Regulation of rainbow trout (*Oncorhynchus mykiss*) mRNA at the innate/adaptive immunity interface in response to Vibrogen 2

by

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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

Aquaculture supplies the world market with over 50% of food fish and is an economically important industry, however limited disease management strategies often lead to significant economic losses and/or poor-quality product. Historically, antibiotics were used prophylactically and as a treatment in disease outbreaks caused by bacterial infections, however, due to antibiotic resistance among pathogens alternative methods for disease control are required and strong vaccination programs are essential. Vibrogen 2, is considered a successful vaccine available for use in aquaculture, however the reasons for this success are poorly understood. Furthermore, while fish appear to have immunological machinery and signaling molecules similar to those found in mammals, evidence suggests fish may use these immune mechanisms differently. In this study, the early immune response elicited by Vibrogen 2 vaccination in rainbow trout head kidney and RTS11 cells was examined through investigation of mRNA expression of two pro-inflammatory cytokines; IL-1β and IFNγ, as well as the mRNA expression of MH class I associated TAP1 and MH class II associate S25-7, to understand the efficacy of the bacterin, as well as, the innate/adaptive immunity interface in teleosts. In RTS11 cells Vibrogen 2 up-regulated IL-1β, S25-7 and TAP1 but not IFNγ in the first 24h post-stimulation and in rainbow trout head kidney IL-1β, IFNγ and TAP1 but not S25-7 was up-regulated in the first 24h after vaccination. Moreover, at 24h S25-7 expression was trending down in head kidney. The results suggest Vibrogen 2 is initiating antibody production in rainbow trout via the pro-inflammatory pathway while the MH class I pathway is also activated. Further, it is possible that 14-1 or INVX rather than S25-7 is involved in MH class II presentation in rainbow trout head kidney. It appears both MH class I and MH class II may be involved in the development of long term immunity in fish and thus, both pathways should be considered when developing vaccines in the future.

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Dedication

To my family, friends and fish lovers everywhere.

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List of Abbreviations

AARS	Alma Aquaculture Research Station
Ala	Alanine
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CLIP	Class II associated invariant chain peptide
cm^2	Centimetre squared
Cp	Crossing point
^oC	Degrees Celsius
DAMPs	Danger associated molecular patterns
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
EF1α	Elongation factor 1 alpha
FBS	Fetal bovine serum
g	grams
gDNA	Genomic deoxyribonucleic acid
g.i.	Gastrointestinal
ICE	Interleukin converting enzyme
IFNs	Interferons
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M

Igs	Immunoglobulins
IgT	Immunoglobulin T
IgZ	Immunoglobulin Z
Ii	Invariant chain
IL-1β	Interleukin 1 beta
IL-1F	Interleukin 1F
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-10	Interleukin 10
IL10R	Interleukin 10 receptor complex
IL10R1	Interleukin 10 receptor 1
IL10R2	Interleukin 10 receptor 2
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-18	Interleukin 18
i.p.	Intraperitoneal
γIP-10	Interferon gamma inducible protein 10
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
LPS	Lipopolysaccharide
MH	Major histocompatibility
MHC	Major histocompatibility complex
min	Minutes
mL	Millilitre
mRNA	Messenger ribonucleic acid
NK	Natural killer
NLRs	Nucleotide-binding and oligomerization domain-like receptors

NTC	No template control
PAMPs	Pathogen associated molecular patterns
PBLs	Peripheral blood leukocytes
PCR	Polymerase chain reaction
PHA	Phytohaemagglutanin
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
rIFNγ	Recombinant interferon gamma
rIL-1β	Recombinant interleukin 1 beta
rTNFα	Recombinant tumor necrosis factor alpha
RAG	Recombination activation gene
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
rps	Rotations per second
RT	Reverse transcriptase
spp.	Species
TAP	Transporter associated with antigen processing
TAP1	Transporter associated with antigen processing 1
TGF-β	Transforming growth factor beta
Th1	T helper cell 1
x g	Times gravitational force
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
USA	United States of America
v/v	Volume/volume

Chapter 1
General Introduction

1.1 Nutritional Value of Fish

Fish is an important food source that provides high quality protein that contains all of the essential amino acids for a healthy diet (Food and Agriculture Organization of the United Nations, 2016; Health Canada, 2011). In 2013, fish was a key source of protein for over 3.1 billion people, providing them with approximately 20 percent of their average per capita animal protein (Food and Agriculture Organization of the United Nations, 2016). Overall, this per capita animal protein value accounted for 17 percent of the global animal protein intake and 6.7 percent of total protein intake in 2013 (Food and Agriculture Organization of the United Nations, 2016). Additionally, fish is an excellent source of essential fatty acids such as long chain omega-3-fatty acids and a good source of vitamins, such as vitamins, D, A and B, as well as minerals, such as, calcium, iodine, zinc, iron and selenium (Food and Agriculture Organization of the United Nations, 2016; Health Canada, 2011). Species identified by Health Canada, (2011) to be very high in omega-3 fats include rainbow trout, salmon, char, herring, mackerel, and sardines. Due to the health benefits of fish, particularly those with high levels of unsaturated fat, which plays a role in reducing the risk of cardiovascular disease and supports fetal and infant neural development, Health Canada, (2011) recommends at least two servings of fish each week (Food and Agriculture Organization of the United Nations, 2016). Furthermore, fish can be valuable in correcting unbalanced diets and reducing saturated fat intake to help manage obesity and weight gain (Food and Agriculture Organization of the United Nations, 2016). In the past, capture fisheries supplied the global market with enough food fish to meet consumer demands (Food and

Agriculture Organization of the United Nations, 2016). However, today over 50 percent of food fish is being produced by the aquaculture industry (Food and Agriculture Organization of the United Nations, 2016).

1.2 World Aquaculture

Hundreds of millions of people worldwide rely on fisheries and aquaculture not only for food and nutrition but also as a livelihood (Food and Agriculture Organization of the United Nations, 2016). However, while capture fishery production has remained relatively static since the late 1980s, aquaculture production has continued to grow, and now supplies half of all food fish to the global market (Food and Agriculture Organization of the United Nations, 2016). This reflects substantial growth of aquaculture considering the industry supplied the global market with only seven percent of food fish in 1974, and 2014 marked a record high world per capita fish supply, which reached an impressive 20kg per year (Food and Agriculture Organization of the United Nations, 2016). This is coupled with a significant growth in fish consumption that reflects an enhanced diet for people around the world, not only providing important nutrition but also diversification (Food and Agriculture Organization of the United Nations, 2016).

The top ten countries involved in exportation of fish and fishery products in 2014, and the value in US$ millions each country exported are listed in Table 1-1, while the top ten countries involved in importation of fish and fishery products in 2014, and the value in US$ millions each country imported, are listed in Table 1-2. In 2014, the combined value of exported fish and fishery products from the top ten exporters in US$ millions was 77 801, while all remaining countries involved in aquaculture product export accounted for 70 346 US$ millions (Food and Agriculture Organization of the United Nations, 2016). The 2014 combined value of

Table 1-1: Top Ten Countries in 2014 Exporting Fish and Fishery Production

Rank	Country	Value US$ Millions
1	China	20 980
2	Norway	10 803
3	Viet Nam	8 029
4	Thailand	6 565
5	United States of America	6 144
6	Chile	5 854
7	India	5 604
8	Denmark	4 765
9	Netherlands	4 555
10	Canada	4 503

(Food and Agriculture Organization of the United Nations, 2016)

Table 1-2: Top Ten Countries in 2014 Importing Fish and Fishery Production

Rank	Country	Value US$ Millions
1	United States of America	20 317
2	Japan	14844
3	China	8501
4	Spain	7051
5	France	6670
6	Germany	6205
7	Italy	6166
8	Sweden	4 783
9	United Kingdom	4 638
10	Republic of Korea	4 271

(Food and Agriculture Organization of the United Nations, 2016)

imported fish and fishery products from the top ten importers in US$ millions was 83 447, while all remaining countries that import fish and fishery products accounted for only 57 169 US$ millions (Food and Agriculture Organization of the United Nations, 2016). Moreover, fish has become a leading export for developing countries and in 2014 was valued at over 35 billion US dollars compared to coffee export which was valued at only about 15 billion US dollars in 2014 (Food and Agriculture Organization of the United Nations, 2016).

At the top of the list of species being traded on the world market is salmon, trout and smelt, which account for 16.6 percent of share by value and 7.2 percent share by live weight in world trade for 2013 (Food and Agriculture Organization of the United Nations, 2016). In fact, salmon, along with shrimp, bivalves, tilapia, carp and catfish, is a key species involved in driving global demand and consumption due to the shift in supply from primarily wild-caught to farmed product (Food and Agriculture Organization of the United Nations, 2016). Additionally, the increased production of salmon, trout and freshwater fishes, has led to a significant growth in yearly per capita consumption of freshwater and diadromous species, which reached 7.3kg in 2013, up from only 1.5kg in 1961 (Food and Agriculture Organization of the United Nations, 2016). Over the next ten years, it is anticipated that the production of salmon, particularly Atlantic salmon, and trout, will continue to grow, and new markets for processed products as well as a higher demand for salmon over sashimi tuna, will be valuable support for this growth (Food and Agriculture Organization of the United Nations, 2016). By 2025, it is expected that world fish consumption will increase by 31 million tonnes, reaching 178 million tonnes (Food and Agriculture Organization of the United Nations, 2016). Figure 1 summarizes the predicted 2025 increase in fish consumption by continent, while Figure 2, illustrates the predicted 2025 relative production of capture fisheries and aquaculture, compared to the predicted relative consumption of fish supplied by capture fisheries and aquaculture.

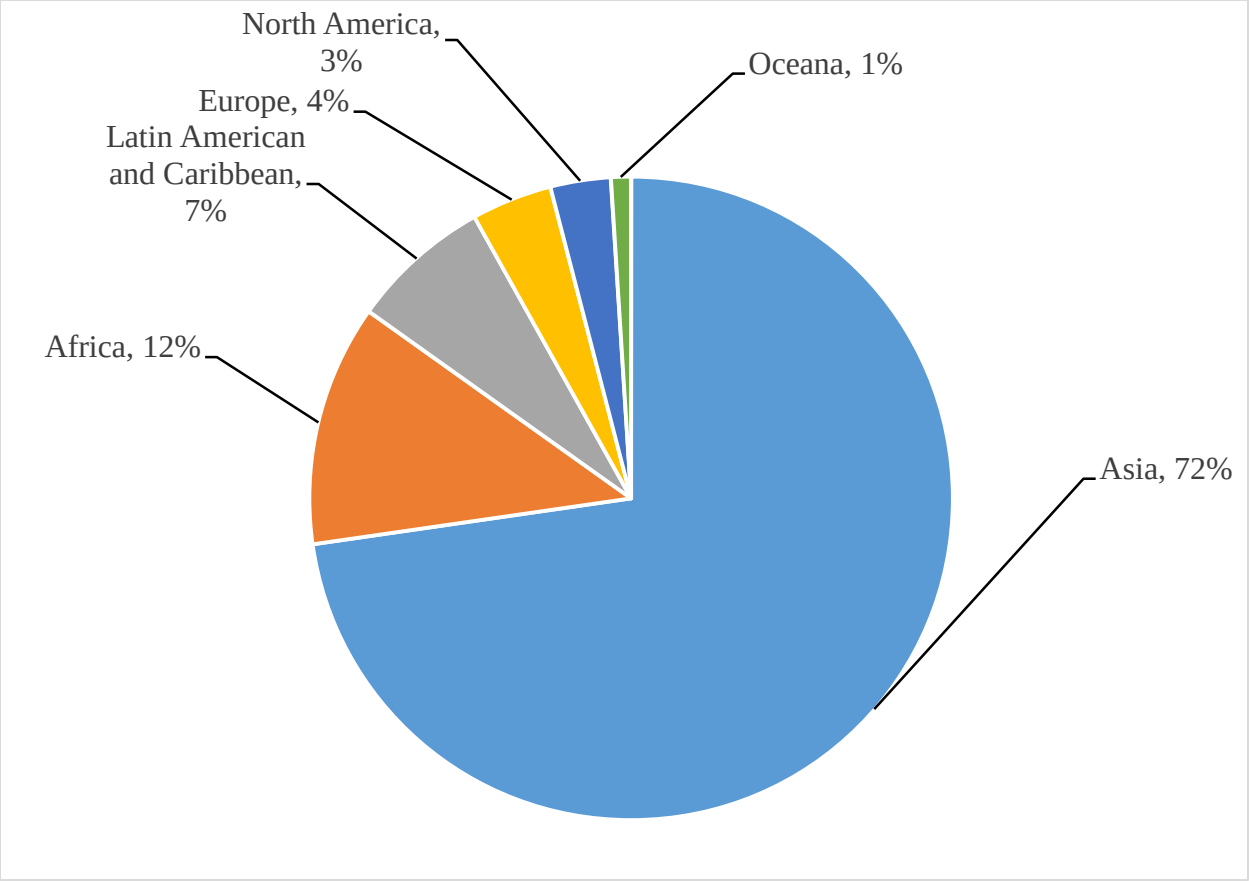

Figure 1-1: Predicted additional consumption of fish in 2025 by continent. (Food and Agriculture Organization of the United Nations, 2016).

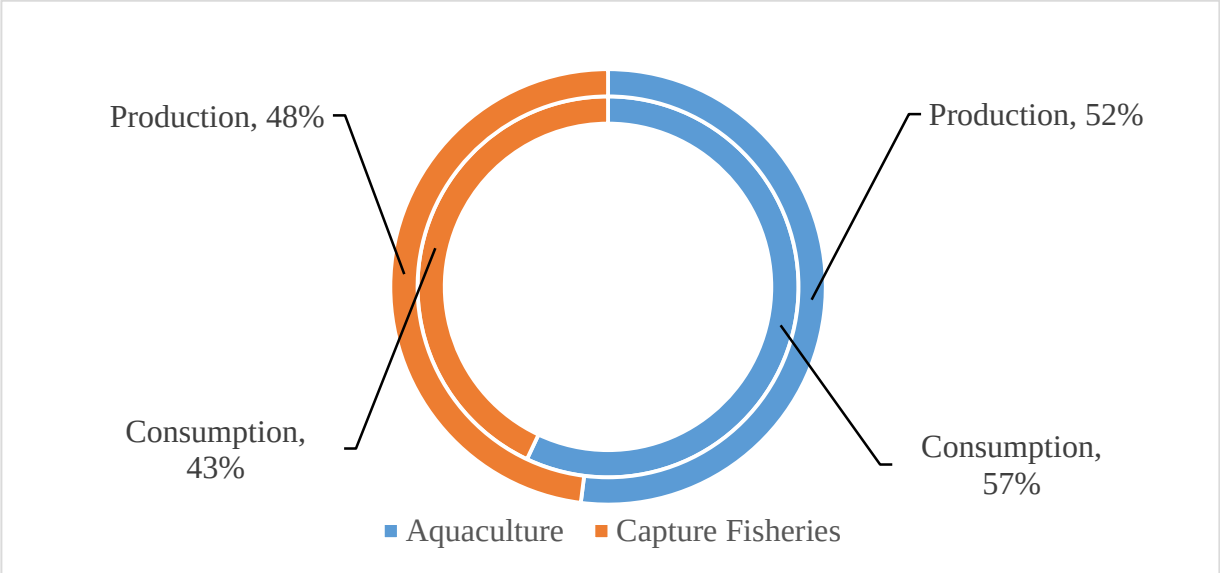

Figure 1-2: Predicted relative global fish production and consumption by aquaculture and capture fisheries for 2025. (Adapted from Food and Agriculture Organization of the United Nations, 2016).

Together these figures illustrate the need to support the growth of the aquaculture industry to meet the food needs of the future. Canadian aquaculture has played an important role in the rapid growth of the aquaculture industry and has the potential to be a leading supplier of food fish for the future.

1.2.1 Canadian Aquaculture

The Canadian aquaculture industry utilizes the oceans and freshwater lakes and rivers, as well as, land-based ponds and tanks to produce farmed products including about 45 different species of finfish, shellfish and marine algae (Fisheries and Oceans Canada, 2014). All ten Canadian provinces, as well as the Yukon Territory, are involved in aquaculture; however, the 2015 value added report by Fisheries and Oceans Canada (2017), lists only seven provinces: Newfoundland, Prince Edward Island, Nova Scotia, New Brunswick, Quebec, Ontario and British Columbia (Fisheries and Oceans Canada, 2014; Food and Agriculture Organization of the United Nations, 2013). These seven provinces are making an important contribution to the Canadian economy, and in 2015, not only contributed $826.6 million to the gross value, but also supported employees and their families paying out a total of $112.2 million in salaries and wages (Fisheries and Oceans Canada, 2017). In 2013, the Canadian aquaculture industry represented about a third of the country's total fisheries value, produced about 20 percent of Canada's total seafood, and was valued at $962 million (Fisheries and Oceans Canada, 2013). This marks an increase of 63 percent over a ten year span as the industry was valued at only $591 million in 2003, and overall a four-fold increase since the early 1990s (Fisheries and Oceans Canada, 2013).

On the global market Canada is number ten on the 2014 export list (Table 1-1). Notably, the United States of America (USA) is Canada's biggest salmon export market and the majority of rainbow trout produced in Canada are exported to the USA as well (Fisheries and Oceans Canada, 2015a, 2015b). In terms of the Canadian economy, the gross value of finfish production by the Canadian aquaculture industry in 2015 was $877.9 million (Fisheries and Oceans Canada, 2017). Total production value reflects production of salmon, including Atlantic, Chinook and Coho, trout, primarily rainbow and brook, as well as, steelhead, which are rainbow trout that spend a portion of their life in saltwater, and a small number of other species currently farmed in Canada (Fisheries and Oceans Canada, 2015a, 2017). Overall, salmon farming is economically important to Canada as it is the third largest seafood export by value, and plays a key role in the economy of coastal and rural communities on both the east and west coast (Fisheries and Oceans Canada, 2015a).

Canada is a top salmonid producing country and in 2013 was the number four producer of salmon species (Fisheries and Oceans Canada, 2013, 2015a). Indeed, the top Canadian aquaculture export is Atlantic salmon, the majority of salmon produced in Canada is farmed in British Columbia, and salmon is the top agriculture export from this province (Fisheries and Oceans Canada, 2015a). However, while salmon is the top saltwater fish farmed in Canada, rainbow trout and brook trout are the most commonly farmed freshwater salmonids (Fisheries and Oceans Canada, 2015b). Trout are produced in all ten Canadian provinces: Alberta, British Columbia, Manitoba, New Brunswick, Newfoundland and Labrador, Nova Scotia, Ontario, Quebec, Prince Edward Island, and Saskatchewan (Fisheries and Oceans Canada, 2015b). Markedly, Ontario is the largest producer of trout species in Canada with the majority of grow-out sites situated in the Northern part of the province, primarily in Georgian Bay and the North

Channel of Lake Huron around Manitoulin Island (Fisheries and Oceans Canada, 2015b). Additionally, Quebec is the second largest producer of trout in Canada, and notably, rainbow trout farming is an important industry in Saskatchewan (Fisheries and Oceans Canada, 2015b). Across Canada aquaculture is an active industry that, over the past two decades, has experienced rapid growth (Fisheries and Oceans Canada, 2013). Considering Canada has the longest coastline in the world and wise use of our water resources alone, will allow growth of the Canadian aquaculture industry, especially in coastal provinces, the nation is poised to play an integral part in meeting the food needs of the future (Natural Resources Canada, 2015). However, water resources are not the only consideration in the development of sustainable aquaculture.

1.2.2 World Population Growth and the Importance of Sustainable Aquaculture

Climate change, economic and financial uncertainty and an ever-increasing competition for natural resources, combined with the expectation that the world population will continue to grow, and by 2050 is predicted to reach over 9.5 billion, presents one of the greatest challenges faced today: How will future generations be fed (Food and Agriculture Organization of the United Nations, 2016)? Currently, aquaculture is the fastest-growing food production sector and recent recognition that the oceans, inland lakes and waterways found all over the world hold great potential to make a significant contribution to food security and nutrition, not just today but in the future, is encouraging (Food and Agriculture Organization of the United Nations, 2016). However, there are hurdles to overcome, such as, control of disease before sustainability of the aquaculture industry will be possible (Food and Agriculture Organization of the United Nations, 2016).

1.3 Disease in Finfish Aquaculture

Disease occurs in both saltwater and freshwater production and can affect fish at any stage of development (Ingerslev, Hyldig, Przybylska, Frosch, & Nielsen, 2012). Infectious agents causing disease in finfish aquaculture include viruses, parasites, bacteria and fungi (Dhar, Manna, & Thomas Allnutt, 2014; Ingerslev et al., 2012). Of these the most prevalent disease causing agent is bacteria which accounts for 54.9 percent of disease in finfish production, while viruses account for 22.6 percent, parasites account for 19.4 percent and fungi account for 3.1 percent of disease in farmed fish (Dhar et al., 2014). Certainly, disease can result in mortality however, even when fish survive, fillet quality is reduced by the presence of parasites, and both viral and bacterial disease can cause bleeding and irreversible damage in the musculature (Ingerslev et al., 2012; Lafferty et al., 2014). Thus, disease frequently results in significant losses in aquaculture and it has been reported that over the past 20 years disease outbreaks have cost the industry tens of billions of dollars (Food and Agriculture Organization of the United Nations, 2016). Despite lower prevalence of viral disease, some have argued that viral diseases are more difficult to control due to lack of treatment options, such as, anti-viral agents (Dhar et al., 2014). However, continued use of antibiotics, the main treatment for bacterial infection is no longer a viable option, due to antibiotic resistance among pathogens (Austin & Austin, 2016a; Haenen et al., 2014; Ingerslev et al., 2012; Magnadottir, 2010; Pridgeon & Klesius, 2012; Tuševljak et al., 2013). According to a survey conducted by Tuševljak et al., (2013) nine antimicrobial drug classes: aminoglycosides, tetracyclines, macrolides, nitrofurans, penicillins, phenicols, potentiated sulphonamides, quinolones and sulphonamides, are in use for treatment of diseases in finfish such as salmon and trout. Of these, resistance to four treatments, tetracyclines, potentiated sulphonamides, penicillins and phenicols, was frequently reported for

several pathogens affecting a variety of farmed species (Tuševljak et al., 2013). This study also surveyed the antimicrobial resistance for six aquatic pathogens and reported that the two most frequently resistant bacterial strains were *Aeromonas spp*. and *Vibrio spp*. (Tuševljak et al., 2013). In fact, for five of the nine antimicrobials 20 percent or more of respondents indicated frequent observation of resistance by *Aeromonas spp*. and for three of the nine drugs 20 percent or more of respondents indicated frequent observation of resistance by *Vibrio spp*. (Tuševljak et al., 2013). The resistance of *Vibrio spp*. to antimicrobials, together with the impact vibriosis, the disease caused by *Vibrio spp*., has on survival and product quality is alarming (Food and Agriculture Organization of the United Nations, 2016; Ingerslev et al., 2012; Lafferty et al., 2014; Le Roux et al., 2015). It is evident that in the years ahead bacterial diseases in aquaculture will likely become increasingly problematic given the growing number of antibiotic resistance strains (Food and Agriculture Organization of the United Nations, 2016; Tuševljak et al., 2013). This, along with the sizeable economic impact disease has on the aquaculture industry, highlights the need for appropriate biosecurity and health management practices to support resilience within the industry and strong vaccination programs will be at the heart of sustainable aquaculture (Food and Agriculture Organization of the United Nations, 2016).

1.3.1 *Vibrio anguillarum* and *Vibrio ordalii*: Causative Agents of Salmonid Vibriosis

Two *Vibrio spp.*; *Vibrio anguillarum*, and *Vibrio ordalii*, have a long history of causing vibriosis, the most serious and significant disease in marine fish, and are responsible for severe economic losses in aquaculture (Avci, Birincio Lu, & Çagirgan, 2012; Busschaert et al., 2015; Frans et al., 2011; Poblete-Morales et al., 2013; Ruiz, Poblete-Morales, Irgang, Toranzo, & Avendaño-Herrera, 2016; Schiewe, Trust, & Crosa, 1981; Steinum et al., 2016). In fact, disease

in marine fish caused by *Vibrio spp*., has been reported as far back as the early eighteenth century, and while *Bacillus anguillarum* was first linked to diseased eels in 1817, in 1909 *V. anguillarum* was identified as the causative agent of the disease in eels known as Red Pest (Actis, Tolmasky, & Crosa, 2011; Schiewe et al., 1981). In 1972 a second *Vibrio spp*. strain was identified as the causative agent of atypical vibriosis and was named *V. anguillarum* biotype II, however, in 1981 Schiewe et al., provided data supporting the separation of the two strains and *V. anguillarum* biotype II was renamed *V. ordalii* (Ruiz et al., 2016; Schiewe et al., 1981; Steinum et al., 2016). Over the years nomenclature used to identify these organisms was further confused as *V. anguillarum* was also named *Listonella anguillarum* for a time, however, today *V. anguillarum* is the accepted and most commonly used terminology (Austin & Austin, 2016b; Avci et al., 2012; Frans et al., 2011; Silva-Rubio et al., 2008).

There are 23 different *V. anguillarum* serotypes and two are responsible for the majority of vibriosis outbreaks in fish: O1 and O2, while a third, O3, also plays a small role in disease (Busschaert et al., 2015; Frans et al., 2011; Steinum et al., 2016). The remaining serotypes are mainly non-pathogenic organisms found in environmental samples such as sediment, plankton and sea water (Busschaert et al., 2015; Frans et al., 2011; Poblete-Morales et al., 2013). *Vibrio anguillarum* serotypes O1 and O2 are responsible for the majority of vibriosis disease in salmonid species and Larsen, Pedersen, & Dalsgaard, (1994), identified 302 of 322 strains isolated from salmonids as type O1 or O2. In fact, of the 322 strains isolated 70.2 percent belonged to the O1 serotype while 20.2 percent belonged to the O2 serovar (Larsen et al., 1994). *Vibrio anguillarum*, which can infect several different fish species including Atlantic salmon, Pacific salmon and rainbow trout, is a Gram-negative, 0.5 × 1.5μm rod, with a shape resembling a comma (Austin & Austin, 2016b; Frans et al., 2011; Rodkhum, Hirono, Crosa, & Aoki, 2005).

This organism is motile and has a polar flagella that can rotate up to 1 700 rotations per second (rps) allowing the bacterium to swim up to 60µm/s (Mccarter, 2001). Additionally, *V. anguillarum*, is a non-spore forming facultative anaerobe that is halophilic, meaning it grows well in high salt environments, and grows in a temperature range of 15-37^{o}C (Austin & Austin, 2016b; Frans et al., 2011). Furthermore, rapid growth of this bacterium has been observed between 25-30^{o}C (Actis et al., 2011; Frans et al., 2011). *Vibrio ordalii*, which has been isolated from coho, chum and spring Chinook salmon, is a Gram-negative, 2.5–3.0 × 1.0µm, curved rod that like *V. anguillarum* is motile by a polar flagella (Actis et al., 2011; Austin & Austin, 2016b). The temperature range for growth of *V. ordalii* is more limited than that of *V. anguillarum* and is 15-22^{o}C (Austin & Austin, 2016b). Both *V. anguillarum* and *V. ordalii* degrade gelatin, however, haemolysins and proteases have been identified in *V. anguillarum* but not *V. ordalii* (Austin & Austin, 2016b; Rodkhum et al., 2005). Additionally, metalloproteases, dermatotoxin, hemagglutinin and cytotoxin, are secreted exotoxins that have been associated with *V. anguillarum* strains and are correlated with disease pathogenesis and some strains carry a 65 kilo base (kb) pJM1 or pJM1-like plasmid allowing these strains to synthesis and transport the virulence factor, siderophore anguibactin (Naka et al., 2011; Rodkhum et al., 2005). In comparison, little is known about the virulence factors associated with *V. ordalii* infection however, recent work by Ruiz, Balado, et al., (2016), has demonstrated that this organism has two different iron acquisition systems; one involves synthesis of siderophore, and a second involves direct binding of haeme, and this iron uptake is related to pathogenicity (Actis et al., 2011). There is some uncertainty regarding the mode of infection of *V. anguillarum* and *V. ordalii*, however it is clear that infection is a result of bacterial colonization and tissue penetration and proposed sites of entry include the skin, fins, anus, intestine and rectum (Austin

& Austin, 2016b; Kanno & Nakai Muroga, 1990; Ruiz et al., 2016). Additionally, the temperature and quality of the water as well stress and strain virulence play a role in infection and disease outbreak (Actis et al., 2011).

1.4 Vibriosis

Vibriosis outbreaks have been reported in nearly 50 different fish species in multiple countries on both the Atlantic and Pacific coasts and have occurred in both fresh and saltwater fish (Actis et al., 2011; Frans et al., 2011; Kanno & Nakai Muroga, 1990). Indeed vibriosis, caused by *Vibrio spp*., has been recognized as one of the most widespread fish diseases and is among the most important diseases in aquaculture (Actis et al., 2011; Busschaert et al., 2015). Vibriosis, caused by *V. anguillarum* is characterized by a haemorrhagic septicemia and red spots on the ventral and lateral tissues (Actis et al., 2011; Busschaert et al., 2015; Frans et al., 2011; Munn, 1977; Poblete-Morales et al., 2013). Infected fish lose weight, become lethargic, develop skin discolouration and dark swollen necrotic skin lesions that abscess and bleed (Actis et al., 2011; Austin & Austin, 2016b; Frans et al., 2011; Li, Mou, & Nelson, 2013; Munn, 1977). Bloody patches, or erythema, is observed around the fins and vent as well as in the mouth and haemorrhaging occurs in the gills, gut and muscle (Austin & Austin, 2016b; Ransom, Lannan, Rohovec, & Fryer, 1984). Lesions of the eye may also develop first resulting in opacity followed by the development of ulcers and finally abnormal protrusion or bulging of the eye (Actis et al., 2011; Frans et al., 2011). Additionally, distention of the intestines may be observed due to accumulation of a clear viscous liquid and high bacterial cell counts are obtained from blood, haematopoietic tissues, such as kidney, and from the spleen, liver and gut as well as the heart and connective tissues (Actis et al., 2011; Austin & Austin, 2016b; Frans et al., 2011; Ransom et al., 1984). Notably, in acute disease outbreaks infection spreads rapidly and

mortalities occur in fish infected but without any clinical signs of disease (Actis et al., 2011; Frans et al., 2011). Atypical vibriosis, caused by *V. ordalii*, may also present with haemorrhagic septicemia however the development of bacteremia generally occurs later in disease progression than it does with *V. anguillarum* infection (Austin & Austin, 2016b; Ransom et al., 1984). Instead, *V. ordalii* tends to localize in muscle and skin tissues aggregating and forming micro-colonies in the heart, skeletal muscles, loose connective tissues as well as gill tissue, gut and pyloric caeca (Actis et al., 2011; Austin & Austin, 2016b; Ransom et al., 1984). Due to this localization and aggregation bacteria are not uniformly distributed in tissues in *V. ordalii* infection as they are with *V. anguillarum* infection (Ransom et al., 1984). In some instances bacterial aggregates may completely replace host tissue however, necrosis of the area may or may not be present (Actis et al., 2011; Ransom et al., 1984). Additionally, Ransom et al., (1984), did not observe *V. ordalii* in the kidney of infected fish and only found occasional bacterial colonies in the liver and spleen (Actis et al., 2011). Vibriosis outbreaks generally occur in salt or brackish water during late summer and are most problematic in shallow water (Actis et al., 2011). In general, *V. anguillarum* is the primary pathogen encountered by salmonids such as, Atlantic salmon, Pacific salmon and rainbow trout during their first year at sea (Jansson & Vennerstrom, 2014). However, *V. ordalii*, has been responsible for recent disease outbreaks in aquaculture of all three species in Chile (Actis et al., 2011; Ruiz et al., 2015, 2016; Silva-Rubio et al., 2008). In addition to *V. anguillarum* and *V. ordalii*, several other *Vibrio spp*. are responsible for vibriosis outbreaks in fish and shellfish including, *Aliivibrio salmonicida* (formerly *Vibrio salmonicida*), the causative agent of cold water vibriosis in Atlantic salmon, rainbow trout and cod and *Vibrio vulnificus* biotype II, the causative agent of vibriosis in cultured eel (Actis et al., 2011; Haenen et al., 2014). Vibriosis has high morbidity and a

mortality rate that can be as high as 100%, thus, antimicrobials are often used as a therapeutic treatment however, fish in poor health often stop eating reducing antimicrobial uptake and recovered fish produce lower quality fillets due to histopathogenesis (Austin & Austin, 2016b; Busschaert et al., 2015; Hickey & Lee, 2017; Li et al., 2013; Munn, 1977; Poblete-Morales et al., 2013).

1.4.1 Vibriosis in Canada

While *Vibrio spp*. outbreaks have been reported all over the world since the early 1900s and throughout the early 1900s were problematic in the USA, the first cases of vibriosis in Canada were not reported until in the late 1960s (Evelyn, 1971). In 1968 on July 22nd vibriosis was first observed in Canada in a stock of under-yearling chum salmon housed at the Nanaimo Research Station and two days later disease was observed in a second stock of fish; two year old sockeye salmon (Evelyn, 1971). At a second site, the Pacific Environment Institute, Fisheries Research Board of Canada, West Vancouver an outbreak of vibriosis occurred in yearling pink and Chinook salmon in August through September of that same year (Evelyn, 1971). The approximated water temperature at both locations at the time of disease outbreak was at the annual maxima expected; 15^{o}C at Nanaimo Research Station and 12^{o}C at the Pacific Environment Institute and confirmatory testing identified *V. anguillarum* as the causative agent at both sites (Evelyn, 1971). Throughout the early 1970s vibriosis outbreaks among cultured salmonids on the Canadian West Coast remained problematic and in 1976 *V. ordalii* was isolated for the first time from nearby cultured Pacific salmon on the USA west coast in Puget Sound, Washington (Evelyn, 1984; Harrell, Novotny, Schiewe, & Hodgins, 1976). Indeed, in the early 1970s vibriosis outbreaks were occurring around the world, but it was the constraint of disease

outbreaks faced by culturists on the West Coast of North America that spurred the development of preventative vibriosis vaccines (Evelyn, 1984).

1.4.2 Prevention and Treatment of Vibriosis

Currently, there are several practices implemented by fish farmers to control bacterial disease in aquaculture including: adequate husbandry and management practices such as preventing movement of infected stock, use of genetically resistant fish strains, providing suitable diets and appropriate use of dietary supplements; water treatments including use of disinfectants such as benzalkonium chloride, chlorine and formalin and use of antibiotics such as Florfenicol, Flumequine and potentiated sulphonamides when fish refuse feed as well as; use of probiotics, prebiotics, antimicrobial compounds and/or vaccines (Austin & Austin, 2016a). The use of antibiotics such as Florfenicol, to control vibriosis has met with some success however, treatment is generally administered as a food additive thus, it is essential that antibiotics be provided early in the disease cycle as loss of appetite ensues as the disease progresses (Austin & Austin, 2016b). While the use of antibiotics has historically been at the heart of strong disease management practice in aquaculture, the use of antibiotics as a preventative measure is now discouraged because, as described above, antibiotic over use leads to resistant pathogens leaving antibiotics ineffective as a treatment (Austin & Austin, 2016a; Haenen et al., 2014; Ingerslev et al., 2012; Magnadottir, 2010; Pridgeon & Klesius, 2012; Tuševljak et al., 2013). Additionally, in a survey of aquaculture professionals asked to identify observed resistance associated with six different aquatic pathogens, *Vibrio spp.* were the second most frequently reported pathogen associated with antibiotic resistance (Austin & Austin, 2016b; Tuševljak et al., 2013). Furthermore, the primary causative agent of finfish vibriosis, *V. anguillarum*, is a pathogen with high potential risk of antibiotic resistance as plasmids or R factors associated with some strains

confer resistance to multiple antibiotics including chloramphenicol, streptomycin, sulphonamides and tetracycline (Austin & Austin, 2016b). Therefore, appropriate alternate preventative measures are essential for control of vibriosis in aquaculture. While probiotics such as *Kocuria SM1* have been shown to be effective in preventing vibriosis in rainbow trout, vaccines may be a more effective means of disease control and vaccine development has garnered much attention (Austin & Austin, 2016a, 2016b; Dixon, 2012; Pridgeon & Klesius, 2012; Sharifuzzaman & Austin, 2010).

1.5 Aquatic Vaccine Development and Methodologies

The first attempt to develop a vaccine to a bacterial fish pathogen was made by Duff, in 1942 and in the 75 years since, vaccines to only about half of existing bacterial fish pathogens have been developed (Austin & Austin, 2016a). Certainly research has moved forward investigating potential vaccine components such as cell lysates, inactivated whole cells, live-attenuated and DNA subunit vaccines, as well as, purified sub-cellular components such as lipopolysaccharides (LPS) and inactivated soluble cell extracts such as toxins, however, it has been difficult to identify a specific preparation that out performs the rest in terms of long term immunity (Austin & Austin, 2016a; Pridgeon & Klesius, 2012). A variety of inactivation methods for whole cell preparations have also been considered including heat, pressure and electric current, as well as, chemicals (Austin & Austin, 2016a). Researchers have used 3 percent (v/v) chloroform, 0.3-0.5 percent (v/v) formalin and 0.5-3.0 percent phenol in whole cell preparations (Austin & Austin, 2016a). Commercially formalin inactivation is favoured given successful results in vaccine trials against several fish pathogens including *V. anguillarum* and *V. ordalii* (Austin & Austin, 2016a). Even though formalin inactivated whole cell preparations have met with some success, data comparing inactivated whole cell vaccines prepared with differently inactivated whole cells is

lacking, therefore, it is not clear why formalin inactivation has been successful while other inactivation methods have failed (Austin & Austin, 2016a).

The best method of administration for fish vaccines has also been considered and a wide variety of methodologies have been investigated (Austin & Austin, 2016a). These include: injection, oral uptake via food, immersion and bathing, spaying and showering, hyperosmotic infiltration, anal or oral intubation and ultrasonics/ultrasound (Austin & Austin, 2016a; Evelyn, 1984). While it is difficult to determine the most effective method most evidence suggests oral administration is the least effective vaccination method although some oral booster doses have been successful (Austin & Austin, 2016a). While immersion has been shown superior to injection with some preparations, injection, with and without adjuvant, has been studied and in some cases may lead to higher antibody titres and thus, presumably better protection, although, antibody titre may not correlate directly with level of protection in all instances (Austin & Austin, 2016a). Additional consideration for injection, as a vaccination method, include the need to anaesthetize fish prior to vaccination and although mass injection techniques are available this process is generally slow (Austin & Austin, 2016a). Notably, vaccines against *V. anguillarum* and *V. ordalii* have been the focus of several studies due in large part to the commercial success of formalin-inactivated whole cell bacterins against these pathogens (Austin & Austin, 2016b).

1.6 *Vibrio anguillarum* and *Vibrio ordalii* Bacterins and Immunogenicity Research

Formalin inactivated *V. anguillarum* and *V. ordalii* bacterins are commercially available and the success of these vaccines in Atlantic halibut, African catfish, and sea bass has led to widespread use in aquaculture (Austin & Austin, 2016b). However, a limited number of studies have been conducted to understand either the efficacy of these vaccines or the immune response

they elicit. One study in Atlantic salmon conducted by Acosta, Lockhart, Gahlawat, Real, & Ellis (2004) demonstrated that a *V. anguillarum-V. ordalii* bacterin induced the Type I interferon inducible Mx gene following intraperitoneal (i.p.) injection and that both purified deoxyribonucleic acid (DNA) and LPS from *V. anguillarum* serotype O1 induced Mx as well (Austin & Austin, 2016b). In another study Joosten, Kruijer, & Rombout, (1996) demonstrated bacterin supernatant contains the most immunogenic component of the vaccine and it has been suggested that this immunogenicity is a an indication of heat-stable LPS in the cell wall that has been released into the culture supernatant (Austin & Austin, 2016b). Furthermore, a 100kDa LPS is thought to be involved in the development of protection, although, two minor outer membrane proteins, approximately 49-51kDa in size, with strong antigenicity, as well as, a weakly antigenic protein, approximately 40kDa in size, may also contribute to bacterin immunogenicity (Austin & Austin, 2016b; Chart & Trust, 1984; Evelyn, 1984). It is unclear if *V. anguillarum* or *V. ordalii* produce other non-LPS immunogens, however it has been demonstrated that preparations of O-antigen induce an immune response in a number of fish species including rainbow trout, and some work has suggested that both heat-labile and heat-stable antigenic components play a role in immunogenicity, since formalin-killed vaccines provided better protection than heat-killed preparations in some studies (Austin & Austin, 2016b; Evelyn, 1984). Potential flagella protein immunogenicity has also been evaluated in Japanese flounder (*Paralichthys olivaceus*) (Austin & Austin, 2016b). Additionally, some research has focused on developing live-attenuated vaccines and while these have met with some success and may confer cross-protection against other pathogens, licensing these products for use in fisheries may be problematic (Austin & Austin, 2016b). Consequently, LPS is one of the most important

vaccine components in *V. anguillarum-V. ordalii* bacterins and the majority are prepared from formalin-killed rather than heat-killed cultures (Colquhoun & Lillehaug, 2014; Evelyn, 1984).

Moreover, the majority of vibriosis vaccines are bivalent *V. anguillarum-V. ordalii* inactivated whole cell bacterins, prepared for immersion or i.p. injection methods despite the convenience of oral vaccination (Austin & Austin, 2016b; Colquhoun & Lillehaug, 2014). Certainly many studies have focused on orally administered vaccines, including encapsulation to protect the vaccine from breaking down in the stomach and intestines, however, immersion and injection methods continue to out-perform oral vaccination (Austin & Austin, 2016b; Evelyn, 1984). In general, i.p. vaccination against vibriosis provides better and more durable protection than immersion vaccination, and it has been reported that vaccination by injection in a group of salmonids reduced mortalities to zero percent (Austin & Austin, 2016b; Colquhoun & Lillehaug, 2014).

1.6.1 Intraperitoneal Injection for Vibriosis Vaccination in Fish

While i.p. injection of vaccines against vibriosis leads to superior and longer-lasting immunity this method is relatively costly in comparison to other methods and increases fish stress due to handling (Colquhoun & Lillehaug, 2014; Evelyn, 1984). Additionally, it is inconvenient for vaccination of small fish, generally less than 20g, requires a large workforce for mass vaccination and is a slower process in comparison to alternative methods (Austin & Austin, 2016b; Colquhoun & Lillehaug, 2014; Evelyn, 1984). However, it is generally more feasible for large and/or valuable fish, it allows for even distribution and effective use of antigen, as well as, provides an opportunity to include adjuvants that enhance and prolong immunity (Austin & Austin, 2016b; Evelyn, 1984). Additionally, some vaccines are only effective when administered via injection and this method has also been linked with an increase in skin mucus

antibody levels (Evelyn, 1984). Furthermore, while alternative vaccination methods such as, immersion, often require repeated immersion vaccination and/or boosters, i.p. vaccination, especially with vaccines containing oil adjuvants, generally provides protection through to harvest in anadromous fish after a single i.p. injection (Colquhoun & Lillehaug, 2014).

Another consideration, although one to bear in mind for any vaccination method, is the effect of temperature on the immune response of fish which, for *V. anguillarum* vaccination, is best documented for injection (Evelyn, 1984). In general it seems antibody development is slower at lower temperatures following injection with killed *V. anguillarum* cells, while antibodies develop more quickly at warmer temperatures (Evelyn, 1984). Thus, the effect of temperature must be taken into consideration when determining the best time to vaccinate fish to ensure immunity before exposure (Evelyn, 1984). Generally, for commercially available vaccines a set of instructions lists the appropriate dose, method of administration, and details regarding any additional considerations. For example, Vibrogen 2, a formalin-killed *V. anguillarum serotypes I and II-V. ordalii* bacterin, produced by Elanco Canada Limited, and licensed for use prior to 2000 indicates the bacterin can be administered to healthy salmonids by i.p. injection in fish 10g or larger or by bath immersion in a one part bacterin to ten parts water bath for 30 seconds (Canadian Food Inspection Agency, 2017; Pridgeon & Klesius, 2012). Additional information includes details concerning vaccine efficacy and states that vaccination is most effective when carried out at least 250 degree-days, calculated by multiplying the number of days post-vaccination by the mean water temperature (^{o}C), post-vaccination and if immunity is required for greater than 300 days fish can be revaccinated.

1.7 Challenges for Successful Fish Vaccine Development

Despite the noted success of *V. anguillarum* and *V. ordalii* bacterins, little is understood regarding the immune response triggered in fish post-vaccination and many research programs for vaccines to important aquatic pathogens have not led to commercial products (Austin & Austin, 2016a). This is likely due in large part to the general lack of available knowledge describing fish immunity (Dixon, 2012). For example, little is known about the immunological pathways, such as inflammation, initiated as part of the innate immune response to pathogens, and how these activated pathways transition to the adaptive immune response for development of long term immunity. However, what has been made clear from research conducted to date is that while the immune system of fish have machinery and immune components analogous to those found in mammals, the way the machinery and immune components work together differs in fish (Dixon, 2012). Therefore, it is important fish vaccines be developed based on an understanding of the fish immune system rather than the mammalian system, and careful consideration of both the innate and adaptive immune pathways are critical for a complete understanding of long term immunity in fish (Dixon, 2012).

1.8 Innate Immunity in Fish

The innate immune response involves germ-line encoded and generally non-specific recognition of pathogens and is present in both invertebrates and vertebrates (Magnadottir, 2010; Rasmussen, Reinert, & Paludan, 2009). This response is a rapid first line of defence and has adapted over time in response to environmental factors and pathogenic associations (Castro, Zou, Secombes, & Martin, 2011; Magnadottir, 2010; Rasmussen et al., 2009). Thus, the innate immune response to pathogens is dependent on the evolutionary lineage and genetics of an

organism and any specificity gained over time is heritable (Magnadottir, 2010). Despite a finite arsenal of machinery capable of pathogen recognition the innate immune response provides a strong and efficient defence against pathogens (Magnadóttir, 2006). In fact, invertebrates rely solely on efficacy of innate immunity for protection against numerous pathogens across diverse environmental conditions (Magnadóttir, 2006). There are three main components of innate immunity, these include: external physical barriers such as skin and mucous; humoral including antimicrobial peptides, complement and cytokines; and cell mediated including myeloid phagocytic cells (Romo, Perez-Marinez, & Ferrer, 2016). Indeed, innate immunity relies heavily on white blood cells, such as macrophages and neutrophils, to kill pathogens via phagocytosis and simultaneously synthesize a broad range of inflammatory mediators and cytokines coordinating the additional responses necessary to combat invading pathogens (Aderem & Ulevitch, 2000). Additionally, most vertebrates elevate body temperature by 1-4^{o}C in a complex neuroendocrine and behavioural response known as fever, to aid the immune system by improving leukocyte efficacy and impairing microbial growth (Grans, Rosengren, Niklasson, & Axelsson, 2012). Moreover, the innate immune system is necessary for activation of acquire or adaptive immunity (Magnadóttir, 2006; Rasmussen et al., 2009). The characteristics of teleost fish innate immunity are, for the most part, shared by invertebrates as well as higher vertebrates and the innate immune response of fish is generally divided into three: the epithelial/mucosal barriers including the skin, gills and alimentary tract; the humoral parameters expressed as cell receptors and soluble secreted forms including complement; and the cellular component including important innate immune cells such as monocytes/macrophages (Magnadottir, 2010; Magnadóttir, 2006). Notably, while mammals and birds increase body temperature via a complex internal thermoregulatory process to elicit fever, fish, including rainbow trout, have

been shown to seek out warmer water to induce a behavioural fever during immunostimulation and in goldfish (*Carassius auratus*) increased water temperatures improved survival of infected fish (Grans et al., 2012).

1.8.1 Pattern Recognition Receptors

Once a pathogen has penetrated the physical protective barriers of the innate immune system such as the skin, the germ-line encoded pattern recognition receptors (PRRs) are responsible for recognizing exogenous conserved motifs, known as, pathogen associated molecular patterns (PAMPs) such as LPS and flagellin, as well as, endogenous cellular debris and components generated by cellular damage, known as, danger associated molecular patters (DAMPs) (Castro & Tafalla, 2015; Chettri, Raida, Holten-Andersen, Kania, & Buchmann, 2011; Rebl, Goldammer, Fischer, Köllner, & Seyfert, 2009; Tanekhy, 2016). PRRs are primarily located on dendritic cells (DCs) and macrophages, however, they are also present on additional cell types including, B cells and endothelial cells, and once activated PRRs trigger several intracellular activation pathways that ultimately all lead to activation of pro-inflammatory genes or anti-microbial genes (Castro & Tafalla, 2015). In teleosts, five PRRs types have been identified; C-type lectin receptors, NOD-like receptors, RIG-I-like receptors and peptidoglycan recognition proteins, however, Toll-like receptors (TLRs) are the most studied and well known (Castro & Tafalla, 2015). TLRs are important to both innate and adaptive immunity with activation leading to the production of inflammatory cytokines, which are small glycoproteins or simple polypeptides of less than 30kDa, responsible for regulating immune function by mediating cell signaling, as well as, induction of major histocompatibility complex (MHC) class II in mammals (Castro & Tafalla, 2015; Secombes, Hardie, & Daniels, 1996; Tanekhy, 2016). More than 17 TLRs have been identified in fish and while some appear similar to those in mammals some are

unique to teleosts (Castro & Tafalla, 2015; Tanekhy, 2016). Indeed, a high degree of TLR recognition and function appears to be conserved between mammals and fish, however, notable differences include TLR4, that in mammals recognizes LPS, however in some fish TLR4 is unable to recognize LPS and in other fish TLR4 is absent altogether, and TLR3 in fish, appears to recognize both bacterial and viral PAMPs, while mammalian TLR3 recognizes only viral PAMPs (Castro & Tafalla, 2015; Tanekhy, 2016). Additionally, while both mammalian and fish TLR5 appear to recognize bacterial flagellin, in several fish species including rainbow trout, a TLR5-like soluble protein that binds flagellin has also been reported and this soluble form is absent in mammals (Tanekhy, 2016; Tsujita et al., 2006). Irrespective of these difference TLRs in fish are a key part of the innate immune response and are involved in initiating the pro-inflammatory response.

1.8.2 Inflammation

The inflammatory response involves a variety of cell types, such as, macrophage and DCs, as well as, the attraction of leukocytes to an inflamed area in a complex and multi-step process under strict regulatory control by cytokines (Secombes et al., 2001; van der Aa, Chadzinska, Golbach, Ribeiro, & Lidy Verburg-van Kemenade, 2012). Indeed, the acute inflammatory response is characterized by the transport of blood components such as plasma and leukocytes to sites of microbial infection via a process triggered by innate immune receptors (Medzhitov, 2008). This initial pathogen recognition event is mediated by macrophage and mast cells present in the tissue at the site of infection and leads to the production of pro-inflammatory mediators such at chemokines and cytokines (Medzhitov, 2008). That is, when PRRs such as TLRs bind PAMPs, a series of intracellular signaling pathways that lead to production and release of pro-inflammatory cytokines such as, tumor necrosis factor alpha (TNFα) and interleukin 1β (IL-1β)

are initiated (Fierro-Castro et al., 2012). The primary and direct effect of these pro-inflammatory cytokines, along with other pro-inflammatory mediators such as chemokines, vasoactive amines, eicosanoids and proteolytic cascade products is to induce an inflammatory exudate at the site of infection allowing plasma proteins and leukocytes such as neutrophils access to the extravascular tissues at the infection site (Medzhitov, 2008). Once neutrophils reach the site of infection they are activated by the invading pathogen or cytokines and will attempt to eliminate the invading pathogen (Medzhitov, 2008). However, neutrophil activity relies on the release of toxic contents, such as reactive oxygen species and reactive nitrogen species, from their granules and this process results in damage to host tissues as neutrophils are unable to differentiate between microbial and host targets therefore, a resolution and repair phase, mediated by recruited and tissue-resident macrophages, follows clearance of the infectious agent as part of a successful acute inflammatory response (Medzhitov, 2008). It has been demonstrated that the pro-inflammatory response of teleosts is similar to the response observed in mammals and is biphasic, beginning with an influx of neutrophils followed by the arrival of monocytes and/or macrophages, however, it appears to be less intense and both slower to appear and resolve (Finn & Nielson, 1971; Reite & Evensen, 2006). Additionally, it is well understood that in mammals, activation of the inflammatory response leads to a cytokine cascade that begins with the release of TNFα, and is followed by IL-1β production which is followed by release of interleukin 6 (IL-6) (Secombes et al., 2001). Characterization of rainbow trout TNFα bioactivity demonstrated that recombinant TNFα (rTNFα) induces expression of several pro-inflammatory genes, including IL-1β, in both head kidney leukocytes and macrophages, as well as, enhances leukocyte migration *in vitro* and regulates phagocytosis of head kidney leukocytes, while characterization of rainbow trout IL-1β showed recombinant IL-1β (rIL-1β) increases the

expression of important downstream genes including MH class II β chain, as well as, the phagocytic activity of head kidney leukocytes (Hong et al., 2001; Zou et al., 2003)

1.8.3 Interleukin 1β

Interleukin 1β was the first cytokine cloned in fish and has been identified in several teleost species including salmonids (Angosto et al., 2014). In mammals, IL-1β has a range of biological effects, a broad range of target cells and plays a fundamental and central role in both the initiation and regulation of inflammation (Peddie, Zou, Cunningham, & Secombes, 2001). The mammalian IL-1β protein is characterized by an IL-1 converting enzyme (ICE) cut site where caspase-1 cleaves the IL-1β inactive precursor to generate the biologically active mature form (Hong, Zou, Collet, Bols, & Secombes, 2004). Additionally, in mammals, it is understood that activation of inflammatory caspases, such as caspase-1 is dependent on cytosolic multiprotein platforms called inflammasomes (Angosto et al., 2012).

Four distinct inflammasome compositions have been identified in mammals three of which contain the nucleotide-binding and oligomerization domain-like receptors (NLRs): NLRP1B, NLRP3 and NLRC4 (Angosto et al., 2012). The NLRP3 inflammasome is well characterized and the best studied inflammasome and it has been shown that this inflammasome is not evolutionarily conserved and is specific to mammals (Ogryzko, Renshaw, & Wilson, 2014). In fact, Angosto et al., (2012) demonstrated that stimulated or infected gilthead seabream (*Sparus aurata*) macrophages process and release IL-1β independent of caspase-1, and while the IL-1β cytokine identified in fish has similar biological function to mammalian IL-1β, including regulation of the inflammatory response, fish IL-1β lacks the ICE cut site (Hong et al., 2001; Hong et al., 2004; Zou, Grabowski, Cunningham, & Secombes, 1999). However, despite the absence of the ICE cut site in the fish IL-1β sequence Zou et al., (1999) predicted, based on

multiple sequence alignment, that the rainbow trout IL-1β precursor is cleaved at Ala^{95} (alanine 95) and it has been demonstrated that although fish appear to lack NLRP3, the NLR protein expansion of teleosts is similar to a family of proteins found in sea urchin (Ogryzko et al., 2014). Nevertheless, strong evidence suggests that rainbow trout IL-1β is processed by macrophage cells, and cleavage of precursor IL-1β occurs within the cell to produce the mature active form, which is released from the cells as part of the processing event or after processing has occurred (Hong et al., 2004).

1.8.4 Macrophages

Macrophages are present in nearly every cell where they are responsible for maintaining balance and are generally among the first cells to recognize invading pathogens (Hodgkinson, Grayfer, & Belosevic, 2015). Additionally, macrophages are responsible for coordinating appropriate immune responses (Hodgkinson et al., 2015). In mammals four different macrophage subset populations have been described including one classically activated population type and three alternatively activated types (Hodgkinson et al., 2015; Wiegertjes, Wentzel, Spaink, Elks, & Fink, 2016). Classically activated macrophages (M1) are stimulated by interferons (IFNs) and TNFα and alternatively activated macrophages are activated by; interleukin 4 (IL-4) and interleukin 13 (IL-13) (M2a) or, immune complexes or apoptotic cells (M2b), or are deactivated by interleukin 10 (IL-10), transforming growth factor β (TGF-β) or glucocorticoids (M2c) (Hodgkinson et al., 2015; Wiegertjes et al., 2016). In fish, macrophages appear to be the primary antigen presenting cell although DCs have been identified in some fish species, and activation of teleost macrophages comparable to M1 classically activated macrophages in mammals, is the most studied and well characterized macrophage population type in fish (Hodgkinson et al., 2015; Magnadottir, 2010). Macrophages in fish are responsible

for rapidly killing invading pathogens by phagocytic activity, a process where pathogens are engulfed and toxic reactive intermediates are produced, by phagolysosomal acidification and by restriction of nutrient availability (Hodgkinson et al., 2015; Wiegertjes et al., 2016). In addition to pathogen clearance phagocytosis is also required for antigen presentation (Castro & Tafalla, 2015). Moreover, classically activated macrophages are responsible for producing cytokines, as well as, chemokines and lipid mediators that enhance and regulate both the inflammatory response and the adaptive immune response (Hodgkinson et al., 2015). Furthermore, in mammals, interferon γ (IFNγ) plays an important role in macrophage activation and is considered an important pro-inflammatory cytokine responsible for M1 macrophage activation (Hodgkinson et al., 2015; Wiegertjes et al., 2016). In fact, M1 polarization of macrophages is achieved primarily by IFNγ activity, however, to fully stimulate M1 macrophage activity, co-stimulation with TNFα is required (Hodgkinson et al., 2015).

1.8.5 Interferon γ

Interferon γ, a type II interferon, is primarily produced and secreted by natural killer (NK) cells and T cells, is generally considered a T helper cell 1 (Th1) cytokine, and is responsible for macrophage activation, mediating leukocyte migration, enhancing antigen presentation and inducing T cell differentiation in mammals (Martin, Zou, Houlihan, & Secombes, 2007; Zou, Carrington, Collet, Dijkstra, Yoshiura, Bols, & Secombes, 2005). Additionally, Darwich et al., (2009) demonstrated that human macrophages, stimulated by interleukin 12 (IL-12) and interleukin 18 (IL-18) produce IFNγ in the absence of both a T cell response and NK cells. This cytokine has been identified and characterized in fish and Zou et al. (2005) demonstrated that recombinant IFNγ (rIFNγ) enhances respiratory burst activity in macrophages and induces expression of IFNγ inducible protein 10 (γIP-10) and MH class II β chain suggesting the

bioactivities of rainbow trout IFNγ are similar to that of mammalian IFNγ. IFNγ has also been shown to upregulate antiviral effector genes such as Mx in fish and is a strong inducer of pro-inflammatory cytokines such as IL-1β (Wang & Secombes, 2013; Zou & Secombes, 2016). Furthermore, in addition to evidence supporting IFNγ involvement in MH class II antigen presentation, global transcriptome analysis has revealed IFNγ up-regulates genes involved in MH class I presentation (Wang & Secombes, 2013; Zou & Secombes, 2016). Overall, the evidence suggests that IFNγ in teleosts plays an important regulatory role in both the innate and adaptive immune response (Zou & Secombes, 2016)

1.9 Adaptive Immunity in Fish

The adaptive immune response is considered an evolutionarily more recent defence system, and is thought to have first appeared in the jawed vertebrates, primitive fishes, about 400 to 500 million years ago (Magnadottir, 2010). The main components of the adaptive immune system that have developed through evolutionary time are the thymus, B-cells and T-cells, and the recombination activation gene (RAG) which is responsible for generating immunoglobulin superfamily diversity through gene rearrangement (Magnadottir, 2010). The immunoglobulin superfamily includes B-cell receptors, T-cell receptors and MHC receptors, all of which are generated somatically during ontogeny and are therefore not germ-line encoded (Magnadottir, 2010). The nature of the adaptive immune system allows for a specific response that reflects the immune experience of an individual and thus is not heritable (Magnadottir, 2010). While the adaptive response is specific, this response system generally develops slowly and requires a series of events including specific receptor selection, cellular proliferation, as well as, protein synthesis for activation, however, the adaptive response is also characterized by memory and therefore provides long term immunity (Findly, Zhao, Noe, Camus, & Dickerson, 2013;

Magnadottir, 2010; Wilson, 2017). The immunoglobulins (Igs), or antibodies, are essential to humoral adaptive immunity and are expressed as B-cell receptors or are secreted in the plasma however, both B-cells and T-cells, which are involved in cell mediated immune responses, are responsible for specifically recognizing pathogens and initiating an adaptive immune response (Magnadottir, 2010). While B-cells are activated following recognition of either soluble antigen or antigen associated with MHC on an antigen presenting cell, T-cells only recognize antigen associated with MHC receptors on antigen presenting cells (Magnadottir, 2010). It appears that fish have the basic machinery and features of the vertebrate adaptive immune system although there are some differences observed, for example the antibody repertoire of fish which includes IgM and IgD, as well as IgT, or IgZ in some species, seems to lack IgG, IgA and IgE isotypes and thus appears to be limited in comparison to mammals (Findly et al., 2013; Magnadottir, 2010; Mashoof & Criscitiello, 2016; Wilson, 2017). Additionally, isotype switching or class-switch recombination does not occur in bony fish and while MH genes have been identified in several fish species the genes are not found as a complex on a single chromosome in teleost fish as they are in mammals, and instead are spread over three or four genomic locations, therefore in fish, they are designated MH genes rather than MHC genes (Findly et al., 2013; Fujiki, Smith, Liu, Sundick, & Dixon, 2003; Magnadottir, 2010; Wilson, 2017). Furthermore, immunological memory in teleost fish is a topic of contention and the role of teleost B-cells and plasma cells in adaptive memory is poorly understood (Findly et al., 2013; Parra, Reyes-Lopez, & Tort, 2015).

Overall, the adaptive immune response of fish appears to be slower than that of mammals and, following vaccination, specific antibody titres remain below detectable levels until week three or four in fish (Parra et al., 2015). Additionally, Findly et al., (2013) observed that in channel catfish infected and challenged with *Ichthyophthirius multifiliis* antigen specific plasma

cells do not persist for more than about one year while IgM, memory B-cells are sustained for at least three years. Moreover, while affinity maturation of teleost B-cells exists, as does clonal expansion, leading to protection by memory response for several years post-vaccination, antibody affinity increases marginally in fish compared to antibody affinity in mammals which is known to increase logarithmically (Parra et al., 2015).

1.9.1 Major Histocompatibility Class I

In mammals MHC class I molecules are present on almost every nucleated cell and are responsible for binding endogenous proteins that are generated through degradation by proteasomes in the cytosol (Castro & Tafalla, 2015; Sever, Vo, Bols, & Dixon, 2014). Peptides produced by proteasome degradation enter the lumen of the endoplasmic reticulum via the transporter associated with antigen processing (TAP) as part of the MH class I antigen presentation pathway (Sever, Vo, Bols, et al., 2014). Therefore, the MH class I receptor generally presents intracellular antigens produced during an infection by virus or intracellular bacteria or those produced as a result of cellular damage or dysfunction, to $CD8^+$ T-cells (Castro & Tafalla, 2015; Sever, Vo, Bols, et al., 2014). However, evidence of cross-presentation, or the presentation of exogenous antigen by MHC class I, has been reported (Goodridge et al., 2013). In fish MH class I is up-regulated in response to pathogens and immunostimulants, and expression of important MH class I accessory proteins and genes including TAP1, tapasin, calnexin, calreticulin and ERp57, primarily in response to viral pathogens or viral mimics, have also been investigated, however, many of the details surrounding MH class I antigen presentation in fish remains a mystery (Castro & Tafalla, 2015; Martin et al., 2007; Sever, 2014; Sever, Vo, Bols, et al., 2014; Sever, Vo, Lumsden, Bols, & Dixon, 2014)

1.9.2 Major Histocompatibility Class II

Mammals only express MHC class II receptors on professional antigen presenting cells such as macrophages, DCs, and B-cells, and present phagocytosed extracellular proteins to $CD4^+$ T helper cells (Castro & Tafalla, 2015; Fujiki et al., 2003). This generally occurs in the spleen or lymph nodes of mammals (Castro & Tafalla, 2015). In mammals MHC class II is associated with invariant chain (Ii) in the endoplasmic reticulum where the class II associated invariant chain peptide (CLIP) binds the MHC class II receptor groove ensuring the receptor does not bind intracellular proteins (Fujiki et al., 2003). Once the Ii-MHC class II complex fuses with a lysosome that contains exogenous antigen the Ii chain is fragmented by proteases and the CLIP is replaced with exogenous antigen while a dedicated molecular chaperone, HLA-DM in humans, removes the active component of the Ii chain from the peptide binding region and stabilizes the molecule (Fujiki et al., 2003; Wilson, 2017). Finally, the MHC class II receptor bound with extracellular antigen moves to the cell surface where it presents the bound antigen to T helper cells and initiates the adaptive humoral immune response (Fujiki et al., 2003). In fish up-regulation of MH class II following exposure to pathogens or immunostimulation has been observed and multiple invariant chain genes have been identified while a DM analog is absent in teleosts (Castro & Tafalla, 2015; Christie, 2007; Fujiki et al., 2003; Wilson, 2017). Three invariant chain genes have been identified in rainbow trout: 14-1, S25-7 and INVX, however, it is unclear the role each may play in the adaptive immune response of salmonids (Christie, 2007; Fujiki et al., 2003).

1.10 Reverse Transcriptase Relative Quantitative Polymerase Chain Reaction

Amplification of complementary deoxyribonucleic acid (cDNA) prepared by reverse transcription (RT) of messenger ribonucleic acid (mRNA) by quantitative real-time polymerase chain reaction (qPCR) is a tool in molecular biology commonly used to study low abundance gene expression (Pfaffl, 2001). This popular technique is highly sensitive allowing quantification of both rare transcripts and small changes in the expression of genes, and provides desired levels of accuracy, as well as, rapidly generates results (Pfaffl, 2001). The use of SYBR Green I, a fluorescence dye that binds to the minor groove double stranded DNA, is a simple technique for detecting products that have been synthesized by qPCR (Pfaffl, 2001). There are two types of quantification generally conducted by RT-qPCR; relative quantification, which is based on the relative expression of a gene of interest compared to a reference gene, and absolute quantification, which is based on the expression of a gene of interest compared to an internal or external calibration curve (Pfaffl, 2001). Given that development of absolute quantification is time consuming and requires the design and production of standard material along with optimization and validation of the calibration curve, as well as, normalization to an endogenous reference gene, relative quantification is an attractive alternative (Pfaffl, 2001). A calibration curve is not required for relative quantification and a relative expression ratio is calculated from the qPCR efficiency and the difference between the crossing point (Cp) of the unknown sample verses the Cp of a control (Pfaffl, 2001). Thus, relative qPCR using the Roche LightCycler and SYBR Green I is a sensitive and rapid method for detecting low levels of mRNA transcript and provides a convenient method for elucidating the mRNA expression levels of important immune genes (Pfaffl, 2001).

1.11 Purpose

Vibrogen 2, is among the few commercially available vaccines licensed for use in aquaculture and confers immunity to *V. anguillarum* and *V. ordalii*, the causative agents of the economically devastating fish disease, vibriosis. Use of this vaccine, as well as other, *V. anguillarum*-*V. ordalii* bacterins, plays a key role in successful control of vibriosis in aquaculture, however, the reason behind the success of these vaccines is poorly understood. Furthermore, the immunological pathways leading to long-term immunity remain unclear in fish. The main goals of this work are to determine the type of immune response triggered by the commercially available bacterin, Vibrogen 2, and investigate the early response of rainbow trout at the innate/adaptive immunity interface to better understand how immunity to *V. anguillarum* and *V. ordalii* is conferred by Vibrogen 2, as well as, the transition from innate to adaptive immunity in fish through the investigation of some of the genes up-regulated during the first 24h post-vaccination.

Chapter 2

Induction of IL-1β, IFNγ, S25-7 and TAP1 in RTS11 Cells by the Vibrogen 2 Vaccine

2.1 Introduction

The monocyte/macrophage like RTS11 cell line has been widely used for *in vitro* studies aiming to understand the immune response of rainbow trout. This cell line, established from a haemopoietic rainbow trout spleen culture, was characterized by Ganassin and Bols, (1998) and is comprised of two cell types including a population of small round non-adherent cells and a population of larger granular cells that are both adherent and non-adherent, however the culture primarily consists of the latter. Characterization of the larger cell type suggests that these cells are macrophages while the smaller round cell population represents cells at an earlier stage of development and these cells are thought to be macrophage precursor cells (Ganassin & Bols, 1998). Immunological studies investigating the gene expression of pro-inflammatory cytokines including IL-1β and IFNγ, as well as, the expression of MH class I, MH class I accessory proteins, including TAP1, and MH class II and MH class II accessory proteins, including S25-7, in RTS11 cells, have been described previously (Christie, 2007; Fujiki et al., 2003; Hong et al., 2004; Martin et al., 2007; Sever, 2014; Sever, Vo, Bols, et al., 2014; Sever, Vo, Lumsden, et al., 2014; Zou, et al., (2005). Notably, despite reports of IFNγ production by human macrophage cells expression of IFNγ by RTS11 cells has not been reported to date (Darwich et al., 2009). The aim of this study is to understand the innate/adaptive immune response interface in fish macrophages in response to the Vibrogen 2 vaccine to elucidate the immunological response triggered in rainbow trout following vaccination and to better understand the immune response

of fish. As this vaccine is among the few successful and commercially available vaccines understanding the effect of this vaccine on macrophages may shed light on the immunological pathways initiated and provide information useful for production of effective vaccines to additional pathogens. It is hypothesized that after stimulation with Vibrogen 2, RTS11 cells, will first transiently up-regulate the pro-inflammatory cytokine IL-1β gene leading to up-regulation of the IFNγ gene, a key cytokine of the adaptive immune response. This will be followed by up-regulation of S25-7 transcript, an MH class II associated invariant chain, but not TAP1 transcript, an MH class I associated transporter protein.

2.2 Materials and Methods

2.2.1 Maintenance of RTS11

RTS11 cells were maintained in Leibovitz's L-15 media (Hyclone) with 20% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Hyclone) at room temperature. Routine maintenance was carried out according to the methods previously described by Sever, Vo, Bols, & Dixon, (2014). Briefly, RTS11 cells were grown in 25cm^2 tissue culture flasks (BD Falcon) and sub-cultured without the use of trypsin at 1:2 where half the conditioned medium with suspended cells was passaged to a new flask and equal volumes of fresh media added to each of the two flasks. Once flasks reached confluency, approximately 2ml of fresh media was added about every three to four weeks.

2.2.2 Cell Collection and Plating

RTS11 cells from multiple confluent 25cm^2 flasks were collected by transferring conditioned media and cells to a sterile 50mL conical tube with a sterile plastic transfer pipette. Adherent cells were removed by washing conditioned media over the surface of the bottom of

the flask using the transfer pipette. Approximately, 1mL of sterile Dulbecco's phosphate buffered saline (DPBS, Lonza) was then added to the flask and a transfer pipette was used to further wash cells from the flask and transfer the DPBS to the 50mL conical tube. The RTS11 cells were pelleted by centrifugation at 4^{o}C for 5min at 550 x g. Cells were then washed once with fresh media supplemented with 2% FBS plus 1% penicillin/streptomycin and pelleted by centrifugation as described above. Washed cell pellets were resuspended in fresh media containing 2% FBS and 1% penicillin/streptomycin and counted with a hemocytometer. Cells were plated in three wells of a four well cell culture plate (Nunc) at 7 X 10^6 cells per well. Plates were then placed at 18^{o}C for 48h.

2.2.3 *In vitro* RTS11 Trials

After 48h at 18^{o}C, RTS11 cells had become adherent and conditioned media was removed by aspiration and replaced with 3mL of fresh media containing 2% FBS and 1% penicillin/streptomycin with or without an immunostimulant. Three control wells and three wells for each treatment were prepared for each of three time points: 4h, 8h, and 24h. Control wells received 3mL of fresh media containing 2% FBS and 1% penicillin/streptomycin and treatment wells received 3mL of 1:100 Vibrogen 2 or 1:500 Vibrogen 2 diluted in fresh culture media containing 2% FBS and 1% penicillin/streptomycin.

At each time point, cells were photographed and conditioned media and cells were collected. Briefly, 1mL of conditioned media was removed and transferred to a low protein binding 1.5mL microfuge tube and was placed on ice. Then a clean cell scraper was used to mechanically lift the cells from the bottom of the well. The conditioned media containing cells was transferred to a 15mL conical tube, 1mL of DPBS was added to the well, and the cell scraper

was used to collect remaining cells. After the 1mL of DPBS containing cells was transferred to the appropriate 15mL conical tube, the tube was centrifuged at 4°C for 4min at 500 x g to pellet the cells. The supernatant was poured off and the pellet was washed twice with 5mL of DPBS. Centrifugation between washes was carried out as above. After the second wash, DPBS was poured off and the cell pellet was resuspended by pipetting up and down in the residual DPBS, a volume of about 100µl, and transferred to a low protein binding 1.5mL microfuge tube. Samples were then centrifuged at 4°C for 5min at 500 x g, the DPBS was pipetted off and the tubes were transferred to ice. Conditioned media and cell pellets were then stored at -80°C until processing.

2.2.4 Ribonucleic Acid and Protein Extraction

Total ribonucleic acid (RNA) was extracted from cell pellets using the Norgen RNA and Protein Plus purification kit according to the manufacturers' specifications except that, RNA was eluted in 30µl of molecular biology grade water (Fisher) rather than 50µl of elution buffer. In brief, each cell pellet was homogenized in lysis buffer and column purification was carried out to remove genomic deoxyribonucleic acid (gDNA). RNA was then column purified and the flow through containing protein extract was retained for future analysis. Both RNA pellets and protein extracts were placed on dry ice immediately after isolation and then transferred to a -80°C freezer for storage.

2.2.5 Ribonucleic Acid Quantification and Purity

RNA pellets were removed from storage at -80°C and placed on ice. Samples were left to thaw on ice and were then mixed by flicking and pulse centrifugation. The purity and quantity of each RNA sample was determined using a BioTek microplate spectrophotometer Take3. In brief, 3µl of molecular biology grade water was loaded in the first well as a blank and 3µl of

each RNA sample was loaded in subsequent wells followed by RNA quantification using the BioTek RNA nucleic acid quantification program for the Take3. The 260/280 ratio was recorded and the quantity of RNA was reported in ng/µl.

2.2.6 cDNA Synthesis

cDNA was prepared using the Maxima H Minus First Strand cDNA Synthesis Kit, with dsDNase, (Thermo Fisher Scientific) according to the manufacturer directions for downstream use in qPCR. Briefly, 1µg of total RNA was added to 2µl dsDNase mix and nuclease free water was added to bring the final reaction volume to 10µl. Tubes were placed in a T100 thermocycler (BioRad) for 2min at 37^{o}C to remove any residual gDNA. Samples were pulse centrifuged and returned to ice and the random hexamer primer and dNTP mix, along with nuclease free water, were added, bringing the reaction volume to 15µl. The optional GC rich incubation step was performed and samples were incubated at 65^{o}C for 5min in the T100 thermocycler. Samples were once again pulse centrifuged and returned to ice for the addition of RT reagents, including 1µl of Maxima H Minus enzyme mix and 4µl of 5X RT buffer. The 20µl reactions were loaded into the T100 thermocycler for cDNA synthesis and incubated for 10min at 25°C followed by 15min at 50°C and then the reaction was terminated by heating at 85°C for 5min. For each run, a negative control without the RT enzyme was included. Samples were stored at -80^{o}C for future use.

2.2.7 Quantitative Polymerase Chain Reaction

cDNA was removed from storage at -80^{o}C and placed on ice to thaw. cDNA was flicked to mix and then pulse centrifuged. SYBR reactions were prepared for each sample using LightCycler 480 SYBR Green Master I (Roche). Briefly, a reaction master mix was prepared for

each primer set to be included on a 96-well plate and included 5µl of 2X SYBR Master Mix, 2.5µl of 2µM primer stock containing both forward and reverse primers (Table 3-1), and 1.5µl nuclease free PCR-grade water. The final concentration of primer in the mix was 0.5µm. To each 9µl aliquot of reaction master mix, 1µl cDNA template was added. A stable reference gene was identified using BestKeeper (Pfaffl, Tichopad, Prgomet, & Neuvians, 2004) and a standard curve for the best reference gene, elongation factor 1 alpha (EF1α), as well as each target gene was generated to determine primer efficiency. Serial dilutions of pooled cDNA from head kidney sample V8B were used to generate each standard curve. Primer pairs used for EF1α, as well as, target genes: IL-1β and IFNγ; were previously published by Chettri, Raida, Holten-Andersen, Kania, & Buchmann, (2011) however to confirm specificity in RTS11 cells a qPCR product for each primer pair was run on a 2% agarose gel containing GelRed (Biotium) and visualized using ultraviolet light. All unknown samples were run in triplicate and each 96-well plate included all samples for one time point with each sample being run with the reference gene, EF1α, primers as well as one set of target gene primers. Controls included no template control (NTC) negatives and a positive calibrator on each 96-well reaction plate. The LightCycler 480 (Roche) SYBR Green I 96-II template provided by the LightCycler 480 software was used according to the default parameters except the pre-incubation time was increased to 10min.

Table 3-1: Reference and target gene primers and expected product size of each amplicon

	Forward	Reverse	Amplicon (bp)	Accession Number
EF1α	5'ACCCTCCTCTTGGTCGTTTC	5'TGATGACACCAACAGCAACA	63	AF498320
IL-1β	5'ACATTGCCAACCTCATCATCG	5'TTGAGCAGGTCCTTGTCCTTG	91	AJ223954
IFNγ	5'AAGGGCTGTGATGTGTTTCTG	5'TGTACTGAGCGGCATTACTCC	68	AY795563
S25-7	5'GTTCCTGGCCAACCTACAGA	5'CCAATTACGTGCCCAAGTCT	82	AY065836
TAP1	5'CCATGAGTCGCATACACACC	5'AGTGACCCGCATGAAGTACC	188	AF115536

2.2.8 Analysis

For all reaction plates, advanced relative quantification using the LightCycler 480 software was performed using the primer efficiency of the reference and target genes as determined by the standard curve for each primer pair. This was followed by normalization of the control group to one and calculation of fold-change for each treatment group relative to the control. Additionally, statistical and graphical analysis was performed using GraphPad software. Two-way ANOVA analysis was used to determine the interaction between treatments over time.

2.3 Results

2.3.1 Morphology Changes, and Proliferation and Differentiation of RTS11 Cells in Response to Vibrogen 2

Qualitative observations reveal that stimulation of RTS11 cells with Vibrogen 2 leads to minimal changes in the morphology of cells stimulated with either a 1 in 100 or 1 in 500 dilution of Vibrogen 2 at 4h and 8h respectively (Figure 2-1). However, at 24h post-stimulation it appears that wells containing cells stimulated with either dilution have a greater number of elongated and adherent cells in comparison to the 24h control where cells appear to predominantly remain in suspension and maintain a rounded monocyte-like morphology (Figure 2-1). The elongated and adherent cells observed are likely monocyte-like cells that have undergone differentiation. Thus, the greater number of cells exhibiting these features in wells 24h post-stimulation appears to correlate with the addition of Vibrogen 2 combined with sufficient time post-stimulation for differentiation to occur (Figure 2-1). However, it is important to note that the observations in different fields of view may not reflect the same abundance of differentiated cells at 24h, and at both 4h and 8h, fields of view with varied

abundance of differentiated cells were observed. Additionally, it appears that there is a greater number of cells present at 24h, than that observed at 4h and 8h respectively, and that there is a greater number of cells present in stimulated wells compared to control wells at 4h and 8h respectively. However, the semi-adherent nature of RTS11 cells, along with the design of the four-well tissue culture dish affected the distribution of cells in the well and cells were not evenly distributed in the well. In fact, cells were consistently more abundant around the edges of the well and became less abundant moving towards the centre of the well. Additionally, during preliminary work cell counts were carried out and it was determined that 72h post-stimulation there was no significant difference in the number of cells present in the 72h control well versus the 72h, 1 in 100 Vibrogen 2 stimulated well (Appendix A).

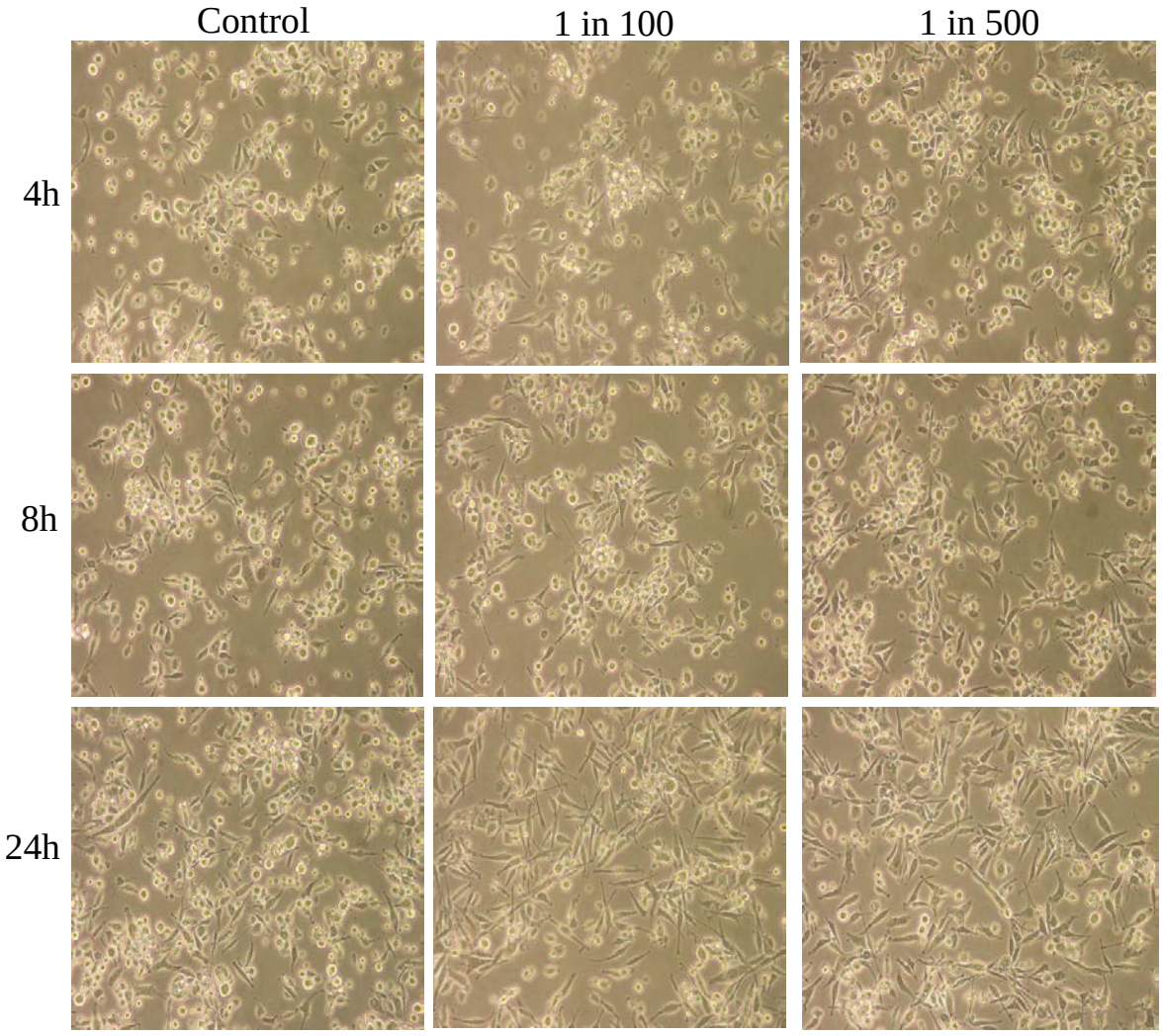

Figure 2-1: Control and stimulated RTS11 cells. Photographs at 100X magnification of control RTS11 cells at 4h, 8h and 24h, and RTS11 cells stimulated with Vibrogen 2 diluted in culture media to 1 in 100 or 1 in 500 at 4h, 8h and 24h, for visual comparison of cell morphology, cell abundance and cell differentiation.

2.3.2 Normalized ratios of IL-1β, IFNγ, S25-7 and TAP1 in RTS11 Cells Stimulated with Vibrogen 2 Demonstrate Patterns in Gene Expression Profiles

Relative qPCR focused on expression of four genes of interest: IL-1β, IFNγ, S25-7, and TAP1, in RTS11 cells stimulated with Vibrogen 2, diluted in culture media to 1 in 100 or 1 in

500, provides a normalized ratio representative of the expression of each gene of interest relative to the expression of the EF1α reference gene. These normalized ratios when plotted graphically (Figure 2-2) demonstrate the generally low variability of gene expression from well to well, allow for the identification of outliers and illustrate the gene expression trends for each gene over time. In Figure 2-2, the normalized ratios for IFNγ expression group together across control cells and both Vibrogen 2 treatments and it appears there is one outlier, that is approximately 1.1 normalized ratio units above the next nearest control normalized ratio, in the control group at 4h. For IL-1β, S25-7 and TAP1 replicate wells grouped consistently across treatments and time (Figure 2-2). Stimulation with the more concentrated 1 in 100 Vibrogen 2 resulted in higher levels of expression for IL-1β at 4h, 8h and 24h, at 24h for S25-7, and at 8h for TAP1, compared to stimulation with the higher 1 in 500 Vibrogen 2 dilution which resulted in lower expression levels. The difference between the expression levels of cells stimulated with 1 in 100 Vibrogen 2 versus 1 in 500 Vibrogen 2, appears to be consistent and translates to nearly a fivefold change in expression for IL-1β at 4h, 8h and 24h, at 24h for S25-7, and at 8h for TAP1, thus, the results demonstrate the concentration of Vibrogen 2 is generally proportional to the expression of these genes. At 24h post-stimulation the expression of TAP1 in RTS11 cells was consistent across both the 1 in 100 and 1 in 500 Vibrogen 2 treatments and appeared to reach a maximum (Figure 2-2). This is suggested by the limited increase in expression observed between 8h and 24h for the 1 in 100 Vibrogen 2 stimulated cells and the increase in expression of TAP1 from 8h to 24h in 1 in 500 Vibrogen 2 stimulated cells combined with the observed grouping of TAP1 normalized ratios for cells from both stimulation groups at 24h. Further, the difference in normalized ratio values between the four genes is demonstrated by Figure 2-2. IL-1β normalized ratios range from about three in control cells to over 800 in 1 in 100 Vibrogen 2 stimulated cells,

IFNγ normalized ratios range only from about 0.1 in control cell samples to approximately 0.9 in 1 in 100 Vibrogen 2 stimulated cells, S25-7 normalized ratios ranging from about 0.1 in control cells to around 0.8 in 1 in 500 Vibrogen 2 stimulated cells is clearly illustrated and TAP1 normalized ratios range from about four in control cells to approximately 25 in 1 in 100 Vibrogen 2 stimulated cells (Figure 2-2

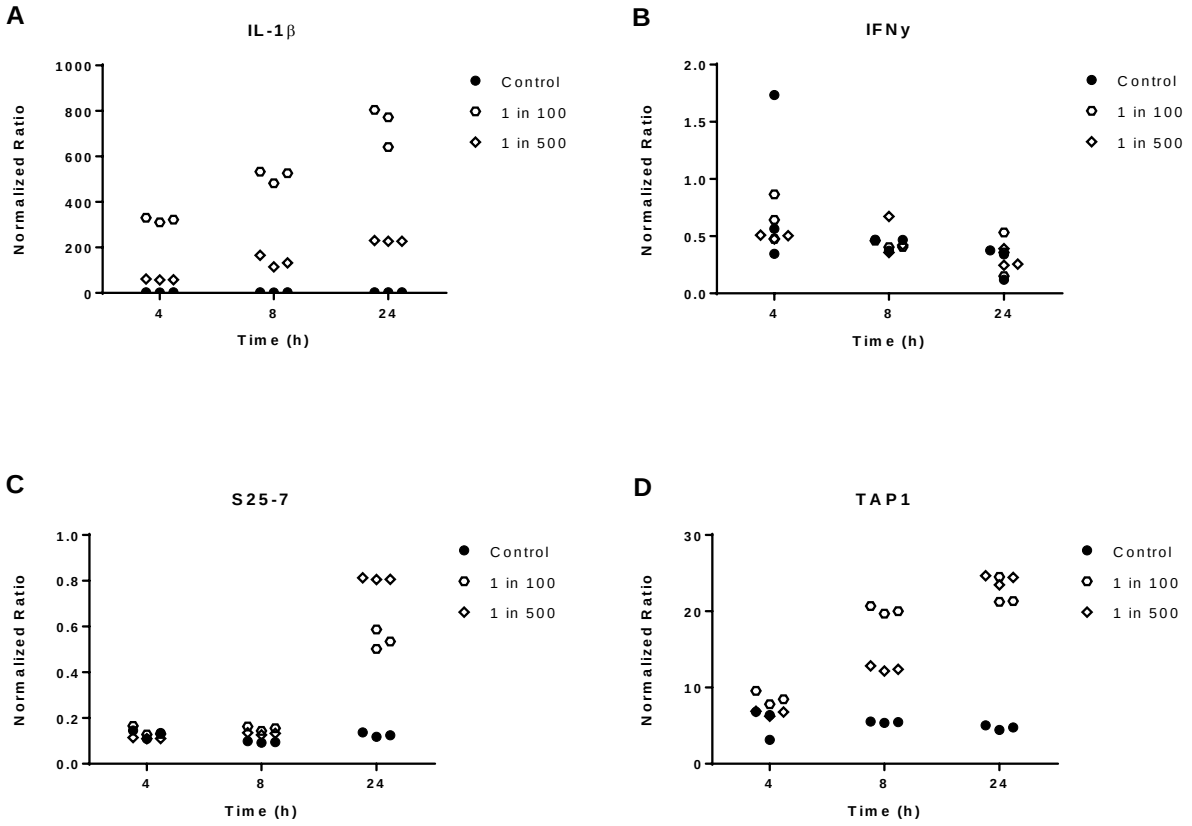

Figure 2-2: IL-1β, IFNγ, S25-7 and TAP1 normalized ratios in RTS11 cells. Normalized ratio of each RTS11 replicate well for control wells and wells stimulated with Vibrogen 2 diluted in culture media to 1 in 100 or 1 in 500 at 4h, 8h and 24h obtained using the Roche LightCycler 480 and calculated using the advanced relative quantification method are illustrated graphically to demonstrate variability of gene expression from well to well and gene expression trends over time where A illustrates normalized ratios for the IL-1β gene, B illustrates normalized ratios for the IFNγ gene, C illustrates normalized ratios for the S25-7 gene and D illustrates normalized ratios for the TAP1 gene. The qPCR reactions were all performed in triplicate and normalized against EF1α expression.

2.3.3 The First 24h of IL-1β, IFNγ, S25-7 and TAP1 Gene Expression in RTS11 Cells Stimulated with Vibrogen 2 Expressed as Fold-Change

The normalized ratios obtained by relative qPCR for the immune relevant genes, IL-1β, IFNγ, S25-7, and TAP1, in RTS11 cells stimulated with Vibrogen 2, diluted in culture media to 1 in 100 or 1 in 500 were used to determine the fold-change in expression of each gene. The normalized ratios of control samples for each gene were normalized to one and the expression of each gene under each treatment condition and at each time point was calculated relative to its respective normalized control (Figure 2-3). It was then determined by two-way ANOVA analysis followed by Tukey's multiple comparisons test that significant differences between unstimulated control cells and stimulated cells exist for IL-1β, S25-7 and TAP1 but not IFNγ. At all three time points the fold-change expression of IL-1β was significantly different between control RTS11 cells and both the 1 in 100 and 1 in 500 Vibrogen 2 stimulated cells (Figure 2-3). The p-value for all IL-1β versus control comparisons is <0.0001 except the 8h control cells versus 8h cells stimulated with 1 in 500 Vibrogen 2 which has a p-value of <0.0006. For S25-7 fold-change expression in RTS11 control cells versus cells stimulated with 1 in 100 or 1 in 500 Vibrogen 2 a significant difference between the 8h control cells and 1 in 100 Vibrogen 2 stimulated cells with a p-value of 0.0058 is reported while the difference in fold-change expression of S25-7 at 24h is extremely significant and control RTS11 cells versus both the 1 in 100 and 1 in 500 Vibrogen 2 stimulated cells reflect a p-value of <0.0001 (Figure 2-3). At 4h post-stimulation the fold-change expression of TAP1 when control RTS11 cells are compared to the 1 in 100 Vibrogen 2 stimulated cells is significantly different and has a p-value of 0.0422 while at both 8h and 24h the fold-change expression of TAP1 in cells stimulated with either 1 in 100 or 1 in 500 Vibrogen 2 versus control cells is significantly different and each comparison has

a p-value of <0.0001 (Figure 2-3). In addition to significant differences observed between control cells and stimulated cells significant differences between 1 in 100 Vibrogen 2 and 1 in 500 Vibrogen 2 stimulated cells exist for IL-1β, S25-7 and TAP1 (Figure 2-3). At all three time points, there is a significant difference between cells stimulated with 1 in 100 Vibrogen 2 versus 1 in 500 Vibrogen 2 for IL-1β (p-value <0.0001), while a significant difference between cells stimulated with 1 in 100 Vibrogen 2 versus 1 in 500 Vibrogen 2 is observed only at 24h for S25-7 (p-value <0.0001) and only at 8h for TAP1 (p-value <0.0001) (Figure 2-3). Additionally, the interaction between treatment and time is considered extremely significant (p-value <0.0001) and the effect of time (p-value <0.0001) and the effect of treatment (p-value <0.0001) are extremely significant for IL-1β, S25-7 and TAP1. However, there is no significant interaction between treatment and time (p-value 0.7464) nor is the effect of treatment (p-value 0.9012) or time (p-value 0.2696) significant for IFNγ.

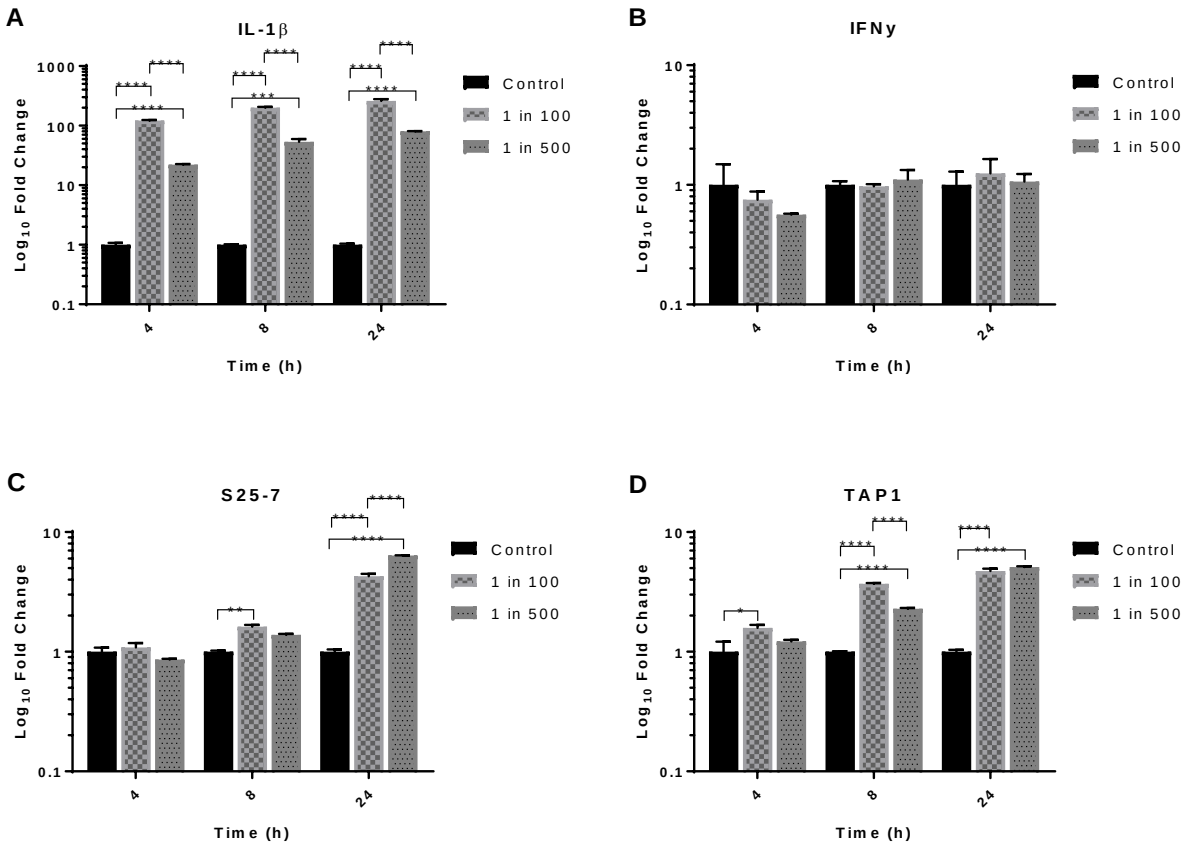

Figure 2-3: IL-1β, IFNγ, S25-7 and TAP1 fold-change gene expression in RTS11 cells following vaccination compared to control expression levels. Fold-change expression, calculated from normalized ratios obtained by relative qPCR, of IL-1β, IFNγ, S25-7 and TAP1 genes in RTS11 cells at 4h, 8h and 24h post stimulation with Vibrogen 2 diluted in culture media to 1 in 100 or 1 in 500 compared to unstimulated controls, where A illustrates fold-change in expression of the IL-1β gene, B illustrates fold-change in expression of the IFNγ gene, C illustrates fold-change in expression of the S25-7 gene and D illustrates fold-change in expression of the TAP1 gene. The qPCR reactions were performed in triplicate and normalized against EF1α expression. Control results were normalized to one and treatment groups were compared to control to determine fold-change expression. Analysis by two-way ANOVA followed by Tukey's multiple comparisons test was used to determine if significant differences between controls and treatments or between treatments at each time point exist. Results are shown as mean ± SEM, n=3.

Further analysis by two-way ANOVA followed by Tukey's multiple comparisons test shows significant differences in fold-change gene expression between time points within a treatment exist for IL-1β, S25-7 and TAP1 but not IFNγ (Figure 2-4). The fold-change

expression of IL-1β in 1 in 100 Vibrogen 2 stimulated cells at 8h and 24h is significantly different than fold-change expression at 4h (p-value <0.0001, <0.0001) and fold-change expression at 24h is significantly different than fold-change expression at 8h (p-value 0.0001) (Figure 2-4). For the 1 in 500 Vibrogen 2 stimulated cells there is only a significant difference in fold-change expression of IL-1β at 24h post-stimulation compared to fold-change expression at 4h (p-value<0.0002) (Figure 2-4). For S25-7 significant differences between 4h and 8h, 4h and 24h and 24h and 8h are observed for both the 1 in 100 stimulated cells and the 1 in 500 stimulated cells (Figure 2-4). The p-values for the 1 in 100 Vibrogen 2 stimulation group are 0.0058, <0.0001 and <0.0001 respectively while the p-values for the 1 in 500 Vibrogen 2 stimulation group are 0.0066, <0.0001 and <0.0001 respectively. RTS11 cells stimulated with 1 in 100 Vibrogen 2 or 1 in 500 Vibrogen 2, after 8h and 24h have significantly higher TAP1 gene fold-change expression than 4h cells (p-value <0.0001, <0.0001) and cells stimulated with 1 in 100 Vibrogen 2 and 1 in 500 Vibrogen 2 after 24h have significantly higher TAP1 fold-change expression than cells 8h post-stimulation (p-value 0.0002, <0.0001) (Figure 2-4).

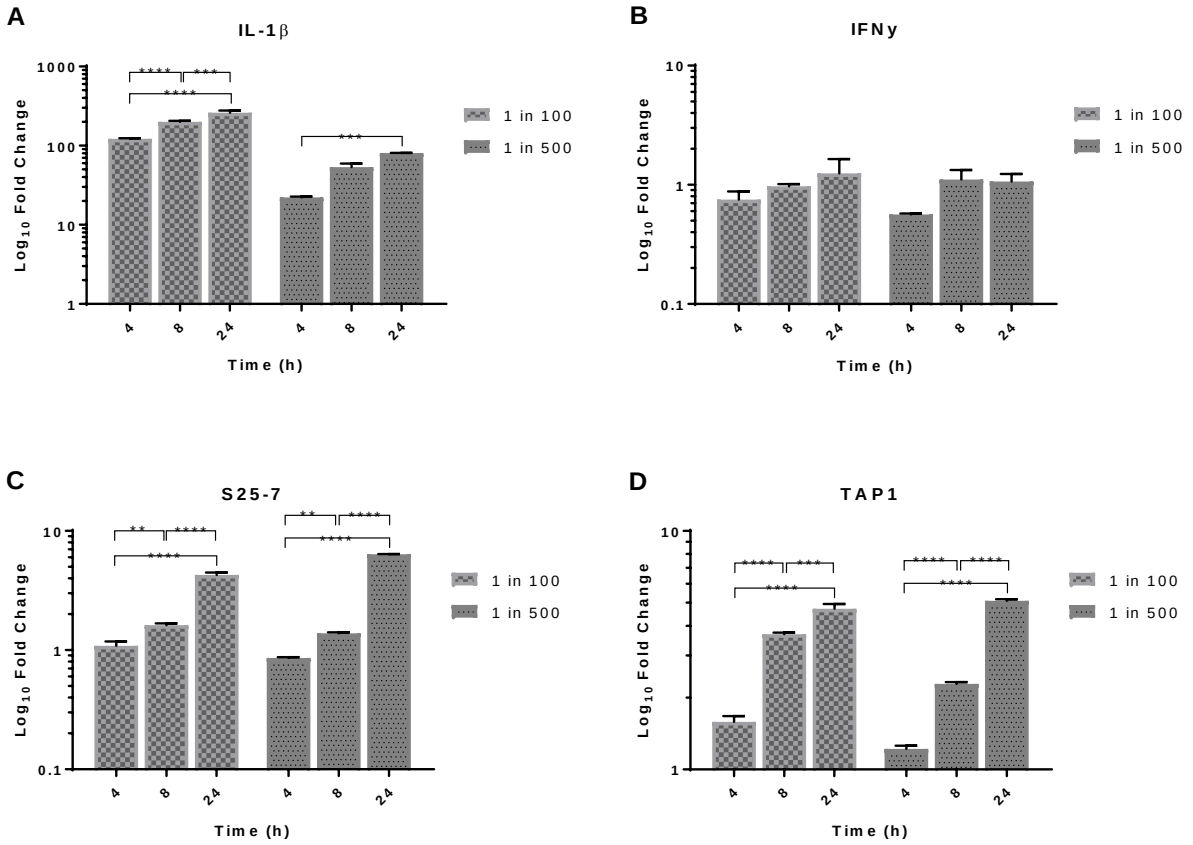

Figure 2-4: IL-1β, IFNγ, S25-7 and TAP1 fold-change gene expression in RTS11 cells at 4h, 8h and 24h. Comparison of fold-change gene expression, calculated from normalized ratios obtained by relative qPCR, of IL-1β, IFNγ, S25-7 and TAP1 in RTS11 cells at 4h, 8h and 24h post stimulation with Vibrogen 2 diluted in culture media to either 1 in 100 or 1 in 500, where A illustrates fold change in expression of the IL-1β gene, B illustrates fold-change in expression of the IFNγ gene, C illustrates fold-change in expression of the S25-7 gene and D illustrates fold-change in expression of the TAP1 gene. The qPCR reactions were performed in triplicate and normalized against EF1α expression. Control results were normalized to one and treatment groups were compared to control to determine fold-change expression. Analysis by two-way ANOVA followed by Tukey's multiple comparisons test was used to determine if significant differences between 1 in 100 Vibrogen 2 and 1 in 500 Vibrogen 2 stimulated RTS11 cells across time points. Results are shown as mean ± SEM, n=3.

Comparison of the fold-change gene expression of IL-1β, IFNγ, S25-7 and TAP1 in cells stimulated with 1 in 100 or 1 in 500 Vibrogen 2 demonstrates the differential expression of these four immune relevant genes (Figure 2-5). Of the four genes IL-1β consistently has the highest

level of fold-change expression across time points while IFNγ has the lowest level of fold-change gene expression across time points for cells stimulated with either 1 in 100 Vibrogen 2 or 1 in 500 Vibrogen 2 (Figure 2-5). The only gene with significantly different fold-change gene expression compared to the fold-change gene expression of the remaining three genes is IL-1β (p-value <0.0001) (Figure 2-5). This difference is observed across time points for both 1 in 100 Vibrogen 2 and 1 in 500 Vibrogen 2 stimulated cells. There is no significant difference between the fold-change gene expression of IFNγ, S25-7 and TAP1 for cells stimulated with either 1 in 100 Vibrogen 2 or 1 in 500 Vibrogen 2 at any of the time points investigated (Figure 2-5).

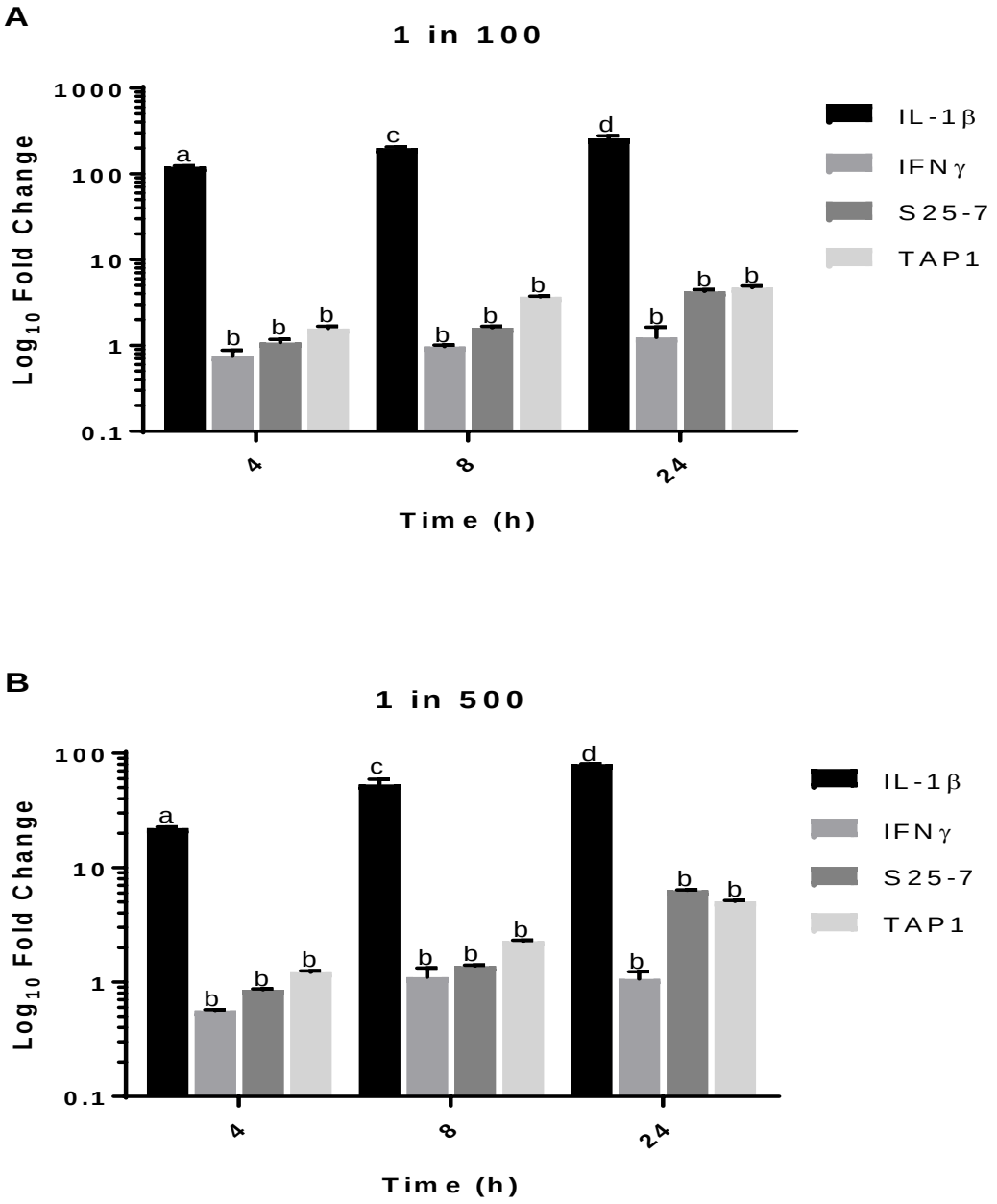

Figure 2-5: Summary of IL-1β, IFNγ, S25-7 and TAP1 fold-change gene expression in RTS11 cells. Fold-change gene expression, calculated from normalized ratios obtained by relative qPCR, of IL-1β, IFNγ, S25-7 and TAP1 for RTS11 cells stimulated with 1 in 100 Vibrogen 2 or 1 in 500 Vibrogen 2 prepared in cell media, for comparison of fold-change expression levels between different genes where A illustrates 1 in 100 Vibrogen 2 stimulated cells and B illustrates 1 in 500 Vibrogen 2 stimulated cells. The qPCR reactions were performed in triplicate and normalized against EF1α expression. Control results were normalized to one and treatment groups were compared to control to determine fold-change expression. Analysis by two-way ANOVA followed by Tukey's multiple comparisons test was used to determine if significant differences between genes over time exist. Results are shown as mean ± SEM, n=3.

2.4 Discussion

The RTS11 cell line is a combination of two cell types, and while there are a small number of adherent macrophage-like cells the majority of cells are small, round monocyte-like cells (Ganassin & Bols, 1998). The qualitative observations presented in Figure 2-1 provide limited support to suggest RTS11 cells undergo morphological changes and differentiation of monocyte-like cells to mature macrophage-like cells due to stimulation with Vibrogen 2. It is possible that the vaccine is stimulating the differentiation of immature monocytes into mature macrophage-like cells however, these results are inconclusive and further work is necessary to better understand the process involving changes in cell morphology and differentiation of RTS11 cells after Vibrogen 2 stimulation. It does appear clear from the results that the vaccine does not have a significant impact on cell number, that is, cells are neither stimulated to proliferate nor do they undergo cell death after Vibrogen 2 stimulation at these doses, as there was no significant difference between the number of cells in control wells versus stimulated wells at 72h post stimulation and there were no observed cytopathic effects (Figure 2-1). This ensures that differences in gene expression observed between control and stimulated cells within a time point are not affected by the number of cells tested. While it is unclear what effect Vibrogen 2 has on the physical characteristics of RTS11 cells it is possible to understand what is occurring within the cell after Vibrogen 2 stimulation through RT relative qPCR assays.

Previously, Martin, Zou, Houlihan, & Secombes, (2007) demonstrated that RTS11 cells exposed to rIL-1β up-regulated genes involved in the pro-inflammatory response, while RTS11 cells, exposed to rIFNγ up-regulated genes associated with antigen presentation. The goal of this study was to understand the immune response triggered, in rainbow trout RTS11 cells, by the commercially available vaccine, Vibrogen 2. It was expected that, in RTS11 cells, IL-1β would

be transiently up-regulated followed by up-regulation of IFNγ which would lead to initiation of the adaptive immune response including up-regulation of MH class II associated genes, such as S25-7 but not MH class I associated genes, such as TAP1. In contrast to expected results, IL-1β was significantly up-regulated, in response to both 1 in 100 and 1 in 500 Vibrogen 2, at 4h and the level of gene expression continued to increase through 8h and 24h and did not return to basal levels (Figures 2-2, 2-3 and 2-4). It is difficult to predict the reason IL-1β does not return to basal levels and why the level of expression continues to increase, in RTS11 cells following Vibrogen 2 stimulation, without further investigation of the regulation of IL-1β antagonist genes such as interleukin-1F (IL-1F) or pro-inflammatory regulatory genes such as the potent anti-inflammatory cytokine IL-10 (Piazzon, Lutfalla, and Forlenza, 2016; Wang et al., 2009; Zou & Secombes, 2016). However, in a previous study Castro, Zou, Secombes, & Martin, (2011) demonstrated the up-regulation of IL-10 in response to cortisol in RTS11 and it is well understood that mammalian macrophages, when activated via the classical pathway, secrete high levels of IL-12 but only modest levels of IL-10 (Mosser, and Edwards, 2008). Thus, RTS11 cells may up-regulate the IL-10 gene in response to Vibrogen 2 but the level of protein expression may be too low to create the necessary negative feedback message to bring IL-1β expression back to basal levels. Further, in mammals IL-10 is responsible for a range of activities including inhibiting pro-inflammatory cytokine expression and MHC class II expression, as well as, stimulating B-cells and the secretion of antibodies and the anti-inflammatory pathway is only activated after the IL-10 receptor complex (IL10R), formed by IL-10 binding two IL-10 receptor 1 (IL10R1) molecules and two IL-10 receptor 2 (IL10R2) molecules, is activated (Piazzon, Lutfalla, and Forlenza, 2016). Therefore, expression of IL-10 alone may not be sufficient to turn off IL-1β gene expression and other proteins that are not

present may be required to initiate the anti-inflammatory pathway and turn off IL-1β gene expression. Another possible reasons IL-1β is expressed at high levels could be that it is necessary to dramatically increase IL-1β gene expression as the gene is not constitutively expressed and the gene expression levels are zero or near zero in control cells (Figure 2-2). Further, this high level of expression may be required to produce sufficient IL-1β protein in the required time frame to initiate an appropriate immune response and a 24h period may not be sufficient for this high level of expression to receive the necessary signals to lower expression and finally return to basal levels (Figure 2-2). Another consideration is that additional cell types, not part of the RTS11 cell line, are required to complete the feedback loops necessary for down-regulation of IL-1β gene expression.

The results also show that IFNγ gene expression is not significantly up-regulated despite the high level of IL-1β gene expression however, it does appear that the expression of S25-7, one of the MH class II associated invariant chain genes, is significantly up-regulated in response to 1 in 100 Vibrogen 2 at 8h and in response to both 1 in 100 and 1 in 500 Vibrogen 2 stimulation at 24h (Figures 2-2 and 2-3). Interestingly, the MH class I associated gene, TAP1, was also significantly up-regulated at 4h in 1 in 100 Vibrogen 2 stimulated RTS11 cells and in response to both 1 in 100 and 1 in 500 Vibrogen 2 at 8h and 24h (Figures 2-2 and 2-3). While the results for IL-1β, IFNγ and TAP1 are contrary to those expected the expression of S25-7 does appear to follow the anticipated trend and expression is increasing over time suggesting up-regulation of MH class II. In mammals, it is well established that MHC class II is required for production of an effective antibody response and plays an important role in T-cell/B-cell collaboration as well as thymocyte education, therefore, evidence of MH class II up-regulation suggests that the

Vibrogen 2 vaccine is leading to the stimulation of an adaptive immune response in RTS11 cells which may lead to antibody production (Cosgrove et al., 1991).

The up-regulation of S25-7, along with the results of Martin et al., (2007), that demonstrate TAP1 expression in RTS11 cells is up-regulated at 24h by rIFNγ, but not rIL-1β, provide evidence that although IFNγ gene expression does not appear to be up-regulated in RTS11 cells after stimulation with Vibrogen 2, the expression of IFNγ protein may be up-regulated which in turn leads to up-regulation of genes involved in antigen presentation. The lack of significant difference in IFNγ gene expression may reflect the need to closely regulate the immune response to ensure proper control and limit unnecessary damage caused by over expression of IFNγ or it could simply reflect the fact that the basal level of IFNγ gene expression is maintained at the level required for an effective immune response to avoid any delay in producing sufficient protein for an appropriate immune response. Another consideration is that the IFNγ gene was transiently up-regulated and the time-point at which the expression of this gene occurred was not investigated or perhaps, given that RTS11 is a mixed culture of primarily immature monocyte-like cells, the number of differentiated IFNγ producing cells may have been low and thus any significant increase or decrease in gene expression remained hidden. Figure 2-4 does provide some evidence to support these theories given the expression of IFNγ does appear to be trending up over time but does not increase to significant levels. The possibility also exists, that in rainbow trout macrophage cells, there is an alternate pathway for the activation of antigen presentation that does not involve IFNγ and this response was induced by the Vibrogen 2 vaccine.

It is unclear why TAP1 gene expression is up-regulated in response to Vibrogen 2 given MH class I is thought to be responsible for presentation of antigens that have entered a cell, such

as viral pathogens or intracellular bacteria (Magnadottir, 2010; Martin et al., 2007). It is possible that the RTS11 cells received a higher dose of Vibrogen 2 or may have been in direct contact with the vaccine longer than cells typically would *in vivo* as they may not possess the machinery a fish does for processing all the bacterin components however, in a previous study Acosta et al., (2004) demonstrated up-regulation of the antiviral effector gene Mx, in Atlantic salmon, following vaccination with a *V. anguillarum*-*V. ordalii* vaccine, as well as, after injection with *V, anguillarum* LPS or DNA. Therefore, up-regulation of TAP1 may be evidence of cross-presentation in teleosts, either triggered by *V. anguillarum* or *V. ordalii* derived molecules such as nucleic acids, proteins, LPS and/or toxins or by added vaccine components. Cross-presentation by macrophages and DCs has been demonstrated in mammals and it has been suggested that exploiting this pathway could lead to more effective vaccines (Basta & Alatery, 2007; Cruz, Colbert, Merino, Kriegsman, & Rock, 2017). Despite evidence that suggests TAP is not involved in the cross-presentation pathway in mammals and that the pathway involves the non-classical MHC class I, HLA-F associated with MHC class I open conformers the cross-presentation pathway in teleost may involve classical MH class I or non-classical MH class I that utilizes TAP1 (Goodridge et al., 2013; Grimholt, 2016). It is also possible that in salmonids both MH class I and MH class II are up-regulated as part of an effective adaptive immune response or that MH class I is responsible for presentation of both endogenous and exogenous antigen which may support the loss of MH class II machinery in cod (Grimholt, 2016; Star, Nederbragt, Jentoft, Grimholt, Malmstrøm, Gregers, Rounge, Paulsen, Solbakken, Sharma, Wetten, Lanzén, Winer, Knight, Vogel, Aken, Andersen, Lagesen, & Tooming-Klun, 2011). However, it is also possible that MH class I associated genes are up-regulated solely in preparation for mounting a response in the event one is necessary but do not go on to present antigen.

The results of this study also indicate that dose may play an important role in the strength of immune response triggered. While the difference in expression is not always significant, when the expression of IL-1β, S25-7 and TAP1 in response to a 1 in 100 dilution versus a 1 in 500 dilution of Vibrogen 2 are compared, a higher concentration of vaccine correlates with a higher level of expression except for at 24h for TAP1 gene expression, where both treatments stimulate the same level of gene expression (Figure 2-4). This would suggest that correct and accurate dosing is necessary to stimulate an appropriate immune response and that overdose could lead to serious side effects that may even be fatal as inflammation, which leads to tissue damage, when not carefully controlled can cause irreparable damage and over expression of regulatory cytokines can lead to dangerous over expression of immunological pathways leading to increased morbidity and mortality (Bystrom et al., 2008; Rieger et al., 2012; Teijaro, Walsh, Rice, Rosen, & Oldstone, 2014).

Overall, these results show IL-1β gene expression is significantly up-regulated in comparison to IFNγ, S25-7, and TAP1 at all time points for RTS11 cells treated with either 1 in 100 or 1 in 500 Vibrogen 2 (Figure 2-5). While, the expression of TAP1 generally trends higher than S25-7, except at 24h in the 1 in 500 Vibrogen 2 stimulated cells, and both TAP1 and S25-7 gene expression trend higher than IFNγ gene expression, there is no significant difference between the expression of these three genes (Figure 2-5). Thus, it appears that the Vibrogen 2 vaccine has a dramatic effect on the expression of IL-1β but the effect on downstream gene expression, although significant when compared to control cells for S25-7 and TAP1, is far less impressive (Figures 2-2 and 2-5). This may suggest that although the vaccine is stimulating the MH class II pathway, demonstrated by the significant up-regulation of S25-7, the vaccine may be preferentially driving a pro-inflammatory response (Figures 2-2 and 2-5). However, it may also

be possible that S25-7 gene expression will increase at later time points. The expression of the TAP1 gene may also reach peak levels at a later time point and the increased expression of this gene raises many questions regarding the role of the MH class I pathway in rainbow trout long term immunity. Certainly, the results presented here provide strong evidence that Vibrogen 2 is not only responsible for macrophage activation but also up-regulation of MH class II associated genes, as well as, the MH class I associated genes suggesting MH class I may play a role in both endogenous and exogenous antigen presentation in rainbow trout. Therefore, it may be important to consider both MH class I and MH class II presentation pathways when developing vaccines for use in aquaculture and exploiting cross-presentation may lead to more effective vaccines and bacterins.

Chapter 3

Induction of IL-1β, IFNγ, S25-7 and TAP1 in Rainbow Trout Head Kidney by the Vibrogen 2 Vaccine

3.1 Introduction

The rainbow trout is a member of the Salmonidae family and while the species is native to the Pacific coast of North America and Russia, it has been introduced around the world to areas with cooler water temperatures and is a popular food and sport fish (Thorgaard et al., 2002). A breadth of basic biological knowledge has been collected about the rainbow trout and the rainbow trout can serve as a proxy for other salmonid species including Atlantic salmon, Pacific salmon and charr (Thorgaard et al., 2002). Additionally, rainbow trout have a larger size relative to other common fish models and are generally more amenable to surgery than smaller species and their larger size also permits collection of larger tissue samples and isolation of a greater number of cells for biochemical, immunological and molecular biological testing (Thorgaard et al., 2002). Thus, rainbow trout are an important model organism and have been widely studied in a diverse range of research areas (Thorgaard et al., 2002).

Immunological studies of rainbow trout not only provide data for comparison of the human and salmonid immune system for biomedical and evolutionary studies but also provide data relevant and of immediate importance to the aquaculture industry (Thorgaard et al., 2002). The primary immune organ in rainbow trout is the head kidney which is a haematopoietic organ morphologically similar to mammalian bone marrow (Press & Evensen, 1999; Soulliere & Dixon, 2017). It is believed that the stroma of the head kidney, in addition to providing support to the haematopoietic tissue, is involved in non-specific immunity and clearing cell debris as

well as damaged cells (Press & Evensen, 1999). Additionally, it has been demonstrated that 70 percent of injected radiolabeled bacteria localize to the head kidney where sinusoidal macrophages and endothelial cells are involved in capturing substances and debris from the blood (Press & Evensen, 1999). Furthermore, the head kidney is responsible for production and maturation of B cells, as well as, progenitor T cells (Soulliere & Dixon, 2017). It has also been suggested that the head kidney acts as a secondary lymphoid organ and may be involved in antibody production (Press & Evensen, 1999; Soulliere & Dixon, 2017). A handful of immunological studies investigating the gene expression of pro-inflammatory cytokines in rainbow trout head kidney and/or primary head kidney leukocytes, as well as, the gene expression of MH class I and MH class I accessory proteins and the gene expression of MH class II and MH class II accessory proteins, including S25-7, have been described previously (Chettri et al., 2011; Christie, 2007; Fujiki et al., 2003; Jørgensen, Hetland, Press, Grimholt, & Gjøen, 2007; Sever, Vo, Bols, et al., 2014; Sever, Vo, Lumsden, et al., 2014). The aim of this study is to understand the innate/adaptive immune response interface in rainbow trout head kidney in response to vaccination with Vibrogen 2, to reveal the immunological response triggered in rainbow trout following vaccination and to better understand the immune response of fish. Given the success of this vaccine in comparison to other commercially available vaccines it is important to understand the immune response triggered in vaccinated fish, as this may be useful for production of effective vaccines to additional pathogens. It is hypothesized that after stimulation with Vibrogen 2, the pro-inflammatory cytokine IL-1β transcript will be transiently up-regulated in head kidney leading to up-regulation of the IFNγ gene, a key cytokine of the adaptive immune response, in the head kidney. This will be followed by up-regulation of the

S25-7 gene, an MH class II associated invariant chain but not TAP1, an MH class I associated transporter protein, in rainbow trout head kidney.

3.2 Materials and Methods

3.2.1 Rainbow Trout Transport

Sexually immature rainbow trout weighing approximately 190 to 200 grams were obtained from Alma Aquaculture Research Station (AARS) (University of Guelph). Approximately 360 rainbow trout were transported by AARS staff in an oxygenated tank from AARS to the University of Waterloo aquatics facility. Upon arrival, all 360 fish were transferred to an outdoor holding tank by net. Of these 260 fish were transferred, using fish nets, to a 450-gallon holding tank for future trials and 100 fish were equally distributed at random into four 180-gallon treatment tanks; Tank 1, Tank 2, Tank 3 and Tank 4, in Biology 1 (B1) room 377B (Figure 3-1).

3.2.2 *In vivo* Rainbow Trout Trials

The photoperiod in B1-177B was 18h light to 6h dark and fish were acclimated for four and a half weeks prior to the start of the trial. Each tank was checked daily and fish were fed three-point floating trout chow (Martin Mills) to satiation once daily Monday to Friday and were not fed on Saturday and Sunday. At the time of the trial trout in all tanks had been feeding well and appeared to be in good health.

Figure 3-1: Aquatic facility trial room. Photo of Biology 1 room 377B treatment room tank set up

Beginning three days before the trial feed was withheld. On day one, each tank was assigned a treatment according to Table 3-2. Control fish were not treated or handled at the time of injection. Saline control or "sham injected" and Vibrogen 2 (Novartus) treated fish were anesthetized in an anesthetic bath containing 25-40mL of Benzocaine, prepared by

Table 3-2: Treatments in each tank for each *in vivo* trial

	Tank 1	**Tank 2**	**Tank 3**	**Tank 4**
Trial A Treatment	Control No Treatment	Saline	NA	Vibrogen 2 Vaccine
Trial B Treatment	Vibrogen 2 Vaccine	NA	Control No Treatment	Saline

NA-Treatment group was used for a second study not described here

dissolving 50g of ethyl 4-aminobenzoate in 500mL of anhydrous ethanol, and received an i.p. injection of 100µl of DPBS or Vibrogen 2 respectively (Figure 3-2). Two different individuals carried out i.p injections simultaneously with a 25-gauge needle and 1mL syringe. Immediately following injection fish were transferred to a recovery bath, one per treatment, supplied with oxygen. After the last fish was injected all fish in that treatment group were returned to the appropriate 180-gallon tank.

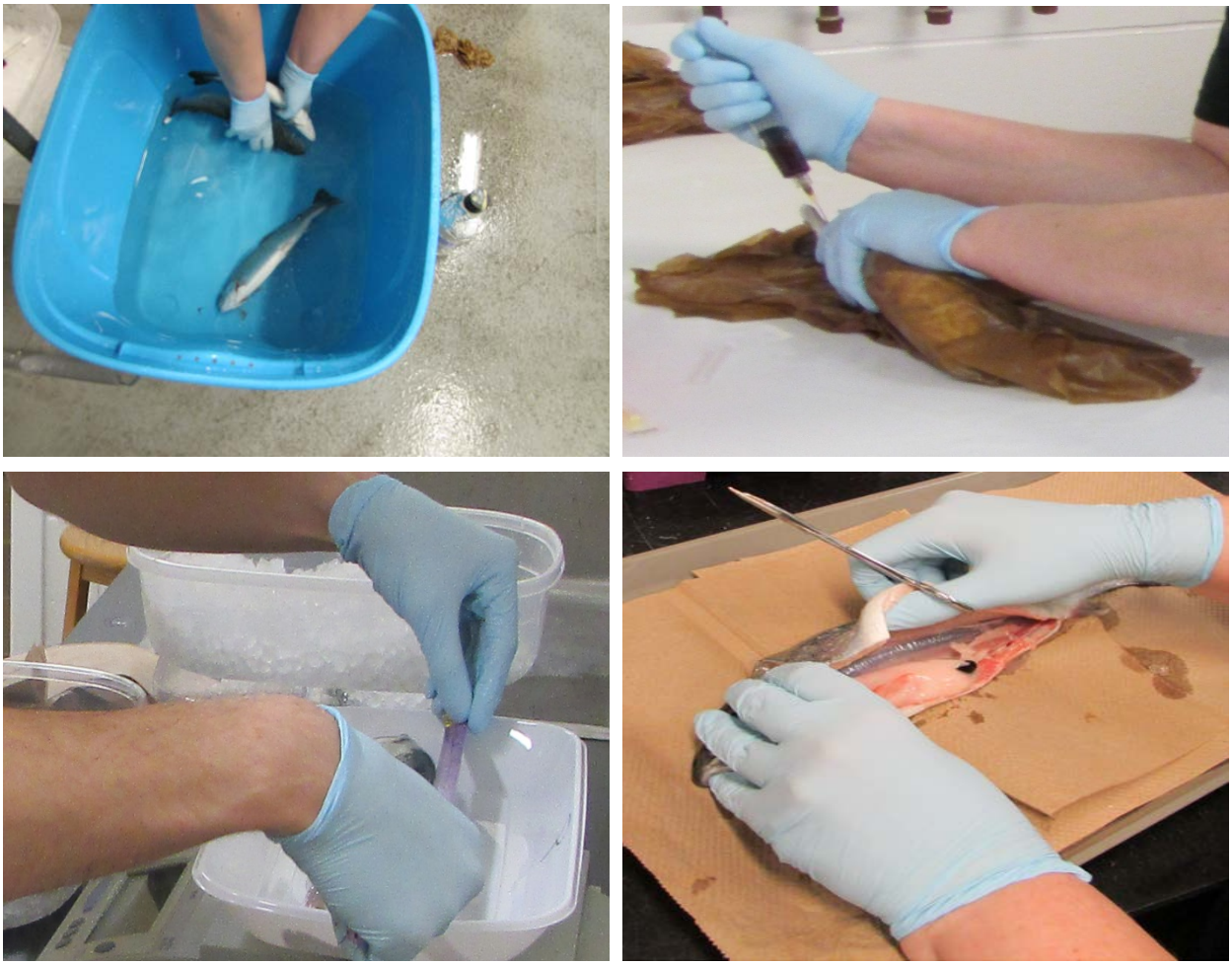

Figure 3-2: *In vivo* trial sampling. Photo panel of sampling steps showing anesthetizing, bleeding, weighing and measuring and dissecting rainbow trout.

One hour after all fish were returned to their respective treatment tank and at 4h, 8h, 12h, 24h and 48h three fish from each tank were collected and anesthetized as described above (Figure 3-2). Blood was drawn from the caudal blood sinus with a 25-gauge heparinized needle and 10ml syringe until exsanguination (Figure 3-2). Blood was then transferred to 15ml conical tubes and placed on ice for transport and subsequent peripheral blood leukocyte (PBL) extraction and plasma collection. The fish were euthanized in a Benzocaine bath and then placed on ice. Weight and length were recorded and head kidney, spleen and muscle tissue was collected from each fish (Figure 3-2). The gender of the fish was recorded when possible and any unusual markings and signs of infection or poor health were noted. Tissues were dissected, placed in labeled 5ml Eppendorf tubes and immediately flash frozen by immersion in liquid nitrogen (Figure 3-2). Once frozen tubes containing tissue were transferred to dry ice and transported to a -80^{o}C freezer for storage until processing.

The trial was repeated and all steps were carried out as describe above except each tank was assigned a different treatment (Table 3-2). Additionally, for the second trial 100 rainbow trout from holding were moved by net and evenly distributed between the four treatment tanks in B1-177B. Fish were acclimated for two and a half weeks before the start of the trial. This trial was conducted seven and a half weeks from the start of the first trial.

3.2.3 Ribonucleic Acid and Protein Extraction

RNA and protein were extracted from tissue samples using the Norgen RNA and Protein Plus purification kit according to the manufacturers' specifications. In brief, 25µg of tissue was homogenized in lysis buffer. gDNA was removed, RNA was purified and the flow through containing the protein extract was retained as described above for RTS11 cells.

3.2.4 Ribonucleic Acid Quantification and Purity and cDNA Synthesis

RNA purity and quantification of each RNA sample was determined as described above. cDNA was prepared using the Maxima H Minus First Strand cDNA Synthesis Kit, with dsDNase (Thermo Fisher Scientific) as described above for all samples except S8C. Quantification of sample S8C RNA indicated that the RNA concentration was 114.4ng/µl therefore the cDNA reaction volume was adjusted to ensure that 1µg of total RNA was added. For this reaction 2.18µl dsDNase mix was added and nuclease free water was excluded as 8.74µl of RNA sample were required to obtain the necessary 1µg of RNA. Thus, the dsDNase reaction volume was 10.92µl. After completing the dsDNase step, random hexamer primer and dNTP mix plus water were added bringing the reaction volume to 16.38µl and the optional GC rich incubation step was performed. Lastly, the RT reagents were added bringing the final reaction volume to 21.84µl and cDNA synthesis was completed as previously outlined. All samples were stored at -80^{o}C for future use.

3.2.5 Quantitative Polymerase Chain Reaction

All head kidney cDNA samples, except S8C, were handled as described above. As the final reaction volume of sample S8C was modified to account for the lower RNA concentration of this sample, it was necessary to modify the SYBR reaction. In summary, the volume of nuclease free PCR-grade water was reduced to 1.41µl and the volume of cDNA template was increased to 1.09µl, while all other reagent volumes were added as outlined above. The final reaction volume for all samples was 10µl and the qPCR protocol was performed as described above for RTS11 samples.

3.2.6 Analysis

All analysis' were performed as described above.

3.3 Results

3.3.1 Size of Rainbow Trout

After sacrifice, each rainbow trout was weighed and length was recorded. These values were used to determine if the size of fish varied from treatment to treatment or from sample time point to sample time point. Two-way ANOVA analysis followed by Tukey's multiple comparisons test shows that no significant difference between treatment or time points was observed for fish weight or fish length (Appendix B).

3.3.2 Normalized Ratios of IL-1β, IFNγ, S25-7 and TAP1 in Head Kidney of Rainbow Trout Stimulated with Vibrogen 2 Demonstrate Patterns in Gene Expression Profiles

Relative qPCR investigating the expression of four genes: IL-1β, IFNγ, S25-7, and TAP1, in rainbow trout head kidney of unvaccinated control fish and fish vaccinated by i.p. injection with 100µl Vibrogen 2 or injected with 100µl saline, provides a normalized ratio representative of the expression of each gene of interest relative to the expression of the EF1α reference gene. These normalized ratios when plotted graphically (Figure 3-3) demonstrate the variability of gene expression from fish to fish, allow for the identification of outliers and illustrate the gene expression trends for each gene over time. In Figure 3-3, the normalized ratios for IL-1β demonstrate that the expression of this gene is not upregulated in the head kidney of saline injected fish or fish from the control group and the saline injected group and control group

cluster together across all time points. In contrast, expression of IL-1β is upregulated in fish injected with Vibrogen 2 at 4h and 8h but not at 24h (Figure 3-3). At 4h all three fish injected with Vibrogen 2 differentially upregulate the expression of IL-1β in the head kidney while two of the three fish injected with Vibrogen 2 upregulated the expression of IL-1β in head kidney at 8h (Figure 3-3). Thus, Figure 3-3 clearly demonstrates that the up-regulation of IL-1β varies from fish to fish. Additionally, it appears that at 4h IL-1β reaches higher levels of expression in the head kidney of Vibrogen 2 injected fish, than it does at 8h, and by 24h the expression of IL-1β in the head kidney of Vibrogen 2 injected fish returns to control levels (Figure 3-3). The expression patterns of IFNγ are less clearly defined than those demonstrated for IL-1β however, it appears that at 4h the normalized ratio for each fish in all three groups; control, saline injected and Vibrogen 2 injected, cluster together (Figure 3-3). At 8h it appears that there is a high level of fish to fish variability in head kidney IFNγ expression and while the IFNγ normalized ratios of saline injected fish appear to cluster together the normalized ratios from control fish and Vibrogen 2 injected fish do not (Figure 3-3). By 24h the normalized ratios of fish injected with Vibrogen 2 group together above the saline injected and control normalized ratios except for one outlier, a saline injected fish with a normalized ratio above the normalized ratios of all other fish (Figure 3-3). Like IFNγ the expression trends of S25-7 in head kidney are difficult to discern. While the normalized ratios of control, saline injected and Vibrogen 2 vaccinated fish cluster together at 8h and 24h at 4h they are spread across a range of normalized ratios however, at 4h one fish from each group has a normalized ratio above any ratio found at either 8h or 24h and the fish with the highest normalized ratio belongs to the Vibrogen 2 stimulated group (Figure 3-3). For TAP1 the normalized ratios of fish from all three treatment groups cluster together at 4h and 8h however at 4h two control fish group together above the larger cluster (Figure 3-3). At 24h

there are two distinct clusters, the lowest represents the normalized ratios of control fish while the second slightly higher cluster represents saline injected fish and the normalized ratios of Vibrogen 2 injected fish are found above this cluster (Figure 3-3). The fish to fish variability that was clearly observed for IL-1β up-regulation is also observed for TAP1 and the results for IFNγ and S25-7 also suggest that the regulation of these genes varies from fish to fish (Figure 3-3). Further, the difference in normalized ratio values between the four genes is demonstrated by Figure 3-3. IL-1β normalized ratios range from about zero in control fish head kidney to over 22 in Vibrogen 2 vaccinated fish head kidney, IFNγ normalized ratios range only from about 0.1 in control head kidney samples to approximately 1.8 in Vibrogen 2 vaccinated fish head kidney, S25-7 normalized ratios ranging from about 0.4 in control fish head kidney to around 1.7 in Vibrogen 2 vaccinated fish head kidney were observed and TAP1 normalized ratios range from about 0.4 in control fish head kidney to approximately 2.4 in Vibrogen 2 vaccinated fish head kidney (Figure 3-3).

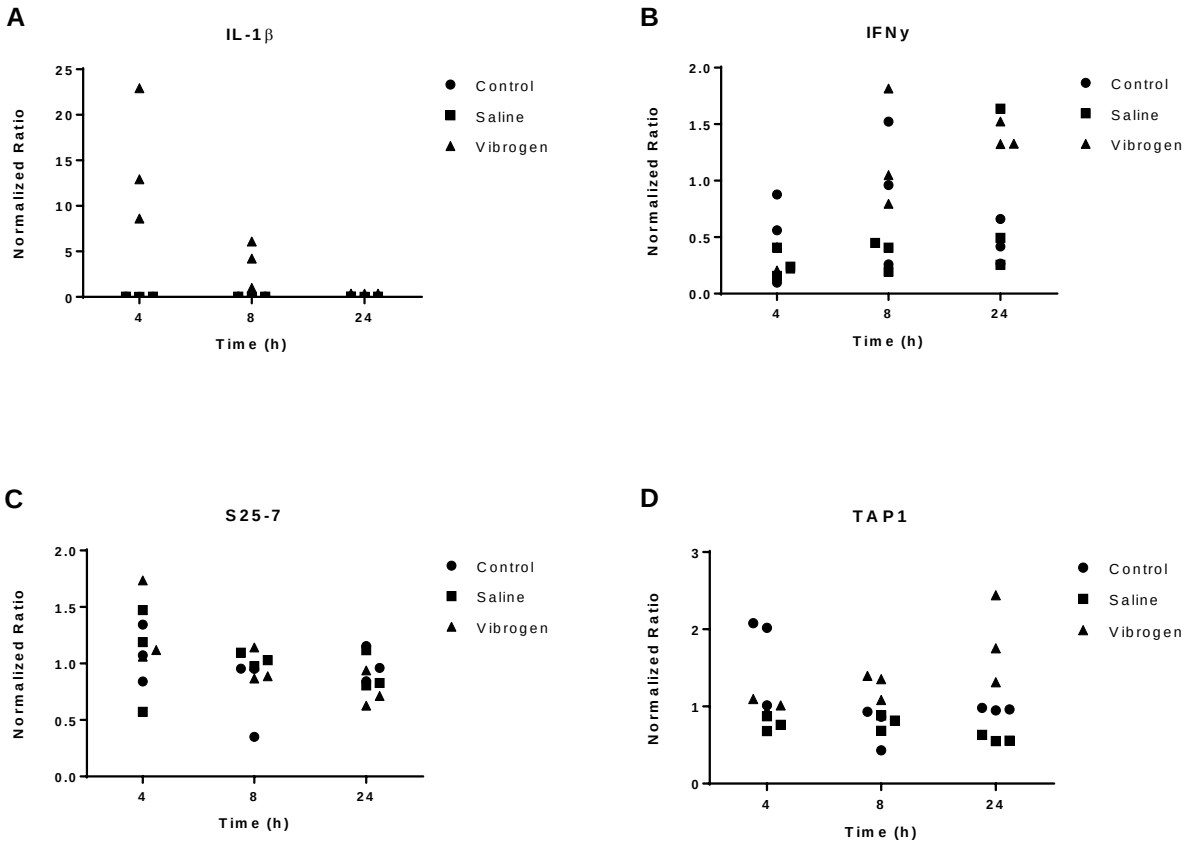

Figure 3-3: IL-1β, IFNγ, S25-7 and TAP1 normalized ratios in head kidney. Normalized ratio, obtained by relative qPCR, of each rainbow trout head kidney for control fish and fish vaccinated by i.p. injection with 100µl Vibrogen 2 or 100µl saline at 4h, 8h and 24h obtained using the Roche LightCycler 480 and calculated using the advanced relative quantification method are illustrated graphically to demonstrate variability of gene expression from fish to fish and gene expression trends over time where A illustrates normalized ratios for the IL-1β gene, B illustrates normalized ratios for the IFNγ gene, C illustrates normalized ratios for the S25-7 gene and D illustrates normalized ratios for the TAP1 gene. The qPCR reactions were performed in triplicate and normalized against EF1α expression.

3.3.3 The First 24h of IL-1β, IFNγ, S25-7 and TAP1 Gene Expression in the Head Kidney of Rainbow Trout Vaccinated with Vibrogen 2 Expressed as Fold-Change

The normalized ratios obtained by relative qPCR for the four genes of interest, IL-1β, IFNγ, S25-7, and TAP1, for rainbow trout head kidney from fish i.p. injected with saline, or vaccinated with

Vibrogen 2 were used to determine the fold-change expression of each gene. For each gene, the normalized ratios of control fish were normalized to one and the expression of each gene under each treatment condition and at each time point was calculated relative to the respective normalized control (Figure 3-4). It was determined by two-way ANOVA analysis followed by Tukey's multiple comparisons test that significant differences in fold-change gene expression between control fish and Vibrogen 2 vaccinated fish exist for IL-1β, IFNγ and TAP1 but not S25-7. At 4h the fold-change expression of IL-1β in head kidney was significantly different between control fish and Vibrogen 2 vaccinated fish (p-value <0.0001) (Figure 3-4). For IFNγ expression in head kidney from control fish versus head kidney from fish vaccinated with Vibrogen 2, a significant difference between the 24h control fish and Vibrogen 2 vaccinated fish with a p-value of 0.0383 is reported (Figure 3-4). At 24h post-vaccination the expression of TAP1 when control fish head kidney fold-change expression is compared to the Vibrogen 2 vaccinated fish head kidney fold-change expression, is significantly different and has a p-value of 0.0176. In addition to significant differences observed between control fish and Vibrogen 2 vaccinated fish significant differences in fold-change gene expression of saline injected fish and Vibrogen 2 vaccinated fish exist for IL-1β and TAP1 (Figure 3-4). At 4h there is a significant difference in fold-change gene expression between head kidney from Vibrogen 2 vaccinated fish versus head kidney from saline injected fish for IL-1β expression (p-value <0.0001), and a significant difference between head kidney from Vibrogen 2 vaccinated fish versus head kidney from saline injected fish at 24h for TAP1 expression (p-value 0.0005) (Figure 3-4). Additionally, for IL-1β fold-change expression in head kidney the interaction between treatment and time is considered extremely significant (p-value 0.0002) and the effect of treatment (p-value 0.0001) is extremely significant while the and the effect of time (p-value 0.0010) is very

significant. The interaction between treatment and time is not quite significant (p-value 0.0609) as is the effect of treatment (p-value 0.0692) however the effect of time (p-value 0.0028) is extremely significant for IFNγ expression in head kidney. For S25-7 expression in head kidney, the interaction between treatment and time is considered not significant (p-value 0.3828) as is the effect of treatment (p-value 0.7194) and the effect of time is not quite significant (p-value 0.0783). The interaction between treatment and time is very significant (p-value 0.0025) and the effect of treatment (p-value 0.0002) and time (p-value 0.0006) are both considered extremely significant for TAP1 expression in head kidney.

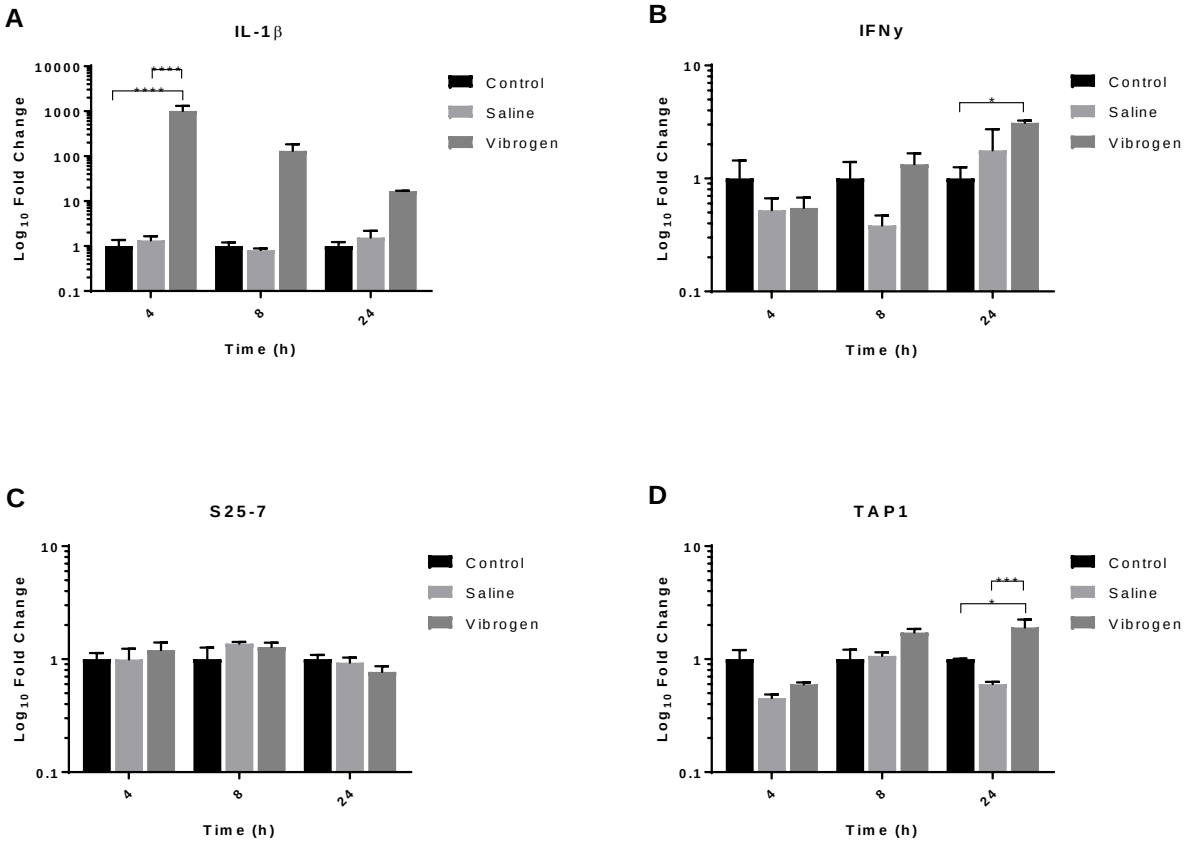

Figure 3-4: IL-1β, IFNγ, S25-7 and TAP1 fold-change gene expression in head kidney following vaccination compared to control expression levels. Fold-change expression, calculated from normalized ratios obtained by relative qPCR, of IL-1β, IFNγ, S25-7 and TAP1 genes in rainbow trout head kidney at 4h, 8h and 24h post-stimulation with 100µl i.p. injected saline or 100µl i.p. injected Vibrogen 2 compared to unvaccinated controls, where A illustrates fold-change in expression of the IL-1β gene, B illustrates fold-change in expression of the IFNγ gene, C illustrates fold-change in expression of the S25-7 gene and D illustrates fold-change in expression of the TAP1 gene. The qPCR reactions were performed in triplicate and normalized against EF1α expression. Control results were normalized to one and treatment groups were compared to control to determine fold-change expression. Analysis by two-way ANOVA followed by Tukey's multiple comparisons test was used to determine if significant differences between controls and treatments or between treatments exist. Results are shown as mean ± SEM, n=3.

Further analysis by two-way ANOVA followed by Tukey's multiple comparisons test show significant differences in fold-change gene expression between time points within a treatment exist for IL-1β, IFNγ and TAP1 but not S25-7 (Figure 3-5). The fold-change expression of IL-1β in the head kidney of Vibrogen 2 vaccinated fish at 8h and 24h is

significantly different than fold-change expression at 4h (p-values 0.0002, <0.0001) and a significant difference between 4h and 24h fold-change expression is observed for IFNγ (Figure 3-5). In the head kidney of fish vaccinated with Vibrogen 2 after 8h and 24h TAP1 fold-change gene expression is significantly higher than TAP1 fold-change gene expression at 4h (p-values 0.0024, 0.0004) (Figure 3-5).

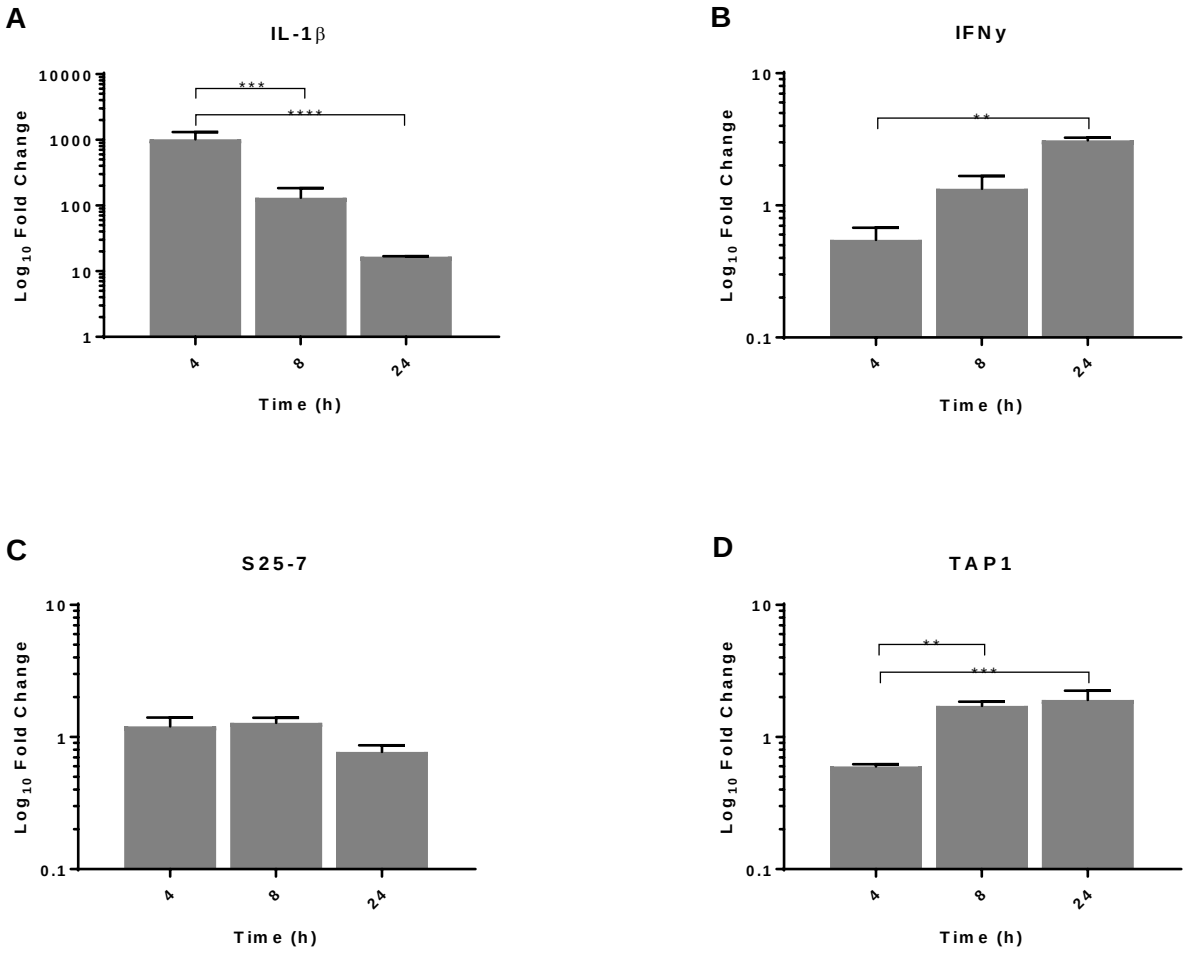

Figure 3-5: IL-1β, IFNγ, S25-7 and TAP1 fold-change gene expression in head kidney at 4h, 8h and 24h. Comparison of fold-change gene expression, calculated from normalized ratios obtained by relative qPCR, of IL-1β, IFNγ, S25-7 and TAP1 in rainbow trout head kidney at 4h, 8h and 24h post-vaccination with Vibrogen 2, where A illustrates fold-change in expression of the IL-1β gene, B illustrates fold-change in expression of the IFNγ gene, C illustrates fold change in expression of the S25-7 gene and D illustrates fold-change in expression of the TAP1 gene. The qPCR reactions were performed in triplicate and normalized against EF1α expression. Control results were normalized to one and treatment groups were compared to control to determine fold-change expression. Analysis by two-way ANOVA followed by Tukey's multiple comparisons test was used to determine if significant differences between time points exist. Results are shown as mean ± SEM, n=3.

Comparison of the fold-change gene expression of IL-1β, IFNγ, S25-7 and TAP1 demonstrates the differential expression of these four immune relevant genes in head kidney (Figure 3-6). Of the four genes IL-1β consistently has the highest fold-change expression across time points while IFNγ, S25-7 and TAP1 fold-change expression is more variable across time points (Figure 3-6).

The only gene with significantly different fold-change gene expression is IL-1β (p-value <0.0001) and this difference is observed only for 4h fold-change expression (Figure 3-6). That is, in comparison to IL-1β fold-change expression at 8h and 24h, as well as, fold-change expression of all other genes across all time points the 4h fold-change expression of IL-1β is significantly different however there is no significant difference in fold-change IL-1β expression at 8h or 24h compared to IFNγ, S25-7 or TAP1 fold-change expression and there is no significant difference between the fold-change expression of IFNγ, S25-7 and TAP1 (Figure 3-6).

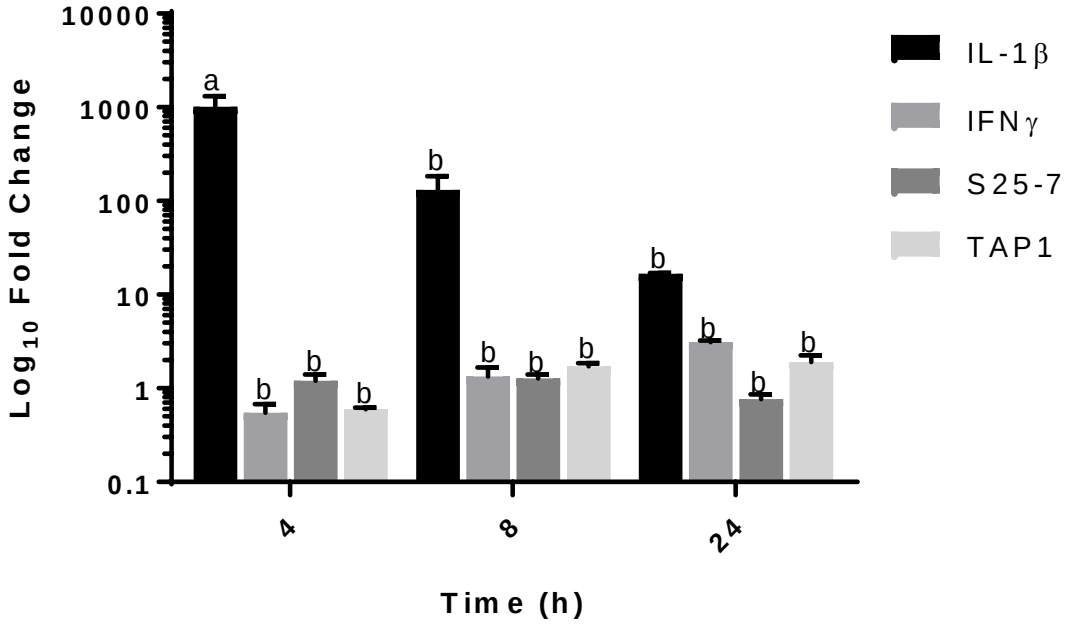

Figure 3-6: Summary of IL-1β, IFNγ, S25-7 and TAP1 fold-change gene expression in head kidney. Fold-change gene expression, calculated from normalized ratios obtained by relative qPCR, of IL-1β, IFNγ, S25-7 and TAP1 in head kidney of fish vaccinated with Vibrogen 2, for comparison of gene expression levels between different genes. The qPCR reactions were performed in triplicate and normalized against EF1α expression. Control results were normalized to one and treatment groups were compared to control to determine fold-change expression. Analysis by two-way ANOVA followed by Tukey's multiple comparisons test was used to determine if significant differences between genes over time exist. Results are shown as mean ± SEM, n=3.

3.4 Discussion

It has been demonstrated that the life stage of rainbow trout effects the immune response an individual can mount (Magnadottir, 2010). The results of this trial are considered unaffected by fish size as the weight and length of each fish was recorded and it was determined that neither the average weight nor length of fish was significantly different between treatment groups or across time points (Appendix B). Additionally, all fish were obtained from the same source at the same time and were sexually immature. Therefore, the life stage of the fish is unlikely to affect the results obtained. Further, it is unlikely that the rainbow trout sampled were previously

exposed to either *V. anguillarum* or *V. ordalii* as they were reared in a freshwater facility. Therefore, these results reflect vaccination of naïve rainbow trout against *V. anguillarum* and *V. ordalii* by i.p injection with the commercially available Vibrogen 2 vaccine.

In a previous study conducted by Boltana et al., (2014) expression of the IL-1β gene in the head kidney of gilthead seabream injected with *V. anguillarum* LPS was investigated and Chettri et al., (2011) isolated rainbow trout head kidney leukocytes and exposed them to a series of substances that mimic the molecular patterns of different pathogens to determine the expression profiles of key immune genes including important pro-inflammatory genes such as IL-1β and IFNγ. While Vibrogen 2 has been licensed for use for over 17 years an investigation of the immune response triggered after vaccination with this vaccine has not been conducted (Canadian Food Inspection Agency, 2017). The goal of this study was to understand the immune response triggered, in rainbow trout head kidney, by the commercially available vaccine, Vibrogen 2. It was expected that, in rainbow trout head kidney, IL-1β would be transiently up-regulated followed by up-regulation of IFNγ, which would lead to initiation of the adaptive immune response including up-regulation of MH class II associated genes such as S25-7, but not MH class I associated genes, such as TAP1.

As expected, IL-1β gene expression appears to be transiently up-regulated in Vibrogen 2 vaccinated fish while saline injected fish do not up-regulate IL-1β gene expression (Figure 3-3). This gene was significantly up-regulated, in Vibrogen 2 vaccinated fish, at 4h but not 8h, and by 24h had returned to basal expression levels (Figures 3-3, 3-4 and 3-5). These results are consistent with trends observed by Boltana et al., (2014) for IL-1β expression in gilthead seabream head kidney where IL-1β peaks at 6h and begins to fall by 12h post *V. anguillarum* LPS injection. However, in isolated head kidney leukocytes exposed to LPS or flagella the

expression of IL-1β has been shown to increase through 12h post-stimulation and expression does not appear to return to basal levels (Chettri et al., 2011). Perhaps the different expression patterns observed when comparing the whole organ expression levels to isolated leukocyte expression is a result of limitations imposed by looking at a group of cells that no longer have all the necessary machinery or cell types to produce all the signals required to regulate the immune response as the whole organ would. This could be an example of a system that lacks IL-1β antagonist genes like IL-1F or pro-inflammatory regulatory genes like IL-10. Although these important regulators of IL-1β were not investigated given that the cells used by Chettri et al., (2011) are a population of all leukocytes isolated from the kidney it is likely that cells able to express IL-1F and IL-10 are part of the leukocyte population. Thus, the difference in expression patterns observed may in fact be the result of macrophage and leukocyte migration from the head kidney to the spleen, a secondary lymphoid organ, or other sites within the fish, leading to fewer cells expressing the IL-1β gene in the head kidney which is observed as a reduction in IL-1β gene expression. While, direct comparison of the rainbow trout and gilthead seabream IL-1β gene expression patterns is somewhat challenging given the time points selected for analysis by Boltana et al., (2014) were 3h, 6h, 12h and 24h and the time points investigated here were 4h, 8h and 24h, and here EF1α rather than 18s was used as a reference gene, it appears that the expression of the IL-1β gene in rainbow trout head kidney from Vibrogen 2 vaccinated fish peaks earlier with a higher expression level than in gilthead seabream injected with *V. anguillarum* LPS (Figures 3-3 and 3-4). While this difference may be attributed to variation in species, dose, or the fact that the LPS injection may lack other important bacterial components it may also reflect the ability of additional bacterin components to stimulate a strong response.

The results for IFNγ gene expression also appear to follow the expected trend and this gene is significantly up-regulated at 24h, but not 4h or 8h post-vaccination, however, at 24h one saline injected fish also up-regulated IFNγ gene expression at a level consistent with vaccinated fish (Figures 3-3 and 3-4). In general, the results for IFNγ are somewhat inconsistent and it appears that fish-to-fish variability may mask significant results particularly at 8h where control fish expression levels mix with Vibrogen 2 vaccinated fish levels (Figures 3-3 and 3-5). Furthermore, at 4h it appears that two of three control fish have higher IFNγ gene expression than either saline injected or vaccinated fish (Figure 3-3). Despite the challenges this fish-to-fish variance creates for understanding IFNγ expression it also suggests that the saline injected fish up-regulating the IFNγ gene expression at 24h is likely a fish that was up-regulating IFNγ for unknown reasons rather than due to the saline injection, and the well-defined grouping of vaccinated fish at 24h suggests that this is a reliable result despite the inconsistencies observed for this gene overall (Figures 3-3, 3-4 and 3-5). Furthermore, in the investigation conducted by Chettri et al., (2011) IFNγ gene expression in head kidney leukocytes was not significantly altered by stimulation with either LPS or flagellin at 1h, 4h or 12h. Since this study did not extend to investigate the expression of the IFNγ gene at 24h it is not possible to compare this result, however, the results of earlier time points appear to be consistent with the results presented here. Notably, just as variability in IFNγ gene expression was observed in this study, the IFNγ gene expression in response to different concentrations of LPS or flagellin also appeared to be variable, and did not seem to follow an expected pattern where expression increased or decreased with volume of stimulant added (Figures 3-3 and 3-4) (Chettri et al., 2011). It is possible that the variability observed for IFNγ gene expression at the earlier time points reflects low gene copy number in the original sample which lead to a variation in copy

number between each pipetted volume, and this in turn lead to variability of the qPCR results due to the limitations imposed by sample preparation and the nature of the sample itself (Klein, 2002). Additionally, if the study of Chettri et al., (2011) extended past 12h it is possible that by 24h IFNγ gene expression may have been significantly up-regulated in head kidney leukocytes in response to LPS and/or flagellin as was observed here in response to Vibrogen 2.

While the results for IL-1β and IFNγ appear to follow the expected expression trends in head kidney after vaccination with Vibrogen 2, in contrast to the expected up-regulation of MH class II associated S25-7, the results indicate that the expression of this gene is not significantly altered in head kidney post-vaccination (Figures 3-3 and 3-4). In fact, although not statistically significant it appears that the expression of S25-7 in rainbow trout head kidney is treading towards down-regulation at 24h post-vaccination (Figures 3-3, 3-4 and 3-5). Given that significant up-regulation of the IFNγ gene is not observed until 24h post-vaccination it is possible that up-regulation of the S25-7 gene occurs at a later time point such as 48h or 72h, however, a previous study of S25-7 transcript levels in head kidney following stimulation of rainbow trout with phorbol myristate acetate (PMA), a stimulator of protein kinase C that involves activation of B cells and T cells, also showed that at 24h post-treatment down-regulation of the S25-7 gene occurs, and at later time points significant down-regulation of the gene is observed (Christie, 2007). Although it is possible that up-regulation of the S25-7 gene may occur after the final 24h time point studied here, this result may in fact, be further evidence that head kidney macrophages, and other activated antigen presenting cells, migrate out of the head kidney, to other sites where they continue the necessary inflammatory and/or adaptive response. Alternatively, other invariant chains reported in rainbow trout, either 14-1, INVX or both, may be up-regulated rather than S25-7, in rainbow trout following vaccination with

Vibrogen 2 (Fujiki et al., 2003). Another possibility is that MH class II in fish may not require invariant chain once an immune response is initiated.

Despite the downward trend of S25-7 expression, TAP1 gene expression is significantly up-regulated in response to Vibrogen 2 at 24h post-vaccination (Figure 3-4). Not only is this result unexpected because MH class I is thought to be responsible for presenting endogenous antigens rather than exogenous antigens, it is also unclear why TAP1 would be up-regulated in rainbow trout head kidney following Vibrogen 2 vaccination, while S25-7 gene expression is not significantly changed and appears to be treading towards down-regulation (Figure 3-3, Figure 3-4 and Figure 3-5) (Magnadottir, 2010; Martin et al., 2007). Nonetheless, these results are consistent with the work of Acosta, Lockhart, Gahlawat, Real, & Ellis, (2004), who demonstrated that vaccination of Atlantic salmon with a *V. anguillarum-V. ordalii* bacterin, as well as, injection with either *V. anguillarum* LPS or DNA leads to up-regulation of Mx, a gene typically associated with anti-viral activity and thus MH class I presentation (Martin et al., 2007; Verhelst, Hulpiau, & Saelens, 2013). However, these results are contrary to other studies that have shown that neither type II interferons nor LPS up-regulate Mx protein in fish (Verhelst et al., 2013). Additionally, if the observed down regulation of S25-7 expression is related to fewer antigen presenting cells in the head kidney due to migration of cells out of this organ, the dichotomy observed between S25-7 and TAP1 gene expression in rainbow trout head kidney, may suggest that different cells are up-regulating the MH class I pathway than are up-regulating the MH class II pathway (Figures 3-3, 3-4 and 3-5). Furthermore, these results may provide evidence that teleosts up-regulate MH class I in response to both endogenous and exogenous antigens and may suggest MH class II has limited function in comparison to MH class I in fish.

Perhaps this is the reason cod have eliminated MH class II machinery in favour of expanding the MH class I repertoire (Grimholt, 2016; Star et al., 2011) .

Overall, these results show IL-1β gene expression is significantly up-regulated in comparison to IFNγ, S25-7, and TAP1 at 4h in fish vaccinated with Vibrogen 2 and the expression levels of the IFNγ, S25-7 and TAP1 genes are comparable across all time points studied (Figure 3-6). While it appears that the Vibrogen 2 vaccine has a dramatic effect on the expression of IL-1β the effect on downstream gene expression, although significant when compared to control cells for IFNγ and TAP1, is far less remarkable (Figures 3-4, 3-5 and 3-6). This combined with the downward trend of S25-7 gene expression may suggest that the vaccine is preferentially driving a pro-inflammatory response and inhibiting the MH class II pathway, or perhaps expression of IFNγ, S25-7 and TAP1 may increase at later time points (Figures 3-4 and 3-5). Additionally, it is possible that either 14-1 or INVX are involved in MH class II presentation or that fish regulate MH class II independent of the invariant chain. Nevertheless, the results presented here provide strong evidence for two alternatives: activated antigen presenting cells involved in the MH class II pathway are migrating out of the head kidney and/or MH class I is responsible for adaptive immune function and responds to both endogenous and exogenous antigen in rainbow trout. If MH class I in fish, is in fact more heavily involved in antigen presentation it may be wise to tailor vaccines for use in aquaculture to stimulate this pathway along with the MH class II pathway, or perhaps instead of the MH class II pathway altogether. However more data is necessary to determine which option is most effective for the development of long term immunity in fish.

Chapter 4
General Discussion, Future Directions and Conclusions

4.1 General Discussion

The aims of this work were to: Develop an understanding of the immune response triggered by Vibrogen 2; Determine the immunological response type driven by vaccination with Vibrogen 2 and; Elucidate the regulation of mRNA at the interface between innate and adaptive immunity in rainbow trout. By investigating four genes, that in mammals play a fundamental role in the development of inflammation and/or long-term immunity, this study set out to map the changes in mRNA transcript levels of two cytokines; IL-1β and IFNγ and the MH class II associated invariant chain, S25-7, as well as, the MH class I associated transporter protein, TAP1, in the first 24h post-stimulation. Two studies; an *in vitro* study using RTS11 macrophage-like cells, and an *in vivo* study focused on the head kidney, were carried out and results from these two studies notably differ for IL-1β, IFNγ and S25-7 but not TAP1.

In both RTS11 and head kidney the IL-1β mRNA transcript is up-regulated however, transient expression of this gene is observed in head kidney but not RTS11 cells and while IFNγ gene expression remains unchanged, at 4h, 8h and 24h, in RTS11 cells following stimulation with Vibrogen 2, expression of this gene in head kidney is up-regulated at 24h post-vaccination (Figures 2-3 and 3-4). In light of previous study results where, isolated head kidney leukocytes were shown to have similar IL-1β gene expression trends to those observed in RTS11 cells, and a second study where similar expression patterns for IL-1β in head kidney was demonstrated in gilthead seabream following injection with *V. anguillarum* LPS, it is possible the expression patterns for the IL-1β gene differ between RTS11 cells and head kidney following stimulation

with Vibrogen 2 because RTS11 cells are either unable to migrate or because the cell line lacks the variety of necessary cells and/or machinery to regulate the response in the same manner a fish with a complete set of arsenal can (Boltana et al., 2014; Chettri et al., 2011). This may also support the lack of change observed for IFNγ gene expression in RTS11 cells as well, however the up-regulation of S25-7 and TAP1 in RTS11 may suggest that IFNγ protein is present therefore, it is possible the time point that IFNγ was up-regulated in RTS11 cells was not sampled or that an initial up-regulation of the gene was not necessary to produce the appropriate levels of IFNγ protein (Martin et al., 2007).

The work of Roher, Callol, Planas, Goetz, & Mackenzie, (2011) who demonstrated that macrophages isolated from LPS-exposed rainbow trout head kidney, secrete TNFα as early as 30min post-stimulation while gene expression is not detected until 3h post-stimulation supports the possibility that IFNγ mRNA transcript may be up-regulated in RTS11 following Vibrogen 2 stimulation later than 24h while stored IFNγ is able to initiate the adaptive immune response. However, recent work in zebrafish has shown that there is a significant positive correlation between the regulation of IFNγ mRNA transcript and IFNγ protein expression following stimulation with phytohaemagglutanin (PHA), a mitogenic plant lectin (Yoon et al., 2016). Therefore, it is increasingly likely that the time point at which IFNγ gene expression was up-regulated is not captured here and although the work of Yoon et al., (2016) indicates that at 4h both IFNγ protein and mRNA transcript reach peak levels it is possible that in the study presented here, IFNγ mRNA transcript in RTS11 cells stimulated with Vibrogen 2 peaked prior to 4h, or was delayed and peaked at a later time that was not described here. Since significant up-regulation of IFNγ was not observed until 24h post-vaccination in rainbow trout vaccinated with Vibrogen 2, it is possible that IFNγ mRNA transcript levels peaked between 8h and 24h or

after 24h in head kidney (Figure 3-4). However, the rapid and strong up-regulation of IL-1β at 4h in RTS11 cells, reached expression levels well above those documented at 4h *in vivo* therefore, IFNγ gene expression is more likely to have peaked prior to 4h in RTS11 cells (Figures 2-2 and 3-3). If IFNγ is up-regulated earlier *in vitro* than *in vivo* in these studies it may explain the reason S25-7 up-regulation was observed in RTS11 cells but not head kidney, and this may also support the higher levels of TAP1 mRNA transcript *in vitro* versus *in vivo* (Figures 2-2 and 3-1). The notion that IFNγ gene expression was up-regulated at an alternate time point is further supported by the observed dose dependent expression of IL-1β, S25-7 and TAP1 in RTS11 when gene expression in cells stimulated with 1 in 100 Vibrogen 2 is compared to expression in cells stimulated with 1 in 500 Vibrogen 2 (Figures 2-2, 2-3 and 2-4).

S25-7 mRNA transcripts in RTS11 cells are significantly up-regulated beginning at 8h for 1 in 100 Vibrogen 2 stimulated cells and at 24h for both 1 in 100 and 1 in 500 Vibrogen 2 stimulated cells however, in rainbow trout head kidney no significant changes in expression are observed and at 24h it appears that gene expression for this gene is beginning to trend down (Figures 2-3 and 3-4). The results of this *in vivo* study are consistent with previous results obtained by Christie, (2007) that demonstrated S25-7 gene expression begins to trend down and is significantly down-regulated at later time points in response to PMA. However, in this same work Christie, (2007) also observed no significant changes in S25-7 gene expression in RTS11 cells. The results obtained in RTS11 cells following stimulation with Vibrogen 2 may differ from those obtained by Christie, (2007) because Vibrogen 2 contains natural pathogen components and the vaccine may have additives that enhance the immunological response. It is also possible that dosing may play a role in the level of expression and perhaps RTS11 cells exposed to a higher concentration of PMA may up-regulate S25-7. Additionally, the earlier

work of Christie, (2007), involved RT-PCR followed by densitometry analysis while RT-qPCR was used in the current study which may have increased sensitivity for detection. Thus, it is expected that in RTS11, S25-7 mRNA transcript levels would continue to increase past 24h post-stimulation with Vibrogen 2, while the results of the *in vivo* study presented here demonstrate down-regulation of S25-7 mRNA transcript levels in rainbow trout head kidney (Figures 2-3 and 3-4). Since two additional invariant chains have been identified in rainbow trout, 14-1 and INVX, it is possible that while S25-7 is down regulated either 14-1, INVX or both are up regulated or that MH class II is expressed independent of increased invariant chain gene expression (Fujiki et al., 2003).

Another possibility is that S25-7 mRNA transcript levels in head kidney are down regulated as rainbow trout turn off the MH class II pathway in favour of an MH class I response either systemically or in the head kidney. One reason rainbow trout may choose to down regulate the immune response in the head kidney is to protect this important hematopoietic tissue from damage and it has been demonstrated by high-throughput RNA-sequencing (RNA-seq) that during the early response of zebrafish immunized with a live-attenuated *Edwardsiella tarda* vaccine MH class I associated genes are up-regulated while MH class II associated genes are down regulated in the liver, an organ thought to have little or no involvement in the immune response (Bystrom et al., 2008; Christie, 2007; Soulliere & Dixon, 2017; Yang et al., 2012). Additionally, Christie, (2007) demonstrated that, in response to PMA, the expression of S25-7 mRNA transcript remains unchanged in a panel of rainbow trout tissues including liver, spleen and gill, as well as, in PBLs, while it is down-regulated in head kidney suggesting that down-regulation of S25-7 may in fact be tissue specific. This along with the results presented in zebrafish may suggest that either species, pathogen or both play an important role in the

regulation of the MH class I and MH class II pathways in fish (Yang et al., 2012). Further evidence of this is presented in the *in vitro* study described here where RTS11 cells, a population of cells derived from rainbow trout spleen, are up-regulating S25-7, suggesting rainbow trout may shut down the MH class II pathway in head kidney but up-regulate this antigen presentation pathway in a secondary immune organ in response to Vibrogen 2. However, the results from the *in vitro* and *in vivo* studies when considered together also provide evidence that Vibrogen 2 activates antigen presenting cells, such as macrophages, and this leads to activation of the MH class II antigen presentation pathway which initiates an immune response that requires the activated cells to migrate from the head kidney (Figures 2-3 and 3-4). Furthermore, evidence of macrophage migration in response to i.p. injection of a *V. anguillarum* bacterin has been documented in rainbow trout. In a previous study it was demonstrated that at 1h post-vaccination a *V. anguillarum* bacterin accumulates in the kidney and spleen and then begins to spread throughout the fish and is found in additional tissues including the mesenteries of the gastrointestinal (g.i.) tract and air bladder (Nelson, Rohovec, & Fryer, 1984). This study further demonstrated macrophages that had phagocytosed bacterin persisted throughout the body until day 7 when they localized in the spleen, kidney, g.i. tract lamellae and mesenteries, gill arches and air bladder however, at day 14 the bacterin was cleared from all sites except the spleen and kidney where it persisted and remained present at trial termination on day 28 (Nelson et al., 1984).

In both RTS11 cells and rainbow trout head kidney TAP1 mRNA transcript levels are significantly up-regulated (Figures 2-3 and 3-4). This is unexpected and it is unclear why the MH class I pathway would be up-regulated in response to Vibrogen 2. Evidence in mammals supporting cross-presentation pathways in macrophages and DCs exists however it suggests that

TAP independent non-classical MHC class I HLA-F associated with MHC class I open conformers is involved (Basta & Alatery, 2007; Cruz et al., 2017; Goodridge et al., 2013; Grimholt, 2016). It is possible that in salmonids both MH class I and MH class II are up-regulated and/or TAP1 dependent MH class I is responsible for presentation of both endogenous and exogenous antigen, which may provide explanation for the loss of MH class II machinery in cod (Grimholt, 2016; Star et al., 2011). However, the *in vivo* results presented here indicate TAP1 gene expression is up-regulated at 24h post-vaccination while S25-7 transcript levels trend down at 24h post-vaccination (Figures 2-4 and 3-5). If S25-7 mRNA transcript is down regulated because activated antigen presenting cells are migrating away from the head kidney as was suggested above, it appears that TAP1 mRNA transcripts are regulated in different cells than S25-7 mRNA transcripts. This suggests that expression of the MH class I and the MH class II pathway may not be occurring simultaneously in the same cell. Since the RTS11 cell line is a macrophage-like cell line the *in vivo* and *in vitro* study results together may provide evidence that at minimum two different macrophage cell population exist in rainbow trout. Therefore, rainbow trout vaccinated with Vibrogen 2 may activate two distinct macrophage populations, and one is a non-migratory population responsible for MH class I presentation while the other is a migratory population responsible for MH class II presentation. However, this may also be evidence in fish of the characteristic plasticity of mammalian macrophages, as well as, the presence of two macrophage populations, where one is responsible for the inflammatory response and is classically activated (M1) leading to up-regulation of the MH class II pathway, while the second is either non-classically activated by, a cytokine such as IL-4 (M2a) or an immune complex and the TLR ligand LPS (M2b) leading to MH class I rather than MH class II up-regulation, or a third possibility is that IL-10 activates a population of resolution-phase

regulatory macrophages (M2c) that are responsible for establishing tissue equilibrium following an inflammatory event and these macrophages up-regulate MH class I antigen presentation to ensure that any compromised cell is eliminated (Bystrom et al., 2008; Castro & Tafalla, 2015; Edholm, Rhoo, & Robert, 2017; Hodgkinson et al., 2015; Sever, Vo, Bols, et al., 2014).

Overall, the results of the *in vitro* and *in vivo* studies when considered collectively show that the immune response initiated by the Vibrogen 2 vaccine leads to classical activation of macrophages that up-regulate MH class II and may migrate out of the head kidney. The vaccine also appears to lead to up-regulation of TAP1 dependent MH class I in cells. It is possible that cells expressing MH class II are presenting antigen leading to activation of the pathway necessary for antibody production while cells up-regulating MH class I are prepared for but do not present antigen. On the other hand, MH class I may also present antigen and thus aid in eliminating cells that may have been damaged or compromised either by the inflammatory response or the invading pathogen.

4.2 Challenges and Future Directions

While the results of this work provide a strong foundation for understanding the immune response triggered in rainbow trout in response to Vibrogen 2 and vaccination in general, as well as, Gram negative bacteria such as, *V. anguillarum* and *V. ordalii*, several challenges encountered during data collection and suggestions for future studies are outlined below. First, the work presented here was produced using previously published primers for IL-1β, IFNγ and TAP1 however, a new primer set was designed for S25-7 (Chettri et al., 2011; Martin et al., 2007). While a blast search was conducted to ensure product specificity for the S25-7 primers and a small subset of samples obtained during quantification by qPCR for all four genes were run on an agarose gel and compared to a 100 base pair (bp) ladder to confirm product size, due to

time constraints the products were not sent for sequencing. Thus, future work with the S25-7 primers should include obtaining confirmation of the product sequence. Additionally, melt curves were generated for each 96-well plate run on the Roche LightCycler 480 and in most cases one peak per well was observed however, some double peaks did occur. These anomalies appeared to occur intermittently and were not reproducible either within triplicates or between repeat plates. The limited time available for optimization of the qPCR protocol used here may contribute to this issue therefore, the double peaks may be eliminated by tweaking the developed protocol. However, given the intermittent nature of this issue it is proposed that the observed double peak may have been the result of duplicate alleles, multiple isoforms, splice variants or were the result of incomplete splicing events that generated mRNA in low copy number. It is also possible that there was low level gDNA contamination in some samples despite including two gDNA removal steps including both column technology and enzymatic elimination. To ensure that sample wells with double peaks were not altering the final crossing point (Cp) value the results were carefully considered and the Cp values from double peak wells were compared to single peak wells for the same sample. It was determined that the Cp values between double peak and single peak wells were consistent however, in the future determining the sequence of the larger bands may be useful. Second, the nature of working with living organisms such as fish, lends to increased variability and reduced sample size. Here, fish-to-fish variability was observed and was most pronounced in the IFNγ results. Additionally, the sample size at each time point for each treatment was three, the smallest sample size that is recommend for statistical analysis. To add strength to the data presented here a second trial was conducted in the same manner as the first trial however time constraints did not permit analysis of these samples. Therefore, data for this second set of samples is not included in this work and a complete set of

samples ready for processing exists that can be included in future analysis and would provide a set of data for comparison to the results presented above. Third, the number of time points assessed here was limited to 4h, 8h and 24h as the *in vitro* study involved only these time points. However, during the *in vivo* study samples were collected at additional time points:1h, 12h and 48h. Therefore, quantifying the mRNA transcript levels of IL-1β, IFNγ, S25-7 and TAP1 at these additional time points may provide additional data relevant to the immunological pathways activated by Vibrogen 2. Additionally, given the results presented here suggest that IFNγ up-regulation in RTS11 may occur prior to 4h future work should include a second RTS11 trail where cells are collected at 1h, 2h and 3h. It would also be beneficial to extend the RTS11 trial and collect 12h, 48h and 72h samples for better assessment of S25-7 and TAP1 expression. In fact, extending the trial in both RTS11 and rainbow trout to include samples up to two weeks post-stimulation may provide data relevant to the innate/adaptive immune interface in fish given macrophage migration may persist throughout the first 14 days post-vaccination (Nelson et al., 1984). Four, during the *in vivo* trial not only was head kidney tissue collected but the spleen of each fish was sampled, however, the limited time available for data collection did not permit analysis of this tissue. Given that Nelson et al., (1984) demonstrated macrophages that phagocytosed *V. anguillarum* bacterin localized to rainbow trout kidney and spleen at 1h, persisted in these organs during widespread macrophage distribution then re-localized to the kidney and spleen at day 14 post-vaccination, and considering the results obtained here for S25-7 mRNA transcript regulation in rainbow trout head kidney and in RTS11 cells, a macrophage-like cell line derived from rainbow trout spleen, investigating the S25-7 transcript levels in rainbow trout spleen following Vibrogen 2 vaccination may prove valuable. Further, in addition to an *in vitro* study in RTS11 cells a second *in vitro* study in RTgutGC cells, a rainbow trout gut

epithelial cell line, was carried out, however, again the limits imposed by time resulted in exclusion of these samples from this work. Therefore, future work may also include an investigation of innate and adaptive immune gene expression in response to Vibrogen 2 in RTgutGC and this could be coupled with an investigation from *in vivo* gut samples that were collected by another investigator during the rainbow trout trial. Since the gut is thought to be one of the main entry points for *V. anguillarum* and *V. ordalii* understanding the immunological pathways induced in this organ by the Vibrogen 2 vaccine may provide important data leading to a better understanding of the defence mechanisms rainbow trout utilize to combat infection and/or develop long term immunity to the bacteria responsible for vibriosis (Austin & Austin, 2016b). Five, since the Vibrogen 2 vaccine is a proprietary product it is difficult to assess the exact components stimulating the observed immune response and previous studies with *V. anguillarum* preparations and/or components is limited. Therefore, future studies should attempt to vaccinate fish with purified formalin-killed bacteria preparations rather than a prepared bacterin and investigators should attempt to assess the response triggered by the different bacterial components of *V. anguillarum* and *V. ordalii* such as the flagellin and LPS alone as well as in multiple combinations to determine the most potent components for immune stimulation and future vaccine development. Additional, considerations for future studies include: Processing *in vivo* trial samples collected from Poly I:C stimulated fish to compare the early immune response of rainbow trout to bacterial versus viral pathogens; Investigating the expression of additional genes that may be involved at the innate/adaptive immune interface such as 14-1, INVX, IL-1F and IL-10 to expand the understanding of immune pathway regulation in fish; Optimizing the Vibrogen 2 dilution used *in vitro* to more closely mimic the dose used in i.p. vaccination: Conducting studies with additional rainbow trout cell lines such at RTgill-W1, a

rainbow trout gill cell line matched to the corresponding rainbow trout tissue to increase understanding of the systemic immune response in fish and; Expanding the investigation of the innate/adaptive immune interface gene regulation to include other salmonid species, as well as, other teleosts such as, cod, to better understand the evolutionary history of immunity as well as the adaptive immune response of teleost fish.

Finally, perhaps the most important future studies will focus of an examination of the protein expression patterns of IL-1β, IFNγ, S25-7 and TAP1 along with other cytokines such as TNFα, IL-1F and IL-10 and other MH associated proteins such as INVX, 14-1 and tapasin that may be involved in regulating the transition from the innate pro-inflammatory response to the antigen presentation pathways of the adaptive immune response in fish. Additionally, it will be important for future studies to follow the adaptive immune response through to the up-regulation of MH class I and MH class II protein itself. Over the years, information about these proteins in fish has trickled in however, with antibodies that recognize these important immune proteins from rainbow trout and other salmonid species at various stages of development it will not be long before studies that permit quantification of fish immune proteins will be more easily accessible (Christie, 2007; Kales, 2006; Roher et al., 2011; Sever, 2014; Yoon et al., 2016). In fact, a small repertoire of antibodies for a handful of immune relevant rainbow trout protein already exist and Christie, (2007) produced antibodies against rainbow trout invariant chain, S25-7 and INVX while both Kales, (2006) and Sever, (2014) worked with antibodies against MH class I proteins. Furthermore, as part of the work conducted for the studies presented here IL-1β, IFNγ and TNFα antibodies were produced and validated. That is, antibodies were titred by enzyme linked immunosorbent assay (ELISA) and reactivity and specificity to recombinant protein was shown by western blot for each antibody. Development of quantitative sandwich

ELISAs and enzyme linked immunosorbent spot assays (ELISpot) for each of these proteins was also initiated however, optimization of these assays proved challenging and due to time constraints, the developmental work has been passed on to another investigator for further optimization.

4.3 Conclusions

In chapter two, results from an *in vitro* investigation with rainbow trout macrophage-like cells (RTS11) indicate that Vibrogen 2 activates rainbow trout macrophages and this activation results in a continuous increase in IL-1β gene expression. While significant up-regulation of IFNγ is not observed it appears a shift occurs and the adaptive immune system is activated. Not only is the MH class II associated invariant chain gene S25-7 up-regulated suggesting initiation of the pathway involved in antibody production but TAP1, the MH class I associated transporter protein is also up-regulated by Vibrogen 2 in RTS11 cells. The up-regulation of TAP1 in rainbow trout macrophages may suggest that the MH class I pathway in teleost fish is involved in cross-presentation and is responsible for responding to both endogenous and exogenous antigens, or that the MH class I associated genes are simply up-regulated in preparation for mounting an immune response if necessary.

In chapter three *in vivo* results indicate that the IL-1β gene is up-regulated and an immune response is initiated via the classical inflammatory pathway. Despite evidence of macrophage activation via the classical pathway the MH class II associated gene S25-7 expression is not significantly altered and trends down while TAP1, the MH class I associated gene is up-regulated. It appears that classically activated macrophages and antigen presenting cells expressing MH class II may be migrating out of the head kidney while a second group of

cells expressing MH class I remains in the head kidney. Thus, the results suggest that the Vibrogen 2 vaccine activates at minimum two separate and distinct cell populations.

Together the results of chapter two and chapter three show that the Vibrogen 2 vaccine initiates an immune response via the pro-inflammatory pathway and may suggest that alternate activation pathways are triggered. These results also indicate that the immune response is progressing towards an adaptive response including antibody production via the MH class II pathway in classically activated macrophage cells while cells expressing MH class I may be preparing for antigen presentation if necessary; may be involved in homeostasis or may be preparing for antigen presentation if endogenous antigens are produced. Additionally, the results may support macrophage plasticity in teleost fish, as well as TAP1 dependent cross-presentation in salmonids. Furthermore, the results indicate that the apparent success of Vibrogen 2 in aquaculture may be related to the ability of the vaccine to activate the adaptive immune response via the pro-inflammatory pathway. This response may be enhanced by activation of different macrophage populations and/or the ability to activate both MH class II and MH class I antigen presentation. Thus, it may be important to consider both MH class I and MH class II activation when developing vaccines for use in aquaculture and taking advantage of cross-presentation may allow for the development of vaccines to additional aquatic pathogens. However, another consideration for the development of more effective fish vaccines may be shifting the response triggered to favour either MH class II presentation or MH class I presentation rather than stimulating both pathways.

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Appendix A

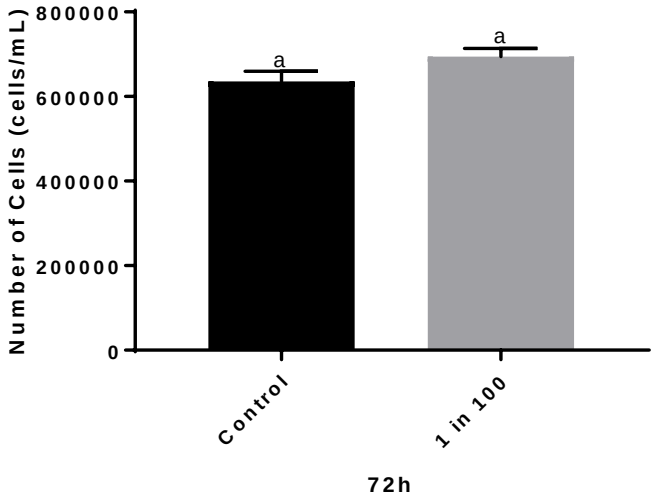

Figure A-1: Average number of RTS11 cells at 72h. Average RTS11 cell counts obtained using a Millipore Scepter Cell Counter in control wells versus wells stimulated with 1 in 100 Vibrogen 2 dilutions 72h post-stimulation to determine if stimulation significantly increases cell number. Analysis by two-way ANOVA followed by Tukey's multiple comparisons test show no significant difference exists. Results are shown as mean ± SEM, n=3.

Appendix B

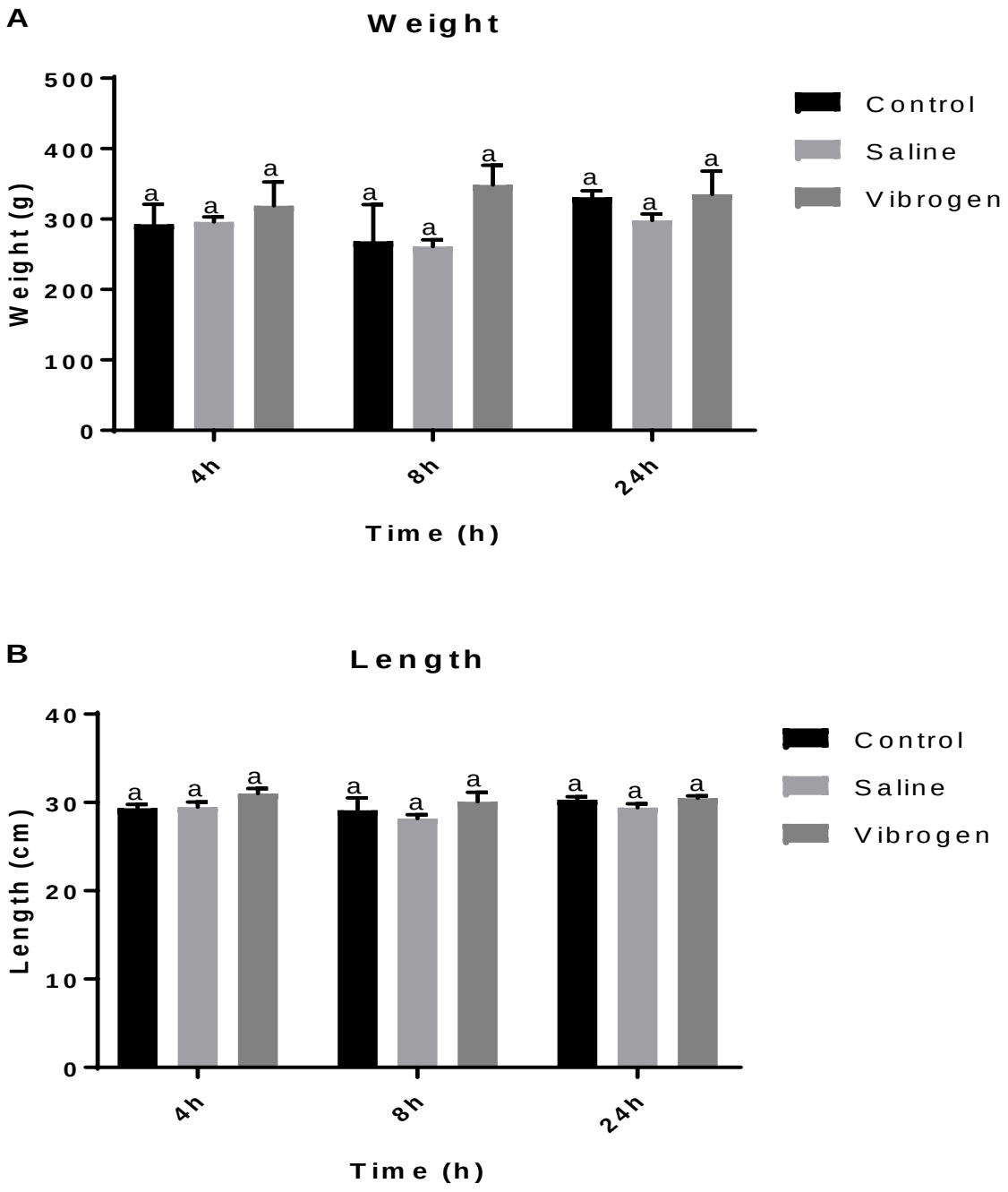

Figure B-1: Average weight and length of rainbow trout. Average size of control rainbow trout versus saline injected and Vibrogen 2 vaccinated rainbow trout obtained using a top loading balance and measuring tape at time of sampling to determine if A) weight or B) length is significantly different between groups or across time points. Analysis by two-way ANOVA followed by Tukey's multiple comparisons test indicates that no significant differences exist. Results are shown as mean ± SEM, n=3.