) were annotated through blastx (Altschul et al., 1990; Camacho et al., 2009; Cock et al., 2015; Götz et al., 2008; Guindon et al.,

2010; Huelsenbeck and Ronquist, 2001; Lynn et al., 2008; Ronquist et al., 2012) searches of public databases (NCBI and SwissProt), domain identification Blast2GO (Conesa et al., 2005) and compared to similar species transcripts that were previously described in primary pathogen-challenge research (Altschul et al., 1990; Camacho et al., 2009; Cock et al., 2015; Götz et al., 2008; Guindon et al., 2010; Huelsenbeck and Ronquist, 2001; Lynn et al., 2008; Ronquist et al., 2012). Annotation refers to those DETs that were blast identifiable through blastx with a protein hit with an E value < $1.0x10^{-5}$. The annotation with the highest percentage similarity was retained. Each annotation was then classified to better understand what types of transcripts were differentially expressed. The data from the DETs was used to create volcano plots with Volcano Plot (Freiburg Galaxy Team, 2021) and a heat map with heatmap2 (Freiburg Galaxy Team, 2021) from Galaxy Europe.

From the SwissProt annotation, the UniProt identification value was used to further classify each of the transcripts into a functional category in UniProt (UniProt Consortium, 2021). The pathway analysis of each DET was determined with the Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server (KAAS) (Moriya et al., 2007).

2.4.5 Modifications to Original Methods for Transcriptome Production

Due to the high number of sequences in the transcriptome, the low number of Assigned_Mapped reads, the high number of Unassigned_Unmapped reads and the high number Unassigned_MultiMapping sequences observed in the featureCounts summary, two modifications were utilized to improve the results: (1) two reference genomes, *Litopenaeus vannamei* (GenBank: ASM378908v1) and *Procambarus virginalus* (GenBank: GCA_002838885.1), were utilized, rather than the *de novo* transcriptome in effort to increase the number of Assigned_Mapped reads and decrease the high number of Unassigned_Unmapped reads; and (2) in the *de novo*

transcriptome creation, the Trinity settings were modified and re-run. As the minimum k-mer coverage default is 1, transcriptomes were generated using the default minimum k-mer coverage value as well as values of 2, 5, 10, 20, 25, 30 and 35, to see if this modification would improve the *de novo* transcriptome using the above analysis (2.4.4).

2.5 Results

2.5.1 Sequencing and *de novo* assembly

The raw reads of each the control (PBS; n = 6), Poly (I:C) (HMW; n = 6) and Poly (I:C)-NanoDendrix™ complex (HN; n = 7) were 147,143,136, 121,451,944 and 157,802,290, respectively (Table 2). The *de novo* transcriptome contained 579, 496 sequences ranging in length from 117 to 42,250 nt, with majority of the sequences being greater than 200 nt (99.98%). The GC content of the transcriptome was 43.0% from the 388,527,635 bp included (Table 3).

Table 2 The data provided by NovoGene from Illumina NovaSeq 6000 sequencing. Full data provided in Appendix 1.

Sample	n	Raw reads	Effective Reads (%)	nt Error (%)	Q20 (%)	Q30 (%)	GC (%)
PBS	6	147,143,136	98.33	0.03	97.41	92.88	45.13
HMW	6	121,451,944	98.12	0.03	97.73	93.78	45.41
HN	7	157,802,290	97.95	0.03	97.77	93.94	45.74

Table 3 The descriptive statistics data for the *de novo* transcriptome created from the *Faxonius propinquus* samples using a minimum k-mer coverage of 1.

Number of Assembled Sequences	579,496
L50	79,144 contigs
Maximum Length	353 nt
N50 Length	1,062 nt
Minimum Length	177 nt
Mean Length	670 nt
Median Length	353 nt

2.5.2 Improving the Quality of the Transcriptome

Two genomes were examined as possible substitutions for the *de novo* transcriptome in this study. Both *L. vannamei* (GenBank: ASM378908v1) and *P. virginalus* (GenBank: GCA_002838885.1) genomes were utilized in an effort to improve the results of the featureCounts (German Cancer Research Center, 2018; Institute of Oceanology, Chinese Academy of Sciences, 2017) (Table 4). The featureCounts output for the reference genome-generated transcriptomes did not result in improvement or the use of the minimum k-mer coverage of 1 *de novo* transcriptome.

Table 4 The featureCounts output from the genomes used to analyze transcriptomes of *Faxonius propinquus* injected with PBS, Poly (I:C) and Poly (I:C)-NanoDendrix™ complex using a minimum k-mer coverage of 1.

Genome	Assigned	Unassigned_Unmapped	Unassigned_MultiMapping
Litopenaeus vannamei	3,000	26,000,000	1,154
Procambarus virginalus	3,500,000	17,000,000	750,000

The second approach involved changing the minimum k-mer coverage setting for Trinity from the default of 1 to 2, 5, 10, 20, 25, 30 and 35. The results of mapping and featureCounts using these transcriptomes found that the minimum k-mer coverage of 25 was ideal for this data set due to the reasonable number of transcripts, the high N50 value and the highest number of assigned reads compared to unassigned reads (Figure 3). The descriptive statistics for the *de novo* transcriptome (minimum k-mer coverage = 25) are displayed in Table 5.

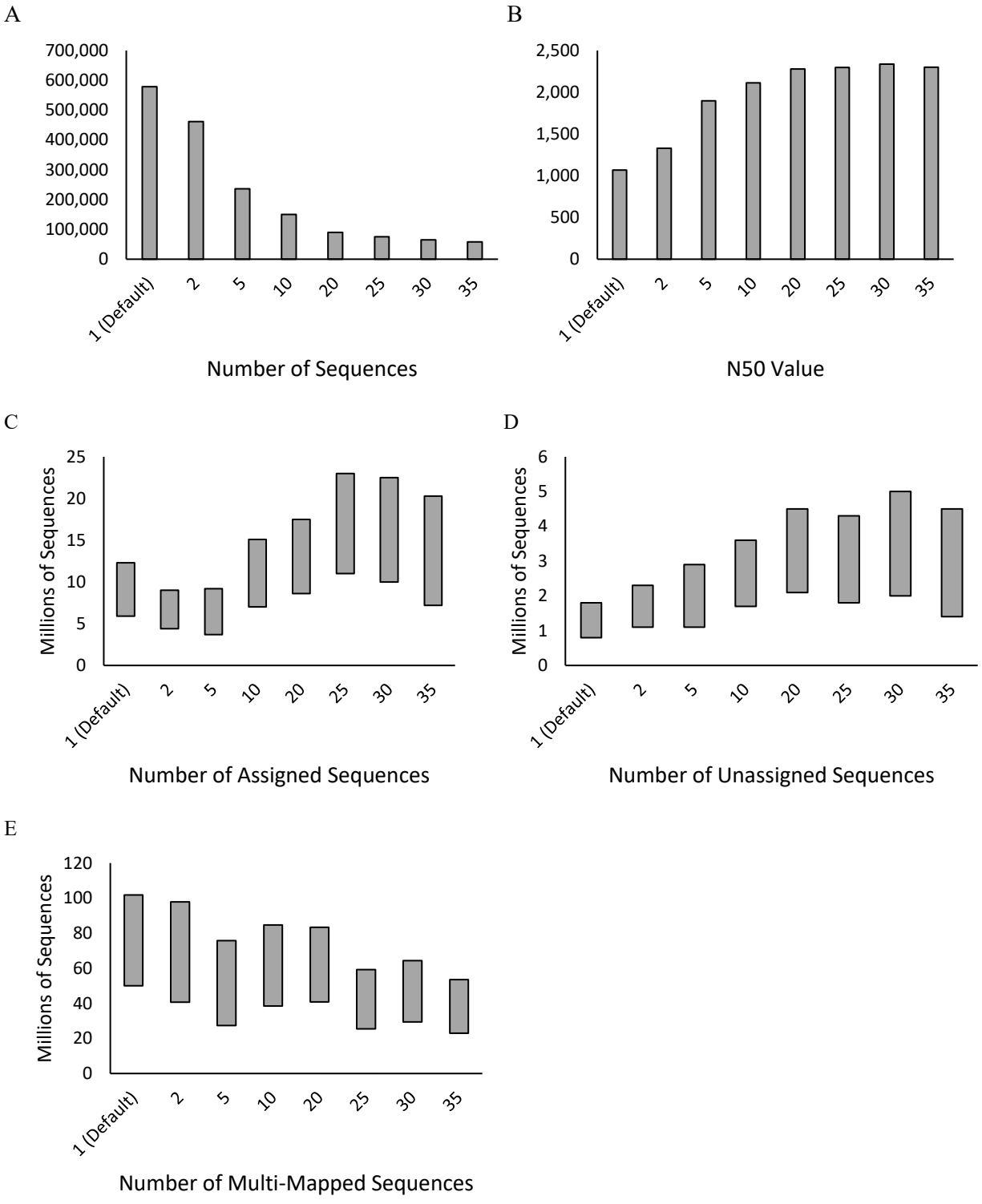


Figure 3 Fasta Statistics and featureCounts output from the various minimum k-mer coverage settings tested in the Galaxy Europe - Trinity pipeline for improvement of the *Faxonius propinquus* transcriptome. (A) The number of sequences. (B) The N50 value. (C) The number of sequences that were assigned. (D) The number of sequences that were unassigned. (E) The number of sequences that were multi-mapped.

Table 5 The descriptive statistics data for the best *de novo* transcriptome (minimum k-mer coverage = 25) created from the *Faxonius propinquus* samples.

Number of Assembled Sequences	75,175
L50	10,966 contigs
Maximum Length	23,710 nt
N50 Length	2,298 nt
Minimum Length	179 nt
Mean Length	1,183 nt
Median Length	581 nt

2.5.3 Functional Annotation and Classification of Transcriptome Sequences

blastx-based similarity searches of publicly available NCBI protein non-redundant (nr) and SwissProt databases resulted in annotation for 48.3% (34,309 transcripts) and 25.9% (19,456 transcripts), respectively (S 2.1; S 2.2). Due to the low annotation percentage within the SwissProt database, the NCBI protein non-redundant (nr) database was used for this analysis.

From the NCBI database, the organisms associated with the annotations were classified into their respective taxonomical groups. The majority of the annotations came from Crustacea (66.0%), followed by Arthropoda excluding Crustacea (9.0%). Annotations that related back to bacteria accounted for 7.0% (Figure 4). The majority of the species hits were from sequences attributed to crustaceans with the exception of the genera *Armadillidium* and *Trinorchestia* (Figure 5).

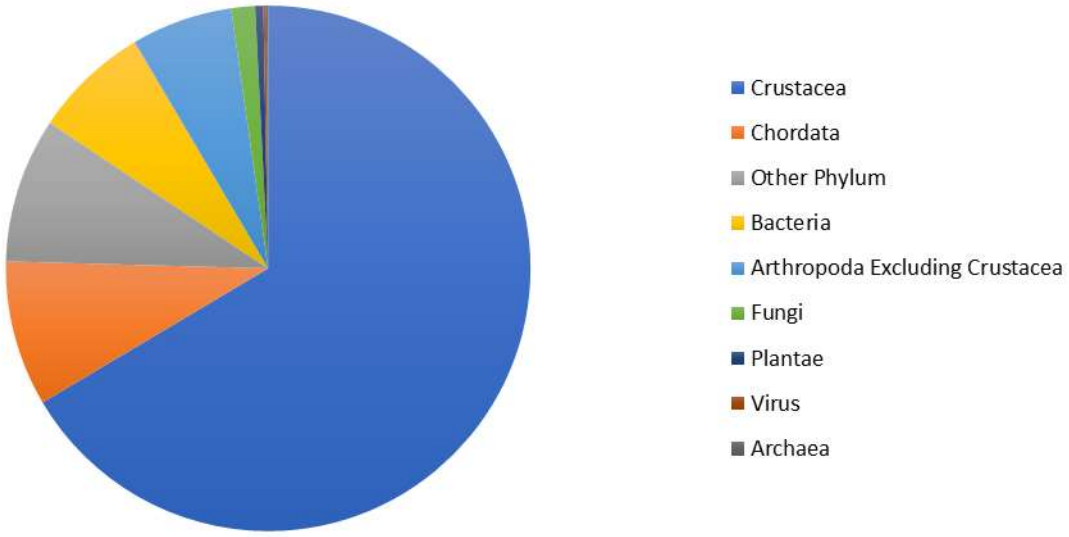


Figure 4 Taxonomic attribution of the sequence homology hits found in the NCBI nr database from the *Faxinious propinquus de novo* transcriptome.

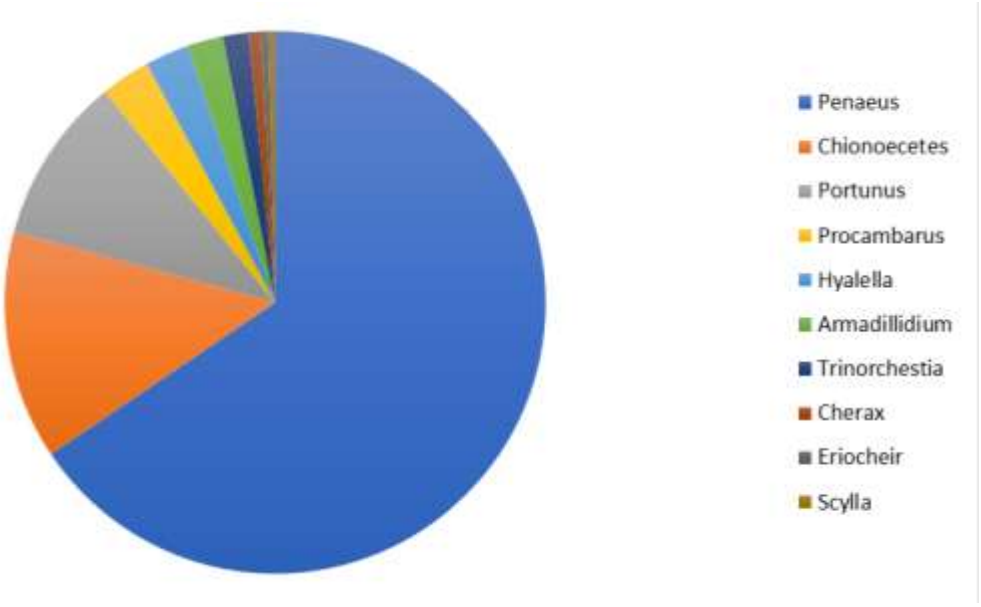


Figure 5 The top 10 crustacean genera annotation hits from the *Faxinious propinquus de novo* transcriptome found in the NCBI (nr) database.

2.5.4 Identification of Differentially Expressed Transcripts from the Improved *de novo* Transcriptome

The *de novo* transcriptome with a minimum k-mer coverage value of 25 was used to determine the DETs through both the edgeR (Robinson et al., 2010) and DESeq2 (Love et al., 2014) pipelines of Galaxy Europe. The total number of DETs from the DESeq2 and edgeR from both the Poly (I:C) vs the PBS control and Poly (I:C)-NanoDendrix™ complex vs the PBS control treatments was zero for edgeR and 404 for DESeq2.

2.5.4.1 DESeq2 Determined Differentially Expressed Transcripts of the Improved Faxonius propinquus de novo Transcriptome

Of the transcripts found to be differentially expressed through DESeq2, 41 transcripts were differentially expressed in the Poly (I:C) only treatment, while Poly (I:C)-NanoDendrix™ complex had 382 DET. In comparing the two trials, 19 DETs were found in both treatments (Figure 6). Of these 19 DET, 12 had no known annotation, four were hypothetical proteins, and the remaining three annotated to the following: *troponin T skeletal muscle isoform*, *unconventional myosin-Va like protein* and *glutactin isoform* (Appendix 2).

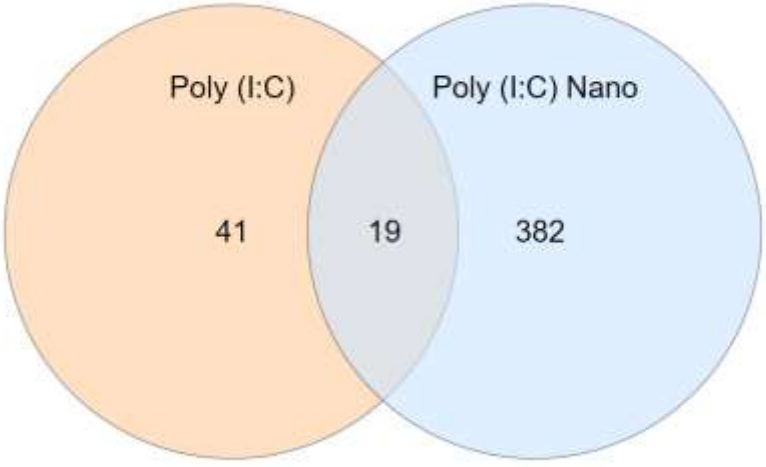
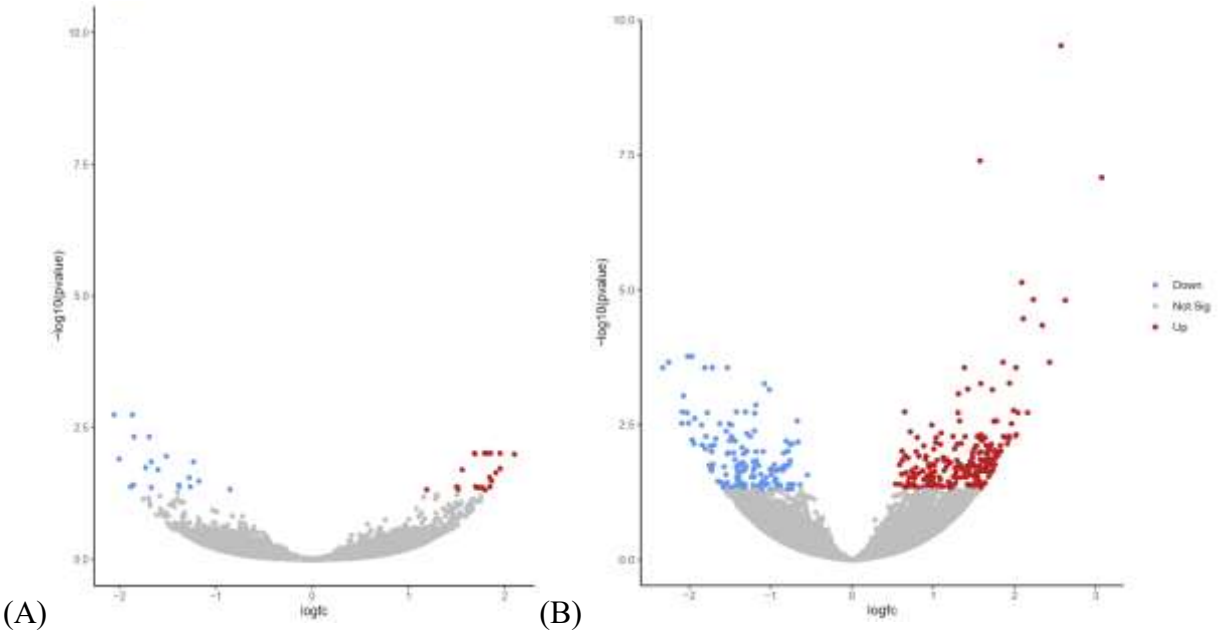


Figure 6 Differentially expressed transcripts from the DESeq2 analysis on the Poly (I:C) and Poly (I:C)-NanoDendrix™ complex treatments compared to the PBS control.

A total of 44% of the 41 DETs from DESeq2 of the control vs. the Poly (I:C) were annotated in the NCBI database and a total of 27% from the SwissProt database (Table 6; Figure 7). The DETs from DESeq2 of the control vs. the Poly (I:C)-NanoDendrix™ complex treatment resulted in the discovery of 382 DETs; 38% were annotated in the NCBI database and 20% annotated in the SwissProt database (Table 6; Figure 7). These DETs were examined across each sample of the three treatments; samples within the same treatment had more similar normalized counts to each other than to those in the other two treatments (Figure 8).

Table 6 The number of successfully annotated differentially expressed transcripts by publicly available databases and pathway analysis (minimum k-mer coverage = 25).

	Total Number of Transcripts	Number of Transcripts Annotated NCBI	Percentage Annotated (%) NCBI	Number of Transcripts Annotated SwissProt	Percentage Annotated (%) SwissProt	Number of KO Value	Percentage KO Value (%)
PBS vs HMW	41	18	43.90	11	26.83	9	21.95
PBS vs HN	382	145	37.96	77	20.16	42	10.99



(A) (B)

Figure 7 The differentially expressed transcripts as determined by DESeq2 for the control to Poly (I:C) (A) and the control to Poly (I:C)-NanoDendrix™ complex (B).

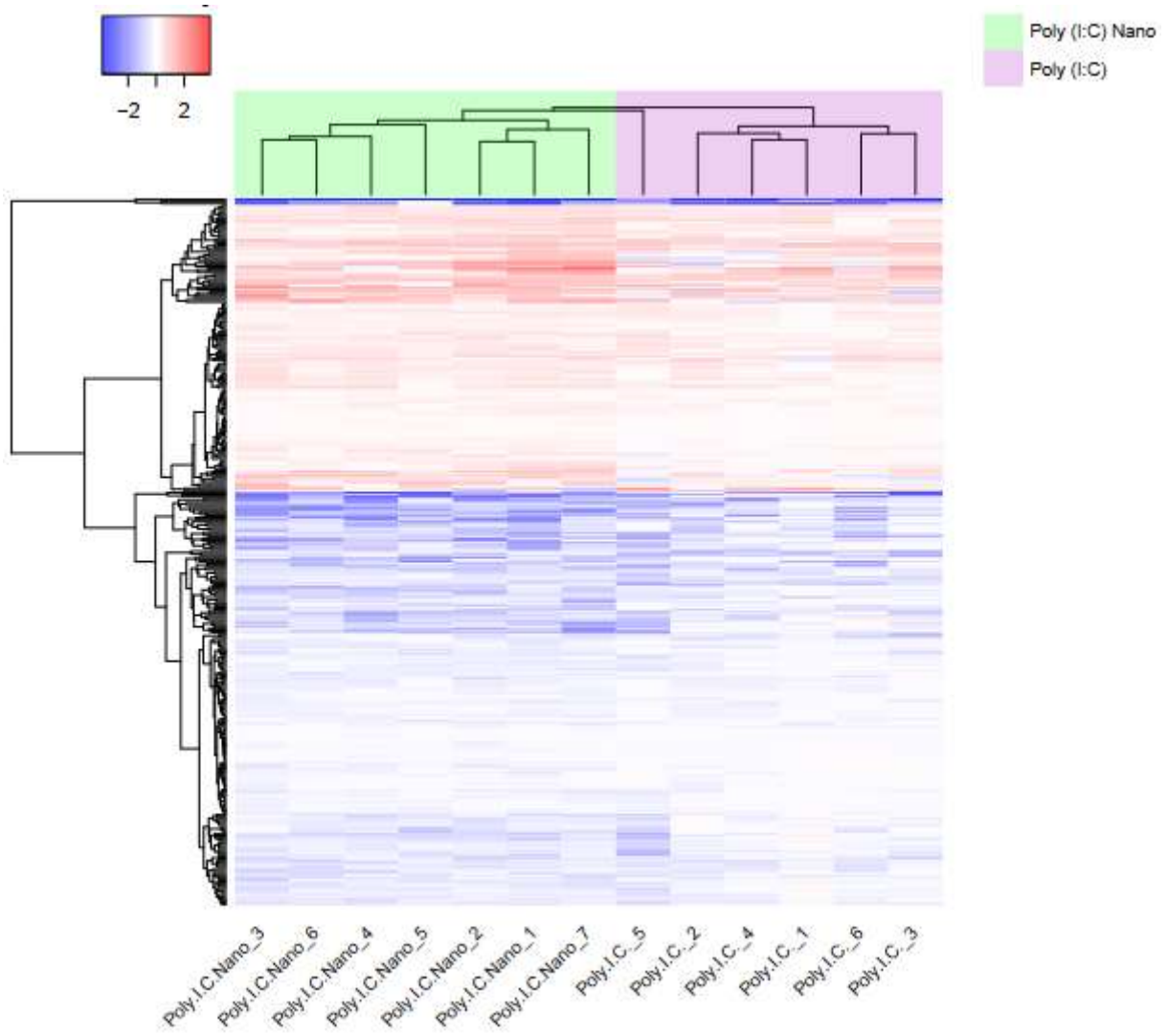


Figure 8 The heat map of the DESeq2 determined differentially expressed transcripts counts ratios (log2) for individual Poly (I:C) (n=6) and Poly (I:C)-NanoDendrix™ complex (n=7) samples compared to the average PBS control. The columns represent samples while the rows represent transcripts.

The DETs were grouped based on their associated biological processes as determined by UniProt from their SwissProt annotations. The DETs were classified on their biological process key words from UniProt (The UniProt Consortium, 2021). Of these DETs, 11 of the 41 DETs from the Poly (I:C) comparison were classified and 77 of the 382 DETs from the Poly (I:C)-NanoDendrix™ complex comparison were classified (Figure 9). The DETs from the Poly (I:C)

alone fell in categories of transcription, metabolism, transport, biosynthesis, ATP production, cytoskeletal processes and regulation. In the Poly (I:C)-NanoDendrix™ complex, the majority of DETs were found to be classified as transcription, metabolism and no known biological function.

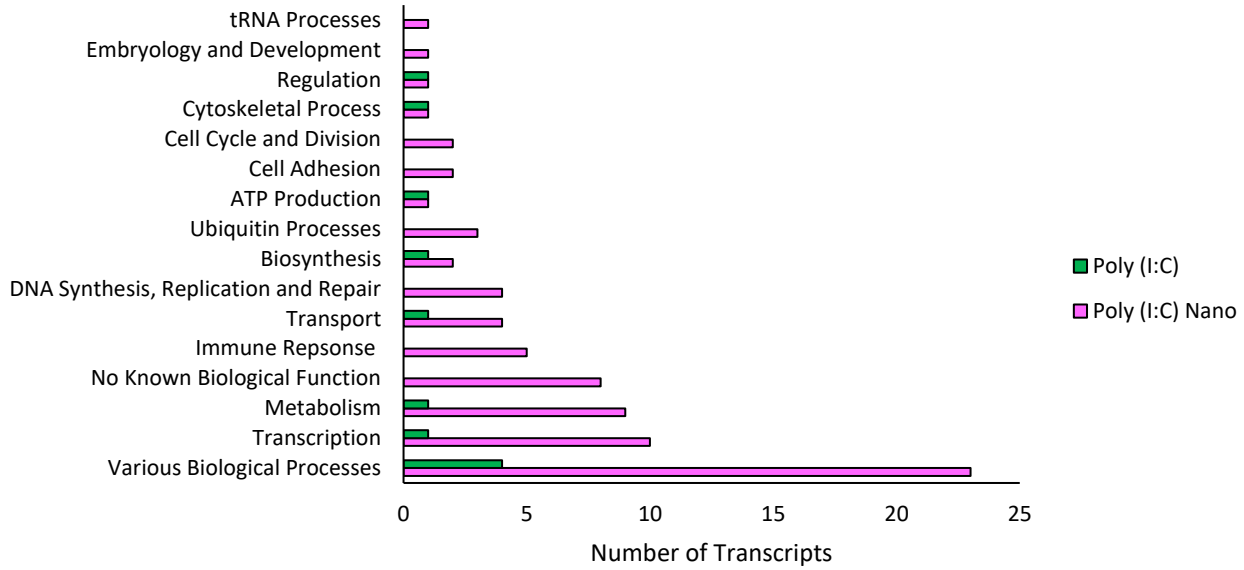


Figure 9 The classifications based on UniProt of the differentially expressed transcripts as determined by DESeq2.

The biological pathways of the DETs were determined using KAAS (Moriya et al., 2007). From the comparison of the control to the Poly (I:C), 34 pathways contained DET. When examining the DETs that were found to have these KASS associated pathways from the Poly (I:C), only five pathways were determined to be relevant to this study based on previous work (Dai et al., 2017; Perdomo-Morales et al., 2020; Saray et al., 2018) (Appendix 3). The pathways are autophagy, peroxisome, protein processing in endoplasmic reticulum, ubiquitin mediated proteolysis and Wnt signaling pathway. These pathways were then used to determine the immune related DETs as well as a review of the other annotated DETs that did not associate to pathways in KAAS. From the KAAS pathway analysis, three transcripts were found to be annotated that were associated to five pathways (Appendix 3). Differentially expressed transcripts with similarity

to *peroxisomal sarcosine oxidase-like* from *L. vannamei* and *ubiquitin-like-conjugating enzyme ATG3* from the red harvester ant (*Pogonomyrmex barbatus*) were both found to be present in one pathway, downregulated in peroxisome and upregulated in autophagy, respectively. A DET with similarity to *Cullin-1* from the turnip sawfly (*Athalia rosae*) was found to be downregulated in the pathways of ubiquitin mediated proteolysis, protein processing in endoplasmic reticulum and Wnt signalling pathway. No other DETs were found related to immune function that were not associated to a KAAS pathway.

The pathways associated to the metabolism of amino acids was examined due to the possible role as modulators of crustacean immunity (Huang et al., 2020). The four amino acid metabolism pathways that were found were: biosynthesis of amino acids, lysine degradation, purine metabolism and glycine, serine and threonine metabolism (Appendix 4; Appendix 5).

From the comparison of the control to the Poly (I:C)-NanoDendrix™ complex, 89 pathways were discovered to contain DET. When examining the DETs that were found to have these KASS associated pathways from the Poly (I:C)-NanoDendrix™ complex treatment, the pathways relevant to this study based on previous work were: autophagy, FoxO signaling pathway, mitophagy, NOD-like receptor signaling pathway, ubiquitin mediated proteolysis, endocytosis, MAPK signaling pathway and Rap1 signaling pathway (Perdomo-Morales et al., 2020; Saray et al., 2018). Some DETs were also associated with more than one pathway (Appendix 6). These pathways were then used to determine the immune related DETs, as well as a review of the other annotated DETs that did not have a KAAS associated pathway. From the KAAS pathway analysis, four DETs were found to be annotated that were associated to eight pathways (Appendix 6). A DET with similarity to *putative autophagy protein 8* from the zigzag leafhopper (*Recilia dorsalis*) was found to be upregulated 1.62 -log2 fold in the KAAS pathways of autophagy, FoxO signaling

pathway, mitophagy and NOD-like receptor signaling pathway. A DET with similarity to *E3 ubiquitin-protein ligase NEDD4 isoform X4* from the red mason bee (*Osmia bicornis*) was downregulated 0.85 -log2 fold in the KAAS pathways of endocytosis, MAPK signaling pathway and ubiquitin mediated proteolysis. A DET with similarity to *hypothetical protein B7P43_G09598* from the drywood termite (*Cryptotermes secundus*) was upregulated 1.93 -log2 fold in the ubiquitin mediated proteolysis pathway. Although these DETs were associated to immune related pathways, other DETs were found to relate to immune function that did not contain a KAAS pathway association. The DETs of interest included two different transcripts, both of which were *C-type lectins*. The transcripts were *C-type lectin* with similarity to *P. monodon* (2.04 -log2 fold) and *antiviral-like C-type lectin* with similarity to the Indian prawn (*Penaeus indicus*) (-1.57 -log2 fold).

As well as examining the immune related DETs, the pathways associated to metabolism of amino acids were examined. In the specific pathways, most of the metabolism could be related to the larger classification of lipid or carbohydrates (Appendix 7). The metabolism of amino acids was examined due to the possible role as modulators of crustacean immunity (Huang et al., 2020). No pathways were found related to amino acid metabolism.

2.6 Discussion

2.6.1 Improvement of the *de novo* Transcriptome

The initial *de novo* transcriptome (minimum k-mer coverage = 1) was not ideal for this study due to the high number of transcripts included in the multi-mapped category and the low number of reads in the assigned category. In an ideal transcriptome, the number non-multimapping assigned reads would be as high as possible. Two reference genomes and adjustments to the minimum k-mer coverage value in the Trinity analysis was performed in an effort to improve the *Faxonius propinquus* transcriptome.

The use of the reference genomes did not improve the transcriptome. The *Litopenaeus vannamei* and *Procambarus virginalus* genomes were unsuccessful for different reasons. The *L. vannamei* genome resulted in a very low count of assigned reads and a very high count of unassigned reads and thus the genomes did not help resolve the issues found with the *de novo* transcriptome (minimum k-mer coverage = 1). The *P. virginalus* genome provided a higher assigned number of reads, however there were still a greater number of reads in the unassigned category than the assigned category. The *P. virginalus* genome should have been more ideal than the *L. vannamei* genome as the two species share an infraorder, however it still did not result in a usable transcriptome.

The default setting for minimum k-mer coverage in Trinity is 1 (Grabherr et al., 2011). Generally, studies do not disclose the minimum k-mer coverage value that they use in their methods (Dai et al., 2017; Ding et al., 2018; Li et al., 2013; Tian and Jiao, 2019; Zhong et al., 2017); however, some studies have started to disclose the k-mer coverage value utilized (Melicher et al., 2014; Polinski et al., 2021; Rana et al., 2016). Utilizing a range of minimum k-mer coverage values (1, 2, 5, 10, 20, 25, 30 and 35) resulted in the minimum k-mer coverage of 25 standing out as the best. This improved transcriptome has a high number of assigned and low number of unassigned reads and a higher N50 compared to the transcriptomes resulting from the other minimum k-mer coverage values.

2.6.2 Differential Expression Analysis

Both edgeR (Robinson et al., 2010) and DESeq2 (Love et al., 2014) were used to identify DETs; however, edgeR found no DETs, while DESeq2 found several hundred. Both of these methods are based on the assumption that most transcripts are not differentially expressed (Dillies et al., 2013). DESeq2 assumes that transcripts that are not differentially expressed should have

similar read counts (CPM) across samples, this would lead to a ratio of one. edgeR in comparison uses a trimmed mean of M values (TMM) factor for this correction, which is based on the weighted mean of log ratios between the reference and the samples. This ratio is determined after the exclusion of the most highly expressed transcripts and the transcripts with the largest log ratios. Although these methods differ, the ratios should both appear close to one (Dillies et al., 2013). The difference in these methods has led to the large differences found in the number of DETs in this study. One way to determine if the DETs are reasonable is to generate a heatmap of the expressed transcripts counts ratios compared to the average of the control, with hierarchical clustering of the samples. If the resulting sample dendrogram separates most of the treatments into different treatment or biological relevant clusters, it can be assumed that the transcripts that were identified as differentially expressed are good indicators of the differences between the treatments. The results of the heatmap (Figure 8) indicate that DESeq2 has identified biologically relevant transcript expression differences between the treatments because of the grouping of the replicates into their treatment groups.

In comparing the two treatments of Poly (I:C) and the Poly (I:C)-NanoDendrix™ complex to the control, the complex had almost 10-fold the number of DETs as the Poly (I:C) alone. This study also found several significant differences from a number of previous studies involving the injection of Poly (I:C) into crustaceans. Other studies in species from the same infraorder and different infraorders, found upregulation of genes and pathways associated with immune and metabolic responses (Dai et al., 2017; Perdomo-Morales et al., 2020; Saray et al., 2018). Although the use of KASS pathway analysis found some pathways associated to immune processes in the Poly (I:C) alone, there were no DETs that were specifically annotated as immune associated. However, the injection of the Poly (I:C)-NanoDendrix™ complex found results similar to the

studies that injected the Poly (I:C) alone, including the presence of immune associated DETs (Dai et al., 2017; Perdomo-Morales et al., 2020; Saray et al., 2018). This indicates that the use of NanoDendrix™ with the Poly (I:C) enhances the immune-related expression more than the Poly (I:C) alone.

Immune response was a classification of DETs that were found in the presence of Poly (I:C) complexed to a NanoDendrix™ but not to Poly (I:C) alone. In the presence of Poly (I:C), an immune response is expected to be observed as Poly (I:C) is a viral mimetic (Caipang et al., 2011; Ding et al., 2018; Inada et al., 2012; Ji et al., 2009; Saray et al., 2018). This immune response is most commonly found to start at around 12 h for Poly (I:C) suggesting that the samples may have been taken prior to the immune response starting; this will be discussed further in 2.6.3. Because an immune response was found with the Poly (I:C)-NanoDendrix™ complex, it may be that the NanoDendrix™ itself, or the combination of the NanoDendrix™ complexed to Poly (I:C) initiated a faster immune response than that of Poly (I:C) alone. Although no transcripts were found to be classified as immune response through the UniProt classification, two DETs discovered were immune-related in crustaceans; both of which were *c-type lectins*.

A *c-type lectin* was a crustacean immune transcript with the highest upregulation within immune DETs having a 2 -log2 fold upregulation. Conversely, *c-type lectin* was also present with a 1.5 -log2 fold downregulation. In comparing these two DETs, they did not appear to be paralogues or splice variants. From the blastp comparison of two sequences (Altschul et al., 1997; Camacho et al., 2009), the percent similarity of the two sequences was determined to be 27.69% ($E = 1.0\times10^{-19}$). With the lack of similarity between the two sequences, it can be determined that these sequences are most likely not paralogues or splice variants. Within crustacean immunity, C-type lectins have a large range of functions including pathogen neutralization (Söderhäll, 2010).

This neutralization includes the role that C-type lectins play as opsonins to encourage phagocytosis, encapsulation and nodule formation of microorganisms. The annotation of this lectin only categorized it as a C-type lectin; however, it would be interesting to further investigate this lectin as different C-type lectins have different roles in crustacean immunity. In *L. vannamei*, a C-type lectin has been discovered that possesses anti-WSSV properties (Zhao et al., 2009). This lectin, LvCTL1 protein, has been found to significantly increase the survival of the shrimp in a WSSV challenge, as well as protect the haemocytes from WSSV infection. It is possible that LvCTL1 is acting as a PRR that recognizes and binds WSSV, thus blocking the virus from entering cells, thereby neutralizing the virus. Expression of C-type lectins in the presence of Poly (I:C) injection has not been noted in any other studies (Caipang et al., 2011; Ding et al., 2018; Du et al., 2017; Inada et al., 2012; Saray et al., 2018; Wang et al., 2018; Zhao et al., 2009). The expression of the C-type lectin in the presence of the Poly (I:C)-NanoDendrix™ complex suggests an immune response at the examined time point, which was not found with the Poly (I:C) alone.

Oxygen transport classified genes were not differentially expressed in the Poly (I:C) trial, and many were downregulated in the presence of the Poly (I:C)-NanoDendrix™ complex. It would be expected that an immune response would cause an increase in oxygen transportation, as most immune response studies have found an increase in the need for oxygen (Ji et al., 2009). This increase in oxygen transport is often accompanied by an increase in haemocyte or haemocyanin production but would require total haemocyte counts and haemocyanin levels to be determined during the study. An increase in haemocytes would be expected as haemocytes are the main cellular component of the crustacean immune system (Johansson and Söderhäll, 1989; Vazquez et al., 2009). Because of the lack of oxygen transport changes in the Poly (I:C) trial, it could be suggested that an immune response had not yet occurred; however, an examination of

haemocyanin would be needed to confirm this. This lack of immune response occurrence at the time point tested could also have resulted in the decreased oxygen transportation in the Poly (I:C)-NanoDendrix™ complex. If the experiment was conducted for a longer period of time, it is possible that more genes associated to oxygen transport would be upregulated. These effects observed from Poly (I:C) intermuscular injections in crustaceans most commonly start at around 12 h, which will be discussed further in 2.6.3.

Genes that code for proteins related to metabolic functions are routinely the biological pathways with the most differentially expressed genes during Poly (I:C) injections in crustaceans (Dai et al., 2017; Perdomo-Morales et al., 2020; Saray et al., 2018). The energy consumption of immune responses is high, and therefore energy producing metabolic processes must increase to produce more energy from energy stores. This increase in metabolic associated DETs result was found in this study when the crayfish was injected with Poly (I:C) complexed to the NanoDendrix™. Although these metabolic pathways could be related to the introduction of the NanoDendrix™, the classification of metabolism was related specifically to carbohydrate and lipid metabolism. In the carbohydrate metabolism, the *alpha-amylase enzyme* encoding transcript was upregulated, which leads to the prediction of an increase in the breakdown of both starch and glycogen (Rodríguez-Viera et al., 2016; Sakai et al., 2006). In the lipid metabolism, the enzyme encoding transcript *cytochrome-b5 reductase* was upregulated, which leads to the prediction of an increase in the denaturation of fatty acids (Chen et al., 2019). Although other pathways were classified to lipid metabolism, their pathway associations were not available through KAAS, or no information was available on the classifications within invertebrates such as *plasmanylethanolamine desaturase*. The relationship of the lipid and carbohydrate metabolism suggests there was a need for energy stores (Kumari et al., 2010). The increase in energy demand

in the crayfish that were injected with Poly (I:C)-NanoDendrix™ complex would be important for future studies when the NanoDendrix™ complex becomes a feed component for distribution, as this could influence the feed composition (Kim and Kang, 2008; Kim et al., 2007; Kumari et al., 2010).

Pathways that were associated to amino acid metabolism were examined as recent studies have found that amino acids and their metabolites play important roles in immune responses of crustaceans (Huang et al., 2020). Amino acid metabolism pathways were observed in the Poly (I:C) treatment only (Appendix 5). The pathway for purine metabolism as identified by KASS was found in the trial containing Poly (I:C) injections. The transcript encoding the enzyme *allantoinase* was downregulated 1.3 -log2 fold. Allantoinase is an important enzyme in the uricolytic system, catalyzing the conversion of allantoin to allantoic acid, leading to the degradation of uric acid through hydrolysis into glyoxylic acid and two urea molecules (Isoe and Scaraffia, 2013; Regnault, 1987; Weihrauch et al., 2004). The uricolytic system requires a variety of enzymes, including urate oxidase, allantoinase and allantoicase, which catalyse the necessary reactions (Isoe and Scaraffia, 2013). There was no change in regulation of allantoinase-encoding transcript noted in the Poly (I:C)-NanoDendrix™ complex as compared to the control. In the annotation of the DETs, majority of the transcripts were not annotated in the NCBI (nr) (61.5%) and SwissProt (79.2%) publicly available databases. All of the DETs annotated in the SwissProt database were also annotated in the NCBI (nr) database. This low annotation rate is likely due to the large number of genes within crustaceans that have not yet been discovered and are not found within these databases (Salzberg, 2019). As more research is completed on crustaceans, the number of known annotated genes within these publicly available databases will increase and some of the DETs that were not annotated may be able to be annotated. This lack of annotation does not mean that these are not important

transcripts but does mean that they have not yet been biologically discovered and are therefore unable to be put into context as immune related or not in this study.

2.6.3 Improvements to be Made to the Study

The injections that were completed on the crayfish did not include an injection of the NanoDendrix™ alone. As the results find that the Poly (I:C)-NanoDendrix™ complex had a larger response than the Poly (I:C) alone, further testing would need to be completed to examine if the NanoDendrix™ alone would have this affect. Histological samples were not taken during this study and may have been beneficial to observe whether changes occurred within the tissues of the crayfish; specifically at the location of injection and the hepatopancreas. Histological samples could also indicate if any other pathogens were present, which may have had an influence on the results. The pathogen status of these individuals was unknown, meaning that some of these crayfish could be at different stages of an immune response. Although the crayfish did not show clinical signs of disease, if they were in an infection state or carrier state, this could have influenced the response of the crayfish to the injected stimuli. Randomization of the animals to different treatments was performed to minimize this but it is still possible. Both the age and sex of the crayfish were unknown. Immune related sexual dimorphisms have been observed in *Drosophila*, showing that different pathogens can affect the different sexes of the same species differently (Belmonte et al., 2020). The age of the crayfish may have influence on the crayfish. Although there are no current described age-related diseases in crustaceans, the age could affect the crayfishes immune capability (Vogt, 2012). As these crayfish were wild-caught rather than cultivated, the genetic variability affecting their immune capabilities may have played a role larger than would be seen in cultivated crayfish (Dunham et al., 2001). Using cultivated crayfish that have a lower genetic variability may also decrease influences on the crayfish response. This information could

provide insight that would have allowed for a stronger understanding to if NanoDendrix™ would be an appropriate candidate for a dsRNA complex for cultivated crayfish populations.

The hepatopancreas tissue samples were taken 6 h after injection making it possible that maximum immune response of the crayfish was missed. The majority of studies conducted on crustacean immune responses to Poly (I:C) are conducted for up to 48 h, with periodic sampling within that time frame (Ji et al., 2009; Saray et al., 2018). In comparing various PAMPs, Poly (I:C) tends to take longer to cause a reaction than other PAMPs such as LPS (Ji et al., 2009). One study found that changes in immune genes, such as putative clotting protein, histone H3, superoxide dismutase and heat shock protein 70 kDa, were not found until 12 h post-injection of *M. rosenbergii* (Saray et al., 2018). Another study of the haemocyte response found that the THC actually increased at the 3 h timepoint and returned back to the original level for the 6 h timepoint before spiking again at 12 h (Ji et al., 2009). This data could suggest that the single timepoint missed the immune response occurring. This same study did find that the immune response to Poly (I:C) occurred after 3 h with significant changes in PO activation, lectin expression and superoxide anion production. From this data, it would be expected to see some DETs at the 6 h timepoint for the Poly (I:C) injection alone. Since these DETs were not found in the Poly (I:C) alone, it could be due to a delayed immune response, which was not observed in the short time point. To determine if this is a case, a longitudinal study would need to be completed that examines the response at regular time intervals; this would also determine the optimal sampling time, post-injection, for future studies.

With the completion of intermuscular injections, hepatopancreas samples were taken for the analysis. Hepatopancreas samples would include both the hepatopancreas tissues as well as the response of the haemocytes, due to the fixed haemocytes present in the hepatopancreas (Factor et

al., 2009; Roth and Kurtz, 2009). As the injections were intermuscular, the immune response may have been better understood with samples of the muscle at the injection site. Intersinus injections, rather than intermuscular injections, may have showed a decrease in the time needed for an immune response to observed in the hepatopancreas due to the quick exposure to haemocytes from intersinus injections (Factor et al., 2009; Roth and Kurtz, 2009). Future studies would need to re-evaluate the tissue samples taken as well as the most appropriate injection site for the best analysis of NanoDendriix™ and an antiviral prophylactic.

2.6.4 Use of NanoDendrix™ as a Nanoparticle Complex for dsRNA

The DETs observed from the use of the NanoDendrix™ compared to the synthetic dsRNA analogue injection alone, suggests that the NanoDendrix™ itself was the cause for the immune responses. This response could be due to receptor clustering in the case of NanoDendrix™, due to the size difference between the nanoparticle and the Poly (I:C). NanoDendrix™ being the cause of the immune response cannot be fully determined until the study is repeated with a longer time point, which may allow for the full response of the crayfish to be understood. A test running an injection of the NanoDendrix™ alone would also allow for a better understanding of the effects of the nanoparticle.

2.7 Conclusion

The use of NanoDendrix™ complexed to Poly (I:C) was completed using two different Galaxy pipeline, one that demonstrated the presence of no differentially transcripts and one that demonstrated differentially expressed immune response transcripts compared to the Poly (I:C) injection alone. The results observed cannot be isolated to the use of NanoDendrix™ complexed with Poly (I:C) or the NanoDendrix™ alone. To fully isolate these effects, future studies should assess NanoDendrix™ injected alone against the Poly (I:C)-NanoDendrix™ complex. In addition,

increasing the length of time for future studies may provide an opportunity for the immune response of the crayfish to be further understood. The use of this experimental design suggests promise for additional research to investigate the immune gene response to NanoDendrix™ in both crayfish and other crustaceans.

Chapter 3: Investigation of the Presence of Crustins in Decapod Crustaceans

3.1 Introduction

3.1.1 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are an important first line of defense in many animal species. They are immunomodulators that actively target Gram-positive and Gram-negative bacteria, fungi, parasites, enveloped viruses and tumor cells (Ghosh et al., 2011). Antimicrobial peptides are able to function with broad specificity, do not require memory and have an important role in innate immunity (Alday-Sanz, 2010; Vargas-Albores et al., 2004).

An antimicrobial peptide is defined as a molecule of less than 10 kDa, that has antimicrobial properties. They are usually a product of a single gene, have a small size allowing them to be easily synthesized without high metabolic cost and be transported quickly to the point of infection (Vargas-Albores et al., 2004). The small size also allows AMPs to be resistant to denaturation from salt or temperature (Alday-Sanz, 2010). Even with this small size, AMPs are very susceptible to trypsin and related proteases (Moncla et al., 2011).

Less than 10% of the known AMPs in the world are from crustaceans. Organisms within the subphylum Crustacea produce a variety of AMPs, which are mainly produced in semi-granular and granular circulating haemocytes (Cerenius and Söderhäll, 2012). Known crustacean AMPs include: callinectin, armadillidin, homarin, defensin, hyastatin, arasin, stylicin, penaeidins, crustins, anti-lipopolysaccharide factors (ALFs) and astacidin; with the two most well known AMPs being penaeidins and crustins (Ghosh et al., 2011; Jiang et al., 2015; Rosa and Barracco, 2010; Vargas-Albores et al., 2004). Antimicrobial peptides can be characterized into groups including those with: single-domain linear α-helical structure, peptides enriched in specific amino

acids, single domain peptides containing cysteine residues engaged in disulfide bonds, multi-domain or chimeric AMPs and unconventional AMPs (Rosa and Barracco, 2010).

Anti-lipopolysaccharide factors are one of the best studied groups of AMPs found in crustaceans. They are a group of small proteins with central β-hairpin loops that allow for binding to microbial membranes (Matos et al., 2018). Anti-lipopolysaccharide factors are released into the haemolymph from haemocytes during rapid degranulation in response to bacterial lipopolysaccharide (LPS) (Tang et al., 2015). These ALFs have been found to have antibacterial activity against some Gram-negative and Gram-positive bacteria, filamentous fungi and enveloped viruses (Rosa et al., 2013). Tharntada et al. (2015) has demonstrated the importance of ALFs in the defense against white spot syndrome virus (WSSV) in crayfish through reduced viral propagation and prolonged haematopoietic survival post-infection.

Penaeidins are restricted to granular haemocytes and an upregulation in expression has been found that corresponds with rapid increases in total haemocyte counts (THC) (Kang et al., 2004; Padhi, 2012; Rosa and Barracco, 2010). Penaeidins have a N-terminal domain rich in proline residues and a C-terminal domain containing six cysteine residues, which form disulphide bridges (Kang et al., 2004). They are active against both Gram-positive bacteria and filamentous fungi and are considered chimeric AMPs that are confined to the suborder Dendrobranchiata (Kang et al., 2004; Padhi, 2012; Rosa and Barracco, 2010).

The mode of action for AMPs is determined by their structural conformation and charge (Rosa and Barracco, 2010). Generally, AMPs act by disrupting the target cell membrane integrity by inserting into the membrane; resulting in membrane destabilization or pore formation. Additional mechanisms can also include translocation into the cytoplasm of the target cell and directly binding to crucial metabolic proteins (Rosa and Barracco, 2010).

3.1.2 Crustins

Crustins are a recently discovered group first identified in 1999 in *C. maenas* (Relf et al., 1999). Crustins have several biological functions including antimicrobial activity, protease inhibitory activity and immune regulation ability (Jiang et al., 2015). The mechanisms behind these biological functions are unknown.

Crustins are defined as cysteine-rich AMPs found in crustaceans, characterized by a signal sequence at the amino terminus and a whey acidic protein (WAP) domain at the carboxyl terminus (Cerenius and Söderhäll, 2012; Jiang et al., 2015; Smith et al., 2008; Vargas-Albores et al., 2004). They are small cationic proteins that occur across Decapoda (Cerenius and Söderhäll, 2012; Smith et al., 2008). The signal sequence at the amino terminus contains 16-24 amino acids, which usually have a high number of valine residues (Jiang et al., 2015; Smith et al., 2008). This signal sequence is not highly conserved in Crustacea. The cleavage site at the end of the signal sequence is most commonly between alanine and glycine residues, but it can also occur between glycine and glutamine, alanine or threonine (Jiang et al., 2015; Smith et al., 2008). At the carboxyl terminus, there is a characteristic four-disulfide core-containing WAP domain. Whey acidic protein domains contain eight cysteine residues in a conserved arrangement that creates the tightly packed four-disulfide core that has antiprotease activity (Smith et al., 2008). The WAP domain is highly conserved between species and types of crustins. The crustin type is determined by the single or multiple domains present between the signal sequence and the WAP domain (Jiang et al., 2015; Smith et al., 2008).

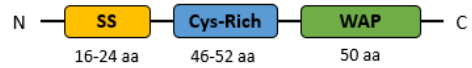
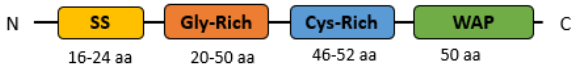
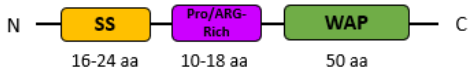
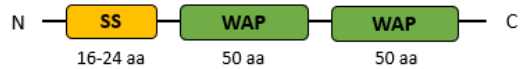
3.1.3 Types of Crustins

Crustins were previously classified into three groups based on amino acid sequence, secondary structure and functional properties (Ghosh et al., 2011). Crustins are currently classified

into four distinct types of crustins, with each containing multiple isoforms that differ in their N-terminal sequence (Table 7) (Destoumieux-Garzón et al., 2016; Jiang et al., 2015). Recent findings have also suggested up to seven crustin types with five subtypes (Li et al., 2020).

Type I crustins have a cysteine-rich domain of variable length in the region between the signal sequence and the WAP domain. This cysteine-rich region is also present in type II crustins, but it is accompanied by a glycine-rich region adjacent to the signal sequence. Type III crustins lack both the cysteine-rich and glycine-rich domains but contain a proline- and arginine-rich domain. Finally, type IV crustins contain two sequential WAP domains adjacent to the signal sequence (Destoumieux-Garzón et al., 2016; Smith et al., 2008). Not all types of crustins have been observed in all decapod crustacean species (Destoumieux-Garzón et al., 2016; Smith et al., 2008).

Table 7 The four types of crustins, structural properties, biological functions and the crustaceans that contain each type (Destoumieux-Garzón et al., 2016; Ghosh et al., 2011; Smith et al., 2008).

Type	Molecular Weight (kDa)	Charge	Biological Function	Species and Groups	Structure
I	9.34-17.79	Anionic and Cationic	Anti-Gram-negative Bacteria-binding Bacterial clearance Haemocyte phagocytosis Antiprotease	Crabs Lobster Crayfish Pleocyemata	
II	11.22-22.45	Cationic	Anti-Gram-positive LPS- and LTA-binding Inhibition of viral propagation Bacterial agglutination	Shrimp Dendrobranchiata King crab Amphipod Copepod	
III	5.96-7.40	Cationic	Anti-Gram-positive Bacteria-binding Antiprotease	Select shrimps *P. monodon* *L. vannamei* *M. japonicus* *F. chinensis*	
IV	10.93-11.14	Cationic	Bacteria-binding Antiprotease	Crabs Shrimp	

3.1.4 Functions of Crustins

Research has begun to show a wider range of biological functions for crustins. Crustins are often regarded as antimicrobial effectors, however they also have activity against both Gram-positive and Gram-negative bacteria, and viruses. In *P. monodon* type III crustin expression was observed post-WSSV infection that suggests a role in antiviral defense (Antony et al., 2011a; Destoumieux-Garzón et al., 2016). Other non-decapod AMPs are known to kill bacterial targets through depolarization and permeabilization of the bacterial cell wall (Smith et al., 2008). Non-decapod AMPs can disrupt the cell metabolisms or interfere with DNA synthesis of the pathogen, resulting in cell death (Smith et al., 2008).

The antimicrobial activity within crustin types, can differ between species; as well as between *in vitro* to *in vivo* experiments. During *in vitro* experiments, crustins of *P. monodon, F. chinensis, C. maenas, H. gammarus, P. leniusculus* and *L. vannamei* were active against Gram-positive bacteria, with few active against Gram-negative bacteria (Ghosh et al., 2011). However, in *in vivo* experiments, the antimicrobial activity of the crustins were variable against both Gram-positive and Gram-negative bacteria. In both cases, the crustins did not respond to fungi (Ghosh et al., 2011).

A crustin from *P. monodon*, crustinPm1, demonstrated specific antimicrobial activity against Gram-positive bacteria, with strong inhibition against *Staphylococcus aureus* and *Streptococcus iniae* (Supungul et al., 2008). Another crustin, Crus-likePm, was discovered in *P. monodon* but had antimicrobial activity against both Gram-positive and Gram-negative bacteria. The study also showed the involvement of Crus-likePm in the resistance to *Vibrio harveyi*, a major pathogenic bacteria in shrimp aquaculture (Amparyup et al., 2008). The crustin CruFc in *F. chinensis* inhibited the growth of Gram-positive bacteria; and was particularly efficient at

inhibiting the growth of *Staphylococcus aureus* (Zhang et al., 2007a, 2007b). In *H. gammarus*, a crustin was discovered that is reactive against Gram-positive bacteria, including the pathogen *Aerococcus viridans* var. *homari* (Hauton et al., 2006). In *L. vannamei*, the crustin LvABP1 was discovered and found to be active against *Vibrio penaeicida* and *Fusarium oxysporum* (Shockey et al., 2009).

3.1.5 Activation of Crustins

Crustin activation is caused by the cleaving of the signal sequence from the peptide, which occurs in the circulating granular or semi-granular haemocytes, where they are produced. The role the signal sequence has remained unknown, however it has been speculated that the signal sequence is directing trans-membrane transportation of the peptide; a phenomenon found in insects and mammals. It is also speculated that the mature protein is released from the haemocytes through exocytosis as is found with penaeidins and other AMPs (Smith et al., 2008).

3.2 Objectives

A better understanding of crustin evolution could demonstrate how different species are able to adapt to their unique environment or pathogenic pressures. This information could be very useful to the global crustacean aquaculture industry, which could use this knowledge to combat specific pathogenic pressures or in broodstock development. Therefore, the main objectives of this study was to examine crustin diversity through: (1) crustin sequence discovery in genomic and transcriptomic datasets, and (2) completion of phylogenetic analyses across different crustacean infraorders.

3.3 Hypothesis

The hypothesis for this study was that the crustin phylogenies would show the crustin sequences grouping into the four types of crustins sequences previously described (Destoumieux-Garzón et al., 2016; Ghosh et al., 2011; Smith et al., 2008).

3.4 Materials and Methods

3.4.1 Sequence Mining and Verification

A known crustin type I protein sequence from Japanese spiny lobster (*Panulirus japonicus*) (ACU25382), as used by Becking et al. (2020), was used to mine the NCBI Gene database and 39 Clark laboratory generated crustacean transcriptomes of different crustacean species (Appendix 8) databases using tBlastn (expected value cutoff: $E = 1.0\times10^{-5}$) (Altschul et al., 1997; Camacho et al., 2009), to find other crustin nucleotide sequences from crustaceans (U.S. National Library of Medicine, 2021). Searches were isolated to organismal groupings: crustaceans, true crabs, lobster and *Homarus*.

All sequences that met these criteria were downloaded and Blast2GO was used to confirm the functional annotation of crustins (BioBam, 2020; Conesa et al., 2005). The sequences were confirmed to be crustins if their annotation suggested: crustin, antimicrobial peptide, WAP containing or carcinin-like protein. All other sequences that did not meet these requirements were removed. The MUSCLE alignment function (Edgar, 2004a, 2004b) of Molecular Evolutionary Genetic Analysis X (MEGA X) (Kumar et al., 2018; MEGA, 2020; Tamura et al., 2021) was used to align the crustin nucleotide sequences. The sequences were then exported as a multifasta file.

3.4.2 Translation

ExPASy – translate tool (SIB Swiss Institute of Bioinformatics, 2020) was used to translate the nucleotide sequences to protein sequences. All sequences were again confirmed to be crustins

based on the presence of a WAP domain as determined in Li et al. (2020). Signal P (Almagro Armenteros et al., 2019) was used to remove the signal peptide sequence on the crustin sequences.

3.4.3 Alignment and Creation of Phylogenies

The sequences of the crustins, and the sequences of the WAP domains alone, were aligned using MUSCLE (Edgar, 2004a, 2004b; Madeira et al., 2019) in MEGA X (Kumar et al., 2018; MEGA, 2020; Tamura et al., 2021). Outlier sequences were fully removed to improve the alignment; however, <2% of sequences had to be deleted from any alignment. Outlier sequences were those which did not align in the eight cysteine residues of the WAP domain. The alignments were then used to determine the best DNA/Protein model (Tamura et al., 2021) to be used for phylogenetic analysis (Appendix 9). The recommended model was used to make a Maximum Likelihood tree with 1000 iterations in MEGA X (Hall, 2013).

The crustacean sequences were separated based on infraorders: Achelata, Anomura, Astacidea, Brachyura, Caridea and Dendrobranchiata (suborder), as specified by Tan et al. 2019. These groupings based on infraorder were then used to create MUSCLE alignments and Maximum Likelihood phylogenies as described above (Edgar, 2004a, 2004b; Guindon et al., 2010).

3.4.4 Phylogenetic Analysis

Once the trees had been constructed, a bootstrap consensus tree was condensed to 50%. The trees were constructed as unrooted phylogenetic trees as the goal was to understand the relationships between the taxa and types of crustins, rather than the evolutionary change. The unrooted trees were described based on the proposed terms for describing unrooted trees (Lapointe et al., 2010; Wilkinson et al., 2007). The crustins were examined based on the crustin type as categorized by their structure and charge (Table 8). Charge was determined using Galaxy Europe's iep (Blankenberg et al., 2007; Rice et al., 2000).

Table 8 The criteria for classification of crustin sequences as based on Barreto et al. (2018).

Type	Number of WAP Domains	Number of Cysteines	Glycine Rich Region	Proline/Arginine Rich Region	Charge
I	1	12	No	No	Positive/Negative
II	1	12	Yes	No	Positive
III	1	8	No	Yes	Positive
IV	2	16	No	No	Positive

3.5 Results

3.5.1 Crustin Sequences and Separation of Crustin Types

From the NCBI blast search using the known crustin type I protein sequence from Japanese spiny lobster (*Panulirus japonicus*) (ACU25382), 688 sequences were found (Becking et al., 2020). Blast2GO determined that 492 of the sequences found from the NCBI blast had functional annotations as crustins. From the laboratory generated transcriptomes, 70 additional sequences were included in the sequence collection. Blast2GO determined that all 70 of the sequences from the laboratory generated transcriptomes had the function annotations as crustins. Along with the identification of the crustins through the Blast2GO functional annotations, the crustins were confirmed through their structure based on the presence of a WAP domain. A total of 16 sequences were removed for not having a complete WAP domain. Subsequently, 430 duplicate sequences were removed. In the end, 116 sequences remained (S 3.1). The most common crustin type found was type I (72 sequences), followed by type II (33 sequences). Very few type III (11 sequences) crustins were found and no type IV crustins were found.

3.5.2 Phylogenetic Analysis of Crustin Sequences

The aligned crustin sequence datasets generated phylogenetic trees that contained similar groupings of crustin isoforms. Each branch was examined in the context of the genus, infraorder and the crustin type (S 3.3). In examining the phylogeny, the most noticeable groupings are the

two type I crustin clans (S 3.3 A and B), both of which were heterogenous for infraorder. Other small clans are observed within the phylogeny for type I, II and III crustins. In all of the clans, the adjacent sequences are often homologous for genus and in some cases species.

A phylogeny was generated from the conserved WAP domain rather than the entire sequence (S 3.4). The WAP domain structure found in the crustins was as follows:

$$C - X_6 - C - X_9 - C - X_5 - C - X_5 - CC - X_3 - C - X_5 - C$$

The phylogeny exhibited a noticeable lack of large clans and a high number of single branches. The largest of the clans was type III containing seven sequences (S 3.4 A). Other small clans were noticed for the type I, II and III crustins. Of the small clans, those heterogenous for genus do not appear to be homologous for their infraorder but tend to group as adjacent groups to their genus. Between these clans are a series of single branches and two-sequence clans that are a mixture of type I and type II crustins, which do not appear to show any specific pattern. The WAP domain sequences were further analyzed with Seq2Logo (Thomsen and Nielsen, 2012). From this analysis, the eight conserved cysteine residues can be easily identified; however, most of the other amino acids are polymorphic (Figure 10).

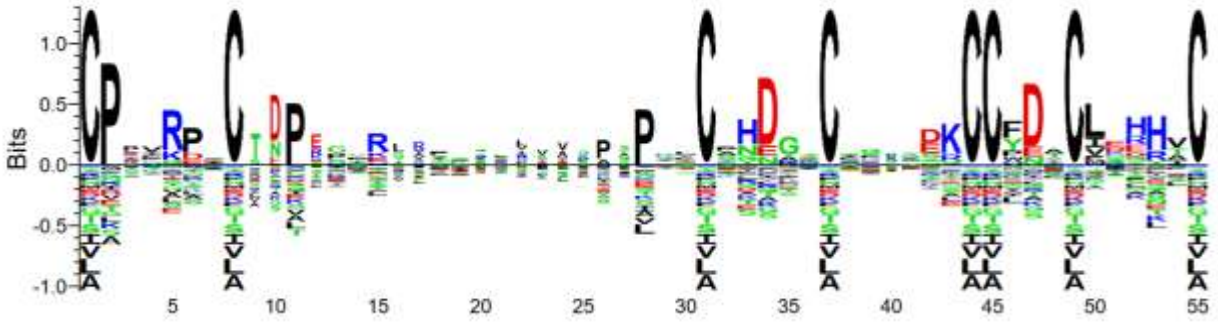


Figure 10 The visualization of the amino acid weighting present in the WAP domain sequences of the examined crustin sequences.

3.5.3 Crustin Sequences for Shrimp vs. Non-Shrimp Species

To expand the phylogeny of the crustins, the sequences from shrimp species (suborder Dendrobranchiata and infraorder Caridea) were separated from those of other species. This resulted in 44 shrimp and 72 non-shrimp sequences for analysis. From the phylogeny examining the shrimp sequences, a large clan was present that was heterogenous for crustin type, containing both type I and II sequences (S 3.5 A). Smaller clans were present in the larger clan that were either heterogenous or homologous for crustin type and genus. Other small clans were also present, homologous for type I, II and III crustins.

The sequences from the non-shrimp species were also collected into a phylogeny (S 3.6). From the top of the phylogeny, two large clans of type I crustins were present, which made up most of the crustin sequences found in this phylogeny (S 3.6 A and B). These clans do not show homology for species, genus or infraorder. Within these clans, adjacent branches group together homologous genus; however, not all of one genus is grouped together. Other small clans of type I, II and III crustins were also present, many homologous for genus or had sequences of the same genus adjacent to one another.

3.5.4 Crustin Sequences by Infraorder

The sequences were further broken down into their infraorders and used to create separate phylogenies. There were six infraorders: Achelata, Anomura, Astacidea, Brachyura, Caridea and Dendrobranchiata (suborder); represented by 2, 5, 3, 62, 24, 20 sequences in each infraorder, respectively.

The Achelata sequence collection contains the reference sequence used to mine the databases as well as one other sequence; both sequences were of the *Panulirus* genus and both

sequences were type I crustins. Two sequences are lower than the requirement for the MEGA X phylogeny, therefore no phylogeny was created for this infraorder.

The Anomura sequence collection contained two species, the long-wristed hermit crab (*Pagurus longicarpus*) and broad-clawed porcelain crab (*Porcellana platycheles*), which contain only type I crustins (S 3.7). Because the collection of sequences was so small and did not contain a high diversity of species or crustin types, it is difficult to find patterns.

The Astacidea were a very small collection of sequences (three) with only one species for examination, *C. quadricarinatus*. Of the three sequences, two were type I and one was type II. Due to the small number of sequences being lower than the requirement for the MEGA X phylogeny, no phylogeny was created for this infraorder. There are no type III crustins within this sequence set.

The Brachyura contain mostly type I crustins, which make up the two largest heterogenous clans at the top of the phylogeny (S 3.8 A and B). Within these large clans, there are small clans homogenous for species type as well as some heterogenous for species type due to single intruders. Other smaller clans are also present for type I, II and III crustins.

The Caridea phylogeny contains a large clan that can be split into two smaller clans, a clan of type I crustins (S 3.9 A) and a clan of type III crustins (S 3.9 B). The type I crustins created a heterogenous clan that contained several smaller homologous clans; this clan however was not perfect or complete. The type III crustin clan was complete, in that is contained all type III crustins.

The Dendrobranchiata did not show a similar pattern to the shrimp phylogeny as expected based on the shrimp phylogeny containing a high number of Dendrobranchiata sequences (S 3.10). No large clans were observed in the phylogeny and of the smaller clans, majority only contained two sequences.

3.6 Discussion

The unrooted phylogeny of all of the crustin sequences found that the sequences did not break into perfect clans at the crustin type or genus level (S 3.3). Although clans were present that were homologous for crustin type, not all of the sequences that belonged to that crustin type remained in that group, in some cases, forming other smaller clans of the same type. This result of multiple clans of each type of crustin suggests the possibility that the subtypes, which have also been found in other studies, do exist in both rooted and unrooted phylogenies (Barreto et al., 2018; Padhi, 2012). The majority of type I crustin sequences did group together within two larger clans, which suggests that these type I crustins are more similar to each other than other types of crustins (S 3.3 A and B). Within the large clans of type I crustins, many smaller clans were found that were homologous for genus. This placement of sequences of the same genus being in close proximity to one another would suggest that the crustin sequences of a single type within a genus are more closely related than to sequences from another genus. The groupings of *Portunus* in both large clans could suggest a gene duplication at the genus level (S 3.3 A and B). Another group of sequences, containing *Charybdis* and *Scylla* in both clans, could suggest a gene duplication at the family level (S 3.3 A and B). The two large type I sequence clans contain similar species, which could suggest a gene duplication event occurred in the common ancestor, resulting in the two separate clans. A notable outlier is the two Australian glass shrimp (*Paratya australiensis*) sequences that are the only sequences within the two clans not of the infraorder Brachyura. These two clans of type I crustins would suggest that these subtypes are found in more crustacean species than just the shrimp, supporting the suggestion by Padhi, (2012) and refuting the suggestions by Amparyup et al., (2008) and Antony et al., (2011b). The presence of two type I crustin clans could indicate the possibility of two subtypes of crustins, which have not yet been classified (S 3.3).

Generally, the phylogeny of the crustins as a whole was not as clearly grouped as expected. This could be due to the long time period between the crustin gene duplication events in a previous common ancestor or from more recent gene duplication events at the family or genus level. The small sample size utilized in this phylogeny could also have influence on phylogenies as more sequences may have showed different patterns.

The phylogenies that contained the WAP domains of the crustin sequences found few large clans, and an increased number of single branch sequences compared to the full crustin sequences examined (S 3.4). As these sequences did not find groupings within the crustin types, these WAP domains alone have been identified as an inefficient way to examine the crustins, which may have further influenced the crustin type classifications. From the analysis of the amino acid sequence weights, the amino acid conservation in the WAP domain was observed to be low with significant conservation only appearing at the eight cysteine residues (Figure 10). It was determined that although the WAP domains are conserved in the cysteines, there does not appear to be enough conservation within the sequences to allow for significant groupings within the unrooted tree.

The phylogenies that separated the shrimp and non-shrimp species found unique patterns in between the two trees. The shrimp sequences results in a large clan that was heterogenous for type of crustin, containing type I and type II (S 3.5). Within this clan, smaller clans were present, which were homologous for genus. These genus homologous groupings suggest that gene duplication took place at the genus level. The gene duplication hypothesis is best observed in the large *Penaeus* grouping within the large clan heterogenous for crustin type (S 3.5 A). However, although this gene duplication appears to be absent in other species demonstrated in this phylogeny, it does not mean that it does not exist. The two distinct *Macrobrachium* groupings suggests the gene duplication event took place in the common ancestor of this genus. There could

be two explanations for the heterogeneity of crustin types in this clan. The first explanation is that the type I sequences are a subtype, which is more closely related to the type II crustins than the type I crustins from the divergence. The next explanation is that the crustins were incorrectly typed, as characteristics that define each crustin type are not well characterized (Barreto et al., 2018; Li et al., 2020). Generally, this clan contains most of the type II crustins, if those type I crustins are considered to be mistyped as a result of this study, this large clan of type II crustins would not match the current understanding of type II crustins, meaning that there should be two distinct subtypes of type II crustins (Barreto et al., 2018; Li et al., 2020; Padhi, 2012). It would be expected that more subtypes would exist for type I crustins as they have the most numerous known subtypes of the classifications, and shrimps have been the most studied (Barreto et al., 2018; Li et al., 2020; Padhi, 2012).

The sequences from the non-shrimp species found two large clans of crustin type I sequences adjacent to one another (S 3.6 A and B). This could suggest the possibility of two crustin subtypes that have previously been described (Barreto et al., 2018; Li et al., 2020; Padhi, 2012). Further examination of these more closely related species, compared to the phylogeny created of all the crustin sequences, suggests that gene duplication did not happen in *Portunus* as suggested by the full crustin phylogeny. As the two clans contained very similar species, the majority within infraorder Brachyura, it is suggested that a gene duplication event occurred at a common ancestor of both clans, which may have been followed by subsequent gene duplication events at the family and genus level. There were no large type II crustin clans in this phylogeny however, there was a section that contained various small type I and II crustins clans. This could be caused by the diversity of the crustins. The divisions between the types are more prominent in the phylogeny containing shrimp species compared to those containing non-shrimp species. This could be due to

the more extensive research and sequencing that has been conducted on shrimp species that results from their high value and significance to the aquaculture industry.

The infraorder separations would have benefited from a wider variety of species and crustin types in each of the infraorders. This may not be possible due to the limited amount of sequencing available for these crustacean species, and the types of crustins that may or may not be present in each species. At the infraorder level, some infraorders did not contain a sufficient number of sequences to be able to generate a phylogeny. This is due to the limited amount of genomic and transcriptomic resources for species within the Achelata and Astacidea infraorders. When examining the infraorders that did contain enough sequences to create a phylogeny, the majority of the infraorders were dominated by one crustin type: the Anomura and Brachyura were dominated by type I crustins, and the Dendrobranchiata were dominated by type II crustins. The Anomura and Brachyura are more closely related to one another than to Dendrobranchiata, which could explain this result (Tan et al., 2019). The Caridea contained the most equal distribution of crustin types, which may be due to the larger genomic and transcriptomic datasets that exist for this infraorder that is important for aquaculture (S 3.9). The large clan of type I and III crustins presents a separation into the two smaller clans based on crustin type but not on evolutionary history, body shape or environment (S 3.9 A and B). Based on the composition of the crustin sequences, it makes sense for the type III crustin to be more closely related to the type I crustin, which would explain the location of the type I and III crustins being adjacent to one another (Li et al., 2020). As the two clans show similar species sequences, it is suggested that gene duplication occurred at the last common ancestor which allowed for these two lineages to diverge resulting in the separation of the two crustin types. Looking at the larger phylogenies created, the Brachyura present a distinct clan of type I crustins that appears to separate into two smaller clans with no

distinguishing features in evolutionary history, body shape or environment, which could account for this separation (S 3.8 A and B). Whether or not the crustins meet the current subtypes based on sequence as defined by the current research is still to be determined (Li et al., 2020). The appearance of subtypes next to each other has been observed in several studies however, separation between them has also been found to occur (Barreto et al., 2018; Padhi, 2012). As the two clans show similar species sequences, it suggests that gene duplication occurred at the last common ancestor, which allowed for these two lineages to diverge and subsequent gene duplication events to occur. The lack of large type I and II clans could again be due to sequencing error or could be due to the large number of crustin subtypes that may exist. The Dendrobranchiata are dominated by type II crustin, which are separated by clans of type I and III crustins (S 3.10). The lack of large clans could be an indicator the long time period between the crustin gene duplication events in an ancient common ancestor or from more recent gene duplication events at the family or genus level. This separation could also be indicating the subtypes of type II crustins, which have been observed in various studies examining *Penaeus* shrimps. However, it must be noted that the current study suggests the presence of three subtypes rather than the two that have been defined (Antony et al., 2011b; Barreto et al., 2018; Li et al., 2020). Generally, some infraorders are found to separate better into crustin types than others. Those that separated better often contained a larger variety of species and crustin types in more equal ratios, which may have been beneficial.

Other studies that have examined crustin phylogenies do not often examine multiple species and multiple crustin types to the extent found in this study (Antony et al., 2011; Barreto et al., 2018). Studies that have examined single species crustin phylogenies have observed the distinct separation of crustin types into their own branches, which is beneficial in helping determine the number of crustin types and the subtypes present in a species (Antony et al., 2011: Barreto et al.,

2018). Studies that examined a variety of species with multiple crustin types utilized far fewer sequences than used in this study (Afsal et al., 2011; Banerjee et al., 2015; Padhi, 2012). These studies found that the crustins would group by crustin type and by species (eg. shrimp, crab, lobster) (Padhi, 2012; Tandel et al., 2018). Some of these trees contained clans that did not have a adjacent grouping that was similar in type or species as they were sporadic appearing in their placement. Other studies found that the types of crustins were grouped in adjacent clans to those of the similar crustin type, regardless of their species (Padhi, 2012; Tandel et al., 2018).

3.6.1 Challenges in Crustin Phylogeny

There were a variety of challenges posed when creating the phylogenies of the crustins including the diversity of the crustin sequences, the search sequence utilized and the certainty of the crustin species utilized. These factors may have contributed to the varying results found in this study compared to those of other studies.

Crustins have been classified into a variety of types and subtypes from different studies in order to better understand the diversity and complexity of the antimicrobial peptide. Originally, crustins were broken into the four subtypes, as used in this study, for classification (Destoumieux-Garzón et al., 2016; Ghosh et al., 2011; Padhi, 2012; Smith, 2011; Smith et al., 2008). More recently, a larger variety of crustin classifications have been introduced that are still being developed with the discovery of new crustins. In *L vannamei* alone, the crustins have been classified into seven distinct types where type I crustins have three subtypes, and type II crustins have two subtypes. Although these crustins all have at least one WAP domain, the rest of the crustin is highly variable in number and presence of a variety of regions including cysteine-rich, glycine-rich, aromatic amino acid-rich or serine/leucine-rich regions; as well as the presence of C-terminal tails and short N-terminal regions (Li et al., 2020). Some studies are also contradictory in

their classification. A majority of these contradictions relate to the type III crustin that have been said to have a proline/arginine rich region or a short N-terminal region, however, there has been no consensus on this (Destoumieux-Garzón et al., 2016; Ghosh et al., 2011; Li et al., 2020; Smith et al., 2008). With this large variety of crustins, classification of the crustins becomes much more difficult. The classifications used in this study were based on the work completed by Barreto et al. (2018), with classifications only occurring for types I-III, as type IV was not present. With the large variety of crustins found, several questions arise such as: are there several subtypes of crustins in each species and at what point do these crustins differ so much from one another that they can no longer be described as crustins?

The search sequence utilized in this study was from Becking et al. (2020) and was typed to be a crustin type I in the previous study. This current study also matched this sequence to be a type I sequence. When looking at the phylogenies where this sequence was present (S 3.3; S 3.4; S 3.6), the sequence was not grouped together with the other type I crustins, but often more closely related to the type II crustins. This could have resulted for a variety of reasons. The sequence was the one sequence of the infraorder Achelata, which could mean that the type I crustins in the Achelata are more similar to type II crustins of other infraorders than type I crustins of other infraorders. It could also mean that this sequence is not a true crustin. Future studies may benefit from a reference sequence from each of the infraorders examined.

Crustin sequences were collected by mining the NCBI database as well as transcriptomes generated in the laboratory of Dr. K. Fraser Clark. The collected sequences went through a variety of tests to determine their classifications as crustins. Blast2GO was used to determine the functional annotation of the crustins based on some key terms identified as possible crustin functional annotations. To further support the idea of these sequences being crustins, the protein

sequences themselves were manually examined. Initial examinations looked for a WAP domain in the crustin. Any sequences with missing or incomplete WAP domains were removed. There is still a chance that due to the large diversity, some sequences were just highly similar to crustins causing them to be included, or that some partial sequences of crustins were included, which would result in mistyping of the crustin.

Due to the challenges in the creation of these crustin phylogenies from to the classification of crustin types, the phylogenies were not ideal for gaining a better understanding of the relationship of crustins and how different species are able to adapt to their unique environment or pathogenic pressures. Instead, this study demonstrated the need for an improved definition and classification system for crustins.

Future studies on crustin phylogenies would be improved with better knowledge on classification of crustins and a standard crustin typing methodology. Along with a better understanding of crustin types, the analysis of a 3D structure of crustins could be beneficial. The analysis of the 3D structure of the crustins after protein folding may provide insight on improved methods for crustin classification (Arockiaraj et al., 2013; Wang et al., 2021).

3.7 Conclusion

Increasing the knowledge on crustins and understanding their phylogeny would help to fill a knowledge gap in crustacean immunology. The phylogenetic analysis of the crustin species found that the crustins did not split into their types as expected. This could be due to a variety of factors including the long periods of time between gene duplication events resulting in the high levels of diversity in the crustin peptides causing complex tree analyses. This high diversity suggests the need for new, more precise classifications of the crustins with a goal of separating true crustins from other crustin-like proteins. Increased understanding of the crustins and their

roles in crustacean immunology is important to understanding the health of such an economically important group of species.

Chapter 4: Conclusion

The main goal of this research was to gain a better understanding of crustacean immune systems through the examination of two studies: the immune response analysis of a crayfish to a nanoparticle-synthetic dsRNA analogue complex and the development of an antimicrobial peptide phylogeny. The use of NanoDendrix™ complexed to Poly (I:C) demonstrated differentially expressed immune response transcripts not found in the Poly (I:C) alone, which cannot be isolated to the use of Poly (I:C)-NanoDendrix™ complex or the NanoDendrix™ alone. To fully isolate these effects, future studies must assess NanoDendrix™ injected alone against the Poly (I:C)-NanoDendrix™ complex. The phylogenetic analysis of the crustin species did not demonstrate the groupings of the types of crustins as expected. This study was challenged with the high levels of diversity in the crustin peptides suggesting the need for new, more precise classifications of the crustins with a goal of separating true crustins from other crustin-like proteins.

Improving the knowledge of crustacean immunology will provide many benefits, which can be realized in the treatment of crustacean viral diseases that severely impact aquaculture. With potential for crustacean aquaculture in Canada, increased knowledge on crustacean immune systems can also lead to development of improved disease management strategies. Creating a sustainable shrimp aquaculture industry in Canada can help in decreasing the need for shrimp importation and increase the Canadian shrimp production. Although wild crustacean species cannot be treated, an increased understanding of how viruses and pathogens affect both wild and cultured crustacean populations can lead to more educated decisions being made on crustacean importation regulations and restrictions. This study increases the knowledge on crustacean immunity, which will benefit the valuable wild and cultured crustacean industry, making the future of food security more certain.

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Appendix 1

Appendix 1 The raw data provided from NovoGene (Beijing, China) on the hepatopancreas (HP) samples sequenced for the control (PBS; n=6), Poly (I:C) (HMW; n=6) and Poly (I:C) complexed to the NanoDendrix™ (HN; n=7).

Sample	Raw reads	Raw data (G)	Effective (%)	Error (%)	Q20 (%)	Q30 (%)	GC (%)
HP_H_N_1	20,528,568	3.1	96.95	0.03	97.50	93.45	45.92
HP_H_N_2	22,389,242	3.4	98.11	0.03	97.82	94.00	46.25
HP_H_N_3	20,796,494	3.1	98.35	0.03	97.68	93.70	45.94
HP_H_N_4	24,799,678	3.7	98.30	0.03	97.88	94.26	47.14
HP_H_N_5	23,860,522	3.6	98.52	0.03	97.89	94.05	44.18
HP_H_N_6	23,572,342	3.5	97.34	0.02	97.98	94.36	46.26
HP_H_N_7	21,855,444	3.3	98.09	0.03	97.67	93.75	44.47
HP_HMW_1	25,793,470	3.9	98.23	0.03	97.56	93.64	44.73
HP_HMW_2	19,925,210	3.0	97.49	0.03	97.50	93.30	47.54
HP_HMW_3	23,233,672	3.5	98.35	0.02	98.00	94.37	44.50
HP_HMW_4	22,932,948	3.4	98.66	0.03	97.87	94.15	46.59
HP_HMW_5	16,271,334	2.4	98.50	0.03	97.73	93.57	44.65
HP_HMW_6	13,295,310	2.0	97.49	0.03	97.69	93.64	44.42
HP_PBS_1	25,002,804	3.8	98.18	0.03	97.44	92.92	46.49
HP_PBS_2	26,784,444	4.0	98.30	0.03	97.32	92.66	45.68
HP_PBS_3	26,096,502	3.9	98.37	0.03	97.21	92.40	44.34
HP_PBS_4	23,801,710	3.6	98.36	0.03	97.35	92.72	45.46
HP_PBS_5	21,633,670	3.2	98.33	0.03	97.31	92.51	42.90
HP_PBS_6	23,824,006	3.6	98.46	0.03	97.83	94.05	45.92

Appendix 2

Appendix 2 The 19 DETs similar between the comparison of the control to the Poly (I:C) and Poly (I:C) complexed to the NanoDendrix™.

Gene ID	NCBI Annotation	Species
XP_022920379.1	troponin T, skeletal muscle isoform X5	*Onthophagus taurus*
MPD00845.1	hypothetical protein	*Portunus trituberculatus*
GCC27648.1	hypothetical protein	*Chiloscyllium punctatum*
GFG37722.1	hypothetical protein Cfor_06961	*Coptotermes formosanus*
KAF2348435.1	hypothetical protein FHG87_020808	*Trinorchestia longiramus*
KAF8788252.1	Unconventional myosin-Va like protein	*Argiope bruennichi*
XP_023704960.1	glutactin isoform X2	*Cryptotermes secundus*

Appendix 3

Appendix 3 The immune KEGG pathways and their associated differentially expressed transcripts from the DESeq2 analysis of the comparison of the control to the Poly (I:C).

Category of Gene ID	**Homologues Function**	**Species**	**log_2FC**
Peroxisome			
XP_027230269.1	peroxisomal sarcosine oxidase-like	*Litopenaeus vannamei*	-1.28
Autophagy			
XP_011646565.1	ubiquitin-like-conjugating enzyme ATG3	*Pogonomyrmex barbatus*	1.50
Ubiquitin mediated proteolysis			
XP_012255442.1	cullin-1	*Athalia rosae*	-1.73
Protein processing in endoplasmic reticulum			
XP_012255442.1	cullin-1	*Athalia rosae*	-1.73
Wnt *signaling* pathway			
XP_012255442.1	cullin-1	*Athalia rosae*	-1.73

Appendix 4

Appendix 4 The metabolic KEGG pathways and their associated differentially expressed transcripts from the DESeq2 analysis of the comparison of the control to the Poly (I:C).

Category of Gene ID	Homologues Function	Species	log_2FC
Metabolic pathways			
XP_027230269.1	peroxisomal sarcosine oxidase-like	*Litopenaeus vannamei*	-1.28
Krebs cycle			
XP_015188895.1	PREDICTED: isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial	*Polistes dominula*	1.85
Carbon metabolism			
XP_015188895.1	PREDICTED: isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial	*Polistes dominula*	1.85
2-Oxocarboxylic acid metabolism			
XP_015188895.1	PREDICTED: isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial	*Polistes dominula*	1.85

Appendix 5

Appendix 5 The amino acid associated KEGG pathways and their associated differentially expressed transcripts from the DESeq2 analysis of the comparison of the control to the Poly (I:C).

Category of Gene ID	**Homologues Function**	**Species**	**log_2FC**
Biosynthesis of amino acids			
XP_015188895.1	PREDICTED: isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial	*Polistes dominula*	1.85
Purine m*etaboli*sm			
KAG1940839.1	allantoinase	*Pimephales promelas*	-1.38
Glycine, serine and threonine metabolism			
XP_027230269.1	peroxisomal sarcosine oxidase-like	*Litopenaeus vannamei*	-1.28

Appendix 6

Appendix 6 The immune KEGG pathways and their associated differentially expressed transcripts from the DESeq2 analysis of the comparison of the control to the Poly (I:C) complexed to the NanoDendrix™.

Category of Gene ID	**Homologues Function**	**Species**	**log_2FC**
Autophagy			
ATV91621.1	putative autophagy protein 8	*Recilia dorsalis*	1.62
Endocytosis			
XP_029034035.1	E3 ubiquitin-protein ligase NEDD4 isoform X4	*Osmia bicornis*	-0.85
FoxO *signaling* pathway			
ATV91621.1	putative autophagy protein 8	*Recilia dorsalis*	1.62
MAPK signaling pathway - yeast			
XP_029034035.1	E3 ubiquitin-protein ligase NEDD4 isoform X4	*Osmia bicornis*	-0.85
Mitophagy			
ATV91621.1	putative autophagy protein 8	*Recilia dorsalis*	1.62
NOD-like receptor signaling pathway			
ATV91621.1	putative autophagy protein 8	*Recilia dorsalis*	1.62
Ubiquitin mediated proteolysis			
XP_029034035.1	E3 ubiquitin-protein ligase NEDD4 isoform X4	*Osmia bicornis*	-0.85
PNF19185.1	hypothetical protein B7P43_G09598	*Cryptotermes secundus*	1.93

Appendix 7

Appendix 7 The metabolic KEGG pathways and their associated differentially expressed transcripts from the DESeq2 analysis of the comparison of the control to the Poly (I:C) complexed to the NanoDendrix™.

Category of Gene ID	Homologues Function	Species	log_2FC
Metabolic pathways			
XP_028038669.1	probable glucosamine 6-phosphate N-acetyltransferase isoform X2	*Bombyx mandarina*	-1.25
CAD7439179.1	unnamed protein product	*Timema bartmani*	-2.09
XP_017550485.2	cytochrome P450 2M1-like	*Pygocentrus nattereri*	1.59
AJN57846.1	glutathione S-transferase	*Rhopalosiphum padi*	-2.01
GCC27648.1	hypothetical protein	*Chiloscyllium punctatum*	1.72
XP_967808.1	PREDICTED: cysteine-rich protein 1	*Tribolium castaneum*	0.69
XP_008912551.1	hypothetical protein PPTG_16800	*Phytophthora parasitica*	-0.93
XP_013383970.1	E3 ubiquitin-protein ligase RNF181	*Lingula anatina*	-1.33
XP_027210161.1	titin-like isoform X1	*Litopenaeus vannamei*	1.23
XP_027238938.1	mitochondrial import receptor subunit TOM70-like, partial	*Litopenaeus vannamei*	1.43
KAA8593891.1	hypothetical protein FQN60_004725, partial	*Etheostoma spectabile*	1.17
Ether lipid metabolism			
CAD7439179.1	unnamed protein product	*Timema bartmani*	-2.09
Glutathione metabolism			
AJN57846.1	glutathione S-transferase	*Rhopalosiphum padi*	-2.01
Fatty acid degradation			
XP_017550485.2	cytochrome P450 2M1-like	*Pygocentrus nattereri*	1.59
Arachidonic acid metabolism			
XP_017550485.2	cytochrome P450 2M1-like	*Pygocentrus nattereri*	1.59
Starch and sucrose metabolism			
XP_027238938.1	mitochondrial import receptor subunit TOM70-like, partial	*Litopenaeus vannamei*	1.43
Carbohydrate digestion and absorption			
XP_027238938.1	mitochondrial import receptor subunit TOM70-like, partial	*Litopenaeus vannamei*	1.43
Sphingolipid metabolism			
XP_027210161.1	titin-like isoform X1	*Litopenaeus vannamei*	1.23
Phosphonate and phosphinate metabolism			
XP_013383970.1	E3 ubiquitin-protein ligase RNF181	*Lingula anatina*	-1.33
Amino sugar and nucleotide sugar metabolism			
XP_028038669.1	probable glucosamine 6-phosphate N-acetyltransferase isoform X2	*Bombyx mandarina*	-1.25
RXG56199.1	NADH-cytochrome b5 reductase 2	*Armadillidium vulgare*	0.96
Oxidative phosphorylation			
XP_967808.1	PREDICTED: cysteine-rich protein 1	*Tribolium castaneum*	0.69
XP_008912551.1	hypothetical protein PPTG_16800	*Phytophthora parasitica*	-0.93

Appendix 8

Appendix 8 The species of the transcriptomes generated in the laboratory of Dr. K Fraser Clark from either hepatopancreas or gill tissue and the number of sequences in each transcriptome. Transcriptomes available upon request.

Transcriptome Species	Number of Sequences
Callinectes sapidus	425,157
Cancer borealis	138,339
Cancer irroratus	199,406
Carcinus maenas	310,254
Charybdis feriatus	418,481
Cherax quadricarinatus	93,618
Chionoecetes opilio	253,368
Dyspanopeus sayi	172,288
Eriocheir sinensis	789,474
Exopalaemon carinicauda	317,085
Gecarcoidea natalis	103,115
Helice tientsinensis	270,622
Homarus americanus	285,259
Hyas araneus	250,157
Hyas coarctatus	375,115
Litopenaeus vannamei	130,543
Macrobrachium nipponense	197,339
Macrobrachium olfersii	110,560
Macrobrachium rosenbergii	74,955
Marsupenaeus japonicus	54,911
Orithyia sinica	41,852
Ovalipes ocellatus	103,873
Pagurus longicarpus	491,486
Pagurus samuelis	176,411
Pandalus borealis	62,733
Panulirus argus	245,761
Paratya australiensis	157,497
Paratya australiensis	157,497
Penaeus monodon	258,073
Porcellana platycheles	364,652
Portunus sanguinolentus	593,742
Portunus trituberculatus	438,806
Portunus trituberculatus	115,741
Sagmariasus verreauxi	112,629
Scylla paramamosain	587,353
Scylla olivacea	345,267
Thenus orientalis	84,815
Uca borealis	18,395

Appendix 9

Appendix 9 The best DNA/Protein Models as determined by MEGA X used for each of the phylogenies created with a Muscle alignment and ExPASy translation.

Sequences for Tree	Best DNA/Protein Model
Full Crustin Sequences	WAG+G
WAP Sequences	WAG+G+I
Shrimp Crustin Sequences	WAG+G
Non-Shrimp Crustin Sequences	WAG+G
Anomura Crustin Sequences	Dayhoff+G
Brachyura Crustin Sequences	WAG+G
Caridea Crustin Sequences	Dayhoff+G
Dendrobranchiata Crustin Sequences	WAG+G+I

Appendix 10

The following electronic supplementary information is available at Dalspace.

S 2.1 The DET as determined from the comparison of the control to the Poly (I:C) from DESeq2 accompanied by the associated metadata.

S 2.2 The DET as determined from the comparison of the control to the Poly (I:C) NanoDendrix complex from DESeq2 accompanied by the associated metadata.

S 2.3 The sequences of the Trinity IDs associated to DET from the DESeq2 analysis of the control compared to the Poly (I:C).

S 2.4 The sequences of the Trinity IDs associated to DET from the DESeq2 analysis of the control compared to the Poly (I:C) NanoDendrix complex.

S 3.1 The crustin sequences examined with their associated crustin type and phylogenetic classification.

S 3.2 Legend of phylogenetic tree groupings.

S 3.3 A bootstrap unrooted phylogeny of the crustin sequences aligned by Muscle and translated by ExPASy. The phylogeny was inferred using the Maximum Likelihood method and Whelan and Goldman model through MEGA X. The bootstrap consensus tree was inferred from 1000 replicates and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

S 3.4 A bootstrap unrooted phylogeny of the crustin sequences WAP domain aligned by Muscle and translated by ExPASy. The phylogeny was inferred using the Maximum Likelihood method and Whelan and Goldman model through MEGA X. The bootstrap consensus tree was inferred from 1000 replicates and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

S 3.5 A bootstrap unrooted phylogeny of the crustin sequences from shrimp species aligned by Muscle and translated by ExPASy. The phylogeny was inferred using the Maximum Likelihood method and Whelan and Goldman model through MEGA X. The bootstrap consensus tree was inferred from 1000 replicates and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

S 3.6 A bootstrap unrooted phylogeny of the crustin sequences from non-shrimp species aligned by Muscle and translated by ExPASy. The phylogeny was inferred using the Maximum Likelihood method and Whelan and Goldman model through MEGA X. The bootstrap consensus tree was inferred from 1000 replicates and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

S 3.7 A bootstrap unrooted phylogeny of the crustin sequences from the infraorder Anomura aligned by Muscle and translated by ExPASy. The phylogeny was inferred using the Maximum Likelihood method and Whelan and Goldman model through MEGA X. The bootstrap consensus tree was inferred from 1000 replicates and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

S 3.8 A bootstrap unrooted phylogeny of the crustin sequences from the infraorder Brachyura aligned by Muscle and translated by ExPASy. The phylogeny was inferred using the Maximum Likelihood method and Whelan and Goldman model through MEGA X. The bootstrap consensus tree was inferred from 1000 replicates and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

S 3.9 A bootstrap unrooted phylogeny of the crustin sequences from the infraorder Caridea aligned by Muscle and translated by ExPASy. The phylogeny was inferred using the Maximum Likelihood method and Whelan and Goldman model through MEGA X. The bootstrap consensus tree was inferred from 1000 replicates and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

S 3.10 A bootstrap unrooted phylogeny of the crustin sequences from the infraorder Dendrobranchiata aligned by Muscle and translated by ExPASy. The phylogeny was inferred using the Maximum Likelihood method and Whelan and Goldman model through MEGA X. The bootstrap consensus tree was inferred from 1000 replicates and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.