The therapeutic application of Pituitary Adenylate Cyclase-Activating Polypeptide and double stranded (ds)RNA as immunostimulants and antiviral agents in crustaceans

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

Dr. Tamiru Alkie and Kayla Samms from the Department of Biology at Wilfrid Laurier University assisted with the three crayfish animal trials. Dr. Alkie injected 50 percent and I injected 50 percent of the animals included in the first and second animal trial. Kayla Samms injected 50 percent and I injected 50 percent of the animals in the third animal trial. I completed 75 percent and Kayla Samms completed 25 percent of the sample analysis in the third animal trial. Kayla Samms contributed additionally with crayfish collection from the Grand River and care of the animals. Dr. Tania Rodriguez-Ramos from the Biology Department at the University of Waterloo contributed guidance and expertise relating to crustacean care and sample analysis. Dr. Brian Dixon from the Biology Department at the University of Waterloo and Dr. Stephanie DeWitte-Orr from the Department of Health Sciences at Wilfrid Laurier University provided all funding for the experiments as well as mentorship, expertise, and knowledge for experimental designs.

Abstract

Currently valued at 28 billion USD per year, the global shrimp market is growing faster than any other aquaculture industry (Food and Agriculture Organization, 2020). The initial fast-paced global industry expansion led to many catastrophic viral shrimp pandemics (Flegel, 1997). White spot syndrome virus (WSSV), a DNA virus with global distribution, is a major causative agent of massive shrimp die-offs worldwide. Shrimp farmers are limited in their ability to manage and reduce disease burden due to the absence of effective antiviral treatments. The present study focuses on the application of therapeutics in crustaceans with the goal of inducing a general immune response to control viral infection. Double-stranded (ds)RNA has been well characterized in vertebrates as an innate immune stimulant and potent inducer of the antiviral response through type I interferons and interferon stimulated genes. Pituitary adenylate cyclase activating polypeptide (PACAP) is a highly conserved, multifunctional, cationic neuropeptide with antimicrobial properties. In vertebrates PACAP has been shown to regulate pro- and anti-inflammatory cytokine production through cAMP signalling cascades and to interfere directly with viral protein transcription. In this study, wild caught Ontario crayfish were injected with 10 µg of High molecular weight (HMW) Polyinosinic:polycytidylic acid (synthetic dsRNA), a low (6 µg) and high (12 µg) dose of PACAP-38 and a combination of 10µg Poly(I:C) HMW and 12µg PACAP-38. The safety of these treatments was first established through exposure of primary hemocytes *in vitro* to concentrations of PACAP between 1.95 and 250nM and Poly(I:C) HMW between 0.0076 and 10µg/mL which did not reduce cell metabolism or membrane integrity. These therapeutics were then delivered to crayfish by both intramuscular and ventral sinus injection. Following treatment for 6-, 24-, 48- and 168-hours immune system activation was measured using functional markers of cellular and humoral responses, including the total number of hemocytes, lectin activity, nitric oxide concentration and activated phenoloxidase

function. Following *in vivo* stimulation by ventral sinus injection, animals treated with Poly(I:C) HMW, PACAP and the PACAP+HMW combination had a higher total number of hemocytes and a significant increase in hemagglutination and phenoloxidase activity between 6 and 168 hours. PACAP treatment for 6 hours resulted additionally in a significant increase in the hemolymph concentration of nitric oxide. This work establishes the immunostimulatory role of dsRNA and PACAP-38 in crustaceans which will benefit the shrimp aquaculture industry by contributing to the development of effective broad-spectrum immune therapies.

Acknowledgments

I would like to thank Dr. Stephanie DeWitte-Orr, my co-supervisor. Thank you for taking a chance on me as an undergraduate. You taught me to become confident in myself as a researcher and person. Without your advocacy and support I would not be where I am today. I am inspired by your kind and compassionate leadership, commitment to research and care for your students. This project has not been without its challenges, but your constant support, encouragement and guidance enabled us to pivot and adapt to any circumstances. I feel so lucky to have you in my corner.

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List of Abbreviations

AHPND- Acute hepatopancreatic necrosis disease
AMP- Antimicrobial peptide
ANOVA- Analysis of variance
ATF-2- Activating transcription factor 2
ADP- Adenosine diphosphate
cAMP- Cyclic adenosine monophosphate
CFDA-AM- 5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester
CFU- Colony-forming unit
CPP- Cell penetrating peptide
CREB- Cyclic AMP responsive element-binding protein
DNA- Deoxyribonucleic acid
dsRNA-double stranded RNA
EDTA- Ethylenediaminetetraacetic acid
GPCR- G-protein-coupled receptors
GTP- Guanosine triphosphate
HIV- Human immunodeficiency viruses
HPV- Human papillomavirus
HMW Poly (I:C)- High molecular weight Polyinosinic: polycytidylic acid
IHHNV- Infectious hypodermal and hematopoietic necrosis
IL-6- Interleukin 6
IMD- Immunodeficiency pathway
IMNV- Infectious Myonecrosis Virus
IRF- Interferon regulatory factors
JAK/STAT- Janus kinases/ signal transducer and activator of transcription proteins
LPS- Lipopolysaccharides
NADH- Nicotinamide adenine dinucleotide
NADPH- Nicotinamide adenine dinucleotide phosphate
NF-κβ- Nuclear factor kappa-light-chain-enhancer of activated B cells
NO- Nitric oxide
PACAP- Pituitary adenylate cyclase activating polypeptide
PAMP- Pathogen associated molecular pattern
PBS- Phosphate-buffered saline
PF4- Platelet factor 4
PGN- Peptidoglycans
PKA- Protein kinase A
PKC- Protein kinase C
PLC- Phospholipase C
Poly I:C- Polyinosinic: polycytidylic acid
PO- Phenoloxidase
PPAE- proPO-activating enzyme
ProPO- Prophenoloxidase
PRR- Pattern recognition receptor
RLR- RIG-I-like receptors
qPCR- Quantitative polymerase chain reaction

RNA- Ribonucleic acid
RNAi- RNA interference
RNS- Reactive nitrogen species
ROS- Reactive oxygen species
SOD- Superoxide dismutase
THC- Total hemocyte count
TIR-Toll/Interleukin-1 receptor
TLR- Toll-like receptor
TME- Tail muscle extract
TSV- Taura syndrome virus
VIP- Vasoactive intestinal protein
WSSV- White spot syndrome virus
YHV- Yellow head virus

Chapter 1. Literature Review

1.1 The importance of crustaceans

The shrimp aquaculture industry

The shrimp aquaculture industry is burdened by frequent incidence of catastrophic disease outbreaks (Walker & Winton, 2010). Industry demand forces high stocking density and cross-boundary movement which incites rampant infections in farmed populations (Alfiansah et al., 2018, Wang et al., 2020). Implementation of strict biosecurity measures and use of selectively screened and bred pathogen free shrimp have reduced the frequency of outbreaks (Liu et al., 2018, Moss et al., 2012). While these measures have been somewhat effective in controlling the pervasiveness of epizootics, viral and bacterial infection precipitates 80% of farmed shrimp mortality (Flegel & Lightner, 2008). Shrimp aquaculture is currently growing faster than any other aquaculture industry. China is the largest contributor, producing 1.76 million tonnes of farmed shrimp a year with other major producers in Indonesia, India, Ecuador, Vietnam, Thailand, and Mexico (White Leg Prawn Production, 2018). Shrimp farmers are limited in their ability to manage and reduce disease burden. Antibiotics - despite being ineffective against viruses - are the most common preventative measure used in shrimp aquaculture (Thornber et al., 2020). Reports from Bangladesh indicate that 80kg of antibiotics were used in a single production cycle at a single hatchery (Hinchliffe et al., 2018). The unregulated use of clinically relevant antibiotics in shrimp aquaculture is driving antimicrobial resistance and contributing to antibiotic pollution (Thornber et al., 2020). This is motivating the identification of alternative antimicrobial compounds. Highly conserved, cationic antimicrobial peptides (AMPs) are a promising alternative. The threat of disease, dependence on antibiotics, and the economic importance of shrimp aquaculture, particularly to developing nations, makes it

paramount that the immune response is understood and novel, effective therapeutics are developed.

Pathogen threats

Crustacean populations are highly susceptible to bacterial and viral disease (Moss et al., 2012, Aguilera-Rivera et al., 2019, Kumar et al., 2014). These are summarized in **Table 1** and include acute hepatopancreatic necrosis disease (AHPND) caused by *Vibrio species*, and viral diseases caused by white spot syndrome virus (WSSV), yellow head virus (YHV), hepatopancreatic parvo-like virus (HPV), infectious myonecrosis virus (IMNV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV) (Walker & Winton, 2010, Flegel et al., 2004, Flegel & Alday-Sanze, 1998, Lai et al., 2015, Knibb et al., 2015, Soto-Rodriguez et al., 2012). Viral disease is caused by the accumulation of circulating virus and stress triggered viral amplification spread through horizontal and vertical transfer (Bu et al., 2017, Mercier et al., 2006, Bonnichon et al., 2006). Shrimp can sustain multiple sequential or simultaneous infections by several viruses or strains of the same virus (Walker & Winton, 2010, Flegel et al., 2004, Bonnichon et al., 2006).

Significant population die-offs due to disease were not observed prior to the unmediated cross-boundary movement of shrimp and cultivation of high stocking densities for aquaculture (Flegel, 2006). Pathogen growth is additionally fostered through the release of untreated human and animal waste and leaching chemicals into shrimp ponds (Food and Agriculture Organization, 2020) . There is also a growing concern for the presence of antibiotic resistant bacteria in shrimp populations. While there is no conclusive evidence for the transfer of viruses from shrimp to humans, there are reports that TSV can be cultured in human and monkey cells (Audelo-Del-Valle, 2003, Flegel, 2009).

Current control methods for prevention of outbreaks in mature populations rely on PCR screening to identify the virus at the hatchery level and euthanizing infected animals. The current measures inadequately control the spread of viruses in shrimp aquaculture. In Japan, 104 *Penaeus monodon* were collected from different hatcheries, PCR screening revealed 12.5% were positive for WSSV alone, while 60.6% were positive for a concurrent (double) infection of HPV, IHHNV, MBV or WSSV (Joseph et al., 2015). In this present project pathogens themselves were not used, but in their stead, immune stimulants were studied.

Table 1. Crustacean infectious diseases OIE, FAO, ICTV (Cotmore et al., 2019) classification

Causative agent	Disease	Genome	Taxonomic classification	Geographic distribution
DNA viruses				
White spot syndrome virus (WSSV)	White spot disease	dsDNA Non-enveloped	*Nimaviridae, Whispovirus*	Asia, South-East Asia, South Asia, India, Middle East, Americas, Mediterranean
Infectious hypodermal and hematopoietic necrosis virus (IHHNV)	Infectious hypodermal and hematopoietic necrosis	ssDNA	*Parvoviridae, Densovirus*	Worldwide
Hepatopancreatic parvovirus (HPV)	Hepatopancreatic necrosis disease	ssDNA	*Parvoviridae, Densovirus*	South-East Asia, Africa, Middle East, Americas
RNA viruses				
Infectious myonecrosis virus (IMNV)	Infectious myonecrosis	ssRNA (+)	*Totiviridae*	South America, Southeast Asia
Taura syndrome virus (TSV)	Taura syndrome	ssRNA (+)	*Picornavirales, Dicitstroviridae*	Americas, South-East Asia, Middle East
Yellow head virus-1 (YHV-1)	Yellowhead disease	ssRNA (+)	*Nidovirales, Roniviridae, Okavirus*	Asia, South Asia, South Ameirca, Australia
Laem-singh virus	Monodon slow growth syndrome	dsRNA (+)	Unclassified, *Luteovirus-like*	South and Southeast Asia
Bacteria				
Vibrio parahemolyticus, Vibrio harveyi, Vibrio owensii, Vibrio campbelli, Vibrio punensis	Acute hepatopancreatic necrosis disease (AHPND)/ Early mortality syndrome (EMS)	pAP1 plasmid (69kB) carrying two toxin producing genes (Pir A/B 12.7 kDA and 50.1 kDA)	*Vibrionaceae*	Asia, South America

Crustacean immune system overview

Invertebrates lack an adaptive immune system and rely on a sophisticated innate immune response based on a combination of physical barriers, signalling cascades, cell mediated and humoral defenses (Figure 1). The first line of defense is the rigid exoskeleton composed of calcium carbonate, carbohydrates, and proteins. The exoskeleton contains hemocyanin in the exocuticle and endocuticle, which has associated humoral responses including reactive oxygen species production, cuticle hardening and melanization (Zheng et al., 2016). In addition to barriers to defend against pathogen infection, crustaceans rely on cellular and humoral responses. The cellular responses of phagocytosis, melanization and encapsulation are achieved through the action of circulating and resident hemocytes. While the humoral response destroys pathogens through reactive oxygen and nitrogen species production, phenoloxidase release and antimicrobial peptide effectors. These responses are initiated following pathogen recognition by pattern recognition receptors, such as Lectin and Toll receptors, and initiation of intracellular signalling cascades.

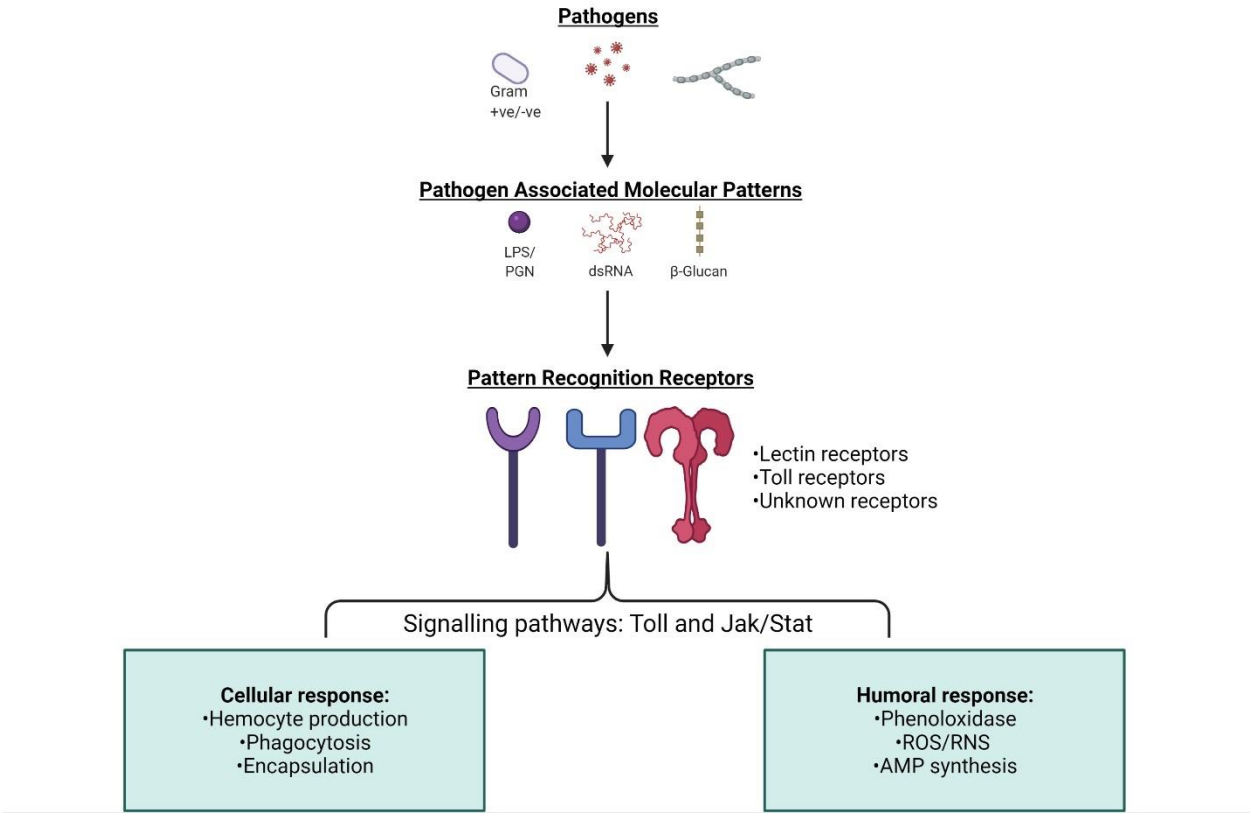


Figure 1. Crustacean immune system overview summarizing recognition of pathogens. The immune system is divided into two arms: cellular and humoral. Following pathogen recognition, pattern recognition receptors (Lectin, Toll and unknown receptors) initiate signalling pathways which stimulate cellular and humoral effector responses. Figure produced in Biorender.

Ontario crayfish as a model

Shrimp and crayfish both belong to the subphylum Crustacea. Pacific white leg shrimp, one of the most economically important shrimp, were inaccessible for this research project due to an ongoing, global outbreak of infectious hypodermal and hematopoietic necrosis virus (IHHNV) and the resulting import bans on non-endemic crustaceans imposed by the Canadian Food Inspection Agency. The present thesis uses local crayfish as a model instead. Crayfish that could be caught from local rivers and streams in Waterloo, Ontario were used because the import ban extended to any species of non-endemic crustacean that is susceptible to the virus. The same cellular and

humoral immune responses have been described across the entire Crustacean subphylum (Bouallegui, 2021, Clark & Greenwood, 2016, Burnett & Burnett, 2015). Shrimp and crayfish are also susceptible to the same globally significant diseases including white spot, yellow head, Taura syndrome, acute hepatopancreatic necrosis, and infectious hypodermal and hematopoietic necrosis (Moss et al., 2012, Aguilera-Rivera et al., 2019, Kumar et al., 2014).

There are nine identified species of crayfish inhabiting Ontario's freshwater streams, rivers, and lakes (Wild Species: Crayfish, 2005). They belong to the family *Astacidae* with six in the genus *Orconectes,* three in the genus *Cambarus* and one in the genus *Fallicambarus.* These crayfish can be distinguished as either open water species or burrowers. Open water species, including *Orconectes virillis, Orconectes propinquus, Orconectes obscurus, Orconectes rusticus, Cambarus bartonii and Cambarus robustus*, never leave the water. These crayfish typically shelter under rocks and vegetation during the day and are most active at night. Native *Orconectes propinquus* and exotic *Orconectes rusticus* are the exception as they are most active during the day. Variation in size (35 and 51mm carapace length, respectively) and markings means they can be easily distinguished (Figure 2). Burrowing crayfish include *Orconectes immunis, Cambarus diogenes, Fallicambarus fodiens*. These crayfish do not depend on open water, living in ditches, meadows, and wetlands where they tunnel through wet soil (Wild Species: Crayfish, 2005).

Figure 2. Crayfish used in this study, collected from the Grand River in Waterloo, Ontario. Suspected to be *Orconectes propinquus* based on behaviour, size, and markings.

1.2 PACAP as an immune stimulant

PACAP structure

Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) is a highly conserved, multifunctional neuropeptide with antimicrobial properties. PACAP exists in two bioactive C-terminal amidated isoforms derived from a 175 amino acid precursor PACAP-38 and PACAP-27 (Miyata et al., 1989). PACAP-38 is the predominant form of PACAP but both forms are found in all tissues, including the brain. The biochemical properties of PACAP are consistent with characterized antimicrobial peptides which are generally cationic and amphipathic (Lai & Gallo, 2009). PACAP is a cationic peptide with a net charge of +9 and an ionization potential of 11.03 at physiological pH (Lugo et al., 2019). The primary structure is composed of 11 basic amino acids (7 Arginine, 4 Lysine) consisting of positively charged side chains (Figure 3).

An analysis of PACAP by circular dichroism (CD) spectroscopy revealed that PACAP is disordered in aqueous solutions and stabilizes into an α-helical conformation in synthetic biological (biomimetic) environments such as organic solvents and lipid micelles (Bourgault et al., 2011). The helix forms between the threonine at position seven to the C-terminus (Figure 4). There is no conformational change between the secondary structures of isoforms PACAP-38 and PACAP-27 and the secondary structures can be directly superimposed. PACAP-38 is composed of a 38 amino acid sequence while PACAP-27 is 27 amino acids in length. On both sides of the helix cationic side chains project (Figure 5). The peptide is amphipathic in nature; hydrophobic regions are involved in membrane binding while hydrophilic regions interact through signalling and membrane permeabilization (Deshayes et al., 2004). The secondary structure of PACAP suggests that it circulates as a soluble molecule, and only adopts a stable α-helix upon high affinity binding with its membrane receptor (Bourgault et al., 2011).

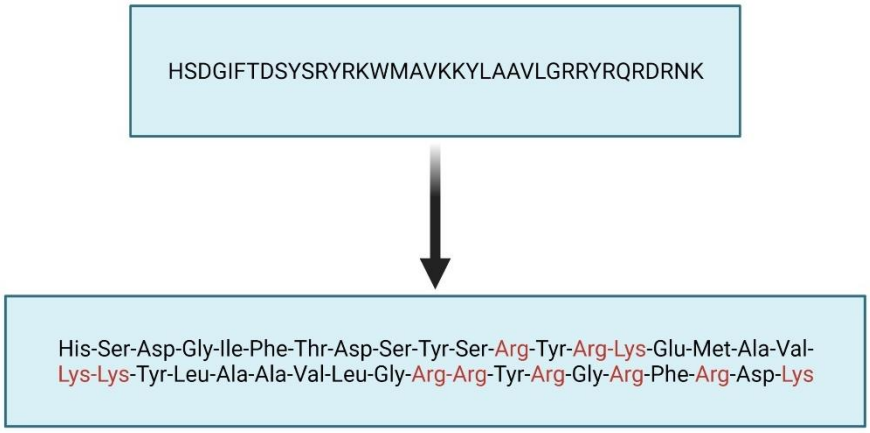


Figure 3. PACAP amino acid translation. Basic amino acids (highlighted in red) establish cationic nature of the peptide. Figure produced in Biorender.

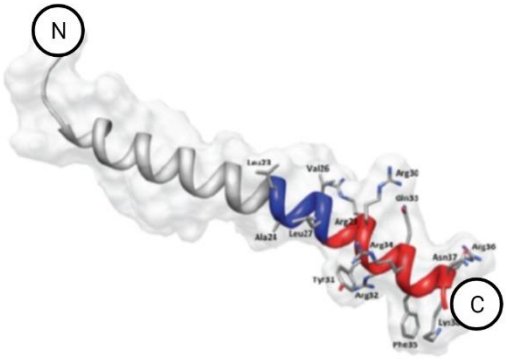


Figure 4. PACAP α-helical secondary structure. Helix has a free N-terminus and cationic side chain projections at the C-terminus (Lugo et al., 2019).

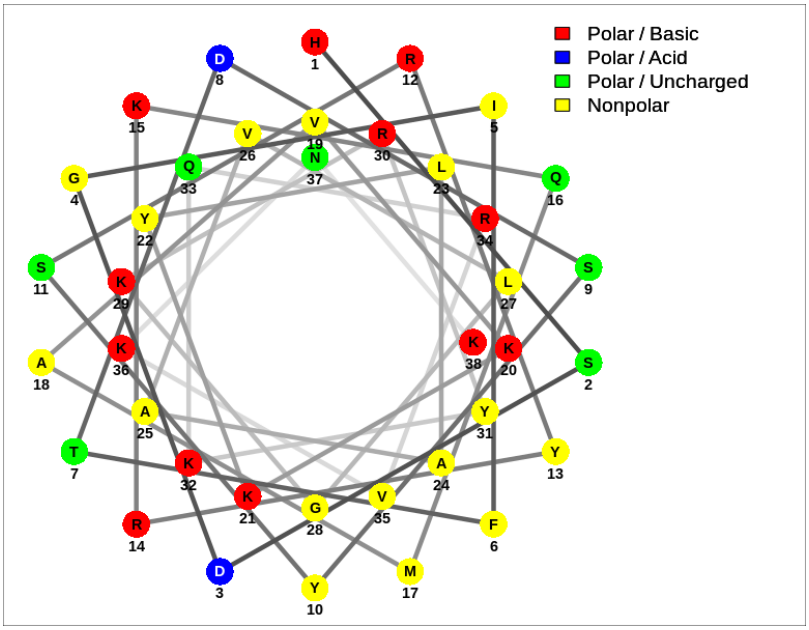


Figure 5. Helical wheel projection demonstrating cationic (basic) amino acids on both sides of the protein structure. Figure produced with "Mól, A. R, Castro, M, S. Fontes, W. NetWheels: A web application to create high quality peptide helical wheel and net projections."

PACAP function

The high degree of evolutionary conservation of PACAP suggests that it plays an important biological role. The sequence of PACAP isolated from a species of shrimp, *Litopenaeus vannamei,* had 87% sequence homology to human and 94% sequence homology to ray finned teleost fish (Lugo et al., 2013). Most substitutions between vertebrate and invertebrate isolates were C-terminal single positively charged amino acids. It is suggested that these changes in the C-terminal extension (amino acid 28-38) serve species specific functions (Lugo et al., 2019).

PACAP functions as a neurotransmitter/neuromodulator, antimicrobial peptide (AMP), cell penetrating peptide (CPP) and immunostimulant. PACAP has been characterized as a neurotransmitter in both invertebrate and vertebrate models. In earth worms, numerous PACAP receptors were discovered in pre and postsynaptic membranes. The study of earthworms also suggests that PACAP functions as a neuromodulator through extra-synaptic release of the peptide (Molnár et al., 2008). Similar characteristics have been identified in humans. PACAP receptors have been identified on postsynaptic hypothalamic, hippocampal, amygdala and autonomic neurons (Reglodi & Tamas, 2016). Furthermore, PACAP functions as a secondary, slow acting neurotransmitter, released from nerve terminals containing fast acting acetylcholine in parasympathetic cardiac and ciliary ganglia. In this role, the release of PACAP can be traced to insulin regulation and stress response (Smith & Eiden, 2012).

PACAP as an antimicrobial peptide

Antimicrobial peptides (AMPs) are soluble effector molecules with the ability to kill a wide range of pathogens, activate signalling cascades and stimulate cytokine production. Fish AMPs are effective at micromolar concentrations against bacteria, viruses and protozoa (Gao et

al., 2012). AMPs also serve as a major defense mechanism in shrimp against bacterial and viral infection (Li et al., 2016, Wang et al., 2014, Wang et al., 2009, Antony et al., 2011, Wang et al., 2011). Consistent with other AMPs, PACAP has been shown to translocate across membranes, forming pores, and causing membrane destabilization and disintegration (Salditt et al., 2006) (Figure 6). The mode of action of AMPs however, is not limited to membrane permeabilization. Many peptides also demonstrate cell-penetrating properties in which they translocate macromolecules across the membrane and interact with intracellular targets such as DNA (Wimley, 2010). C-terminal amino acid sequences consistent with other cell penetrating peptides have recently been identified in PACAP (Lugo et al., 2019).

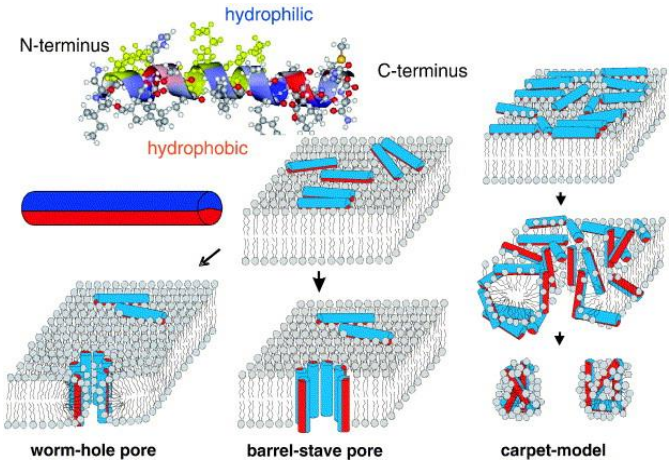


Figure 6. Mechanism of antimicrobial peptide destabilization of lipid bilayer. Transmembrane binding enables formation of pores as hydrophobic side chains anchor into hydrophobic membrane core (Salditt et al., 2006).

PACAP as a signalling and antiviral molecule

PACAPs function has been additionally characterized as an immunomodulator. PACAP can initiate the release of pro- and anti-inflammatory cytokines though activation of the cAMP/PKA stimulating pathway in vertebrates (Bourgault et al., 2011). The PKC signalling

cascade is hypothesized to increase phagocytic ability, as well as superoxide and IL-6 production in macrophages (Nagata et al., 2009, Martinez et al., 1998).

PACAP can modulate the immune system because AMPs do not target host cells. AMPs may not act on eukaryotic cells because of the cholesterol content of eukaryotic membranes. Unlike eukaryotic cell membranes, bacterial cell membranes are free of cholesterol. Because cholesterol is known to cause condensation of phospholipid bilayers, it might prevent AMPs from penetrating the cytoplasmic membrane of eukaryotic cells (Lai & Gallo, 2009, Kang et al., 2014, Augustyniak et al., 2013). Another possible explanation is that the outer monolayer of the host membrane consists of neutral phospholipids without net charge, compared to negatively charged bacterial membranes. The toxicity of AMPs to eukaryotic cells is related to peptide hydrophobicity. An increased hydrophobicity (more nonpolar residues) increases hemolytic activity and decreases selectivity for bacteria (Kang et al., 2014). Furthermore, eukaryotic cells may create sequestered environments in which the ionic composition of the extra cellular matrix protects the host cell (Bourgault et al., 2011).

There is limited research into PACAP's antiviral function. Most research in this area is in humans with very little in aquatic organisms. Treatment of HIV-1 infected macrophages with PACAP resulted in a decrease of viral load due to the activation of β-chemokines and IL-10 (Temerozo et al., 2013). Further evidence suggests that PACAP directly interferes with HIV intracellular replication. One mechanism by which HIV-1 propagates is by hijacking the NF-κβ pathway to elicit transcription of its genome using the transactivation protein TAT and the recruitment of cyclin dependent kinases (CDK). PACAP has been shown to increase production of Cyclin D, which regulates CDK production and interferes with HIV-1 transcription of viral proteins. PACAP increases the concentration of cAMP and subsequent PKA and PKC activation.

It was demonstrated that elevated concentrations of PKA and PKC inhibit HIV-1 replication in infected macrophages (Temerozo et al., 2018). Furthermore, it was determined that PKA and PKC levels regulate expression of a family of IFNα induced antiviral proteins (APOBEC3 proteins). APOBEC3G proteins introduce mutations into viral genomes (Temerozo et al., 2018). PKA can also prevent I-κβ (NF-κβ inhibitor) phosphorylation and disintegration thus preventing NF-κβ transcriptional activity (Delgado & Ganea, 2001, Temerozo et al., 2018). PKA and PKC activated by PACAP phosphorylate and activate the transcription factor CREB which is downregulated by HIV-1 Tat proteins (Delghandi et al., 2005, Schomerus et al., 1996, Temerozo et al., 2018, Zauli et al., 2001).

While antiviral capabilities of PACAP in aquatic organisms have not been well characterized, it was determined that viral infection modulated expression of PACAP and its receptors in brown trout immune tissues. Expression of PACAP and its receptors was upregulated in tissues collected from brown trout challenged with viral haemorrhagic septicaemia virus (VHSV) (Gorgoglione et al., 2015). This suggests that PACAP may play a role in the antiviral immune response of aquatic organisms.

PACAP receptors

PACAP binds to three G-protein coupled receptors (GPCR): PAC1, VPAC-1 and VPAC-2 (Figure 7). G-protein coupled receptors are large transmembrane receptors. In an inactive form, intracellular components of the G-protein coupled receptor are bound to the G-protein-GDP complex (composed of Gα, Gβ and Gγ). Upon extracellular protein binding GTP replaces GDP and the G-protein complex is released from the GPCR and Gα dissociates from Gβ and Gγ. These G-proteins induce a variety of signalling cascades (such as conversion of ATP-cAMP, and

activation of PKA) as well as the formation of ion channels (Bourgault et al., 2011, Hirabayashi et al., 2018).

The two VPAC receptors activate the cAMP signalling cascade (Hirabayashi et al., 2018). PAC1 causes the activation of PKA through cAMP and the activation of PKC through phospholipase C (PLC). PLC bound to the GPCR also opens Ca^{2+} channels (Ganae & Delgado, 2002). PKA regulates the expression of transcription factors NF-κβ, IRF-1, and CREB. Whereas PLC regulates expression of the transcription factor ATF-2 (Bourgault et al., 2011) (Figure 8).

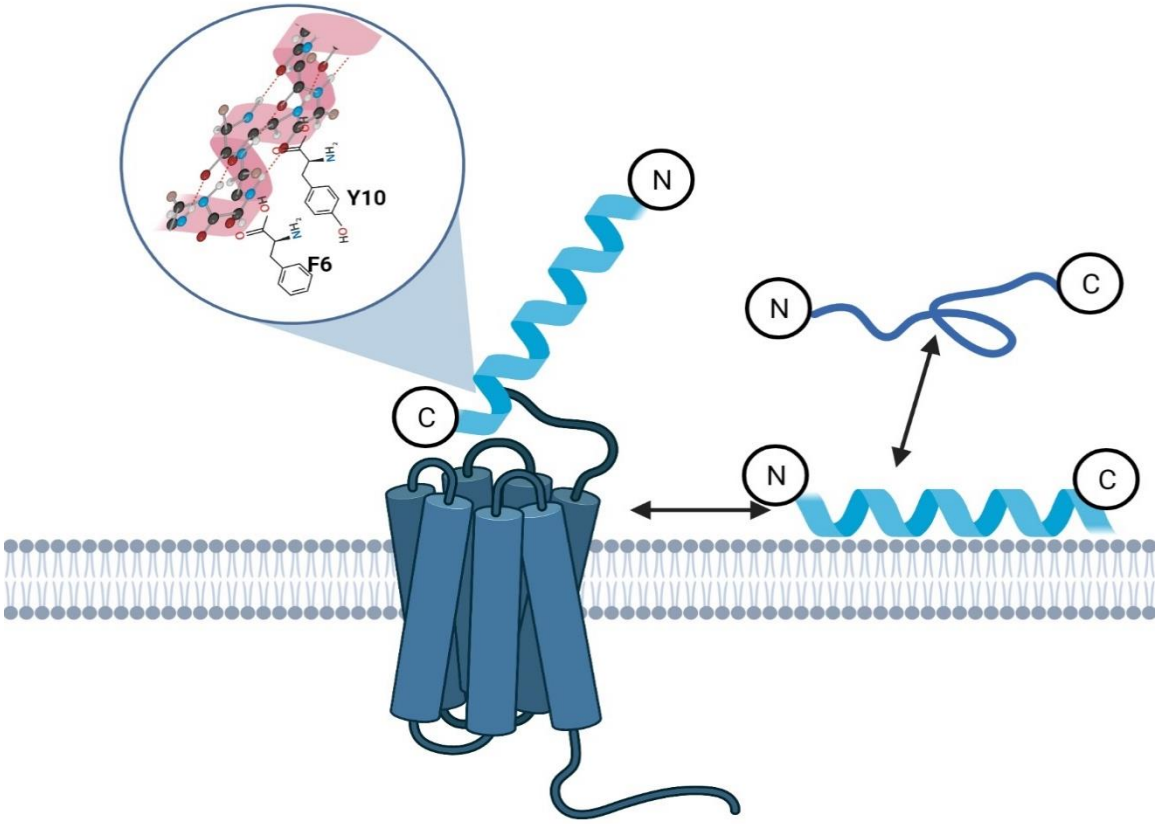


Figure 7. PACAP circulates as a disordered protein and forms an α-helical conformation when it encounters its receptor or extracellular microenvironments. The protein associates with the N-terminal domain of its GPCR. Further conformational changes occur as hydrophobic side chains cluster through the formation of a β-turn. Figure produced in Biorender and adapted from Bourgault et al., 2011.

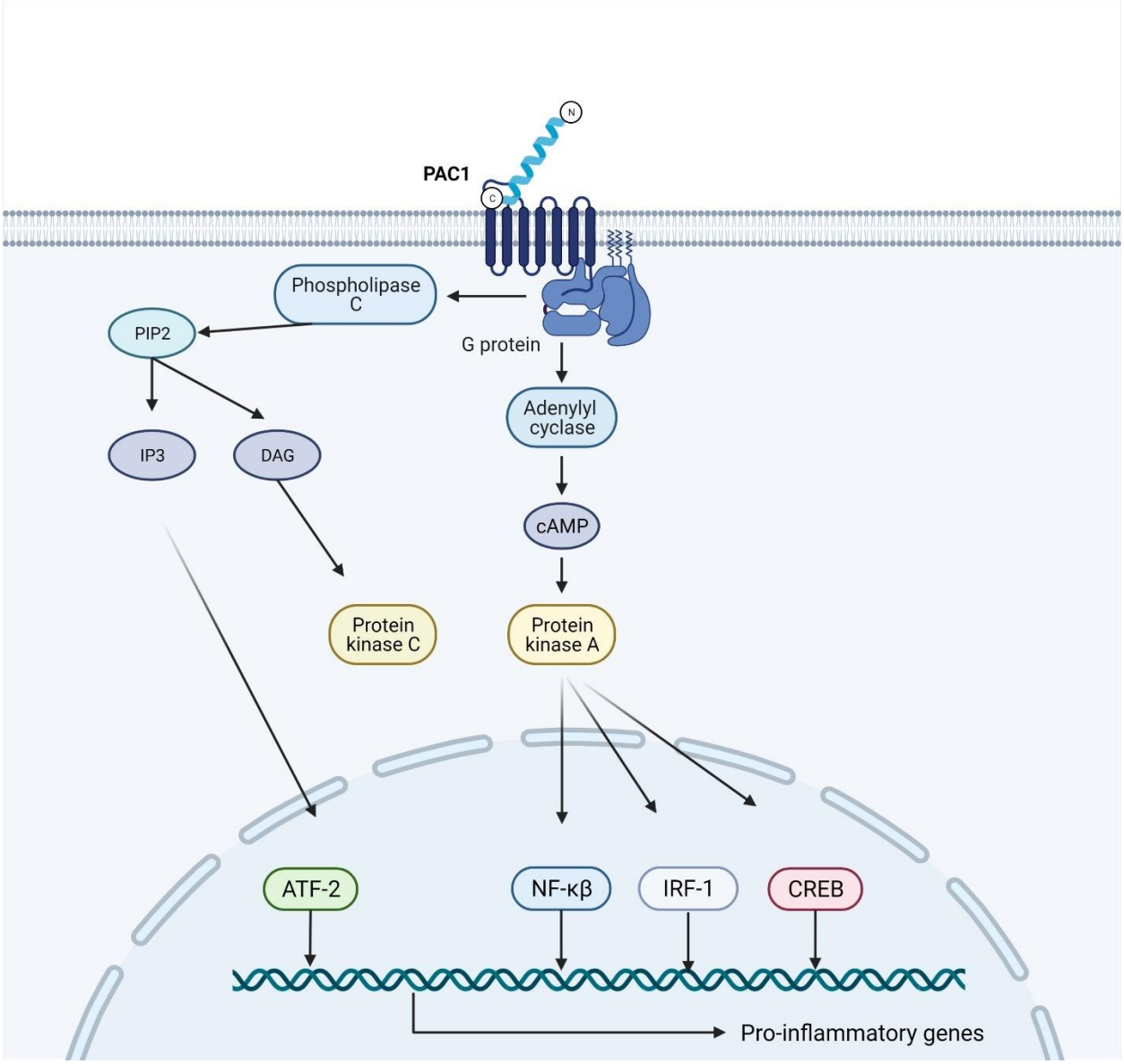


Figure 8. Proposed signalling cascade resulting from PACAP binding. Targets of protein kinase A include transcription factors NF-κβ and CREB. This leads to pro-inflammatory gene transcription (TNFα, IL-6, IL-1β, IFN-γ). Figure produced in Biorender and adapted from Bourgault et al., 2011.

PACAP and crustacean immunity

PACAP has been studied extensively in vertebrates but its role in invertebrates is unclear. Invertebrate PACAP-like peptides share high sequence similarity with human PACAP (>87%) and have been identified in tunicates, cnidarians, protostomes and in three arthropods - crab, white shrimp and cockroach – but PACAP specific receptors and evolution of the ligand-receptor

pair have yet to be determined (Cardoso et al., 2020). The signalling pathways leading to cAMP and mobilization of Ca^{2+} that initiate PACAPs function as a neurotransmitter, antimicrobial peptide and role in metabolism and immune signalling are well conserved in vertebrates and have been described in mammals, birds, fish and amphibians (Cadoso et al., 2020). Although invertebrate GPCR signalling plays an important role in immune modulation, there are currently, minimal functional studies of PACAP in invertebrates (Reboul and Ewbank, 2016). The only described role is in the Pacific white leg shrimp where it boosted innate immunity through increasing the number of hemocytes, lectin activity, nitric oxide metabolite concentration and superoxide dismutase activity (Lugo et al., 2013). Although the receptor has yet to be determined, these results suggest that in crustaceans, PACAP is recognized as a ligand capable of initiating cellular and humoral effector immune responses.

1.3 Double stranded (ds)RNA as an immune stimulant

Double stranded (ds)RNA and viruses

Double stranded (ds)RNA is produced by all viruses at some point in their replicative cycle (Jacobs & Langland, 1996, Doherty et al., 2016,). Whether it originates from the genome - as is the case of dsRNA viruses – or is generated in the host cell during single stranded (ss)RNA and double stranded (ds)DNA viral replication; dsRNA has been established and well characterized as a potent inducer of the innate immune response in all multicellular organisms from plants to humans (Chen & Hur, 2021). dsRNA is composed of two antiparallel strands arranged in a right-handed double α-helix. Contained in its 11-fold helical pitch is a narrow and deep major groove and a shallow minor groove. The molecule interacts with ligands, surface binding proteins and nucleic acids through the 2’- hydroxyl groups of the ribose in the minor groove (DeWitte-Orr & Mossman, 2010).

Commercially available dsRNA-Poly(I:C)

In the late 1950s a synthetic form of dsRNA was developed which is now used commonly in research as a viral dsRNA analogue. Enzymatically produced polymers of polyinsinic acid (Poly I) and polycytidylic acid (Poly C) self-anneal to form a double stranded helix with the same ribose-phosphate backbone as ribonucleic acids (Davies and Rich, 1958). The resulting molecule, Polyinosinic:polycytidylic acid, Poly(I:C), is a dsRNA which contains no viral sequence but functions as an agonist for pattern recognition receptors. Poly(I:C) is available in a high molecular weight (1.5-8 kb) and low molecular weight (0.2-1kb) form.

dsRNA and crustacean immunity

In crustaceans it has been demonstrated that dsRNA can be used to induce sequence-independent antiviral immunity (Robalino et al., 2004). Poly(I:C) stimulation in crustaceans has been shown specifically to upregulate the production of a crustacean interferon regulatory factor (IRF)-like protein leading to an increase in the production of reactive oxygen and nitrogen species and antimicrobial peptides (Serezani et al., 2008). The IRF-like protein was first identified in Pacific white shrimp where it activated transcription of vago by binding to the 5'-untranslated region of the Vago4 gene (Li et al., 2015). Vago functions like an interferon (IFN)-like molecule in invertebrates by initiating antiviral mechanism through the Jak/Stat signalling cascade. Shrimp injected with 5µg of Poly(I:C) had activated expression, dimerization, and translocation of IRF and initiated an antiviral response (Li et al., 2015). Injection of shrimp with 5 µg and 20 µg of Poly(I:C) stimulates a cellular and humoral response (Figure 9). Both doses have been shown to increase the number of hemocytes between 3 and 12 hours, with a decrease at 24 and 48 hours (Ji et al., 2009, Xian et al., 2018). Treatment with 20 µg of Poly(I:C) increases lectin gene expression profiles in hemocytes at 6, 12, 24 and 48 hours post injection (Ji

et al., 2009). While, neither 5µg nor 20 µg of Poly(I:C) impacted phenoloxidase activity, both increased reactive oxygen and nitrogen species in the hemolymph between 6 and 12 hours (Ji et al., 2009, Xian et al., 2018). These results indicate that poly(I:C) can activate the crustacean immune response and, in crayfish, may be able to initiate similar signalling cascades as well as humoral and cellular effector responses.

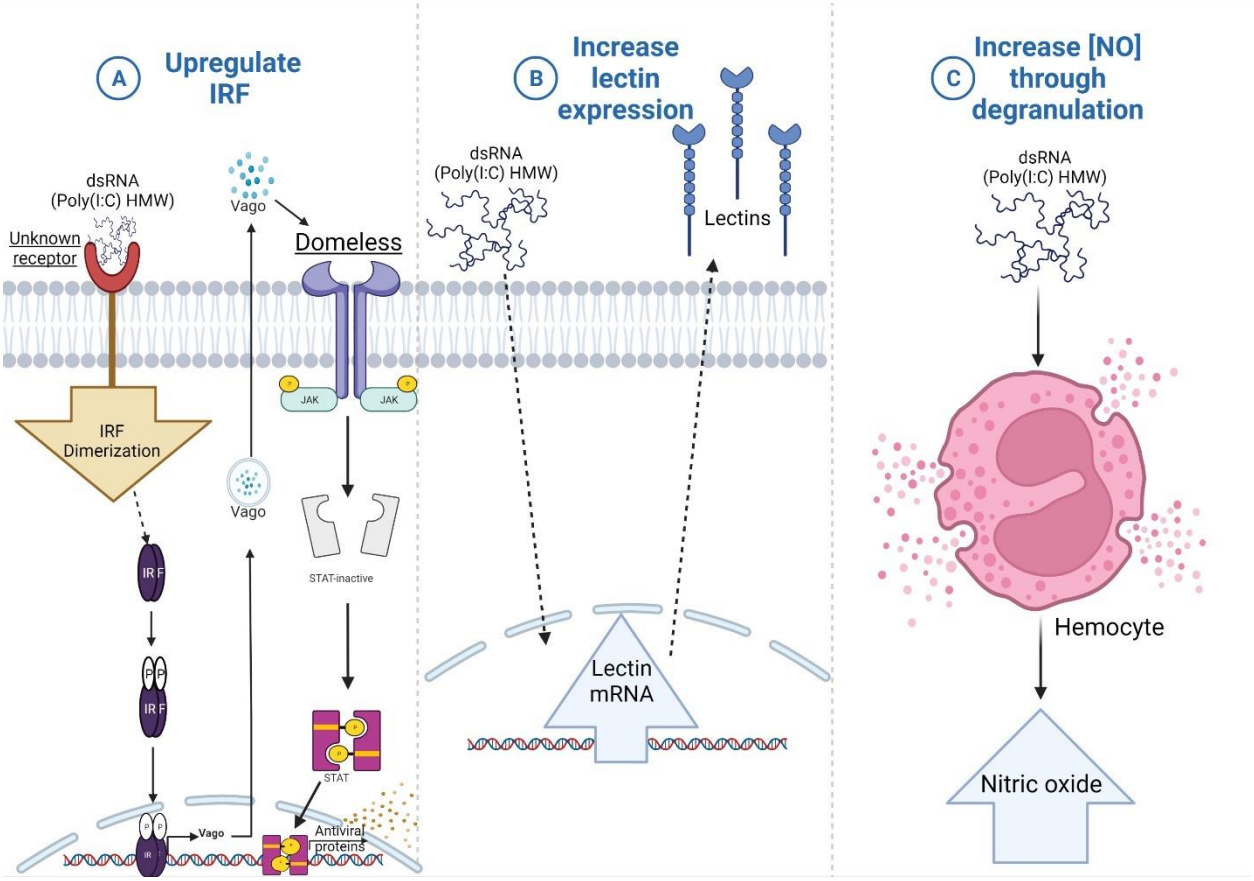


Figure 9. Proposed impact of Poly(I:C) leading to signalling and culminating in effector responses. **A)** Upregulation of antiviral proteins through Vago following increased rate of IRF dimerization. **B)** Promotion of lectin gene transcription by hemocytes following exposure to Poly(I:C) leading to increased lectin receptors in the hemolymph. **C)** Poly(I:C) exposure increases nitric oxide concentration in the hemolymph by promoting degranulation. Figure produced in Biorender.

1.4 Induced effector immune responses in crustaceans

Cell mediated

Hemocytes are the main immune cell in crustaceans. Crustacean hemocytes are classified as either hyaline, semigranular or granular (Lin & Soderhall 2011; Kumar et al. 2013). Hyaline cells are agranular and function as active phagocytes. They are the smallest cell type and least abundant (5%) cell type. Semigranular cells, distinguishable by the presence of small eosinophilic granules, are the most abundant cell type (75%). They are involved in encapsulation, phagocytosis, and coagulation. The action of semigranular hemocytes is co-ordinated through large granular hemocytes which contain the degranulation factor peroxinectin (Lin & Soderhall 2011). The granular hemocytes contain 25-40 large eosinophilic granules which store antimicrobial peptides and pro-phenoloxidase, the inactive zymogen involved in the pro-phenoloxidase activating cascades (Lin & Soderhall 2011; Kulkarni et al., 2020).

The mechanism by which hemocytes are regulated during infection have yet to be fully elucidated in crustaceans (Bouallegui, 2021). Hemocytes are produced by hematopoiesis in the hematopoietic tissue. In crayfish, it has been shown that progenitor cell differentiation in the hematopoietic tissue occurs over 3-4 days, followed by maturation to semigranulocytes and granulocytes while in circulation over 1-3 months (Li et al., 2021). Circulating hemocytes rarely proliferate, however, 10% of the total number of hemocytes are replaced daily following overnight depletion (Soderhall et al., 2003). The number of circulating hemocytes are controlled through hematopoietic cytokines, astakine 1 and astakine 2 (Soderhall et al., 2005). Additionally, it has been shown that clotting proteins, released following PAMP-PRR binding, lead to the recruitment of new hemocytes from the hematopoietic and peripheral tissue (Fagutao et al., 2012). An increase in the number of hemocytes functions to prevent loss of hemolymph, reduce

pathogen spread, promote phagocytosis through hyaline cell recruitment and enhance humoral effectors through co-ordinated degranulation of semigranular and granular cells (Fagutao et al., 2012). Studies have shown that WSSV and bacteria infection in shrimp dramatically reduces the number of hemocytes (Hameed et al., 2006; Wongprasert et al., 2003). During WSSV infection host immunity is weakened as the virus increases the apoptotic rate of non-infected hemocytes (Cui, 2020).

Hemocyte phagocytosis occurs following particle recognition and receptor binding, causing reorganization of the plasma membrane. The pathogen is engulfed into a phagosome and forms a phagolysosome through fusion with lysosomes. Phagocytes destroy internal material through enzyme digestion or through NADPH/NADH reduction of oxygen to H_2O_2 and super oxide dismutase (SOD), and subsequent production of a new oxygen molecule (Aguirre-Guzmán et al., 2009). Phagocytosis is regulated by GTPase binding proteins, ADP ribosylation factors and PRRs (Kulkarni et al., 2020). Lectin receptors are an example of a phagocytic PRR that increase the rate of phagocytosis (Wang et al., 2014). Scavenger receptors are also involved in phagocytosis. The only scavenger receptor so far characterized in shrimp is the class B scavenger receptor, Croquemort, which can function as a phagocytic receptor for gram-positive bacteria and mediates non-opsonic phagocytosis (Wang & Wang, 2013).

Encapsulation is activated when particles are too large for phagocytosis. This cell-mediated response involves pathogen entrapment through hemocyte aggregation and degranulation which kills the pathogen through proPO mediated melanization, free radicals and AMPs (Jiravanichpaisal et al. 2006).

Effector Humoral response

Humoral responses are regulated through signal transduction pathways. The innate immune system utilizes highly conserved pattern recognition receptors (PRRs) to sense pathogen associated molecular patterns (PAMPs) from invading pathogens (Chen et al., 2014). These patterns are essential for microbial survival and include cell wall components such as lipopolysaccharides (LPS), peptidoglycans (PGN), flagellin proteins, fungal β-1,3-glucan and nucleic acids, dsRNA, ssRNA and DNA. These molecules are highly conserved and therefore, despite pathogen diversity, can be sensed by limited receptors (Mogensen, 2009).

Signalling cascades rely on germ-line encoded pattern recognition receptors (PRRs). Currently, Lectin and Toll receptors have been characterized in shrimp and crayfish. PRR: PAMP binding initiates an intracellular signalling cascade regulating transcription factors which move into the nucleus, bind to specific genome sequences and ultimately affect the rate of effector protein transcription. This leads to immune action through respiratory bursts, the pro-phenoloxidase pathway, and the release of NF-κβ dependent AMPs (Borregaard et al. 2000) (Figure 10).

A) Lectin

Lectins are pattern recognition receptors that are involved in bacterial clearance, phagocytosis, the pro-phenoloxidase activating system, signalling cascades and the antiviral response. In crustaceans, lectins have been described as both secreted and membrane bound receptors (Baliarsing et al., 2022, Wang et al., 2014). Lectins promote phagocytosis and pathogen clearance through a carbohydrate recognition domain (CRD) which crosslinks with specific carbohydrate sequences causing bacterial agglutination and opsonization (Wang et al., 2014). Induction of calcium dependent (C-type) lectin expression through NF-κβ signalling

pathways has been established in shrimp challenged with WSSV, *Vibrio parahemolyticus*, LPS and Poly(I:C) (Li et al., 2014, Wang et al., 2014, Zhao et al., 2009, Wang et al., 2020). Lectins protect shrimp from viral infection through high antiviral affinity and direct binding to several WSSV glycosylated envelope proteins (Zhao et al., 2009). Furthermore, knockdown of lectin genes has been shown to increase shrimp susceptibility to WSSV infection (Li et al., 2014).

Lectins are also pattern recognition receptors which are involved in immune signalling cascades capable of inducing the phenoloxidase activating system, release of AMPs and the antiviral response (Kulkarni et al., 2020) (Figure 10). Following viral recognition, lectins have been shown initiate Jak/Stat signalling to induce expression of antiviral factors and antimicrobial peptides. This is achieved through a specific region of the receptor (the cc region) which is not involved in viral recognition but is critical to cell surface binding to Domeless which initiates Jak/Stat antiviral signalling pathways and expression of antiviral effectors (Gao et al., 2021). Without generating intermediates, Lectins are single proteins that both recognize viral PAMPS and stimulates antiviral effectors. Their role is critical to the initiation of a direct and immediate antiviral response.

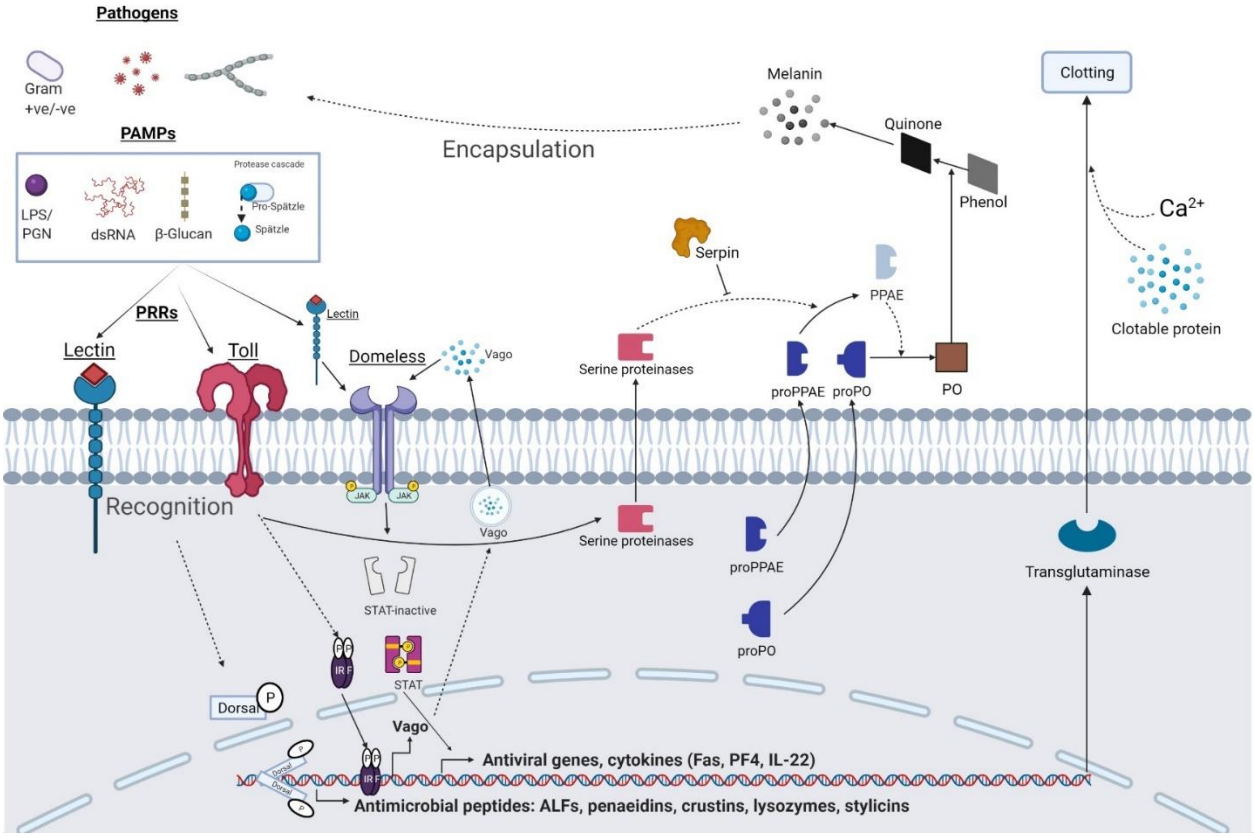


Figure 10. Crustacean immune system overview showing specific pathways relating to lectins. Recognition of PAMPs by lectins leads to signal transduction pathways by interacting with Toll and Jak/Stat pathways. Through direct binding to lectins and Vago. This initiates cytokine and AMP transcription. Additionally, it activates a cell, and humoral effector response leading to encapsulation, the pro-phenoloxidase cascade and the clotting cascade. Figure produced in Biorender and adapted from Tassanakajon et al., 2013.

B) Signalling pathways

Activation of the Toll pathway leads to the transcription factor Dorsal and target effector antimicrobial peptides (AMPs). Toll receptors are comprised of an extracellular domain with leucine rich repeats and an intracellular TIR domain. In crustaceans, extracellular receptor binding occurs directly to pathogens, or the pathogen activates a proteolytic cascade (also involved in clotting and melanization) which cleaves pro-Spaetzle to Spaetzle. Spaetzle is a cytokine-like ligand for Toll receptors. Extracellular binding to the Toll receptor activates intracellular TIR domain dimerization and recruitment of MyD88. MyD88 recruits the secondary adaptor protein, Tube, and protein kinase, Pelle. The MyD88-Tube-Pelle complex recruits tumor necrosis receptor-associated factor 6 (TRAF-6) and Pellino which leads to the phosphorylation of Cactus (a homolog of the NF-κβ inhibitor I-κβ) and the release of Dorsal, the transcription factor that increases antimicrobial peptide (AMP) expression (Figure 11).

A newly proposed antiviral pathway involves an interferon-like system**.** Lectin and Toll receptors have been shown to interact with the Jak/Stat signalling cascade leading to the crustacean IFN-like protein, Vago (Figure 11). Lectins bind directly to Domeless following viral recognition while toll receptors contain a DexD/H-box and helicases, Dicer 1 and 2 (Li et al., 2015). Vago is a viral infection inducible protein that is upregulated through the interferon (IFN) regulatory factor (IRF)-like transcription factor. The Jak/Stat pathway then initiates an antiviral interferon-like response (Li et al., 2015, Yan et al., 2015). In *Drosophila* the Jak/Stat pathway can be activated via binding of the antiviral protein Vago to the unique receptor, Domeless (DOME), which combines with an associated JAK protein (called Hopscotch) which recruit STAT proteins (STAT92E) which leads to transcription of AMPs, antiviral genes, and cytokines such as Fas, PF4 and IL-22 (Tassanakajon et al., 2013) (Figure 11). The presence of the LvDOME has been discovered in *Litopenaeus vannamei*. It has been determined that Poly I:C

stimulation upregulates the production of IRF to establish an antiviral state through the activation of the IRF-Vago Jak/Stat pathway (Yan et al., 2015, Wang et al., 2014).

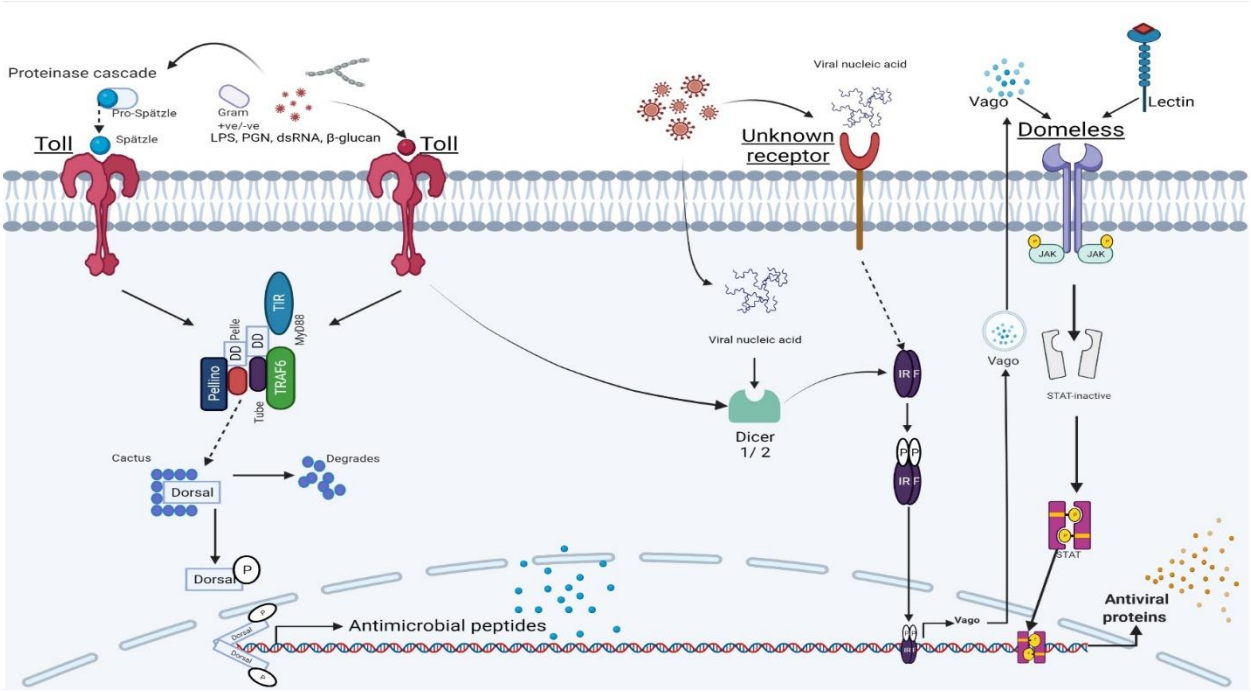


Figure 11. Signalling cascade characterization. PAMP recognition activates Toll receptors leading to TIR domain dimerization and MyD88 recruitment. Interaction Jak/Stat and Toll and Lectin recognition through Vago and direct binding of lectin to Domeless receptor initiates antiviral gene transcription. Figure produced in Biorender and adapted from Li et al., 2019 and Li et al., 2015.

C) Nitric oxide

Reactive oxygen species producing enzymes are stored in hemocyte granules and released during phagocytosis and degranulation, creating characteristic respiratory bursts. Reactive oxygen and nitrogen species (ROS/RNS) production is mediated through these antioxidant enzymes. Expression of the enzymes superoxide dismutase (SOD) and nitric oxide synthase (NOS) indicate the level of ROS/RNS in crustaceans. SOD reduces oxygen to H_2O_2 and catalase reduces H_2O_2 (Tassanakajon et al., 2013). Nitric oxide is a short-lived free radical generated by nitric oxide synthases through conversion of L-arginine to citrulline, which oxidizes to nitrites and nitrates (Figure 12). These enzymes are stored in granules to be released and destroy invading pathogens (Rodriguez-Ramos et al., 2016) It is established that high levels of nitric oxide plays an important role in immune defenses against bacteria, fungal and viral infection (Moncada et al., 1991). In crayfish it has been shown that nitric oxide can enhance bacterial clearance and destruction by promoting adhesion to hemocytes and damaging DNA and proteins (Yeh et al., 2006).

It has been demonstrated that with LPS exposure, nitric oxide is inducible in *Procambarus clarkii* hemocytes and directly reduces *Staphylococcus aureus* colony forming units (CFU) by reacting with O_2^- to $ONOO^-$ which reacts subsequently with H_2O_2 and forms OH which damages cell membranes, proteins, and nucleic acids (Yeh et al., 2006). While cytotoxic reactive intermediates can directly kill invading pathogens, nitric oxide is also a signalling molecule. An elevated NO concentration can be detected by receptors, leading to signalling cascades that enhanced antimicrobial peptide expression profiles (Nappi et al., 2000).

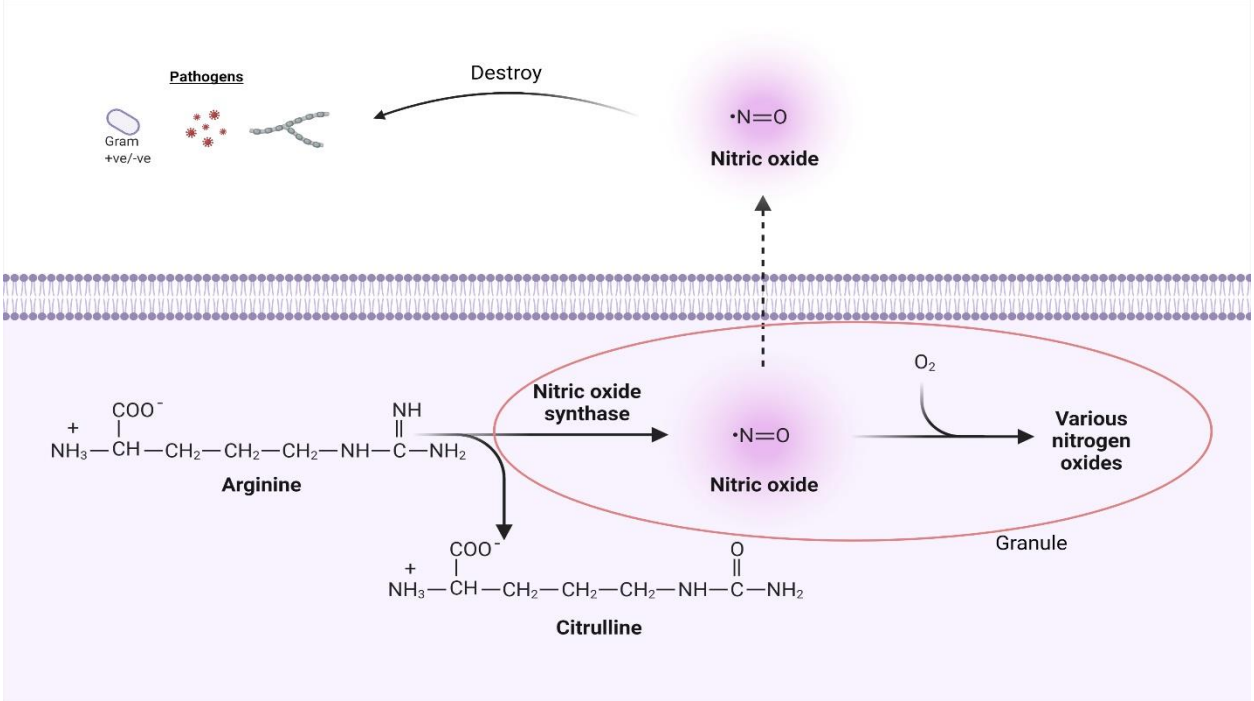


Figure 12. The production of nitric oxide through nitric oxide synthases stored in granules and released during respiratory bursts to destroy invading pathogens. Figure produced in Biorender.

D) Phenoloxidase

The pro-phenoloxidase activating system is critical to crustacean immunity (Kulkarni et al., 2020). The pro-phenoloxidase zymogen is stored in densely packed eosinophilic hemocyte granules. Degranulation and activation to phenoloxidase leads to melanization and destruction of invading pathogens through toxic intermediates. During infection, PAMPs recognized by PRRs triggers the activation cascade of serine proteinases which subsequently cleave proPO-activating enzyme (proPPAE) to active proPO-activating enzyme (PPAE). The inactive prophenoloxidase (proPO) zymogen is then cleaved to active phenoloxidase (PO) by PPAE. Phenoloxidase is a type 3- copper protein that oxygenates monophenols to o-diphenols and o-quinones which cross-link neighboring molecules to form melanin around invading pathogens (Cerenius et al., 2004). The phenoloxidase cascade shares a proteolytic enzyme with the Toll signalling cascade

suggesting that the two responses can be activated simultaneously (Figure 13) (Kulkarni et al., 2020).

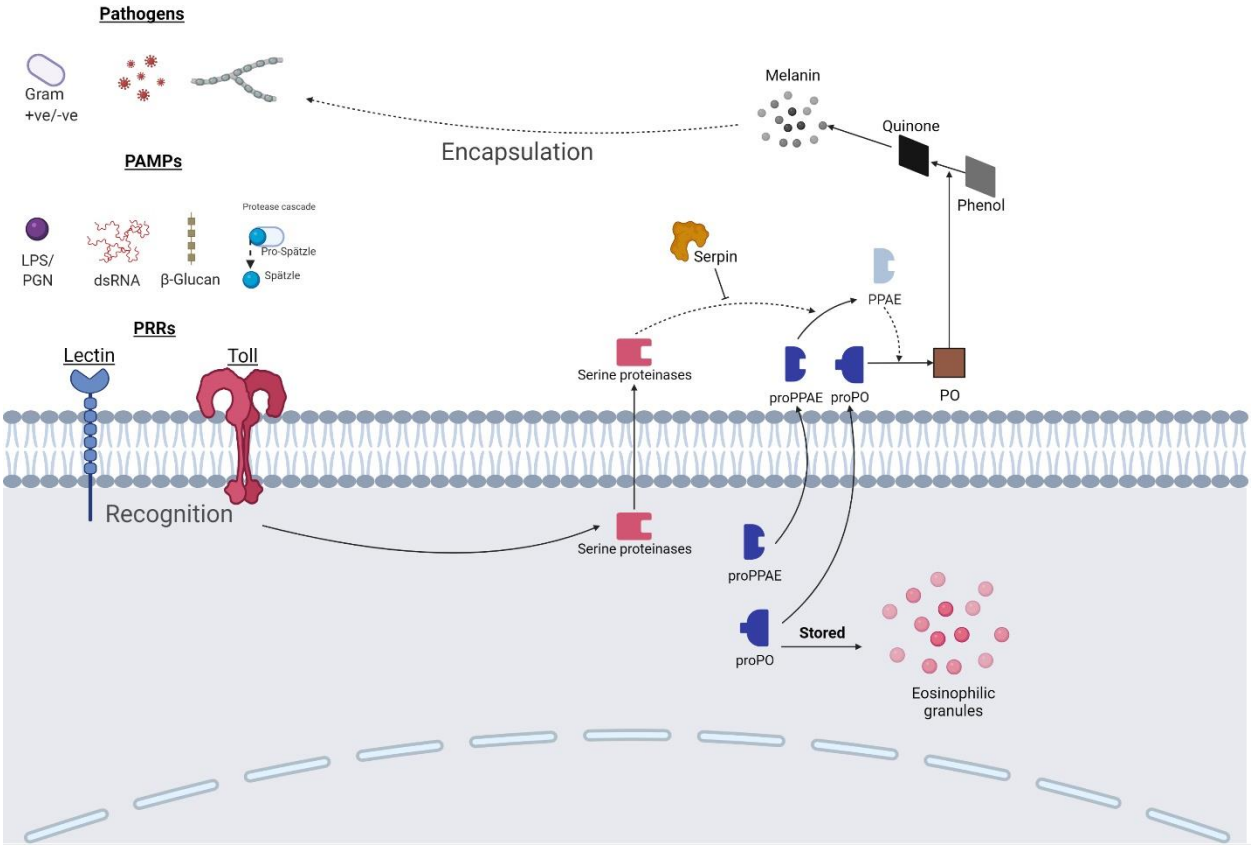


Figure 13. The phenoloxidase cascade. PAMP recognition activates serine proteinases which cleave proPPAE to PPAE and activate the ProPO zymogen leading to melanization. ProPO is also stored in eosinophilic granules to be deployed during degranulation. Figure produced in Biorender and adapted from Kulkarni et al., 2020.

1.5 Research objectives and hypotheses

The purpose of this research project was to determine if dsRNA and PACAP can be used as broad spectrum immunostimulants and antiviral agents to induce immune responses in crustaceans. dsRNA and PACAP safety was first assessed *in vitro* in cultured hemocytes. The impact of *in vivo* treatment with dsRNA and PACAP was measured through a functional analysis of cellular and humoral immune responses. It was hypothesized firstly that Poly(I:C) HMW and PACAP would not be cytotoxic, secondly that intramuscular and ventral sinus injection of crayfish with dsRNA and PACAP would stimulate immunity and finally that this response could be quantified through an increase in the total number of hemocytes as well as through increased lectin activity, nitric oxide metabolite concentration and activated phenoloxidase function.

Chapter 2. Materials and Methods

2.1 Pituitary adenylate cyclase activating polypeptide

Clarias gariepinus synthetic PACAP-38 (HSDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK, with a MW of 4.7 kDa) was purchased from CS Bio (Shanghai) Ltd, China with 85% purity. PACAP was diluted and aliquoted to 2mM stock solution in phosphate buffered saline (Hyclone) and stored at -80°C until used.

2.2 Synthetic dsRNA

A synthetic dsRNA – poly(I:C) HMW (high molecular weight) – (Catalog# tlrl-pic, HMW), was obtained from InvivoGen and resuspended at 1mg/mL in molecular biology grade water according to the manufacture's recommendations. The poly(I:C) HMW aliquots were stored at -20ºC until used.

2.3 *In vitro* stimulation of primary hemocytes

Primary hemocyte collection and culture techniques

a) Media

Crayfish media was prepared by supplementing Leibovitz's L-15 media (Hyclone) with 10% V/V fetal bovine serume (FBS, Corning), 1% V/V penicillin/ streptomycin (10mg/mL streptomycin and 10000 U/mL penicillin, Thermo Fischer Scientific), 1% V/V polyamine (Sigma Aldrich) and 1% V/V Primocin (InvivoGen).

b) Cell collection

Primary hemocytes were extracted from crayfish through the ventral sinus using a 20-gauge needle and 2mL syringe loaded with 300µL of ice-cold anticoagulant (0.45M NaCl, 0.1 M glucose, 60mM trisodium citrate, 26mM HCl, 10mM EDTA in water, pH 5.0). The hemocyte-

anticoagulant mixture was added to a 15mL centrifuge tube (BD Falcon), and the volume was topped up to 5mL with crayfish media.

Tail muscle extract (TME) was prepared by harvesting and freezing tail muscle from 15-20 crayfish. The frozen tail muscle was cut into small (2-5mm) pieces using scissors. The cut tissue was then transferred to 1.5 mL Sterile Screw Cap MicroTubes with Conical Base and O-Ring Cap (Sarstedt, Newton, NC) and zirconium oxide beads were added. Samples were then homogenized using a Fisherbrand™ Bead Mill 24 Homogenizer at 6400rpm for 2-4 cycles of 15 seconds until all muscle pieces disappeared. The homogenized tissue was then left at 4ºC overnight for bubbles to settle. The homogenized tissue samples were then combined and added to 15mL centrifuge tubes (BD Falcon) and centrifuged at 2.0 RCF for 6 minutes. The supernatant was then filter sterilized by passing twice through a 0.45µm syringe filter into a new 15mL centrifuge tube (BD Falcon), centrifuging at 2.0 RCF for 6 minutes between each filtration. The contents were once again centrifuged at 2.0 RCF for 6 minutes before passing through a 0.22µm syringe filter into a sterile 15mL tube. The TME was then stored at 4ºC.

Hemocytes were isolated by centrifugation at 0.4 RCF for 4 min. The supernatant was then removed, and cell pellet was resuspended in crayfish media supplemented with 10% V/V crayfish tail muscle extract (TME).

c) Cell plating

A 96 well plate was coated with Poly-L-Lysine (Sigma Aldrich) by adding 100µL to each well and leaving for 1 hour before removing the solution and letting the plate air dry for 1 hour. The number of cells in the resuspended cell pellet (**section 2.3 b**) was counted using a Neubauer hemocytometer (Hausser Scientific) and cells were plated in the Poly-L-Lysine coated 96 well plate (BD Falcon) at 25,000 cells/well.

2.4 Determination of cell viability following exposure to PACAP and poly(I:C) HMW

Cells were incubated for 24 hours after plating before treating with PACAP and Poly(I:C) HMW. Cells were then stimulated by spiking in PACAP at final concentrations from 1.95nM to 250 nM and Poly(I:C) HMW at concentrations between 0.078µg/mL to 10µg/mL resuspended in full media. Cell viability was measured following 24 hours of treatment. According to manufacturer instructions, a solution containing 10 µL of PBS (Hyclone) and 5% (V/V) alamarBlue (Thermo Fisher Scientific) and 0.05% (V/V) CFDA-AM (Invitrogen) was added to each well. Following incubation in the dark for 1hr at 20°C, fluorescence was measured for alamarBlue (Ex 530 / Em 590 nm) and CFDA-AM (Ex 485 / Em 528 nm) using a Synergy HT plate reader (BioTek). Relative fluorescent units were normalized to the untreated (media) control wells. The fluorescent indicator dye, alamarBlue (Invitrogen, Carlsbad, Ca, USA), was utilized to measure cell metabolism, while the fluorescent indicator dye, CFDA-AM (ThermoFisher), was utilized to measure cell membrane integrity.

2.5 Local Ontario crayfish husbandry

The local Ontario crayfish (5.22 ± 1.68 g) used in all experiments were collected from the Grand River (Waterloo, ON, Canada). The river was accessed via the Claude Dubrick Trailway located at 546 Woolwich Pl (43°30'22.9"N 80°29'40.4"W) in Waterloo. Crayfish were transported to Wilfrid Laurier University's animal care facility where they were maintained in glass aquariums (Aqueon) containing 75L of aerated reverse osmosis fresh water at a temperature matching the river, 19ºC, with continuous circulation and charcoal filtration (Aqueon). Prior to arrival, tanks were sterilized with bleach and new filters were installed. Each day water quality was checked to ensure a constant temperature of 19 ºC, a % dissolved oxygen (DO) of 80% and water pH of 7-7.5. 50% water changes were performed every 4 days. Only

crayfish of similar markings and size were selected from the environment and randomly distributed between tanks. Territorial aggression between animals was eliminated by providing each crayfish with a shelter made of 1 in. x 4 in. pieces of PVC pipe. Crayfish were fed once daily with commercial sinking feed that contained 38% protein, 10% fat, 2% fiber and 8% ash (Omega One). Each crayfish received ~275 mg of feed per day. Prior to experimental use, crayfish were acclimated to the new environmental conditions for a minimum of one week and up to three weeks.

2.6 Treatment formulation for intramuscular and ventral sinus injection

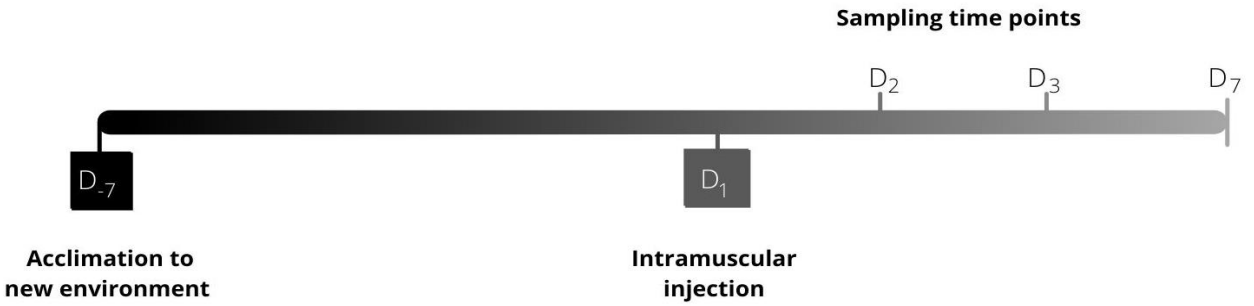
In all injection trials, PACAP stock was diluted in phosphate buffered saline (Hyclone) to a concentration low dose (6µg) or high dose (12µg) per crayfish. Poly(I:C) HMW (InvivoGen) was also diluted in PBS (Hyclone) to a concentration of 10µg per crayfish. For the combination treatment, the cationic PACAPwas combined with the Poly(I:C) HMW which contains a negatively charged phosphate backbone. The dilutions of 12µg/ individual of PACAP and 10µg/ individual of Poly(I:C) HMW were combined and allowed to sit for 10 minutes prior to treatment. Treatments were dispensed using 31-gauge 1mL insulin syringes (Ultra-Fine™, BD).

2.7 Intramuscular injection trial #1

Seventy-two crayfish were acclimated for one week at a stocking density of eighteen crayfish per 75L tank in the animal care facility at Wilfrid Laurier University as described in section **2.5 Local Ontario crayfish husbandry**. Following the acclimation period, all animals were removed from the tanks and a full water change, tank cleaning and filter change was performed prior to beginning treatments. Animals were randomized into four treatment tanks labelled as Mock (PBS), poly(I:C) HMW (10µg), PACAP (6µg) and PACAP (12µg). The treatments were administered by intramuscular injection between the third and fourth abdominal

segment, where each animal received 20µL of the mock (PBS) or a treatment. Six crayfish from each tank were sampled at 24-, 48- and 168 hours post injection. (Figure 14. a, b). At each time point, hemolymph was extracted from the ventral sinus using a 20-gauge needle and 2mL syringe loaded with 300µL of ice-cold anticoagulant (0.45M NaCl, 0.1 M glucose, 60mM trisodium citrate, 26mM HCl, 10mM EDTA in water, pH 5.0), hemocytes were separated from the hemolymph by centrifugation at 3850 rpm for 10 min at 4°C and samples were stored at -20ºC. Portions of the gill, heart and hepatopancreas were taken from each animal and stored in 300 µL of RNAlater (Invitrogen) at -20ºC.

a)



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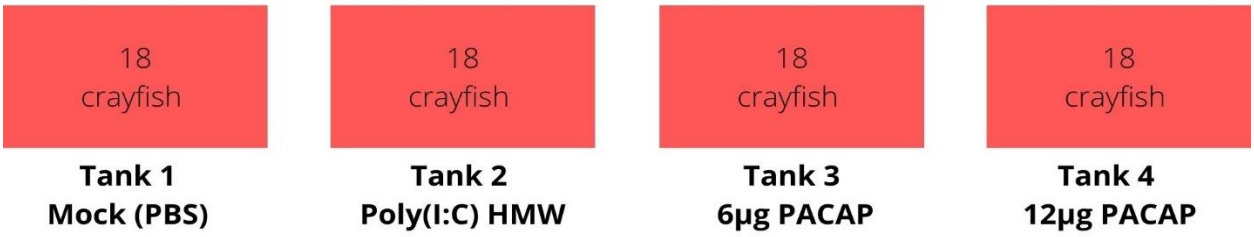
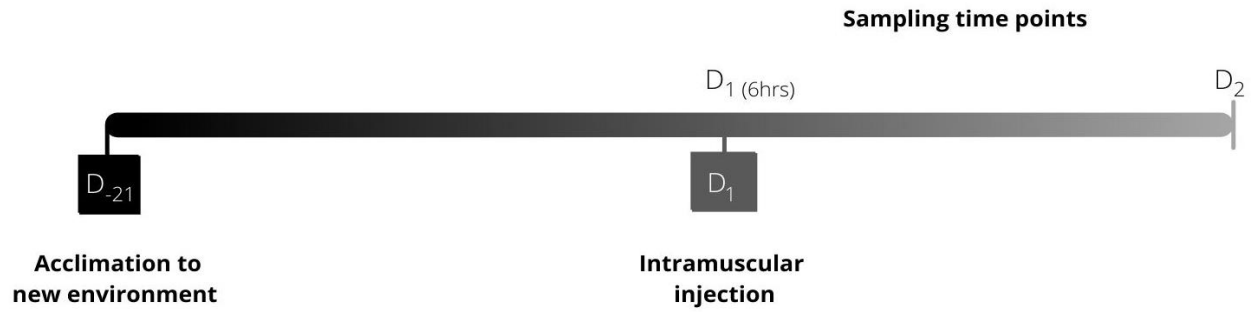


Figure 14. a) Schematic of the first intramuscular injection trial. Crayfish were acclimated to the new environment one week prior to intramuscular injection with mock (PBS), 10µg Poly(I:C) HMW, 6µg PACAP or 12µg PACAP. Six crayfish were sampled at each time point per tank at 24, 48, and 168 hours (days 2, 3 and 7) post injection. **b)** Representation of the tank set up utilized in the first intramuscular injection trial. Four tanks each housed 18 crayfish for the duration of the experiment labelled as mock (PBS), Poly(I:C) HMW, 6µg PACAP and 12µg PACAP.

2.8 Intramuscular injection trial #2

The results from the intramuscular injection trial #1 indicated that cell and humoral responses were impacted by treatment time as markers elevated at 24 hours decreased over time. A 6-hour time point was chosen to detect respiratory bursts and degranulation which may occur rapidly following detection of a stimulant. Additionally, there was substantial variation in the data that could be attributed to different individuals still acclimating to the new environment. This effect was reduced by housing and monitoring the crayfish until the population stabilized. This resulted in a three-week acclimatization period following collection. Forty-eight crayfish were acclimated for three weeks at a stocking density of twelve crayfish per tank in the animal care facility at Wilfrid Laurier University as described in section **2.5 Local Ontario crayfish husbandry**. Following the acclimation period, all animals were removed from the tanks and a full water change, filter change and tank cleaning was performed. Animals were randomized into four treatment tanks labelled as Mock (PBS), poly(I:C) HMW (10µg), PACAP (6µg) and PACAP (12µg). The treatments were administered by intramuscular injection between the third and fourth abdominal segment, where each animal received 20µL of the mock or a treatment. Six crayfish from each tank were sampled at 6- and 24- hours post injection (Figure 15 a, b). At each time point, hemolymph was extracted from the ventral sinus using a 20-gauge needle and 2mL syringe loaded with 300µL of ice-cold anticoagulant (0.45M NaCl, 0.1 M glucose, 60mM trisodium citrate, 26mM HCl, 10mM EDTA in water, pH 5.0), hemocytes were separated from the hemolymph by centrifugation and samples were stored at -20ºC. Portions of the gill, heart and hepatopancreas were taken from each animal and stored in 300 µL of RNAlater (Invitrogen) at -20ºC.

a)



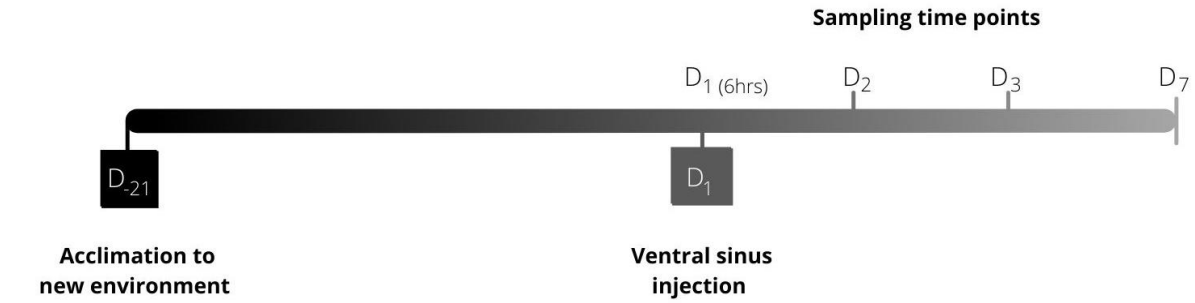
b)

Figure 15. a) Schematic of the second intramuscular injection trial. Crayfish were acclimated to the new environment three weeks prior to intramuscular injection with mock (PBS), 10µg Poly(I:C) HMW, 6µg PACAP or 12µg PACAP. Six crayfish were sampled at each time point per tank at 6 and 24 hours (days 1 and 2) post injection. **b)** Representation of the tank set up utilized in the second intramuscular injection trial. Four tanks each housed 18 crayfish for the duration of the experiment labelled as mock (PBS), Poly(I:C) HMW, 6µg PACAP and 12µg PACAP.

2.8 Ventral sinus injection trial

Three-hundred and twenty crayfish were acclimated for three weeks in the animal care facility at Wilfrid Laurier University as described in section **2.5 Native Ontario crayfish husbandry**. The crayfish were maintained at a stocking density of forty crayfish per 75 L tank. Daily monitoring confirmed that the population stabilized quickly to the new environment with no evidence of aggression or stress caused by a higher stocking density. Following the acclimation period, all animals were removed from the tanks and a full water change, filter change and tank cleaning was performed. Animals were randomized into two replicate tanks of four treatments labelled as Mock (PBS), poly(I:C) HMW (10µg), PACAP (12µg) and PACAP (12µg) +HMW (10µg) combination, eight tanks total. The treatments were administered by injection directly into the ventral sinus, where each animal received 100µL of the mock or a treatment. The volume increased from 20 µL to ensure that each animal was receiving a consistent quantity of the treatment and to minimize losses during administration. Eight crayfish from each tank and replicate were sampled at 6-, 24-, 48- and 168- hours post injection for a total of sixteen animals per time point and treatment (Figure 16 a, b). As previously described, at each time point, hemolymph was extracted from the ventral sinus using a 20-gauge needle and 2mL syringe loaded with 300µL of ice-cold anticoagulant (0.45M NaCl, 0.1 M glucose, 60mM trisodium citrate, 26mM HCl, 10mM EDTA in water, pH 5.0), hemocytes were separated from the hemolymph by centrifugation and samples were stored at -20ºC. The sex and weight of each crayfish was recorded and portions of the gill, heart and hepatopancreas were taken from each animal and stored in 300 µL of RNAlater (Invitrogen) at -20ºC.

a)



b)

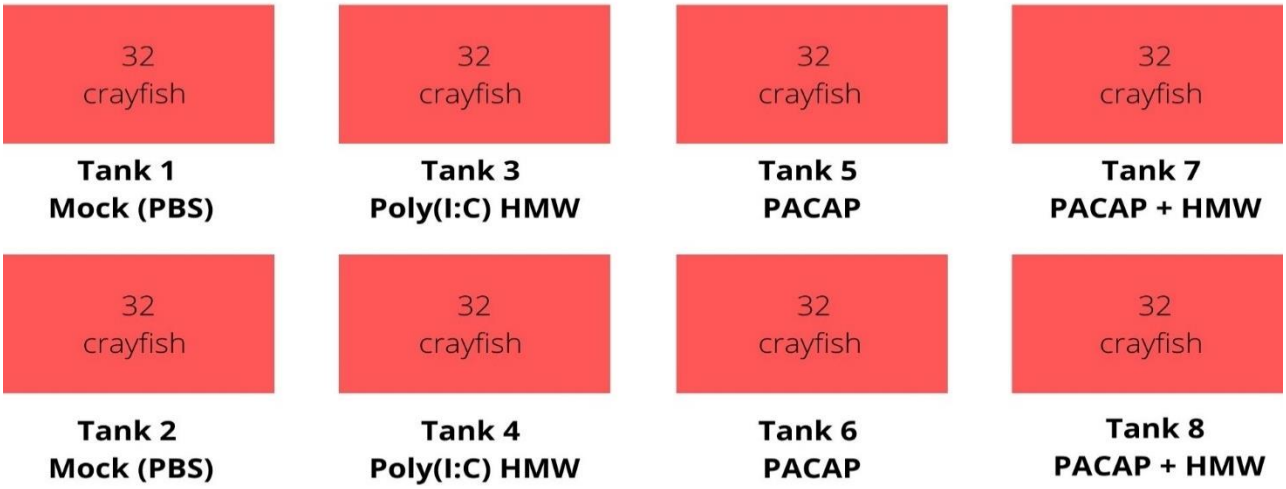


Figure 16. a) Schematic of the ventral sinus injection trial. Crayfish were acclimated to the new environment for three weeks prior to ventral sinus injection with mock (PBS), 10µg Poly(I:C) HMW, 12 µg PACAP or 12µg PACAP + 10µg HMW combination. 8 crayfish were sampled at each time point per tank at 6, 24, 48 and 168 hours (days 1, 2, 3 and 7) post injection. **b)** Representation of the tank set up utilized in the ventral sinus injection trial. Eight tanks each housed 32 crayfish for the duration of the experiment and two replicate tanks were included per treatment labelled as mock (PBS), Poly(I:C) HMW, PACAP or PACAP + HMW combination.

2.9 Preparation of hemolymph

Hemolymph was extracted with 300µL of ice-cold anticoagulant (0.45M NaCl, 0.1 M glucose, 60mM trisodium citrate, 26mM HCl, 10mM EDTA in water, pH 5.0). A volume of 25µL was reserved for determination of the total hemocyte count, either counted immediately or fixed with formalin and stored for later counting. The remaining hemocytes were isolated through centrifugation at 3850 rpm for 10 min at 4°C. Samples were then stored at -20ºC until use.

2.10 Total hemocyte count

i. Formolized hemocytes were prepared by combining 25 µL of hemolymph with 75 µL Alsever's solution (113 mM glucose, 27.2 mM sodium citrate, 2.8 mM citric acid, 71.9 mM sodium chloride, 4% formalin). The mixture is refrigerated at 4 °C for up to three weeks. The total number of hemocytes was determined using a Neubauer hemocytometer (Hausser Scientific) where 10 µL of the sample was added to the chamber. Total number of hemocytes was calculated using the following equation:

THC= (*Total cells counted x Dilution factor x 10,000 cells/ml*) */ Number of squares counted*

ii. For immediate counting, 10 µL of extracted hemolymph and anticoagulant were mixed with 10 µL of Trypan Blue (Invitrogen). Number of cells/mL was measured using the Countess II Automated Cell Counter (Invitrogen). Total number of hemocytes was calculated using the following equation:

THC= (*Number of cells/mL*) * (*mL of hemolymph collected*)

2.11 Functional analysis of lectin activity by determination of the hemagglutination titer:

Rabbit erythrocytes were used for determination of the lectin activity in the hemolymph. Blood was drawn from the ears of two rabbits and collected in 3mL heparin (blood was obtained through Martin Ryan at the University of Waterloo Animal Care Facility). The red blood cell suspension was treated with formaldehyde for storage and use with all samples. Five mL of blood was centrifuged at 2000xG for 10 minutes at 4°C. Plasma was discarded and cells were washed four times by adding 10mL of PBS (Hyclone) and centrifuging at 2000xG for 10 minutes at 4ºC. A suspension of 10% RBC in PBS was prepared by combining 500µL of washed erythrocytes with 5mL of PBS (Hyclone). The cell suspension was then mixed with 5mL of 3% formaldehyde (Sigma-Aldrich). The formolized RBC preparation was then incubated overnight at 37ºC in a water bath. Following overnight incubation, red blood cells were washed four times by adding 10mL of PBS (Hyclone) and centrifuging for 10 minutes at 4ºC. A 4% RBC suspension was then prepared in PBS and sodium azide was added to a final concentration of 0.02%.

To determine hemagglutination activity in the samples, 96 well U-bottom plates were used (Thermo Fisher Scientific). A doubling serial dilution (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64) of hemolymph (described in section **2.9 preparation of hemolymph**) was performed in phosphate buffered saline (Hyclone) to obtain 20µL of the sample or dilution in each well. Negative control wells were composed of 20 µL of PBS (Hyclone). Twenty µL of the formolized RBC suspension was then added to each well and samples were incubated for 2 hours at room temperature (21°C). The titer was determined visually, and its value corresponds to the reciprocal of the highest diluted sample showing hemagglutination.

2.12 Determination of nitric oxide metabolite (nitrate and nitrite) concentration

The concentration of nitric oxide metabolites was determined in the hemolymph samples using 96 well plates (BD Falcon). Chemical reagents were prepared to the following concentrations in milliQ water: hydrazine sulfate (Sigma Aldrich) at 3.605mg/mL, sodium hydroxide (Sigma Aldrich) at 14.17mg/mL, copper sulfate (Sigma Aldrich) at 0.1 mg/mL and phenol (Sigma Aldrich) at 0.0376 mL/mL. Griess reagent A was prepared as 2% sulfanilamide (Sigma Aldrich) and 5% phosphoric acid (Sigma Aldrich) in milliQ water. Griess reagent B was prepared as 0.2% N-(1-Naphthyl ethylenediamine) (Sigma Aldrich) in milliQ water. Sodium nitrate (Sigma Aldrich) and sodium nitrite (Sigma Aldrich) were prepared at a concentration of 250µM for use in the standard curve.

The nitric oxide metabolites were converted to nitrites and the concentration determined based on their reaction with the Griess reagents. This generates a purple-azo dye which can be monitored by spectrophotometer at 540nm. One-hundred µL of hemolymph samples (described in section **2.9 preparation of hemolymph**) were added to a 96 well plate (BD falcon) at a 1:1 and 1:10 dilution in phosphate buffered saline (Hyclone). A 2-fold serial dilution (from 250µM to 3.9µM) of 100µL of sodium nitrate and sodium nitrite in PBS (Hyclone) was used to create the standard curve. The reaction mixtures were then prepared by mixing V/V hydrazine sulfate and copper sulfate and V/V sodium hydroxide and phenol (Rodriguez-Ramos et al., 2016). Forty µL of the phenol and sodium hydroxide mixture was then added to each well of the sample and the nitrate standard, and 20µL of the hydrazine sulfate and copper sulfate mixture was added to each well of sample and the nitrate standard. The nitrite standard curve was included to monitor reduction efficiency, therefore, 60µL of milliQ water was added to these wells to supplement the reaction mixture. Plates were then covered with parafilm and incubated at 37ºC for 2 hours.

Griess A and Griess B were combined V/V and protected from light. Following incubation, 40µL of the Griess A and B mixture was added to all samples as well as the nitrate and nitrite standards. The plate was incubated in the dark for 5 minutes before reading the optical density at 540nm using the Synergy HT plate reader (BioTek). The concentration of nitric oxide metabolites in the samples was determined using the standard curves.

2.13 Determination of activated phenoloxidase function in the hemolymph

To determine activated phenoloxidase in the hemolymph samples, L-Dopa (3, 4-dihydroxy-L-phenylalanine) (Sigma Aldrich) was prepared at a concentration of 1 mg/mL in milliQ water and protected from light. Ten µL of hemolymph (described in section **2.9 preparation of hemolymph**) was added in triplicate to a 96 well plate (BD Falcon). Two-hundred and fifty µL of the prepared L-Dopa (Sigma Aldrich) was added to each well. The samples were then protected from light and incubated for 1 hour at room temperature (21°C). The optical density was recorded at 490nm using the Synergy HT plate reader (BioTek). The phenoloxidase activity in the samples was determined as % of the mock (PBS) control.

2.14 Statistical Analysis

All statistical analyses were performed using STATISTICA v7.0 (StatSoft software, Tulsa, USA) and graphs performed using version 9 of GraphPad Prism (GraphPad Software, La Jolla California USA). All data from the first and second intramuscular injection trial and the ventral sinus injection trial were analyzed using a two-way ANOVA using the different treatments and time-points as factors of variance followed by a Tukey *post-hoc* test to identify differences between groups. A star system was used to indicate the level of significance. Differences were considered significant at $p < .05$ (*), $p < .01$ (**) and $p < .001$ (***). Cell

viability data was analysed through a one-way ANOVA, using a Tukey's post hoc test to determine statistical significance between the treatment concentration.

Chapter 3. Results

3.1 Establishing primary crayfish hemocyte viability following 24 hours of exposure to Poly(I:C) HMW and PACAP

Primary hemocyte viability following 24 hours of *in vitro* exposure to Poly(I:C) HMW and PACAP was determined through an analysis of cellular metabolism and membrane integrity using the fluorescent indicator dyes alamarBlue and CFDA-AM. No treatment caused a significant difference in metabolism or membrane integrity compared to the control. Data is presented as % of untreated (media) control cells set to 100%. Cell metabolism was consistent at 100% of the control following treatment with PACAP between 1.95 and 250 nM (Figure 17. A) and Poly(I:C) HMW between 0.076 and 10 µg/mL (Figure 17. B). Membrane integrity was also not impacted significantly even at high concentrations of PACAP and Poly(I:C) HMW. While not significant, CFDA-AM fluorescent levels were elevated however, beyond 100% of the control and there was evidence of an inverted dose response. When treated with the lowest dose of PACAP (1.95 nM), membrane integrity was measured at 163.4% of the control, which then dropped to 118.3% with 31.25 nM before increasing to 145.58% with the highest dose of 250 nM (Figure 17. C). With Poly(I:C) HMW treatment, fluorescence was detected at 145.7% of the control with the lowest treatment dose (0.078 µg/mL), which decreased to 109.1% at 0.625 µg/mL, before increasing to 122.95% at 1.25 µg/mL and finally declining to 102.8% of the control at the highest dose of 10 µg/mL (Figure 17. D).

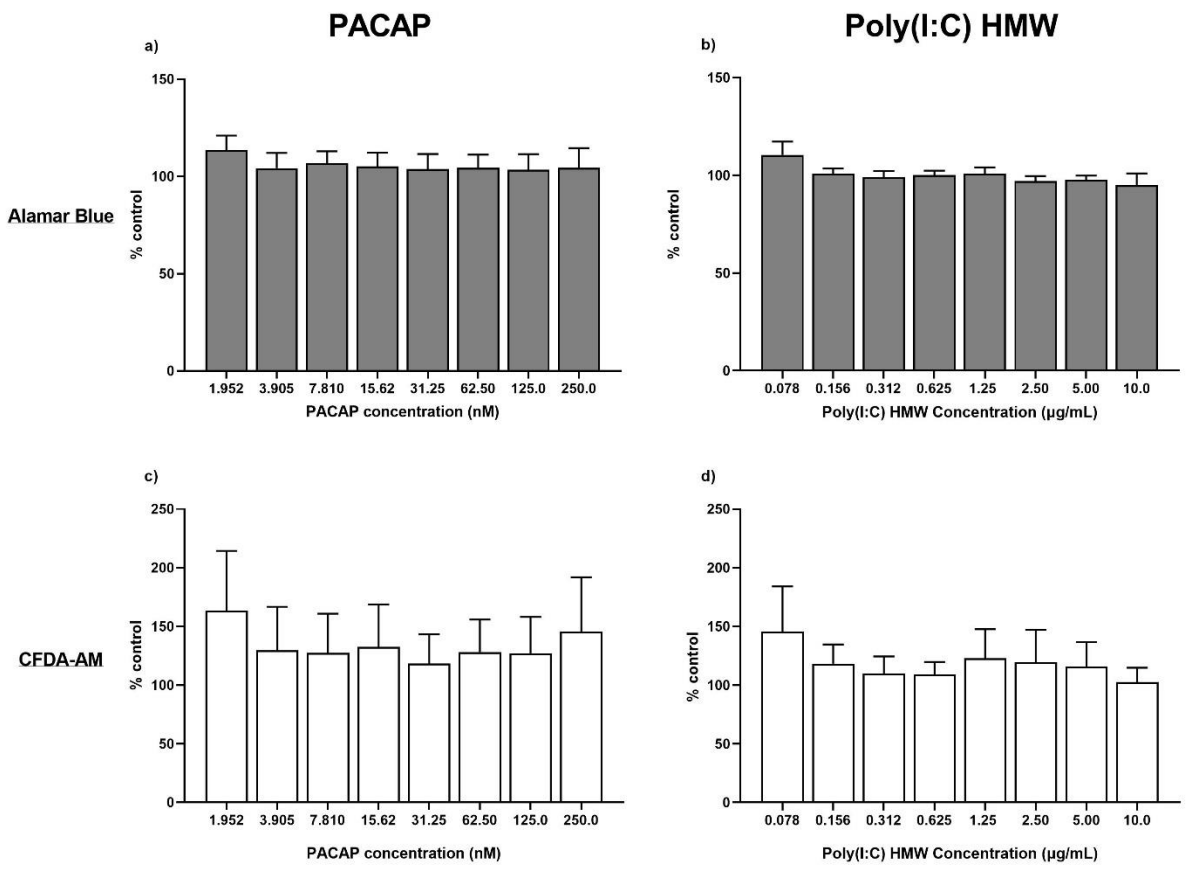


Figure 17. Viability of primary crayfish cells following 24 hours of treatment with either PACAP or Poly(I:C) HMW. Cellular metabolism following treatment a) PACAP and b) Poly(I:C) HMW was measured using alamarBlue cell viability reagent. Membrane integrity c) PACAP and d) Poly(I:C) HMW was determined using CFDA-AM. Data is presented as a % of untreated control cells set to 100%, and statistical differences were tested using a one-way ANOVA with a Tukey's post hoc test (N=3). All statistical analyses were performed using STATISTICA v7.0 (StatSoft software, Tulsa, USA) and graphs performed using GraphPad Prism (GraphPad Software, La Jolla California USA).

3.2 Intramuscular injection trial #1: Functional analysis of cellular and humoral immune responses in crayfish at 24, 48 and 168 hours following intramuscular injection with Poly(I:C) HMW and PACAP

Immune stimulation by Poly(I:C) HMW and PACAP was determined through the functional analysis of cellular and humoral defense systems. The total hemocyte count, lectin, nitric oxide concentration and activated phenoloxidase levels were analyzed by two-way ANOVA considering time and treatment as factors of variance (Figure 18). While there were no statistically significant differences, the results still warrant consideration as they are indicative of an association between treatment and response, indicative of immune activation. These are summarized in Table 2. Following intramuscular injection and treatment for 24, 48 and 168 hours, the number of hemocytes were counted and hemolymph was analyzed to determine lectin activity, nitric oxide metabolite concentration and activated phenoloxidase function. The sex of the animals was not recorded; however, each individual is represented demonstrating high variability following only one week of acclimatization (Figure 18. A). At 24 hours post injection, crayfish treated with 12µg of PACAP had a higher number of hemocytes compared to the average number of hemocytes in crayfish from the mock (PBS), Poly(I:C) HMW (10µg) and PACAP (6µg) treatment groups. The number of hemocytes in the PACAP 12µg treatment group then declined between 48- and 168-hours post injection (Figure 18. A). Treatment with Poly(I:C) HMW resulted in an opposite trend as, compared to the control, the number of hemocytes only started to increase between 48- and 168-hours post injection (Figure 18. A). While treatment with 6µg of PACAP did not result in any differences in the number of hemocytes, it was effective at increasing the hemolymph lectin activity (Figure 18. B). The lectin activity in the PACAP (12µg) group peaked at 48 hours but the lower dose of PACAP resulted in a sustained increase in lectin activity between 24- and 168-hours post injection (Figure 18. B). Poly(I:C) HMW treatment did not cause any

changes in lectin activity between 24 and 168 hours (Figure 18. B). No significant differences were detected in the hemolymph concentration of nitric oxide metabolites but there was a notable decrease in concentration detected in all samples between 48- and 168- hours (Figure 18. C). Phenoloxidase in the hemolymph was detected through the catalytic conversion of L-Dopa to dopachrome. The results showed almost no differences in the treatment groups compared to the control at 24- and 48-hours post injection. Treatment for 168 hours however with both 6µg and 12µg of PACAP effectively elevated phenoloxidase activity beyond 150% of the control (Figure 18. D).

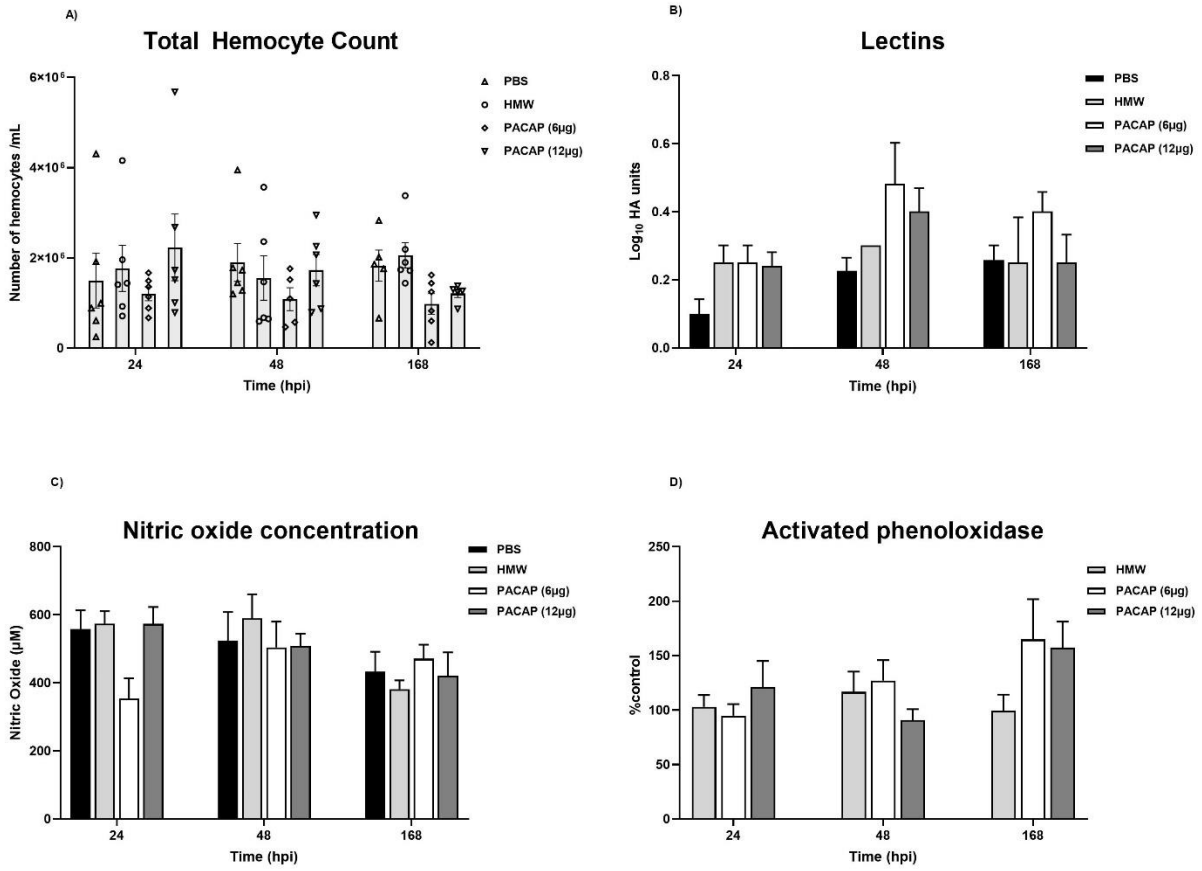


Figure 18. Functional analysis of cellular and humoral immune markers in crayfish hemolymph at 24, 48 and 168 hours following intramuscular injection with mock (PBS), Poly(I:C) HMW, 6µg PACAP and 12µg PACAP. A) total hemocyte count B) hemagglutination activity of lectins C) nitric oxide concentration D) activated phenoloxidase function. All data are shown as the mean ± standard error (SE) (N=6). No significant differences were detected in any of the data. Assumptions of variance, normality, and homogeneity were tested. A two-way ANOVA was performed using the different treatments and time-points as factors of variance, followed by a Tukey *post-hoc* test to identify differences between groups. All statistical analyses were performed using STATISTICA v7.0 (StatSoft software, Tulsa, USA) and graphs performed using GraphPad Prism (GraphPad Software, La Jolla California USA).

Table 2. Summary table of results from intramuscular injection trial #1 for total hemocyte count (THC), Lectin, nitric oxide concentration (NO) and activated phenoloxidase (PO).

	24 hours				48 hours				168 hours			
	THC	Lectin	NO	PO	THC	Lectin	NO	PO	THC	Lectin	NO	PO
Poly(I:C) HMW		√			X				√			
PACAP (6µg)	X	√	X		X	√			X	√		√
PACAP (12µg)	√	√			X	√			X			√

In the table above, a check mark (√) indicates a trend that the response was induced compared to mock (PBS) at each time point and treatment. An X indicates a trend that the response was lower than the mock (PBS) treatment. No symbol is indicative of no trend in the data compared to the mock (PBS).

3.3 Intramuscular injection trial #2: Functional analysis of cellular and humoral immune responses in crayfish at 6 and 24 hours following intramuscular injection with Poly(I:C) HMW and PACAP

The preliminary intramuscular injection trial supported the inclusion of an earlier time point as immune markers that were elevated at 24 hours decreased over time. Therefore, following intramuscular injection with Poly(I:C) HMW and PACAP, crayfish were collected at 6 and 24 hours post treatment. Immune stimulation was determined by counting the number of hemocytes and completing a functional analysis of the hemolymph to determine lectin activity, nitric oxide metabolite concentration and activated phenoloxidase function. The data was once again analyzed by two-way ANOVA considering time and treatment as factors of variance with no statistically significant differences detected (Figure 19). While the findings were not statistically significant, they warrant consideration as they indicate biologically important associations between treatment and response which could be indicative of immune stimulation. These results are summarized in Table 3. Each individual crayfish is represented in the total hemocyte count data showing reduced variability in the control following 3 weeks of acclimatization (Figure 19. A). At 6 hours post injection, crayfish treated with Poly(I:C) HMW and both 6µg and 12µg of PACAP had a higher number of hemocytes compared to the average number of hemocytes in crayfish from the mock (PBS) treatment group. The number of hemocytes detected in all treatment groups dropped considerably at 24 hours, with the PACAP 12µg treatment group exhibiting the steepest decline (Figure 19. A). The hemolymph lectin activity peaked similarly at 6 hours in all treatment groups compared to the control. While treatment with Poly(I:C) HMW and 12 µg of PACAP resulted in the highest levels of detected lectin activity, these treatments were also characterized by a decline at 24 hours. The response to treatment with 6µg of PACAP was distinct as the elevated lectin

activity was sustained from 6- to 24-hours post injection (Figure 19. B). The hemolymph concentration of nitric oxide metabolites was only slightly elevated in the PACAP treatment groups compared to the control. There was, however, an elevated level detected in the Poly(I:C) HMW treated animals at 6 hours post injection which then decreased at 24 hours (Figure 19. C). The function of activated phenoloxidase in the hemolymph of the Poly(I:C) HMW treatment group followed the opposite trend as levels elevated to 150% of the control at 6 hours post injection and increased to almost 200% of the control at 24 hours post injection. PACAP 6μg did not result in any elevated enzyme function compared to the control but the higher dose resulted in a 200% increase at 6 hours which then decreased to approximately 150% at 24 hours post injection (Figure 19. D).

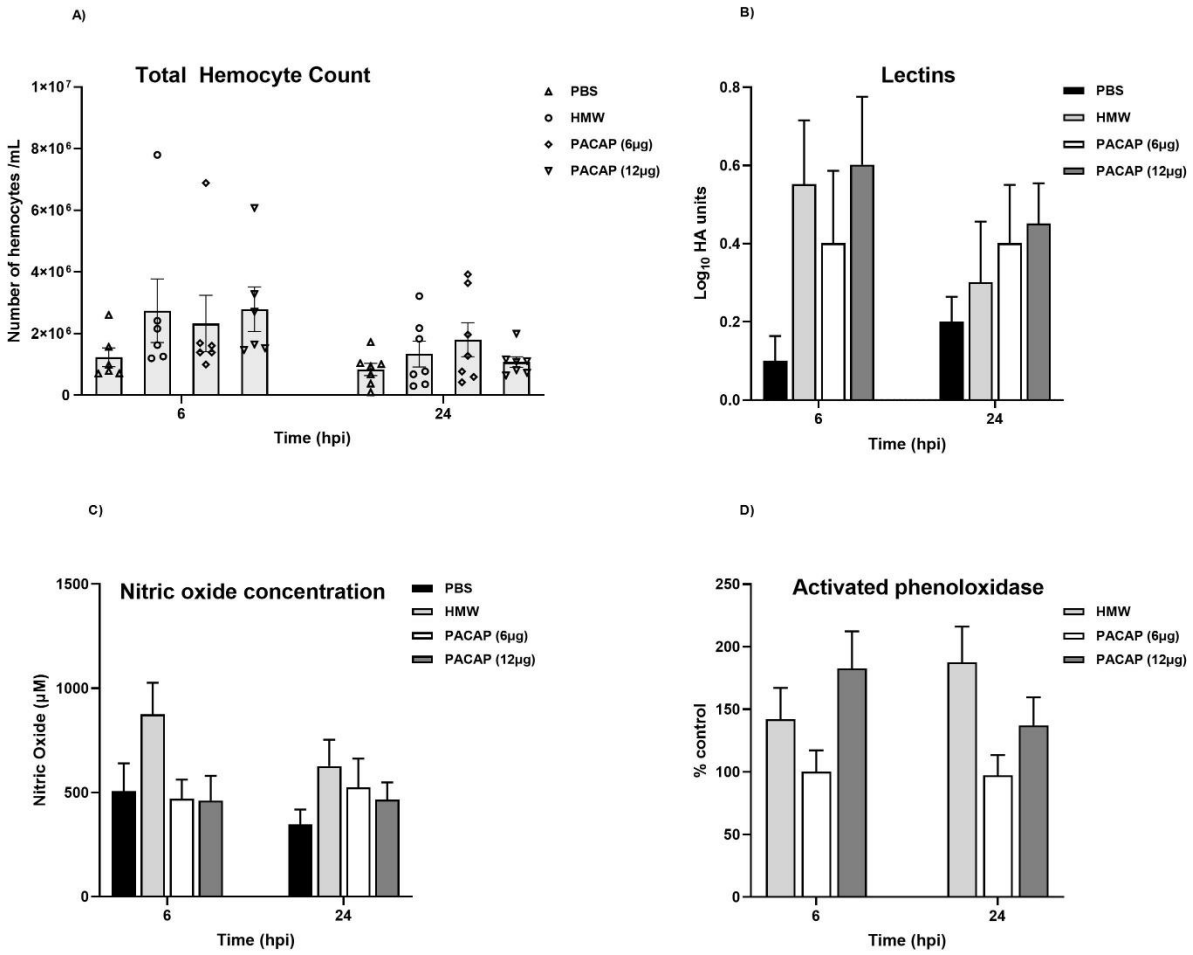


Figure 19. Functional analysis of cellular and humoral immune markers in crayfish hemolymph at 6 and 24 hours following intramuscular injection with mock (PBS), Poly(I:C) HMW, 6µg PACAP and 12µg PACAP. A) total hemocyte count B) hemagglutination activity of lectins C) nitric oxide concentration D) activated phenoloxidase function. All data are shown as the mean ± standard error (SE) (N=6). No statistically significant differences were detected in any of the data. Assumptions of variance, normality, and homogeneity were tested. A two-way ANOVA was performed using the different treatments and time-points as factors of variance, followed by a Tukey *post-hoc* test to identify differences between groups. All statistical analyses were performed using STATISTICA v7.0 (StatSoft software, Tulsa, USA) and graphs performed using GraphPad Prism (GraphPad Software, La Jolla California USA).

Table 3. Summary table of results from intramuscular injection trial #2 for total hemocyte count (THC), Lectin, nitric oxide concentration (NO) and activated phenoloxidase (PO).

	6 hours				24 hours			
	THC	Lectin	NO	PO	THC	Lectin	NO	PO
Poly(I:C) HMW	√	√	√	√			√	√
PACAP (6µg)	√	√			√	√		
PACAP (12µg)	√	√		√		√		√

In the above table, a check mark (√) indicates a trend that the response was induced compared to mock (PBS) at each time point and treatment. An X indicates a trend that the response was lower than the mock (PBS) treatment. No symbol is indicative of no trend in the data compared to the mock (PBS).

3.4 Ventral sinus injection trial: Functional analysis of cellular and humoral immune responses in crayfish following ventral sinus injection with Poly(I:C) and PACAP and treatment for 6-, 24-, 48- and 168-hours

The method of treating crayfish by intramuscular injection is common and has the advantage of ensuring consistent dose administration. This approach, however, requires maneuvering between the carapace to access protected abdominal tissue which negatively impacts the crayfish by causing static abdomen flexion following injection. Delivery of therapeutics through direct injection into the ventral sinus avoids stimulation of the flexion motor nerves and maintains consistency of dose administration (Xu et al., 2022, Larimer & Moore, 2003). Furthermore, due to an open circulatory system, ventral sinus injection can generate a more direct, robust, and systemic response. Crayfish were injected into the ventral sinus with either mock (PBS), 10µg Poly(I:C) HMW, 12µg PACAP, or a combination treatment of 10µg Poly(I:C) HMW and 12µg PACAP (PACAP + HMW). At 6-, 24-, 48- and 168-hours post injection 8 crayfish from each replicate tank, for a total of 16 individuals per treatment and time point, were sampled. Cellular and humoral immune pathways were evaluated through total hemocyte counts and the functional analysis of the hemolymph to determine lectin activity, nitric oxide metabolite concentration and activated phenoloxidase function. These results are summarized in Table 4.

At 24-, 48- and 168-hours post injection, no treatment had a significant impact on the number of hemocytes/mL. Statistical significance was calculated considering different treatments and time points as factors of variance. There were no significant differences detected between treatments in time. While the data is not significant, there were on average a higher number of hemocytes in Poly(I:C) HMW and PACAP treated crayfish at 24-, 48- and 168-hours post injection compared to the mock and PACAP+HMW. At 24 hours the mean number of hemocytes in the mock, Poly(I:C) HMW, PACAP and PACAP+HMW was 7.9 $x10^5$, 1.1 x 10^6, 8.4 x 10^5, and 7.3 x 10^5 respectively. At 48 hours, the mean number of hemocytes in the mock Poly(I:C) HMW, PACAP and PACAP+HMW was 8.6 $x10^5$, 1.0 x 10^6, 9.8 x 10^5, and 8.1 x 10^5 respectively. Finally, at 168 hours, the mean number of hemocytes in the mock Poly(I:C) HMW, PACAP and PACAP+HMW was 5.4 $x10^5$, 9.4 x 10^5, 7.5 x 10^5, and 6.2 x 10^5 respectively (Figure 20). Of the sixteen mock crayfish sampled at 24 hours, only two (one male and one female) had elevated hemocyte counts beyond the mean. Similarly, in the mock treatment at 168 hours there were five (two female and three male) individuals that were recorded with elevated cell counts. While a cluster of low hemocyte counts with few outliers was not observed in the mock treatment group at 48 hours, all treatments for 48 hours, except Poly(I:C) HMW, resulted in an increased average hemocyte count which then decreased at 168 hours post injection (Figure 20). The sex of the animal is represented in order to detect any apparent differences. An equal number of male (3) and female (3) were noted as the highest individual responders. Following 24 hours of HMW Poly(I:C) treatment, one male and one female had hemocyte counts of 1.96 x 10^6 and 1.98 x 10^6 respectively. Also at 24 hours, one female crayfish in the HMW+PACAP combination group had a recorded hemocyte count of 2.61 x 10^6. At 48 hours post injection one female crayfish in the Poly(I:C) HMW group had a hemocyte count of 2.23 x 10^6. Finally, in the PACAP treatment group two male

crayfish had elevated hemocyte counts of 1.82 x 10^6 and 1.86 x 10^6 at 48- and 168-hours post injection respectively (Figure 20). The number of hemocytes were not determined at 6 hours post injection because the reagents necessary for hemocyte preservation were unavailable.

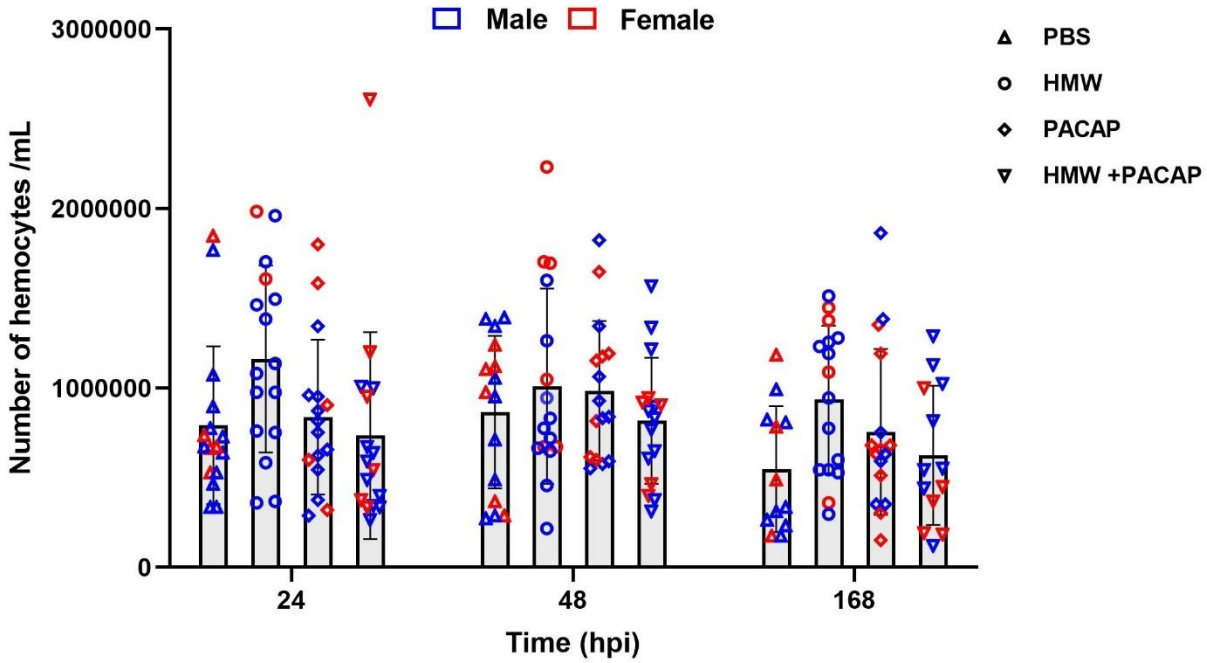


Figure 20. Total number of hemocytes determined in crayfish after 24, 48 and 168 hours of treatment via ventral sinus injection with 10 µg Poly(I:C) HMW, 12 µg PACAP and combination of PACAP+HMW. All data are shown as the mean ± standard error (SE) with individual data points for each crayfish (red indicating female and blue indicating male) (N=16). Assumptions of variance, normality, and homogeneity were tested. A two-way ANOVA was performed using the different treatments and time-points as factors of variance, followed by a Tukey *post-hoc* test to identify differences between groups. All statistical analyses were performed using STATISTICA v7.0 (StatSoft software, Tulsa, USA) and graphs performed using GraphPad Prism (GraphPad Software, La Jolla California USA).

The lectin activity of all treatment groups were significantly higher ($p < 0.001$) than the mock at 6 hours post injection. At 48 hours, treatment with Poly(I:C) HMW resulted in a significantly higher lectin titer ($p<0.05$) compared to the control. At 168 hours, Poly(I:C) HMW treatment also resulted in significantly higher lectin activity ($p<0.001$) compared to the control. Statistical significance was calculated using a two-way ANOVA with different treatments and time points as factors of variance. Considering the effects of time, there was a significant decline in the lectin activity caused by all treatments at 48 hours post injection. Poly(I:C) HMW treated animals exhibited this significant decrease only between 6- and 48-hours post injection ($p<0.05$). PACAP and PACAP + HMW combination treatments on the other hand resulted in a significant decrease in lectin activity between 24- and 48-hours post injection ($p<0.05$). In the PACAP alone treatment group, the significantly lower lectin activity was sustained through 24-168 hours. The detected lectin activity increased in the PACAP+HMW group at 168 hours indicating no significant differences between 24- and 168-hours post injection (Figure 21).

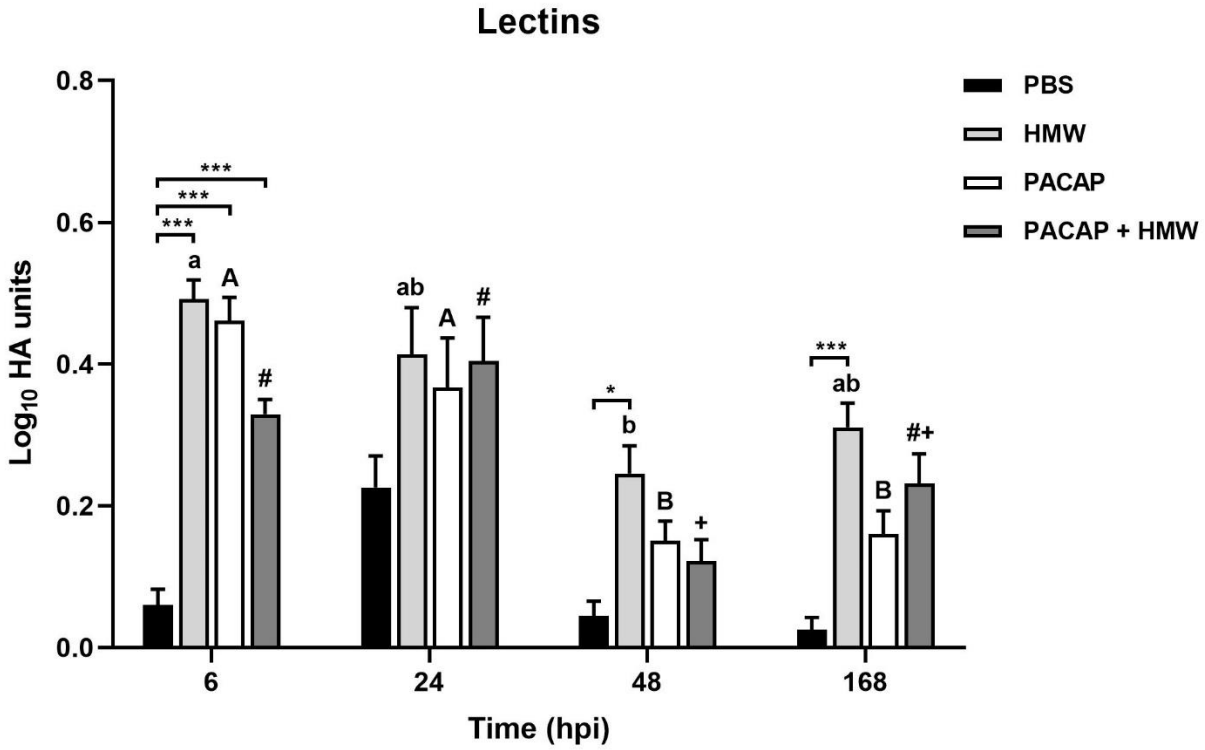


Figure 21. Hemagglutination activity of lectins in crayfish hemolymph after 6, 24, 48 and 168 hours of treatment via ventral sinus injection with 10 µg Poly(I:C) HMW, 12 µg PACAP and combination of PACAP+HMW. All data are shown as the mean ± standard error (SE) (N=16). Assumptions of variance, normality, and homogeneity were tested. A two-way ANOVA was performed using the different treatments and time-points as factors of variance, followed by a Tukey *post-hoc* test to identify differences between groups. Differences were considered significant at $p < .05$ (*), $p < .01$ (**) and $p < .001$ (***). Bars indicate differences among treatments for the specific time-point ($p < .05$ (*),$p < .01$ (**) and $p < .001$ (***)). Letters indicate differences in one specific treatment in time. Lower case letters correspond to HMW treatment, upper case letters to PACAP treatment and #+ symbols for PACAP-HMW combination treatment. All statistical analyses were performed using STATISTICA v7.0 (StatSoft software, Tulsa, USA) and graphs performed using GraphPad Prism (GraphPad Software, La Jolla California USA).

The hemolymph concentration of nitric oxide metabolites was significantly increased in the PACAP treatment group compared to the control and PACAP + HMW treatments at 6 hours post injection ($p<0.01$). The Poly(I:C) HMW treatment was also effective at increasing the concentration of nitric oxide metabolites as it was not significantly lower than the PACAP treatment group (unlike the mock and PACAP+HMW combination). Statistical significance was once again calculated using a two-way ANOVA with different treatments and time points as factors of variance. There were no significant differences in the Poly(I:C) HMW or the PACAP+HMW combination treatments in time. However, as is characteristic of an induced respiratory burst, there was a significant drop in the PACAP treatment group between 6 hours and 24-, 48- and 168-hours post injection ($p<0.05$) (Figure 22).

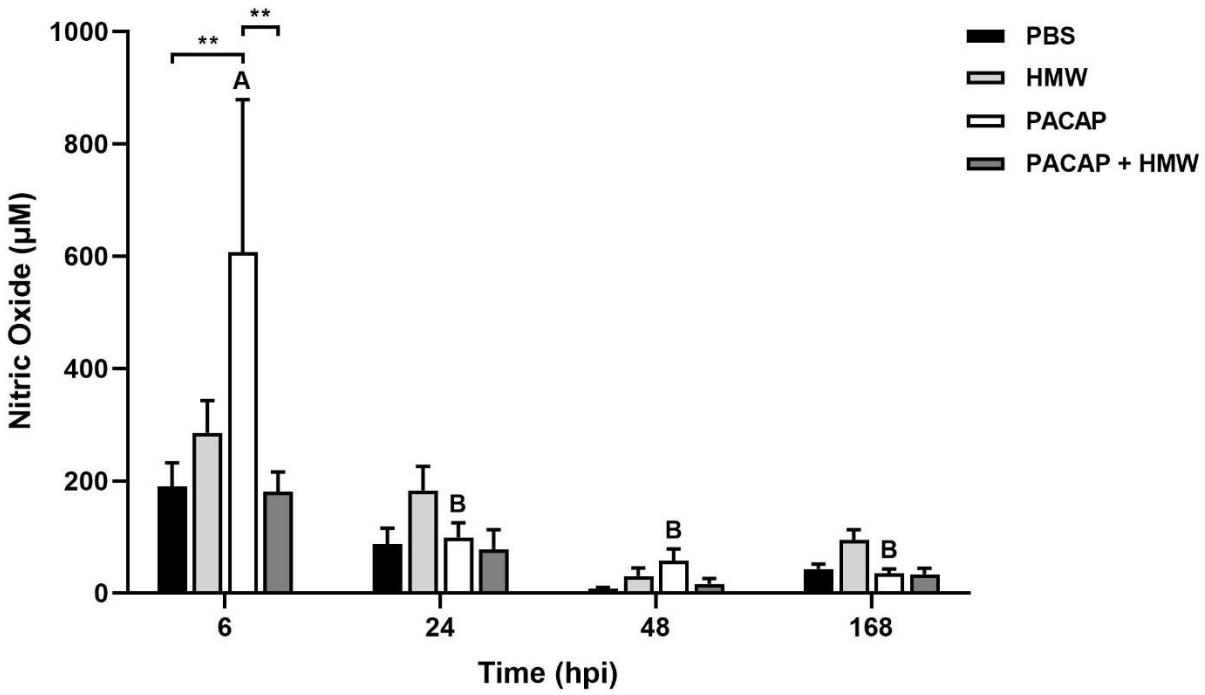


Figure 22. Concentration of nitric oxide in crayfish hemolymph after 6, 24, 48 and 168 hours of treatment via ventral sinus injection with 10 µg Poly(I:C) HMW, 12 µg PACAP and combination of PACAP+HMW. All data are shown as the mean ± standard error (SE) (N=16). Assumptions of variance, normality, and homogeneity were tested. A two-way ANOVA was performed using the different treatments and time-points as factors of variance, followed by a Tukey *post-hoc* test to identify differences between groups. Differences were considered significant at $p < .05$ (*), $p < .01$ (**) and $p < .001$ (***). Bars indicate differences among treatments for the specific time-point ($p < .05$ (*),$p < .01$ (**) and $p < .001$ (***)). Letters indicate differences in one specific treatment in time. Lower case letters correspond to HMW treatment, upper case letters to PACAP treatment and #+ symbols for PACAP-HMW combination treatment. All statistical analyses were performed using STATISTICA v7.0 (StatSoft software, Tulsa, USA) and graphs performed using GraphPad Prism (GraphPad Software, La Jolla California USA).

Phenoloxidase activity was significantly increased in the Poly(I:C) HMW and PACAP treatment groups compared to the control at 48 hours post injection. Data is presented as a % of mock (PBS) control treatment group set to 100%. At 48 hours post injection Poly(I:C) HMW treatment resulted in a 482% increase and PACAP treatment in a 269% increase in active phenoloxidase compared to the control. At 6 hours, Poly(I:C) HMW treatment elevated enzyme function to 131% control and PACAP treatment to 124% control. At 24 hours, Poly(I:C) and PACAP treatment both resulted in elevated phenoloxidase activity, 147% and 116% control respectively. Throughout all time points, PACAP+HMW treatment resulted in a non-significant decrease in enzyme function compared to the control. At 6, 24, 48 and 168 hours of treatment, PACAP+HMW treatment resulted in phenoloxidase activity of 72%, 90%, 94% and 79% of the control respectively. Statistical significance was once again calculated using a two-way ANOVA with different treatments and time points as factors of variance. There were significant differences in the phenoloxidase activity between the HMW and HMW+PACAP treatments at 48 hours ($p<0.01$) and 168 hours ($p<0.05$) post injection. In time, there was a significant difference in the HMW treatment group only between 24 and 48 hours of treatment ($p<0.05$). There were no significant differences in the phenoloxidase activity resulting through PACAP or PACAP+HMW treatment in time (Figure 23).

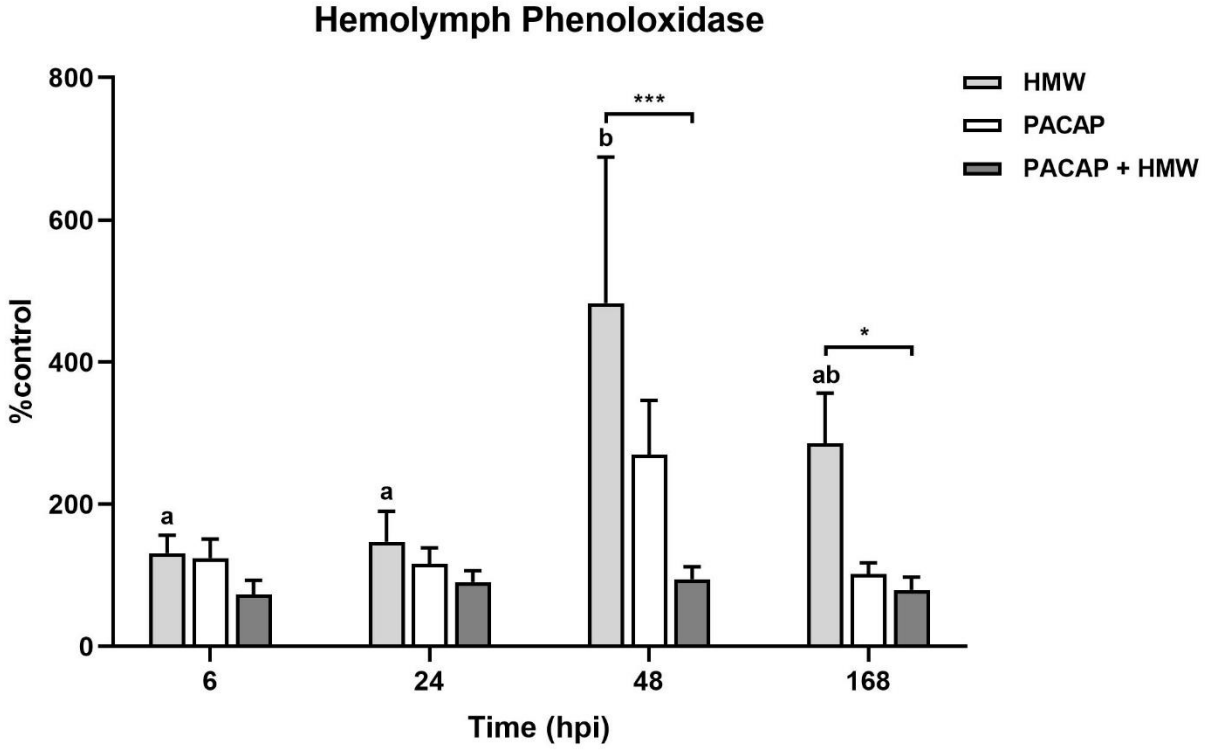


Figure 23. Activated phenoloxidase in crayfish hemolymph after 6, 24, 48 and 168 hours of treatment via ventral sinus injection with 10 µg Poly(I:C) HMW, 12 µg PACAP and combination of PACAP+HMW. All data are shown as the mean ± standard error (SE) (N=16). Data is presented as a % of mock (PBS) control treatment group set to 100%. Assumptions of variance, normality, and homogeneity were tested. An arcsine followed by a square root transformation was performed for percentage data. Then, a two-way ANOVA was performed using the different treatments and time-points as factors of variance, followed by a Tukey *post-hoc* test to identify differences between groups. Differences were considered significant at $p < .05$ (*), $p < .01$ (**) and $p < .001$ (***). Bars indicate differences among treatments for the specific time-point ($p < .05$ (*),$p < .01$ (**) and $p < .001$ (***)). Letters indicate differences in one specific treatment in time. All statistical analyses were performed using STATISTICA v7.0 (StatSoft software, Tulsa, USA) and graphs performed using GraphPad Prism (GraphPad Software, La Jolla California USA).

Table 4. Summary table of results from ventral sinus injection trial for total hemocyte count (THC), lectin, nitric oxide concentration (NO) and activated phenoloxidase (PO).

	6 hours				24 hours				48 hours				168 hours			
	THC	Lectin	NO	PO	THC	Lectin	NO	PO	THC	Lectin	NO	PO	THC	Lectin	NO	PO
Poly(I:C) HMW		√*			√	√		√	√	√*		√*	√	√*		√*
PACAP (12 µg)		√*	√*	√	√	√			√	√*		√	√	√		
HMW+PACAP		√*		X		√		X		√*		X		√		X

In the above table, a check mark (√) indicates a trend that the response was induced compared to mock (PBS) at each time point and treatment. An X indicates a trend that the response was lower than the mock (PBS) treatment. No symbol is indicative of no trend in the data compared to the mock (PBS). A star (*) is indicative of a statistically significant difference in the data compare to the mock (PBS).

Chapter 4. Discussion and limitations of study

4.1. Discussion

In this study, the crustacean immune response was investigated by determining the effects of immune stimulants in crayfish. First, the safety of Poly(I:C) HMW and PACAP treatments was established by demonstrating that exposure of primary hemocytes to the immune stimulants *in vitro* for 24 hours did not lead to reduced cell viability. Primary crayfish hemocytes were exposed to concentrations between 1.95 and 250 nM of PACAP and 0.076 and 10 µg/mL of Poly(I:C). Cell metabolism and membrane integrity was measured using fluorescent indicators alamarBlue and CFDA-AM. No concentration of either stimulant reduced metabolism or membrane integrity below the untreated control (Figure 17). This indicates the treatments were nontoxic and safe to use in the animals.

The fluorescent indicator of metabolism was consistent at 100% control at all doses of both treatments but there was a fluctuation in the results for membrane integrity characterized by an increase in fluorescence at 250 nM of PACAP and 1.25-5.0 µg/mL of Poly(I:C) HMW. This assay measures esterase activity in hemocytes. Membrane integrity is determined as intracellular esterases cleave CFDA-AM to the fluorescent product carboxyfluorescein, only when retained in the cell-membrane (Parish, 1999). Interestingly, esterases are lysosomal enzymes that destroys pathogens. Indeed, esterase activity can be induced in crustaceans with LPS and Poly(I:C) stimulation (Xian et al., 2016; Xian et al., 2018). While there was an increase in CFDA-AM fluorescence following PACAP and Poly(I:C) HMW treatment *in vitro*, the differences were not significant. This theory nevertheless provides an explanation for CFDA-AM fluorescent levels detected at greater than 100% of the control.

Next, the effects of poly(I:C) HMW and PACAP delivery by intramuscular or ventral sinus injection on the cellular and humoral immune responses of crayfish was investigated. Three *in vivo* trials were completed using locally caught crayfish. In the first two trials, crayfish were treated with either mock (PBS), poly(I:C) HMW (10µg), PACAP (6 µg) and PACAP (12 µg) by intramuscular injection between the third and fourth abdominal segment. Intramuscular injection is a common delivery method that ensures consistency in treatment dose and administration between animals (Xu et al., 2022). Though the results were not significant, there was an association between treatment for 6, 24, 48 and 168 hours and an immune response. In trial 1, 6 crayfish in each treatment group were sampled at 24-, 48- and 168-hours post injection. A cellular response was observed through an increase in the number of hemocytes in the PACAP (12 µg) group at 24 hours which then declined at 48- and 168-hours post injection (Figure 18. A). These results complement findings in the shrimp species *Litopenaeus vannamei* which through water bath exposure to PACAP for 35 days did not exhibit any increase in the number of hemocytes compared to the untreated control (Lugo et al., 2013) The Poly(I:C) HMW treated crayfish had a slightly elevated number of hemocytes compared to the control at 24 hours which increased at 48 hours, however observations made in trial 2 at 6 hours suggest that production/recruitment of hemocytes is rapidly induced as there was a dramatic increase in all treatment groups at 6 hours which then decline by more than 50% at 24 hours post injection (Figure 19. A).

The evaluation of humoral responses following intramuscular injection showed a slight increase in lectin activity, nitric oxide concentration and activated phenoloxidase function at 6 hours post injection with Poly(I:C) HMW and PACAP (12 µg) (Figure 19. B-D). This was followed by a decline in lectin activity and NO concentration at 24 hours. NO concentration

continued to decline at 48- and 168-hours post injection whereas lectin activity increased slightly at 48 hours before decreasing at 168 hours (Figure 18. B, C, Figure 19. B, C). Activated phenoloxidase increased in the Poly(I:C) HMW group and dropped in the PACAP group in trial 2 at 24 hours (Figure 19. D). At 48 hours there were no differences in activated phenoloxidase compared to the control, but there was an observed increase at 168 hours post injection with PACAP (Figure 18. D).

It was suggested by Xian et al. (2018) that with high doses (20 µg) of Poly(I:C), nitric oxide – the main toxic reactive oxygen/nitrogen species that kills microorganisms in crustaceans – is induced which then depletes hemocyte counts by inducing apoptosis. The results of Poly(I:C) HMW and PACAP treatment for 6, 24 and 48 hours (Figure 18. A, Figure 19. A) are consistent with this observation.

The results from intramuscular injection trials 1 and 2 were not significant but showed that the immune response in crayfish can be quantified through determining the total number of hemocytes and through a functional analysis of humoral responses. While these trials indicated a response that could be associated with the treatments, none of the results were significant. This is likely the result of individual variation in crayfish collected from a natural environment. These differences could therefore be minimized by increasing the number of individuals in each treatment group.

An additional concern was the injection method. While intramuscular injection has been a standard method for therapeutic delivery (Xu et al., 2022), it was observed in intramuscular injection trials #1 and #2 that following treatment crayfish displayed static abdomen flexion. This was also observed by Panksepp and Huber (2003), where following injection of *Orconectes rusticus* collected from a natural environment, all crayfish that received intramuscular injection

displayed a static posture characterized by flexion of the abdomen, walking legs and claws as well as rigidity that prevented crayfish from righting themselves (Panksepp and Huber, 2003). This behaviour may result from hitting the flexion motor nerves that innervate the crayfish tail muscle and abdomen (Larimer & Moore, 2003).

Furthermore, Xu et al., (2022) recently determined the impact of drug administration method in crayfish. The administration method of a drug (doxycycline) to crayfish had a considerable impact on drug half life and concentration in different tissues. When delivered by intramuscular injection, the drug had a half life of 60.21 hours in the muscle, compared to 23.25 hours in the hemolymph. Whereas delivery by sinus injection resulted in a half life of 25.28 hours in the muscle and 31.20 hours in the hemolymph. Both muscular and sinus injection enabled the drug to circulate systemically, reaching the hepatopancreas after 2 hours following intramuscular injection and 0.5 hours following ventral sinus injection. Most notably, researchers determined that, when the drug was administered through the ventral sinus, the concentration in the hemolymph was higher compared to intramuscular injection over 100 hours with a concentration peak at 6 hours post injection (Xu et al., 2022). These results support delivery of immune stimulants via ventral sinus injection. While a stimulant administered through intramuscular injection may diffuse through capillaries, a majority remains in the muscle tissue. Whereas, with direct administration into the hemolymph, the open circulatory system means that the stimulant can reach different tissues and organs and initiate a more robust effector response. Specifically, it reaches the hepatopancreas, an organ which plays a key role in synthesizing immune factors such as lectins, phenoloxidase, lysozymes, superoxide dismutase and catalase, faster (Ji et al., 2009).

The third injection trial was designed to introduce the immune stimulant more effectively and eliminate the potential of causing static abdomen flexion. When crayfish were injected with each treatment directly into the ventral sinus, no flexion of the abdomen or static posture was observed.

The ventral sinus injection trial also benefited from an increased number of animals per treatment and duplicate tanks to take tank effects into account. In the ventral sinus injection trial, 16 crayfish - 8 from each replicate treatment tank - were sampled at 6-, 24-, 48- and 168-hours post injection and cellular and humoral responses were measured. In contrast to the intramuscular injection trials, the average number of hemocytes in the Poly(I:C) HMW and PACAP groups never dropped below the mock (PBS) control. Furthermore, there were a higher number of responsive crayfish in the Poly(I:C) HMW and PACAP treatment groups compared to the control and PACAP+HMW. Following Poly(I:C) HMW treatment, hemocyte counts remained stable around 1×10^6 between 24-, 48- and 168-hours post injection. Similarly, in the PACAP group, the number of hemocytes were comparable at 24- and 168-hours however they were elevated at 48 hours post injection (Figure 20). While the hemocyte counts were elevated compared to the control following Poly(I:C) HMW and PACAP treatment, there were no statistically significant differences when considering treatment and time as factors of variance.

In trial 3, the sex of the animal was recorded and considered because a weaker immune response has been observed in female perch (Shepherd et al., 2012). There were, however, no differences in the number of hemocytes from male and female crayfish. These results are consistent with findings in several crustacean species. Evaluation of total hemocyte count, phenoloxidase activity and encapsulation in the western rock lobsters, signal crayfish and stone

crayfish showed no correlation between sex and immune response (Jussila et al., 1997, Dragicevic et al., 2021, Lucic and Erben, 2005).

In contrast to therapeutic delivery by intramuscular injection, the number of hemocytes never depleted below the control in the ventral sinus injection trial. Hemocyte counts in the Poly(I:C) HMW and PACAP treatment groups were elevated due to a release of hemocytes into circulating hemolymph however, there was no evidence of humoral responses depleting hemocyte counts over time. This is a notable improvement over results from the intramuscular injection trials. Following treatment with Poly(I:C) and PACAP, hemocyte counts only improved and recovered to control levels despite significant increases in lectin activity, nitric oxide concentration and activated phenoloxidase function (Figure 20-23). This suggests that the stimulants were able to reach the hematopoietic tissue and replenish degranulating hemocytes.

Following 6 hours of treatment with Poly(I:C) HMW, PACAP and a PACAP+HMW combination, there was a significant increase in the hemolymph lectin activity. While Poly(I:C) treatment resulted in a significant decline in the hemagglutination titer between 6 and 48 hours, the response remained significantly higher than the control at 48- and 168-hours post injection. PACAP treatment resulted in a significant decline between 24 and 48 hours, but hemagglutination activity was still elevated compared to the control and not significantly lower than Poly(I:C) HMW (Figure 21). C-type lectin induction through NF-κβ signalling has been established in shrimp exposed to Poly(I:C) (Li et al., 2014). PACAP may act similarly, as binding to its receptor activates PKA through cAMP which regulates expression of NF-κβ. This pathway, however, has not been fully elucidated and requires further study. Lectins are capable of binding to WSSV surface proteins and there is evidence that this leads to activation of antiviral signalling pathways. In the crayfish, *Procambarus clarkii,* lectins were found to

recognize VP28, a glycosylated WSSV envelope protein. WSSV infection significantly upregulated lectin expression profiles between 6- and 48-hours post infection and viral replication increased with expression knockdown (Gao et al., 2021). It was additionally determined that while lectins increase steadily until 48 hours during WSSV infection, this is followed by a dramatic decrease at 72 hours. The decrease in the free protein in the hemolymph may be caused as it initiates an antiviral response through direct binding the virus or to Domeless, the Jak/Stat receptor (Zhao et al., 2009).

It has been demonstrated in *Drosophila* that Jak/Stat signalling activates reactive oxygen/nitrogen species production (West & Silverman, 2018). A significant increase in the concentration of nitric oxide was detected in crayfish hemolymph at 6 hours post injection with PACAP compared to the control. The concentration was also significantly higher in the PACAP treatment group at 6 hours compared to 24-, 48- and 168-hours post injection. The concentration of nitric oxide in the PACAP group at 6 hours was also not significantly higher than the Poly(I:C) HMW group. While not significant, the concentration of nitric oxide in Poly(I:C) HMW injected crayfish was elevated at all time points compared to the control (Figure 22). These results are consistent with previous research as Poly(I:C) HMW and PACAP have been shown to significantly increase the concentration of nitric oxide in shrimp at 6 hours and 14 days respectively (Xian et al., 2019, Lugo et al., 2013). In the ventral sinus injection trial, Poly(I:C) HMW and PACAP administration increased the concentration of nitric oxide but did not induce a depletion of hemocytes. This result indicates that while the therapeutics induced a respiratory burst, the hemocytes lost during degranulation were effectively replaced.

It has been speculated that oxidative stress generated reactive oxygen and nitrogen species may contribute to reduced phenoloxidase activity in crustaceans (Cheng et al., 2002).

One explanation is that the phenoloxidase system results in the generation of cytotoxic reactive oxygen, nitrogen and quinone intermediates and to reduce the concentration of cytotoxic molecules the systems are not activated simultaneously (Nappi et al., 2000). The results from the ventral sinus injection trial are consistent with these observations. The significant increase in the concentration of phenoloxidase following Poly(I:C) HMW and PACAP injection occurred after 48 hours which corresponded to the significant decline in the concentration of nitric oxide (Figures 22 & 23). The phenoloxidase concentration was slightly elevated at 6 hours and 24 hours following Poly(I:C) and PACAP injection (131%, 147% and 124%, 116% respectively) compared to the control (Figure 23). While PACAP treatment resulted in lower levels of activated phenoloxidase compared to Poly(I:C) HMW these levels were not significantly different.

Previous research failed to show any interaction between PACAP and the phenoloxidase system after 35 days of water bath exposure (Lugo et al., 2013). It has been determined however, that injection of *P. monodon* with 20 µg of Poly(I:C) did not induce phenoloxidase between 0 and 48 hours but caused a significant decline between 6 and 12 hours and inhibited its activity by 48 hours (Xian et al., 2018). This has led researchers to conclude that phenoloxidase is not the main immune defense pathway of shrimp against viral infection. The significant increase of activated phenoloxidase following Poly(I:C) HMW and PACAP treatment challenges this claim.

One reason given in support of the theory that phenoloxidase is not essential to protection against viral infection is that WSSV has been shown to significantly reduce phenoloxidase activity (Mathew et al., 2007). It has been determined however that this decline occurs with increased mortality during the late stage of infection as the host immune system becomes overwhelmed (Wang et al., 2012). In fact, it has been shown that priming the immune system

with a polysaccharide solution containing β-glucan and α-mannan peptide prior to WSSV challenge leads to increased phenoloxidase function, enhanced antiviral defenses and a higher survival rate to WSSV (Wang et al., 2012). This suggests that stimulating phenoloxidase production prior to infection can improve the crustacean antiviral response. Furthermore, the phenoloxidase system and Toll signalling cascades share a serine protease (a proteolytic enzyme) leading to a coordinated response (Kulkarni et al., 2020).

The PACAP+HMW treatment did not induce any significant cellular or humoral responses compared to the control, except an increase in the lectin titer at 6 hours post injection (Figure 20). This is likely because PACAP is a cationic peptide and Poly(I:C) HMW is negatively charged. When combined the electrostatic interaction meant that the treatments were bound and delivered at a lower, less bioavailable, dose.

The results demonstrate that Poly(I:C) HMW and PACAP are effective immune stimulants in crayfish when delivered by ventral sinus injection but are less effective with intramuscular injection and in combination. These treatments stimulate lectin activity, increase nitric oxide concentration, and activated phenoloxidase function without depleting hemocytes and with no apparent harm to the animal. These markers indicate the activation of a cellular response, leading to phagocytosis, melanization and encapsulation, as well as humoral signalling cascades, leading to the production of antimicrobial peptides and transcription of antiviral immune effectors. These treatments, therefore, have broad spectrum applications through their ability to stimulate the crustacean immune system with the potential of reducing bacterial and viral infection.

4.2. Limitations of study

Typically, an *in vivo* study of crustaceans involves sourcing animals from commercial farms and including between 100 and 450 individuals in each treatment group (Lugo et al., 2013, Ji et al., 2009, Xian et al., 2018). The practice reduces unpredictable individual variation, which even when animals are sourced from a standardized, sterile environment can impact results (Ji et al., 2009). In the intramuscular injection trial #1 and #2, the data was not significant. The study was limited by the number of individuals collected from the Grand River in Waterloo, Ontario. Only six crayfish were included at each time point in each group, so although there was an observed association between the treatment and response, the study was underpowered, and the small number of replicates meant that individual variation eliminated significance in the results. Furthermore, the animals came from a natural environment. There were no methods for analysis of the animals prior to inclusion in the trial except for visual inspection to confirm apparent health. This was a concern because the mock (PBS) group contained high responders despite no exposure to an immune stimulant. An attempt to standardize the animals was made in intramuscular injection trial #2 and the ventral sinus injection trial through a lengthy acclimatization period (21 days) in a lab environment. The longer acclimatization period did reduce the number of high responders in the mock (PBS) group. This is a comparatively long acclimatization period as shrimp and crayfish are often held only between 2 and 7 days prior to experimentation (Panksepp and Huber, 2016, Ji et al., 2009, Xian et al., 2018).

In intramuscular injection trials #1 and #2, there was no study of the immune response at the injection site. Muscle tissue was sampled, and an attempt was made to analyze lectin and phenoloxidase gene expression by designing primers using conserved regions of crayfish

genomes (species included: *Orconectes virilis, Astacus astacus, Procambarus Clarkii, Pacifastacus leniusculus* and *Cherax quandricarinatus*). Despite several attempts to design and sequence degenerate primers, no product was detected by endpoint or qPCR. The species of crayfish collected from the Grand River is unknown and its genome was not sequenced during the time frame of this project. Designing primers and analyzing gene expression in the tissue from animals in the three *in vivo* trials at the injection site as well as in hepatopancreas, gill and hemocytes is a future direction for this project.

Chapter 5. Concluding Remarks

5.1. Poly(I:C) HMW and PACAP as effective immunostimulants in crayfish

The frequent incidence of catastrophic disease has had a detrimental impact on the economic growth of the shrimp aquaculture industry (Walker & Winton, 2010). The high stocking density and cross-boundary movement has led to rampant bacterial and viral infection of farmed populations (Wang et al., 2020). There are currently no known therapeutics that effectively reduce disease burden in crustaceans. The intent of this study is to show that dsRNA and PACAP can be used as broad spectrum immunostimulants with potential to control disease in aquatic invertebrate species. The results demonstrate that a general immune response could be induced through injection of crayfish with Poly(I:C) HMW and PACAP. Furthermore, immune activation can be quantified by determining the number of hemocytes and through a functional analysis of the hemolymph to determine lectin activity, nitric oxide metabolite concentration and activated phenoloxidase function. Through direct *in vitro* exposure it was shown that neither Poly(I:C) HMW nor PACAP, despite being an AMP, are toxic to crayfish hemocytes.

The results also determined that the most effective delivery method is through ventral sinus injection. Intramuscular injection results in flexion neuron stimulation and static motion of the crayfish. Additionally, it may have stimulated degranulation and humoral cascades that depleted the number of circulating hemocytes. Ventral sinus injection resulted in a more robust, rapid, and direct stimulation of crayfish cellular and humoral responses. The findings show a significant interaction between both stimulants and immune pathways. Following Poly(I:C) and PACAP treatment, crayfish had a higher total number of hemocytes which never depleted below the control, as well as a significant increase in lectin and activated phenoloxidase activity between 6 and 168 hours. PACAP treatment for 6 hours resulted additionally in a significant increase in the

hemolymph concentration of nitric oxide. These results advance the identification and development of effective broad-spectrum immune therapies which can benefit the worldwide shrimp aquaculture industry.

5.2. Future directions

Many crustacean immune pathways have not been fully elucidated and require more research. There is only a limited characterization of receptors and signalling pathways used by crustaceans in response to pathogens. Moreover, there is no knowledge of specific cytokine, AMP, and antiviral effectors in many crustacean species. While functional analysis of the hemolymph provides insights into immune action, further analysis at the transcript level needs to be investigated. A future direction for this project is that following immune system priming with the therapeutic, animals would be challenged with a pathogen of interest such as WSSV or IHHNV. Additionally, since RNAi is an essential component of crustacean immunity, the effects of sequence specific dsRNA should be investigated as it may enhance the observed antiviral response. Finally, these experiments should be replicated in an economically important crustacean species used in aquaculture, such as the Pacific white leg shrimp (*Litopenaeus vannemei*) or the tiger shrimp (*Panaeus monodon*).

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