Development of protein level cytokine assays and assessment of the impact of implanted Acoustic Telemetry Tags on the Rainbow Trout (*Oncorhynchus mykiss*) immune system

by
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Author's declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Telemetry tags are a widely used technology for tracking animals that are difficult to observe in their natural environment. This technology has been increasingly used to monitor and study populations of high value salmonid species in Canadian waters. This study examined the heretofore unexplored impacts of tag implantation on the immune system of Rainbow Trout (*Oncorhynchus mykiss*). Pro-inflammatory cytokines and protein level markers were examined in fish that underwent peritoneal implantation of three tag types as well as a sham surgery control group. The different coating on the tags showed differential immune induction extending over a two-month period. This included peritoneal total protein, IL-1β protein, IgT and IgM, as well as pro-inflammatory transcripts in the spleen. These results are suggestive of a prolonged, costly foreign body response which may be differentially induced by the different types of tag coating.

At day 2 all tagged fish showed less peritoneal IgM and more peritoneal total protein than sham controls. This could indicate the onset of the foreign body response to tag presence. IFNγ, an important immunomodulator was quantified at both the transcript and protein level using a newly developed quantitative ELISA assay. Results suggest that a prolonged foreign body response occurred as a result of tag implantation. We also observed some differences associated with the type of tag coating. Additionally, efforts to develop an ELISPOT assay during this study uncovered a previously unreported serum borne, endogenous alkaline phosphatase enzyme. It is demonstrated that very precise quantification of this potential biomarker is possible with low effort and cost. Thus it may represent a useful metric for the assessment of fish health and vaccine efficacy.

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1.0 - General Introduction

1.1 - Purpose and impact of telemetry tagging

Canada is a country with a variety of geographic blessings, not least of which is it's 250 000km of coastline. The associated coastal waters are home to a wide variety of fish populations that are important to Canadians in terms of ecology, tourism and industry. For all three of the arenas one of the most important fish populations is the salmonids. In both fresh and salt water, salmonids (largely rainbow trout [*Oncorhynchus mykiss]* and Atlantic salmon [*Salmo salar*]) are prized targets for anglers who spend large sums of money to visit and fish Canadian waters. These species also represent the bulk of Canadian fish farming across the country, with Atlantic salmon accounting for greater than 90% of farmed fish in Canada. Canadian salmonid populations also form an important part of aquatic ecosystems, occupying in some cases and, in other cases being food for, the top trophic level of many aquatic food webs.

Salmonids are a family of ray-finned fish that originated in and spread across the northern hemisphere. These animals had diverged into saltwater salmon and freshwater trout with some crosstalk between these populations (Zhivotovsky, 2015). Saltwater salmon are characterized by an anadromous life history, meaning that they are born in freshwater streams, migrate to the ocean when young and then return to their natal freshwater streams to mature and breed. Canadian salmon species often travel long distances from their place of birth to their feeding grounds. With such a life-style, it is unsurprising that efforts to track individual salmon, let alone whole populations, are often confounded by the immensity and inaccessibility of the animal's environment.

Monitoring of salmon populations has benefitted from the introduction of implantable telemetry tags. These tags can be detected by satellites or stationary tracking buoys and are capable of recording depth, home range and various physiological parameters of the fish (Reviewed in Hussey *et al*. 2015). A key assumption of biotelemetry data is that the tagged animal is behaviourally and physiologically equivalent to the untagged animals that make up the wild population being studied. This assumption has been evaluated over the years in many different ways, including how tag implantation affects survival, growth, swimming performance, predation and stress levels (Hall *et al*, 2009; Peake *et al*, 1997; Anglea *et al,* 2004; Brown *et al*, 2013). This literature finds little difference between tagged and untagged fish in the short term (meaning days to a few weeks post implantation). However, it is important to note that most of the metrics were evaluated over short periods with respect to the life of a salmon. Levels of cortisol, glucose and lactate may return to normal in a few days following surgery (Jespen *et al,* 2015), but this

does not mean that the effects of the surgery (nor the presence of the tag) are not having long-term effects. Indeed, studies conducted in field settings have observed increased mortality in juvenile salmonids associated with larger and smaller implanted tags (Brown *et al*, 2013 and Knudsen *et al*, 2009 respectively).

Telemetry tags are largely the same in terms of size and internal components, what varies is the coating material. This is highly important for implant success and the coatings are designed so that they have little to no interaction with the organism's immune system. However, in at least one study, two types of coating (silicone-rubber and paraffin) were equally prone to cause tag expulsion in Channel Catfish (*Ictalurus punctatus*; Marty and Summerfelt, 1986) while previous work suggested that paraffin coating may be a factor in inducing the rare transintestinal route of tag expulsion (Summerfelt and Mosier, 1984). Conversely, some tag coating appear to reduce the incidence of tag expulsion, as was the case with shortnosed sturgeon (*Acipenser brevirostrum*) and elastomer coated tags (Kieffer and Kynard, 2012). Tag expulsion has been associated with formation of a fibrous capsule surrounding the tag in channel catfish (*Ictalurus punctatus*; Marty and Summerfelt, 1986), vundu (*Heterobranchus longifilis*; Baras and Westerloppe, 1999) and rainbow trout (*Oncorhynchus mykiss*; Chisholm and Hubert, 1985; Bunnell and Isely, 1999; Welch *et al.,* 2007). Additionally, Semple and colleagues observed fibrous capsule formation in Rainbow Trout implanted with telemetry tags (Semple et al. 2018). Considering that tag implantation leads to inflammatory responses and tag encapsulation, it stands to reason that the salmonid response to intraperitoneal implantation would be similar to the well documented phenomenon known as the foreign body response (FBR). This study used Starr Oddi tags which have a ceramic shell that is designed to reduce immune activation. There were also two types of Vemco brand tags used in this study, one with the standard plastic surface and the other with an elastomer coating that is supposed to enhance biocompatibility. A comprehensive review of the state of tagging technology was carried out by Cooke and colleagues (Cooke *et al*., 2011).

The history of using foreign materials, from bark, tendons, hair and silk to even ant heads, for suturing and other medical interventions goes back to approximately 600 B.C. where it is found in the writings of the famous Indus valley surgeon Sushruta (Chandorkar *et al*., 2019). Current use of implants made of foreign materials takes advantage of modern materials science to design implants that will avoid recognition by the host immune system and circumvent the FBR. The host immune system is, however, not so easily deceived and even today it is not possible to completely avoid the FBR during implantation.

The FBR is a multistage process that can play out over weeks to months. Necessarily, there is significant overlap between the early FBR and the normal process of wound healing. The first step involves an acute inflammatory response guided primarily by neutrophils (Broughton *et al.*, 2006) which attempt to phagocytose any foreign materials/organisms detected in the wound. The late inflammatory phase follows, mainly because of the action of recruited monocytes which differentiate into macrophages upon arrival at the wound site (Kyriakides *et al.*, 2004). These macrophages are larger phagocytes that also produce many cellular and tissue growth factors and begin the process of provisional extracellular matrix formation (Goldman R, 2004). Up to this point the FBR is almost indistinguishable from normal wound healing aside from having neutrophils, and subsequently macrophages, attempting to phagocytose the implant itself. The deposition of provisional extracellular matrix is the starting point of the physical signs of the FBR, namely an avascular capsule of connective tissue surrounding the implant and thereby cutting it off from the intracellular milieu. The macrophages induce capsule formation by first fusing with other macrophages to form a large multinucleated cell referred to as a foreign body giant cell (FBGC). The FBGC then sends out signals that result in the recruitment of fibroblasts which will encase the implant in collagen (Ratner, 2002). Thus, the development of capsule is directly linked to chronic inflammation (Anderson *et al.*, 2008) as indicated by previous work (Semple *et al.* 2018). The purpose of this experiment was to examine the effects of coated and uncoated tags composed of two different materials, as well as to examine the immune response to implantation using novel assays developed in the Dixon lab.

This study will shed light on the immediate and longer-term impacts of this widely used technology for tracking animals in their native habitat. It represents the first significant attempt to track the immunological impacts of introducing a foreign object into an animal for purposes of tracking life cycle, range and distribution. This is an important gap in our knowledge considering that if tag implantation, through its involvement in inflammation and the FBR, is resulting in alteration of fish behaviour (or indeed, mortality) there is little work done thus far that would even show that signal. To wit, any fish that display tag related morbidity/mortality during a normal life cycle on their home ranges will be at best imperfectly represented in the existing literature, given the impossibility of examining the animals (tagged and untagged) in their natural milieu. Thus, revealing tag related immunological perturbations at the cellular level could point to impacts of tagging that simply cannot be quantified at the population level.

1.2 – Development of protein level assays

Since the development of PCR there have been many important advances in the study of nucleic acids. From advances in transcriptomics capable of discerning the mRNA profile of a single cell to high

throughput sequencing of the gut microbiome the world of nucleic acids has been revealed as never before using this array of new technologies. The world of protein, on the other hand, has proven to be more difficult to examine. In the case of nucleic acids the revolution in technology followed isolation of an enzyme (Taq Polymerase) capable of copying a given DNA molecule by using the molecule itself as a template. However, no similar process is possible with proteins because proteins are not made by copying other proteins, but rather are translated from nucleic acid molecules in a one-way process. As such the exponential amplification of specific protein sequences (which is precisely what underlies much of the nucleic acid technology today) is not possible with today's tools.

Rather than amplifying proteins to study them, much of the current work uses antibodies specifically designed to target an epitope (a small stretch of 6-8 amino acids) or epitopes on the protein of interest. These antibodies are either monoclonal (produced by a single immune cell, targeting a single epitope) or polyclonal (produced by multiple immune cells, targeting several epitopes) and are typically developed by producing a version of the protein of interest in bacterial cells, this is referred to as a recombinant protein. In the context of this study recombinant protein and antibodies were produced to develop an assay capable of detecting a protein involved in immune signalling.

The purpose of the assays developed in this study was to evaluate fish health and so targets in the immune system had to be selected. The vertebrate immune system is made up of diverse organs, tissues and cells distributed all over the body. Many of these parts are not involved in immunity during normal function, but become activated to participate in an immune response upon infection or injury. The main way in which immune reactions are activated and coordinated is through the production of signaling molecules known as cytokines (Turner et al., 2014). These small (5-25 kDa) soluble proteins are incapable of crossing the cell membrane but cause significant signalling cascades within cells upon binding to their membrane bound receptors. This can produce a wide variety of effects on immune cells including: lymphocyte recruitment, growth and differentiation, pro-inflammatory and anti-inflammatory activity, activation of phagocytic activity and polarization of the immune response to antigen (Dinarello, 2007). Cytokines selected were involved in 1) the immune response to the surgical lesion (Interleukin 6 [IL-6]; Heinrich *et al*., 1990), 2) the inflammatory response associated with implantation (Interleukin-1β [IL-1β]; Dinarello, 2018, Tumor necrosis factor alpha [TNFα]; Schottelius *et al*., 2004), or 3) the cross talk between immune cells that could be expected during the FBR (Interferon gamma [IFNγ]; Schroder *et al*., 2004).

IL-1β is a member of a large family (11 members) that share properties and chromosomal location with the first interleukin discovered (Sims *et al.,* 2010). IL-1β is naturally lumped in with IL-1α as they bind the same receptor complex to transduce their signal. However, these two cytokines differ in terms of cellular source, cellular localization (Dinarello, 2009) and induction (Rausch *et al.,* 1994). IL-1β is primarily produced by monocytes and macrophages and, as in IL-1α, is activated by caspase processing. Active IL-1β helps control fever (Horai *et al.,* 1998) and has a powerful pro-inflammatory effect on surrounding cells (Andrei *et al*., 2004). The introduction of a foreign body, aside from any contaminating microorganisms that it might carry, is enough to induce an inflammatory response. Therefore IL-1β is a good candidate for tracking the impact of telemetry tag implantation.

TNFα (also called cachectin) is another potent pro-inflammatory cytokine produced by macrophages. This cytokine is involved in induction of apoptosis (Abrams *et al*., 1999) and endotoxic shock (Tracey, *et al*., 1986). Considering that the surgery or implantation of telemetry tags could result in systemic infection this protein was identified as an immune target.

IL-6 is synthesised by the mononuclear immune cells that are first to arrive at the site of injury. IL-6 then circulates in the blood stream where it has numerous effects including induction of acute phase proteins in the liver (Heinrich *et al*., 1990). Surgical wound closure and healing are costly processes for the tagged animals, so IL-6 was measured to determine if different tag coatings/styles had differential impacts on fish health.

IFNγ belongs to an important group of cytokines, first discovered in 1957, that were and named for their activity in viral inhibition. These cytokines, dubbed 'interferons' interfered with the ability of a virus to infect cells and replicate (Isaacs, A. & Lindemann 1957). Since their discovery further work has revealed two broad types of interferon, Type I and Type II. Type I interferons include seven classes (IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, IFN-τ) along with several IFN-like cytokines (limitin, IL-28A, IL-28β and IL-29; Reviewed in Pestka *et al*., 2004).

The sole type II interferon, IFN-γ, is structurally different from all the Type I IFNs, encoded at a different chromosomal locus and binds to a different receptor (Schroder *et al*., 2004). Unlike Type I IFNs, which can be produced by almost every cell in the body, IFN-γ is only produced by immune cells including: helper T-cells, NK cells, and professional antigen presenting cells such as dendritic cells and monocytes/macrophages (Frucht *et al*., 2001; Gessani & Belardelli, 1998). IFNγ in its active form is a

homodimer which binds to a receptor complex and induces transcription via the intracellular JAK/STAT pathway (Schroder *et al.*, 2004). Although both types of interferon are involved in generating an antiviral reaction they operate somewhat distinctly, to wit, IFN-γ has 10 to 100-fold lower antiviral activity as IFN-α/β (Type I interferons) but 100 -10 000 times greater activity as an immunomodulator (Pace *et al*., 1985). This immunomodulation can take several forms. IFN-γ increases expression of MHC class I and MHC class II on phagocytes, endothelial and epithelial cells (Monde *et al*., 1986). Considering that Type I IFNs also induce MHC class I but do not induce MHC class II proteins this provides further indication of the division of function between these classes of cytokine. This induction of MHC expression reveals IFN-γ's role in the early, inductive phase of immune responses as it promotes antigen presentation of the affected cells (Boss and Strominger, 1986). As such a prime target for IFN-γ is the macrophage, a differentiated myeloid cell responsible for phagocytosis and antigen presentation to T-cells (Schreiber *et al*, 1986). Thus IFN-γ increases macrophage phagocytic/cytolytic activity and also induces pro-inflammatory cytokines to contribute to the early stages of the immune response (Raetz *et al*., 2013). Additionally, the later stages of the immune response (resolution of infection and wound healing) involve a differentially activated form of macrophage. These late-stage macrophages, rather than being induced by IFN-γ, are associated with the Th-2 associated cytokine IL-4 (Kim *et al*., 2019).

The IFN system is evolutionarily ancient and shows significant similarities across vertebrates. With regard to salmonids, several studies have identified IFN genes that fall into the same pattern of structure and function seen in other vertebrates (Robertson *et al*., 2003). One significant difference (commonly encountered in salmonid research) is the presence of duplicate versions of some of the genes deriving from one of the whole genome duplications inferred in the salmonid lineage (Allendorf & Thorgaard, 1984). This was also the case when examining IFN-γ in salmonids, as two versions of the IFN-γ gene have been identified, both being induced by standard stimulants for this cytokine (Purcell *et al*., 2009). This would suggest that both forms of IFN-γ are immunomodulating in the familiar manner.

It should be noted that much of what is known, and referenced above, regarding the actions of cytokines is derived from work studying mammalian models. While there is evidence of structural and functional similarity between mammalian and teleost cytokines (Zou and Secombes, 2016), there is by no means perfect agreement. Thus, this work seeks to examine cytokine dynamics in fish who have undergone tag implantation at both the transcript and protein level. This represents an important limitation to the work at hand, measuring cytokines (at either the protein or transcript level) and inferring the function based on mammalian cytokine expression patterns is bound to introduce discrepancies. The

cytokines selected are good candidates not only based on homology to well characterized mammalian cytokines but also based on studies carried out in related fish species.

Another contribution of this study is the development of protein level assays for salmonid cytokines. At present the study of salmonid immunity is, in most cases, limited to the level of the whole animal (survival, growth etc) or the level of the transcript (qPCR, ESTs, next-gen sequencing). There is little in the way of assays that measure important salmonid immune markers at the protein level. Thus, a focus of this work was to develop polyclonal antibody-based protein assays of both the Enzyme-linked immunsoassay (ELISA) and Enzyme linked-immunsospot assay (ELISPOT) form. ELISA is a technique by which two antibodies (polyclonal mixtures in our case), both designed to be specific to the same antigen (cytokines in our case) are used to measure the amount of the antigen in a given sample. Briefly, one of the antibodies (termed the 'capture antibody') is bound to a membrane usually on a 96 well plate. The protein sample is then applied to the wells and the specific antigen is 'captured' by the membrane bound antibody. The remaining sample is then washed away and the second antigen specific antibody (termed 'primary detection') is added to wells. A secondary detection antibody (which specifically binds to the primary detection antibody) is then added, this antibody having an enzyme (such as Alkaline phosphatase or Horseradish Peroxidase) bound to it designed to produce the final colorimetric signal. The colorimetric signal, reflecting only the amount of antigen captured out of the sample, is then developed using a substrate that is acted on by the enzyme bound to the secondary detection antibody. The signal is then measured on a plate reader in a specific window of absorbance corresponding to the colorimetric product. This amount of absorbance is then compared to a standard curve made up of known amount of recombinant protein to get a quantitative measure of antigen presence in the sample. The ELISA assays developed for this study are of two types: sandwich ELISA and direct ELISA. The assay described above is an example of a sandwich ELISA wherein the antigen of interest is 'sandwiched' between the capture and primary detection antibodies. A direct ELISA assay differs in that the antigen of interest (along with the rest of the protein sample) is diluted in coating buffer and subsequently bound directly to the plate. This means the assay can be carried out using just one antigen specific antibody, although the assay usually has lower sensitivity. In the context of this study the sandwich ELISA method was used for salmonid cytokines and the direct ELISA method was used for salmonid IgM and IgT antibodies.

ELISPOT is an assay that uses the same principal and conditions as the ELISA described above. The major difference is that the sample applied to ELISA assays is a protein homogenate, whereas the sample applied to ELISPOT wells consists of known numbers of living cells. The cells in the sample produce the antigen of interest and it is captured only in the locale of the cell itself. Thus, the final colorimetric

product is a precipitating version of the substrate, resulting in a darkened spot in the location where cells that were producing the antigen of interest were located. This assay has the benefit of being able to quantify antigen production down to the level of a single cell.

This work represents the first attempt, to the author's knowledge, to examine the prolonged impacts of telemetry tag implantation on the immune system. Previous work has typically focused on using short-term stress markers, immediate behavioural disruptions and naturalistic tag recapture experiments to evaluate tag impacts. As outlined above these methods, while providing valuable information, have definite blind spots when it comes to evaluating tag viability. Previous work in our lab had suggested that the immune system of salmonids was interacting meaningfully and lastingly with the surface of an implanted telemetry tag. This study sought to expand on this previous finding by examining multiple tag coating types for differences in biocompatibility. Additionally, this work expanded on the previous tag trial by quantifying tag related immune responses at the level of protein. Toward this aim a novel antibody-based ELISA assays were developed capable of detecting salmonid immune proteins which the previous study had implicated. An attempt was also made to develop an ELISPOT assay using these same antibodies. This would have permitted the detection of important immune protein production down to the level of a single cell. Permitting immune assessment from a small number of non-lethally sampled cells (PBLs) is an exciting prospect.

These assays targeting salmonid cytokine protein are important contributions to the field of comparative immunology. They will enable many aspects of basic research that have heretofore been impossible and improve our ability track interventions that use the immune system (vaccination, selective breeding). Considering that a principal challenge in salmonid aquaculture is combatting disease outbreaks these assays have the potential to make a significant difference. Furthermore, revealing the complete extent and duration of telemetry tag impacts is essential to responsible use of this technology. To whatever extent tag presence is related to morbidity or mortality the literature will be similarly distorted. Add to this the fact that tag related trials provide us with our only means of studying certain aspects of salmonid life and it can be seen that this work will help to clarify what has been a constitutively opaque body of evidence.

2.0 – Coating composition of acoustic transmitters influences the inflammatory response of Rainbow Trout (*Oncorhynchus mykiss*) during long-term implantation

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2.1 – Abstract

Telemetry tags are a widely used technology for tracking animals that are difficult to observe in their natural environment. This technology has been increasingly used to monitor and study populations of high value salmonid species. This study expanded a previous study of the impacts of tag implantation on the immune system of rainbow trout (*Oncorhynchus mykiss*). Pro-inflammatory cytokines and protein level markers were examined in fish that underwent peritoneal implantation of three tag types as well as a sham surgery control group. The different coating on the tags showed differential immune induction extending over a two-month period. This included peritoneal total protein, IL-1β protein, the immunoglobulins IgT and IgM, as well as pro-inflammatory transcripts in the spleen. These results are suggestive of a prolonged, costly foreign body response which may be differentially induced by the different types of tag coating. Examining tag impacts at the level of the immune system enables the development of more biocompatible tags which will improve data fidelity. This will support more effective strategies for the management of this important resource.

2.3 – Introduction

Given their importance to the economic welfare of sport and commercial fishers it is unsurprising that salmonid stocks have been under pressures generated by harvesting. This pressure is compounded by the fact that the saltwater salmonid life cycle involves long migrations over a large area. This makes assessment of salmonid populations a difficult and imprecise task. Moreover, salmonid stocks are under increased pressure due to climate mitigated increases in surface ocean temperature (Perry *et al*., 2005). These pressures can manifest as displacement of existing home range (Rose, 2005), alteration in the timing of seasonal events such as spawning (Sims *et al*., 2005) and disturbance of food availability through effects on prey populations (Beaugrand *et al*., 2002). Each of these disruptions alone can impact salmonid populations and it has been argued that temperature related factors have caused 'regime shift' in ecosystems as large as the North Sea (Beaugrand, 2004). Thus studies of fish movement and behaviour in the wild using tags are of crtical importance.

Tagging of fish, with ribbon in this case, goes back at least to the 17th century (Walton and Cotton, 1921) and now boasts an array of technologies; from inert synthetic tags for visual identification to electronic tags with various capabilities (Lucas and Baras, 2000). Whether inert or electronic, the implantation of a tag allows the researcher to identify and follow an individual fish over a part or all of it's life cycle. More advanced tags can record and transmit a greater range of parameters, both ecological and species related (Reviewed in Cooke *et al*., 2013). These tags can be detected by satellites or stationary tracking buoys and are capable of recording depth, home range and various physiological parameters of the fish (Reviewed in Hussey *et al*. 2015). However, these tags are either attached via surgery to the exterior of the fish or implanted into the body of the fish. This is obviously damaging and stressful for the animal in question and it necessitates an important assumption about tag telemetry data: that the tagged animal is entirely equivalent to their untagged counterparts that make up the wild population being studied.

This assumption has been evaluated over the years in many ways, including studies of how tag implantation affects survival, growth, swimming performance, predation and stress levels (Hall *et al*, 2009; Peake *et al*, 1997; Anglea *et al,* 2004; Brown *et al*, 2013). This literature finds little difference between tagged and untagged fish. However, it is important to note that most of the metrics in these studies were evaluated over short periods with respect to the life of a salmon. The longer term studies that were performed did not evaluate measures of individual response, looking only at general metrics like survival. Levels of cortisol, glucose and lactate may return to normal in a few days following surgery (Jespen *et al,* 2015), but this does not mean that the effects of the surgery (nor the presence of the tag) are not having long-term effects. Indeed, very few studies examine truly long-term effects of tags while those that do have

found increased mortality in juvenile salmonids associated with both larger and smaller implanted tags (Brown *et al*, 2013 and Knudsen *et al*, 2009).

The literature on implantation of telemetry tags is understandably focused on the utility of these tools in assessing population range and dynamics. However, it is reasonable to assume that the insult of surgical implantation, as well as the presence of the tag in the peritoneum will have an impact on the immune system of the animal in question. A previous study from our lab (Semple et al., 2018) demonstrated this impact on salmonid immunity, and showed that the response lasted over the whole two-month period of the study. In particular, the profile of cytokine activation in cells of peritoneum indicated the involvement of macrophages in the salmonid immune response to foreign material. This, along with the observed formation of fibrous capsule surrounding the implanted tag, suggests the involvement of a well characterized immune process known as the foreign body response (FBR).

The FBR is a multistage process that can play out over weeks to months. Necessarily, there is significant overlap between the early FBR and the normal process of wound healing. The first step involves an acute inflammatory response guided primarily by neutrophils (Broughton *et al.*, 2006) which, through production of reactive oxygen species (Winterbourn *et al*, 2016), attempt to degrade any foreign materials/organisms detected in the wound. Neutrophils also produce several proinflammatory cytokines, among them tumor necrosis factor alpha (TNF-α), interleukin 1 alpha (IL-1α) and interleukin 1 beta (IL-1β; Cassatella, 1999). These cytokines, along with others, recruit monocytes and induce their differentiation into macrophages on site. The late inflammatory phase follows, mainly because of the action of recruited monocytes which differentiate into macrophages upon arrival at the wound site (Kyriakides *et al.*, 2004). These macrophages are larger phagocytes that also produce many cellular and tissue growth factors and begin the process of provisional extracellular matrix formation (Goldman R, 2004). Macrophages also produce proinflammatory cytokines including IL-1α/β and interleukin 6 (IL-6: Duque & Descoteaux, 2014). These cytokines, being indicative of an inflammatory response, were upregulated in the previous trial and were examined in the study at hand (Semple *et al.*, 2018). Up to this point the FBR is almost indistinguishable from normal wound healing aside from having neutrophils, and subsequently macrophages, attempting to phagocytose the implant itself. If the implant does not degrade there is a shift in the FBR, to a phase characterized by a fibrotic process. Macrophages will begin to recruit fibroblasts which secrete a variety of extra-cellular matrix proteins onto the implant (Ignotz and Massague, 1986). Over time this combination of macrophages, fibroblasts and extracellular-matrix forms a fibrous capsule, covering and sealing off the implant from the rest of the body.

The deposition of provisional extracellular matrix is the starting point of the physical signs of the FBR, namely the avascular capsule of connective tissue surrounding the implant that cuts it off from the intracellular milieu. Thus, the development of capsule is directly linked to proinflammatory signals from macrophages (Anderson *et al.*, 2008) as was indicated by previous work in the Dixon lab (Semple *et al.* 2018)

The purpose of this expanded tag trial was to examine the effects of uncoated tags composed of two different materials: epoxy (Vemco) and ceramic (Star Oddi) and coated tags made of epoxy covered with paralyene (coated Vemco), as well as examining the immune response to implantation using novel assays developed in the Dixon lab to provide tools that can be used to assess the biocompatibility of future materials used to tags.

2.4 – Methods

2.4.1 – Fish

Rainbow Trout of an average length of 48cm and 1559 +/- 328g were obtained from and housed at the University of Guelph hatchery located at the Alma Research Center in Alma, ON. Water was supplied from a ground well at a constant 9°C (+/- 0.5°C). Fish were held under a constant 12h light and 12h dark cycle in the Alma Hatchery. Empty tag casings were provided by the manufacturers as the surface was the key component interacting with the immune system. Thus the implants here did not exceed the 2% rule, meaning that the implant does not exceed 2% of the dry body weight of the fish (Winter, 1983). The fish were divided into four tanks representing the experimental groups needed to compare three tag-types (coated Vemco tags, Vemco tags and Star Oddi Tags, all 23-26 mm in length and 9 mm in width) along with a control group which would undergo a tagging surgery without tag implantation (Sham surgery). In addition, a no surgery control group was sampled prior to surgery and implantation. All tanks received flow through water from the hatchery well and the hatchery standard day-night cycle. Animals were handled according to Canadian Council on Animal Care guidelines on a permit from the University of Waterloo Animal Care Committee (#30048).

2.4.2 – Surgery procedure and sample collection

Tag implantation surgery was carried out in the same manner as previously described (Semple *et al.*, 2018). In brief, following anaesthesia with benzocaine, a small incision was made in the mid-ventral surface anterior to the pelvic girdle and a non-active telemetry tag (of either Vemco or Starr Oddi manufacture) was inserted into the peritoneal cavity. The incision was then closed using silk sutures and the fish was

placed in a recovery tank prior to being returned to their original tank. The surgery was short (~2 minutes) and there was no post-surgical mortality. Spleen, muscle and peritoneal lavage was collected from 12 fish for each group at days 2, 14 and 70 following surgery. Spleen and muscle samples were flash frozen in liquid nitrogen before being transferred to -80°C for storage. Peritoneal lavage and lavage supernatant was collected as previously described by Semple et al. (2018). Pictures were taken of the wound site as well as the internal location of the implanted transmitter for each fish sampled at each timepoint.

2.4.3 – qRT-PCR primer design and reaction details

Sequences of Rainbow trout cytokines were identified in Pubmed and qRT-PCR primers designed (see table 2.1). Primers were used to amplify targeted regions from Rainbow trout cDNA with the following conditions: 95° C for 3 min followed by 32 cycles of 95° for 30 sec, 60°C for 10 sec, 72° for 45 sec, ending the entire process with a final step of 72° for 5 min. The bands were electrophoresed on a 1.5% agarose gel, stained with GelRed (Biotium), visualized on a Blue Light Transilluminator (Pearl Biotech) and excised using a scalpel. Bands were purified using a QIAquick gel extraction kit (Qiagen) and blunt ligated into pGEM-T easy vector (Promega). These vectors were transformed into *E. Coli* XL1-Blue supercompetent cells (Aligent Technologies) which were then grown on LB media with 0.1 mg/ml of ampicillin (Fisher Scientific). White colonies were selected, grown in 1ml of LB plus ampicillin, miniprepped and sent for sequencing (using T7 and Sp6 primers) at TCAG sequencing facility at Toronto Sick Kids hospital. qPCR primer sets were validated via sequencing and then not modified further.

Each sample was plated in triplicate on a 96 well plate (Nunc, ThermoFisher) for qRT-PCR analysis using primers specific for three genes of interest (IL-1β, IL-6 and TNFα) and one reference gene (EF1α). Each well contained a 10ul reaction consisting of 2.5ul of sample cDNA, 2.5ul of forward and reverse primer mix (to a final working concentration of 0.25 μM) and 5ul of SYBRgreen qRT-PCR mix (Wisent Bioproducets, Quebec). The sequences for all primers sets are outlined in Table 1.1. All qPCR reactions were completed on the LightCycler® 480 II (Roche). Each experimental sample was run in triplicate. The program used for all qRT-PCR reactions was as follows: pre-incubation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 5 sec and extension at 72°C for 8 sec. A melting curve was completed for every run from 65°C to 97°C with a read every 5 sec. Product specificity was determined through single PCR melting peaks. All selected qPCR primer sets had amplification efficiencies between 1.9 and 2. Data were analyzed using the ΔΔCt method while incorporating individual primer efficiencies into the equation as described by Pfaffl (2001). Gene expression was normalized to the reference gene (EF1α) and expressed as a fold change over the no surgery control group.

Table 2. 1 – Table of qRT-PCR and protein production primers used in this study, along with Accession # for each gene studied.

Primer set	Organism	Forward	Reverse	Amplicon (bp)	Accession #
IL-1B qPCR	Rainbow Trout	ccacaaagtgcatttgaac	gcaacctcctctaggtgc	151	NM_001124347.2
IL-6 qPCR	Rainbow Trout	cttctacacgctatctctcactc	cgtctgtcccgagct	124	NM_001124657.1
TNF-α qPCR	Rainbow Trout	gtgcaaaagatacccacc	cactgcacggtgtcag	109	AJ278085.1
EF1-α qPCR	Rainbow Trout	gcgcacagtaacaccgaaactaattaagc	gcctccgcacttgtagatcagatg	132	NM_001124339.1
IL-1B Cloning	Rainbow Trout	aggatccgattttgagtcaaactacag	gaagcttcatcaggacccagcacttgttc	354	NM_001124347.2
IgT Cloning	Rainbow Trout	acagtcccaaagtctccaac	cctaaggagatgagtgtgt	913	AAW66979.1

2.4.4 – Recombinant protein expression primer design

A Primer set for protein production (IgT, IL-1B; See table 2.1) were modified to include restriction enzyme cut sites designed to facilitate cloning into the protein expression vector pRSET (Forward primers – Xho1, Reverse primers – EcoR1). These modified primers were verified as above with blue-white screening in pGEM vector. Verified clones (along with purified pRSET A vector) were then double digested with Xho1 and EcoR1 restriction enzymes according to manufacturer's instructions (Thermofisher). Digests were separated on a 1% agarose gel and bands excised corresponding to the insert in the case of pGEM and the digested vector in the case of pRSET. Following gel extraction with QIAquick Gel extraction kit (Qiagen) and PCR cleanup with QIAquick PCR Purification kit (Qiagen) the insert and pRSET were ligated using T4 DNA ligase (Fisher Scientific). Resulting plasmids were used to transform BL-21 competent cells which were subsequently sequenced (as before) to confirm the sequence, orientation and reading frame of pRSET constructs.

2.4.5 – Recombinant protein production and purification

The BL-21 bacteria harboring pRSET construct described above were inoculated into 50ml of SOB broth with 50mg/ml ampicillin and 35mg/ml chloramphenicol and grown overnight at 37°C shaking at 200 RPM. This 50ml culture was used to inoculate 1L of SOB broth with 10mM $MgCl_2$ and no antibiotics. This culture was grown at 37°C shaking at 200 RPM until an optical density between 0.4 and 0.6 was reached at which point and inducer of the LAC operon - Isopropyl β-D-1-thiogalactopyranoside (IPTG) –was added to 1mM. Cultures were incubated at 37°C shaking at 200 RPM for 4hr at which point

the bacteria were pelleted and the supernatant (media) removed. Bacterial pellets were lysed overnight in 8M urea (pH 8) with protease inhibitor (Roche) on a rotary mixer at 4°C. Insoluble material was pelleted and the supernatant was incubated for 1hr with Ni-NTA resin (Qiagen). Following binding the resin-protein mix was poured onto glass columns. Once settled successive washes of 8M urea with decreasing pH were used to initially wash away contaminating proteins and finally to elute the resin bound protein of interest. Protein was quantified using BCA assay and examined on an SDS-PAGE gel (15% polyacrylamide). Total recombinant protein was visualized using SeeBand Forte (GeBA) staining of the SDS-PAGE gel.

2.4.6 – Development of polyclonal antibodies specific to IL-1B

One milligram of recombinant IL-1β the protein was sent to Cedarlane labs (Burlington, ON) for production and purification of antibodies targeting the recombinant protein. In brief, recombinant protein was injected into both rabbit and goat host animals. After an appropriate amount of time for the animal to mount an immune response serum was collected. A column was then prepared by attaching some of the recombinant protein to a matrix and the serum was passed over the column. Any antibodies formed in the immune response that were specific to the injected recombinant protein would therefore bind to the matrix in the column. Following wash steps to remove any non-specific antibodies a buffer was used to separate the bound the antibodies from the column matrix. The result is a purified fraction of the serum borne antibodies that is enriched for antibodies targeting the recombinant protein of interest.

2.4.7 – Development of polyclonal antibodies specific to IgT

One milligram of recombinant IL-1β the protein was sent to Cedarlane labs (Burlington, ON) for production and purification of antibodies targeting the recombinant protein. In brief, recombinant protein was injected into both rabbit and goat host animals. After an appropriate amount of time for the animal to mount an immune response serum was collected. A column was then prepared by attaching some of the recombinant protein to a matrix and the serum was passed over the column. Any antibodies formed in the immune response that were specific to the injected recombinant protein would therefore bind to the matrix in the column. Following wash steps to remove any non-specific antibodies a buffer was used to separate the bound the antibodies from the column matrix. The result is a purified fraction of the serum borne antibodies that is enriched for antibodies targeting the recombinant protein of interest.

2.4.8 – Detection of IL-1β protein in peritoneal lavage supernatant

PVDF bottom 96 well plates (Mabtech) were coated with 100ul of 2ug/ml Goat αIL-1β antibody in coating buffer overnight at 4°C. All subsequent steps were interspersed with three washes of all wells using TBS+0.01% Tween 20, each wash lasting at least 5 minutes. Following coating wells were blocked for 1hr at RT with TBS-T plus 5% skim milk. Following this blocking step 75ul of General Assay Diluent (Immunochemistry Technologies) was added to each well in order to enhance binding. Peritoneal lavage samples were diluted 1:20 with PBS and 100ul was plated for each sample in triplicate wells. Additionally, a recombinant protein standard curve was created (and plated) extending down to the detection threshold of this assay, namely 12.5pg/ml. The plate with samples and standard curve was incubated overnight at 4°C. Next the samples/standards were decanted and 100ul of the primary detection antibody (1ug/ml Rabbit αIL-1B) diluted in TBS-T was added to each well and incubated for 1hr at RT. This was then decanted and the secondary detection antibody Goat αRabbit Ig-HRP (100ul at 1:1000 in TBS-T; Abcam) was added to each well. Following a 1hr incubation at RT plates were decanted and a final colorimetric development was carried out with 100ul per well of TMB (Thermofisher) for 30minutes at RT in the dark. The colorimetric development was halted by adding 100ul of a 0.3M HCl stop solution to each well. Plates were then loaded into a Synergy H1 plate reader (Biotek) and each well was measured for absorbance at 450nm.

2.4.9 – Detection of IgM and IgT protein in peritoneal lavage supernatant

An indirect ELISA was developed to measure the concentration of IgT in the intraperitoneal lavage of rainbow trout. Samples were diluted 1:1 with PBS, transferred to the wells of 96 well plates (Immulon® 2 HB Flat Bottom MicroTiter® Plate, Thermofisher) and incubated overnight (~16h) at 4ºC. The following day the plates were washed with 300 µL of TBS-T. Washes using TBS-T were repeated two more times with the inclusion of a 5-minute incubation between the final two washes. This wash procedure was completed after all subsequent incubation steps unless otherwise specified. Following the washes, the plate was blocked with 5% skim milk-TBS and incubated at 37ºC for 1 hour. Subsequently, a chicken anti-IgT antibody (100 µL at 2 µg/mL in 5% skim milk TBS-T, produced in-house) was transferred to the wells and incubated at room temperature (25 ºC) for 2 hours. Then, biotin-conjugated rabbit anti-chicken antibody (100 µL at 0.05 µg/mL in 5% skim milk TBS-T; Arigo) was transferred to the wells and incubated at room temperature in the dark for 1 hour. The plate was then washed according to the procedure above, except that from this step and until the end of the protocol, the wash-incubation steps were performed in the dark. Thereafter, horseradish peroxidase-conjugated streptavidin (100 µL at 0.5 µg/mL in 5% skim milk TBS-T; BioLegend, USA) was transferred to each well and incubated in the dark at room temperature for 1 hour. Finally, TMB SENS (100 µL, ECO-TEK) was transferred to each well

and incubated in the dark at room temperature for 30 minutes, and this reaction was stopped with 100 µL of 0.3 M H2SO4. The absorbance at 450 nm was measured immediately using a Synergy H1 plate-reader (BioTek Instruments, USA).

The same procedure was followed to determine the levels of IgM in the intraperitoneal lavage except that the samples were diluted 1:3 in PBS, that a mouse anti trout IgM was used as the primary antibody (5 µg/mL, Cedarlane) and that an anti mouse-HRP (0.1 µg/mL, Sigma) was used as the secondary antibody.

2.4.10 – Detection of total protein using Bicinchoninic acid (BCA) assay

Total proteins in the peritoneal lavage was quantified with the Pierce™ BCA Protein Assay Kit (Thermofisher Scientific) following the manufacturer's protocol.

2.4.11 – RNA collection and CDNA synthesis

Spleen and muscle tissue (~50mg) was placed in Trizol reagent (Thermofisher) and homogenized using a homogenizer for 5 seconds. Extraction of nucleic acids was carried out following manufacturer's instructions for Trizol reagent. 5ug of nucleic acid was treated with 5ul of DNase (Sigma) according to manufacturers instructions in order to remove genomic DNA contamination. Following DNase digestion 500ng of RNA was loaded into a, 30ul total, cDNA synthesis reaction (Sigma).

2.4.12 – Statistical analysis

After verifying normal distribution and homogeneity of variances by Kolmogorov Smirnov and Levene's test respectively, and equal variance for each individual timepoint (Statistica: Statsoft, Tulsa, OK), a one way ANOVA (∀= 0.05) was performed on qPCR (and protein) values with a Dunnets post test comparing all treatment groups to the no surgery control.

2.5 – Results

2.5.1 – Upregulation of pro-inflammatory cytokines with tag type and presence

Pro-inflammatory cytokines measured in the control muscle tissue were not significantly affected by sham surgery or any of the implanted tags at day 14 and day 70 following surgery (Figure 2.1 A,C,E). Compared to both no surgery and sham surgery controls levels of IL-1β, IL-6 and TNFα in muscle did not vary by even a 1-fold difference as measured by qPCR. However, levels of these cytokines in spleen tissue did show upregulation in response to tag implantation across the board, with some upregulation also being observed in fish undergoing the sham surgery. Overall, the Vemco tags (coated and uncoated) induced a significant increase in IL-1β expression at day 14, while Star Oddi tags induced no significant

increase in expression at this time, similar to fish undergoing the sham surgery (Figure 2.1 B, $P<0.05$). IL-6 expression was upregulated at day 14 in only the Coated Vemco group (Figure 2.1 D, $P<0.05$). At day 70, for both IL-1β and IL-6 both Vemco tag groups were still significantly upregulated and Star Oddi remained elevated relative to no surgery controls with regard to TNFα expression (Figure 2.1 B, D, F, $P<0.05$) On day 70, IL-6 expession levels for the Starr Oddi group were too low to detect.

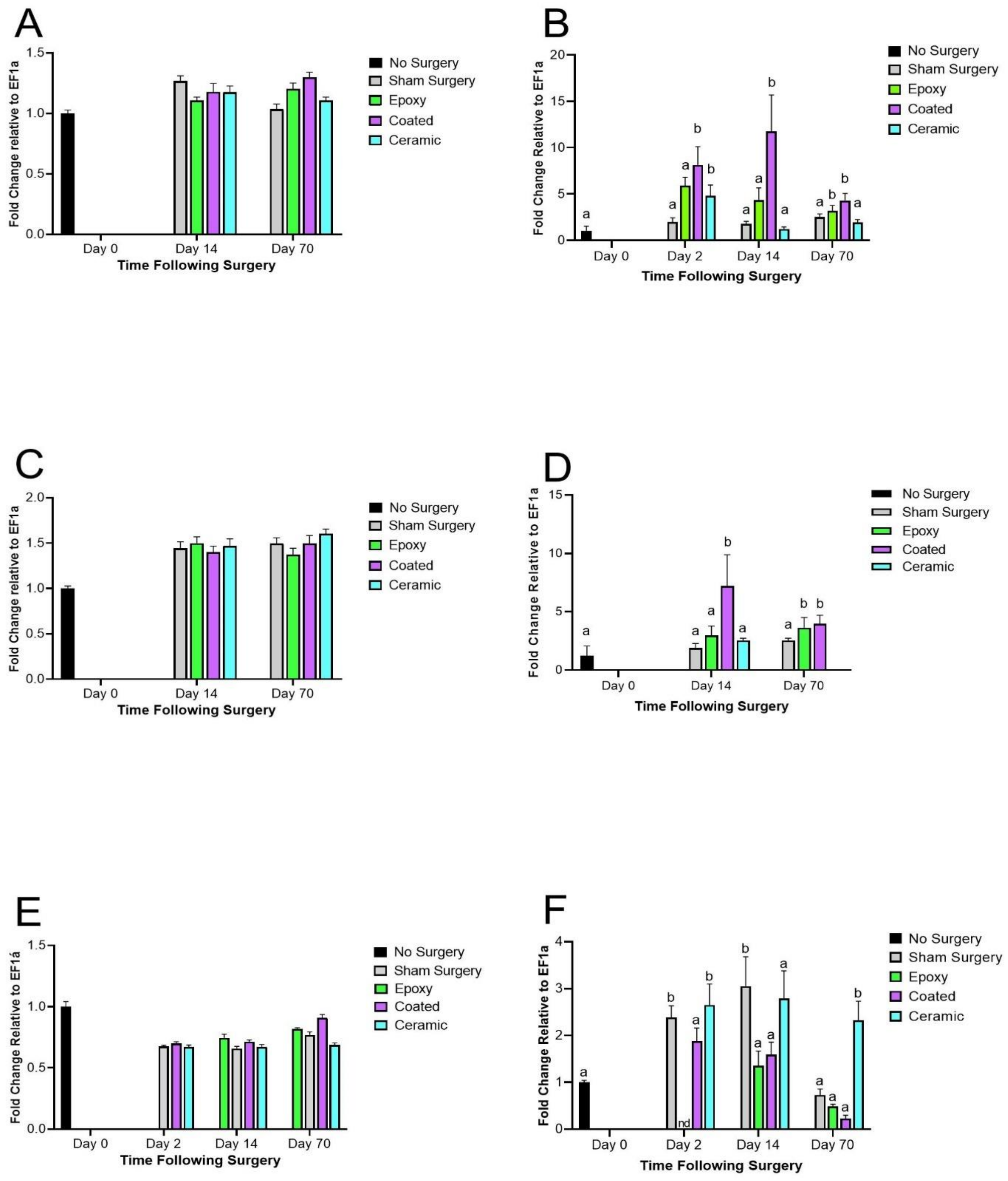

Figure 2.1 – Transmitter coating influences pro-inflammatory cytokine expression in Rainbow Trout. Real-time PCR measuring pro-inflammatory cytokines in Rainbow Trout spleen and muscle tissue. Graphs show group mean plus standard error of the mean (SEM). qPCR levels are presented relative to reference gene elongation factor 1α (EF1α) with the No Surgery group set to 1. A) Interleukin 1β in muscle B) Interleukin-1β in spleen. C) Interleukin-6 in muscle D) Interleukin-6 in spleen. E) Tumor necrosis factor-α in muscle F) Tumor necrosis factor-α in spleen. nd denotes missing samples. Different letters denote statistically different samples by One-way ANOVA ($p<0.05$). n=6

2.5.2 – IgT and IgM ELISAs/BCA

Increased protein levels in the peritoneal lavage supernatant indicate a significant and long-term immune response to tag introduction. Total protein was initially (day 2) increased in all groups relative to no surgery controls. Additionally, for the two earlier sampling points (day 2, 14) all tag groups were elevated relative to the sham surgery group (Figure 2.2 A, $P<0.05$). At day 70 total protein was significantly ($p>0.05$) increased in both Vemco tag types relative to sham surgery and Star Oddi groups which were not significantly different from no surgery controls. The day 70 samples showed a diminishing level of induction over the course of the experiment.

Contrastingly, levels of the mucosal IgT antibody as measured by ELISA were significantly reduced in all groups at the earlier sampling points. At day 2 coated Vemco and Star Oddi tags showed significantly less IgT than no surgery controls (Figure 2.2, B $P<0.05$). At day 14 all four treatment groups were significantly reduced relative to no surgery, with coated Vemco being statistically lower than the other three (Figure 2.2, B $P<0.05$). 70 days post-surgery IgT in all groups had returned to the levels of the no surgery control group. The exception to this was the Star Oddi tag group which showed significantly lower IgT levels than the rest of the treatment groups (Figure 2.2 B, $P<0.05$).

Due to significant individual variation in measured levels of peritoneal IgM most tag groups were not different from each other. At all time points sham surgery and tag groups showed greater IgM levels in the peritoneum compared with the no surgery control group (Figure 2.2 C). At day 2 the Star Oddi tag group showed significantly less IgM compared with the sham surgery group in the peritoneal lavage (Figure 2.2 C, $P<0.05$). Additionally at day 14 the coated vemco group showed significantly higher IgM compared with the no surgery control (Figure 2.2 C, $P<0.05$). At day 70 the Star Oddi tag treated animals had statistically more IgM than the no surgery control group (Figure 2.2 C, $P<0.05$).

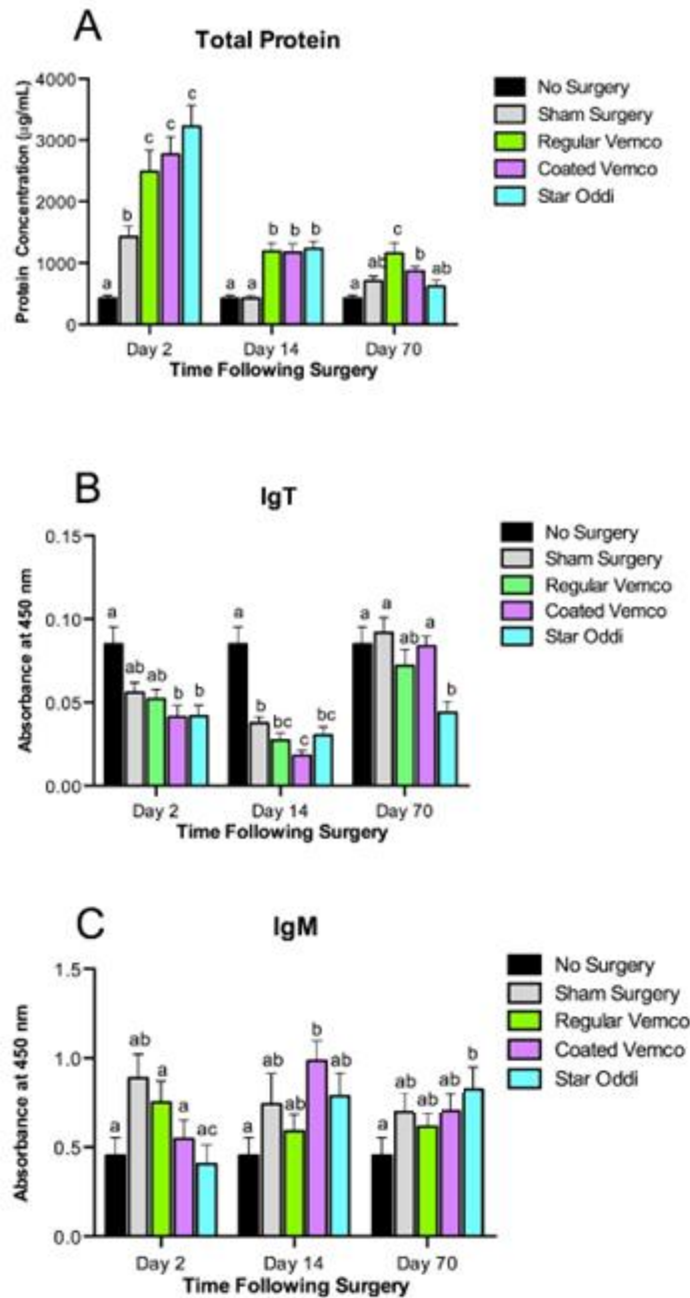

Figure 2.2 – Transmitter coating influences peritoneal proteins in Rainbow Trout. Assays measuring total protein and immunoglobulin levels in peritoneal lavage samples. Graphs shows mean plus standard error of the mean (SEM). A) Total protein as measured by BCA. B) Salmonid IgT as measured by ELISA. C) Salmonid IgM as measured by ELISA. Different letters denote statistically different samples by One-way ANOVA ($p<0.05$). n=6-12

2.5.3 – IL-1β protein levels in peritoneum

Compared with the no surgery control IL-1β protein in the peritoneum was significantly downregulated in the majority of experimental conditions (Figure 2.3). At day 2 all tag types and sham surgery fish showed significant reductions in peritoneal IL-1β. At day 14 the sham surgery condition returned to control levels of IL-1β protein while all three tag types still showed reduced peritoneal IL-1β. This pattern was again observed at day 70 following surgery except that the Star Oddi brand tag group had, by this time, returned to levels of peritoneal IL-1β similar to the sham surgery (Figure 2.3).

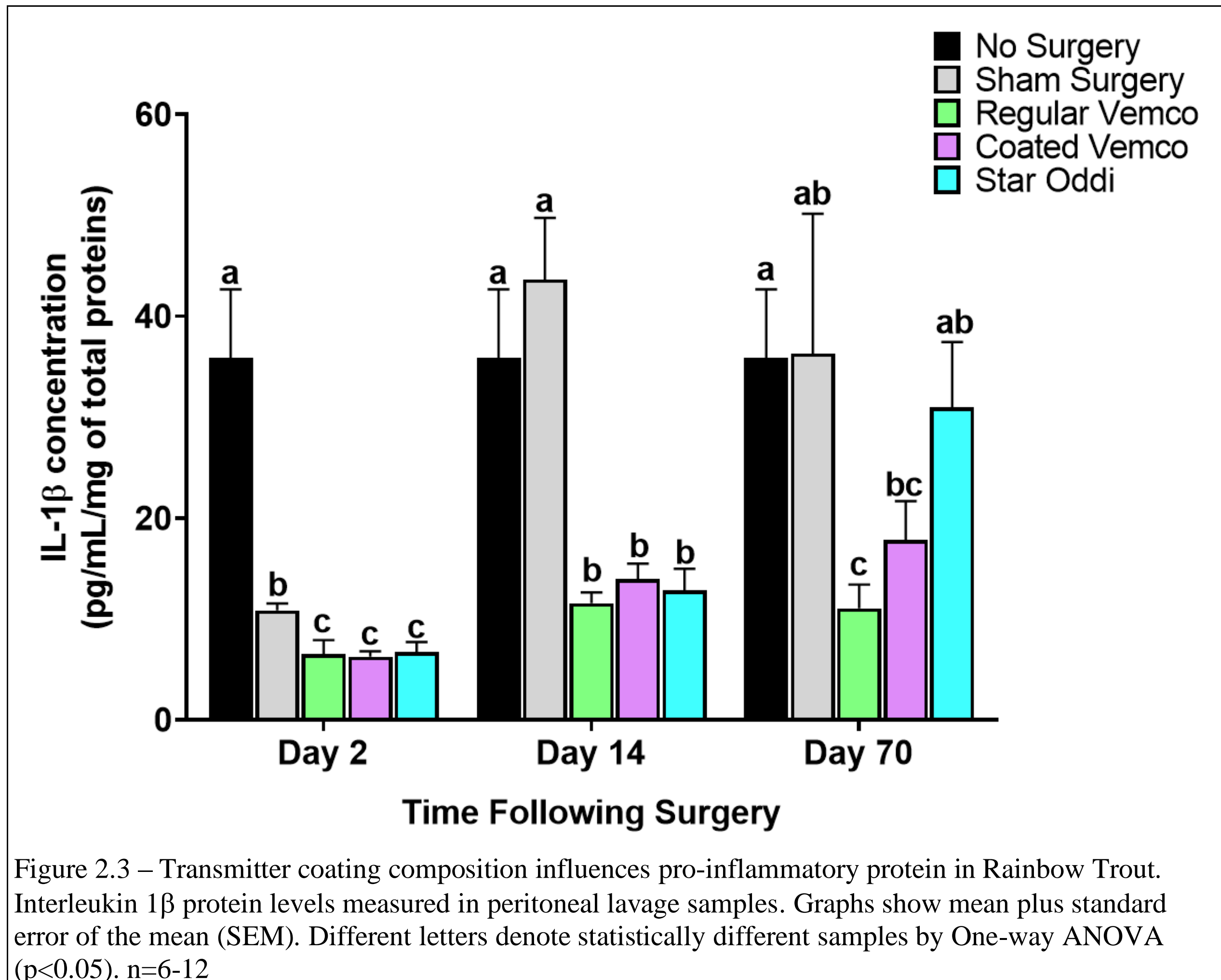

Figure 2.3 – Transmitter coating composition influences pro-inflammatory protein in Rainbow Trout. Interleukin 1β protein levels measured in peritoneal lavage samples. Graphs show mean plus standard error of the mean (SEM). Different letters denote statistically different samples by One-way ANOVA ($p<0.05$). n=6-12

2.5.4 – Wound healing/Tag encapsulation

Healing of the surgical wound seemed to occur quickly following implantation procedure. Wounds appeared sealed and showed little external evidence of inflammation at day 2 and day 14 time points. Day 70 samples, however, showed evidence of infection and inflammation of surgery wound especially around the stitches (Figure 2.4). This healing did not appear to be affected by tag presence or type as the sham surgery control appeared to follow the same healing progress as that of tagged fish.

Tag encapsulation was observed to progress over the course of the experiment for all tag types. Tagged fish showed no tag encapsulation at 2 days post surgery. At later time points all tagged fish showed significant encapsulation of telemetry tags, with the most extensive encapsulation being observed in day 70 samples (Figure 2.5). Tags were most frequently found encapsulated by a fibrous growth bound to the viscera, and in one case of (Day 70 Sham Surgery) visceral tissue was attached to the surgical wound (Figure 2.5).

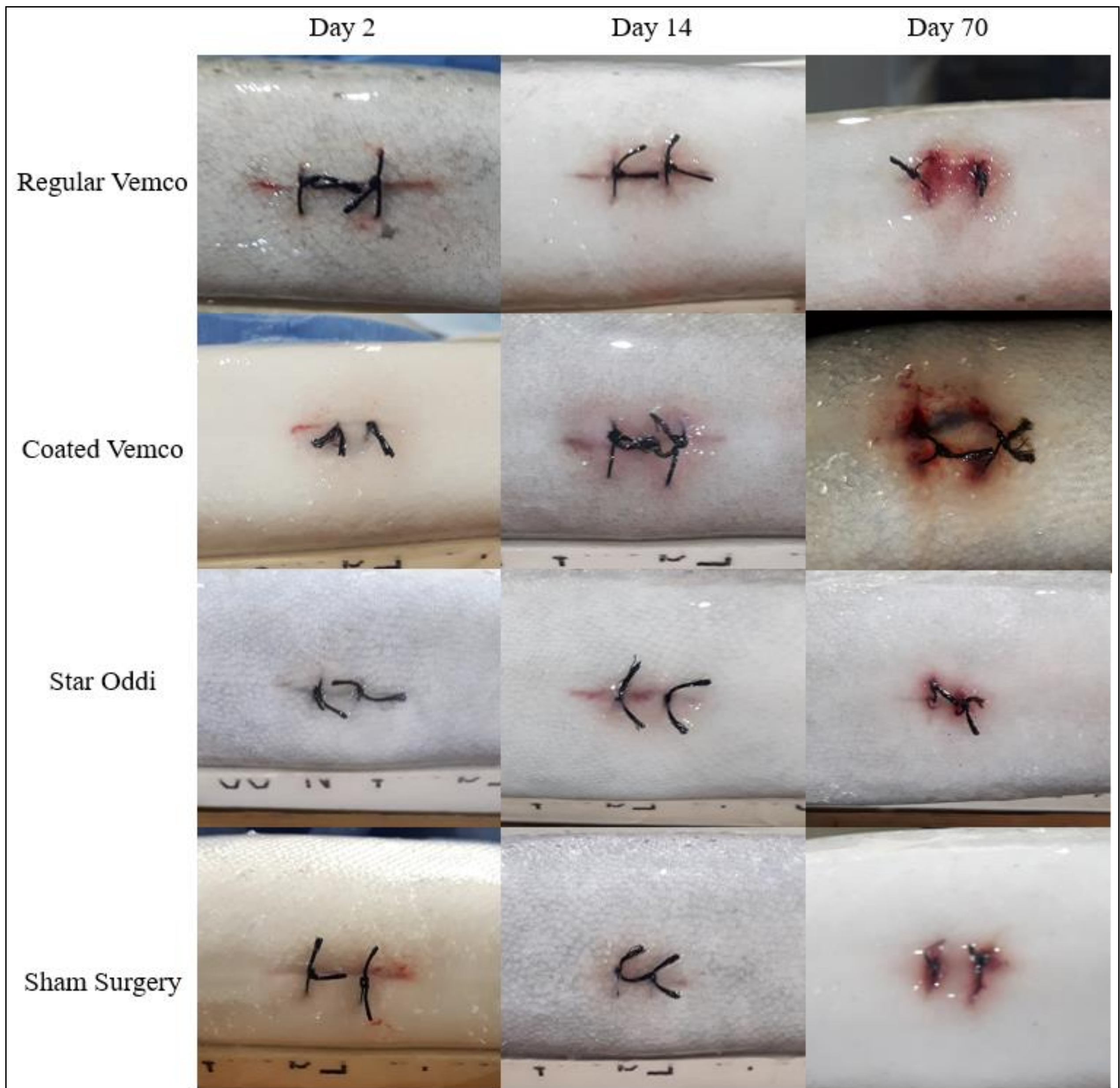

Figure 2.4 – Progression of surgical wound healing over the course of tag implantation experiment. Photos are representative samples from each treatment group at each time-point, post surgery, for all conditions.

Figure 2.5 – Progression of tag encapsulation over the course of tag implantation experiment. Photos are representative samples from each treatment group at each time point for all conditions. Arrow indicates tag position in photo.

2.6 – Discussion

The current understanding of foreign body response to implanted materials is focused on biomaterials being implanted subcutaneously, as opposed to intraperitoneally as in this study. The dynamics and duration of FBR are thus most thoroughly understood in the context of a biomaterial inserted into a surgery wound and surrounded by somatic tissue. In the case of this study the biomaterials (sterilized telemetry tags) were inserted *through* a wound and into the sterile, open space of the peritoneum. The

cellular and humoral reactions to the tags in this anatomical milieu can be expected to differ somewhat from the inter-tissue case typically described.

The proinflammatory cytokine mRNA expression levels measured at the surgical site (muscle) were unchanged relative to no surgery and sham surgery controls. This means that the surgery itself did not greatly contribute to the proinflammatory signal that was detected. Being that there was little to no inflammatory signal deriving from the surgical site it can be assumed that most of the signal observed in the spleen is due to interactions between the tag and cells of the peritoneum. However, this underscores a key limitation of the study at hand; namely that wound healing was not explicitly monitored. Pictures were taken, but scoring the healing process was beyond the scope of this work. Additionally, only a small subset of wound associated tissue was examined (muscle near surgical site). As such it is possible that the surgical wound itself was contributing to the disruption of cytokine levels observed. Moreover, recent work implanting telemetry tags in the peritoneum without surgical closure has shown significant effects of suture presence on tag retention (Kelican *et al*., 2021). It is therefore possible that the method of wound closure selected for this study was contributing to the inflammatory signals detected in this study.

The tag associated induction of pro-inflammatory cytokines measured in the spleen suggests that circulating cells were reacting to the implantation. This suggests that, apart from the surgery itself, the presence of the foreign body (the tag) is recruiting leukocytes to the peritoneum and activating inflammatory signals. Monocytes and macrophages make up the bulk of cells in the peritoneal cavity and these cells are primary producers of proinflammatory cytokines (Heel and Hall, 1996). These results suggest that when the coating on Vemco brand tags (coated or uncoated) is introduced into the peritoneal cavity it has the effect of activating peritoneal macrophages. This activation of proinflammatory signals has been previously observed just following surgery (Riese *et al*., 2004) but not at such long intervals post surgery. It is possible that the FBR observed to be occurring in the tagged fish is the main source of these cytokine signals. The Star Oddi manufactured tags were less inductive of this immune response following the expression pattern of the sham surgery control without exception.

The inflammatory markers associated with the FBR were still somewhat elevated even 70 days post-surgery. When implanting beads into the peritoneum of mice it was found that cell populations characteristic of the acute stage of the FBR had peaked and then gone to zero at day 14, being the latest time-point taken in that study (Christo *et al*., 2016). This disagreement could be attributable to either the size of the implants in this study (the mouse study implanted only 200 ug of beads while the tags in this study were several grams) and/or the slower, delayed nature of the salmonid immune response compared

to mammals. Encapsulation of beads that are micrometers in size is a distinctly different prospect for phagocytic cells when compared with an implant the size of a telemetry tag. Extension of the inflammatory response time course could be an important consequence of implanting such a large foreign body, whether in fish or otherwise.

The elevation of total protein levels above no surgery controls at all time points also suggests a longer time course of systemic response to the implanted tags. At day 2, the sham surgery control showed elevated protein levels in the peritoneum. This is much too early for antigen specific antibodies to be present and therefore likely reflects a general infiltration of immune cells and humoral factors from the immune response to the surgery. The further elevation in peritoneal protein levels associated with the presence of tags at day 2 suggests that the acute phase of the FBR is engaged at this point. This elevation associated with tag presence continued, albeit at a reduced level, to day 14 and somewhat to day 70. This could indicate that the recruitment and activation of peritoneal neutrophils (Jhunjhunwala *et al*., 2015), macrophages and fibroblasts (and the resulting accumulation of fibrin) was still occurring even two months following surgery, similar to studies in mice with peritoneal macrophages (Mooney et al. 2010).

The observed decrease in peritoneal IgT, at days 2 and 14 in all treatment groups likely indicates a alteration in peritoneal cell population associated with the surgical implantation. The resident peritoneal cells may be producing mucosal antibodies at a rest state (Zhan *et al*., 2010), only to be replaced by monocytes being recruited by the injury associated with implantation. The peritoneum also contains resident IgM producing cells (Stoermann *et al.,* 2007) that seem to have been activated (or increasingly recruited) simply in response to the surgery itself. Tag associated IgM levels were not significantly upregulated when compared to the sham surgery. Therefore IgM producing cells are unlikely to be major drivers of the FBR in the peritoneal cavity.

IL-1β protein in the peritoneum appears to be negatively affected by the surgery alone if only for a short window afterward. Following this window IL-1β levels return to normal in the fish with no tag, but remain depressed in tagged fish. Even at two months post surgery the levels in both Vemco tags were still significantly reduced while the Star Oddi tagged fish had returned to control levels. This suggests that there is an early alteration of peritoneal cell population as a result of surgery. The altered cell population persists up to two months in Vemco tagged fish, but returns to normal in fish with Star Oddi tags or no tag at all. It is possible that this reflects a quicker resolution of tag associated disruption when fish are implanted with Star Oddi coated tags. The different pattern of IL-1β peritoneal protein and spleen transcript is likely due to the different cell populations being sampled. Spleen samples reflect circulating

proinflammatory cells, which could be induced anywhere in the body. Whereas the peritoneal protein derives from tissue resident cells, recruited and localized in the peritoneum. Considering that IL-1β is secreted and circulates systemically (Sims *et al*., 2010) it is possible that peritoneal cells are producing IL-1β which circulates and collects in the spleen. If presence of a foreign body increases the exchange of peritoneal fluid components this could help explain why IL-1β transcript was elevated in the spleen of some fish while the protein level in the peritoneum was significantly lower than controls. Another possibility relates to the observation that macrophages can repress the release of IL-1β protein from cells that are otherwise showing high IL-1β transcript (Ipseiz *et al*., 2020). Taking this view, cells in the peritoneum have the release of IL-1β protein repressed by the action of resident macrophages while IL-1β transcript is building up. This increase in mRNA is then found in the spleen when those cells end up there. This could help explain why IL-1β transcript was high in spleen tissue even when protein levels remained low in the peritoneum.

Regardless of tag type (or presence) wound healing progressed at the same pace over the course of this experiment. Despite an early appearance of closure, the surgical wounds were frequently infected and inflamed at the two-month time point. This lack of complete healing was unrelated to tag presence as the sham surgery control fish still had internal lesions at the surgical site. Additionally, all tag types were readily encased in a fibrous capsule at the end of the experiment, pointing directly to an active FBR. Encapsulation was most rapid and complete in the case of Star Oddi tags, again suggesting that use of the Star Oddi coating leads to a quicker resolution of injury.

2.7 – Conclusion

All three tag types resulted in the same immune response and healing process in the salmonid peritoneum. However, the Star Oddi tags appeared to be the least immunogenic, permitting an apparently quicker resolution of the immune response to implants. This suggests that the binding properties of the ceramic material that the Star Oddi tags were made of are superior to those of the Vemco expoy and Vemco paralyene tags.

This study seeks to fill an important gap in current knowledge regarding the impact of the increasingly widely used technology of telemetry tags. The immune reactions observed in the present study are unlikely to be the only result of implantation surgery or the complex interaction between the fish immune system and the implanted tag. These disruptions are likely to impact the behavior and fitness of the animals that are taken to stand as examples of the population at large particularly since the effects last 70 days, which indicates a chronic response that will use energy. Using these animals as though they are representative of the population may lead to distortions in data and misguided, inappropriate management

practices. Only by investigating the immune level consequences of tag implantation, alongside the physiological and behavioral consequences, can this technology be perfected.

3.0 – Development of a Rainbow Trout IFNγ Protein Assay

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3.1 – Abstract

Canadian salmon farming faces challenges associated with high density and disease outbreaks. In order to facilitate the rapid evaluation of stock and vaccine candidates this study developed a novel protein level assays for detecting the cytokine interferon gamma. The antibodies used in this assay detect rainbow trout IFNγ, as determined by blocking assays, and the final assay is capable of detecting IFNγ protein levels down to the picogram range. This assay was used to test levels of IFNγ in peritoneal lavage supernatant from rainbow trout implanted with telemetry tags over time. It was found that peritoneal IFNγ was slightly reduced by the presence of some tag types and unaffected by others. The ability to measure IFNγ at the protein level provides aquaculturalists an opportunity to better track stock viability and vaccine effectiveness. This will improve practices in this expanding realm of Canadian industry.

Keywords: Interferon gamma, Rainbow Trout, ELISA, Peritoneal Lavage, Western Blot. Blocking

3.2 – Introduction

Fish protein is a major component of human diets across the world and throughout time. Fish populations, even those of little economic import, also play important roles as participants in the support and maintenance of aquatic ecosystems (Holmlund and Hammer, 1999). As a result of human harvesting practices some estimates deem 70% of marine fish resources to be overharvested or depleted in population status (World Resources Institute, 1996). These overharvested fish stocks are not only more susceptible to population crashes from disease but also destabilize the ecosystems they are in, possibly contributing to a broader ecosystem collapse. Given the deep importance of maintaining and rehabilitating existing fish stocks there has been an increasing interest in studying the pathogenesis of fish diseases as well as the structure and function of the teleost immune system.

The study of salmonids, which include many ecologically and financially important species, has been limited by the availability of diagnostic tools. While there are numerous new and emerging technologies that deal with nucleic acids, tools to study proteins remain scarce. It is therefore necessary to expand the variety of protein level assays and in-so-doing permit wider exploration of important aspects of teleostean immunity. A particularly important subset of proteins for study is the master regulators of the immune system, the cytokines (Dinarello, 2007).

Cytokines are small, soluble proteins that, even at very low levels, govern the many and varied activities of the immune system. As such they are excellent targets for assessment when the goal is to characterize the nature and effectiveness of immune responses to pathogens. Considering that a major threat to the health of both farmed and wild salmonids is disease outbreaks it can be anticipated that protein level assays targeting cytokines will be useful for industry and environmental studies. The cytokine of interest in this study is a member of the interferon family, Interferon Gamma (IFNγ). This type II interferon does not possess the direct antiviral activity from which the interferons get their name. Rather IFNγ is more involved in the recruitment and activation of other immune cells, facilitating the cell dependant aspects of an immune response (Pace *et al.*, 1985).

Prior work studying IFNγ in salmonids is focused on the transcript level, which is easy to measure and gives some indication of bioactivity. However, there are examples (IL-1β in the previous paper among others) that show a disconnect between transcript expression levels and actual functional protein levels. This suggests that a protein level validation of large swaths of transcript data is needed in order to evaluate the veracity of findings from said data.

The purpose of this study was to expand the toolkit for working with salmonids by developing antibody-based assays capable of measuring important salmonid cytokines. These proteins are largely produced by immune cells and are highly active even at very low levels. Frustratingly, this means that they are difficult to visualize in typical protein assays (small/invisible bands on a western blot) and, at the same time, they are intricately involved in many aspects of general fish health as well as response to disease. In order to quantify these important proteins an assay based on antibody binding must be developed, namely the Enzyme Linked Immunosorbent Assay (ELISA).

ELISA assays come in two varieties, direct and sandwich. A direct ELISA uses antibodies derived from a single host animal to detect protein that has been coated directly onto a plate. This type of ELISA is simpler to construct and optimize but is less sensitive than its counterpart. A sandwich ELISA utilizes antibodies derived from one type of host animal (perhaps a rabbit) to 'capture' the protein of interest from a sample. Then, antibodies derived from a second type of host animal (perhaps a goat), and targeting a different epitope from the first, is used to detect the captured protein. This type of ELISA can be much more sensitive and capable of detecting proteins present in picogram amounts.

Salmonids have a delayed adaptive immune response and are sometimes considered to be dependent on proper function of their innate immune defenses such as inflammation. IFNγ has been implicated in aspects of both early (Fruct *et al*., 2001) and later adaptive immune function (Sen, 2001). The ELISA assay developed in this study will permit researchers to directly examine the active form of IFNγ. Tracking the actual protein itself, rather than a proxy for it, is essential to understanding the exact role of this important salmonid cytokine. The assay developed in this study will improve the fidelity of findings in the world of salmonid immune research.

3.3 – Methods

3.3.1 – Fish

Unless otherwise described, rainbow trout were held at the University of Guelph associated hatchery located in Alma, ON. All fish were maintained in 450 L flow-through tanks at 10°C. Animals were handled according to Canadian Council on Animal Care guidelines on a permit from the University of Waterloo Animal Care Committee (#30048). Rainbow trout averaged 47cm in length and 1350g in weight.

3.3.2 – Species alignment of known salmonid IFNγ genes

Identification and cloning of Rainbow Trout IFNγ began with the identification of IFNγ sequences from the NCBI online database. The sequences for Rainbow Trout, Atlantic Salmon and Zebrafish were obtained and aligned. This study was designed to produce polyclonal antibodies that would cross react with both of the salmonid species targeted and therefore included Zebrafish in the alignment as a close outgroup.

3.3.3 - IFNγ gene expression in Rainbow Trout

3.3.3.1 – PHA stimulation of Rainbow Trout PBLs

To ensure that the primer sequences developed from the gene alignment were specific for a functional region of the IFNγ gene, qPCR analysis was completed on immune stimulated rainbow trout peripheral blood leukocytes (PBLs). Rainbow trout were sedated were using a 1:1000 dilution of benzocaine in fresh water. Blood was drawn from the caudal sinus into a sterile, heparin coated syringe and placed on ice. PBLs were isolated from the whole blood via a hypotonic lysis procedure carried out in a sterile flow hood on campus. In brief, whole blood samples were diluted 1:10 with sterile, cold, distilled water to cause lysis of red blood cells and shaken gently for 20 seconds. Normal osmolarity was restored by adding an appropriate volume of sterile 10x PBS to each sample. Samples were then passed over a 20µm Falcon cell strainer (Thermofisher) to remove debris. The remaining, intact PBLs were then pelleted by centrifugation for 5 minutes at 200xg and supernatant was decanted. Cells were then washed with 5ml of sterile PBS and then pelleted again as before. Cells were resuspended in 500 µl of sterile PBS and cell concentrations were determined using a hemocytometer.

PBLs were plated with the IFNγ stimulant Phytohemagglutinin (PHA) and collected at three timepoints over two days. Subsequently PBLs were plated with PHA and co-stimulatory proteins in order to discern their effect on IFNγ production.

All cell stimulation experiments used 6-well Nunc cell culture plates (Thermofisher) and were carried out in a sterile environment. Triplicate wells of Rainbow Trout PBLs were plated in 1.5ml of L-15 media at 3.0 x105 cells per well. After seeding, 1.5ml of media containing stimulant (or just media in the case of control) was added immediately to appropriate test wells. The stimulants used were 5µg/ml of Phytohemagglutinin (PHA, Roche) for the timepoint stimulation and 5µg/ml of PHA with 2.5µg/ml of the following costimulants: Salmonid CD28 antibody (gift of the Sunyer Lab, University of Pennsylvania), recombinant Interleukin 2 (IL-2), recombinant interleukin 1β (IL-1β) and recombinant protein expression control PR[E] consisting of proteins collected from a bacteria transformed with our recombinant expression vector [pRSET] without insert). Cells were harvested with gentle scraping to ensure collection of adherent cells and then pelleted and stored at -80°C for RNA extraction.

3.3.3.2 – RNA extraction and cDNA synthesis

Cell pellets were resuspended in 500µl of Trizol reagent (Thermofisher) and the extraction of total RNA was carried out following the manufacturers instructions. To remove any contaminating genomic DNA, RNA samples were treated with DNase I (Thermofisher) according to the manufacturer's instructions. RNA samples were then quantified using the Take3 plate of a Synergy H1 plate reader (BioTek Instruments) and were stored at −80 °C until further use. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA using the qScript cDNA Supermix (Quanta Biosciences) as described by the manufacturer. For a no template control, 500 ng of RNA suspended in 20µl of DEPC water was included in the cDNA synthesis reaction without reverse transcriptase

3.3.3.3 – qRT-PCR

All qRT-PCR reactions were 10 µl and contained: 2.5 µl of cDNA (25 ng/µl diluted 1:10 in RNase free water), 5µl SYBRgreen qRT-PCR mix (Wisent Bioproducts), and forward and reverse primers (Sigma Aldrich) to a final working concentration of 0.25 µM. The sequences for the IFNγ and EF1α primer set used are outlined in Table 3.1. All qPCR reactions were completed on the LightCycler® 480 II (Roche) and each experimental sample was run in triplicate. For each plate, triplicate wells of a calibrator, no template control and RNA only control were also present. The program used for all qRT-PCR reactions was as follows: pre-incubation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 5 seconds and extension at 72°C for 8 seconds. A melting curve was completed for every run from 65°C to 97°C with a read every 5 seconds. Product specificity was determined through single PCR melting peaks. All primer sets used had amplification efficiencies between 1.9 and 2. Data were analyzed using the ΔΔCt method while incorporating individual primer efficiencies into the equation as described by Pfaffl (2001) and is presented as the average of cells from 3

fish with the standard deviation. Specifically, gene expression was normalized to the reference gene (EF1α) and expressed as fold change with time zero control group set to 1.

Table 3. 1 – Table of qPCR and cloning primers used in this study, along with Accession # for each gene studied.

Primer set	Organism	Forward	Reverse	Amplicon (bp)	Accession #
IFN-γ qPCR	Rainbow Trout	ATGATTGAGAGTCT GAAATAT	TGTCCTCAGCTCAGGTA TCCT	87	NM_001160503
IFN-γ Cloning	Rainbow Trout	ATGGATGTGTTATC AAGGGCT	AGGCTCAAATCACACA TCATG	540	NM_001160503
IFN-γ Subcloning	Rainbow Trout	AGGATCCATGGATG TGTTATCAAGGGCT	GAAGCTTCATGATGTG TGATTTGAGCCT	540	NM_001160503
EF1-α	Rainbow Trout	GCGCACAGTAACAC CGAAA CTAATTAAGC	GCCTCCGCACTTGTAG ATCAGATG	186	NM_001123629.1

3.3.4 – Development of recombinant Rainbow Trout IFNγ (rIFNγ)

3.3.4.1 – Cloning of Rainbow Trout IFNγ

RNA was extracted and cDNA synthesized as described above in section 3.3.3.2. Primers were used to amplify targeted regions from Rainbow trout spleen cDNA with the following conditions: 95° C for 3 min followed by 32 cycles of 95° for 30 sec, 60°C for 10 sec, 72° for 45 sec, ending the entire process with a final step of 72° for 5 min. The bands were electrophoresed on a 1.5% agarose gel, stained with GelRed (Biotium), visualized on a Blue Light Transilluminator (Pearl Biotech) and excised using a scalpel. Bands were purified using a QIAquick gel extraction kit (Qiagen) and blunt ligated into pGEM-T easy vector (Promega). These vectors were transformed into E. Coli XL1-Blue supercompetent cells (Aligent Technologies) which were then grown on LB media with 0.1 mg/ml of ampicillin (Fisher Scientific). White colonies were selected, grown in 1ml of LB plus ampicillin, miniprepped and sent for sequencing (using T7 and Sp6 primers) at TCAG sequencing facility at Toronto Sick Kids hospital. QPCR primer sets were validated via sequencing and then not modified further. Primer sets for protein production were modified to include restriction enzyme cut sites designed to facilitate cloning into the pRSET A bacterial expression vector (Forward primers – BamHI, Reverse primers – HindIII). These modified primers were verified as above with blue-white screening in pGEM vector. Verified clones (along with purified pRSET A vector) were then double digested with BamH1 and HindIII restriction enzymes according to manufacturer's instructions (Thermofisher). Digests were separated on a 1% agarose gel and bands excised corresponding to the insert in the case of pGEM and the digested vector in the case of pRSET. Following gel extraction with QIAquick Gel extraction kit (Qiagen) and PCR cleanup with QIAquick PCR Purification kit (Qiagen) the insert and pRSET were ligated using T4 DNA ligase (Fisher Scientific).

Resulting plasmids were used to transform BL-21 competent cells which were subsequently sequenced (as before) to confirm the sequence of pRSET constructs.

3.3.4.2 Protein productin and purification

The BL-21 bacteria harbouring pRSET constructs described above were inoculated into 50ml of SOB broth with 50mg/ml ampicillin and 35mg/ml chloramphenicol and grown overnight at 37°C shaking at 200 RPM. This 50ml culture was used to inoculate 1L of SOB broth with 10mM MgCl2 and no antibotics. This culture was grown at at 37°C shaking at 200 RPM until an optical density between 0.4 and 0.6 was reached at which point and inducer of the LAC operon - Isopropyl β-D-1-thiogalactopyranoside (IPTG) –was added to 1mM. Cultures were incubated at at 37°C shaking at 200 RPM for 4hr at which point the bacteria were pelleted and the supernatant (media) removed. Bacterial pellets were lysed overnight in 8M urea (pH 8) with protease inhibitor (Roche) on a rotary mixer at 4°C. Insoluble material was pelleted and the supernatant was incubated for 1hr with Ni-NTA resin (Qiagen). Following binding the resin-protein mix was poured onto glass columns. Once settled successive washes of 8M urea with decreasing pH were used to initially wash away contaminating proteins and finally to elute the resin bound protein of interest. Protein was quantified using BCA assay and examined on an SDS-PAGE gel (15% polyacrylamide). Total protein was visualized using SeeBand Forte (GeBA) staining of the SDS-PAGE gel.

3.3.5 – Antibody development

Following production/purification of recombinant protein 1mg of the protein was sent to Cedarlane labs (Burlington, ON) for production and purification of antibodies targeting the recombinant protein. In brief, recombinant protein was injected into both rabbit and goat host animals. After an appropriate amount of time for the animal to mount an immune response serum was collected. A column was then prepared by attaching some of the recombinant protein to a matrix and the serum was passed over the column. Any antibodies formed in the immune response that were specific to the injected recombinant protein would therefore bind to the matrix in the column. Following wash steps to remove any non-specific antibodies a buffer was used to separate the bound the antibodies from the column matrix. The result is a purified fraction of the serum borne antibodies that is enriched for antibodies targeting the recombinant protein of interest.

3.3.6 – Western blot analysis of stimulated Rainbow Trout PBLs

Rainbow trout PBLs were collected as described using hypotonic lysis. PBLs from individual fish were plated (as above) and exposed to PHA. Samples were collected at time zero as well as 1h and 4h post stimulation.

For sampling, cells were harvested with gentle scraping to ensure the collection of adherent cells. The cell suspension was then pelleted and resuspended in 500µl of T-per protein extraction reagent (Thermofisher). This mixture was homogenized using a 24-gauge needle and passing the sample through the needle 10 times. As per the T-per protocol the samples were then centrifuged at 12 000 RPM for 5 minutes after which supernatant (containing only soluble proteins) was collected and frozen in a -80°C freezer.

SDS-PAGE was carried out using 40µg of total protein loaded into a 15% polyacrylamide gel for size separation. This gel was run at 80V for 15 minutes followed by 160V for approximately 1hr to achieve proper separation. Following this the separated protein was transferred to a PVDF membrane in a Trans-Blot Turbo machine (BioRad) and the membrane was probed with recombinant αIFNγ antibodies. Prior to SDS-PAGE samples were denatured by addition of 2.5% β-mercaptoethanol and heated at 95°C for 10 minutes.

Western blot protocol involved an initial blocking step of 1hr with 5% Skim milk in TBS-T, followed by an overnight incubation with a 1:1000 dilution of Rabbit αIFNγ in TBS-T + 5% Skim milk. This was followed by a 1hr incubation with an αRabbit immunoglobulin antibody conjugated to an alkaline phosphatase enzyme. The final step was addition of an NBT+BCIP development solution which causes a colorimetric product to be deposited where bands corresponding to αIFNγ binding occur. Each of the steps above was separated from the other by washing the membrane 3 times with TBS+0.05% Tween 20 (Sigma). Blots were photographed on a ChemiDoc imaging system (Bio Rad).

3.3.7 – Creating a quantitative sandwich ELISA assay using rIFNγ

3.3.7.1 – Collection of samples for western blocking experiment

Rainbow trout from farms located in the Central Highlands of Peru (Juaja, Huaraz, Huancayo, Huaura and Concepción provinces) were sampled during a pathogen outbreak. Animals showing clear signs of infection with *Yersinia ruckeri* (lethargy, swimming near the surface, exophthalmia, melanosis, distended abdomen and hemorrhages in the oral cavity and at the base of the fins) were sampled along with putatively pathogen free fish. Infection with *Y. ruckeri* was confirmed via 16S RNA sequencing of the

pathogen as per Gibello et al., 1999. Spleen tissue was collected and preserved in RNAlater and returned to the lab for further analysis. Seventy milligrams of spleen tissue was homogenized in 1ml of PBS with protease inhibitors (Thermofisher). Samples were then put through a standard (1:1, V:V) Chloroform extraction protocol to further remove lipid contaminants . Following this, samples were quantified using a Pierce BCA assay (Thermofisher). Spleen protein samples from 3 infected fish and 3 uninfected fish were pooled for use in the western blot blocking test of IFNγ antibodies.

PBLs used in blocking experiments were collected as outlined above. After being isolated PBLs were exposed to 5ug/ml PHA and sampled over a four-hour time course. PBL samples were then collected by gentle scraping, pelleted and then put through T-per protein isolation (as outlined above). PBL protein samples were quantified using Pierce-BCA assay (Thermofisher).

3.3.7.2 – Blocking experiments to verify specificity of αIFNγ antibodies

Western blot blocking experiments were carried out using the same conditions outlined above with one alteration. This alteration was that, for the blocked membrane only, the αIFNγ antibody mixture used to probe the western blot was mixed with 20ug/ml of recombinant IFNγ and incubated for 12hr at 4°C. Colorimetric development was identical between all blots.

3.3.7.3 – Assessing IFNγ protein levels in Rainbow Trout experiencing a foreign body response

Samples for testing IFNγ ELISA were collected in coordination with a trial investigating the effects of different types of acoustic telemetry tags surgically implanted into the peritoneum of rainbow trout. In brief, the fish were sedated and underwent implantation surgery followed by surgical wound closure. A population of control fish were sampled (no surgery) at day 0, while at subsequent time points (Day 2, Day 14 and Day 70) samples were taken from the four other groups. These included a sham surgery control, which underwent the implantation surgery but had no tag implanted, as well as 3 different types of commercial telemetry tags representing three different types of tag coating (Vemco uncoated, Vemco coated and Star Oddi coated). Peritoneal lavage was collected as described by Semple et al., 2018 and the supernatant was isolated by centrifuging the total lavage at 400 x g for 15 minutes at 4°C. The supernatant of the peritoneal lavage was collected, aliquoted and stored at -80°C for future use.

PVDF bottom 96 well plates (Mabtech) were coated with 100ul of 2ug/ml Goat αIFN-γ antibody in coating buffer overnight at 4°C. All subsequent steps were interspersed with three washes of all wells using TBS+0.01% Tween 20, each wash lasting at least 5 minutes. Following coating wells were blocked

for 1hr at RT with TBS-T plus 5% skim milk. Following this blocking step 75ul of General Assay Diluent (Immunochemistry Technologies) was added to each well in order to enhance binding. Peritoneal lavage samples were diluted 1:20 with PBS and 100ul was plated for each sample in triplicate wells.

Additionally, a recombinant protein standard curve was created (and plated) extending down to the detection threshold of this assay, namely 12.5pg/ml. The plate with samples and standard curve was incubated overnight at 4°C. Next the samples/standards were decanted and 100ul of the primary detection antibody (1ug/ml Rabbit αIFN-γ) diluted in TBS-T was added to each well and incubated for 1hr at RT. This was then decanted and the secondary detection antibody Goat αRabbit Ig-HRP (100ul at 1:1000 in TBS-T; Abcam) was added to each well. Following a 1hr incubation at RT plates were decanted and a final colorimetric development was carried out with 100ul per well of TMB (Thermofisher) for 30minutes at RT in the dark. The colorimetric development was halted by adding 100ul of a 0.3M HCl stop solution to each well. Plates were then loaded into a Synergy H1 plate reader (Biotek) and each well was measured for absorbance at 450nm.

3.3.8 – Statistical analysis

After confirming normal distribution and equal variance (Statistica; Statsoft, Tulsa, OK), a one-way ANOVA was performed on qPCR results with a Dunnett's post test comparing all means to time zero control. For peritoneal lavage samples a 1-way ANOVA was performed with a Dunnett's post test comparing all means within a timepoint to the no surgery control.

3.4 – Results

3.4.1 - IFNγ Alignments

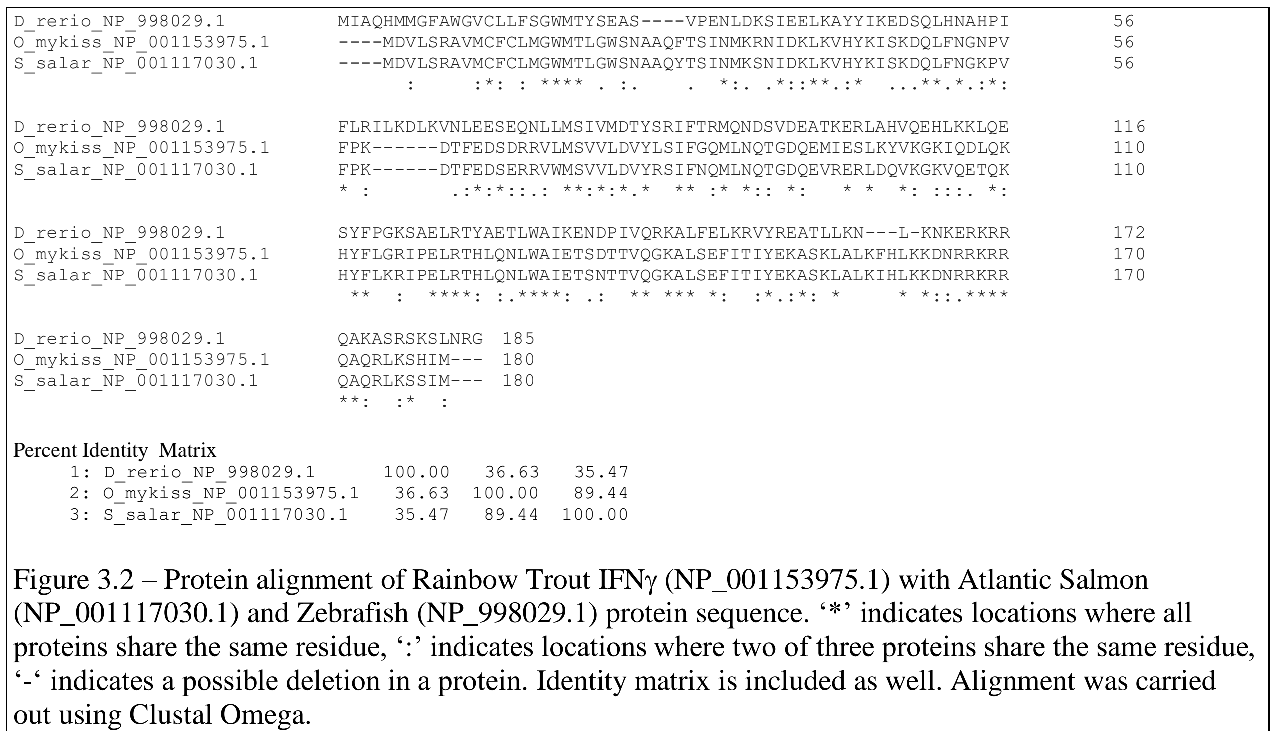
Primers for both qPCR and recombinant protein production were designed based on Rainbow Trout sequences (See Table 3.1). Rainbow Trout and Atlantic Salmon share 94% of residues at the nucleotide level and 89% of residues at the protein level (Figure 3.1, 3.2 respectively). This similarity between salmonid lineages is contrasted with Zebrafish (*D. rerio*) as an outgroup (56-57% shared nucleotides, 35-36% shared amino acids) sequence. This is encouraging for the utility of the IFNγ primers in both Atlantic Salmon and Rainbow Trout as well as the potential for cross reactivity of the polyclonal antibodies developed during this study.

```
D_rerio_NM_212864.1           ATGATTGCGCAACACATGATGGGCTTTGCCTGGGGAGTATGTTTGCTGTTTTCGGGATGG      60
O_mykiss_NM_001160503.1       ATGGATGT----GTTATCAAGGGC------T--GTGATGTGTTTCTGCTTGATGGGCTGG      48
S_salar_NM_001123558.1        ATGGATGT----GTTATCAAGGGC------T--GTGATGTGTTTCTGCTTGATGGGCTGG      48
                              ***  **        ** * ****      *  *   * *****    **   *** ***

D_rerio_NM_212864.1           ATGACATACAGTGAAGCCAGTGTGCCCGAG------------AACCTAGACAAGAGCATC      108
O_mykiss_NM_001160503.1       ATGACTTTAGGATGGAGTAATGCTGCTCAGTTCACATCAATTAACATGAAGAGAAACATA      108
S_salar_NM_001123558.1        ATGACTTTAGGATGGAGTAATGCCGCTCAGTACACATCAATTAACATGAAGAGCAACATA      108
                              ***** *   *       * **   *  **            *** *  * *  * ***

D_rerio_NM_212864.1           GAAGAGCTCAAAGCTTACTATATTAAAGAAGATTCTCAGCTACATAATGCACACCCCATC      168
O_mykiss_NM_001160503.1       GACAAACTGAAAGTCCACTATAAGATCTCCAAGGACCAGCTG------------------      150
S_salar_NM_001123558.1        GACAAACTGAAGGTCCACTATAAGATCTCCAAGGACCAGCTG------------------      150
                              **  * ** ** *   ******  *      *    *****

D_rerio_NM_212864.1           TTCCTGCGAATCCTGAAAGATTTAAAGGTGAATCTTGAGGAAAGTGAGCAGAATCTACTG      228
O_mykiss_NM_001160503.1       TTCAACGGAAACCCTGTTTTCCCCAAGGACACGTTTGAGGACAGTGATCGGAGGGTGTTG      210
S_salar_NM_001123558.1        TTCAACGGAAAACCTGTTTTCCCCAAGGACACGTTTGAGGACAGTGAGCGGAGGGTGTGG      210
                              ***    ***  *           ****  *   ******* ***** * **   *   *

D_rerio_NM_212864.1           ATGAGCATTGTAATGGACACATACAGTAGGATATTCACTCGCATGCAGAATGACAGCGTG      288
O_mykiss_NM_001160503.1       ATGAGTGTGGTTCTGGACGTGTATCTGAGTATCTTCGGCCAGATGCTGAACCAGACGGGG      270
S_salar_NM_001123558.1        ATGAGTGTGGTTCTGGACGTGTATCGGAGTATCTTCAACCAGATGCTGAACCAGACGGGG      270
                              *****  * **  *****   **    ** ** ***   *  **** ***  * *  * *

D_rerio_NM_212864.1           GATGAAGCTACAAAGGAGAGGCTGGCACATGTTCAAGAGCATTTGAAAAAGCTGCAAGAA      348
O_mykiss_NM_001160503.1       GACCAGGAAATGATTGAGAGTCTGAAATATGTCAAGGGGAAAATTCAGGATCTCCAGAAA      330
S_salar_NM_001123558.1        GACCAGGAAGTGAGGGAGAGGCTGGACCAGGTCAAGGGGAAGGTTCAGGAGACCCAGAAA      330
                              **  * *     *  ***** ***    * **  * * * *  *  *  *    **  **

D_rerio_NM_212864.1           AGCTACTTTCCAGGCAAGAGTGCAGAGCTCAGGACGTATGCAGAAACGCTATGGGCGATC      408
O_mykiss_NM_001160503.1       CACTATTTCCTGGGGAGGATACCTGAGCTGAGGACACACCTGCAGAACCTGTGGGCCATC      390
S_salar_NM_001123558.1        CACTACTTCCTGAAAAGGATACCTGAGCTGAGGACACACCTGCAGAACCTGTGGGCCATC      390
                                *** ** *     * **   * ***** *****  *     * *  ** ***** ***

D_rerio_NM_212864.1           AAGGAAAACGACCCGATCGTCCAGCGAAAGGCTTTGTTTGAGCTGAAGCGTGTCTATAGA      468
O_mykiss_NM_001160503.1       GAGACCAGCGACACCACAGTCCAGGGGAAGGCTCTGTCCGAGTTCATTACCATCTACGAG      450
S_salar_NM_001123558.1        GAGACCAGTAACACCACAGTCCAGGGGAAGGCTCTGTCCGAGTTCATTACCATCTACGAG      450
                               **   *   ** * *  ****** * ****** ***  *** * *      ****

D_rerio_NM_212864.1           GAAGCAACACTCCTGAAAAAC---T------TAAAGAATAAAGAA---CGCAAGAGACGG      516
O_mykiss_NM_001160503.1       AAAGCCTCCAAACTGGCCCTTAAGTTCCATCTAAAGAAGGACAACCGCAGGAAGAGACGG      510
S_salar_NM_001123558.1        AAAGCCTCCAAACTGGCCCTTAAGATCCATCTAAAGAAGGACAACCGCAGGAAGAGACGG      510
                               ****  *    ***                *******  *  *     * *********

D_rerio_NM_212864.1           CAGGCTAAAGCATCAAGAAGCAAAAGTCTAAATAGAGGTTGA      558
O_mykiss_NM_001160503.1       CAAGCCCAG--------AGGCTCAAATCACACATCATGTAG-      543
S_salar_NM_001123558.1        CAAGCCCAG--------AGGCTCAAATCAAGCATCATGTAG-      543
                              ** **  *         * **  ** **       * ** *
```

Percent Identity Matrix

```
     1: D_rerio_NM_212864.1       100.00   54.72   53.95
     2: O_mykiss_NM_001160503.1    54.72  100.00   94.11
     3: S_salar_NM_001123558.1     53.95   94.11  100.00
```

Figure 3.1 – Nucleotide alignment of Rainbow Trout IFNγ (NM_001160503.1) with Atlantic Salmon (NM_001123558.1) and Zebrafish (NM_212864.1) *ifnγ* mRNA coding sequence. ‘*’ indicates locations where all transcripts share the same residue, ‘-‘ indicates a possible deletion in a transcript. Identity matrix is included as well. Alignment was carried out using Clustal Omega.



```
D_rerio_NP_998029.1          MIAQHMMGFAWGVCLLFSGWMTYSEAS----VPENLDKSIEELKAYYIKEDSQLHNAHPI     56
O_mykiss_NP_001153975.1      ----MDVLSRAVMCFCLMGWMTLGWSNAAQFTSINMKRNIDKLKVHYKISKDQLFNGNPV     56
S_salar_NP_001117030.1       ----MDVLSRAVMCFCLMGWMTLGWSNAAQYTSINMKSNIDKLKVHYKISKDQLFNGKPV     56
                                   :     :*: : **** . :.    .  *:. .*::**.:*  ...**.*.:*:

D_rerio_NP_998029.1          FLRILKDLKVNLEESEQNLLMSIVMDTYSRIFTRMQNDSVDEATKERLAHVQEHLKKLQE     116
O_mykiss_NP_001153975.1      FPK------DTFEDSDRRVLMSVVLDVYLSIFGQMLNQTGDQEMIESLKYVKGKIQDLQK     110
S_salar_NP_001117030.1       FPK------DTFEDSERRVWMSVVLDVYRSIFNQMLNQTGDQEVRERLDQVKGKVQETQK     110
                             * :       .:*:*::.: **:*:*.*  ** :* *:: *:   * *  *: :::. *:

D_rerio_NP_998029.1          SYFPGKSAELRTYAETLWAIKENDPIVQRKALFELKRVYREATLLKN---L-KNKERKRR     172
O_mykiss_NP_001153975.1      HYFLGRIPELRTHLQNLWAIETSDTTVQGKALSEFITIYEKASKLALKFHLKKDNRRKRR     170
S_salar_NP_001117030.1       HYFLKRIPELRTHLQNLWAIETSNTTVQGKALSEFITIYEKASKLALKIHLKKDNRRKRR     170
                              **   :  ****: :.****: .:   ** *** *:   :*.:*: *     * *::.****

D_rerio_NP_998029.1          QAKASRSKSLNRG  185
O_mykiss_NP_001153975.1      QAQRLKSHIM---  180
S_salar_NP_001117030.1       QAQRLKSSIM---  180
                             **:  :*  :
```

Percent Identity Matrix

```
     1: D_rerio_NP_998029.1      100.00   36.63   35.47
     2: O_mykiss_NP_001153975.1   36.63  100.00   89.44
     3: S_salar_NP_001117030.1    35.47   89.44  100.00
```

Figure 3.2 – Protein alignment of Rainbow Trout IFNγ (NP_001153975.1) with Atlantic Salmon (NP_001117030.1) and Zebrafish (NP_998029.1) protein sequence. '*' indicates locations where all proteins share the same residue, ':' indicates locations where two of three proteins share the same residue, '-' indicates a possible deletion in a protein. Identity matrix is included as well. Alignment was carried out using Clustal Omega.

3.4.2 - IFNγ qPCR quantification

IFNγ transcript levels in stimulated PBLs, as measured by qPCR, were elevated at 14h post stimulation and declined over the subsequent timepoints (Figure 3.3A). Although there was a clear upregulation, high individual variation resulted in no statistically significant differences being observed. IFNγ levels at 14h were significantly affected by the co-stimulants IL-1β and IL-2 when compared to PHA stimulation alone (Figure 3.3B). While IL-1β as a co-stimulant reduced IFNγ expression compared with PHA alone, the addition of IL-2 as a co-stimulant increased IFNγ expression at 14h post stimulation ($P \leq 0.05$).

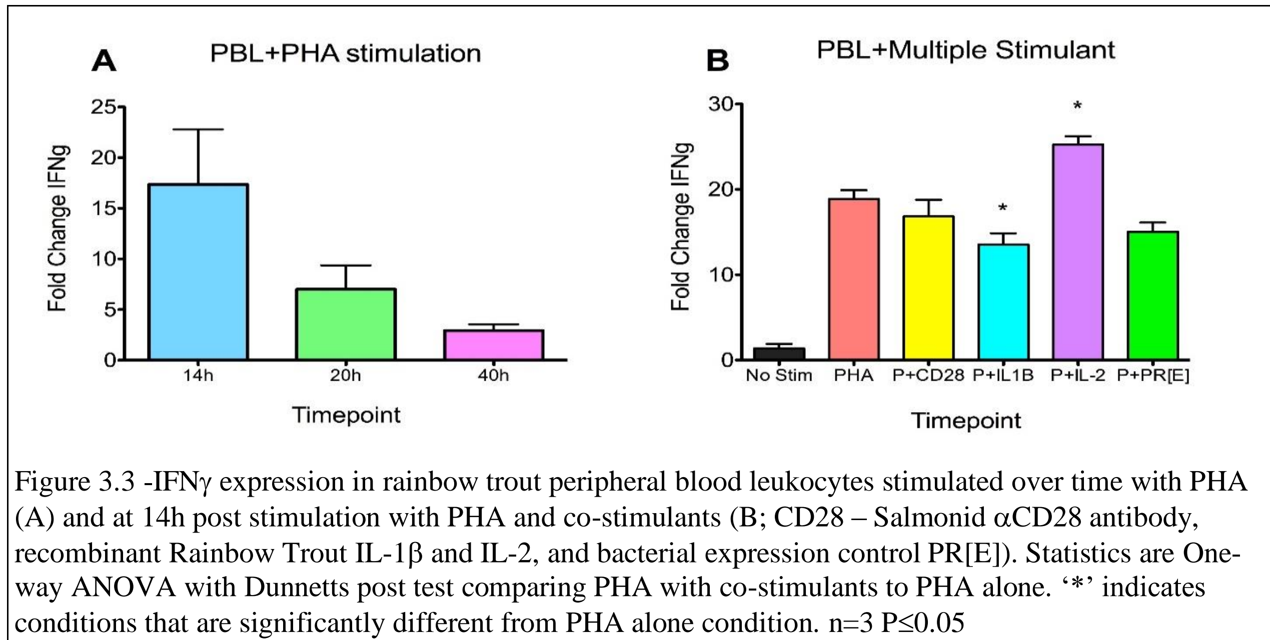


Figure 3.3 -IFNγ expression in rainbow trout peripheral blood leukocytes stimulated over time with PHA (A) and at 14h post stimulation with PHA and co-stimulants (B; CD28 – Salmonid αCD28 antibody, recombinant Rainbow Trout IL-1β and IL-2, and bacterial expression control PR[E]). Statistics are One-way ANOVA with Dunnetts post test comparing PHA with co-stimulants to PHA alone. '*' indicates conditions that are significantly different from PHA alone condition. n=3 $P \leq 0.05$

3.4.3 - IFNγ Western Blots

Recombinant Rainbow Trout IFNγ protein showed a strong band at 25kDa on western blots (Figure 3.4). This band disappeared when the polyclonal αIFNγ antibodies that was developed were pre-incubated with 50ug/ml of recombinant IFNγ for 12hr at 4°C prior to use in the western blot (Figure 3.5). This disappearance of recombinant banding pattern also held for native protein drawn from spleen and PBL protein samples.

Native protein samples showed a more complicated banding pattern depending on the sample tissue/cell. For spleen samples (Figures 3.4, 3.5), two large bands approximately 45kDa and 65kDa were detected by our antibody in both infected and uninfected fish (Figure 3.4). Infected fish and PHA stimulated PBLs showed an additional three smaller bands at slightly different sizes (infected spleen ~28, 20, 10kDa [Figure 3.4], stimulated PBLs ~38, 23, 10kDa [Figure 3.6]). All of the bands observed disappeared in blocking experiments along with the recombinant IFNγ band (Figure 3.5, 3.6).

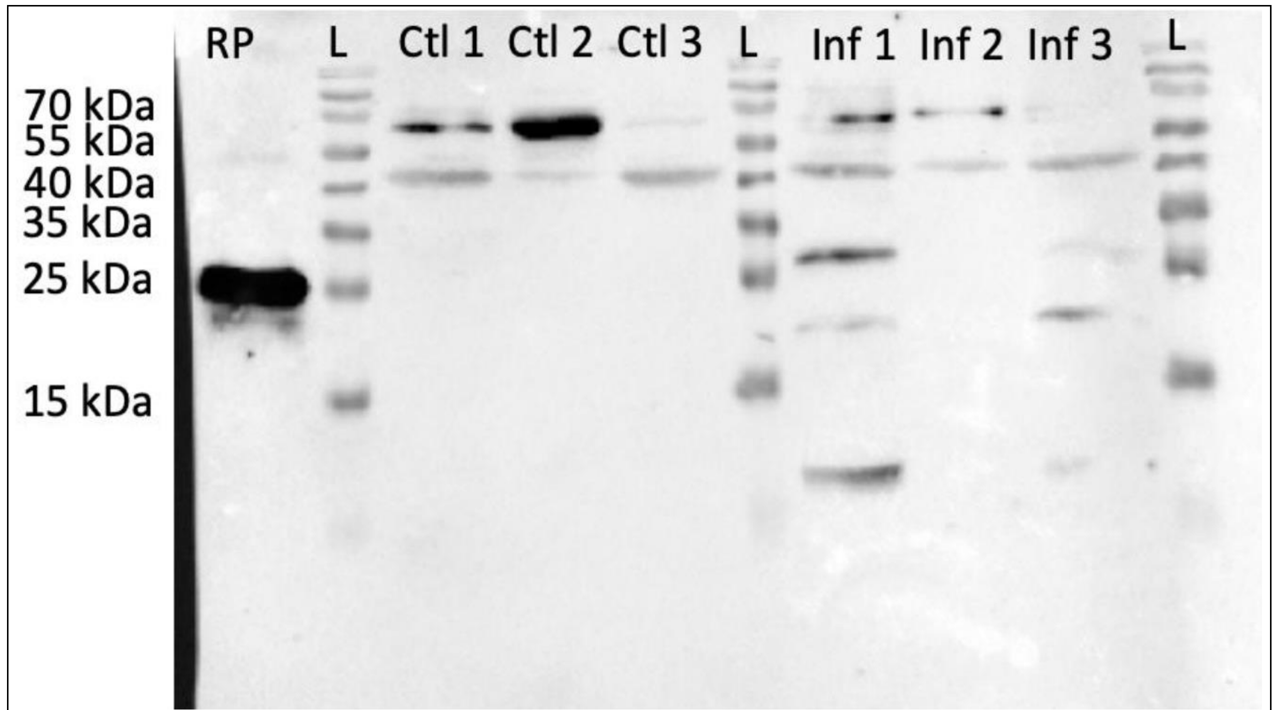


Figure 3.4 – Western blot showing recombinant IFNγ (RP) along with spleen protein from *Yersinia ruckeri* infected (Inf) and disease free (Ctl) Rainbow Trout.

In a PHA stimulation of PBL cells 10kDa band was prominent 1hr after stimulation and had faded somewhat at 4hr post stimulation (Figure 3.6). A blocking experiment conducted with PBL protein samples showed the same result. Recombinant and three native (10kDa, 23kDa and 38kDa) bands were absent or greatly reduced on the blocked membrane (Figure 3.7). Some non-specific cloudiness resulted in the native protein lanes on the blocked membrane. It was determined that this non-specific binding did not impact the blocking experiment as it did not obscure the reduction in antibody binding to the native bands of interest. Additionally, the cloudiness was present in the ladder lane on the blot, where no fish protein had been present.

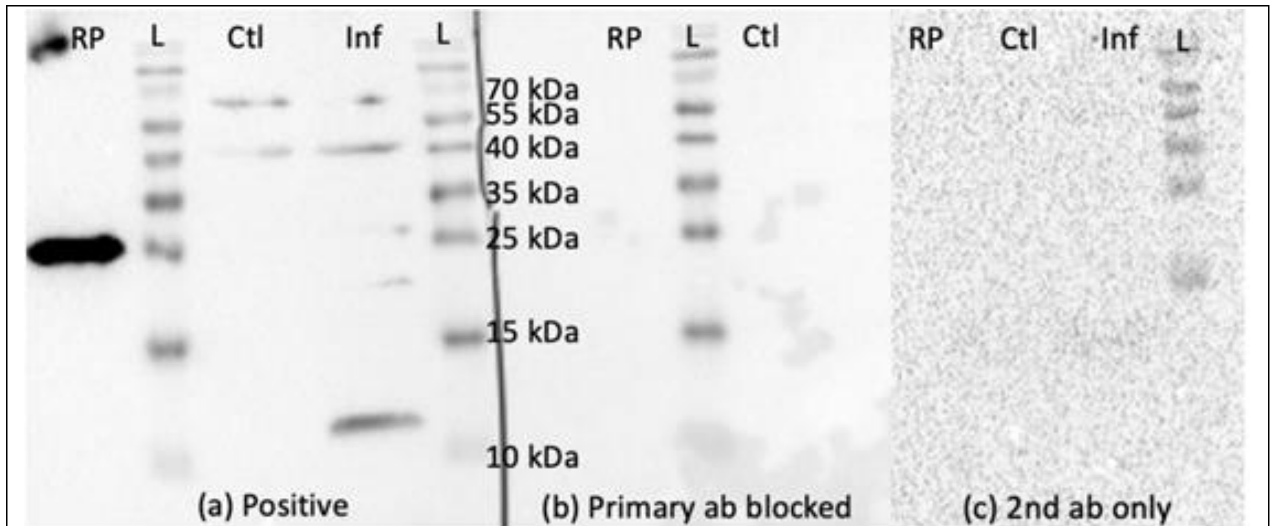


Figure 3.5 – Western blot demonstrating blocking of αIFNγ polyclonal antibody. Lanes are Molecular ladder (L) and Recombinant IFNγ (RP) along with spleen protein from *Yersinia ruckeri* infected (Inf) and disease free (Ctl) Rainbow Trout. A) No antibody blocking B) Primary antibody blocked C) No primary antibody.

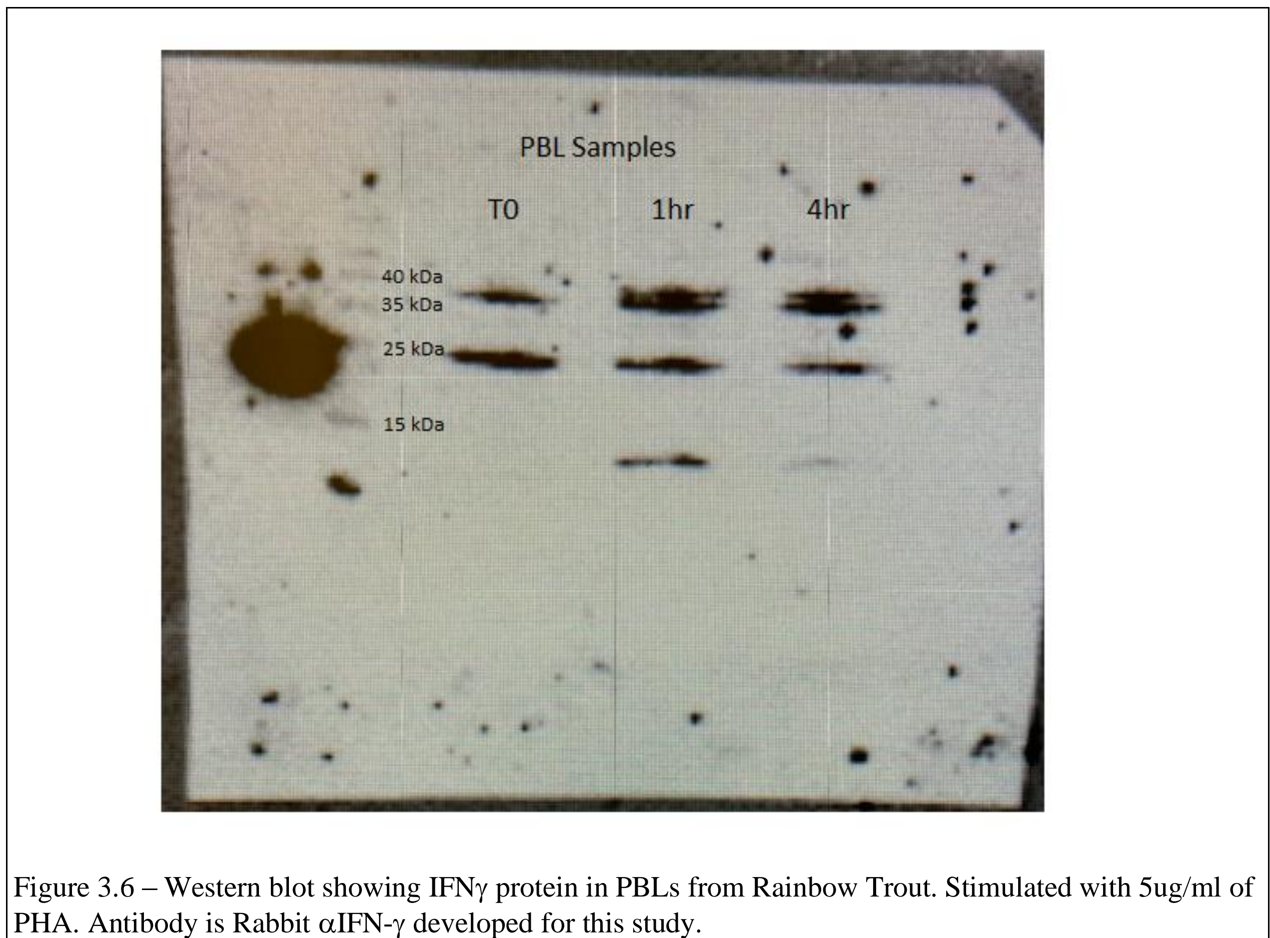


Figure 3.6 – Western blot showing IFNγ protein in PBLs from Rainbow Trout. Stimulated with 5ug/ml of PHA. Antibody is Rabbit αIFN-γ developed for this study.

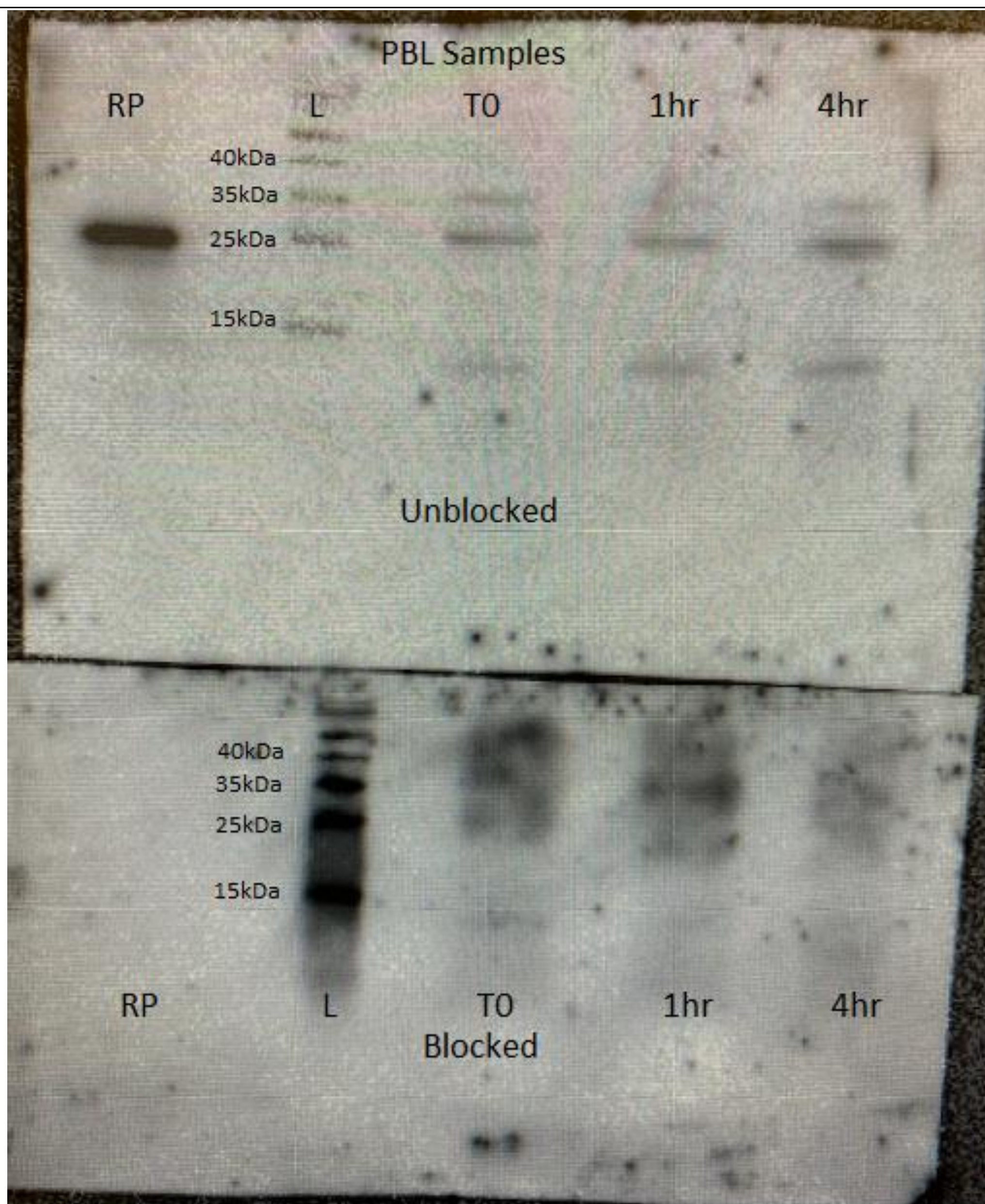


Figure 3.7 – Western blot demonstrating blocking of αIFNγ polyclonal antibody in PBL samples. Lanes are recombinant protein (RP), unstimulated (T0) PBLs along with PHA stimulated PBLs at 1h and 4h post stimulation.

3.4.4 - IFNγ ELISA

IFNγ protein levels in lavage supernatant did not differ significantly between tag types at day 2 of the sampling timepoints. Levels in the picogram per milliliter range were measured for all tag types and time points. 14 days post surgery significant reductions in IFNγ were observed in the groups implanted with Regular Vemco and Star Oddi tag types. This effect in these groups was maintained at day 70, where all

groups including the sham surgery group showed significantly reduced peritoneal IFNγ protein compared with the no surgery control (Figure 3.8, P≤0.05).

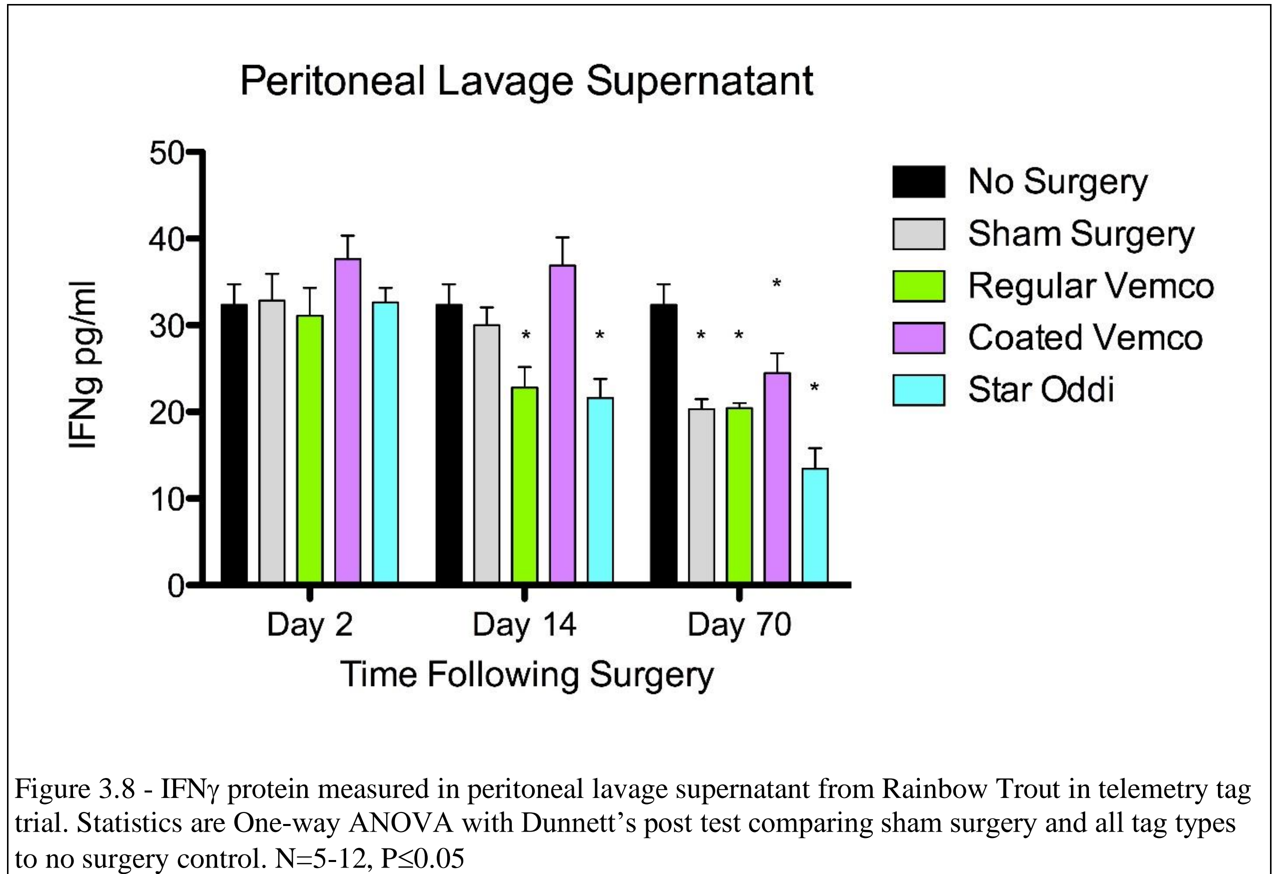


Figure 3.8 - IFNγ protein measured in peritoneal lavage supernatant from Rainbow Trout in telemetry tag trial. Statistics are One-way ANOVA with Dunnett's post test comparing sham surgery and all tag types to no surgery control. N=5-12, P≤0.05

3.5 - Discussion

Identification of Rainbow Trout IFNγ transcript was facilitated by the presence of two related fish species in which IFNγ had been recently characterized. At the transcript and predicted protein level the putative Rainbow Trout IFNγ is more similar to Atlantic salmon sequences than it is to Zebrafish sequences. This is encouraging as it is directly in line with what is expected given the established phylogenetic distance between these species.

Primer validation, through sequencing of the resulting amplicon, verified that the primers were selectively targeting Rainbow Trout IFNγ. The gene at this locus was induced in a manner consistent with what is known about the salmonid IFNγ induction. This IFNγ was inducible by PHA in salmonid cells (Zou *et al*., 2005) and zebrafish (Yoon *et al*., 2016). The presence of co-stimulants (alongside PHA) did not significantly alter peak IFNγ expression at 14h in PBLs except in the case of IL-1β and IL-2. Although the identity of the recombinant IL-1β and IL-2 used in this co-stimulation is not entirely verified these proteins appear to have acted in a familiar way. PHA stimulation of T cells elicits IFNγ expression, which functions to recruit and activate other immune cells such as macrophages, which then produce IL-1B. The presence of IL-1β in the PHA stimulant may have served as a signal that activated macrophages were already present, thereby reducing the need for IFNγ production (Figure 1.2B). IL-2, on the other hand, is a stimulator of T cell activity and proliferation. Thus addition of IL-2 to the PHA stimulant could be expected to upregulate IFNγ expression in T cells (Figure 1.2B).

Recombinant Rainbow Trout IFNγ appeared as a single band of about 25kDA as shown in multiple blots. The polyclonal rabbit αIFNγ antibody detected multiple bands in protein samples from both spleen and PBL samples. Two of these bands (45kDa and 65kDa) were larger and present in only infected and control spleen samples (Figure 1.3). These bands were not observed in any of the PBL protein samples (Figure 1.5, 1.6) leading to the conclusion that they are the result of cross reactivity between our polyclonal antibodies and spleen proteins that share epitopes with IFNγ. It is also possible that these bands represent our target, IFNγ, bound strongly enough to a chaperone, receptor subunit or other protein that the western conditions used were unable to cause it to dissociate.

Interestingly, both stimulation with PHA (PBL samples, Figure 1.5) and infection with *Y. ruckeri* (spleen samples, Figure 1.3) induced three smaller bands that were detected by our antibodies. These bands appear to be different sizes in spleen preparations when compared to PBL preparations, possibly representing an artifact of the SDS-PAGE process. The banding pattern seen in infected spleen samples

(Figure 1.3) is a match for that seen in stimulated PBL samples (Figure 1.5, 1.6) except for the size as estimated by the molecular ladder. This could be due to differences between SDS-PAGE runs (spleen samples were run for longer), in which case the ladder could lead to distorted size estimates. It is also possible that IFNγ is differentially produced/processed in cells that reside in the spleen, as opposed to circulating cells. Indeed, at the gene level, two different isoforms of IFNγ have been identified in Rainbow Trout (Purcell *et al*., 2009) which could help explain the size difference observed with our antibodies.

The agreement in size along with the inducible nature of these bands suggest that these antibodies detect IFNγ in its secreted and active form. Indeed, IFNγ cloned from Atlantic salmon is predicted to be 18kDA in size (Robertsen *et al*., 2003) and a study in Zebrafish showed two IFNγ bands, one unreduced 40kDA band, and one 20kDA band under reducing conditions (Yoon *et al.,* 2016). The 38kDa band observed in our PBL protein would correspond to the incompletely reduced 40kDa band seen in Zebrafish leukocytes and the 20kDa band observed would correspond to the 20kDa band in the reduced condition. The persistence of the unreduced-like band in Rainbow Trout western blots may be due ineffective reducing conditions. Yoon *et al*., placed protein samples under 5% β-mercaptoethanol conditions at 95°C for 30 minutes in order to fully remove the 40kDa band. Thus the 38kDa band may be dimerization (38kDa band) as was observed in Zebrafish (Yoon *et al*., 2016). If so the 20kDa band observed in Rainbow Trout corresponds to the reduced, monomeric form of IFNγ. Finally, the 10kDa band, being absent (Figure 3.9) or faint (Figure 3.10) prior to stimulation is likely a degradation product of IFNγ that retains epitopes capable of being recognized by our polyclonal antibodies.

Following this interpretation, it would seem that the circulating leukocytes sampled in this study were already producing IFNγ in its active form prior to stimulation with PHA (at time 0). In the case of the Peruvian hatchery fish, the fish were already infected or recovering from infection when spleens were collected so expression of immune genes could be anticipated. Alternatively, this could point to a baseline amount of IFNγ expressing cells present in Rainbow Trout blood. Indeed, the bands corresponding to the active homodimer (38kDa, Figures 1.5) show some induction and a dual banding following PHA stimulation. This is some indication that, whether time zero is showing a baseline or prior induction, our putative IFNγ is inducible via canonical stimulation. This stimulation also produces the degradation product that is detectable by our antibodies.

This interpretation also helps explain the results seen in spleen samples. Uninfected fish show only the larger, non-specific bands while infected fish show those bands along with the three smaller bands that correspond to those observed in PBLs. Thus, during infection, circulating leukocytes are activated, begin expressing IFNγ and some proportion of these collect in the spleen. This brings the smaller three bands to the spleen, which are only seen in the infected spleen samples as these results demonstrate.

IFNγ produced by immune cells in the peritoneum varied both by tag type (at day 14) and treatment as a whole (at day 70. Sham surgery). It is anticipated that lower peritoneal IFNγ protein means less (or less active) T cells being recruited to the peritoneal cavity in response to implantation. However at day 70 the Sham surgery group, with no implant, also showed significantly reduced IFNγ protein levels. The day 14 protein levels indicate that both the Regular Vemco and Star Oddi tags negatively affected leukocyte recruitment and/or activation.

Another possible explanation for the reduction in peritoneal IFNγ is related to the previously mentioned suppression of inflammation by tissue resident macrophages (Ipseiz *et al*., 2020). Although IFNγ was not a target of that study it is involved in early inflammatory cell recruitment and activation (Schroder *et al*., 2004). Thus, it is possible that the reduction in IFNγ protein is a signal of IL-10 mediated resolution of the inflammatory response. In this view the Star Oddi tags performed best in terms of biocompatibility while the coating on the Vemco tag appeared to make the immune response worse, at least at 14 days.

3.6 - Conclusion

The development of an ELISA assay capable of quantifying IFNγ protein at the picogram level is a significant advancement in the study of teleost immunity. Being able to quantify cytokines, which are expressed and active at low levels, will benefit many aspects of salmonid research, culture and management. IFNγ ELISA assays could be used to test for alloimmune responses (Augustine *et al*., 2012), immunity to viral infections (Altfield *et al*., 2001) and vaccine efficacy (Smith *et al*., 2003). Indeed, being able to quantify IFNγ protein derived from PBL cells following vaccination will provide a level of resolution that was previously unachievable. This combined with the fact that the assay is possible to carry out on non-lethally obtained samples means that vaccines can be evaluated more quickly and at a higher resolution. This will enable better selection of vaccines, adjuvants, vaccination schedules and fish stocks that are more responsive to current vaccination protocols.

3.7 – Acknowledgements

The authors would like to acknowledge the assistance provided by the entire crew at Alma Hatcheries in southern Ontario. Special thanks go to Marcia Chaisson at Alma for all her help organizing the sampling trips to her hatchery site. Additionally, thanks are owed to Tania Rodriguez-Cornejo for her work sampling outbreaks at fish farms in the Central Regions of Peru. Starr Oddi gave free tags and Vemco gave us free coated tags,

4.0 – Chapter 4 –Discovery of a novel, blood-borne endogenous alkaline phosphatase in Rainbow Trout (*Oncorhynchus mykiss*)

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4.1 – Abstract

Provision of fish protein through sea cage aquaculture faces significant challenges from disease outbreaks, which reduce protein available for food and cause economic losses. Disease protection via vaccination has proven difficult to achieve as current vaccination programs are only moderately effective at preventing disease losses. Understanding of vaccine efficacy and function is fish is limited due to the lack of protein level and functional assays that can guide developers in inducing appropriate immune responses. This study aimed to develop assays to circulating protein markers and revealed a novel, blood-borne endogenous alkaline phosphatase (AP) detectable by ELISPOT techniques. This endogenous AP was observed mainly in suspended peripheral blood leukocytes (PBLs) as opposed to adherent PBLs, suggesting a T-cell origin. Additionally, the novel AP function was impaired by the common AP inhibitor Levamisole. Moreover, this novel AP is measurable from samples that can be non-destructively obtained. This could enable testing of an animal's immune status without having to sacrifice the animal, greatly improving our ability to test vaccines and adjuvants. Finally, this work could potentially contribute to improved selective breeding programs designed to foster more disease resistant salmonid stocks.

Keywords: Alkaline phosphatase, Artefact, ELISPOT, Levamisole, PBL

4.2 – Introduction

Aquaculture in Canada is an expanding industry, bringing upwards of 1 billion dollars per year in economic value. A major economic issue is that high density of animal stocking results in greater losses to disease than is preferable. Salmon farmers combat these losses primarily by feeding antibiotics to their stock and acting swiftly to cull emerging outbreaks. Another strategy for reducing disease loss is vaccination of the fish stocks against regionally common pathogens. This includes vaccines that have proved effective against bacterial pathogens such as *Vibrio* (Hayashi *et al*., 1964), *Yersinia ruckeri* (Tebbit *et al.,* 1981), *Aeromonas salmonicida* (Midtlyng, 1997), as well as for viral pathogens (reviewed in Biering *et al*., 2005). These advances in vaccine technology had the added benefit of significantly reducing the need for, and therefore use of, antibiotic treatments in aquaculture (Midtlyng *et al*., 2011). Despite this progress it still remains the case that most vaccine evaluation requires either the sacrifice of experimental animals for tissue collection or a longer-term study which tracks the performance of vaccinated versus unvaccinated populations and simply compares mortality over time. Both of these strategies are expensive and time consuming. It is possible that an assay examining protein production from circulating white blood cells (termed peripheral blood leukocytes) could provide much of the information needed to assess vaccine efficacy. As these cells can be collected without having to sacrifice the fish this approach would be superior in two ways. Firstly, this would reduce the overall costs compared to approaches that involve sacrificing the animals, as every animal is a valuable product in itself. Secondly it would permit the researchers to follow an individual animal’s response to vaccination over an extended period, as multiple non-lethal blood samples could be collected from the same fish over the course of vaccination. This has the added benefit of increasing the fidelity of experimental findings since individual variation among even closely related fish makes it difficult to draw conclusions from studies wherein each timepoint represents distinct individual fish due to the limitations of lethal sampling. An assay capable of detecting an important immune marker, using a non-lethally obtained sample would enable significant improvements to both vaccine and stock selection processes. Therefore, this study initially targeted the salmonid cytokine Interleukin 2.

Cytokines are the master regulators of the vertebrate immune system (Turner *et al.,* 2014). Interleukin 2 is expressed by activated T-cells and plays a central role in T-cell dependent immune responses. Specifically, IL-2 is potent inducer of T-cell proliferation which leads to populations of differentiated T-helper cells. These specialized T-helper cells are essential for proper activation and maturation of antibody expressing plasma cells (Malek, 2008). The antibodies produced by plasma cells have many functions (pathogen neutralization, recruiting of complement factors, activation of phagocytes, etc) and are represent the main targeting tool of the adaptive immune response. Considering that antibody

production is the entire purpose of vaccination against disease we sought to examine IL-2 protein in circulating salmonid leukocytes. Moreover, being that cytokines are expressed at low levels while still being bioactive this work attempted to develop a more exact protein level assay, the ELISPOT. This assay, using the same antibodies designed for ELISA, has a much higher resolution. ELISPOTs use live cells as opposed to protein homogenates as their sample material and measure soluble protein expression down to the level of a single cell. It was anticipated that being able to quantify this important marker of antibody production in circulating PBLs could help evaluate and improve vaccination candidates (different formulations, different adjuvants) and vaccination schedules. Additionally, it was hoped that vaccination success would differ between different type of hatchery reared salmonids. Toward this end the ELISPOTs were designed using two type of rainbow trout, the normal diploid stock (closer to the wild type) and the increasingly popular triploid stock. Triploid fish, while they occur very rarely in the wild, can be produced by several methods in the hatchery, all of which cause retention of the polar body during early stages of embryogenesis resulting in an extra set of genetic material (taking 2N diploid and turning it into 3N triploid; Arai, 2001). These stocks are popular in aquaculture because they do not reproduce (maturation of stock being a massive issue for fish farmers) but they appear to suffer decreased overall immune function (Weber *et al.*, 2013).

However, this study's exploration of protein products derived from PBLs yielded a new blood-borne alkaline phosphatase protein detectable via a modified ELISpot assay.

Alkaline phosphatase (AP) is a family of membrane bound glycoproteins present in prokaryotes and eukaryotes. There are four versions of AP in mammalians distinguished by their location in the body: Intestinal, Placental, Germ-cell and Liver/Bone/Kidney. Although AP enzymes are widely distributed and long studied, little is known about the function of some isotypes. Some role in bone mineralization is supported for the Liver/Bone/Kidney version of AP (Wuthier RE & Register, 1985), while the intestinal AP (IAP) has been implicated in the acquisition of the gut microbiome and proper functioning of gap junctions (Lallès, 2020). IAP appears to be involved immunologically in the removal of pro-inflammatory signals (Yang *et al.*, 2012) in fish. Indeed, in germ-free zebrafish there is no IAP activity measured but exposure of these animals to a bacterially derived immunogen (Lipopolysaccaride [LPS]) or bacteria upregulates IAP activity (Bates, 2007). Additionally, IAP activity reduces the impact of LPS exposure on rainbow trout intestinal epithelial cells (Kawano *et al.*, 2011).

Given the potential involvement of circulating AP in fish health an ELISPOT assay was developed for the purpose of quantifying AP activity in fish blood. This has the potential to provide a powerful new tool for the assessment of fish health. This tool uses a non-lethally obtained sample to rapidly measure AP

activity in fish blood, potentially revealing aspects of the animal's immune response that were previously opaque. Such clarity stands to benefit selective breeding programs, therapeutic regimes along with vaccination candidates and schedules. To the extent that AP levels reflect immune activation this assay could greatly accelerate the testing, selection and improvement (via different adjuvant mixtures) of vaccine programs.

4.3 – Methods

4.3.1 - Development of recombinant rainbow trout Interleukin-2/IFN-γ protein

4.3.1.1 – Cloning of Rainbow Trout IL-2/IFN-γ

The sequence for rainbow trout IL-2 was identified in Pubmed (NM_001164065.2) and primers were designed to amplify the coding region (see table 4.1). Primers were used to amplify targeted regions from rainbow trout spleen cDNA with the following conditions: 95° C for 3 min followed by 32 cycles of 95° for 30 sec, 60°C for 10 sec, 72° for 45 sec, ending the entire process with a final step of 72° for 5 min. The bands were electrophoresed on a 1.5% agarose gel, stained with GelRed (Biotium), visualized on a Blue Light Transilluminator (Pearl Biotech) and excised using a scalpel. Bands were purified using a QIAquick gel extraction kit (Qiagen) and blunt ligated into pGEM-T easy vector (Promega). These vectors were transformed into E. Coli XL1-Blue supercompetent cells (Aligent Technologies) which were then grown on LB media with 0.1 mg/ml of ampicillin (Fisher Scientific). White colonies were selected, grown in 1ml of LB plus ampicillin, miniprepped and sent for sequencing (using T7 and Sp6 primers) at TCAG sequencing facility at Toronto Sick Kids hospital.

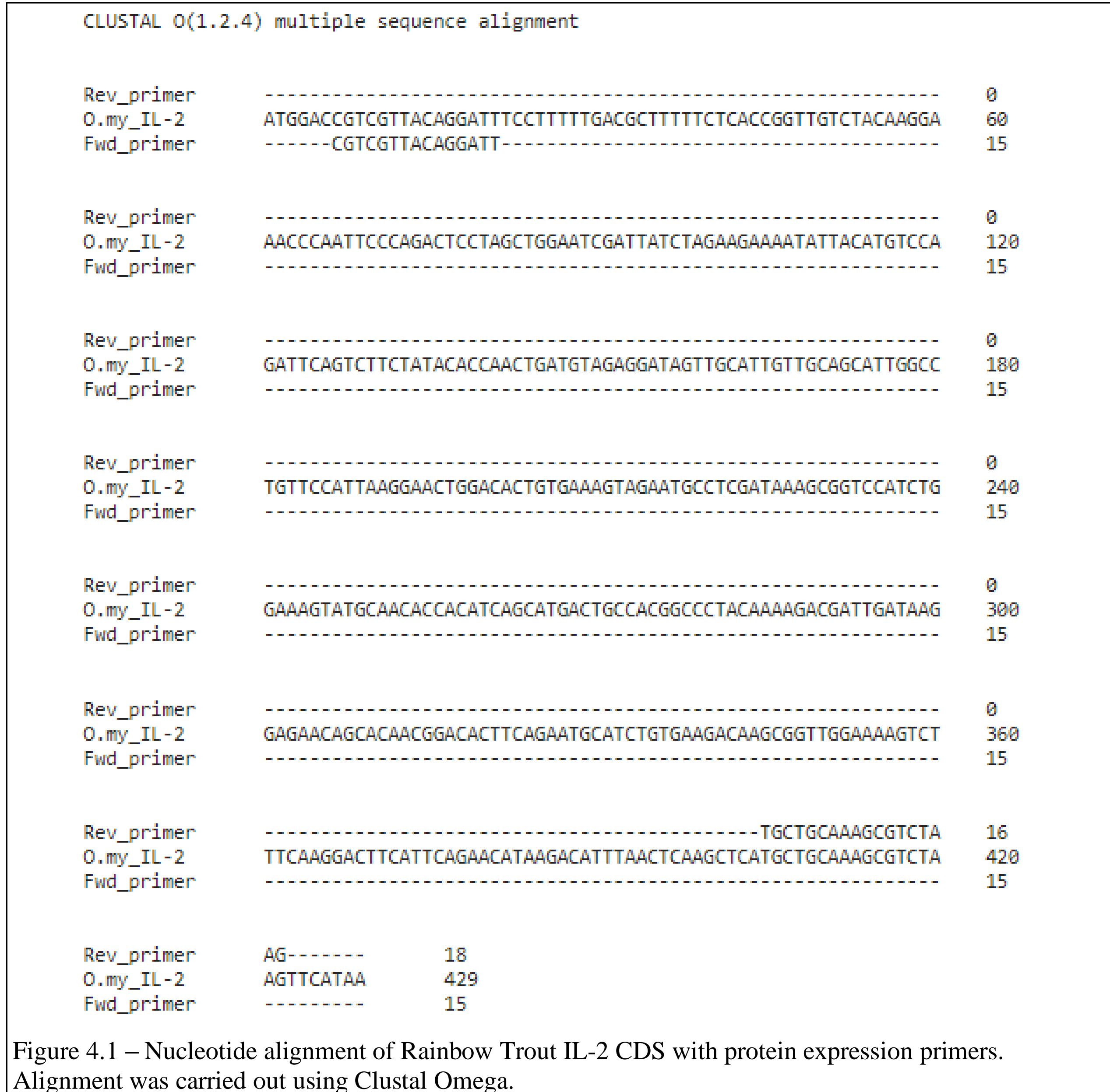
Table 4. 1 – Table of PCR and cloning primers used in this study, along with Accession # for the target gene.

Primer set	Organism	Forward	Reverse	Amplicon (bp)	Accession #
IFN-γ	Rainbow Trout	GTCCACTATAAGATCTCCA	GTCTGGTTCAGCATCTG	152	NM_001160503
IFN-γ Cloning	Rainbow Trout	ATGGATGTGTTATCAAGGGCT	CATGATGTGTGATTTGAGCCT	540	NM_001160503
IL-2 Cloning	Rainbow Trout	CGTCGTTACAGGATT	CTTAGACGCTTTGCAGCA	261	NM_001164065.2
IL-2 Subcloning	Rainbow Trout	AGGATCCCGTCGTTACAGGATT	GAAGCTTCTTAGACGCTTTGCAGCA	261	NM_001164065.2

4.3.1.2 – Development of IL-2/IFN-γ expression construct

The primer sets for protein production (Figure 1; see chapter 3 for IFN-γ) were modified to include restriction enzyme cut sites designed to facilitate cloning into the pRSET A bacterial expression vector (Forward primers – BamHI, Reverse primers – HindIII). These modified primers were verified as above with blue-white screening in pGEM vector. Verified clones (along with purified pRSET A vector) were then double digested with BamH1 and HindIII restriction enzymes according to manufacturer's instructions (Thermofisher). Digests were separated on a 1% agarose gel and bands excised corresponding to the insert in the case of pGEM and the digested vector in the case of pRSET. Following gel extraction with QIAquick Gel extraction kit (Qiagen) and PCR cleanup with QIAquick PCR Purification kit

(Qiagen) the insert and pRSET were ligated using T4 DNA ligase (Fisher Scientific). Resulting plasmids were used to transform BL-21 competent cells which were subsequently sequenced (as before) to confirm the sequence of pRSET constructs.



```
CLUSTAL O(1.2.4) multiple sequence alignment


Rev_primer      ------------------------------------------------------------    0
O.my_IL-2       ATGGACCGTCGTTACAGGATTTCCTTTTTGACGCTTTTTCTCACCGGTTGTCTACAAGGA    60
Fwd_primer      ------CGTCGTTACAGGATT---------------------------------------    15


Rev_primer      ------------------------------------------------------------    0
O.my_IL-2       AACCCAATTCCCAGACTCCTAGCTGGAATCGATTATCTAGAAGAAAATATTACATGTCCA    120
Fwd_primer      ------------------------------------------------------------    15


Rev_primer      ------------------------------------------------------------    0
O.my_IL-2       GATTCAGTCTTCTATACACCAACTGATGTAGAGGATAGTTGCATTGTTGCAGCATTGGCC    180
Fwd_primer      ------------------------------------------------------------    15


Rev_primer      ------------------------------------------------------------    0
O.my_IL-2       TGTTCCATTAAGGAACTGGACACTGTGAAAGTAGAATGCCTCGATAAAGCGGTCCATCTG    240
Fwd_primer      ------------------------------------------------------------    15


Rev_primer      ------------------------------------------------------------    0
O.my_IL-2       GAAAGTATGCAACACCACATCAGCATGACTGCCACGGCCCTACAAAAGACGATTGATAAG    300
Fwd_primer      ------------------------------------------------------------    15


Rev_primer      ------------------------------------------------------------    0
O.my_IL-2       GAGAACAGCACAACGGACACTTCAGAATGCATCTGTGAAGACAAGCGGTTGGAAAAGTCT    360
Fwd_primer      ------------------------------------------------------------    15


Rev_primer      --------------------------------------------TGCTGCAAAGCGTCTA    16
O.my_IL-2       TTCAAGGACTTCATTCAGAACATAAGACATTTAACTCAAGCTCATGCTGCAAAGCGTCTA    420
Fwd_primer      ------------------------------------------------------------    15


Rev_primer      AG-------       18
O.my_IL-2       AGTTCATAA       429
Fwd_primer      ---------       15
```

Figure 4.1 – Nucleotide alignment of Rainbow Trout IL-2 CDS with protein expression primers. Alignment was carried out using Clustal Omega.

4.3.1.3 - Protein Production and Purification

The BL-21 bacteria harboring pRSET constructs described above were inoculated into 50ml of SOB broth with 50mg/ml ampicillin and 35mg/ml chloramphenicol and grown overnight at 37°C shaking at 200 RPM. This 50ml culture was used to inoculate 1L of SOB broth with 10mM $MgCl_2$ and no

antibiotics. This culture was grown at 37°C shaking at 200 RPM until an optical density between 0.4 and 0.6 was reached at which point and inducer of the LAC operon - Isopropyl β-D-1-thiogalactopyranoside (IPTG) –was added to 1mM. Cultures were incubated at 37°C shaking at 200 RPM for 4hr at which point the bacteria were pelleted and the supernatant (media) removed. Bacterial pellets were lysed overnight in 8M urea (pH 8) with protease inhibitor (Roche) on a rotary mixer at 4°C. Insoluble material was pelleted and the supernatant was incubated for 1hr with Ni-NTA resin (Qiagen). Following binding the resin-protein mix was poured onto glass columns. Once settled successive washes of 8M urea with decreasing pH were used to initially wash away contaminating proteins and finally to elute the resin bound protein of interest. Protein was quantified using BCA assay and examined on an SDS-PAGE gel (15% polyacrylamide). Total protein was visualized using SeeBand Forte (GeBA) staining of the SDS-PAGE gel.

4.3.2 - αIL-2 antibody development

Following production/purification of recombinant protein 1mg of the protein was sent to Cedarlane labs (Burlington, ON) for production and purification of antibodies targeting the recombinant protein. In brief, recombinant protein was injected into both rabbit and goat host animals. After an appropriate amount of time for the animal to mount an immune response, the animal was bled and serum collected. A column was then prepared by attaching a portion of the recombinant protein to a matrix and the serum was passed over the column. Any antibodies formed in the immune response that were specific to the injected recombinant protein would therefore bind to the matrix in the column. Following wash steps to remove any non-specific antibodies a buffer was used to separate the bound the antibodies from the column matrix. The result is a purified fraction of the serum borne antibodies that is enriched for antibodies targeting the recombinant protein of interest.

4.3.3 – Western blot with polyclonal αIL-2

The antibodies described above were used to probe a SDS-PAGE gel loaded with protein homogenates derived from Rainbow Trout spleen tissue as well as a PBLs. Prior to SDS-PAGE protein samples were denatured by the addition of 2.5% β-mercaptoethanol and heated at 95°C for 10 minutes. SDS-PAGE was carried out using 40ug of total protein loaded into a 15% polyacrylamide gel for size separation. This gel was run at 80V for 15 minutes followed by 160V for approximately 1hr to achieve proper separation. The separated protein was then transferred to a PVDF membrane in a Trans-Blot Turbo machine (BioRad) and the membrane was probed with recombinant αIL-2 antibodies.

4.3.4 - Development of rainbow trout IFNγ protein and αIFNγ antibodies

See chapter 2 for the details of recombinant rainbow trout IFNγ production and development and purification of αIFNγ antibodies.

4.3.5 - Collection of peripheral blood leukocytes (PBLs)

Rainbow Trout from the University of Guelph associated hatchery located in Alma, ON were sedated using a 1:1000 dilution of benzocaine in fresh water. Blood was drawn from the caudal sinus into a sterile, heparin coated syringe and placed on ice. PBLs were isolated from the whole blood via a hypotonic lysis procedure carried out in a sterile flow hood on campus. In brief, whole blood samples were diluted 1:10 with sterile, cold, distilled water to cause lysis of red blood cells and shaken gently for 20 seconds. Normal osmolarity was restored by adding an appropriate volume of sterile 10x PBS to each sample. Samples were then passed over a 20um Falcon cell strainer (Fisher Scientific) to remove debris. Remaining, intact PBLs were then pelleted by centrifugation for 5 minutes at 200xg and supernatant was decanted. Cells were then washed with 5ml of sterile PBS and then pellet again as before. Supernatant was removed with a pipette and then the PBLs were resuspended in 500ul of T-per protein extraction reagent (Thermofisher) and homogenized with a 24-gauge needle and 1ml syringe by passing the sample through the needle 10 times. As per the T-per protocol the samples were then centrifuged at 12 000 RPM for 5 minutes after which supernatant (containing only soluble proteins) was collected and frozen in a -80°C freezer.

4.3.6 - Separation of adherent from suspended PBLs

PBL isolation was carried out in the same manner as described above (see section 3.2.1) with slight variation in order to keep the cells alive for plating in the ELISPOT assay. Following the wash step that comes after primary cell pelleting the PBLs were resuspended in sterile L-15 media with only 1% pen/strep added (Thermofisher) and enumerated using a hemocytometer and light microscope. Dilution series of cells were prepared and plated onto ELISPOT plates with PVDF membranes (Mabtech) using 100ul of cell dilution and 100ul of sterile L-15 media per well. The sterile media that was added to the plates prior to addition of cells was used to ensure that the appropriate concentration of stimulant (phytohemagglutinin [PHA], or phorbol 12-myristate 13-acetate [PMA]) was present when the cells were plated into their respective wells. All sample dilutions were plated in triplicate. Following PBL isolation from Rainbow Trout whole blood the cells were placed in 75cm^2 sterile cell culture flasks (Falcon) and incubated at 14°C for approximately 24h. Following incubation, media (along with suspended PBLs) was removed and replaced with fresh L-15 media (1% pen/strep). Vigorous tapping was used to dislodge adherent PBL cells from the flask bottom and this cell portion was then removed from the flask. Cells were then enumerated via hemacytometer and diluted to plating densities.

4.3.7 - ELISPOT with rainbow trout PBLs

ELISPOT plates (Mabtech) were coated with 100ul of 2ug/ml of capture antibody in coating buffer overnight at 4°C (Rabbit αIFNγ, Rabbit αIL-2). Plates were then decanted and washed three times with 300ul of TBS-T (0.01% Tween 20). Following isolation of PBL cells, separation of the two fractions (adherent and suspended) and quantification cells were diluted to plating concentrations and then plated in 200ul total of either LB media (0% FBS, 1% pen/strep) or LB media plus stimulant (PHA and PMA) at three different concentrations. Plated cells were then incubated overnight at 14°C. Up to this point all solutions and cell mixtures were kept in sterile conditions and only manipulated in a sterile flow hood. Following this the cell/stimulant mixtures were decanted and the plates were washed with TBS-T three times. Plates were then incubated for 1hr at RT with 100ul of 1ug/ml of primary detection antibody (Goat αIFNγ, Goat αIL-2) in TBS-T with 5% skim milk and then washed as before. This was followed by incubation for 1hr at RT with 100ul of 1:1000 of Rabbit αGoat IgG in TBS-T with 5% skim milk. Following washes, a final spot development step was carried out for 20 minutes at room temperature with an NBT/BCIP mixture as per manufacturers protocol. Spot development was arrested by removing the underdrain from the plate and rinsing the plate with water. Plates were then left to dry overnight to enhance spot visibility and then quantified on an Immunospot C.T.L. plate reader (Ohio, USA). Following an indication that the spots observed were non-specific an ELISPOT plate was prepared with sample PBLs and an ingredient removal panel. This involved a set of four wells (in triplicate) with the same number of PBLs plated wherein one of the set contained all antibody ingredients (capture, primary detection and secondary detection) with the other three sets having one of the three antibodies left out of the procedure.

Subsequent ELISPOT plates testing the effectiveness of the alkaline phosphatase inhibitor Levamisole were carried out as above with the addition of an inhibition step prior to addition of the NBT/BCIP mixture at the end. In this step 2 replicate wells were treated with 10uM of levamisole for 10 minutes before the addition of NBT/BCIP substrate.

4.3.8 - Statistical analysis

Statistical analysis was not possible as only two technical replicate wells were performed for each cell dilution treated with Levamisole. There was insufficient time to repeat the experiment, however the dramatic nature of the inhibition is readily apparent.

4.4 – Results

4.4.1 – Interleukin 2 cloning and Western Blot

Rainbow trout interleukin 2 was cloned into pGem and then subcloned into the protein expression vector. The recombinant protein expressed was used to generate antibodies used in subsequent assays. Western blots carried out using rabbit αIL-2 detected the recombinant IL-2 as a strong 17kDA band, as well as two lesser bands 25 and 38kDa in size (Figure 4.2). No distinct bands were present in protein prepared from spleen samples, however a strong 14kDA band was observed in the PBL protein preparations (Figure 4.2).

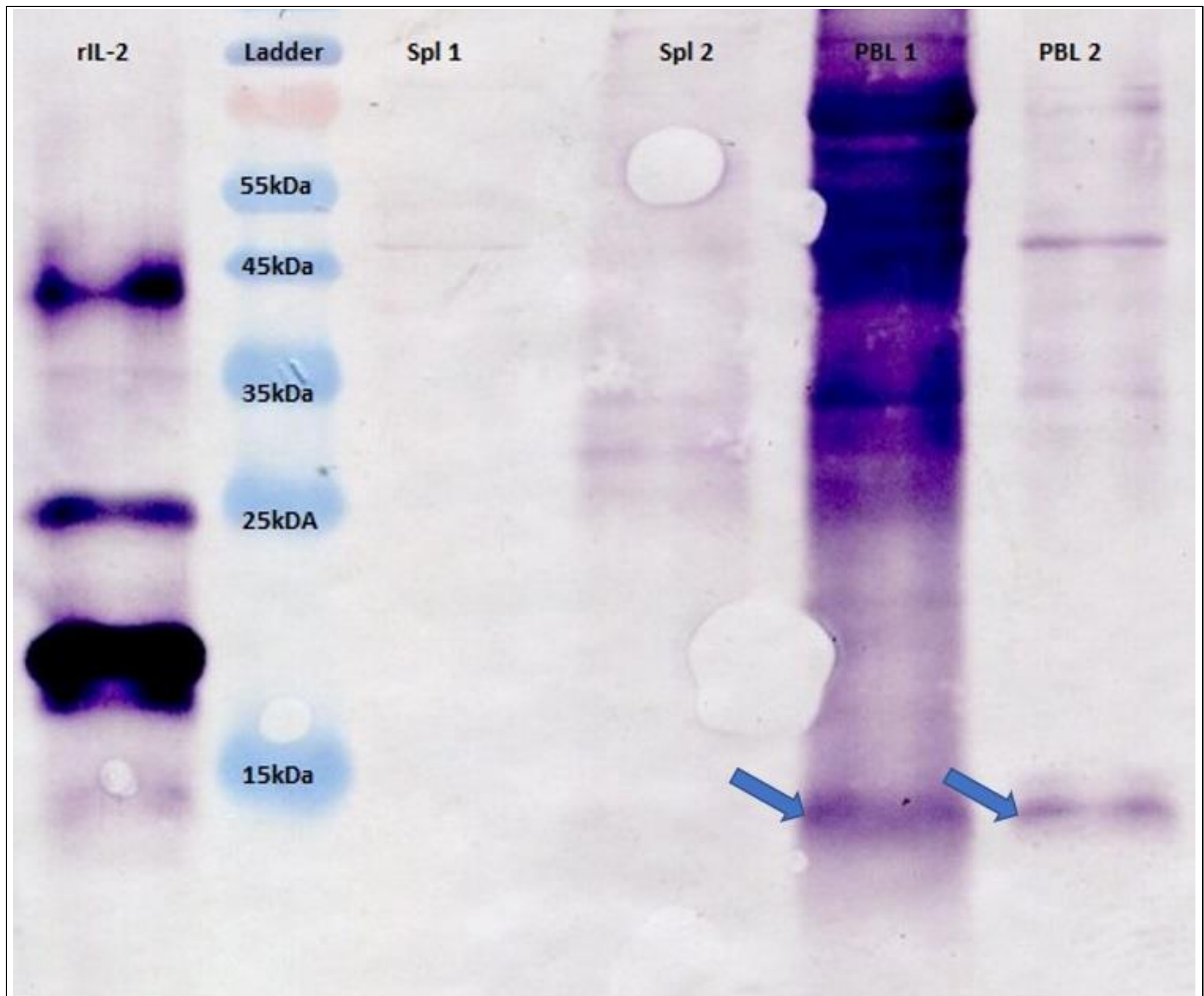


Figure 4.2 – Western blot of protein prepared from rainbow trout spleen tissue and PBL cells. Lanes are recombinant interleukin 2 (rIL-2), Ladder, Spleen sample 1, Spleen sample 2, PBL 1, PBL 2. Blot was probed with Rabbit αIL-2 antibody for 1hr at room temperature. Arrows indicate 14kDA IL-2 band. Detection was carried out using NBT/BCIP substrate for an AP conjugated secondary detection antibody.

4.4.2 – Exploratory ELISPOT Assays

Initial ELISPOT assays were carried out using capture and detection antibodies targeting rainbow trout cytokines. As can be seen in figure 1.2 panels A and C the stimulant conditions did not produce significantly more spots than was observed in the control wells. Otherwise the dilution series did return a progressively lower number of spots on the plate as the number of cells plated decreased (Figure 4.3 A,C vs B,D). Without exception the highest stimulant concentrations, for both stimulants, resulted in spot numbers equal to or less than the control wells. It should also be noted that spot numbers were highly similar for both the IFNγ and IL-2 specific antibody pairs across both stimulants used (Figure 4.3).

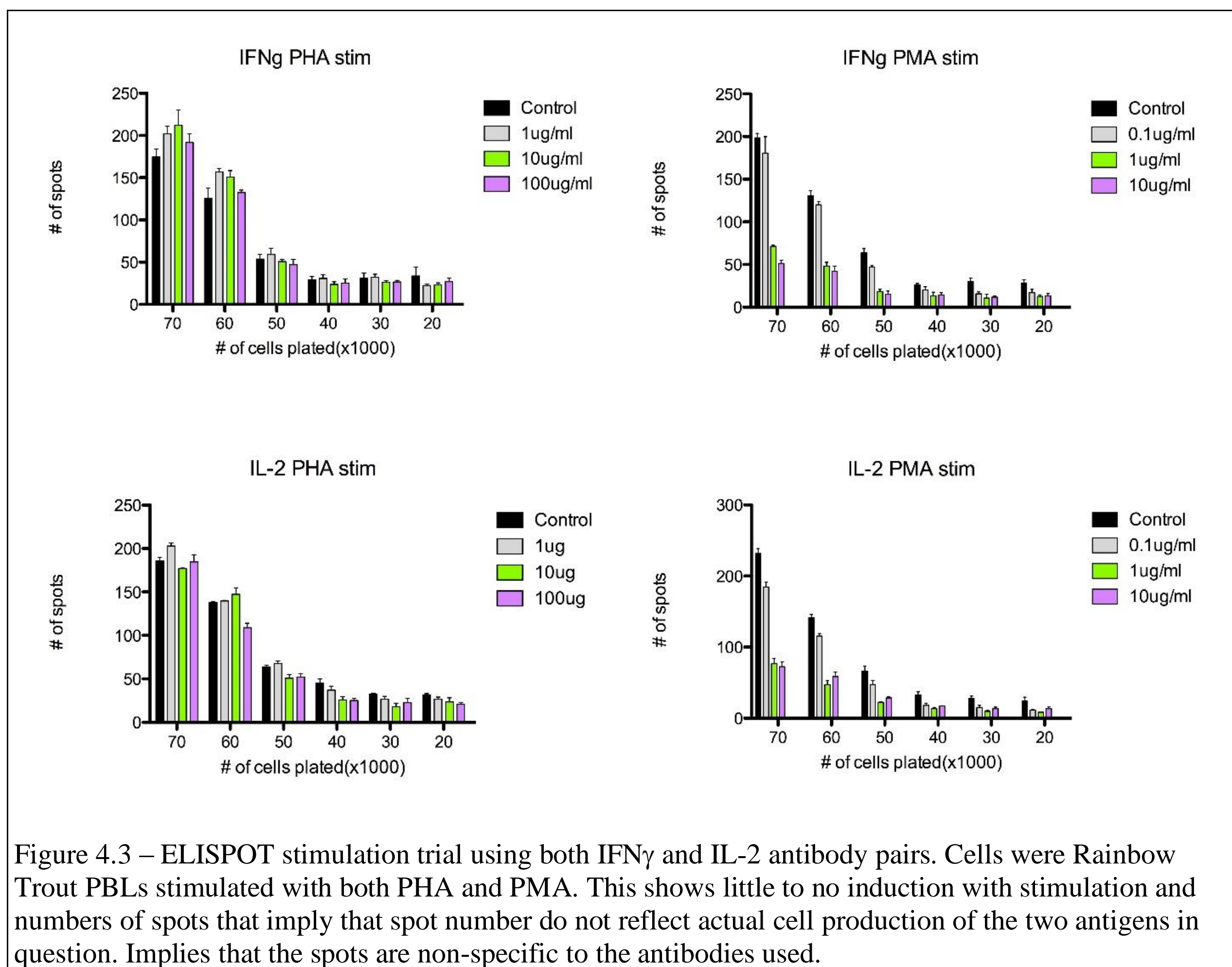


Figure 4.3 – ELISPOT stimulation trial using both IFNγ and IL-2 antibody pairs. Cells were Rainbow Trout PBLs stimulated with both PHA and PMA. This shows little to no induction with stimulation and numbers of spots that imply that spot number do not reflect actual cell production of the two antigens in question. Implies that the spots are non-specific to the antibodies used.

4.4.3 – Ingredient removal panel

The ingredient removal panel showed clearly that the removal of any one of the antibodies involved in the ELISPOT assay had no impact whatever on the quality or number of spots developed. Importantly, even the triplicate wells which did not receive any secondary detection antibody (and therefore no added

alkaline phosphatase enzyme) still showed the same number of spots as the control (Figure 4.4 D and A respectively).

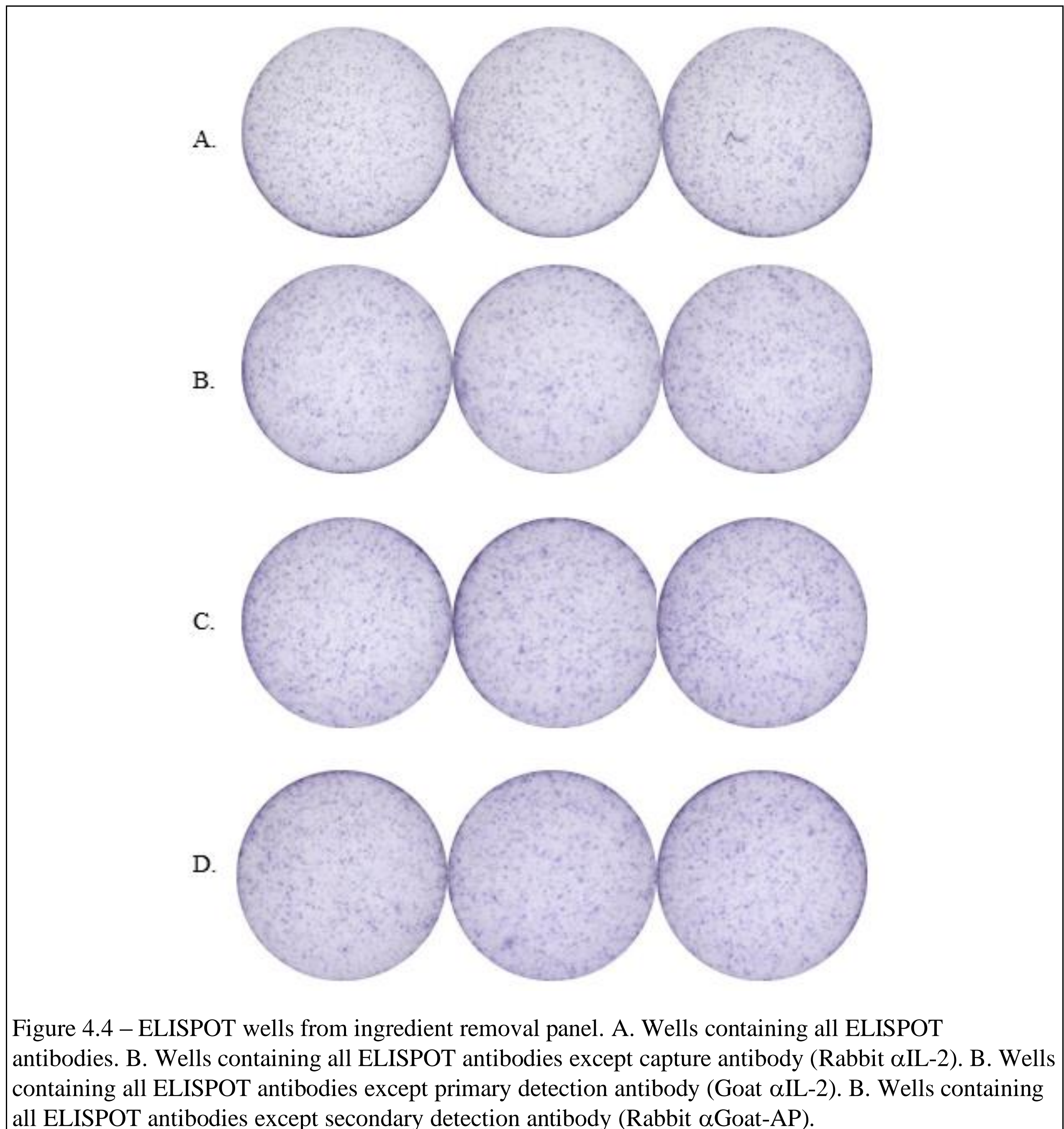


Figure 4.4 – ELISPOT wells from ingredient removal panel. A. Wells containing all ELISPOT antibodies. B. Wells containing all ELISPOT antibodies except capture antibody (Rabbit αIL-2). B. Wells containing all ELISPOT antibodies except primary detection antibody (Goat αIL-2). B. Wells containing all ELISPOT antibodies except secondary detection antibody (Rabbit αGoat-AP).

4.4.4 – Alkaline phosphatase ELISPOTs

Subsequent ELISPOT assays were carried out to confirm 1) that the spots were related to an alkaline phosphatase enzymatic reaction 2) whether there was differential production of AP between suspended

and adherent populations of leukocytes and 3) whether there was differential production of AP between diploid and triploid fish. Mixed PBLs (suspended and adherent) from both diploid and triploid fish showed significant reduction of spots in wells treated with the alkaline phosphatase inhibitor levamisole (Figure 4.5 A and D). For both types of fish it was observed that the remaining spots were exclusively derived from the adherent population of cells. A ten minute inhibition step was sufficient to remove all spot formation from diploid and triploid suspended PBL cells, which accounted for the great majority of spot formation overall (Figure 4.5 B and E). However, the adherent cell populations from both types of fish showed lower numbers of spots overall and had greater persistence of spot formation even in the presence of the AP inhibitor (Figure 4.5 C and F). Adherent cells derived from diploid fish still showed significantly lower spot numbers in the levamisole treated wells, while the numbers did not consistently differ across the plated dilutions of triploid adherent cells (Figure 4.5 C and F).

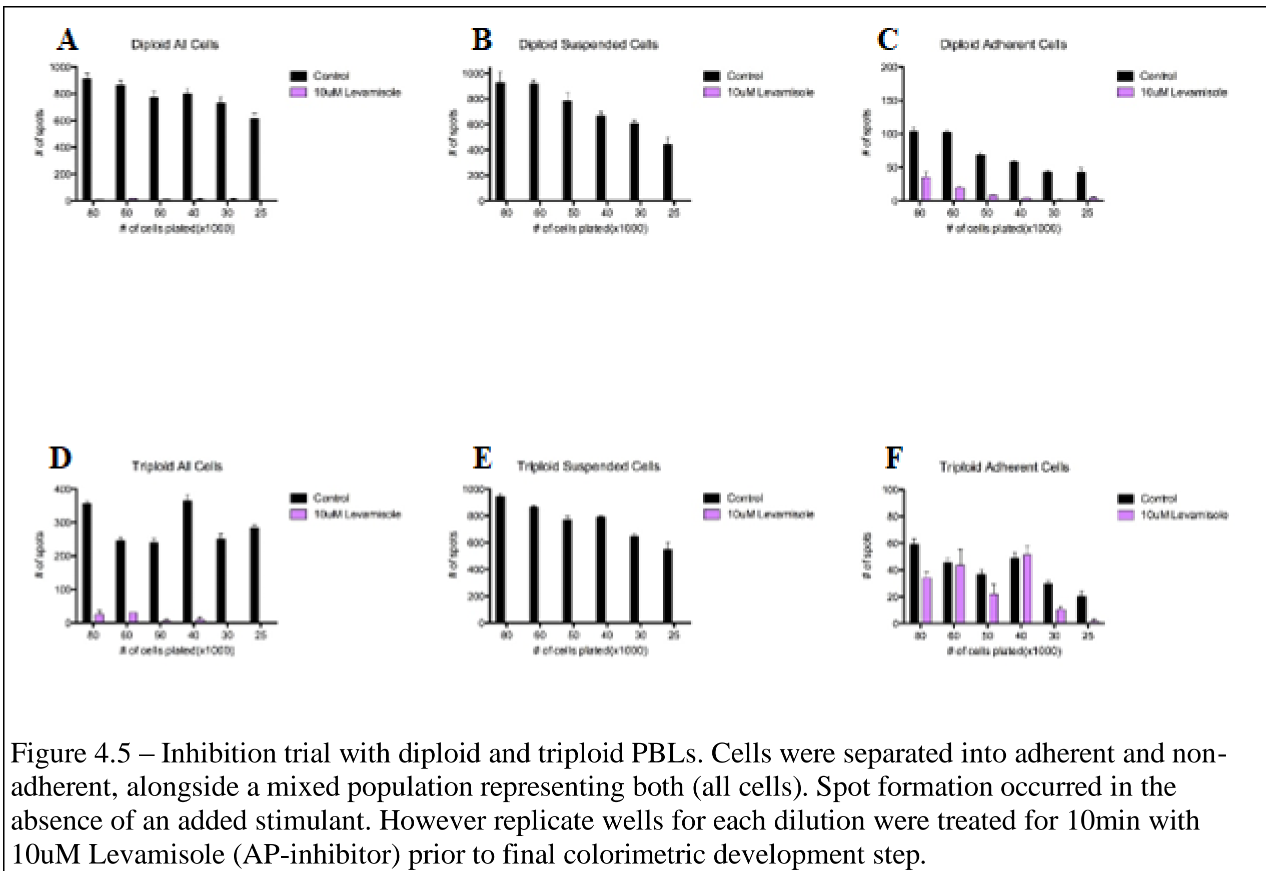


Figure 4.5 – Inhibition trial with diploid and triploid PBLs. Cells were separated into adherent and non-adherent, alongside a mixed population representing both (all cells). Spot formation occurred in the absence of an added stimulant. However replicate wells for each dilution were treated for 10min with 10uM Levamisole (AP-inhibitor) prior to final colorimetric development step.

4.5 - Discussion

The endogenous AP enzyme observed in this study was clearly derived from the white blood cell population obtained from fish blood. The spots it produced occurred in proportion to the number of PBL cells plated in each well. This indicates that a subset of the rainbow trout PBLs was producing the excreted, soluble AP enzyme. The likely cellular source of this endogenous AP enzyme is an immune cell (neutrophil). Neutrophil derived AP is typically localized in the cytoplasm and responsive to multiple disease states (Kubota & Haruta, 2006; Reviewed in Kaplow, 1971). Considering that all cells were removed from the ELISPOT prior to addition of AP substrate no cytosolic (cell derived) AP would be detectable in this ELISPOT. One possibility is that during the overnight incubation dying cells ruptured and deposited functional cytoplasmic AP onto the membrane. However, PBL cell survival was in the range of 90% following 24hr incubation under normal lab conditions when using rainbow trout, making it unlikely that the observed spots were derived from dead cells. I observed significantly more spot formation in suspended cells than in adherent cells at the same plating densities, but less complete inhibition of AP derived from adherent cells. This could indicate that either a) there are actually two sources of serum AP in Trout PBLs, with one producing a different isoform from the other, or B) neutrophils at different stages of activation produce different levels of endogenous, secreted AP. It has been demonstrated that the inhibitor used (Levamisole) is a potent inhibitor of one type of endogenous AP while having little effect on others (Harris, 1990). Thus, if a second circulating source of AP is present it may be enriched in the adherent cell subset and producing a different isoform of endogenous AP. This second isoform would be one of the forms upon which Levamisole is ineffective. Alternatively, it is possible that all of the spots observed were derived from neutrophils with suspended cells producing less secreted AP (and therefore showing more complete inhibition) and adherent, activated neutrophils producing so much more AP that inhibition was incomplete. If this were the case it would appear that activated neutrophils produce more AP from a smaller subset of cells, helping to explain why spot formation from adherent cells was so low comparatively.

A candidate for the second cellular source of AP would be the bone remodeling cell, the osteocyte. These cells are mainly involved in regulating bone formation and remodeling but have been implicated in arterial calcification associated with atherosclerosis (Wannamethee *et al*., 2013). Atherosclerosis of blood vessels is a well-documented and life-long aspect of the salmonid circulatory system (Farrell, 2002). Thus it is possible that our ELISPOT assay allowed us to visualize the mechanism by which salmonid circulatory health decays over their short lives. If so the level of serum AP enzyme could be an important biomarker of cardiac health in salmonid fish. Returning to the previous explanation, if the observed serum AP is derived from immune cells it could still be an important biomarker of immune status and function. The activity of neutrophils is related to acute inflammation, bacterial infection and other aspects of the

immune response. This easily, inexpensively measured parameter could be reflective of infection status and therefore vaccine effectiveness as well. If so, this assay has the added benefit of utilizing a non-lethal sample (a blood sample drawn from the caudal vein), which would enable better evaluation of vaccination protocols.

Another important consideration emerging from this AP adventure is the necessity of proper blocking during any Rainbow Trout (and likely other teleosts) ELISPOT assay. The spots emerged when the blocking step was omitted from the novel fish cytokine ELISPOT protocol. This omission was adopted from a human cell based ELISPOT protocol that apparently did not suffer from non-specific spot formation. In the event that cells sampled from other animal models also produce endogenous, serum-borne AP enzyme, any ELISPOT assay utilizing these cells would need one of three modifications at least. The first would be to replace the omitted blocking step as this would likely prevent the secreted AP enzyme from adhering to the ELISPOT membrane. The second option is to embrace the levamisole blocking step carried out in this study in order to ensure that spot formation only takes place where secondary detection antibody bound AP enzyme is located (levamisole is highly effective at blocking the action of one type of AP enzyme, so another isoform of the enzyme bound to detection antibody should not be affected). Finally, the secondary detection antibody could be conjugated to a different reporter enzyme, this would remove the necessity for AP development substrate and thus prevent the non-specific spot formation that was observed in this study.

A final consideration is the impact of this endogenous AP enzyme on the cells and solutions used in the assay. It is unclear whether the secreted AP may be activating cells, killing cells, changing pH or otherwise interacting with other components present in the plated cell mixture. As the cells must be left on the plate for extended periods in order to produce enough cytokine to form spots (especially in the case of salmonid PBLs) there is ample time for a secreted AP enzyme to have an impact. Also, seeing as the enzyme is ongoingly secreted from the cells it is very difficult to completely remove it during incubation. It may be necessary to include the inhibitor for the entire duration of cell incubation in order to obtain a true reporting of the cell's cytokine secretion profile.

4.6 – Conclusion

This study represents the first example of an ELISPOT assay being used to characterize endogenous AP in salmonids. This AP enzyme was uncovered due to the artefacts that it produced in an assay designed to target another protein. Further study is needed to identify the specific isoform of this enzyme, track down its cellular and tissue origin and evaluate its role in infection and fish health more broadly. While these results point to the possible utility of this enzyme they also suggest that AP based artefacts

could be a larger issue in ELISPOT and ELISA assays for fish in general. Many of these assays utilize AP as their final step and it is possible that without proper blocking or inhibition these AP enzymes could be contributing to noise in the assay's results. Uncovering this enzyme in this context may have identified an assay flaw that has flown under the radar for too long.

Further characterization of this possible immune marker is necessary. The cellular origin of this AP enzyme could be further characterized by stimulation or cell sorting experiments. Macrophage cell lines exist (RTS 11) that could be probed for this AP expression. Levels of this enzyme could be compared between young and old fish along with tests of vascular integrity in order to explore the possible link to atherosclerosis. Finally, it is possible that the AP derived spots detected in our (and possibly others) ELISPOT assays were such prevalent artefacts that they were entirely frustrating our ability to detect cytokines from cellular sources. Therefore, a repeat of the ELISPOT optimization process including Levamisole at each step (or using a different colorimetric enzyme) may yield success.

4.7 Acknowledgements

The authors would like to acknowledge the assistance provided by the entire crew at Alma Hatcheries in southern Ontario. Special thanks go to Marcia Chaisson at Alma for all her help organizing the sampling trips to her hatchery site. The assistance of Xiao-qing Dang was invaluable for obtaining samples and prepping cells for plating. Extra thanks belong to Dr Dixon for allowing the primary author to explore this interesting artefact of an assay designed for another purpose.

5.0 – General Discussion

5.1 – Impact of Telemetry Tag Implantation

Tagging of animals remains the only option for data gathering much of the long term, natural setting research that goes on today. It is important to reconsider our assumptions, revisit previous studies and re-evaluate the conclusions that lead us to trust the data from such research. This work explores a largely uncharted section of scientific inquiry. The author has discovered a paucity of literature exploring the long-term impacts of telemetry tagging at the cellular or molecular level. While this complicates the task of placing our findings in the context of current literature it also points to the innovative nature of the work described here.

In this study we sought to evaluate a previously unexplored aspect of tag (and implantation) impact. As revealed previously (Semple *et al*., 2018), tag implantation turns out to carry with it a significant immune impact related to tag presence and coating type. This cost, associated with the immune response to tag implantation is piled on top of the significant energetic challenges faced by migrating salmonids (Hinch *et al.,* 1996). The implications of this work are significant for the evaluation of tag studies and the conclusions reached. Indeed, this work calls into question the accepted view that telemetry tag implantation does not significantly alter the behavior of tagged animals. This view is based on literature that did not include examination of the immune parameters that were studied here. The evidence of immune activation, even after a much longer period than most tag experiments cover, suggest strongly that the tagged animals are different from the natural population. If nothing else, at the level of energetics, some trade-off must be occurring in tagged animals between immune response and some other aspect of fish survival (Garland, 2014). Even if the only impact is a reduction in energy stores, due to the costly immune induction, this would still be anticipated to influence overall viability. Furthermore, certain aspects of teleost immunity, such as behavioral fever, indicate a direct link between immune status and behavior patterns (Reynolds *et al*., 1976). It is possible that there are more subtle interactions between immunity and behavior that are being played upon by the implantation of a large foreign object into the peritoneum of salmonids. If so, this would represent a previously unrecognized alteration to fish behaviour that has been distorting the results of experiments that use telemetry tags. This is an important discovery for two reasons, first it suggests that previous work with telemetry tags needs to be re-evaluated and second it underscores the necessity of further tag optimization.

In the course of the tag trial we explored the idea of tag optimization by including multiple types of tag coating in our experiment. The results suggest that there are differences in how tag coating composition interacts with the immune system. Circulating leukocytes showed lasting upregulation of pro-inflammatory cytokines, likely induced by a prolonged FBR in the peritoneum. Levels of peritoneal

immunoglobulin (IgM) and total protein were elevated in tagged fish and remained so even after two months. Contrastingly, peritoneal IgT and IL-1β proteins were depressed at the early time point and rebounded to control values in only Star Oddi and sham surgery fish. As discussed previously there was a disconnect between the IL-1β protein in the peritoneum (downregulated) and the *il1b* mRNA transcript in spleen (upregulated). This is likely due to a macrophage dependant repression of mature IL-1β release. Indeed, IL-1β protein (and therefore it's mRNA precursor) typically build up in cells prior to inflammasome formation and the subsequent caspase-1-dependant processing and release of mature IL-1β protein (Dinarello, 2018). This action of tissue resident macrophages (Ipsiez *et al*., 2020) is marked by expression of IL-10, an important anti-inflammatory signal of immune response resolution rather than activation (Sabat *et al*., 2010). In this context, we identify Star Oddi tags as being presenting the most biocompatible surface in this study. Star Oddi tag implantation induced less pro-inflammatory cytokine in the circulating leukocytes and saw levels of intraperitoneal IL-1β protein return to control levels more rapidly than any other condition aside from sham surgery. This finding underscores the importance of the protein level assays developed for this expanded tag trial. Previous work in fish immunity was unable to examine the active form of cytokines – the protein – due to lack of assays (Dixon *et al*.,2018). Without the protein level data in this study, it would difficult to make any inference regarding the progression of the inflammatory process, as evidenced by the contradictory signals from *il1b* mRNA and protein. The development of these protein level assays represents and new tool in the study of salmonid immunity and it should be no surprise that they reveal previously hidden patterns. One important assumption throughout this work has been that the antibodies used in these protein level assays are specifically detecting the cytokine protein that they are targeting. The antibodies were used to detect both recombinant and native cytokine proteins. Further, in the blocking experiments both recombinant and native protein samples behaved in a manner that indicates antibody specificity. In an ideal world these antibodies would be used on a column to collect the protein they bind to and then this protein would be, in effect, sequenced using mass spectrometry.

Surgical wound closure appeared to progress without issue all the way to two weeks post surgery. However, between two and ten weeks (day 70) most of the surgical wounds began showing signs of infection and inflammation. Although this was not mirrored in the qPCR data deriving from muscle samples (it is possible that sampling epithelial tissue from the surgical site would be better) the infection seemed to be associated with the stitches used to close the wounds. This represents another finding that has not been prominent in the literature on tag implantation, literature which usually follows animals for between 10 and 15 days post surgery. This is perhaps unsurprising as, in our study, at day 14 the surgical wounds appeared closed from the outside. It is likely that the skin on the exterior surface is better adapted

to closure of wounds, whereas the interior surface of peritoneum is not. Taking this view, the exterior wound closed over a normal time frame but the peritoneal side of the wound closed more slowly. This is supported by observation that, when checking peritoneal tag location in tagged fish, the wound on the peritoneal surface was unresolved in all fish. Indeed, in several cases, parts of the pleura were attached to the surgical site, even in sham surgery controls. In mammals, injuries that open the peritoneal space have been associated with something called 'abdominal compartment syndrome' (Roberts *et al*., 2016). This syndrome is characterized by an inflammatory response that affects intra-abdominal pressure (Leng *et al*., 2014) and contributes to impairment of organ function (Kirkpatrick *et al*., 2014). Considering that this prolonged syndrome is mammals occurs because of the wound to the gut wall, and not the epithelial wound, a similar process may be induced in tagged fish. If so, the increased intra-abdominal pressure may be impacting fish behavior, as the fish's abdominal muscles are essential to swimming. Thus, this work suggests that behavioral alteration in tagged fish may only arise later as a result of the surgical wound, which appears closed upon exterior inspection.

Encapsulation of the implanted tags occurred in all tag types, implicating a peritoneal foreign body reaction. Expression of pro-inflammatory cytokines was consistent with aspects of the foreign body response, suggesting the presence of neutrophils and macrophages that attempt to engulf the foreign body. Increases in total protein, both immediately following implantation and still at day 70, could reflect the deposition of the fibrous capsule by active cells in the peritoneum. Again, it would stand to reason that the energy involved in this FBR must be borrowed from some other aspect of the fish's homoeostasis, putting the fish at an energetic disadvantage relative to conspecifics.

5.2 – IFNγ Protein Assay

The IFNγ protein assay utilized polyclonal antibodies designed to target epitopes on recombinant salmonid cytokines produced in *E. coli*. The successful ELISA assay, capable of detecting picogram amounts of native protein represents an important contribution to the study of salmonid health (Dixon *et al*., 2018). The polyclonal antibodies used were validated using assays testing binding to native protein binding and competitive blocking assays. Observed patterns of native IFNγ detection corresponded with expected protein size, cellular origin and timing of induction (Yoon *et al*., 2016). IFNγ was detectable, using these antibodies, in western blots using a non-lethally obtained sample of blood from fish.

IFNγ protein was measured in the peritoneum following a telemetry tag implantation experiment using our novel protein level assay. This pro-inflammatory cytokine was, interestingly, unaffected at the early time point but then downregulated in both tagged individuals and sham surgery controls. This again suggests the macrophage dependent suppression of pro-inflammatory signal in the peritoneum (Ipseiz *et al*., 2020). In as much as this is related to resolution of the inflammatory response it suggests that the

uncoated tags (Star Oddi and Regular Vemco) possessed the most biocompatible surface for peritoneal implantation. Another interesting finding from this ELISA is the fact that sham surgery controls also showed reduction in IFNγ at the day 70 timepoint. This points to a late onset or chronic effect of the surgical wound, quite apart from tag presence. There are two distinct possibilities when it come to surgical wound related issues, inflammation (Capobianco *et al.*, 2017) and infection (Mais, 2009). Inflammation resulting from the physical surgical wound would be expected to arise sooner and resolve more quickly than inflammation arising from the immune system's response to infection which requires formation of inflammasome structures (Christo *et al*., 2016). Thus, the delayed nature of the IFNγ reduction in sham surgery controls suggests that infection, resulting from microorganisms accidentally introduced in the surgery process, was still occurring at this later timepoint. Although one would expect induction of IFNγ expression if infection was occurring (Borre *et al.*, 2006) it is possible that drastic alterations in peritoneal cell populations were occurring even in response to surgery alone (Capobianco *et al*., 2017). Combined with the changes in peritoneal cell populations that are associated with FBR activity (Littikhuizen *et al*., 2006) this could have resulted in the pattern of IFNγ reduction observed in this study. It would appear that more than one source of inflammatory signal is involved in the response to surgery and tag implantation, making it hard to identify the specific culprit. Regardless, the clear indication from this work is that there are effects on fish health (detectable, apparently, only at the level of immune activation) that result from the implantation surgery, the presence of large telemetry tags and the surface material of the tag that is encountered by cells of the peritoneum.

A future direction for this would be to track the anti-inflammatory cytokine IL-10 (Sabat *et al*., 2010) in an effort to describe the resolution of the early inflammatory process. This could help delineate between the immediate inflammatory response to surgery/implantation and the more prolonged foreign body response occurring in the presence of tags.

Moreover, the ability to measure IFNγ protein in a non-lethal sample has significant ramifications for the study of salmonid immunity. This cytokine is involved in both innate (rapid) and adaptive (delayed) immunity (Schoenborn and Wilson, 2007), both of which are important for overall fish health. The ability to track immune system activation at the level of protein could lead to improvements in feeding and feed additives, optimization of microbiome flora as well as vaccine candidates and additives. All of this would improve the health of salmonids in aquaculture, preventing both economic loss and the loss of food security.

5.3 – Endogenous alkaline phosphatase ELISPOT

This study also yielded an unexpected circulating protein that could be used for assessing salmonid health. Circulating alkaline phosphatase level in blood has been used an easily measurable indicator of

health status (McComb, 1979). The utility of blood-borne AP as a health indicator in salmonids remains to be demonstrated. However, in other models, AP levels in blood are related to skeletal, hepatobiliary and cardiac health (Gutman, 1959; Tonelli *et al*., 2009). If the same is true in fish, the endogenous AP measuring ELISPOT developed in this study could improve our understanding of the challenges faced by the aquaculture industry, specifically an emerging issue related to cardiac pathology (Brun *et al*., 2003).

Many protein-based assays utilize an alkaline phosphatase as their final color development step. Even so, this study is the first, to the author's knowledge, to identify the interaction between an endogenous AP enzyme and the color development substrate. Specifically in ELISA or ELISPOT assays that involving plating live cells there is a significant danger of mistaking signal coming from cells depositing endogenous AP (noise) and AP enzyme that is present because it is conjugated to a detection antibody (signal). Any assay that uses cell capable of producing endogenous AP could have it's signal confused by the presence of this, previously unrecognized, noise in the system. Indeed, the work presented here suggests that extra controls should carried out alongside the normal set of controls designed to remove this noise. These controls would take the form of a selective inhibitor (different types of AP, including the one conjugated to detection enzymes are inhibited by different compounds [Sharma *et al*., 2014]) and a set of wells with appropriate blocking to eliminate the non-specific binding of secreted AP enzyme to the plate. Another possibility is to utilize a different enzyme for colorimetric development in the terminal steps of the assay. In lieu of these controls it remains unclear if spots (or increased absorbance) is really due to increased interaction between antibody and target, or simply due to an unforeseen product deposited by the cells in question. Development of truly effective antibody-based assays for salmonids will have to account for this endogenous AP enzyme.

5.4 - Conclusion

The information gleaned from the ELISA and ELISPOT assays developed for this study is an important indicator that previous experiments suffer from a lack of protein level information. mRNA transcripts are easy to obtain, amplify and quantify, however mRNA is not, typically, a bioactive molecule. Since protein is the level at which most immune cells actually interact with the world it is clearly of prime importance to understand the function of any gene in question. This is especially true of the cytokines for a couple of reasons: 1) cytokines are constitutively expressed at low levels 2) cytokines are extremely bioactive, even at low concentrations and 3) because of 1) and 2) cytokines are tightly regulated and can occur in unbalanced ratios of mRNA transcript to active protein – as seen with IL-1β here.

This study was conceived with the purpose of revealing the important, and largely ignored, problem with studying the transcript level of physiology while avoiding the more difficult exploration of the

protein level. This study not only expanded the availability of tools for studying the protein level in teleost fish, but it also demonstrated the lack of perfect cohesion between transcript and protein level actions of the immune system. It should be anticipated that most other aspects of physiology will show a similar pattern of occasional disagreement between results from these two levels of analysis. This disagreement is certain to encompass information that is vitally important to solving the problems faced by aquaculture.

This work addresses a question that was deemed answered. Information on impacts of tag implantation is scarce and only addresses metrics that are easily obtained (body condition, growth [Klinard *et al.,* 2018], tag retention, swimming speed [Curtis *et al*., 2019] and stress markers [Jepsen *et al*., 2001]). With the assays developed in this work the study of immune impacts (tag related and otherwise) is greatly empowered. Much of the work that only examined the mRNA transcript of a candidate gene should be revisited with an eye to confirming what was found (at the transcript level) at the level of protein. It is anticipated that protein dynamics will differ from transcript dynamics in ways that are meaningful to whatever study is being carried out. This study represents not only advancement on the frontier of understanding the actual agents of molecular and physiological action (proteins) but also an illustration of the importance of this new stream of information. This work suggests that telemetry tag technology, from implantation to surface material, can be further optimized in a system that is more precisely tracking negative impacts. Better information about the impacts of tags will lead more precise data and guide the search for true biocompatibility.

6.0 – References

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