Characterizing the Immune Function of the Brown Bullhead (*Ameiurus nebulosus*) from Less Contaminated and Highly Contaminated Locations along the Detroit River

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Biology

Waterloo, Ontario, Canada, 2011

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Some fish populations are able to adapt and thrive in contaminated habitats. Survival of populations depends on the ability of the organism to elicit resistance, either due to genetic adaptation or physiological acclimations. Brown Bullheads (*Ameiurus nebulosus*) are able to survive in very contaminated areas and their benthic and philopatric characteristics make them a model organism to study chronic exposure. This research assesses the immune function of brown bullhead collected at four pre-determined sites along the Detroit River, which are characterized by high or low concentrations of environmental toxicants. Clean and contaminated sediment used for contaminant exposure was collected by ponar at designated sites of the river. The bullheads were vaccinated with heat-killed *V. anguillarum* in order to induce an immune response, before the vaccinated bullheads were randomly divided into corresponding contaminant exposure tanks. Respiratory burst assays to assess innate oxygen radical production 24hrs post vaccination and sediment exposure identified an inhibition of neutrophil oxidative activity in adult 6 month cleared of contaminant bullheads collected from a clean (Peche Island) site exposed to contaminated sediment, and of F1 raised populations from a contaminated (Trenton Channel) site. Results also showed overall inhibition on contaminated sediment in both PI and TC recently captured fish. Enzyme linked immunosorbant assay (ELISA) to assess antibody production revealed no difference between those fish exposed to either sediment. Results did show a lower expression of total antibody in chronically contaminant exposed bullheads (acute adults). Real time PCR to assess immune gene expression was conducted using cloned Major Histocompatibility Class II Beta (MHIIB), Interleukin-8 (IL-8) and Interleukin-1 Beta (IL-1B) 24hr post vaccination and sediment exposure. No contaminant induced immunosuppression of MHIIB was observed, while a reduction in IL-8 and IL-1B in acute adults may signify a delayed response due to chronic sediment exposure or of a normal functioning delayed response in wild bullheads. Results of the present study indicate negative environmental impacts on the innate immune response, leading to physiological adaptations in the brown bullhead, which can be reversed upon removal of the contaminants.

Acknowledgements

Over the past two and a half years I have not only met a tremendous amount of amazing people, but I have made friendships that will continue on. Many of these people had a great impact in my life and I would like to express my gratitude.

I would like to thank my immediate supervisor and great lecturer Dr. Brian Dixon for allowing me the opportunity to work under him and in his lab. From the trip to Belize to the trips to Windsor, you gave me opportunities to see other aspects involved in research that I know not everyone had the chance to learn. You allowed me to make my own decisions with the project, and in turn made me feel trustworthy, adequate and capable of completing the work. And I know I am very hard on myself and seem unsatisfied with the work I have done at times, but that was because I admired you and your work so much that I just wanted everything to meet and surpass your expectations. I have also always admired your uncanny ability to captivate a full auditorium of people, to a room of just our lab staff whenever you present research or discuss immunological related issues. Your lecturing enthusiasm has always impressed me, and I have been inspired by you in that way. I thank you for this opportunity and I hope the best for you and your family in the future.

I would like to thank my committee members Dr. Neils Bols and Dr. Daniel Heath for their support and guidance throughout the project. I would like to thank Dr. Heath for the initial collaboration with Dr. Dixon which has allowed myself to work on the project, and for all the help throughout the many field seasons. I would like to thanks Dr. Bols for his help with the respiratory burst assay, and allowing me access to his lab on those late nights. I would also like to thank him for the inspired Canuck discussions, as they make their run in this years playoffs!

I would like to thank all of those in the Biology Department that have helped me throughout the years. I would like to send a special thanks to Dr. Dragana Miskovic for everything she has done for me. You first provided me the opportunity to teach, and I would like to thank you as it inspired me to follow my current career path. You were always there wanting not only research but life updates and I thank you for that. A very special thank you will also go to Mishi Savulescu for all of her help, and for being a person I could always talk to. To both Linda Zepf and Mila Kaufman, I appreciate everything you two amazing ladies have done for me over that past couple of years.

I would like to thank each member of the Brian Dixon lab that I have met over the past few years. You are all remarkable individuals and wish each of you success in your futures. I will miss our “man cave”

and the not always insightful discussions that took place. I thank each of you who have helped me along the way with different aspects of my project.

I would like to thank Rebecca Williams, Linda Soderberg, and Michelle Farwell for all of their help and expertise in field season and related issues. Thanks to Todd Leadley for everything he did for us. You allowed us to take over your farm in order to perform our experiments and conduct our research. Thanks for everything. Thanks to Rebecca and to her supervisor Dr. Hubbersty for the help and use of the qPCR machine. I would also like to thank Steve Cho and Mike Burtynk for their tremendous help during field season. Steve you were a great roomie and colleague while in the field, and thanks for all your help with your fish capturing skills! Mike, thanks for being our navigator, boat captain and electroshocking/seining master! You were always willing to brave the elements for someone else's research. We all thank you for that.

I would like to thank my amazing family, my sister Lynsey, brother Kyle, mother Debby and my father George. You have always been supportive of my education and have always pushed me on. Even when I missed those thanksgiving dinners, or birthdays, or family get together's because of field work or lab work, you have always been supportive of me. I thank you all for that, and I love you all. Lynsey, thanks for always being there as not only a sister but as a best friend. Kyle, I thank you for all those years of heated debates and discussions ranging from vaccines to a platypus. You always pushed me to learn more and read more so that I could try to prove you wrong, even when you were so darn persuasive! Mom, you are an amazing woman in everything you do. I thank you for all those prepackaged meals, the laundry, and all the love and support you have provided. To my dad, you are the best father a guy could ever ask for. You have pushed me to better myself in every aspect for as long as I can remember, from hockey to school to being a better person in life. I thank you every day for everything you have done for me, and I would not be the person I am today if it wasn't for you and mom. I love you both more than you know.

Lastly I would like to thank my partner Nadene Fischer. You have been an amazing girlfriend and have been supportive throughout my Masters. Even when times were tough and field season or schooling brought us apart, you were always there for me. I thank you for driving and picking me up from school, even on those late nights and early mornings when I needed to get things done. I thank you for bringing in food and meals, because you always know I don't stop to eat when I'm focused and busy. I thank you for doing the laundry and housework when I was busy, always keeping me smelling fresh and looking presentable! I love you, and always will. You hold a special place in my heart and I thank you for everything.

I apologize to anyone that I have forgotten, as there have been too many people who have had an impact and have helped me to mention.

Thank you!

Sincerely, your little skinny buddy

Kevin

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List of Abbreviations

Peche Island	**PI**
Trenton Channel	**TC**
Belle Isle	**BI**
First generation offspring	**F1**
Cleared adults	**Cl. Ad**
Acute Adults	**Ac. Ad**

General Introduction

1.1 Aquatic Pollution and Toxicity

Aquatic pollution from dumping and aerial fallout has contaminated numerous aquatic ecosystems such as rivers, lakes and oceans (Zelikoff, 1993). In recent history, it has been documented that exposure to aquatic contaminants can have detrimental effects on the growth and development of aquatic organisms. It has also been shown that these contaminants can interfere with the proper functioning of a healthy immune response (Sweet & Zelikoff, 2001). These contaminants include metals, polycyclic aromatic hydrocarbons (PAH), and chlorinated synthetic organics (Zelikoff, 1993). PAHs are immunotoxic and carcinogenic environmental pollutants entering the environment through incomplete combustion of fossil fuels and release of petroleum, which have contaminated both marine and freshwater systems (Reynaud & Deschaux, 2006; Reviewed in Bols et al, 2001). The best studied of these contaminants are 7,12-dimethylbenz(a)anthracene (DMBA), benzo(a)pyrene (BaP) and 3-methylcholanthrene (3-MC) (Reynaud & Deschaux, 2006). Halogenated aromatic hydrocarbons (HAHs) include dioxins and furans (by-products of industrial usage), bipheyls and naphthalenes (Regala et al, 2001). Polychlorinated biphenyls (PCBs) which were manufactured for industrial purposes are the most widespread of the HAHs, are resistant to degradation, and bioaccumulate with ease (Rice et al, 1998; Reviewed in Bols et el, 2001). Heavy metals can originate from many sources including industrial discharge, municipal wastes and surface runoff (Reviewed in Bols et al, 2001).

1.2 Detroit River

The Detroit River is located along the southernmost tip of Ontario Canada, bisecting the border between Canada and USA. The 51km long river runs from Lake St. Clair south to Lake Erie, contains 12 islands and is the busiest commercial waterway of the Great Lakes (USGS, 2002; Manny & Kenaga, 1991). The river is an important migration route for fish, birds, and insects, and provides a habitat for 27 species of waterfowl, 65 species of fish, 300 species of macrozoobenthos, 31 species of aquatic macrophytes and at least 82 species of phytoplankton (USGS, 2002; Manny et al, 1988). For many years the Detroit River was a dumping ground for the disposal of toxic wastes, and in 1985 was designated as an Area of Concern (AOC) by the International Joint Commission (USGS, 2002). Power plants, steel mills, and petroleum refineries among other industries, along with effluent outputs have greatly contaminated the river. Aquatic habitats have been destroyed by both toxic contaminants and shoreline and channel modifications (Manny & Kenaga, 1991). More than 200 different chemical compounds have been identified in the river (Arcand-Hoy & Metcalfe, 1999), with many persistent organic pollutants, including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) highly contaminating the sediments (Drouillard et al, 2006).

1.3 Mode of action of Contaminants

1.3.1 Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a class of hydrophobic organic chemicals containing three or more fused benzene rings, and include more than 10,000 possible individual compounds (Cerniglia, 1992; Logan, 2007). PAHs often occur as complex mixtures that can vary

in physical and chemical properties, affecting their movement and fate in the environment. Four different classes of PAH mixtures exist, based on their environmental source; pyrogenic are formed by combustion of organic matter, petrogenic are formed in the earth by geological processes, diagenic are formed in sediments from biogenic compounds, and biogenic are formed by animals, plants, fungi and bacteria (Logan, 2007). PAHs are one of the major environmental pollutants and occur in automobile exhaust, cigarette smoke, cooking of foods, and by-products of industrial processes (Cerniglia, 1992; Tauchi et al, 2005). Due to their hydrophobic nature and low water solubility, PAHs rapidly enter sediments and persist within ecosystems until they are degraded, resuspended, bioaccumulated or removed by dredging (Cerniglia, 1992). Fish in particular are constantly exposed to either PAH-contaminated sediment for benthic species, contaminated prey for top predator fish species, and suspended water particles, due to their behaviour, life cycles and habitat. Exposure can occur through respiration (uptake by the gills), ingestion (uptake by the gut from contaminated food or sediment) or through the integument (absorption by the skin) (Reviewed in van der Oost et al, 2003; Logan, 2007). Once absorbed, PAHs are carried through the bloodstream to the most lipid-rich tissues such as the liver and bile (Logan, 2007).

PAHs are known to be both carcinogenic and immunotoxic to fish (Reynaud & Deschaux, 2006). Narcosis, mortality in all life stages, decrease in growth, edema, cardiac dysfunction, lesions and tumors of the skin and liver, cataracts, and compromised immunity have all been attributed to PAH exposure. Most fish species biotransform PAHs through enzymatic systems, metabolizing and detoxifying PAHs mainly in the liver (Reviewed in van der Oost et al, 2003; Logan, 2007). The metabolism of PAHs in fish can induce phase I enzymes, while most

PAHs induce the cytochrome P4501A (CYP1A) gene (Reviewed in Reynaud & Deschaux, 2006, Reviewed in van der Oost et al, 2003). Phase I enzyme induction results in the addition or removal of reactive functional groups through oxidation, reduction or hydrolysis by microbial monooxygenase (MO) enzymes, ultimately transforming lipophilic xenobiotics to more water soluble compounds (Reviewed in van der Oost et al, 2003). Phase I enzymes are a membrane bound family of heme proteins located in the endoplasmic reticulum of the liver. Phase I enzymes metabolize certain PAHs to hyrdrophilic products like phenols, dihydrodiols, quinines and epoxides that are excreted as polar metabolites in bile (Reviewed in van der Oost et al, 2003). Most PAHs induce phase II enzymes, including CYP1A1, before being excreted through the bile. CYP1A1 protein induction is initiated by the binding of PAHs to the intracellular aryl hydrocarbon receptor (Ah-R) as shown in Figure 1.1 (Reviewed in Reynaud & Deschaux, 2006). The AhR is a member of the steroid-hormone receptor family, and increases or decreases levels of gene products through gene induction due to contaminant exposure (Carpenter, 2006; Reviewed in Reynaud & Deschaux, 2006). Ah-Rs are most often located in the liver but can also be found in extra-hepatic tissues. The Ah-R receptor is located within the cytoplasm of cells, and in inactive form is in a complex with molecular chaperone proteins; heat shock protein 90 (hsp90), immunophilin XAP2 and cochaperone P23. Once bound to a PAH ligand, the receptor detaches from the chaperone proteins, and translocates to the nucleus where it dimerizes with AhR nuclear translocator (arnt). The Ah-R–arnt heterodimer can then freely bind to xenobiotic response elements (XRE) in target genes (CYP1A1) (Reviewed in Reynaud & Deschaux, 2006). Transcription factors are now able to freely bind, increasing the mRNA and protein expression of CYP1A1.

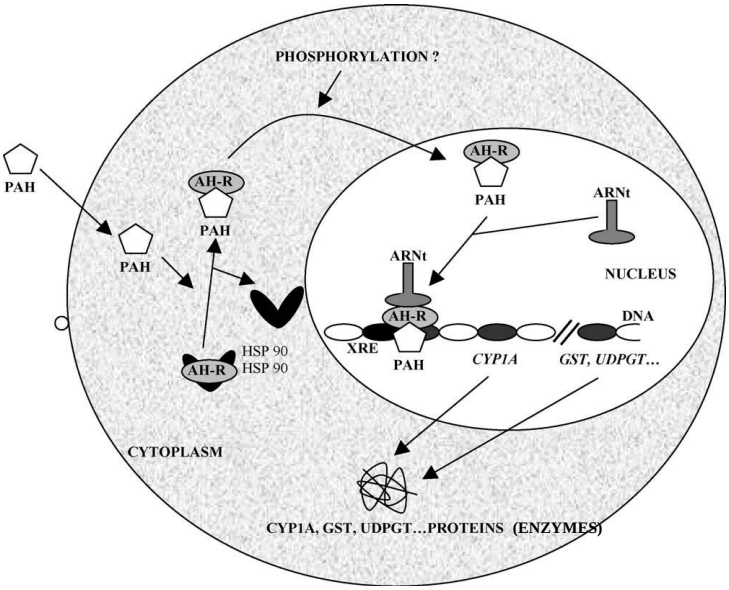

Figure 1.1 Induction of CYP1A1 expression induced by PAH ligand binding (Modified from Reynaud & Deshaux, 2006)

Although both phase I and phase II enzymes are essential in the detoxification of PAHs, these processes also create reactive intermediates that are highly toxic, mutagenic or carcinogenic to the fish. The highly toxic products include certain oxides that can bind to cellular DNA, RNA and proteins resulting in increased DNA adduct formation or enhanced oxidative DNA damage (Reviewed in van der Oost et al, 2003). In mammals, immunosuppression by PAHs depends on metabolic activation. However, the mechanism that elicits immunosuppression in fish is still unknown (Reviewed in Reynaud & Deschaux, 2006). It appears with increasing reports that PAHs have varying effects in different species of fish, at different life stages of the fish, as well as different PAHs may elicit different effects in the same fish species (Logan, 2007).

1.3.2 Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are synthetic chlorinated organic chemicals, made from the biphenyl molecule and two six-carbon rings linked by a single carbon-carbon bond (Beyer & Biziuk, 2009; Carpenter, 2006). They are widespread throughout the environment and are found in air, water, sediments, and soils. The molecule is made of 12 carbon atoms bound to chlorine atoms which substitute hydrogen atoms at any of the possible 10 positions (Carpenter, 2006) and encompass 209 different possible structures (See Figure 1.2).

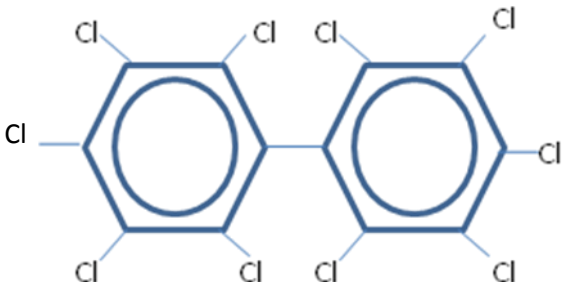

Figure 1.2 Structure of polychlorinated biphenyls (Modified from Beyer & Biziuk, 2009)

PCBs are used for many industrial applications due to their low reactivity and chemical stability. They have been used as fluid insulators in high-voltage electric transformers, in high-capacity condensers, as heat exchangers, pesticide extenders, adhesives, dedusting agents, components of cutting oils, flame retardants, hydraulic lubricants, and components of plasticizers in paints, inks, toners, and printing inks (Reviewed in Beyer & Biziuk, 2009; Erickson 1997) . The qualities that make PCBs ideal for industrial purposes also make them less environmentally favourable. The high lipid solubility prevents PCBs from being degraded, resulting in bioaccumulation throughout the environment and food chain (Duffy et al, 2002). The only source

of PCB production is through the activities of humans, leading to highly contaminated sites located in industrialized areas. PCBs bind to organic particles in the water, atmosphere and sediment, and those that are present in water or sediments are filtered by fish, crustaceans, or mollusks via their gills. Due to their lipophilic properties, PCBs are absorbed into the fatty tissues of these animals (Reviewed in Beyer & Biziuk, 2009; Larson, 2006). PCBs' persistence, resistance to metabolism and bioaccumulation in aquatic organisms are an increasing concern to the impact of PCB exposure on fish populations (Duffy et al, 2003).

Recently it has been discovered that PCBs can be dehalogenated in freshwater and in estuarine sediments, resulting in detoxification. Volatilization and colloidal absorption of PCBs to dissolved organic matter are important pathways to reduce its bioavailability (Reviewed in Beyer & Biziuk, 2009; Bedard & Van Dort 1997). The only known process of PCB biodegradation in aquatic environments occurs through the action of bacteria or other microorganisms by either aerobic oxidative dechlorination or hydrolytic dehalogenation, and anaerobic reductive dechlorination. The degradation of PCBs occurs by the removal of chlorine from the biphenyl ring, ring cleavage, and oxidation of the compound resulting in the production of CO_2, chlorine, and water (Reviewed in Beyer & Biziuk, 2009).

PCBs, like PAHs also induce the CP1A1 genes through the Ah-R receptor as described above. In mammals, different PCB congeners induce different CYP1A isoenzymes. These isoenzymes are responsible for the oxidative metabolism (Phase I activation) of many drugs, steroids, and carcinogens. Dioxins and coplanar PCBs induce the activity of CYP 1A1, 1A2, 2A1, and 1B1. These gene inductions result in the proliferation of endoplasmic reticulum in the liver, resulting in an increase in liver size and an alteration in many aspects of liver function

(Carpenter, 2006). As with PAHs discussed above, studies identifying the role of immune function in correspondence with CYP1A activity are limited, with most research focussed on dioxin-like coplanar PCB congeners due to their affinity for Ah-R and CYP1A-induction.

1.3.3 Metals

Heavy metals are a source of contamination that has affected the aquatic environment for many years. Heavy metal contamination is a result of industrial and mineral mining sources, municipal wastes and surface runoffs (Zelikoff, 1993). Metals at low concentrations are essential for biological processes, but in excess elicit toxicity. Metals such as copper, zinc, iron, cobalt, selenium, and manganese are essential for the health of most organisms. These metals form important components of proteins that are involved in biological functions (Bury et al, 2003) such as the participation in the control of metabolic and signalling pathways (Valko et al, 2005), cellular respiration, oxygen transport, protein stability, free radical scavenging, action of many cellular enzymes, and DNA transcription (Bury et al, 2003)**.** The breakdown of any of these mechanisms or excess exposure to these metals, can result in metals inappropriately binding to protein sites or forming dangerous free radicals (Bury et al, 2003) that can result in DNA damage, lipid peroxidation, or depletion of protein (Valko et al, 2005). Most vertebrate species have mechanisms that reduce the damaging effect of excess metals. Proteins that are capable of binding metals within the organism include the metallothioneins (Monserrat et al, 2007). Metallothioneins are a cystiene rich metal binding family of proteins that are essential for the intracellular processing of metal ions (Sweet and Zelikoff, 2001). Metallothioneins can limit the availability of toxic cations at undesirable sites, decreasing the sensitivity of the organism to metal exposure (Monserrat et al, 2007).

Metals have also been found to have adverse effects on the immune system of fish exposed to high levels. Metals directly bind or readjust the tertiary structure of active molecules, or act as stressors and modify corticosteroid levels in the fish (Zelikoff, 1994). Metals can also have major impacts on the structure and function of the immune response at sublethal doses during disease outbreaks (Arunkumar, 2000). Metals depress the humoral immune system by inducing DNA strand breaks and altering lymphocyte proliferation (Arunkumar, 2000). Metal toxicity appears to depend on many environmental factors. It has been identified that metals induce greater toxicity at low dissolved oxygen, are less toxic in hard water due to competitive binding from other ions, and leach different metals to become more bioavailable in acidic environments (Witeska & Jezierska, 2003). Macrophage activity, cell number and humoral immunity have been shown to be suppressed as a result of metal toxicity, ultimately leading to an increased susceptibility to disease (Zelikoff, 1994). The primary routes of metal exposure in fish are uptake from the gills, dermal absorption and ingestion (Sweet & Zelikoff, 2001).

1.3.4 Pesticides

Pesticides are toxic chemicals that are released into the environment, used to control pest species such as insects, weeds or fungi. They contaminate aquatic habitats mainly through aerial drift, accidental release and surface runoff (Zelikoff, 1994). Organochlorine pesticides are persistent in the environment and their use has been banned since the 1970's, when DDT and their metabolites were found to bioaccumulate and cause adverse health effects (Galloway & Handy, 2003). DDT is carcinogenic, and is associated with tumour formation (Zelikoff, 1994). Organochlorine pesticides were replaced with organophosphorous pesticides due to their low persistence but high acute toxicity (Galloway & Hardy, 2003). Recent studies have shown that

the exposure of organophosphorous pesticides modulate the immune function by altering lymphocyte proliferation, affecting disease resistance, reduction in oxidative burst and resulting in histopathological changes to immune tissues and organs (Galloway & Handy, 2003; Albert et al, 2007; Gilbertson et al, 2003). Organophosphorus and carbamates, are known to inhibit cholinesterase (ChE) activity (Monserrat et al, 2007), but also act through non-choligenic pathways (Galloway & Hardy, 2003). Organophosphous pesticides are neurotoxic agents and act by inhibiting acetylcholinesterase leading to an increase in acytlcholine that disturbs transmission across synapses. They can also inhibit serine hydrolase enzymes that are vital to immune function (i.e. complement) (Galloway & Handy, 2003). Organochlorines have been found to induce CYP1A expression (Quirós et al, 2007) through the Ah-R receptor, as discussed above.

1.4 Adaptations in aquatic wildlife to contaminants

Despite the contaminated habitat, aquatic species populations are still able to thrive. Survival of populations depends on the ability of the organism to elicit resistance, either due to genetic adaptation or physiological acclimations (Wirgin and Waldman, 2004). Fish can be one of the most significant aquatic species affected by contaminated waters, due to both their ecological role in the aquatic food web and the fact that they can be found almost everywhere in the aquatic environment (Reviewed in Van der Oost et al, 2003). Studies demonstrating such adaptations in fish species exposed to toxicants have previously identified that the enzyme CYP1A (induced by and catalyses the metabolism of organic contaminants), attains resistance to induction after chronic exposure to PCB compounds in the mummichogs, (*Fundulus heteroclitus*) (Elskus et al, 1999) and in rainbow trout (*Oncorhynchus mykiss*) (Celander &

Forlin, 1995). Mummichogs collected from dioxin-contaminated and from PAH contaminated waters have been less sensitive to toxicological chemical challenges compared to reference site collected fish (Reviewed in Nacci et al, 1999, Prince and Cooper 1995; Elskus et al. 1999). It has also been shown that perch (*Perca fluviatilis)* obtained from waters containing high levels of PCBs had decreased P450 1A and enzyme activity (Förlin & Celander, 1995). Genetic adaptation has been recently identified in Atlantic tomcod (*Microgadus tomcod*) showing a high frequency of the AHR receptor allele consisting of a 6bp deletion resulting in impaired binding to TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) (Wirgin et al, 2011).

1.5 Immune Function in Fish

The immune system is essential for defending the host against pathogenic bacteria, viruses and parasites, and is pivotal for survival. It is a highly complex defence mechanism that can be organized into innate and adaptive or acquired responses that collectively work to prevent disease and infection (Bowden, 2008). The innate immune response precedes the adaptive, and is non specific against foreign particles. It recognizes proteins and receptors of microbes by identifying the molecular patterns by the variety of pattern recognition receptors (Purcell et al, 2006; Reviewed in Magnadottir, 2006). Phagocytic cells such as granulocytes (neutropihls), monocytes and macrophages, along with non-specific cytotoxic cells, epithelial and dendritic cells are all involved in the innate response (Shen et al, 2002; Neumann et al, 2001). Innate immunity is the first line of defence, and fish scales, mucous surfaces and the epidermis are all the initial barriers preventing infection in fish (Aoki et al, 2008; Ellis, 2001). Innate immune cells provide mechanisms to remove the invading pathogen internally and can also aid in the

initiation of the adaptive immune response. Respiratory burst is an innate defence mechanism used by macrophages, esonophils and neutrophils against bacterial infections by producing a series of reactive oxygen species (ROS) (Lilius and Marnila, 1992; Serada et al, 2005). When activated through a phagocytic or soluble stimulus, there is an increase in oxygen consumption and glucose metabolism by the cell. Phagocytes contain the enzyme NADPH-oxidase that will reduce oxygen molecules to superoxide molecules, which in turn can form hydrogen peroxide, catalyzed by the enzyme superoxide dimutase within the cell. Hydrogen peroxide is the substrate for the myeloperoxidase reaction, in which different toxic metabolites are formed. Phagocytes use the generated oxygen radicals to cause oxidation of the microorganisms surface and decarboxylation of cell membranes to elicit bacterial injury (Reviewed in Fantone, J., 1982). Phagocytic innate immune cells are also capable of presenting portions of the pathogen to adaptive immune cells, specifically lymphocytes, through the Major Histocompatibility (MH) peptide grooves (Cuesta et al, 2006; Nath et al, 2006). MH class I presents peptides to cytotoxic T lymphocytes when confronted with intracellular pathogens (Dijkstra et al, 2007), while MH class II presents peptides to helper T cells dealing with extracellular pathogens (Cuesta et al, 2006). Innate responses are mediated by the secretion of many different cytokines. Cytokines act to both attract and activate initial innate immune cells in response to infection or injury (Magnadottir, 2006). Interleukin 1-beta (IL-1β) is part of the IL-1 gene family and is an early response pro-inflammatory cytokine, and plays a role in both the innate and adaptive immune response by stimulating the release of inflamatory and chemotactic eicosanoids (Wang et al, 2006, Ellis, 2001). IL-1β is secreted by a variety of cells including neutrophils and both T and B lymphocytes, but is mainly secreted by monocytes and macrophages (reviewed by Bird et al,

2002). Interleukin 8 (IL-8) is also an early response cytokine, attracting and activating migratory neutrophils to sites of damage or inflammation (Zhang et al, 2002). Interferons (IFNs) are proteins secreted by a variety of cells and have anti-viral activity. Type 1 IFNs (α and β) are secreted by innate immune cells (i.e macrophages; reviewed in Pestka et al, 2004), and have been linked to both innate and adaptive immunity in response to viral activity, and specifically activates T-lymphocytes (reviewed in Pestka et al, 2004).

Innate immunity is also influential in both activating and determining the nature of the adaptive response (Fearon and Locksley, 1996). Adaptive immunity consists of T, B, and antigen presenting cells. It is stimulated through the activation of innate phagocytes, increases in cytokine and chemokine production, and also the activation of the complement system (Shen et al, 2002; Klesius, 1992; Aoki et al, 2008). Antibody producing cells (analogous to mammalian B cells and plasma cells) express the immunoglobulin on the cell surface, and their proliferation can be induced by the mitogen lipopolysaccharide (Zelikoff, 1994). Fish B cells secrete IgM that can opsonize and induce complement activation (Sakai, 1984). Fish T cells can be induced by phytohemagglutin and concanavalin A. These T cells are involved in the cell mediated immunity in fish (Zelikoff, 1994).

1.6 Immunotoxicity

1.6.1 Immune Function in Response to PAH Exposure

Several studies identifying the relationship between several immune functions and PAHs have been conducted, with varying results. These differences may be due to the different compound mixtures of PAHs or to the ability of the fish to metabolize them (Logan, 2007). Kennedy and Farrell (2008) measured the respiratory burst of activity in fish macrophages

exposed to high and low concentrations of hydrocarbons for short and long term durations, identifying that short term exposure at the highest concentrations enhanced macrophage ROS production. Activated macrophages in mummichog collected from contaminated sites were also found to have elevated COX-2 levels. The increased COX-2 levels may be a result of enhanced metabolism of PAHs through cooxidation-peroxidase pathways (Frederick et al, 2007). On the other hand, oxidative burst was inhibited by creosote exposure in rainbow trout, returning to near control levels on day 28 exposure (Karrow et al, 2001). Sub-chronic exposure to hydrocarbons was also shown to reduce the respiratory burst of Pacific herring, (*Clupea pallasi)* (Kennedy & Farrell, 2008). Plasma lysozyme concentrations were reduced in short term exposure to high hydrocarbon concentrations (Kennedy & Farrell, 2008), but were also elevated in mummichog collected from a contaminated site compared to a reference site along the Elisabeth River, Virginia (Frederick et al, 2007). Phagocytic activity was elevated before returning to near control levels by day 28 of creosote exposure in rainbow trout (Karrow et al, 2001). Mummichog collected from a contaminated site compared to reference site showed a decrease in plasma IgM compared to those of a reference site (Frederick et al, 2007). Short term exposure to high hydrocarbon concentrations increased the resistance to *V. anguillarum*, but sub-chronic exposure increased the susceptibility (Kennedy & Farrell, 2008). To date, there are only a limited number of studies reporting the effects of PAHs on the immune response by the induction of cytochrome P450 (CYP1A). These studies mainly focussed on the response to two known PAHs, 3-methylcholanthrene (3-MC) and benzo[*a*]pyrene (BaP).

It has been demonstrated that *in vivo* exposure of both 3-methylcholanthrene and benzo[*a*]pyrene significantly depress both innate and humoral immunity (Carlson et al, 2004;

Reynaud et al, 2002). Benzo[*a*]pyrene suppressed lymphocyte proliferation in medaka (*Oryzias latipes*) at concentrations below those that activated CYP1A expression (Carlson et al, 2002). At higher concentrations, exposed medaka significantly induced CYP1A expression or activity within lymphoid tissue. The induction was observed specifically within distinct sub-populations of kidney mononuclear cells (Carlson et al, 2004). CYP1A and EROD expression in the head kidney and liver of rainbow trout was also elevated in response to injection of BaP (Nakayama et al, 2008). BaP also suppressed antibody-forming cell numbers, superoxide production by phagocytes and host resistance against bacteria when exposed to higher concentrations (Carlson, 2002). 3-MC, on the contrary, caused a rapid increase in the macrophage respiratory burst that was also shown to coincide with the induction of CYP1A and EROD activity in liver and head-kidney of carp (*Cyprinus carpio*)(Reynaud et al, 2002). In a separate study, carp head kidney macrophages incubated with 3-methylcholanthrene were also shown to increase respiratory burst when stimulated with PMA (Reynaud et al, 2001). However, similar to BaP, 3-methylcholanthrene also inhibited both B- and T-lymphocyte proliferation in carp (Reynaud & Deschaux, 2005). When stimulated with α-naphthoflavone (AhR antagonist and CYP1A inhibitor), the suppression of antibody forming cells was decreased (Carlson, 2002), the inhibition of renal EROD activity was observed (Carlson et al, 2004) and the reversal of 3-methylcholanthrene effect on respiratory burst activity was shown (Reynaud et al, 2002). However, the reversal of suppressed T and B cell proliferation was not identified (Reynaud and Deschaux, 2003), indicating that BaP and 3- methylcholanthrene may act on different cells or influenced by different metabolic metabolites. It was also identified that inhibition of CYPlA-mediated metabolism also reverses the immunotoxicity induced by benzo[*a*]pyrene-7,8-

dihydrodiol, but not benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE)(Carlson et al, 2004). BaP induced DNA-adducts have also been localized to the kidney, liver and spleen of the mummichog (Rose et al, 2001). B lymphocytes and granulocytes expressing inducible CYP1A protein have recently been identified as likely sites of BaP metabolism in the head kidney (Nakayama et al, 2008). Results of the work correlating BaP induced CYP1A expression and immune function have indicated that BaP suppression of medaka humoral immunity is dependent on the CYP1A production of BaP metabolites (Carlson et al, 2004).

1.6.2 Immune Function in Response to PCBs

PCB 126 exposure at high doses increased unstimulated production of superoxide anions in both juvenile (4-6 month old) and adult (12 -15 month old) Japanese medaka, but after stimulation decreased superoxide production in juvenile animals after 3 days exposure, and increased production after 14 days exposure. The exposure to PCB 126 had no impact on stimulated adult medaka phagocytic cells (Duffy et al, 2003). Non-specific cytotoxic cell activity was reduced at high doses of PCB 126, and respiratory burst by phagocytes was also reduced at both low and high concentrations in the channel catfish (*Ictalurus punctarus*) (Rice & Schlenk, 1995). These results were obtained with the induction of CYP1A expression in fish treated with high exposure concentrations (Duffy et al, 2003). Japanese medaka also showed an initial suppression in respiratory burst in stimulated phagocytes after PCB 126 exposure at day 3, before recovering and increasing respiratory burst at day 7 (Duffy et al, 2002). At low doses of PCB 126, antibody forming cell production increased in the channel catfish, but was unaffected at higher doses (Rice and Shlenk, 1995). PCB 153 exposure in bluegill sunfish (*Lepomis macrochirus*) increased the production of superoxide radicals while suppressing lymphocyte

proliferation (Duffy & Zelikoff, 2006). These results were obtained in the absence of CYP1A induction, identifying a second mechanism, in addition to the Ah-R pathway, that elicits immunotoxicity in fish exposed to PCBs.

1.6.3 Immune Function in Response to Metals

Chromium (Cr) is a heavy metal that is found in the effluent of many industries. It has been found to react with lymphocyte surface proteins and alter the response to stimuli, as well as reacting with replication proteins within the cell (Arunkumar, 2000). Exposure of carp leukocytes to chromium decreased both lymphocyte activation and phagocytic function of neutrophils. Changes in cell shape and depressed respiratory burst were also demonstrated in exposed neutrophils (Steinhagen et al, 2004). It was also shown that the antibody response to chromium is suppressed in the African mouth breeder (*Oreochromis mossambicus)* exposed to two forms of chromium. Spleen weight, splenocyte number and blood lymphocytes were also reduced in chromium exposed fish *(*Arunkumar, 2000).

Copper (Cu) has important roles in vertebrate neuro-endocrine functions, but can also act as an endocrine disrupting metal in the aquatic environment (Handy, 2003). Copper accumulates in the haemopoietic and lymphoid tissue in fish, inhibiting their growth, metabolic activity, fecundity, gonadal development and immune function (Sayer et al. 1992; Reviewed in Shariff et al, 2001). Acute exposure of copper to fish affects the gills, as well as respiratory and ion regulation functions (Handy, 2003). Sub-lethal concentrations of copper were found to increase oxidative activity of phagocytes and total immunoglobulin levels in carp (Dautremepuits et al, 2004), but were also found to produce no significant difference at low concentrations in a separate study (Shariff et al, 2001). Carp were also found to have increased white blood cell

counts and total immunoglobulin levels at higher concentrations, but a decrease in oxidative phagocyte activity (Shariff et al, 2001).

Mercury (Hg) is present in normal geochemical cycles such as erosion from soils or rocks, volcanic activity, flooding, and ocean sources. It is also found as an additive to dental amalgams, industrial emissions, electrical sources, mining and many other sources. It has been used for pigments in paint, pesticide and pulp and paper manufacturing, battery production and therapeutic medicines (Sweet & Zelikoff, 2001). Hg has been found in the lysosomes of kidney and liver cells as well as in the basal lamina of kidney proximal tubules in exposed fish (Sweet & Zelikoff, 2001). It has been shown that Hg affects serum C-reactive proteins, which may alter opsonization and complement activation. Hg also inhibits acetylcholinesterase activity through altering membrane permeability (Sweet & Zelikoff, 2001).

Zinc (Zn) is an essential nutrient to fish (Clearwater, 2002), and has been identified as an inducer of carp lymphocyte proliferation (Reviewed in Secombes et al, 1996) It has been known to reduce the toxicity of cadmium and mercury by increasing the production of metallothionein. When present in high concentrations however, Zn can cause growth retardation, histopathological alterations and death (Sanchez-Dardon et al, 1999). Carp exposed to zinc demonstrated an initial increase in leukocyte count, before decreasing in number due to reduction in lymphocyte and neutrophil numbers. A reduced phagocytic activity was also evident (Witeska & Kosciuk, 2003). Rainbow trout exposed to ZnCl showed inhibited phagocytosis of head kidney macrophages, and lymphocyte stimulation, but increased lysozyme activity (Sanchez-Dardon et al, 1999). Cytotoxic cell activity and lymphocyte cell counts were reduced, but

macrophage activity was enhanced by the exposure of Zn to Zebrafish (*Brachydanio rerio*) (Rougier et al, 1994).

Arsenic (Ar) is naturally found in soil, air and water. It can also be formed through the combustion of fossil fuels, metal smelting and from glass industries. Arsenic is also used in wood preservatives, insecticides, herbicides and different pigments (Reviewed in Lage at al, 2006). Arsenic has been implicated in the apoptosis of fin cells, liver inflammation, hyperplasia and necrosis, gall bladder inflammation and edema, abnormal lysosomes in fish hepatocytes and inactivation of the JAK-STAT pathway (Reviewed in Lage at al, 2006). Chronic exposure to arsenic has also been demonstrated to suppress B cell proliferation and reduce immunoglobulin levels in the Philippine catfish (*Clarias batrachus)* (Ghosh et al, 2007).

1.6.4 Immune Function in Response to Pesticides

Japanese medaka exposed to sublethal concentrations of the organophosphorous pesticide malathion, showed no significant effect on leukocyte count or T cell proliferation, but significantly decreased antibody plaque forming cell numbers in a dose-dependent manor (Beaman et al, 1999). At high concentration of organophosphate chlorpyrifos and insecticide esfenvalerate exposure in chinook salmon (*Oncorhynchus tshawytscha*) led to complete mortality, but high survival rate at low exposure concentrations (Eder et al, 2004). Exposure to naphthalene (main constituent of moth balls) increased respiratory burst in short term exposure, but decreased upon longer term exposure in European eel (*Anguilla anguilla L*). Lipid peroxidation as a result of respiratory burst was also significantly increased in both short and long term exposures of the same study (Ahmad et al, 2003). The main metabolite of

organchlorine DDT, *p,p′*-DDE, reduced cell viability and proliferation, as well as increasing apoptosis in exposed Chinook salmon (Misumi et al, 2005).

1.7 Brown Bullhead (Ameiurus *nebulosus*)

The brown bullhead is native to North America, common to the lower Great Lakes, and is abundant in Lake Erie and connecting rivers (DNR, 2010; Yang & Bauman, 2006). Brown bullheads spawn in the late spring and early summer in the months of May and June (Blumer, 1985). They are benthic fish and their diet consists of insects, plants, algae and molluscs, and can be found in ponds, swamps, lakes and rivers (Haws & Goodnight, 1962; DFO, 2010). These fish have been shown to tolerate polluted waters better than most other fish species, and as a result have been widely used as indicator species when studying effects of toxicants on fish and their sensitivity to contaminated sediment (Steyermark et al, 1999; Yang & Bauman, 2006; Arcand-Hoy & Metcalfe, 1999). The philopatric and benthic nature of the brown bullhead results in direct physical contact with sediment contaminants, making this fish a model for toxicant exposure.

1.8 Objectives of the Research

This research project is based on the hypothesis that fish populations exposed to high contaminant levels will attain resistance through acclimatization, tolerance or the ability to adapt to contaminant induced effects to survive. Acclimatization is becoming more resistant in response to earlier exposure at the individual level (Wirgin and Waldman, 2004), adaptation is a result of selection against individuals with lowest tolerance to contaminants (Ownby et al, 2002).

Immune responses were assessed in Brown bullhead (*Ameiurus nebulosus*) either chronically exposed or not exposed to high levels of sediment containing environmental toxicants. Brown bullheads were collected from various pre-determined locations along the Detroit River, prior to transport and sediment exposure at the University of Waterloo. This project can provide an understanding of genetic adaptations or physiological acclimatizations of the brown bullhead immune function to prolonged contaminant exposure. The project will also provide an understanding of the impacts of chronic exposure to contaminants on the ability of the immune function to recover when removed from contaminant exposure. This information can be used in the understanding of remediation projects and the effects on currently affected populations of aquatic species, along with providing essential information concerning the critical levels of contaminants requiring clean up measures.

Materials and Methods

2.1 Cloning of Brown Bullhead (*Ameiurus nebulosus*) Immune Related Genes

2.1.1 Initial Tissue Extraction

Brown Bullhead purchased from the commercial fishery Kendall Dewey in Bay of Quinte (Picton, Ontario) were housed at University of Guelph Aquatic Facility (Guelph, Ontario). Two bullheads were euthanized in an overdose of 2mL/L 2-phenoxyethanol (Sigma, #P1126, Oakville Ontario). The spleen, head kidney, liver and gill were removed from each fish and placed in RNA later (2.5mM Na citrate, 5.3M $(NH_4)_2SO_4$, 0.01M EDTA, pH 5.2) in 1.5mL large cap graduated microtubes (Diamed, #AD151-N500, Mississauga Ontario) on ice. Two millilitres of blood was drawn from the caudal sinus using 5mL syringes (BD, #309602, Mississauga Ontario) and 23 gauge needles (BD, #305193, Mississauga Ontario) with a final concentration of 30U/mL of ammonium heparin (Sigma, #H6279, Oakville Ontario). Blood was transferred to sterile 15mL falcon tubes (BD, #352096, Mississauga Ontario) and stored on ice. Samples were stored on ice during transport back to University of Waterloo before long term storage at -80^{o}C.

2.1.2 Peripheral Blood Leukocytes (PBL) Extraction from Blood

Whole blood in 15mL conical centrifuge tubes (BD, #352096, Mississauga Ontario) was centrifuged at 200xg for 10 minutes at room temperature in an Eppendorf swing-bucket rotor 5810R (Eppendorf, Mississauga Ontario). The white buffy coat containing blood leukocytes was removed using a sterile Pasteur pipette (VWR, 14672-380, Mississauga Ontario) and transferred to a 50mL sterile falcon tube (BD #352070, Mississauga Ontario). The volume was brought to 45mL with collection media Leibovitz-15(L-15) (Sigma, #L1518, Oakville) and the cell

suspension was split equally into 6 sterile 15mL falcon tubes (BD, #352096, Mississauga Ontario). Three milliliters of Histopaque 1077 (Sigma, #10771, Oakville) was added to the bottom of each tube, and centrifuged for 30 minutes at 400xg at room temperature in swing-bucket rotor 5810R centrifuge (Eppendorf, Mississauga Ontario). The white leukocyte layer located at the media:histopaque interface was collected from each 15mL falcon tube using sterile Pasteur pipettes and pooled into a new sterile 15mL falcon tube. Equal volume of L-15 media was added to the collected leukocyte layer, and cells were pelleted by centrifugation at 400xg for 5 minutes at room temperature. The supernatant was aspirated and the pellet was washed using 1mL of 1X phosphate buffered saline (PBS) (137mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4. 7H2O, and 1.4 mM KH2PO4; pH 7.4) followed by centrifugation again at 400xg for 5 minutes at room temperature. The PBS was aspirated and the pellet was stored at -80^{o}C or used for RNA extraction.

2.1.3 RNA Extraction and cDNA Synthesis

Extraction of RNA followed a revised procedure of (Chomczynski and Sacchi, 1987). A maximum of 80mg tissue sample or cell pellet was weighed and quickly placed into 500uL of Trizol (Invitrogen #15596-018, Burlington) contained in a 1.5mL large cap graduated microtube (Diamed #AD151-N500, Mississauga Ont.). Using a plastic pellet pestle the tissues were homogenized before the addition of another 500uL of Trizol (Invitrogen #15596-018, Burlington Ontario). DNA and proteins were removed from the mixture through the addition of 200uL chloroform (Fisher Scientific #C574-1, Ottawa Ontario) and subsequent vigorous vortex followed by centrifugation at 12000xg for 15min at room temperature. The supernatant of the

suspension was collected and transferred to a new 1.5mL microcentrifuge tube and placed on ice. Total RNA was precipitated by the addition of 500uL isopropanol (EMD Chemicals #PX1835-2, USA) to the supernatant, mixed by inversion and allowed to stand for 10 minutes on ice before centrifugation at 12000xg for 10 minutes at room temperature. The supernatant was removed and the pellet was rinsed with 1mL of 75% Ethyl alcohol (Commercial Alcohols Inc, Brampton Ontario) made with diethyl pyrocarbonate (DEPC) (Sigma #D5758, Oakville Ontario) treated water. The pellet was centrifuged at 7500xg for 5 minutes at room temperature, the ethanol was aspirated and the pellet was allowed to dry at room temperature in the fume hood for 30 minutes. Thirty microliters of DEPC treated water was added to re-suspend the pellet and placed in a heating block incubator at 70^{o}C for 10 minutes. The RNA solution was mixed by pipetting and the concentration measured on a NanoDrop spectrophotometer ND 1000 (NanoDrop Technologies Inc, Wilmington Delaware). RNA samples were diluted to a final concentration of 0.5ug/mL in DEPC treated water and used for first strand cDNA synthesis using Fermentas RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, #K1632, Burlington Ontario), following the manufacturer's instructions.

2.1.4 Sequence Alignment and Primer Design

Channel catfish immune related gene sequences were obtained through NCBI database. Species-specific primers were designed for Channel catfish gene sequences in anticipation of sequence similarity to Brown bullhead. Each primer was based on a specific gene region of interest, and primer specifications were determined using Integrated DNA Technologies Oligo Analyzer 3.1. Specific immune related genes are listed in Table 2.1. Primers were obtained through Sigma Aldrich (Oakville, Ontario).

Table 2.1: Primer Design for Channel Catfish Immune Related Gene Sequences

Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Region of Interest	Template RNA
Elongation Factor 1 alpha (Ef1a)	GGAGGTATTGGAACTGTACCC	GAGAAT TCACTTGGTCTTGGC	EST CB940917 Nucleotide 64-689 of coding region	PBL cDNA
Beta 2 microglobulin (B2M)	CAGGTTTACAGTCGTAACCCTGG	TTCAGGTGTCTGACTCTGCAGG	gi:3779214 nucleotide 79-314 of coding region	Liver cDNA
Interleukin 8 (IL-8)	ATGAAGGCTGCAACTCTCACAG	GGCAGACCTTCATTCCTTGC	gi: 24496449 nucleotide 1 to 240 of coding region	PBL cDNA
Interleukin 1 Beta type b (IL-1Bb)	AATGTTGTAATCGCTTTGCAGAG	CTGACTCGAAGGTGTTAAGGG	gi:78707324 nucleotide 196-761 in coding sequence	PBL cDNA
Interleukin 1 Beta type a (IL-1Ba)	CAATGTCTTCATTGAGAATGTG	CCTCTGCACCAAAGCCCT	DQ157743.1 Nucleotide 78-217 in coding sequence	SPLEEN cDNA
Major Histocompatibility Class 1 alpha (MHIA)	CTCTGGAGAGGAAAGTTCCTCC	TGTGTTTCTGCAGATCCTCAG	gi:2995928 The α3 domain starts at nucleotide 549 – 846.	Liver cDNA
Major Histocompatibility Class II Alpha (MHIIA)	GAACACACTTATCTGCCACT	GTGCAGGTGTACACGTCTCCTTC	gi:7831247 α2 domain (exon 3) starts at nucleotide 270 to534. Used DAA allele.	Liver cDNA
Major Histocompatibility Class II Beta (MHIIB)	GTTGGGAATTACTACAGCGGC	GATCTCCTCTCCAGATTCAGG	gi:1763554 beta-2 domain to cytoplasmic tail is from nucleotide 279 – 558.	PBL cDNA
Immunoglobulin Mu (IgM)	TTGAGAGAGAGCTCCATGCATC	GGTTCTTCTGCTGGAATTCGC	X52617 Catfish Igh for immunoglobulin heavy chain (mu) CH2	PBL cDNA

2.1.5 Polymerase Chain Reaction (PCR)

Amplification of partial Brown bullhead gene sequences was accomplished by PCR. Briefly, 17.7uL autoclaved milliQ water, 1.5uL of 25mM $MgCl_2$ (MP Biomedicals, #EPMG, Solon Ohio), 2.5uL Incubation mix T.pol without $MgCl_2$ (MP, EPXMG), 0.5uL 10mM dNTP mix (Fermentas, #R0192, Burlington Ontario), 0.1uL 5U Taq (MP Biomedicals, #EPTQA025, Solon Ohio), 1uL for each 10nmol primer (Sigma, Oakville Ontario) and between 25-50ng template DNA were mixed on ice in 0.2mL flat-cap PCR tubes (Thermo Scientific, #AB-0620, Rochester New York). PCR amplifications were performed with a single annealing temperature on the BioRad DNA Engine thermocycler (Biorad, Mississauga Ontario), or varying annealing temperatures on the Mastercycler gradient (Eppendorf, Mississauga Ontario) using the profile of 95^{o}C for 5 minutes followed by 35 cycles of 95^{o}C for 40 seconds, annealing temperature for 30 seconds, and 72^{o}C for 30 seconds, ending with 72^{o}C for 15 minutes. Amplified products were run on 1-1.5% agarose gel (Bioshop, #AGA001, Burlington) and visualized using GelRed nucleic acid gel stain (Biotium, #41003, Hayward California). Sigma GenElute Agarose Spin Columns (#5-6500, Oakville) were used to purify DNA following manufactures protocol. DNA was eluted with autoclaved milliQ water and stored at -20^{o}C.

2.1.6 Ligation and Transformation

PCR fragments were cloned using the pGEM-T easy vector kit (Promega, #A1360, Madison Wisconsin). On ice, 3uL of the purified PCR product was combined in a 1.5mL centrifuge tubes on ice with 1uL pGEM-T Easy vector (50ng/uL), 5uL 2X ligation buffer (Promega, #C671A, Madison Wisconsin), and 1uL T4 DNA ligase (Promega, #M180A, Madison

Wisconsin). The ligation mixture was incubated for 1 hr at room temperature. XL1-Blue MRF competent cells were thawed on ice and 45uL were added to the ligation product and left on ice for 30 minutes. The cells were heat-shocked by placing the tubes into a water bath at 42^{o}C for 55 seconds before quickly returning them to ice for 3 minutes. Two hundred and fifty microliters of SOC media (2% yeast extract, 0.5% tryptone, 8.5 mM sodium chloride, 2M magnesium chloride, 2.5 mM potassium chloride and 2M glucose) was added and the mixture was placed in a shaking water bath at 37^{o}C, shaking at 225rpm for 1 hour. Luria Bertani (LB) (0.1% bacto tryptone, 0.5% bacto yeast extract, 0.15% bacto agar and 0.17M NaCl) media coated plates containing an ampicillin (Amp) concentration of 100ug/mL were prepared by the addition of 20ul of 4% XGAL(Fermentas,#R0401, Burlington Ontario) and 100uL 0.1M IPTG (Fermentas, #R0391, Burlington Ontario). Transformed products were transferred to prepared LB/Amp plates and spread in volumes of 10% and 90% of total volume. LB/AMP plates were left for 15 minutes to allow absorption of transformed cells before incubation overnight at 37^{o}C.

2.1.7 Colony selection and Alkaline Lysis Mini-Prep

Blue-white selection was used to pick inserts containing ampicillin resistant colonies before inoculating 4mL LB media (0.1% bacto tryptone, 0.5% bacto yeast extract, 0.17M NaCl pH 7) containing a concentration of ampicillin of 100ug/mL. Media was incubated overnight for 14-18 hours in a 37^{o}C shaking incubator. Plasmid DNA was collected using a modification of Daniel's modified alkaline miniprep (Sambrook *et. al.* 1989). Briefly, 1.5mL of overnight culture was transferred to a new 1.5mL large cap graduated microtubes (Diamed #AD151-N500, Mississauga Ont) and centrifuged at 15,000xg for 5 minutes at room temperature in an IEC

Micromax bench top microcentrifuge (International Equipment Company, Needham Massachusetts). With the supernatant decanted, 200uL of Solution 1 (100mM Tris-HCl pH 8, 10mM EDTA, 400ug.mL RNase A) was added and the bacterial pellet completely re-suspended. Lysis of the bacterial cells was accomplished by the addition of 200uL of Solution 2 (200mM NaOH, 1%SDS) with gentle inversion of the tubes. The cells were neutralized by the addition of 200uL of Solution 3 (3M K-Acetate, 5M Acetic Acid), mixed and incubated on ice for 5 minutes. Tubes were centrifuged at 15,000xg for 5 minutes at room temperature to remove cellular proteins. Supernatant containing plasmid DNA was transferred to a new 1.5mL microcentrifuge tube and precipitated by the addition of 420uL isopropanol alcohol (EMD Chemicals #PX1835-2, USA) and incubated for 10 minutes at room temperature. Precipitated plasmid DNA was centrifuged for 10 minutes at 15,000xg at room temperature, supernatant decanted and pellet was washed by the addition of 500uL 70% Ethyl alcohol (Commercial Alcohols Inc, Brampton Ont.). Tubes were centrifuged for 5 minutes at 15,000xg at room temperature, ethanol completely removed before the addition of 18uL autoclaved milliQ water to re-suspend the DNA. DNA quantity and quality was measured on a NanoDrop spectrophotometer ND 1000.

2.1.8 Sample Digest and Sequencing

Upon completion of alkaline lysis mini-prep, a digest was conducted to confirm the presence of a DNA insert within pGEM T easy plasmid vector. One microliter of either EcoRI (Fermentas, #FD0274, Burlington Ontario) or NOTI (Fermentas, #FD0614, Burlington Ontario) Fast Digest enzymes were added to 2uL 10X Fast Digest Buffer (Fermentas, Burlington Ontario) and 0.5ug/uL plasmid DNA, incubated at 37^{o}C for 1 hour before running on 1-1.5% agarose

(Bioshop, #AGA001, Burlington Ontario) gel with 1-1.5uL GelRed nucleic acid stain (Biotium #41003, Hayward California) for insert confirmation. Plasmid DNA containing brown bullhead DNA insert were sent to TCAG Sequencing Facility (Toronto, Ontario) to determine gene sequence. Plasmid DNA was diluted to a total of 250-300ng in a total volume of 7uL in autoclaved milliQ water. Once DNA clones were sequenced, an NCBI BLAST was conducted to determine identity of sequence. Sequences were aligned with channel catfish related sequences to determine species differences using default settings of Clustal W as a function in BioEdit software.

2.2 Field Collection and Sediment Exposure Study

2.2.1 Collection and Transportation

Brown bullheads were collected from pre-designated sites along the Detroit River (Windsor, Ontario) by boat electrofishing. Two sites represented "clean" sites; Belle River and Peche Island, and two represented contaminated sites; Trenton Channel and Belle Island. Map of designated sites can be seen below in Figure 2.1.

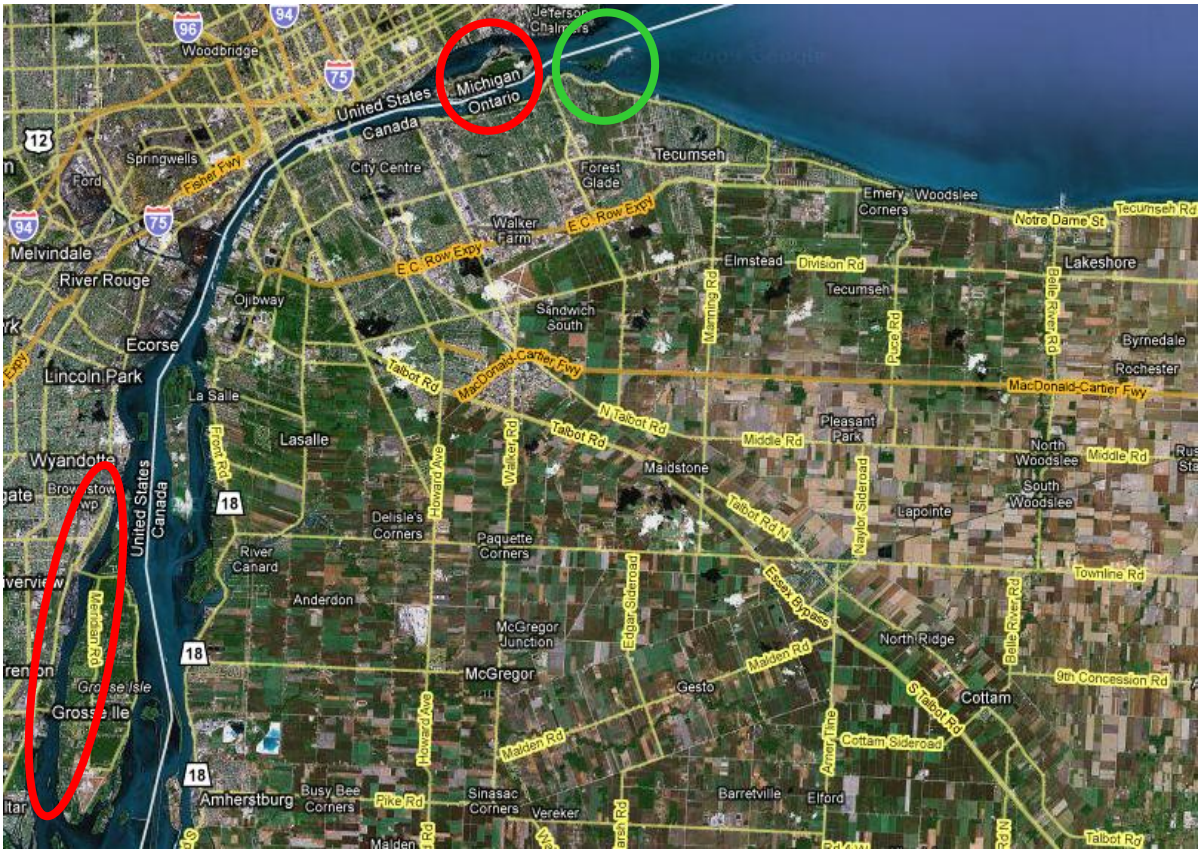

Figure 2.1 Map of pre-determined sites along the Detroit River. Trenton Channel (bottom left circled in red), Belle Island (top left circled in red) and Peche Island (top middle circled in green).

Bullheads were captured and injected with a Passive Integrated Transponder (PIT tag) in spring 2008 and held in clay ponds at Leadley Environmental Inc. (Essex, Ontario) for 1 year to generate an F1 population. Bullheads were captured from the designated sites in spring 2009 to be held for 4 months again in clay ponds at Leadley Environmental Inc (Essex, Ontario). These fish were identified as "Cleared Adults" and were each PIT-tagged for identification. In Fall 2009, the capture of bullheads from each site was attempted with limited success. Bullhead capture in Fall 2009 were identified as "Acute Adults". Spring 2010 Acute Adults were collected

from each site and held at Leadley Environmental Inc. for 2 weeks. In November of 2009, 20 F1 bullheads were tagged with coloured fishing line sewn into their gill plates from each population of Belle Island (clear), Trenton channel (green), Peche Island (blue) and Belle River (black) and were transported back to the University of Waterloo along with 20 Cleared Adults from each population from Belle Island, Trenton channel and Peche Island. Bullheads were transported via an aerator equipped wet tank. Due to the number of fish delivered, bullheads were divided into two holding tanks (4ft long X 4ft wide X 4ft deep and 6ft long X 6ft wide X 3ft deep), with constant flow, aeration and 12^{o}C water temperature. Bullheads were maintained in holding tanks to acclimate for a one week period with standard daylight savings time. Holding tanks were covered with insulation to provide protection for bullheads.

Spring 2010 Acute adults were held for 2 weeks prior to transportation back to the University due to requirements to complete the field sampling. Twenty acute bullheads from Peche Island and 19 acute bullheads from Trenton Channel were delivered to Waterloo in coolers with portable aerators (MengJin, #AP-1502, China).

2.2.2 Vaccination

Brown bullheads were removed from holding tanks upon completion of one week acclimatization and anesthetised in 1mL/L 2-phenoxyethanol (Sigma #P1126, Oakville Ontario). At which point 200ul blood was withdrawn from the caudal sinus of the F1 bullheads, and 500ul was withdrawn from the adults into a 1mL syringe (BD, #309602, Mississauga) with a 23 gauge needle (BD, #305193, Mississauga Ontario). Blood was transferred to 1.5mL large cap graduated microtubes (Diamed #AD151-N500, Mississauga Ontario) and allowed to clot overnight at 4^{o}C. Clotted blood was centrifuged at 1000xg for 10 minutes and serum was collected into new

labeled 1.5mL large cap graduated microtubes (Diamed #AD151-N500, Mississauga) and stored at -20^{o}C. One hundred microliters of *Vibrio anguillarum-ordalii* bacterin, Vibrogen 2 (Novartis Animal Health Canada, Ser 011540, Charlottetown PEI) was intraperitoneal injected into bullheads directly after blood removal. Bullheads from each population were divided and placed onto either sediment collected from PI representing a clean environment or contaminated collected from Trenton channel representing contaminated sediment for the exposure study.

2.2.3 Exposure Study

Two 4ft x 4ft x 4ft plastic tanks were equipped with sediment collected from Peche Island in Fall 2009 ("clean") or sediment collected from Trenton Channel in spring 2008 ("contaminated"), a slow flow through, water temperature of 12^{o}C, aeration, and insulation for cover protection. Bullheads were maintained for 24 days in each exposure tank, fed 5mm trout floating chow (Martin Mills, Elmira Ontario) at 3% body weight every third day. Bullheads were sampled for tissue extraction at 24 hours, 96 hours and 24 day time points, collected for respiratory burst assay at the 24 hour time point and bled for serum collection at the 14 and 21 day time points.

2.2.4 Respiratory Burst Assay

In order to determine neutrophil activity in whole blood, a luminol-enhanced chemiluminescence assay was used. Briefly, 90ul whole blood was removed from the caudal sinus of each bullhead into 1mL syringes (BD, #309602, Mississauga Ontario) using 23 gauge needles (BD, #305193, Mississauga) with a final concentration of ammonium heparin (Sigma, #H6279, Oakville Ontario) of 20 units/mL and placed on ice. Each well of the 96 well plates (Fisher Scientific, #3917, Ottawa Ontario) was pre-filled with 110ul Hanks balance salt solution

(Sigma, #H9394 Oakville Ontario), 10ul of whole blood from each individual bullhead was added in triplicates and to each well, and 60ul of 1mM working stock of luminol (Sigma, #A8511, Oakville Ontario) diluted in HBSS was added. Luminol stock was made at a concentration of 100mM by dissolving 17.7mg in 1mL cell culture grade dimethyl sulfoxide DMSO (Sigma, #D2650, Oakville Ontario). Finally, to each test well 20ul of 20mg/mL zymosan A (Sigma, #Z4250 Oakville Ontario) was added, and 20ul HBSS added to each control well. Zymosan A was prepared by first boiling 100mg zymosan A in 5mL HBSS for 30 minutes before centrifugation at 3000rpm for 10 minutes. The zymosan pellet was washed with 5mL HBSS and again centrifuged at 3,000 rpm for 10 minutes. The pellet was washed a second time before re-suspension in 5mL HBSS and storage at 4^{o}C. The wells of the plate were mixed by pipetting and the emissions were determined using a Victor3V 1420 Multilabel Counter (Perkin Elmer, Woodbridge Ontario). The program used had 99 repeated readings, 115 seconds between readings, and 5 second shaking of the plate directly before reading. No filter was used , and the emission readings were recorded in counts per second (CPS). Each well was measured for 0.1 seconds, and each bullhead test and control was done in triplicate. For each bullhead, the difference between the control and the test wells was determined, and the peak emission reading was used for comparison studies.

2.2.5 Enzyme Linked Immunosorbant Assay (ELISA)

In order to determine increases in antibody titre to the Vibrogen 2 vaccine administration, serum samples collected at time 0 prior to vaccination, and at Day 14 and 21 post-vaccination were used in an enzyme linked immunosorbant assay. *Vibrio anguillarum-ordalii* bacterin,

Vibrogen 2 (Novartis Animal Health Canada, Ser 011540, Charlottetown PEI) was placed in autoclaved 2mL screw cap microcentrifuge tubes (Diamed, #2340-00, Mississauga Ontario) along with 0.8mL of 0.1mm zirconia/silica beads (BioSpec Products, #11079101z, Bartlesville Oklahoma). The vaccine/bead mixture was used to disrupt the bacterial cell wall upon vigorous mixing at full speed using a mini bead-beater (BioSpec Products, Bartlesville Oklahoma) for 45 seconds before returning to ice for 1 minute incubations. The procedure was repeated 5 times for each tube of vaccine. Bead-beated vaccine was diluted 30% in coating buffer (35 mM NaHCO3, 15 mM Na2CO3, and 3 mM NaN3, pH 9.6) and 100uL was added to each well of NUNC 96 microwell plates (VWR, #62409-068, Mississauga Ontario) and allowed to incubate overnight at 4^{o}C. A 1/1000 dilution of Keyhole limpet hemocyanin (KLH) (Sigma, #H7017, Oakville Ontario) in coating buffer was added to positive control wells. One hundred microliters of coating buffer was added to one set of negative control wells to reflect non-specific binding of serum. Other control wells contained vaccine coated with serum, no secondary but tertiary and substrate to indicate tertiary non-specific binding (Goat anti-mouse IgG), and vaccine coated with no serum, secondary, tertiary and substrate to indicate non-specific binding of secondary antibody (11A2). Plates were blocked in 300ul of 5% skim milk powder in T-TBS (0.14 M NaCl, 2.7 mM KCL, 25 mM Tris, 0.5% Tween 20, pH 8) for 1 hour at 37^{o}C. Wells were washed 3 times in TBS-T before the addition of 100uL of serum diluted 1/50 in 1% BSA made in TBS-T and incubated for 1 hour at room temperature. Each serum sample was plated in triplicate. The monoclonal antibody 11A2 specific for the light chain of catfish IgM was added at a volume of 100uL and dilution of 1/100 in 1% BSA in TBS-T and incubated for 1 hour at room temperature after washing the wells with TBS-T. Goat anti-mouse IgG (whole molecule) Alkaline

Phosphatase conjugate antibody (Sigma, #A3562, Oakville) was added to probe each well at a dilution of 1/500 and incubated for additional 1 hour at room temperature. The plate was washed 3 times with TBS-T before the addition of 100uL of Sigma Fast p-nitrophenyl phosphate (p-NPP) tablet set (Sigma, #N2770, Mississauga Ontario) and incubated in the dark for 30 minutes. The reaction was stopped by the addition of 0.03M NaOH and the absorbance was measured at 405nm using Versamax microplate reader (Molecular Device, California USA). For each plate the wells containing only coating buffer to identify non specific binding of serum were averaged and subtracted from each reading to account for background, and the average of the triplicate absorbance were used to create a plot bar graph.

2.2.6 Tissue collection and RNA Extraction

Tissue extraction was completed at 24 hours, 96 hours and 24 days post vaccination and sediment exposure. Bullheads were euthanized in an overdose of 2mL/L 2-phenoxyethanol (Sigma, #P1126, Oakville Ontario). The spleen, gill, liver, head kidney, muscle and blood were removed and placed in RNA later (2.5mM Na citrate, 5.3M $(NH_4)_2SO_4$, 0.01M EDTA, pH 5.2). Tissue samples were stored in -20^oC for 1 month before being transferred to -80^oC. RNA extraction followed the same protocol listed above in section 2.1.3 RNA Extraction and cDNA Synthesis. RNA quantity and quality was measured in combination by NanoDrop spectrophotometer ND 1000 (NanoDrop Technologies Inc, Wilmington Delaware) and RNA agarose gels. Agarose gels were made with 110uL DEPC treated water, 16uL formaldehyde (Fisher Scientific, BP531, Ottawa Ontario) and 24mL 10x MOPS (0.2M MOPS pH 7.0, 20mM Sodium Acetate, 10mM EDTA pH 8). Running buffer consisted of 1X MOPS in milliQ water,

and gel was run for 3 hours at 60 volts using Owl B2 EasyCast Mini Gel System (Thermo Scientific, Rochester New York).

2.2.7 DNase Treatment and First Strand cDNA Synthesis

All RNA samples were treated with DNase for the removal of any genomic DNA contamination using Fermenatas DNase I, RNase-free kit (Fermentas, #EN0521, Burlington Ontario). A total of 2ug of extracted RNA was combined with 2uL of 10X reaction buffer with $MgCl_2$, 2uL DNase I RNase free (#EN0521), and DEPC-treated water (#R0601) to a total volume of 20uL in 0.2mL flat-cap PCR tubes (Thermo Scientific, #AB-0620, USA). The reaction was mixed by pipetting and incubated at 37^{o}C for 30 min. DNase treated RNA was transferred onto ice prior to the addition of 2uL 50mM EDTA, before incubating at 65^{o}C for 10 minutes. DNase-treated RNA preparation quantity and quality was measured again on a NanoDrop spectrophotometer ND 1000 (NanoDrop Technologies, Wilmington Delaware), and 0.5ug was used for first strand cDNA synthesis using Fermentas RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, #K1632, Burlington Ontario). Five hundred nanograms of template RNA was combined with 1uL oligo (dT)18 primer in a total volume of 12uL in 200uL PCR tubes (Thermo Scientific, #AB-0620, USA). The mixture was incubated at 65^{o}C for 5 minutes before returning to ice. To the mixture 4uL 5X Reaction Buffer, 1uL RiboLock RNase Inhibitor (20U/uL), 2uL 10mM dNTP Mix and 1uL RevertAid H Minus M-MuLV Reverse Transcriptase (200U/uL) were added to a total volume of 20uL. Mixtures were incubated for 60 min at 42^{o}C using the BioRad DNA Engine thermocycler (Biorad, Mississauga Ontario).

2.2.8 Brown Bullhead Real Time PCR Primer Design

Gene specific primers and probes for the cloned brown bullhead genes were developed using Primer Express 3.0 software. The primers can be found in Table 2.2.

Table 2.2 qRT-PCR Primer design including forward, reverse and probe sequences, and fragment size.

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'-3')	Fragment Size
EF1a	GTTCCTGGCAAGCCCATGT	AGCAACGGTCTGCCTCATGT	CTACCTATCCTCCTCTTGG	89
IL-8	TCTCACAGTGCTGCCCTTGA	TGGTACAAACAACGCACTGCT	CATCTTTGCACTGACTGC	90
IL-1B	GCTTTGGTGCAGAGGGAAAA	TAGGCCGAAAGGTTGAACCAT	CTGGCCTTTACTCTGAAGG	95
MHIa	TTCTTCCCCAAAACAGTGATG	CCATCCTGGTTGGGTAACGT	AGGACGTGCATGAGG	95
MHIIA	CGGTGTGGATGTGACGGATA	GTGAAGGTCAGGTGGGAGAA	TCAGTATTATCCGAATGAGG	89
MHIIB	GATGTGCAGCGCTTACAGATT	GTGGACGTCACACCTCCTTTA	ACCATCTCGGTGACCTG	90
IgM	TGAAAACGTGGACCTCAATAA	TTCACCGGCGCTGAGTTC	CCAGAATAAAGGTAACTTC	99
B2M	CCACCCCCCAGACATTACAA	ACTTCCAGCCCTTCTCAAAGG	CGCGGTGATTCCA	94

2.2.9 Brown Bullhead Real Time qPCR

Real time PCR 6,000 pmol Taqman probes (Applied Biosystems, Carlsbad California) for MHIIB, IL-8, IL-1B and EF1a as identified in Table 2 were custom ordered. Probes were MGB, all 5' labeled with VIC dye, with the exception of EF1a labeled with 6FAM dye. Probes were diluted 1:50 with RNAse/DNAse free water (Qiagen, Mississauga Ontario). Primers from Table 2 (Sigma, Oakville Ontario) were diluted to 10mmol. Each qPCR reaction contained endogenous control EF1a and one target gene primers and probe. One microliter of each EF1a primer (10mmol) and probe (1:50) were added to the qPCR master mix along with 1uL of the target gene primers (10mmol) and probe (1:50). Twelve and a half microlitres of IQ Supermix (BioRad, #170-8862, Mississauga Ontario), 0.4uL ROX (BioRad, #172-5858, Mississauga Ontario), and 1uL of cDNA template transcribed as stated in section 2.2.7 were added to the qPCR reaction to a final reaction volume of 20uL. MicroAmp Optical ninety six well clear PCR

plates (#N8010560, Applied Biosystems, Carlsbad California) were used, sealed using MicroAmp Optical adhesive film (#4360954, Applied Biosystems, Carlsbad California). Relative quantification qPCR program followed had initial cycles of 2 minutes at 50^{o}C and 10 minutes at 95^{o}C, followed by 40 cycles of 15 seconds at 95^{o}C and 1 minute at 60^{o}C using the Applied Biosystems 7300 qPCR machine. Each plate contained a positive control for plate normalization containing a cDNA template of fish 133 gill, and normalized to EF1a to allow for comparative studies between populations.

2.2.10 Relative Quantification (RQ) Study

RQ studies were completed for each gene of interest through the normalization of each target gene Ct to the Ct of Ef1a. Using the Applied Biosystem 7300 software, automatic Ct and average dCt were calculated before exported to Microsoft Excel. Target gene threshold cycles were normalized to elongation factor-1 alpha threshold cycle (dCt), and sample means were calculated before the creation of a clustered column bar graph.

2.2.11 Statistical Analysis

Statistical analysis was conducted using SPSS Statistics 17.0. Statistical outliers were removed by performing a linear regression and calculated by Cooks Distance outside of 2 standard deviations. The effects of age, sediment exposure and site for each assay were determined using a univariate full factorial ANOVA, corrected using Bonferroni post hoc tests. Data was split at the level of sediment, age or site to determine specific interactions. Normality was assessed by the Shapiro-Wilk test, and homogeneity of variance between groups was assessed by Levene's test.

Results

3.1 Cloning of Brown Bullhead (*Ameiurus nebulosus*) Immune Related Genes

3.1.1 Major Histocompatibility Class I Beta

Cloning of brown bullhead immune genes was accomplished using cDNA created from RNA isolated from spleen, gill or peripheral blood leukocytes (PBLs). The PCR reaction using primers specific for channel catfish Major Histocompatibility class II beta chain, flanking exon 2 (Beta 2 region) is outlined in Figure 1.4. The PCR products were separated on 1.5% agarose gel before visualization using Fluorochem 8000 imager, which uses Alpha Innotech imaging software (San Leandro California). Upon ligation into pGEM T-easy vector, transformation into E. coli XL1-Blue competent cells and miniprep sequencing of PCR DNA, the brown bullhead sequence was first subjected to a BLAST search using NCBI database. The BLAST search reviled a 98% identity to *Ictalurus punctatus* MHC class II beta chain (IcpuDAB*01 allele) (U77597.1) [e value 1e-131]. A sequence alignment of the brown bullhead partial clone of MHIIB and the coding region of channel catfish MHIIB (U77597.1) was created using Clustal W Alignment with default settings on the Bioedit software. The alignment is shown in Figure 3.2.

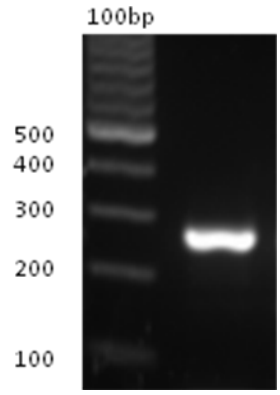

Figure 3.1 PCR amplification of brown bullhead MH II beta exon 2 amplicon of 267bp.

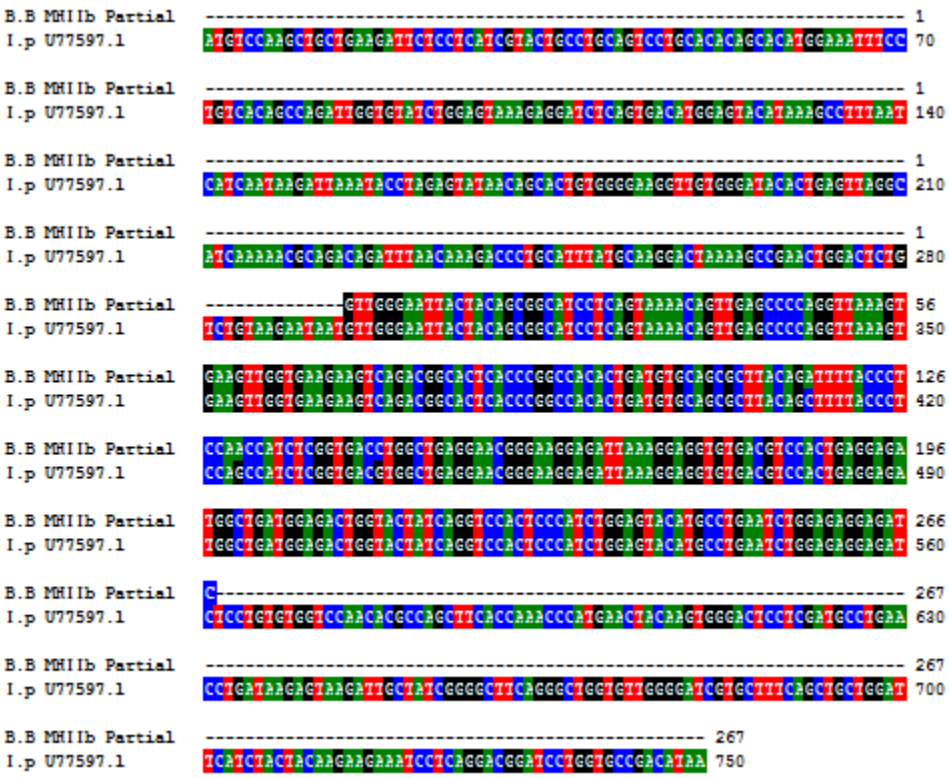

Figure 3.2 Nucleotide sequence alignment of cloned brown bullhead MH class II beta partial clone with the channel catfish MH class II sequence (U77597.1).

3.1.2 Major Histocompatibility Class II alpha

Figure 3.3 shows the PCR amplification of a brown bullhead MHII alpha chain fragment. The sequence alignment of *Ictalurus punctatus* isolate GASC2 MHC class IIA antigen (*Icpu*-DAA) gene, *Icpu*-DAA*10 allele, partial coding sequence (DQ811108.1)[e value 8e-73] and *Ameiurus nebulosus* isolate FLEBR4 MHC class IIA antigen (*Amne*-DAA) gene, *Amne*-DAA*08 allele, partial coding sequence (DQ811106.1)[e value 8e-68] showed 98% and 96% sequence identity respectively (figure 3.4).

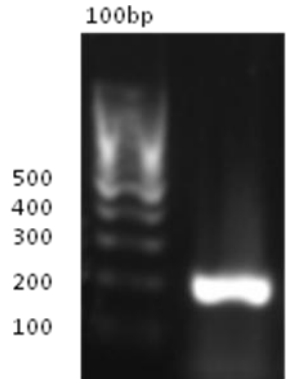

Figure 3.3 MH class II alpha PCR gradient amplification. Amplicon size is 186bp.

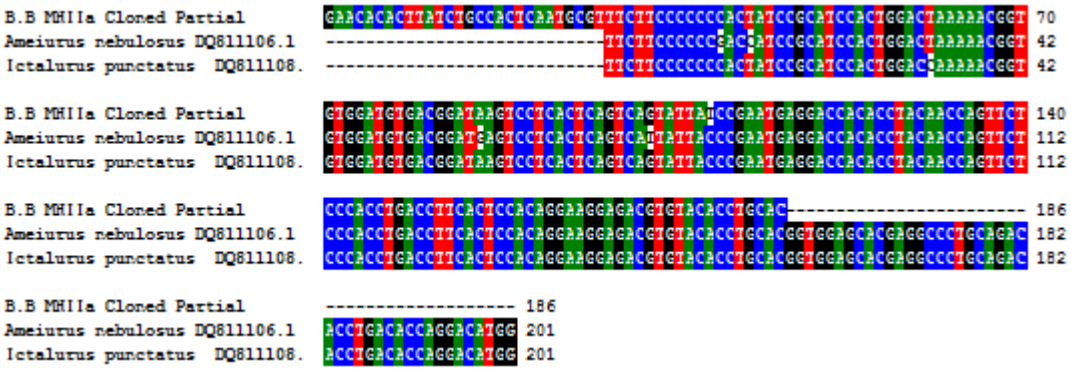

Figure 3.4 Sequence alignment of brown bullhead (B.B) partial clone, brown bullhead allele (DQ811106.1) and channel catfish MHIIa (DQ811106.1).

3.1.3 Major Histocompatibility Class I Alpha

The amplification of major histocompatibility class I alpha chain, specifically the alpha 3 region, is shown in figure 3.5. A BLAST search revealed a 97% identity to *Ictalurus punctatus* MHC class I alpha chain *Icpu*-UBA mRNA (AF053548.1) [e value 1e-106], and the sequence alignment is depicted in figure 3.6.

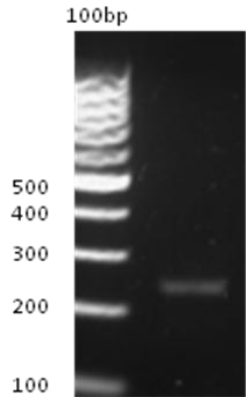

Figure 3.5 PCR amplification of a 231bp fragment of the brown bullhead MHIa gene.

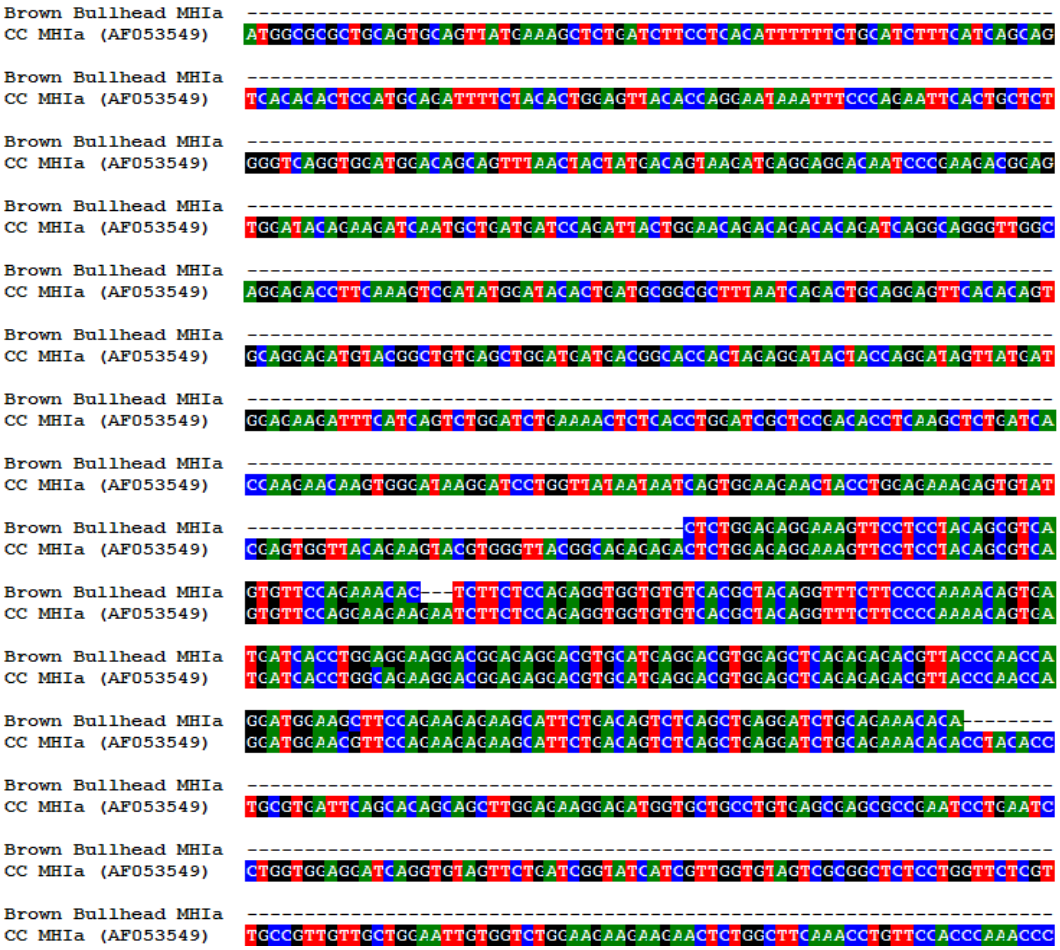

Figure 3.6 Sequence alignment of brown bullhead cloned MHIa alpha 3 region and channel catfish MHIa gene (AF053549).

3.1.4 Interleukin 8 (IL-8)

PCR amplification of the brown bullhead partial Interleukin 8 (IL-8) gene is shown in figure 3.7. A BLAST search indicated 93% identity to channel catfish interleukin 8 variant 3 (AY140804.1). Alternative splicing has been observed in channel catfish IL-8 sequence (Chen et al, 2005), but this was not identified in the brown bullhead. A ClustalW alignment was performed within the Bioedit program for the sequenced brown bullhead and channel catfish IL-8 genes as seen in Figure 3.8. The alignment identifies the 3 known alternative splicing variants of channel catfish IL-8, the only identified brown bullhead IL-8 partial sequence, and the qPCR amplicon for brown bullhead IL-8.

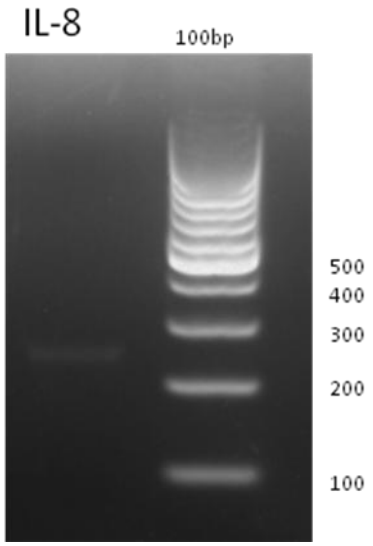

Figure 3.7 The 226bp brown bullhead fragment of interleukin-8 amplicon.

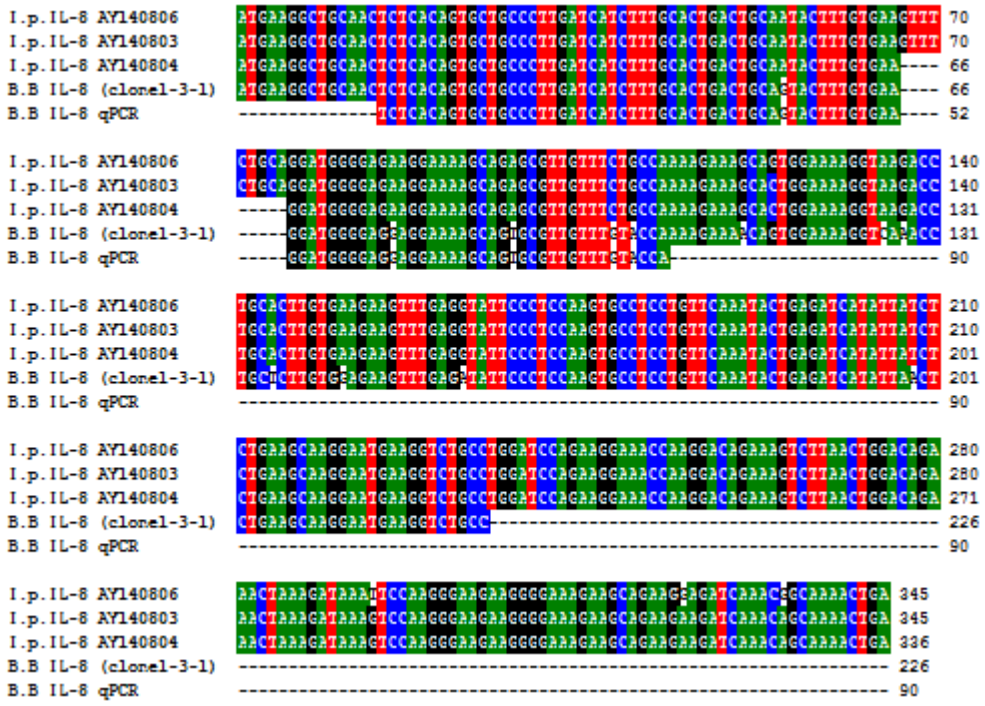

Figure 3.8 Sequence alignment of brown bullhead (B.B) partial interleukin 8 sequences and channel catfish Interleukin-8 alternative splicing variants.

3.1.5 Beta 2 Microglobluin (B2M)

The brown bullhead beta 2 microglobluin (B2M) PCR product can be viewed in figure 3.9, showing amplicons from the gill, liver and spleen in respective wells. The cloning of brown bullhead B2M revealed an 89% identity, which may in part be due to ambiguous sequence data to *Ictalurus punctatus* beta-2 microglobulin precursor mRNA (AF016041.1) [e value 4e-77] and the sequence alignment is graphically shown in figure 3.10.

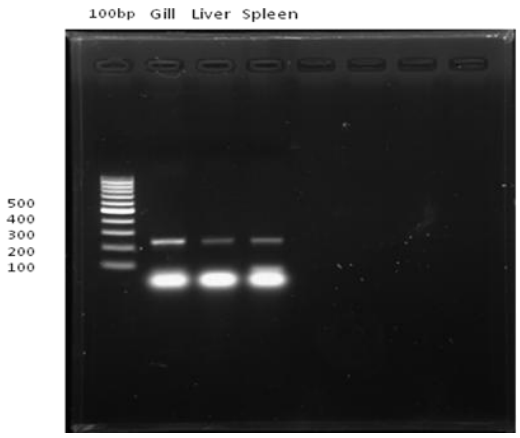

Figure 3.9 PCR amplification of the 234bp brown bullhead partial B2M fragment from gill, liver and spleen.

```
Brown Bullhead B2M   ------------------------------------------------------------
CC B2M (AF016041.1)  ATGAAGTTTCTGCTTTCCTTCGTCGTTCTCGCTGTTTTCAGCGCAAGTGCCTTTGCTAAG

Brown Bullhead B2M   ------------------CAGGTTTACAGTCGTAANC-TGGNAAGTTCGGCAAAGCCAAC
CC B2M (AF016041.1)  GAGTCTCCACCAAAAATCCAGGTTTACAGTCGTAACCCTGGTGAGTTTGGCAAACACAAC

Brown Bullhead B2M   ACACTCATCTGCNATN-GACAGACACTTCCACCCCCCAGACATTACAATCGACCTGCTCAAG
CC B2M (AF016041.1)  ACACTCATCTGCCACGTCAGTCACTTCCACCCCCCAGACATTACAATTGACCTGCTCAAG

Brown Bullhead B2M   AACGGCGCGGTCATTCCAGGTGCACAACACACCGACCTGGCCTTTCAGAAGGGCTGCAAG
CC B2M (AF016041.1)  AATGGCCAGGTCATTCCAAATGCAGAACACACCGACCTGGCCTTTCAGAAGGCATGCAAG

Brown Bullhead B2M   TTTCATCTCACCAAGAGCGTCGGTTTCACTCCCACCAGCGGCCACACATTCGCCTGCACA
CC B2M (AF016041.1)  TTTCATCTCACCAAGAGCGTCTCCTTCACTCCCACCAGCAATCACAAATTCACCTGCACA

Brown Bullhead B2M   GTCAGACACCTCAA----------------------------------------
CC B2M (AF016041.1)  GTCAGACACCTCAAGCAAACCAACAACATCTCCTGGCAGCCAGACATGTAA
```

Figure 3.10 Sequence alignment of cloned brown bullhead partial B2M and channel catfish (CC) B2M gene.

3.1.6 Immunoglobulin mu (IgM)

PCR amplification of immunoglobulin mu (IgM) constant region 2 (Exon 2) is shown in figure 3.11. The resulting partial cloned sequence shared 95% identity to catfish Igh DNA for immunoglobulin heavy chain (mu) (X52617.1) [e value 7e-120] and the sequence alignment can be seen in figure 3.12.

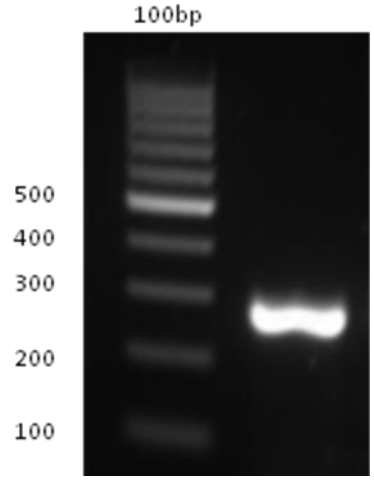

Figure 3.11 PCR amplification of brown bullhead IgM constant region 2. Amplicon size is 279bp.

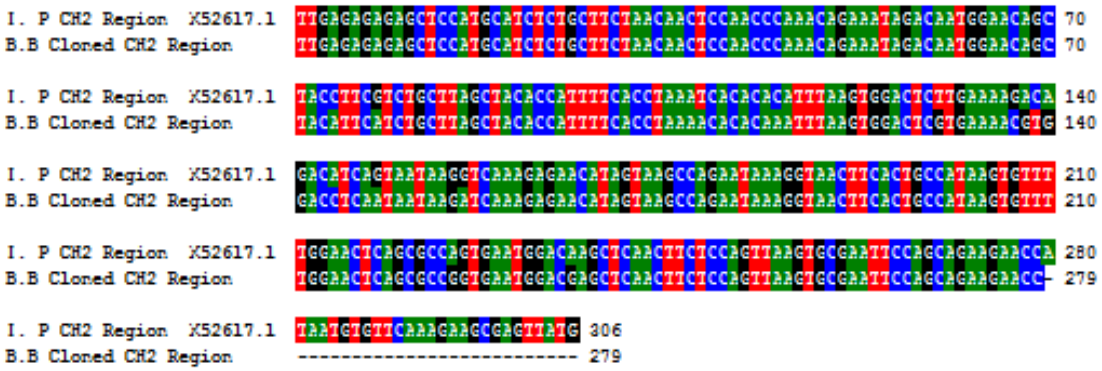

Figure 3.12 sequence alignment of brown bullhead (B.B) CH2 region and channel catfish CH2 region of IgM heavy chain (X52617.1).

3.1.7 Interleukin 1 beta (IL-1B)

The brown bullhead interleukin 1 beta (IL-1B) gene fragment amplified by PCR is shown in figure 3.13. A BLAST search indicated a 96% identity to *Ictalurus punctatus* interleukin 1 beta type a mRNA, complete cds (DQ157741.1)[e value 0] and 96% to *Ictalurus punctatus* interleukin 1 beta type b mRNA, complete cds (DQ157742.1)[e value 0]. Re-design of the primers allowed for the cloning of both type a and type b interleukin 1 beta genes. Amino acid sequence alignments and cNDA sequence alignments of brown bullhead b and partial channel catfish type a IL-1B can be seen in figure 3.14 and 3.15 respectively.

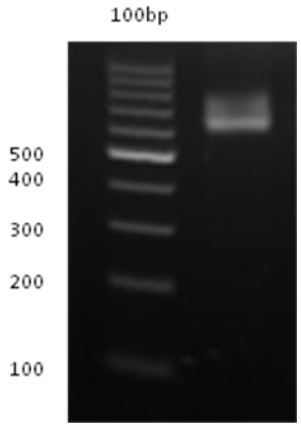

Figure 3.13 PCR amplification of brown bullhead interleukin 1 beta gene. Amplicon size is 523 bp.

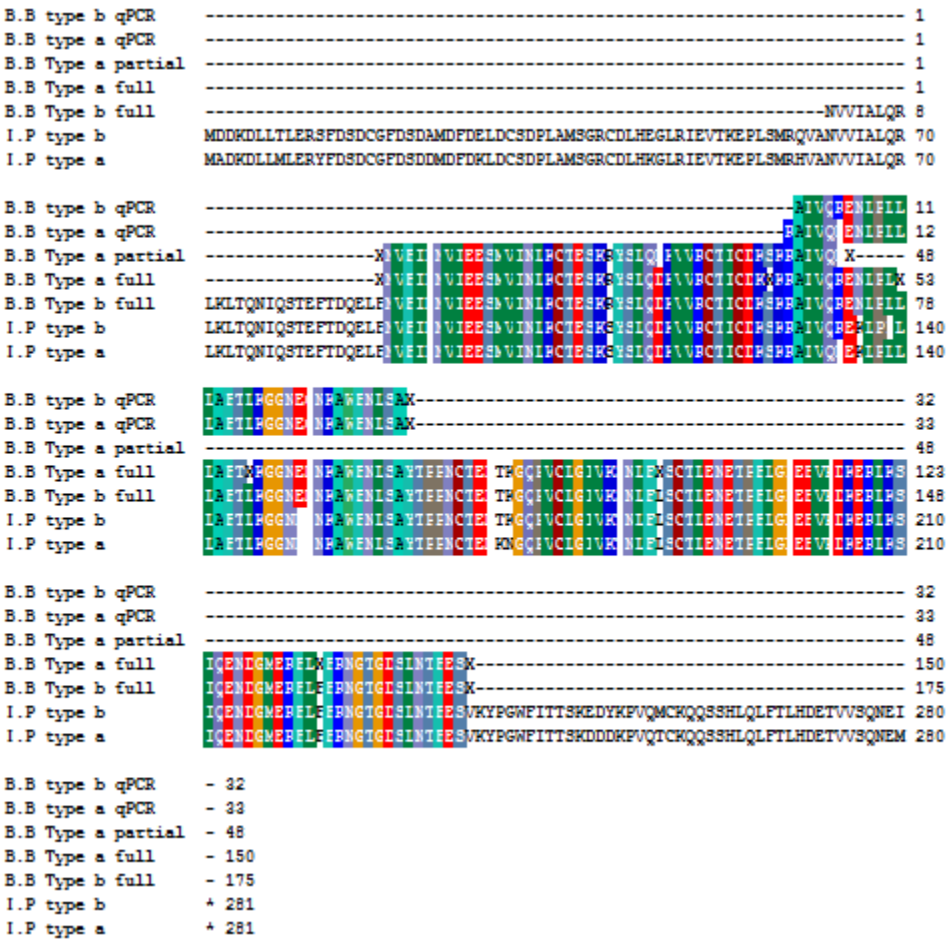

Figure 3.14 Amino acid sequence alignment of cloned brown bullhead partial IL-1B type b gene (B.B Type b full), brown bullhead partial IL-1B type a gene (B.B Type a partial), a longer fragment of type a (B.B Type a full) and channel catfish IL-1B type a (I.P type a: DQ157741.1) and type b (I.P type b: DQ157742.1). Also included in the alignment are the brown bullhead IL-1B type a and b qPCR sequences.

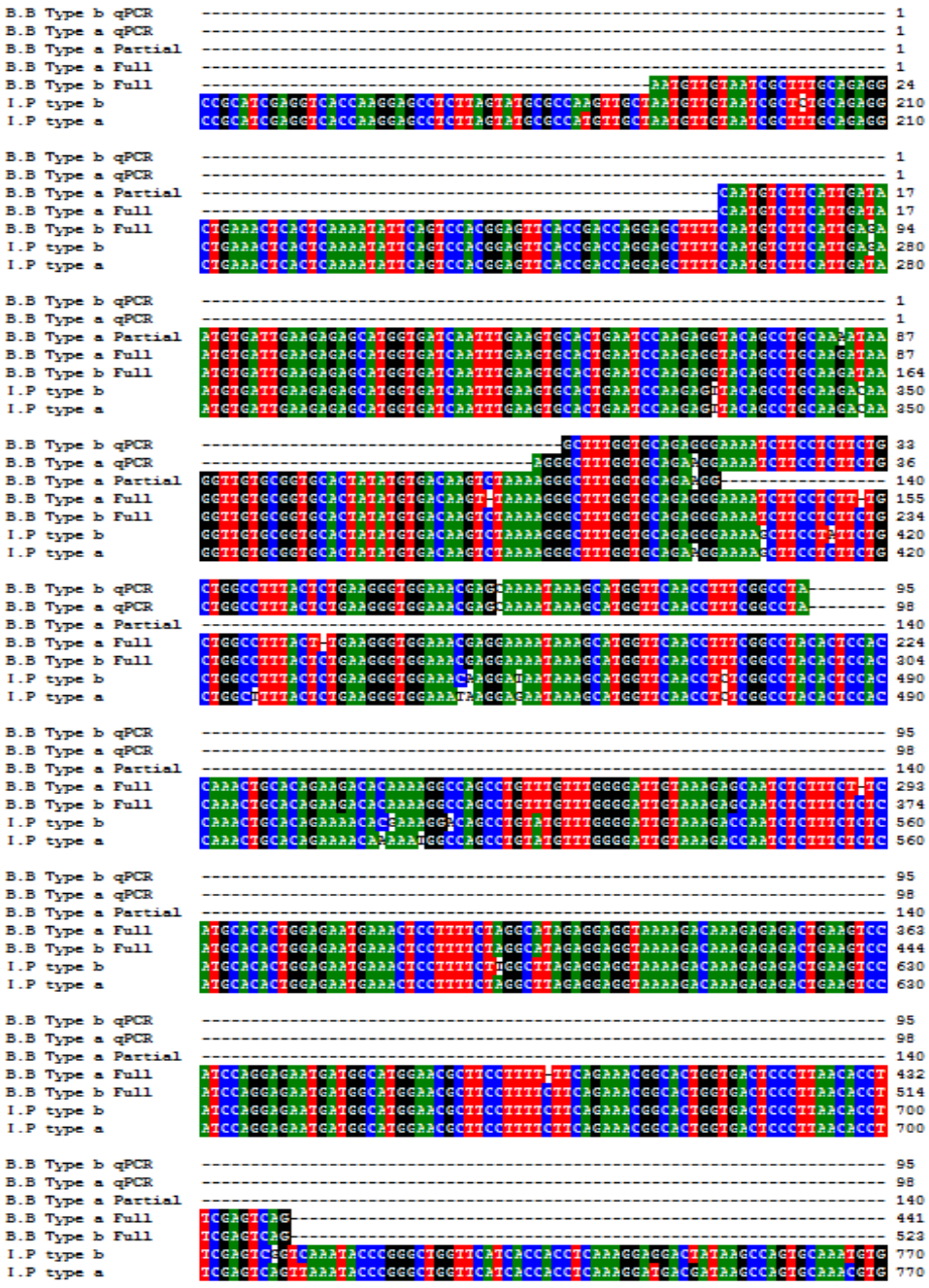

Figure 3.15. cDNA sequence alignment of cloned brown bullhead partial IL-1B type b gene (B.B Type b full), brown bullhead partial IL-1B type a gene (B.B Type a partial), a longer fragment of type a (B.B Type a full) and channel catfish IL-1B type a (I.P type a: DQ157741.1) and type b (I.P type b: DQ157742.1). Also included in the alignment are the brown bullhead IL-1B type a and b qPCR sequences.

3.1.8 Elongation Factor -1 Alpha (EF1a)

Lastly, housekeeping gene elongation factor -1 alpha (EF1a) was cloned in the brown bullhead and is depicted in figure 3.16. A BLAST search indicated 99% identity to *Ictalurus punctatu*s isolate C94SB10 elongation factor 1-alpha mRNA (DQ353797.1), [e value 0] and the resulting sequence alignment can be viewed in figure 3.17.

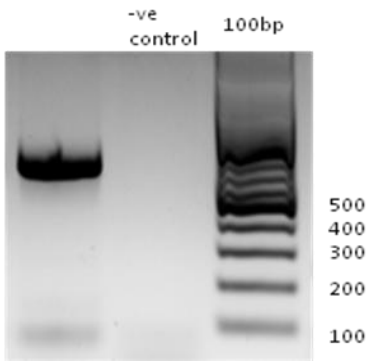

Figure 3.16 PCR amplification of Brown bullhead EF1a partial sequence. Amplicon size 627bp.

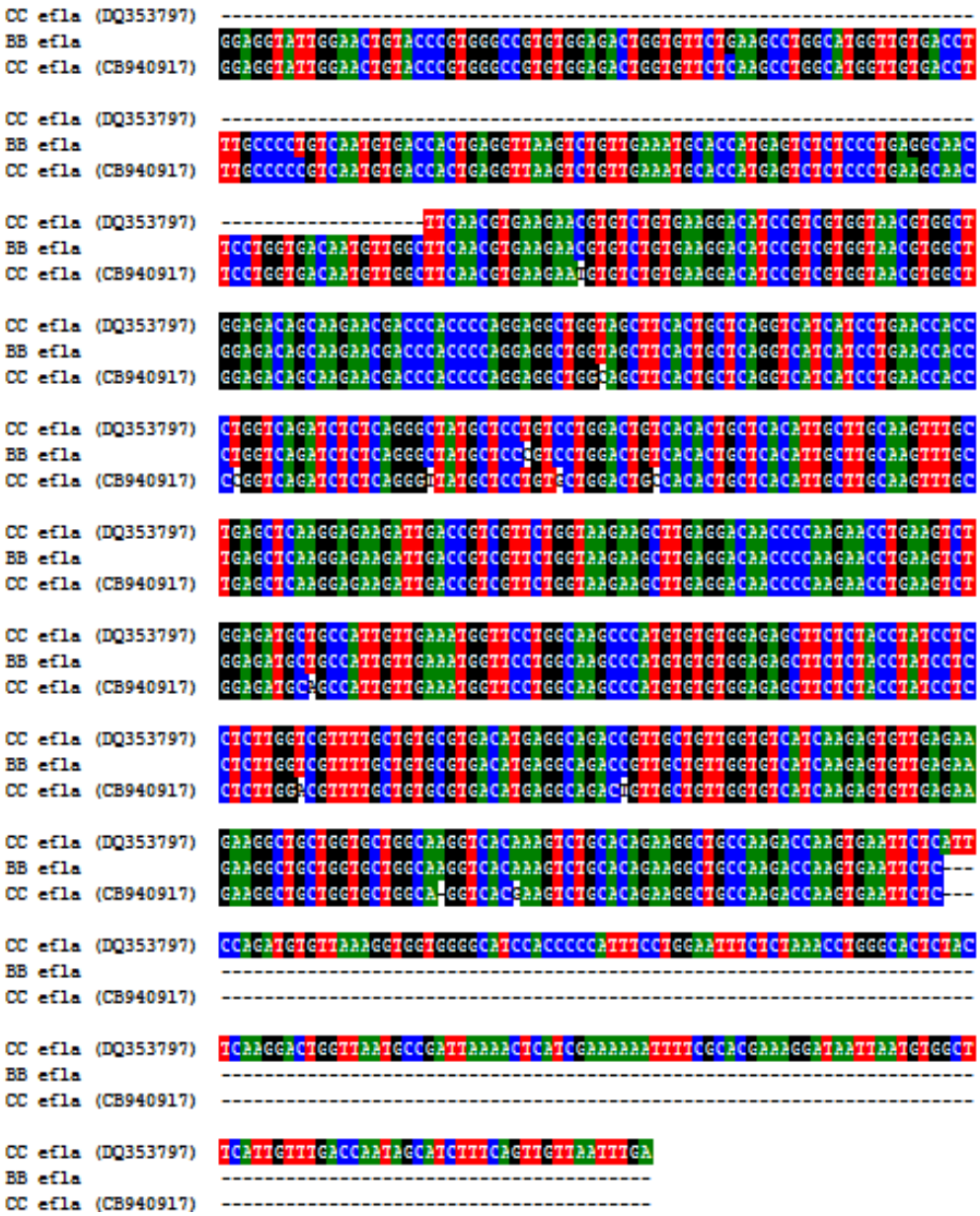

Figure 3.17 Nucleotide sequence alignment of partial cloned brown bullhead EF1a sequence and channel catfish partial CDS (DQ353797) and EST (CB940917).

3.2 Luminol Enhanced Zymosan induced Whole Blood Chemiluminescence

The ability of blood leukocytes to perform the innate immune function respiratory burst was determined using luminol enhanced chemiluminescence. For each bullhead the peak chemiluminescence reading was determined and used for comparison studies. Statistical analysis was conducted using SPSS, outliers removed using Cook's Distance testing.

Table 3.1 Statistical outliers removed from data based on linear regression at the level of Site, Sediment and Age, using case wise diagnostics of 2 standard deviations.

Fish ID	Site	Sediment	Age	Chemiluminescence (cps)
39	PI	Clean	F1	177
62	PI	Clean	F1	2997
50	BI	Clean	F1	323
55	BI	Clean	F1	363
59	TC	Clean	F1	3410
16	PI	Clean	Cl. Ad.	2417
25	BI	Clean	Cl. Ad.	2630
67	BI	Contaminated	F1	2457

Table 3.2 Sample sizes for the respiratory burst assay conducted 24hr post vaccination and post sediment exposure upon outlier removal.

	PI			TC			BI	
	F1	Cl. Ad	Ac. Ad	F1	Cl. Ad.	Ac. Ad.	F1	Cl. Ad.
Clean Sed.	6	9	10	8	10	7	5	9
Contaminated Sed.	6	6	10	6	8	8	6	8

Figure 3.18 identifies the mean peak chemiluminescence for each group, at each site. In order to determine effects of age, sediment exposure and site on the chemiluminescence, a univariate full factorial ANOVA was conducted. Chemiluminescence emission was determined to be normally distributed within each site exposed to either sediment as assessed by the Shapiro-Wilk test. There was homogeneity of variance between groups as assessed by Levene's test for equality of error variances. Chemiluminescence results are demonstrated in figures 3.18 to figure 3.21.

A significant interaction between the effects of site and age on the chemiluminescence emission, $F(2,106)= 4.99$, $P=.009$ was identified. A significant difference in sites on chemiluminescence is demonstrated ($P=.001$), as is a significant difference in sediment ($P<0.001$) and age ($P<0.001$). Simple interactions show a decrease in chemiluminescence on contaminated sediment ($P=<0.001$). Site specific sediment interactions identified a reduction in chemiluminescence on contaminated sediment within each site, showing a 36% decrease of PI ($P=.023$), a 31% decrease in TC ($P=.025$), and a 28% decrease in BI ($P=.044$).

Age specific interactions were identified within each site, as acute adults of both PI and TC had reduced chemiluminescence compared to that of the F1 and cleared adults of each site ($P<0.001$). Acute adults of PI demonstrated a 74% decrease in chemiluminescence compared to F1, and a 68% decrease compared to cleared adults.TC acute adults showed a 77% reduction compared to F1 and a 74% decrease in chemiluminescence compared to cleared adults of TC.

No difference was observed between the F1 and cleared adults of any site, PI ($P=.829$), BI ($P=.412$), TC ($P=1.00$). Acute adults of TC were identified to have a reduced chemiluminescence response when exposed to both clean and contaminated sediment ($P<0.001$)

compared to that of the F1 and cleared adults. The acute adults of PI showed a reduction in chemiluminescence on clean sediment to both F1 and cleared adults (P <0.001), but only compared to F1 when exposed to contaminated sediment (P=.011) and not cleared adults (P=0.159). BI identified no interaction between age and chemiluminescence when exposed to clean sediment (P=0.859) or contaminated sediment (P=0.386).

Site specific interactions between age and sediment identify a reduction of 55% in chemiluminescence of cleared adults of PI exposed to contaminated sediment to that of the cleared adults of PI exposed to clean sediment (P<0.001). F1 of TC exposed to contaminated sediment also had a 44% reduction in chemiluminescence compared to F1 exposed to clean sediment (P=0.013). Site interactions also identified a reduced chemiluminescence of F1 and cleared adults of BI compared to F1 and cleared adults of PI (P=0.004 and P=0.011 respectively) and TC (P<0.001 and P=0.039 respectively).

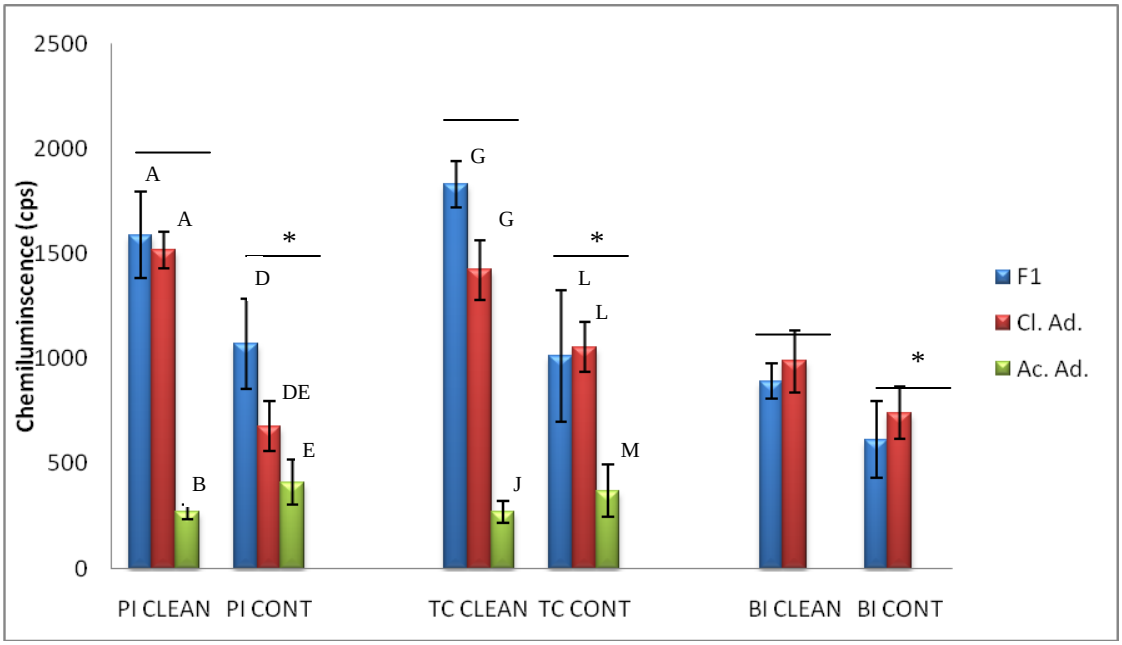

Figure 3.18 Whole Blood Chemiluminescence showing each group from each site on either clean (PI) or contaminated sediment (TC). First generation bullheads (F1), adult bullheads captured from the wild and allowed to clear contaminants in clean environment (Cl. Ad) and adult bullheads captured from the wild with limited time to remove contaminants (Ac. Ad) collected from Peche Island (PI), Trenton Channel (TC) and Belle Isle (BI) are shown.

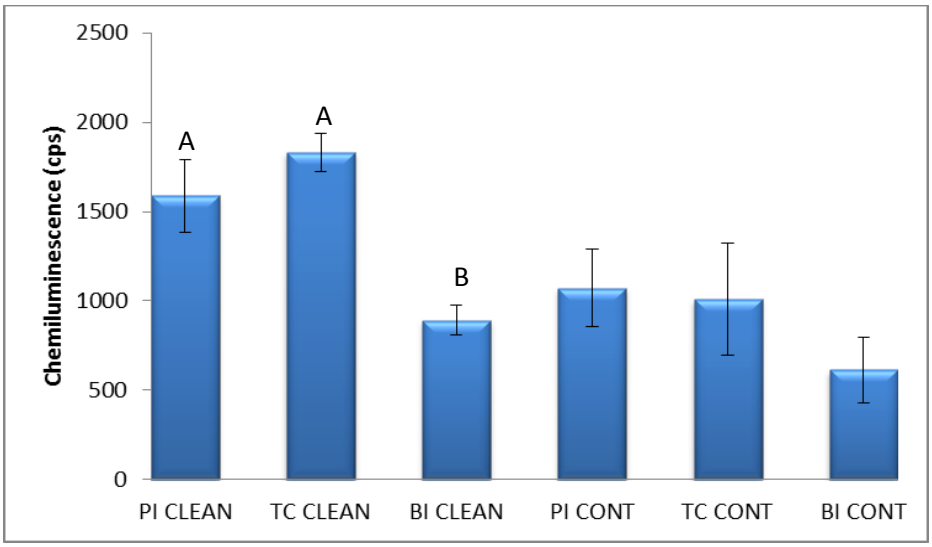

Figure 3.19 Whole Blood Chemiluminescence in F1 populations from each site placed on either clean (PI) or contaminated (TC) sediment.

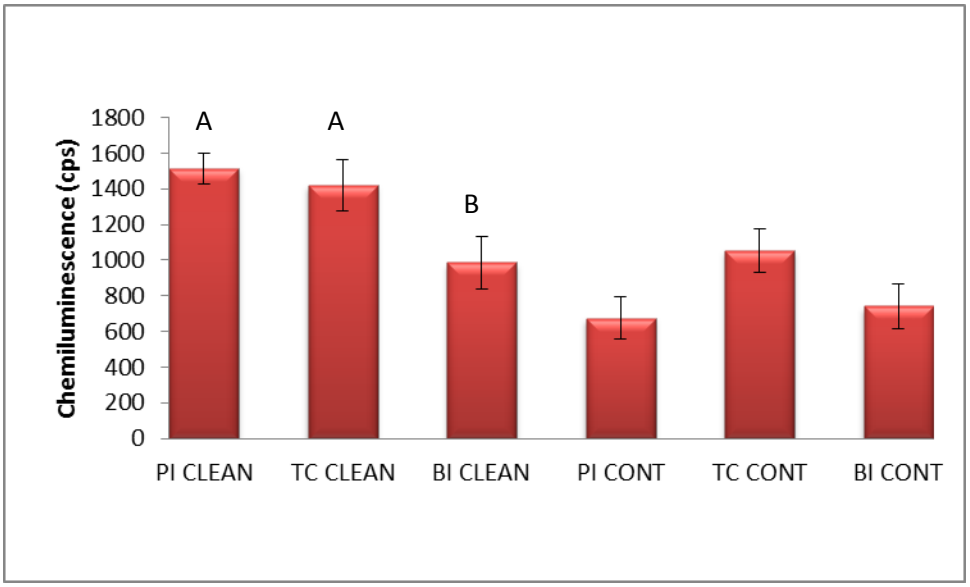

Figure 3.20 Whole Blood Chemiluminescence in cleared adult populations from each site placed on either clean (PI) or contaminated (TC) sediment.

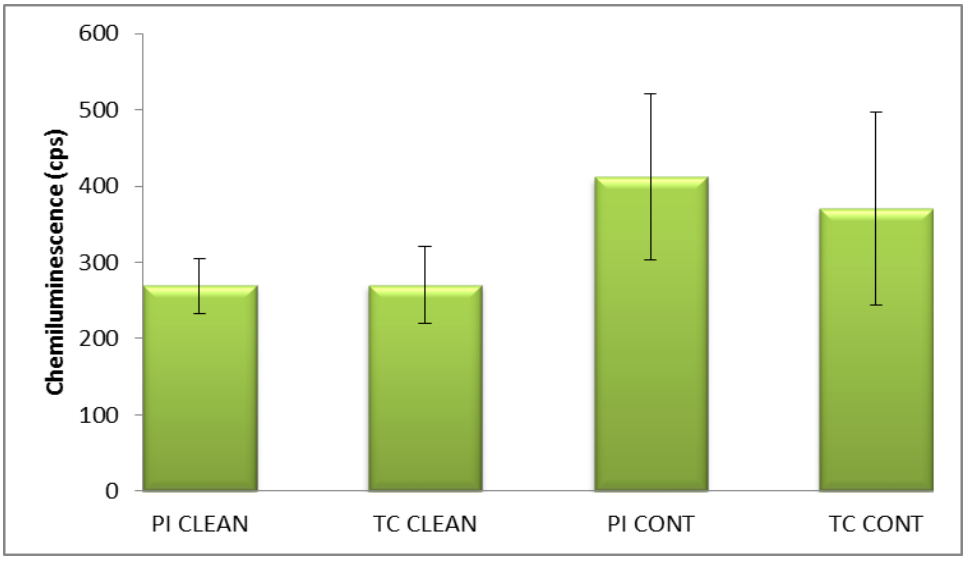

Figure 3.21 Whole Blood Chemiluminescence in acute adult populations from each site placed on either clean (PI) or contaminated (TC) sediment.

3.3 Enzyme Linked Immunosorbant Assay (ELISA)

The increases in antibody titre in response to Vibrogen 2 vaccine administration was measured to examine the adaptive immune response. Plate variation was accounted for by normalizing each plate to the KLH positive control on plate 6. The mean of the triplicate absorbances were organized into each group, and used to create a plot bar graph as demonstrated in figure 3.21. Statistical analysis was conducted using SPSS, outliers removed using Cook's Distance testing.

Table 3.3 Statistical outliers removed from data based on linear regression at the level of Site, Sediment and Age, using case wise diagnostics of 2 standard deviations.

Fish ID	Site	Sediment	Age	Time-Point	Absorbance (405nm)
Juv 2	TC	Clean	F1	Day 0	0.704
Juv 2	PI	Clean	F1	Day 21	1.204
Juv 3	TC	Contaminated	F1	Day 21	1.146

Table 3.4 Sample sizes for ELISA assay conducted 0, 14 and 21 day post vaccination and post sediment exposure upon outlier removal.

	PI								
	F1			Cl. Ad.			Ac. Ad		
	D0	D14	D21	D0	D14	D21	D0	D14	D21
Clean Sed.	3	2	1	3	3	3	4	4	4
Contaminated Sed	3	2	5	4	4	4	2	2	2
	TC								
	F1			Cl. Ad.			Ac. Ad		
	D0	D14	D21	D0	D14	D21	D0	D14	D21
Clean Sed.	2	3	3	4	4	4	2	2	2
Contaminated Sed	3	4	3	4	4	4	1	1	1
	BI								
	F1			Cl. Ad.					
	D0	D14	D21	D0	D14	D21			
Clean Sed.	3	3	2	4	4	4			
Contaminated Sed	3	5	4	4	4	4			

To determine effects of age, sediment exposure, time-point and site on the antibody production measured by absorbance, a univariate full factorial ANOVA was conducted using logarithm (base 10) transformation data. Absorbance at 405nm was determined to be normally distributed within each time-point at the level of sediment as assessed by the Shapiro-Wilk test. There was homogeneity of variance between groups as assessed by Levene's test for equality of error variances. Results of the ELISA can be seen in figure 3.22.

A significant interaction between the effects of age and time-point on the absorbance values, $F(4,102)= 3.13$, $P=.018$, is identified. There are also significant differences in age and absorbance ($P<0.001$), and in time-point and absorbance ($P<0.001$). Simple interactions identify no difference in sediment exposure on absorbance ($P=0.328$), no differences between sites

(*P*=801), a reduction in antibody titre in acute adults compared to F1 and cleared adults (*P*=0.001 and *P*<0.001 respectively), and an increase in antibody titre from day 0 to day 21 (*P*<0.001).

Site specific interactions show increased antibody titre of both PI and TC cleared adults exposed to contaminated sediment (*P*=0.013 and *P*=0.040 respectively). Acute adults of both PI and TC had reduction in antibody titre compared to F1 (*P*=0.028 and *P*=0.021 respectively) and cleared adults (*P*<0.001 and *P*=0.001 respectively). Antibody titre also increased from day 0 to day 21 at all sites, PI (*P*<0.001), BI (*P*=0.033) and TC (*P*=0.010). Antibody titre of PI exposed to clean sediment and contaminated sediment increased from day 0 to day 21 (*P*=0.007 and *P*=0.002 respectively). The same increase was observed in TC exposed to clean sediment (*P*=0.032) but no increase was observed on contaminated sediment (*P*=0.069). There was no increase in titre from day 0 to day 21 of BI exposed to either clean (*P*=0.325) or contaminated *P*=0.201) sediment. Within each site, age specific interactions identified only an increase of PI and TC F1 from day 0 to day 21 (*P*=0.003 and *P*=0.018 respectively). It was also determined that at day 0 prior to vaccination, the antibody titre of cleared adults was higher than that of both the F1 and the acute adults (*P*=0.001 and *P*=0.004 respectively).

Site specific interactions of sediment exposure identify increased antibody titre in cleared adults compared to F1 of PI (*P*=0.019) exposed to clean sediment, reduced antibody titre of acute adults compared to F1 (*P*=0.013) and cleared adults (*P*=0.001) of PI exposed to contaminated sediment. TC acute adults exposed to clean sediment had a reduced titre compared to F1 (*P*= 0.007) and cleared adults (*P*=0.033), the acute adults of TC exposed to contaminated sediment had a reduced titre compared to the cleared adults (*P*=0.044).

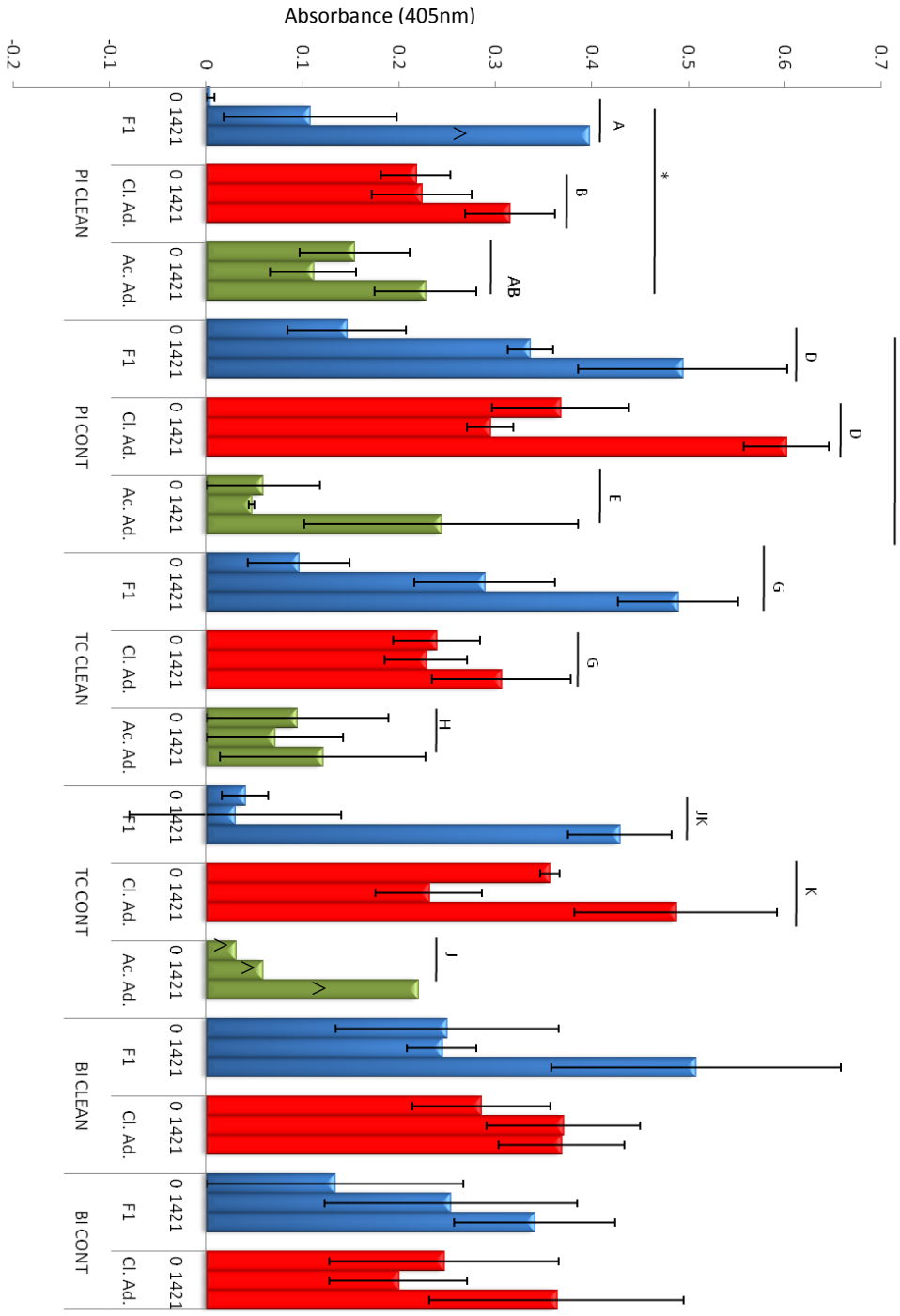

Figure 3.22: Antibody titre against *v. anguillarum*, collected at day 0, 14 and 21 post vaccination exposed to clean (PI) or contaminated (TC) sediment. Cl.Ad refers to cleared adults, Ac. Ad refers to acute adults. ^ refers to n=1. TC cont "J" identifies a significant reduction in acute adults compared to cleared adults. PI cont "E" and TC clean "H" indicate a significant reduction in acute adults compared to both cleared adults and F1.

3.4 Quantitative Real Time PCR

3.4.1 Major Histocompatibility Class II Beta (MHIIB) Gene Expression in both Gill and spleen normalized to housekeeping gene Elongation factor-1 alpha (EF1A).

In order to determine effects of age, sediment exposure and site on the expression level of major histocompatibility class II beta (MHIIB) in gill and spleen tissue, a univariate full factorial ANOVA was conducted using inverse of square root transformation data for gill and using logarithm (base 10) transformed data for spleen. Expression levels relative to endogenous control elongation factor-1 alpha (EF1A) was determined to be normally distributed for gill tissue within each site as assessed by the Shapiro-Wilk test. Spleen transformed data was determined to be normally distributed by the combined effects of age, site and sediment as also assessed by the Shapiro-Wilk test. There was homogeneity of variance between groups as assessed by Levene's test for equality of error variances. Expression levels of MHIIB normalized to EF1a can be seen in figures 3.23 and figure 3.24.

Table 3.5 Statistical outliers removed from data based on linear regression at the level of Site, Sediment and Age, using case wise diagnostics of 2 standard deviations.

Fish ID	Tissue	Site	Sediment	Age	dCt
31	Gill	BI	Clean	Cl. Ad.	14.52
136	Gill	TC	Clean	Ac. Ad.	14.41
141	Gill	TC	Contaminated	Ac. Ad.	13.22
136	Spleen	TC	Clean	Ac. Ad.	13.48
141	Spleen	TC	Contaminated	Ac. Ad.	16.05

Table 3.6 Sample sizes for the expression levels of MHIIB conducted 24hr post vaccination and post sediment exposure upon outlier removal.

Gill								
	PI			TC			BI	
	F1	Cl. Ad	Ac. Ad	F1	Cl. Ad.	Ac. Ad.	F1	Cl. Ad.
Clean Sed.	3	3	3	2	3	2	2	2
Contaminated Sed.	2	3	3	2	3	2	3	3
Spleen								
	PI			TC			BI	
	F1	Cl. Ad	Ac. Ad	F1	Cl. Ad.	Ac. Ad.	F1	Cl. Ad.
Clean Sed.	3	3	3	2	3	2	2	3
Contaminated Sed.	2	3	3	2	3	2	3	3

A significant interaction between the combined effects of age, sediment exposure and site on the expression levels of MHIIB in gill tissue was identified $F(3,25)= 3.56$, $P=.026$. Simple interactions identify no difference in sediment exposure on expression ($P=0.914$), no differences between sites ($P=0.494$), and no differences between age ($P=0.425$).Within site interactions identified F1 of TC had an increased expression compared to cleared adults of TC on clean sediment ($P=0.023$). F1 of TC also had increased expression when exposed to clean sediment then exposed to contaminated sediment ($P=0.018$). Cleared adults of TC also showed higher expression of MHIIB in the gill exposed to contaminated sediment compared to clean sediment ($P=0.035$).

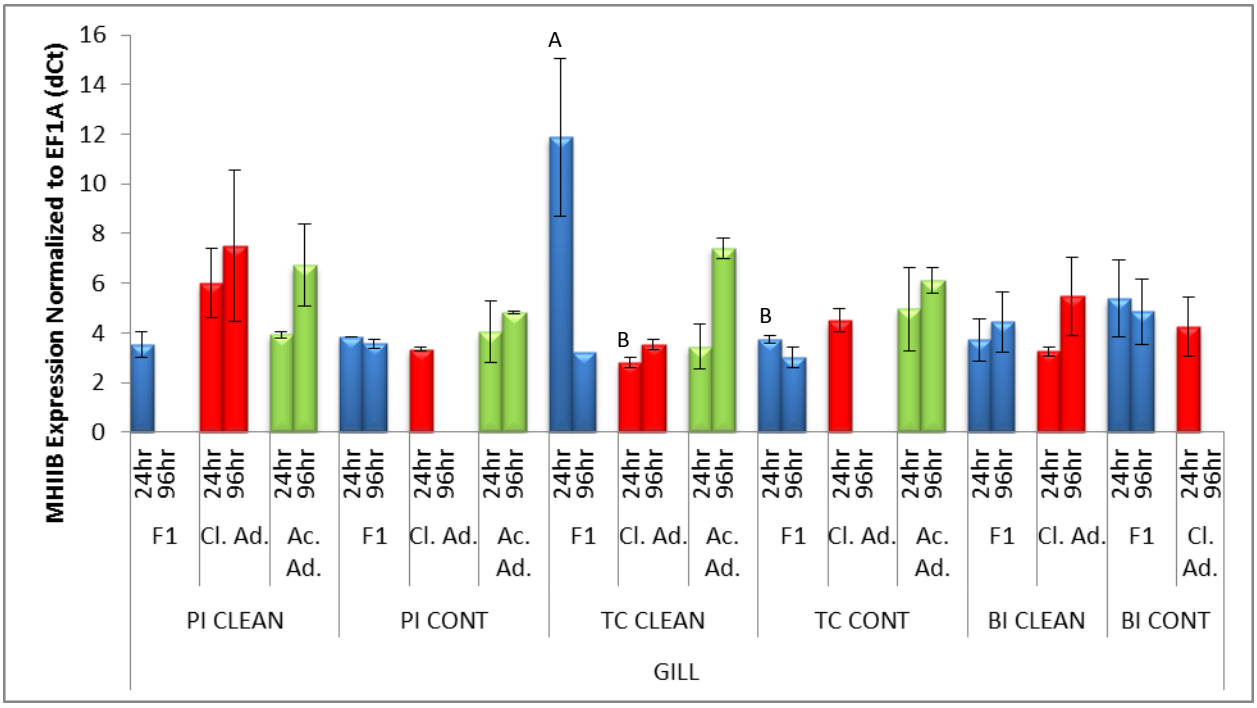

Figure 3.23 Gene Expression of MHIIB 24hr and 96hr after vaccination and sediment exposure of either clean or contaminated sediment in gill tissue. *note 96hr samples for cleared adults exposed to contaminated sediment are not available for analysis. PI Clean refers to fish captured from PI and placed on clean sediment, PI cont refers to bullheads captured from PI and exposed to contaminated sediment. Cl.Ad= Cleared adults, Ac.Ad= Acute Adults. "A" represents significantly higher expression of TC F1 on clean then cleared adults exposed to clean, and F1 exposed to contaminated sediment of TC.

A significant interaction between the effect of age on the expression levels of MHIIB in spleen was observed $F(2,25)= 4.79$, $P=.017$. No interaction between sediment ($P=0.531$), site ($P=0.906$), or the combined effect ($P=0.211$) were identified. F1 had increased expression levels of MHIIB relative to both cleared adults ($P=0.04$) and acute adults ($P=0.012$). Within site interactions identified an increased expression level in F1 of TC compared to cleared and acute adults ($P=0.039$ and $P=0.021$ respectively). TC cleared adults were also found to have increased expression levels on contaminated sediment compared to clean sediment ($P=0.015$).

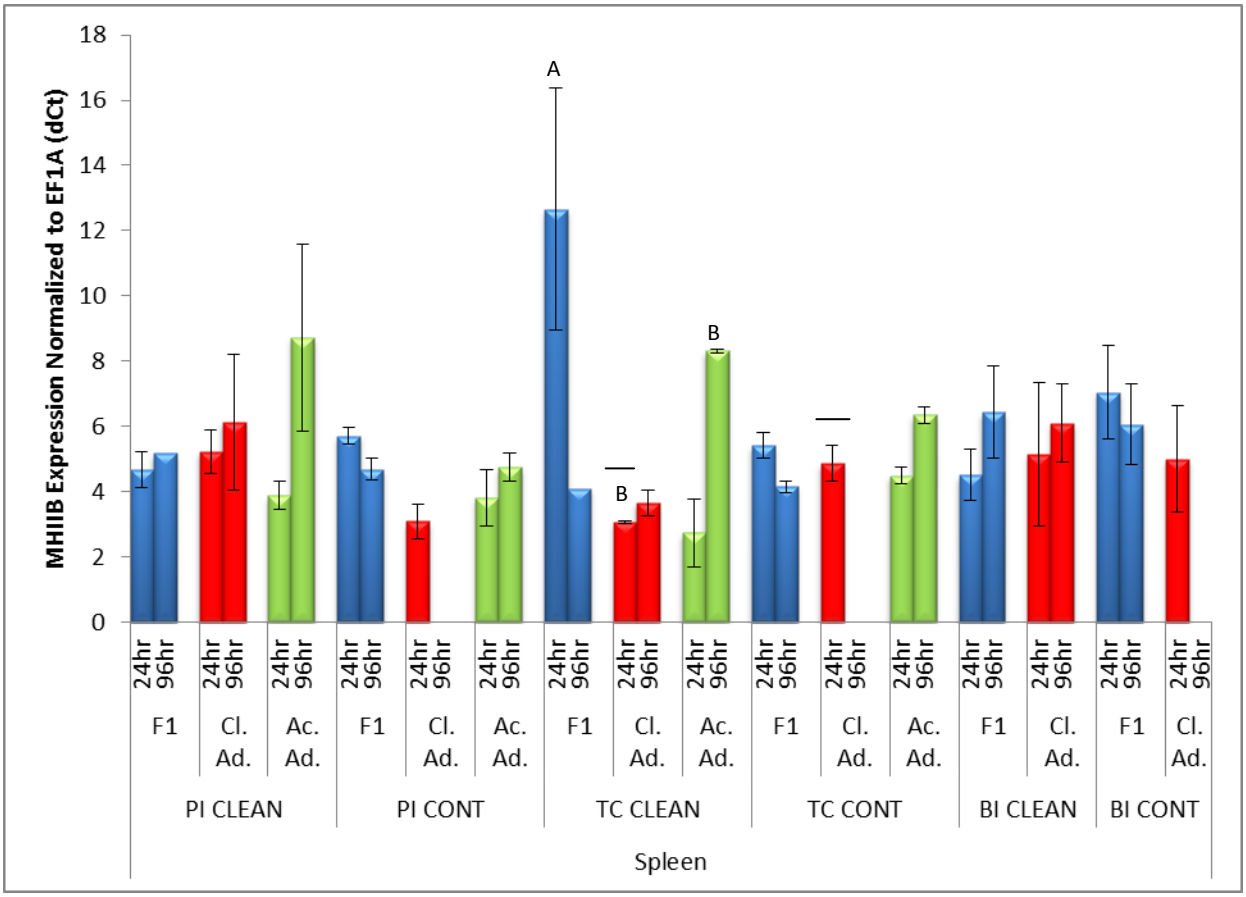

Figure 3.24 Gene Expression of MHIIB 24hr and 96hr after vaccination and sediment exposure of either clean or contaminated sediment in spleen tissue. *note 96hr samples for cleared adults exposed to contaminated sediment are not available for analysis. PI Clean refers to fish captured from PI and placed on clean sediment, PI cont refers to bullheads captured from PI and exposed to contaminated sediment. Cl.Ad= Cleared adults, Ac.Ad= Acute Adults. "A" represents significantly hihger expression of F! of TC exposed to clean sediment compared to both cleared adults and acute adults of TC exposed to clean sediment.

3.4.2 Interleukin-8 like cytokine (IL-8) gene expression in gill and spleen normalized to housekeeping gene Elongation factor-1 alpha (EF1A).

In order to determine effects of age, sediment exposure and site on the expression level of the cytokine interleukin-8 (IL-8) in gill and spleen tissue, a univariate full factorial ANOVA was

conducted using logarithm (base 10) transformed data for gill and untransformed data for spleen. Expression levels relative to endogenous control elongation factor-1 alpha (EF1A) was determined to be normally distributed for gill tissue within each site at levels of sediment and age as assessed by the Shapiro-Wilk test. Spleen untransformed data was determined to be normally distributed within each site at the level of sediment also as assessed by the Shapiro-Wilk test. There was homogeneity of variance between groups as assessed by Levene's test for equality of error variances. Expression levels of IL-8 normalized to EF1a can be seen for gill in figure 3.25 and for spleen in figure 3.26.

Table 3.7 Statistical outliers removed from data based on linear regression at the level of Site, Sediment and Age, using case wise diagnostics of 2 standard deviations.

Fish ID	Tissue	Site	Sediment	Age	dCt
35	Gill	TC	Clean	Cl. Ad	11.61

Table 3.8 Sample sizes for the expression levels of IL-8 conducted 24hr post vaccination and post sediment exposure upon outlier removal.

Gill								
	PI			TC			BI	
	F1	Cl. Ad	Ac. Ad	F1	Cl. Ad.	Ac. Ad.	F1	Cl. Ad.
Clean Sed.	3	3	3	2	2	3	2	3
Contaminated Sed.	2	3	3	2	3	3	3	3
Spleen								
	PI			TC			BI	
	F1	Cl. Ad	Ac. Ad	F1	Cl. Ad.	Ac. Ad.	F1	Cl. Ad.
Clean Sed.	3	3	3	2	3	3	2	3
Contaminated Sed.	2	3	3	2	3	3	3	3

A significant interaction between the effect of age on the expression levels of IL-8 in gill was observed $F(2,25)= 6.50$, $P=.005$, but not observed in the spleen $F(2,28)=.096$, $P=.908$. No interaction between sediment in gill ($P=0.701$) or spleen ($P=0.449$), site in gill ($P=0.907$) or spleen ($P=0.227$), or the combined effect in either tissue ($P=0.923$ and $P=0.963$) were identified. Simple interactions identified lower expression of IL-8 in gill tissue in Acute adults compared to F1 ($P=0.004$) and cleared adults ($P=0.016$).

Within site interactions, PI acute adults displayed a reduced expression of IL-8 in gill tissue than seen in both F1 and cleared adults ($P<0.001$). F1 of PI exposed to contaminated sediment had increased expression levels of IL-8 in spleen tissue relative to both cleared adults and acute adults ($P=0.008$ and $P=0.015$ respectively).

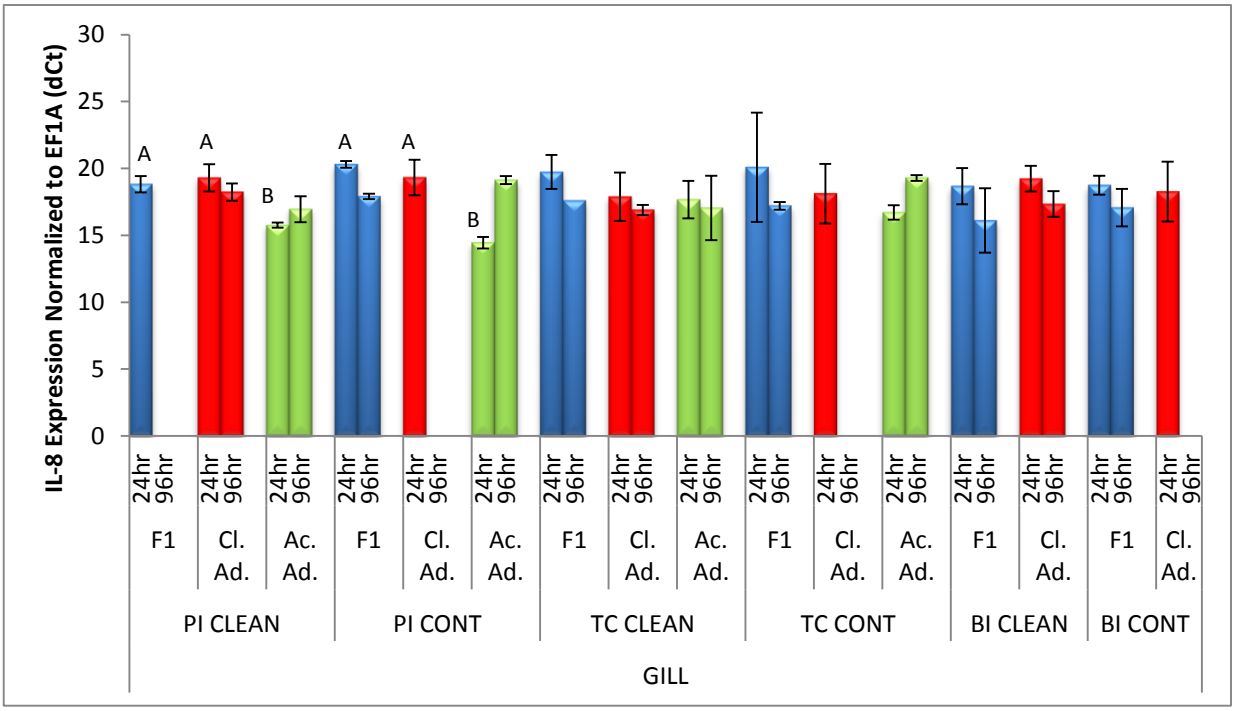

Figure 3.25: Gene Expression of IL-8 24hr and 96hr after vaccination and sediment exposure of either clean or contaminated sediment in spleen tissue. *note 96hr samples for cleared adults exposed to contaminated sediment are not available for analysis. PI Clean refers to fish captured from PI and placed on clean sediment, PI cont refers to bullheads captured from PI and exposed to contaminated sediment. Cl.Ad= Cleared adults, Ac.Ad= Acute Adults. “B” respresents a reduced expression level in acute adults of PI compared to F1 and cleared adults of PI.

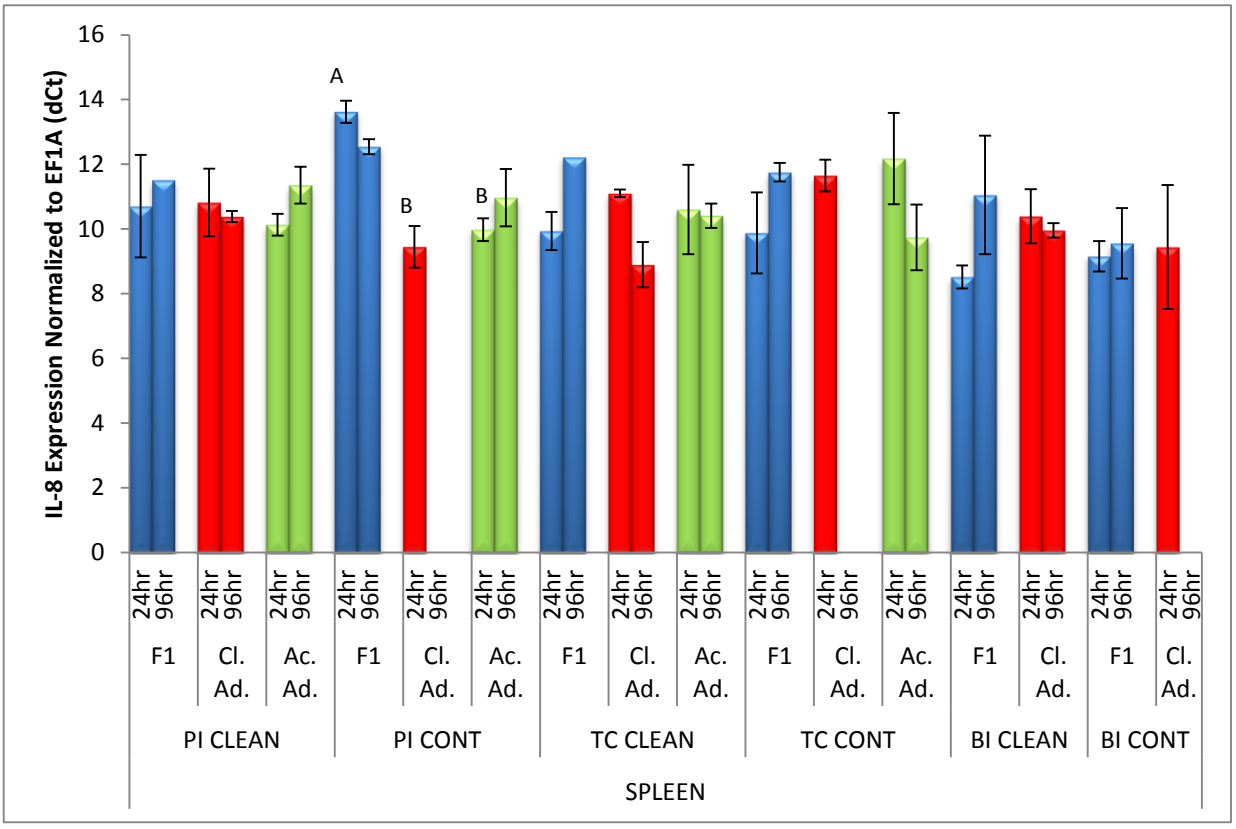

Figure 3.26: Gene Expression of IL-8 24hr and 96hr after vaccination and sediment exposure of either clean or contaminated sediment in spleen tissue. *note 96hr samples for cleared adults exposed to contaminated sediment are not available for analysis. PI Clean refers to fish captured from PI and placed on clean sediment, PI cont refers to bullheads captured from PI and exposed to contaminated sediment. Cl.Ad= Cleared adults, Ac.Ad= Acute Adults."A" represents significantly higher expression levels of F1 of PI exposed to contaminated sediment then both cleared adults and actue adutls of the same site and sediment exposure.

3.4.3 Interleukin 1 Beta (IL-1B) type b Gene Expression in both Gill and Spleen normalized to housekeeping gene Elongation factor-1 alpha (EF1A)

The effects of age, sediment exposure and site on the expression level of the pro-inflammatory cytokine interleukin-1B (IL-1B) in gill and spleen tissue were determined using a univariate full factorial ANOVA. Untransformed data was used for both gill and for spleen analysis. Expression levels relative to endogenous control elongation factor-1 alpha (EF1A) was determined to be normally distributed for gill tissue within each site at levels of sediment and age as assessed by the Shapiro-Wilk test. Spleen untransformed data was determined to be normally distributed at the levels sediment, site and age also as assessed by the Shapiro-Wilk test. There was homogeneity of variance between groups as assessed by Levene's test for equality of error variances. Expression levels of IL-1B normalized to EF1a can be seen for gill in figure 3.27, and for spleen in figure 3.28.

Table 3.9 Sample sizes for the expression levels of IL-1B conducted 24hr post vaccination and post sediment exposure.

Gill								
	PI			TC			BI	
	F1	Cl. Ad	Ac. Ad	F1	Cl. Ad.	Ac. Ad.	F1	Cl. Ad.
Clean Sed.	3	3	3	2	3	3	2	3
Contaminated Sed.	2	3	3	2	3	3	3	3
Spleen								
	PI			TC			BI	
	F1	Cl. Ad	Ac. Ad	F1	Cl. Ad.	Ac. Ad.	F1	Cl. Ad.
Clean Sed.	3	3	3	2	3	3	2	3
Contaminated Sed.	2	3	3	2	3	3	3	3

For both the gill and spleen tissues a significant interaction was observed between the expression of IL-1B and age $F(2,28)= 31.43$, $P<0.001$ for gill and $F(2,28)= 21.27$, $P<0.001$ for spleen. No effects of sediment exposure on IL-1B expression for either gill or spleen ($P=0.049$ and $P=0.686$ respectively), and no effect of the interaction of site on expression for either tissue ($P=0.303$ and $P=0.427$ respectively) was observed. Simple interactions identified acute adults having lower expression in both tissues compared to both F1 and cleared adults ($P<0.001$ in all cases). In gill tissue, there is a 36% decrease in expression of acute adults compared to F1 and a 30% decrease compared to cleared adults. Spleen tissue has a higher decrease of expression in acute adults of 49% compared to F1 and a 47% decrease compared to cleared adults.

Site specific interactions identified an increase in IL-1B expression in both gill and spleen tissues of BI compared to PI ($P=0.001$ and $P=0.005$ respectively), and to TC ($P=0.008$ and $P=0.030$). Interactions of age and site identified a lower expression of acute adults compared to F1 and cleared adults of both PI ($P=0.001$) and $P=0.002$) and TC ($P=0.003$ and $P=0.030$) in gill tissue. The same combined age and site interactions were shown for the spleen tissue, in which acute adults had a lower expression compared to F1 and cleared adults ($P=0.016$ and $P=0.034$) of PI and of TC ($P=0.003$ and $P=0.002$).

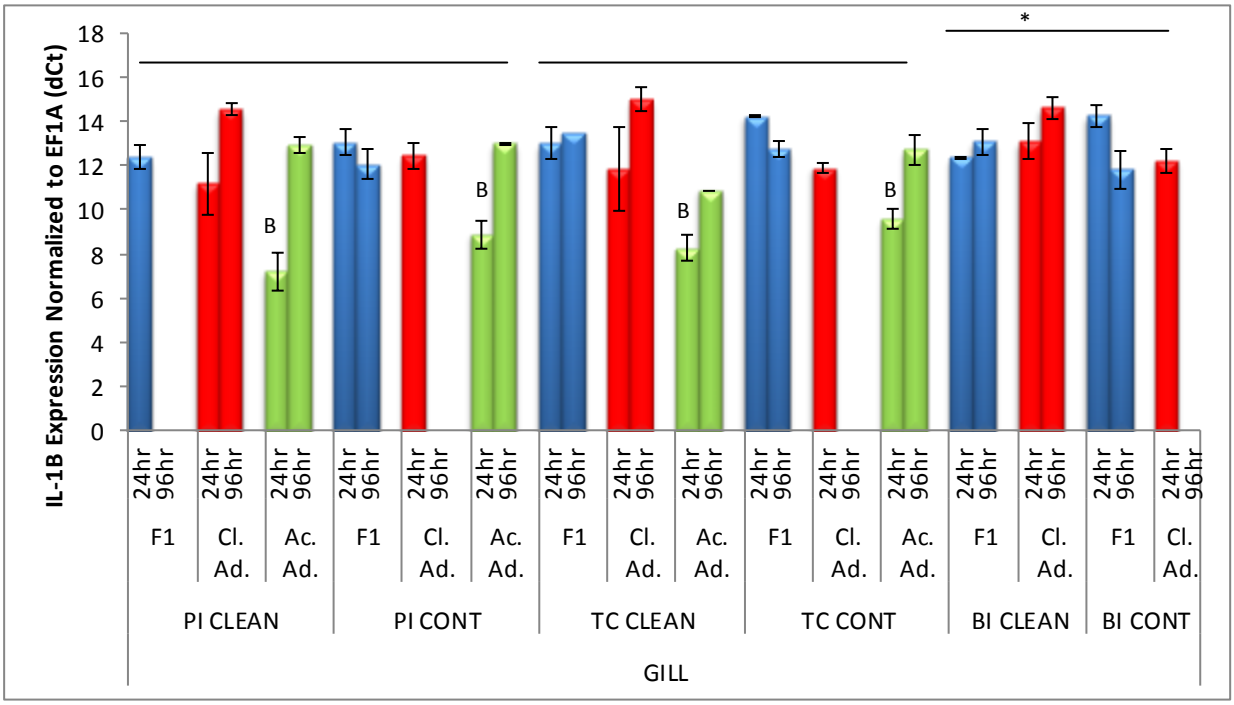

Figure 3.27 Gene Expression of IL-1B 24hr and 96hr after vaccination and sediment exposure of either clean or contaminated sediment in gill tissue. *note 96hr samples for cleared adults exposed to contaminated sediment are not available for analysis. PI Clean refers to fish captured from PI and placed on clean sediment, PI cont refers to bullheads captured from PI and exposed to contaminated sediment. Cl.Ad= Cleared adults, Ac.Ad= Acute Adults. "B" respresents a reduced expression level in acute adults compared to F1 and cleared adults of both PI and TC. "*" represents increased expression level of BI compared to that of PI and TC. Note BI do not have acute adults.

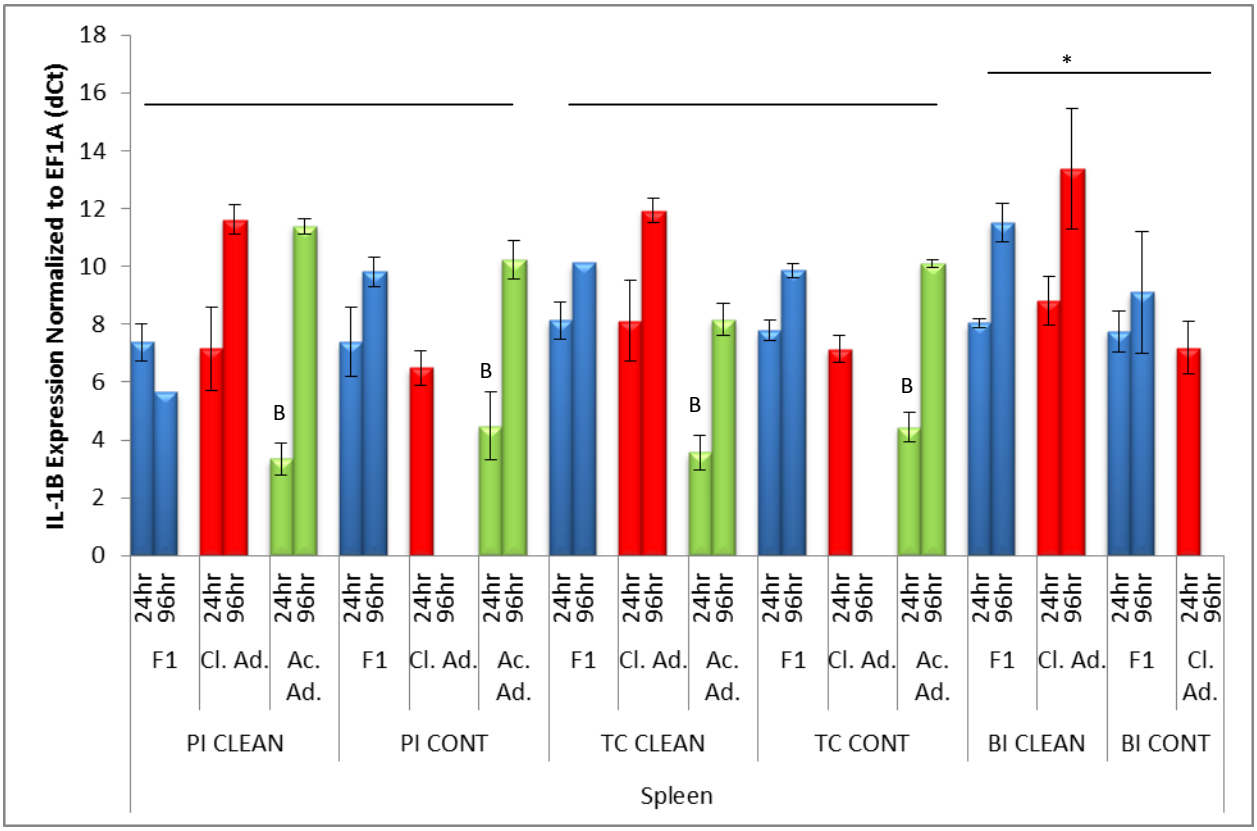

Figure 3.28 Gene Expression of IL-1B 24hr and 96hr after vaccination and sediment exposure of either clean or contaminated sediment in spleen tissue. *note 96hr samples for cleared adults exposed to contaminated sediment are not available for analysis. PI Clean refers to fish captured from PI and placed on clean sediment, PI cont refers to bullheads captured from PI and exposed to contaminated sediment. Cl.Ad= Cleared adults, Ac.Ad= Acute Adults. "B" respresents a reduced expression level in acute adults compared to F1 and cleared adults of both PI and TC. "*" represents increased expression level of BI compared to that of PI and TC. Note BI do not have acute adults.

Discussion

4.1 Cloning of immune related genes

The cloning of brown bullhead immune related genes resulted in the acquisition of cDNA sequence for partial fragments of 8 different genes (Section 3.1). The sequences obtained were verified through the BLAST program of the NCBI database. Species differences were observed within each sequence when compared to channel catfish (*Ictalurus punctatus*), and splice variants were also examined. Channel catfish are known to possess different splice variants of IL-8, resulting in larger and smaller mRNAs. It has been noted in previous RT-PCR studies that the shorter channel catfish alternatively spliced fragment of IL-8 was more abundant than the larger fragment (Chen et al, 2005). The larger fragment was not identified in brown bullhead cloning (seen in figures 3.5 and 3.6), allowing for related comparisons only between the smaller alternatively spliced fragment in channel catfish and the cloned fragment of brown bullhead. There appears to be a higher expression levels of IL-8 in channel catfish spleen compared to gill (Chen et al, 2005), also corresponding to the present research (Figures 3.24 and 3.25). Further studies should be initiated to locate an alternatively spliced form of bullhead IL-8, but to date this has been unsuccessful. IL-1B in channel catfish has been identified to have 2 forms, type a (DQ157741.1) and type b (DQ157742.1). In an attempt to identify and clone each type in brown bullhead, multiple primer pairs were created. An aspartic acid (D) substitution at amino acid 94 instead of glutamic acid (E) and a lysine (K) instead of arginine (R) at amino acid 134 (Figure 3.11) are indicative of type a IL-8 in channel catfish and these are found in the cloned brown bullhead sequence, so it was designated type a. It was not possible to isolate a full length clone of

type a in the brown bullhead, and further studies will need to be undertaken to completely characterize both the sequence and expression of bullhead IL-8.

4.2 Respiratory Burst Assay

A decrease in chemiluminescence signifies a decreased ability of circulating blood phagocytes to mount an immune response when stimulated *in vitro* by zymosan. The successful aerobic destruction of microbes by phagocytic capable cells depends on the consumption of oxygen to form reactive metabolites in a process known as respiratory burst (Secombes & Fletcher, 1992). Many different possible explanations may account for this observed decrease, as many studies have demonstrated the immunosuppressive ability of contaminants (Iwanowicz et al, 2009; Rice et al, 1996; Chaves-Pozo, 2005; Zelikoff, 1993). Results of chemiluminescence studies in teleosts have not conclusively shown acute environmental impacts on the ability of phagocytes to create ROS. The studies have demonstrated increases, decreases and even no impact on the production of ROS.

In agreement with the results obtained from the sediment exposure of brown bullhead (see Figure 3.18), channel catfish (*Ictalurus punctatus*) exposed to IP injected doses of tributylin (TBT) showed a reduction in non-specific cytotoxic cell (NCC) activity at high doses and in phagocyte oxidative burst activity when challenged with *Edwardsiella ictaluri* (Rice et al, 1995). Channel catfish also showed a moderate decrease in oxidative burst in response to *Vibrio anguillarum* challenge after IP injection exposure to a PCB mixture (Regala et al, 2001). Carlson et al (2002) also verified suppression of superoxide production by kidney phagocytes at high doses of IP injected benzo[a]pyrene in Japanese medaka (*Oryzias latipes*). Tilapia (*Oreochromis*

mossambicus) showed decreased phagocytic cell counts and decreased chemiluminescene at high doses of IP injected DMBA (Hart et al, 1998). Although these studies indicate an effect of injected contaminants, few studies have reported the immune impact of contaminated sediment exposure, or chronic exposure.

Hutchinson et al (2003) was able to identify a reduction in hydrogen peroxide production of fish in short term exposures to PCB and PAH spiked sediments. A chronic exposure study conducted by Rice et al (1996) using red-breasted sunfish from a contaminated stream, showed that the oxidative burst activity was suppressed in the fish collected closer to the head waters which contained the highest concentrations of contaminants, and was relatively normal downstream. The data from these two studies correlate with the results of the present research, indicating a combination of contaminants contained in sediment, and chronic exposure to contaminants do have immunosuppressive capabilities. This immunosuppression may account for the reduced chemiluminescence identified in the PI cleared adults and TC F1 on contaminated sediment, as those bullheads exposed to clean sediment did not show inhibition of the respiratory burst defence by the contaminants.

Alternatively, Rice et al (1998) demonstrated an increase in channel catfish (*Ictalurus punctatus*) phagocytic oxidative burst after 14 days of exposure to low levels of PCB in feed and also after 21 days exposure to low levels of PCB mixed with nonyl-phenyl in feed. A study conducted by Kelly-Reay and Weeks-Perkins (1994), also identified an increase in macrophage bactericidal oxygen free radicals in common mummichog (*Fundulus heteroclitus*) upon chronic exposure in the Elizabeth River, Virginia, suggesting low levels of contaminants will provide enough stimulatory effects to increase immune function. However, brown bullheads receiving an

intraperitoneal injection of the PCB mixture Aroclor 1248 were shown to have no effect on the production of ROS when stimulated with LPS, but the bactericidal activity was decreased (Iwanowicz et al, 2009). Although several studies suggest respiratory burst in fish is elevated due to stimulatory effects of contaminants, the results of the present research correlate with the ample studies indicating immunosuppression effects.

The data for the BI bullheads indicates that not only the fish exposed to contaminated sediment, but also those exposed to clean sediment were significantly reduced compared to F1 and cleared adults from TC and PI exposed to clean sediment. A possible explanation of the reduction in BI chemiluminescence in both F1 and cleared adults is that the immune response of these fish may be slightly delayed compared to PI or TC, and may not be reduced in effectiveness. A simple explanation of the timing of the assay may account for this observable and significant difference. It has been shown that within 24hr after infection with *v.anguillarum* in the gilthead seabream (*Sparus aurata*) results in the significant increase in acidophilic granulocytes but does not alter the chemiluminescence. The significant increase in chemiluminescence was observed after 48hr infection and lasted until 72hr post infection (Chaves-Pozo, 2005). These results may indicate a priming effect of gilt seabream granulocytes, and may also provide an insight into the response of not only the bullheads of BI, but of the acute adults. Acute adults may also undergo a priming effect that in some way may be delayed, resulting in the observable significant decrease in respiratory burst. If this assay was conducted at further time points, a clearer indication of innate immunosuppression may have been identified.

The current research may also indicate that although sediment collected around Belle Isle has been shown to contain reduced levels of contaminants compared to TC (Kannan et al, 2001; Leadley et al, 1998), updated studies indicate continual turnover of contaminants results in elevated levels in this area (Drouillard et al, 2006). Prevention of increased deposition of contaminants of BI surrounding area can be attributed to the fast flowing waters of the shipping channels (Tsanis *et al*. 1996). As a result, bullheads collected at BI could potentially have a greater uptake of contaminants than expected from sediment concentrations, resulting in the observed lower respiratory burst levels than seen other groups. It has been shown in previous work that bullheads collected from PI had higher muscle contaminant levels than expected from the levels of contaminant in the sediment (Leadley et al, 1998), supporting this possibility. Further evidence could also be high levels of PAHs and PCBs in the sediments surrounding BI (Drouillard et al, 2006) resulting from the point source of Connor's Creek just upstream (Kannan *et al*. (2001).

Along with increases in sediment contaminants in the surrounding area of BI, the habitat in which the bullheads were collected was quite different from that of PI and TC. BI bullheads were captured within and surrounding two marinas, one abandoned and one active, whereas TC were captured from an open channel, and PI bullheads within an isolated natural habitat around the island. It has been suggested that shoreline development for the construction and function of marinas may alter water circulation and increase contaminant accumulation (Hinkey et al, 2005). Elevated levels of contaminants have been reported as a result of marina activities (Reviewed in Turner et al, 1997; Hinkey et al, 2005). Increases in trace metals such as copper due to leaching of antifouling paints, has also been shown to increase toxicity in marinas (Schiff et al, 2007).

Although sediment collection within certain areas of the marina in combination with muscle contaminant concentrations would be needed to provide further evidence, it may be a reasonable explanation of the reduced respiratory burst activity seen in the bullheads collected at BI. Further investigations into the contamination of both the site and fish of Belle Isle will need to be conducted.

Figure 3.18 identifies a decrease in respiratory burst activity in acute adults. These fish were removed directly from the wild, held in holding tanks for a total of 16 days before the exposure study. These bullheads were only removed from chronic exposure to natural sediment contaminants for a short period of time, thereby limiting the amount of acquired contaminants able to be excreted or cleared. It has been identified that a period of 3 weeks significantly reduces the acute effects of stress due to contaminants as well as being sufficient time to clear certain pesticides, some heavy metals, and certain PAHs and PCBs (Reviewed in Breckles and Neff, 2010). It should also be noted that there may be many other contaminants within the sediment of the Detroit River with unknown clearing times. This theory may also help to support the much higher levels of respiratory burst seen in F1 and cleared adults exposed to clean sediment.

Theoretically, these TC acute fish should be least affected by contaminated sediment exposure, as they have been chronically exposed without a clearing interval. Bullheads collected from TC were expected to have a reduced immune function as a result of contaminant exposure, which correlates to the observed results of the present research (Figure 3.18). Alternatively, acute fish collected from PI were expected to produce a response representing a “clean” environment, allowing for normal immune function when placed on clean sediment. This however was not

observed. These bullheads showed the same trend as those of TC, a decrease in chemiluminescence when exposed to both clean and contaminated sediment. It has been shown previously that fish collected from Peche Island had much higher muscle contaminants than found in the sediments (Leadley et al, 1998). This may explain the observed immunosuppression, as these bullheads may have been exposed to higher levels of contaminants than expected, and in a short period of acclimatization in holding tanks were not able to overcome the immunosuppressive effects. The results therefore may indicate a delayed and or a reduced immune response of the blood granulocytes that can be a result of either direct of indirect contaminant exposure.

The F1 and cleared adults respiratory burst response from PI and TC also seem to indicate an environmental impact of exposure to contaminants, since they showed a reduction in respiratory burst when exposed to contaminated sediments. This environmental impact may also be indicative of physiological adaptation that these fish undergo when exposed to contaminants in the wild (Meyer et al, 2002). Due to the decreased respiratory burst of the acute fish, the results indicate that once contaminants are cleared, immune function can increase. Conversely, while an environmental physiological adaptation may account for bullheads of PI and TC, the F1 and cleared bullheads from BI may seem to exhibit a form of genetic adaptation. BI bullheads exhibit the same trend of mild immunosuppression regardless of sediment, indicating a possible genetic factor carried through the generations. Such genetic adaptations to contaminants have been proposed in bullheads collected from the Detroit River in locomotion and aggressive behaviours, as no difference was exhibited between cleared or F1 populations (Breckles and Neff, 2010). However, before a claim of genetic resistance can be made, it is essential to note

the small population sizes used in this research (Table 3.2). This sample size may not be indicative of the wild population, and will need to be further analyzed.

Contaminants have been identified to not only suppress immune function directly, but impair other responses such as metabolic, hormonal, and behavioural, that can ultimately affect the species survival (Reviewed in Couillard et al, 2008). An alternative explanation to the immunosuppression of the fish placed on contaminated sediment is that the contaminants had an indirect affect on their immune function. The contaminants may have increased energetic costs within the individuals, placing more of a demand on the detoxification and elimination of the acute exposure, therefore increasing individual stress (Reviewed in Couillard et al, 2008). It has been shown that contaminants directly affect aerobic processes and respiration (Couture and Rajotte 2003), hormonal control (Hontela et al, 1992; Liney et al, 2006), and metabolic processes (Reviewed in Couillard et al, 2008). There appears to be well documented evidence supporting that increase stress results in immunosuppression in fish (Wang and Belosevic, 1995; Reviewed in Harris and Bird, 2000). Supporting the idea of chronic stress inducing suppression of respiratory burst of acute adults and those exposed to contaminated sediment are studies identifying reduction in respiratory burst in stressed fish (Angelidis et al, 1987), and cortisol suppressing nitric oxide production in phagocytes (Watanuki et al, 2002; Wang and Belosevic, 1995).

Another possible indirect effect of contaminants is tissue related damage. Non-specific tissue damage due to pollutant induced oxidative stress has been well documented (Farombi et al, 2007, Sanchez et al, 2005). A study of exposure to naphthalene indicated increased lipid peroxidation as a result of respiratory burst activation, potentially leading to increased membrane

damage of vital immune organs (Ahmad et al, 2003). Cell viability can be directly related to cell membrane viability (Darzynkiewicz and Robinson, 1994), and is therefore essential to the proper functioning of immune cells. Lipid peroxidation is also a characteristic of apoptosis (Darzynkiewicz and Robinson, 1994), which may indicate another possible effect of contaminants. The acute fish could potentially be exhibiting the chronic effects of membrane damage, and this may account for the decrease in immune function.

The immunosuppression seen in acute adults may also be reflective of the gender of the fish and season in which they were captured. The acute adult fish were captured during June 2010, nearing the end of the bullhead breeding season of May to June (Blumer, 1985). The immunocompetence handicap hypothesis states that the production of testosterone in males during breeding may contribute to the reduction of male immune defences (Folstad & Karter, 1992), allowing only certain individuals with the best immune genes to maintain immunity during this process. This has been well demonstrated in birds, but to date has been unclear in fish (Reviewed in Kurtz et al, 2007). It has been shown that innate immunity in male sticklebacks is reduced with the injection of 11-ketotestosterone (11-kT) which mimics naturally increased androgen expression and allows increased expression of secondary sexual characteristics (Kurtz et al, 2007). It may be plausible that this interaction between 11-kT and immune function was occurring within the acute male bullheads, explaining the immunosuppression. It has also been suggested that increases in the female hormone estradiol during the breeding season may suppress phagocytic capability of phagocytes (Yamaguchi et al, 2001). Both B- estradiol and 11-kT have been shown to specifically suppress components of the respiratory burst superoxide anion and nitric oxide in carp (Watanuki et al, 2002). It could therefore be proposed that this

evidence may support the reasons behind the acute adult immunosuppression. However, the gender of the bullheads was not determined for the present research, and the involvement of sex as a determining factor of immunosuppression can therefore only be hypothetical.

Not only may the sex have an impact on the results presented here, but the breeding period itself may have the ability to cause physiological stress, indirectly suppressing the immune function (Reviewed in Buchanan, 2000). This down regulation of the immune function in the reproductive period has been identified in captive fish studies (reviewed in Kortet et al, 2003). An increase in parasitic infections in spawning fish compared to non-spawning fish in both Artic charr and Roach species have been identified (Skarstein *et al.*, 2001, Kortet et al, 2003), and may also provide further evidence for immunosuppression during breeding seasons. Spleen size in roach (*Rutilus rutilus*) decreased in both male and female sexes during breeding, and the migratory ability of head kidney granulocytes decreased in both sexes shortly after breeding (Kortet et al, 2003). The spleen is a vital major lymphoid organ in fish (Press and Evensen, 1999), capable of storing immune cells (Kocabas et al, 2002). Spleen size has also been shown to decrease in Actic charr during breeding periods (Reviewed in Kortet et al, 2003). The spleen size of the brown bullheads were not measured, therefore this is only speculation of a plausible reason behind the acute adult immunosuppression.

The results of the respiratory burst as shown in Figure 3.18 clearly indicate an environmental response to contaminant exposure in both F1 and cleared adults collected from TC and PI. The immunosuppressive effects identified in bullheads collected at BI may be indicative of a genetic response to chronic contaminant exposure. Further evidence supporting a reduction in immune function as a result of chronic contaminant exposure is the reduced

respiratory burst response identified in acute adults. The immunosuppressive effects of contaminants has been well documented, the results obtained in the present research indicate the detrimental effects of chronic exposure, and the ability for immune function recovery when removed from contaminated environments.

4.3 Enzyme Linked Immunosorbant Assay (ELISA)

Contrary to what was observed for the respiratory burst assay (Figure 3.18), the ELISA data does not reveal differences in antibody production between sites, or a reduction in antibody production when exposed to contaminated sediment. A study conducted by Powell et al, (2003) seemed to correlate with these results showing that juvenile Chinook salmon (*Oncorhynchus tshawytscha*) orally dosed with various concentrations of a PCB mixture did not significantly alter disease resistance or innate immune response when challenged with *Vibrio anguillarum*. Rice et al (1998) also demonstrated no effect on the channel catfish humoral immune system 21 days after oral injection with a PCB.

However, the acute adults of TC and PI were shown to have a reduced antibody titre to that of the cleared adults and the F1 correlating with the respiratory burst data, indicating an immunosuppressive environmental effect on acute adults. Immunosuppressive effects due to acute exposure to contaminants have been demonstrated in channel catfish IP injected with PCB mixture when assessed by *Vibrio anguillarum* challenge (Regala et al, 2001), and to IP injected TBT when challenged with *Edwardsiella ictaluri* (Rice et al, 1995). It has also been demonstrated that the IP injection exposure to a mixture of PCB and TBT did not suppress antibody production beyond the effect of TBT alone in the channel catfish (Regala et al, 2001), indicating a lack of synergistic effects. Secondary antibody production was suppressed in

juvenile Chinook salmon (*Oncorhynchus tshawytscha*) when injected with DMBA and a PCB mixture (Arkoosh et al, 1994). Japanese medaka (*Oryzias latipes*) exposed to IP injected benzo[a]pyrene at low doses also suppressed T and B lymphocyte proliferation (Carlson et al, 2002). Tilapia have also shown suppressed B cell production in response to BaP injection (Smith et al, 1999), and carp have shown similar results after exposure to 3-MC (Reynaud et al, 2003). Aroclor 1248 exposure also led to suppression in antibody titres at both low and high doses in the brown bullhead (Iwanowicz et al, 2009). Along with the reduction in the respiratory burst seen in acute adults stated above, the acute bullheads reduced antibody titres may be a direct result of chronic exposure and stress due to contaminant exposure in the wild with a limited clearing interval. They may also be in part due to sex, age and seasonal effects.

The cleared adults and the acute adults did not show a significant increase in antibody production from day 0 to day 21 regardless of the sediment exposure or site (Figure 3.22). This may be indicative of antibodies remaining from previous exposures. Although *V.anguillarum* has been primarily associated as a salmonid affecting bacterium, it has been reported as the causative agent in channel catfish disease in the past (Lewis, 1985). The vaccination may not have been strong enough to elicit a large antibody response, as the existing antibodies may have functioned to opsonize the bacteria, eliminating the pathogen without the need for adaptive immunity. Supporting this hypothesis is the high levels of antibody seen at day 0, in cleared adults compared to that of the F1 and the acute adults. This may indicate a lack of pre-exposure in the F1 population to the bacteria, and the demands of contaminant exposure placed on the acute bullheads reduced the ability to produce antibodies. The cleared adults on the other hand may

have been pre-exposed to the bacteria in the wild, and upon clearing the contaminants in the clay ponds they may have been able to again increase energy demands to immune function.

Similar to what was demonstrated in the respiratory burst assay (Figure 3.18), bullheads collected from BI did not significantly differ in antibody response at day 21 regardless of the type of sediment exposure. As stated above, it may be a result of the natural habitat from which they were collected or it may be due to an increased level of contaminant exposure, all resulting in the suggested genetic adaptations carried on to the F1 population. As with the respiratory burst, sample size and the lack of acute adults limits testing of the validity of this proposed theory. Further investigation will be required.

The results of the ELISA may be indicative of acclimatization to the contaminants over a 21 day period, whereas the respiratory burst assay may signify acute environmental impacts to certain individuals (Reviewed in Brekles and Neff, 2010). It may be plausible that the bullheads showed a reduction in immune function after 24hr exposure due to increased acute stress, but upon the completion of 21 days post exposure the bullheads acclimatized and therefore no observable difference in antibody titre could be detected. Recent research has demonstrated in Atlantic salmon an increase in cortisol levels reduces innate immune responses but has no suppressive effect on antibody titres after vaccination (Skinner et al, 2010).

In conclusion, the same trend of acute adult immunosuppression as demonstrated in the respiratory burst was also evident in the ELISA data. These results indicate chronic contaminant exposure have immunosuppressive effects. Due to limited numbers of bullheads for the sediment exposure study, a very small number of bullheads were used for antibody determination as

demonstrated in table 3.4 and therefore limiting the variance between groups. Further research should employ a larger sample size, accounting for variability between groups.

4.4 Reverse transcriptase Real time PCR expression analysis

4.4.1 Major Histocompatibility Class II Beta expression in gill and spleen tissues

The results of the expression study of MHIIB indicate a lack of a contaminant effect after 24hr exposure. The reduced expression levels of MHIIB in bullheads exposed to contaminant sediment was only evident in F1 and cleared adults of TC, and was not observed within any other age groups of other sites. Down regulation of MHII genes have been identified in flounder exposed for 3 days to high levels of oil (Nakayama et al, 2008), correlating with an expected down regulation to contaminant exposure. However, this was only evident in one site of the presented research.

An increase in expression within the F1 of TC in spleen tissue, and the increase in cleared adults of TC in gill tissue may be indicative of different immune responses. The increased levels of MHIIB in the spleen of F1 may represent more circulating MHIIB presenting cells within the blood, and an increased ability to develop an antibody response. This data correlates with the results of the ELISA, showing the increase in antibody titre from day 0 to day 21 in F1. The increased expression observed in the gill of TC cleared adults may be a result of previous exposure to a higher level of bacteria in the wild, leading to an increased ability of phagocytes to ingest and express antigens within the MHII groove. It may also be a result of other bacteria or pathogens remaining from previous exposure or a response to possible pathogens within the sediment.

Previous studies have identified mRNA expression of MHIIB in both the spleen and gill of the gilthead seabream (*Sparus aurata*) (Cuesta et al, 2006), correlating with the expression observed in the present research. Relative expression levels in comparison to baseline expression levels would have allowed for a more thorough analysis, as the response to the vaccination could have been monitored. Increased expression of MHII has previously been identified at the site of vaccine administration (Reviewed in Tonheim et al, 2008) as it is required for the activation of other essential immune cells. It has also been identified that an increase expression is not always apparent in response to immunizations in fish (Reviewed in Nakayama et al, 2008).

4.4.2 Interleukin-8 (IL-8) gene expression in gill and spleen tissues

The present research identified an increase in IL-8 expression in the gill compared to that of the spleen, but an immunotoxicological effect upon exposure was not shown (figure 3.25 and figure 3.26). There was no observable difference between sites or of those exposed to contaminated sediment, correlating with the results of the ELISA. However, the reduced expression of gill IL-8 in acute adults identified an environmental effect of contaminant exposure, and also correlated with what was identified in the respiratory burst and ELISA.

An increase in expression of IL-8 has been shown in flounder exposed to oil in seawater (Nakayama et al, 2008), suggesting the importance for IL-8 in combating bacterial infection even in contaminated waters. It therefore may not be surprising that IL-8 may not have been affected by acute contaminant exposure in the case of the cleared adults and the F1. It may also be plausible that IL-8 expression is not inhibited by acute stress inducing events or acute

contaminant exposure, as also demonstrated in the present research. Further studies involving stress related design may be able to account for such a claim.

The immunosuppressive effects of IL-8 in acute adults may signify a normal response, as it has been identified that a significant increase in spleen IL-8 expression in rainbow trout was evident 4 day post infection (Sigh et al, 2004). However, *in vitro* stimulation indicated a much faster response to LPS stimulation of RTS-11 cell line of IL-8 after only 2 hours, and increasing after 4 hr (Laing et al, 2002). It has also been identified that acidophilic granulocytes accumulate in the spleen 2hr post infection and peak at 4 hr post infection (Reviewed in Chavez-Pozo et al, 2005). Due to the importance of IL-8 in attracting acidophilic granulocytes to inflammatory sites (Chen et al, 2005), it would appear that the observed decrease in IL-8 expression in acute adults may more strongly be a result of chronic contaminant exposure.

4.4.3 Interleukin 1 Beta (IL-1B) expression in gill and spleen tissues

Figures 3.27 and 3.28 identify a decrease in interleukin 1B expression in acute adults in both the gill and the spleen at 24hrs, correlating with the results of the respiratory burst, ELISA and interleukin-8 expression suggesting environmental effects of chronic contaminant exposure leads to immunosuppressive effects.

It has been observed that the expression of IL-1B is significantly increased 24hr post infection in rainbow trout (Sigh et al, 2004) and zebrafish (Rojo et al, 2007), and after 2 days in Atlantic salmon (Haugland et al, 2005). Studies have also indicated the expression of IL-1B by leukocytes within 1-2 hours post stimulation (Zou et al, 2000). The results of the present research may be indicative of a strong early innate response in the F1 and the cleared adults. The

bullheads may have expressed IL-1B early after vaccination, leading to the involvement of other innate cells and cytokines, and the subsequent return of IL-1B to relatively normal levels. The decreased expression in the acute adults at 24 hr may signify a delayed innate response in affected bullheads. IL-1B is known to be an early inflammatory cytokine (Wang et al, 2006), and the decreased expression in acute adults compared to that of the F1 and cleared adults may be indicative of chronic contamination exposure, or even stress inducing properties of living in the contaminated habitats.

IL-1B was increased after a 20 day exposure to the insecticide esfenvalerate in juvenile Chinook salmon, after first being down regulated at 4 days exposure (Eder et al, 2008), identifying a direct contaminant effect. A similar trend of the over expression of IL-1B was demonstrated by Perez-Casanova et al (2008) in cod exposed to the stress of increased temperature, increasing significantly at 45 days. The same study identified an initial increase in plasma cortisol before a reduction to control levels at day 30, prior to the observed increase in IL-1B. An increased IL-1B expression has also been identified in Atlantic salmon up to 9 days post infection when IL-1B inhibitor Prostaglandin E2 was also significantly expressed (Fast et al, 2006). These studies may suggest the delayed immune response of IL-1B due to stress inducting events leading to the secretion of the stress response hormones. The present research has identified a reduced expression level in acute adults chronically exposed to contaminated study, that correlate with previous work identifying a delayed expression in response to stressful conditions. Plasma cortisol levels were not measured in this present study, limiting the ability to correlate stress induced immunosuppression.

The results indicate that no significant difference was observed in IL-1B type b expression when exposed to either clean or contaminated sediment. Contrary to what was observed after 24 hour vaccination in the respiratory burst, no contaminant induced suppression of IL-1B expression was observed. Statistical analysis also revealed an increased expression level in BI compared to that of TC and PI. However, the results of the respiratory burst showed a reduced ability of BI granulocytes to produce ROS. Taken together, the results may lead to an adaptation of the BI bullheads, limiting the number of available cells to respond to cytokines, or a limited ability of the activated cells to respond. The bullheads of BI may therefore have to increase expression of essential early response cytokines in order to activate the immune response, whereas the bullheads of TC and PI do not have to express higher levels as their innate immune system may respond to fewer signals.

Taken together with the results of the immunosuppression in the respiratory burst and the ELISA, the results of the gene expression may indicate that the immune response appears to be reduced upon chronic exposure to contaminated sediment or habitats. Results of the gene expression study do indicate contaminant related effects, varying between sites and age groups. The study also identified reduced levels of IL-1B type b and IL-8 of acute adults. These reduced cytokines may be a direct cause of chronic exposure to contaminants in the wild, or a delayed immune response influenced by stress induction. Further analysis of other immune related genes, specifically adaptive immune system genes, may provide insight into a physiological adaptation to contaminants.

General Conclusions

5.1 Conclusions

The exposure study conducted clearly demonstrates an environmental effect of contaminants on the immune system of the brown bullhead captured from three locations along the Detroit River. The results of the respiratory burst, along with the gene expression of IL-1B and IL-8 indicate a significantly reduced activity of blood phagocytic cells capable of performing the respiratory burst and a decreased production of IL-1B and IL-8 in acute adults 24hr post vaccination and sediment exposure. The ELISA data indicates the same acute adult immunosuppression. Further investigation into other immune aspects will provide more information regarding the impact of the contaminants, but the present sediment exposure study suggests chronic exposure to environmental contaminants has immunosuppressive effects on the brown bullhead innate immune response which reverses when the contaminants are removed.

5.2 Future directions

In order to gain a better understanding of the contaminant induced immunosuppression observed in the respiratory burst assay (Figure 3.18), it may be important to collect and test individuals directly from the river in order to better assess “normal” physiological respiratory burst responses to compare to the affected populations. A determination of “normal” and what it considered reduced may also provide insight, and can be accomplished through the collection of bullheads from an alternative clean site (potentially Belle River). Respiratory burst data is difficult to compare between studies due to variability in reagents, measurements and techniques. If the same assay employed here was to be conducted on fish collected directly from the wild, a

better understanding of the effects due to captivity stress versus natural environment stress may be demonstrated. The artificial environment in the present study supplied constant water, food, shelter and other fish for company. Collecting baseline levels may show variable responses depending on food intake, stress, among other factors, but would give a better indication of the natural immune response of these fish from the collected sites. It would provide more information and a comparison to the collected data to determine if what we found is correlated to the wild.

It may also be beneficial to conduct the respiratory burst at different time points after vaccination and sediment exposure to try to identify the peak time of chemiluminscence. It may be plausible that the acute adults exhibited the same chemiluminescence response, but may have been delayed. Using a control of non-vaccinated bullheads may also provide insight into normal circulating levels of blood neutrophils. A direct impact of this can also be focused on by looking at cell counts, performing leukocyte cell smear and determining leukocyte cell numbers.

In order to determine the uptake of contaminants upon 24, 96 and 24 day sediment exposure, it may be of interest to determine contaminant levels within the muscle tissue. This process was undertaken, but the results to this date have not been completed. This information could potentially help explain any impact of the immunosuppression on contaminated sediment, as the levels of contaminants may be shown to be much higher. If this is not the case, the reason behind the difference is unknown and may have to deal with other factors. Muscle contaminant levels may act as a covariate, providing insight into the amount of contaminants the fish contain and explaining the immunosuppression and delayed response of the acute bullheads.

To look at the potential effects of the marina on the BI fish leading to the proposed genetic adaptation, sediment within different sites of the marina should be collected and analyzed. This information may provide insight into a difference between certain chemicals or contaminants that may be found in greater quantity there than at TC, which was believed to be the highly contaminated site.

Future studies into obtaining the full length cDNA sequence of bullhead IL-1B type a and type b, and identifying the distinct difference between these two in bullheads as seen in channel catfish is of importance. Once these studies are conducted, and two different types are identified, expression studies could also be conducted to determine expression levels of each transcript in different tissues. qPCR analysis can also be conducted, as sequence differences were identified in the qPCR amplicon for type a (Figure 3.11).

5.3 Concluding Remarks

The present study is the first to look at the immune function of different generations of brown bullhead catfish chronically exposed to contaminated sediment. This study clearly indicated a significant reduction in immune function of bullheads collected from the wild, and the subsequent improvement of the immune functioning when the bullheads are removed from contaminated habitats. The reduction in immune function will impact the fitness of any species, and the present data indicates with increased effort towards clean up measures, the fitness of the brown bullhead may increase. This data signifies the importance of chronic contaminant exposure in fish species, and their ability to elicit some form of resistance. The brown bullhead serves little economic importance to the aquaculture industry, but the ability of this species to evolve ways to withstand contaminant exposure can impact the aquatic ecosystem. The evolved

ability of the brown bullhead to increase contaminant loads and remain unaffected can have detrimental consequences through biomagnification of contaminants up the food chain. The ability of the brown bullhead to recover immune function when removed from contaminants may also have implications to other fish species, as the fitness to not only the brown bullhead, but other aquatic species may also increase in the absence of contaminants. The data presented can be of significant use to those managing fish stocks in contaminated waters, and to those of aquatic regulatory agencies creating policies concerning industrial and urban allowable contaminant discharge.

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