Stony Brook University

The Effects of Dietary Immunostimulation on Atlantic Salmon (*Salmo salar*) Immune Response to Sea Lice (*Lepeophtheirus salmonis*) Infection

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Sarah Ellen Friend

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Sarah Ellen Friend

We, the thesis committee for the above candidate for the

Master of Science degree, hereby recommend

acceptance of this thesis.

Mark D. Fast, M.Sc., Ph.D. – Thesis Co-Advisor
Adjunct Assistant Professor
School of Marine and Atmospheric Sciences
Stony Brook University

and

Novartis Research Chair in Fish Health
Department of Pathology and Microbiology
Atlantic Veterinary College, University of Prince Edward Island

Anne E. McElroy, Ph.D. – Thesis Co-Advisor
Associate Professor
School of Marine and Atmospheric Sciences
Stony Brook University

Ian R. Bricknell, Ph.D – Reader
Director, Aquaculture Research Institute
Libra Professor of Aquaculture Biology
Cooperating Professor Animal &Veterinary Sciences
School of Marine Sciences, University of Maine

This thesis is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Thesis

The Effects of Dietary Immunostimulation on Atlantic Salmon (*Salmo salar*) Immune Response to Sea Lice (*Lepeophtheirus salmonis*) Infection

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The ectoparasitic copepod, *Lepeophtheius salmonis,* is considered to be the most economically damaging parasite in commercial Atlantic salmon (*Salmo salar*) culture, causing serious disease outbreaks which cost the industry nearly half a billion dollars annually. While other salmonid species show some resistance to this parasite, Atlantic salmon have previously shown very little in the way of inflammatory response to sea lice infection. The objective of this study was to enhance the immune response of Atlantic salmon to sea lice infection through the administration of in-feed immunostimulants. The efficacy of dietary additives to stimulate the immune response of Atlantic salmon against *L. salmonis* was evaluated over three trials. Trial 1 tested three immunostimulants: ProVale (β-glucan), All Brew/ Nupro (commercial yeast extracts) and CpG ODN during a low-level sea lice exposure in Atlantic salmon. Fish fed CpG ODN showed the greatest reductions in sea lice, which was accompanied by an increase in inflammation observed in histological sections of the lice infection. Up-regulation of IL-1β and MMP 9 was also

observed in CpG ODN fed fish following sea lice exposure. All Brew/ Nupro was also found to be effective at reducing the final sea lice burden though these reductions did not correlate to changes in tissue response or gene expression. The ProVale additive was not effective at reducing infection levels at the dose administered. Trial 2 investigated whether CpG ODN, administered at half the original dose and for a shorter time, could enhance acquired immune responses following experimental re-infection with *L. salmonis.* Both the previously infected control and CpG ODN fed fish showed greater reductions in sea lice abundance indicating that prior exposure to sea lice offered some protective benefits during re-infection. Sea lice reductions in the CpG ODN fed group exceeded the previously infected control indicating that treatment with CpG ODN conferred protection beyond prior exposure alone and may contribute to the activation of adaptive immunity in Atlantic salmon. The objective of Trial 3 was to compare the effects of dose during a low level sea lice infection using a commercial mixture of immunostimulants, administered at a high and low dose. The low dose reduced the sea lice burden in infected salmon by 48% while the high dose provided little protective benefit. No differential gene expression was observed in this trial, thus leaving the mechanism for dose related responses of these treatments unknown. Overall, this study demonstrated the effectiveness of CpG ODN as a dietary immunostimulant during both primary and secondary exposure to sea lice and illustrated the importance of dose to the effectiveness of boosting innate immunity. Furthermore, the immune genes IL−1β and MMP 9 were identified as being involved in the early inflammatory response and wound healing and are likely associated with increased resistance following oral administration of CpG ODN. Incorporation of immunostimulants into future sea lice reduction protocols, either alone or in conjunction with existing methods and treatments is likely to have direct benefits to Atlantic salmon farm management strategies.

DEDICATION PAGE

This work is dedicated in memory of my mother,

Janet S. Friend,

whose love, guidance and friendship will never be forgotten.

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INTRODUCTION

As human populations continue to grow, this year (2011) exceeding 7 billion worldwide, tremendous pressure has been placed on global fisheries to meet the demands for food fish. In many regions, as the demand for high quality fish exceeds what capture fisheries can supply, commercially cultured fish now make up a significant portion of the seafood market. The United Nations Food and Agriculture Organization (FAO) reports that in 2006, of the 110 million tons of harvested fish, nearly half was supplied by aquaculture (FAO, 2008). Among food fish species, the Atlantic salmon (*Salmo salar*) represents a significant share of this industry. As a result of over exploitation of the fishery, in 2000, the US Fish and Wildlife service listed the status of wild Atlantic salmon as endangered in the state of Maine. While wild fish stocks have remained in decline, the Atlantic salmon farming industry has grown dramatically over the last 30 years to accommodate the increasing desire for this popular food fish (Costello, 2005; Costello, 2009; Pike and Wadsworth, 2000). Atlantic salmon culture now represents a highly valuable industry, with over 1.4 million tons produced through aquaculture in 2008, worth approximately $7.2 billion USD (FAO Fisheries and Aquaculture Statistics, 2010).

Atlantic salmon are primarily cultured in sea pens in the North Atlantic, however culture operations have now spread to regions in the Pacific Northwest United States and South America. Norway, Chile, Scotland, Ireland, Iceland, Australia and Canada are currently responsible for production of the majority of cultured Atlantic salmon, though smaller industries also exist, such as in the state of Maine. Despite our growing

dependence on aquaculture, infectious diseases persist as an obstacle to the successful production and economic profitability of commercial fish culture (Carrington and Secombes, 2006). Over reliance on chemotherapeutic compounds such as antibiotics and parasiticides has, in many cases, led to resistance and decreased efficacy of many common treatments against economically important pathogens. Pathogens of salmonids have been receiving increasing research interest, mainly due to the value of the industry and the associated costs of disease prevention, control and losses.

The Problem

Sea lice are recognized as important ectoparasitic arthropods impacting marine aquaculture of finfish, including salmonids. The global economic cost of sea lice control to the salmonid farming industry remains high, with estimated costs near US$480 million dollars in 2006 (Costello, 2009). Sea lice is a general term applied to several species of parasitic copepods within the Family Caligidae, including *Lepeophtheirus salmonis*, the most economically important parasite of Atlantic salmon, *S. salar* (Johnson et al., 2004). *Lepeophtheirus salmonis* has been a major disease in salmon aquaculture since the beginning of major production in the 1960s (Pike and Wadsworth, 2000). This crustacean has a direct life cycle consisting of 10 development stages. Adult female *L. salmonis* carry two strings of eggs that hatch into non-feeding, planktonic nauplius larvae. A second naupliar stage is followed by an infectious copepodid stage. After the free-swimming copepodid infects a salmonid host, four attached chalimus stages precede two mobile pre-adult stages. After this succession of molts, the reproductive adult stage is finally reached (Johnson and Albright, 1992) (Figure 1).

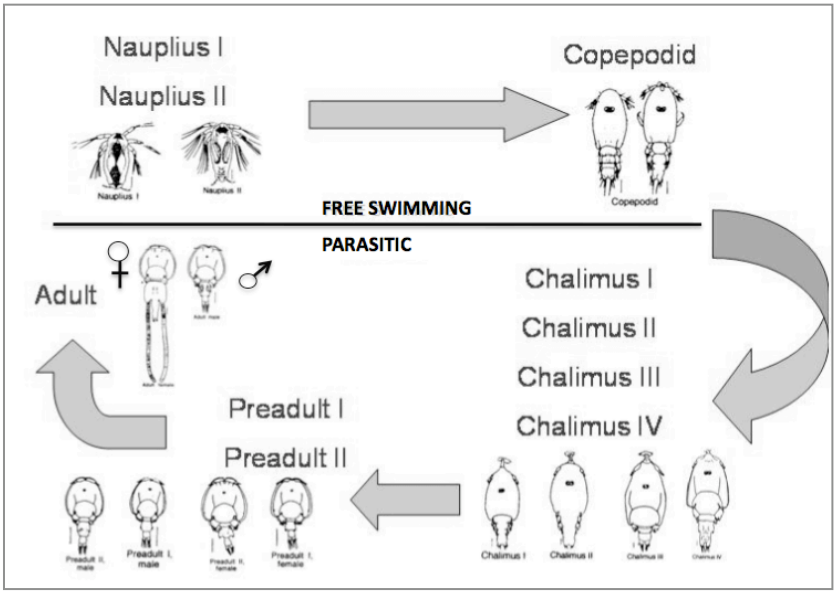

Figure 1. Life cycle of *L. salmonis* (Adapted from Burka et al., 2011)

Sea lice feed on the skin, mucus and occasionally the blood of their hosts, causing damage that ranges from minor skin irritation to serious epithelial erosion, osmoregulatory stress and death in susceptible hosts [i.e. Atlantic salmon] (Grimnes and Jakobsen, 1996; Johnson and Albright, 1992). Infections have traditionally been managed through chemical parasiticide applications. The avermectin treatment, emamectin benzoate, known commercially as SLICE® has been the most common and effective treatment against sea lice since 2002 (Burridge et al, 2010). Unfortunately, recent over and potentially improper use has lead to sea lice resistance (Lees et al., 2008). This has major implications for sea lice control in aquaculture and ecological repercussions as uncontrolled sea lice infection may spread to wild salmonids.

Inflammation and Innate Immunity in Fish

In jawed vertebrates, the immune response is generally divided into two mechanisms of defense: innate and adaptive (or acquired) immunity. The primary features that differentiate them are the types of receptors used to recognize pathogens (Alvarez-Pellitero, 2008; Medzhitov, 2007). Innate immune cells, including macrophages, monocytes, and neutrophils, contain pattern recognition receptors (PRRs) that can identify and link to pathogen associated molecular patterns (PAMPs) associated with microbes (Carrington and Secombes, 2006; Magnadottir, 2010). These are greatly conserved among specific classes of microbes. PRRs are involved in executing particular tasks including phagocytosis and initiation of the complement cascade (Alvarez-Pellitero, 2008). The adaptive immune response relies on recognition by antigen receptors found on B and T lymphocytes, which are specific to particular pathogens and form the basis for immunological memory. The innate and adaptive immune responses are not discrete and independent from each other but are connected through many immunological pathways. However, in poikilotherms, such as fish, the adaptive immune reaction is slow to respond to pathogen insult taking up to several weeks to confer resistance against infectious organisms (Jones, 2001). Therefore, the innate immune system represents the first and sometimes most important line of defense against infectious pathogens.

Innate immunity can be further divided into three components: the mucosal epithelia, humoral, and cellular factors (Alavarez-Pellitero, 2008; Magnadottir, 2010). Mucus, scales and skin create the first barrier of innate defenses that pathogenic organisms, including ectoparasites, must breach (Magnadottir, 2006). The mucosal layer not only forms a physical barrier, but also contains receptors of both innate and adaptive

systems, as well as humoral and cellular components with bactericidal activity, such as lysozyme, complement and antimicrobial peptides. When epithelial tissues are damaged by infection, mucus secretion tends to increase, often in association with cellular hyperplasia (Jones, 2001). This signals the initiation of the inflammatory reaction intended to eliminate the injuring pathogen and seal the epithelial break.

Inflammation is seen primarily as a cellular response but is mediated by many secreted humoral factors. When the epidermal layers are broken, such as through infection with ectoparasites, tissues typically display a marked epithelial hyperplasia at the site of injury as well as an influx of neutrophils, eosinophils, macrophages, and, to a lesser extent, lymphocytes. This can lead to the formation of granulomas, which in some cases may encapsulate the parasite. Phagocytes that have been activated through the linking of PRRs with PAMPs demonstrate respiratory or oxidative burst through the release of reactive oxygen species (ROS) or nitric oxide (Alvarez-Pellitero, 2008; Magnadottir, 2010). As the innate immune cells are activated through interaction of PRRs with PAMPs, signaling molecules known as cytokines are produced. These molecules are crucial to induction of both the innate and adaptive responses (Magnadottir, 2010). Production of pro-inflammatory cytokines, such as Interleukin 1β (IL-1β) and Interleukin 8 (IL-8), set off an immune response that results in recruitment of leukocytes, as well as antigen presentation and the activation of T cells (Fast et al., 2006a). IL-1β stimulates a variety of immune responses such as initiating apoptosis, as well as inducing the multiplication of macrophages and recruitment of leukocytes. IL-8 is involved in mobilizing neutrophils to the site of parasite attachment. Its up-regulation

has been indicated as important in rejection of parasites by infected hosts (Jones et al., 2007).

Following the elimination of invading pathogens or other sources inflammation, tissue remodeling and wound repair processes are initiated to seal the epithelial breaks. Because fish are immersed in their aqueous environment, rapid wound healing after injury or infection is crucial to avoiding osmotic imbalance. Matrix metalloproteinases (MMPs) are typically inactive when fish are healthy and uninjured. However, following damage to the epithelial layers, they become stimulated and induce multiple processes including cytokine release, remodeling and destruction of the extracellular matrix as a precursor to wound repair (Skugor et al., 2008). The MMP family contains at least 25 related enzymes found in vertebrates (Parks et al., 2004). MMP 9 has been associated with the remodeling and repair of tissues in both mammals and fish and is thought to be involved with both the initiation and resolution of inflammation in these animals (Chadzinska et al., 2008, Sutherland et al., 2011).

Host responses to Infection

While *L. salmonis* is known to infect all salmonids, some host species display more resistance than others (Fast et al., 2002; Johnson and Albright, 1992; Jones et al., 2007). Specifically, Atlantic salmon appear more susceptible to infection than other salmonids such as coho (*Oncorhynchus kisutch)*, Chinook (*Oncorhynchus wytscha)*, rainbow trout (*Oncorhynchus mykiss)* and pink salmon (*Oncorhynchus gorbuscha)* (Fast et al., 2002; Johnson and Albright, 1992; Jones et al., 2007). The ability to mount a strong inflammatory response has been correlated with the ability to resist infection (Johnson

and Albright, 1992). In their study of the susceptibility of different salmonids to *L. salmonis* infection, Johnson and Albright (1992) observed that coho salmon, *O. kisutch*, display heavy epithelial hyperplasia on their fins and gills leading to the loss of the parasite. In severe cases of infection, by 10-20 days post infection (dpi) the epithelial hyperplasia progressed to partial or complete encapsulation of the copepod. The interstitial spaces surrounding the encapsulated parasite were filled with a tissue debris and mixed inflammatory cells consisting mainly of neutrophils, macrophages and some lymphocytes. In contrast, Atlantic salmon exhibited only a minor tissue response in reaction to *L. salmonis* infection resulting in heavier infestation levels. At feeding sites, breaks in the epidermis exposed the underlying dermis and fin rays to the external environment with only minor inflammation present. Secondary bacterial infections were present at the location of the fin lesions. Chinook salmon showed similar susceptibility to infection as Atlantic salmon with the exception that sea lice developed at a slower rate on Chinook salmon indicating that they may be somewhat intermediate between coho and Atlantic salmon in their susceptibility to *L. salmonis* infection (Johnson and Albright, 1992).

Similar results showing that more resistant species show greater tissue and inflammatory responses accompanied by lower lice prevalence and slower maturation have been reported by other studies (Fast et al., 2002; Jones et al., 2007). Fast et al. (2002) also found Atlantic salmon to be less resistant than other related species, in this case coho salmon and rainbow trout. This was accompanied by a significant reduction in phagocyte activity and suppression of respiratory burst activity from the head kidney macrophages of Atlantic salmon. No significant differences were observed in the blood

physiology between species leading the authors to conclude that differences in resistance to sea lice infection are present in the mucus and epithelium of infected fish (Fast et al., 2002).

A rapid reduction in sea lice abundance on resistant species has been reported by multiple studies (Fast et al., 2002; Johnson and Albright, 1992) indicating that innate immune factors may be responsible for rejection of parasites. Differential expression of pro-inflammatory genes between resistant and susceptible species seems to back this hypothesis. Jones et al. (2007) reported a more rapid loss of sea lice in pink salmon over chum salmon. When pro-inflammatory gene expression was compared between the two species, significant up-regulation of IL-8 and tumor necrosis factor (TNFα-1) was observed in pink salmon over non-exposed control fish. No difference was seen in IL-8 expression in chum salmon and TNFα-1 was found to be down-regulated in response to infection.

Gene expression studies in Atlantic salmon infected with *L. salmonis* indicate that sea lice can have an overall suppressive effect on immunity in this species. Recently, Skugor et al. (2008) found that many anti-inflammatory genes were up regulated in Atlantic salmon while pro-inflammatory genes and genes involved in wound healing were down regulated in response to heavy sea lice infection. Sea lice stage is considered to be an important factor in the observed immune response. While expression levels of inflammatory genes may show early up-regulation shortly after lice attachment, depression of levels is observed during later stages of infection (Fast et al., 2006a). The down-regulation of pro-inflammatory gene response tends to be maintained during the attached chalami stages of infection, then increase again following the molt to the mobile

pre-adult stages (Fast et al., 2006a; Skugor et al., 2008). At this point in the infection, an increased host immune response is not likely to be effective against a mobile parasite, which emphasizes that it is essential to control the infection and stimulate the host immune response during the early stages of infection.

Similar to other ectoparasitic arthropods, *L. salmonis* has been found to secrete multiple chemical compounds that may be involved in modulation of the immune response in its hosts. Trypsin and Prostaglandin E_2 (PGE_2), as well as other undescribed molecules secreted by *L. salmonis*, may be responsible for the poor inflammatory response seen in Atlantic salmon. Immunomodulation by these chemicals has been recorded both *in vitro* (Fast et al., 2004; 2007) and *in vivo* (Fast et al., 2002). Trypsin is a protease molecule that can inhibit phagocytosis and limit respiratory burst in leukocytes in *S. salar* (Fast et al., 2002; 2003). Prostaglandin E_2, a common component of arthropod salivary secretions, has been shown to inhibit MH class I and II gene expression, down regulate inflammatory cytokines (IL-1β and TNF α), and modulate the response of T_h lymphocytes (Fast et al., 2004; 2005). Host mucus, the primary component of the diet of *L. salmonis*, may also be increased by the presence of PGE_2 (Fast et al., 2004).

Immunostimulation in Fish

Immune system activating compounds, known as immunostimulants, may provide an alternate means of improving disease resistance in cultured fish (Bricknell and Dalmo, 2005; Magnadottir, 2010). Many known immunostimulants are compounds derived from potential pathogens, such as lipopolysaccharide (LPS) from bacterial cell walls and β-glucan from yeast cells (Bricknell and Dalmo, 2005; Magnadottir, 2010). These

compounds have found increasing use in many economically important species, often combined as an adjuvant during vaccine delivery. Though many adjuvants have been found to improve immune response in fish, they may cause damage to tissues when injected, reducing the usefulness in commercial culture situations where tissue quality is important to consumer satisfaction (Carrington and Secombes, 2006).

While immunostimulants may be derived from a variety of natural sources, such as bacterial, fungal, or parasitic origins, synthetic compounds have also been found to have strong immunostimulatory action. Cytosine-guanine oligodinucleotides (CpG ODNs) are a group of synthetic immunostimulants that mimic the activity of bacterial DNA, thus engaging both the adaptive and innate immune systems (Lacroix-Lamonde et al., 2009; Magnadottir, 2010). CpG ODNs show potential to be potent immune stimulants, without inducing tissue lesions at the site of injection (Rhodes et al, 2004). Recent work testing CpG ODN as a dietary immunostimulant in mice found that it is also promising as an adjuvant for oral vaccine delivery (Lacroix-Lamonde et al., 2009). This is important in food fish as it not only avoids the negative tissue reactions often observed after vaccine injection but also circumvents the stressful and labor intensive process of injecting thousands of fish. Furthermore, dietary immunostimulation can be maintained over a longer period as opposed to a one-time injection treatment. β-glucans, derived from yeast cells, are one of the most commonly used immunostimulants (Guselle et al., 2010; Magnadottir, 2010), both as a feed additive and intraperitoneal (IP) injection. While IP injection of β-glucans have been shown to be more effective than feed additives in controlling infections of gill microsporidians in salmon, the feed additive was still able to reduce infection levels by 50% (Guselle et al., 2010)

Immunostimulants generally function by stimulating activation of innate immune cells, which do not possess the specific antigen receptors found on T and B cells of the adaptive immune response. Toll-like receptors (TLR) are an important group of PRRs in vertebrates, with as many as 17 different groups identified in teleost fish (Rebl et al, 2010). TLRs are involved in initiating the innate immune response with different groups responding to particular patterns conserved among specific types of microbes (Bricknell and Dalmo, 2005; Carrington and Secombes, 2006; Rebl et al., 2010). β-glucan is bound by several different receptors including TLR 2 and TLR 6, which are also known to form dimer complexes (Bricknell and Dalmo, 2005;Dalmo and Bogwald, 2008). TLR 2 is reported to bind to wide variety of compounds, including LPS and yeast derivatives (Bricknell and Dalmo, 2005). In mammals and fish, TLR 9 is the PRR associated with binding to bacterial DNA with CpG motifs, as well as the synthetic CpG ODN (Bricknell and Dalmo, 2005; Carrington and Secombes, 2006; Rebl et al., 2010). Compounds that stimulate the PRRs of fish immune cells, such as β-glucan and CpG ODN, show good potential as immunostimulants.

Considerable evidence exists for CpG ODNs, β-glucans and other yeast compounds to elicit immune responses in fish including Atlantic salmon. β-glucans have been extensively tested and reviewed for their immunostimulatory effects. Varied immune responses to oral administration of β-glucans range have been reported including elevated complement and lysozyme activity, increased respiratory burst, improved phagocytic activity, and higher numbers of leukocytes (Dalmo and Bogwald, 2008). Gene expression studies have observed elevated IL-1β expression from the spleens of IP injected rainbow trout (Lovoll et al., 2007) and kidney macrophages of carp (Selvaraj et

al., 2005). Similarly, CpG ODNs have been shown to elicit diverse immune responses in fish that includes activation of macrophages, increased levels of ROS, enhanced phagocyte activity, and proliferation of leukocytes (Carrington and Secombes, 2006). CpG ODNs have also been reported to stimulate the expression of IL-1β and MMP 9, (Iliev et al., 2010; Jorgensen et al, 2001).

Objectives:

The objective of this study was to examine the efficacy of dietary immunostimulants in enhancing the immune response of Atlantic salmon against sea lice infection. This objective was addressed in three trials. Oral administration of immunostimulants presents the opportunity to provide better protection during intensive cage culture production. This is a high-risk time when fish may be stressed by crowding and sea lice transmission may be optimized.

The first trial tested the ability of three different immune stimulants, β-glucan, CpG ODN, and a commercial yeast extract (All Brew/Nupro), to activate the innate immune response. The immunostimulants in this trial were administered prior to sea lice infection. We hypothesized that priming the inflammatory response before *L. salmonis* exposure would lead to greater protection against sea lice as compared to infected fish receiving a control feed. The effects of treatments were compared across three different measures: sea lice stage and abundance, histopathological changes and expression in spleen tissues of a number of immunological genes (IL-1β, IL-8, TLR9 and MMP9).

The second trial, carried out as a continuation of the first trial, looked at the effectiveness of CpG ODN during heavier re-infection with sea lice. The purpose of this

trial was to investigate whether CpG ODN treatment is an effective means of stimulating the acquired immune defenses of Atlantic salmon. During sea cage culture, multiple infections are likely to occur over time before harvest. Therefore, it is important to assess whether oral administration of CpG ODN increases the both the innate and adaptive responses against sea lice re-infection. CpG ODN has previously been shown to enhance adaptive immune responses; therefore we expected oral administration of CpG ODN to increase the immune response of Atlantic salmon above what might be exhibited merely due to prior exposure.

The third trial, conducted separately from Trials 1 and 2, compared the effects of a feed prepared with a complex mixture of immunostimulants administered at either a low or high dose. This is a patent-pending formulation ready for commercial production. If found to be effective, this feed could quickly be made available to salmon farmers to help ameliorate the effects of sea lice infection on commercial salmon culture.

The work for Trials 1, 2, and 3 was accomplished through a collaborative effort between staff at the University of Prince Edward Island's Atlantic Veterinary College and Stony Brook University's School of Marine and Atmospheric Sciences. For all three trials, the sea lice infection experiments including the sea lice abundance and stage analysis and the histological analysis occurred at the Atlantic Veterinary College's Aquatic Animal Facility under the direction of Dr. Mark Fast. My thesis work focused on gene expression analysis in fish evaluated in this study. All gene expression analysis, data analysis and interpretation of all components of these studies were conducted at Stony Brook University.

MATERIALS AND METHODS

Fish source and maintenance

Atlantic salmon (*Salmo salar*) were acquired as smolts from Cooke Aquaculture's Buckman's Creek Hatchery (New Brunswick, Canada). The fish were transferred to the Atlantic Veterinary College's aquatic facility at the University of Prince Edward Island, Charlottetown, PEI and acclimated for three weeks in circular flow-through tanks containing 250 L of 11^{o}C freshwater. Following the initial acclimatization period, tank systems were changed from flow-through to recirculation and the salinity slow raised from 0 to 33ppt over the course of 14 days. The salmon were then allowed to acclimate to the seawater for an additional three weeks.

During the acclimatization period, the fish were fed a control diet (described below) at 1% body weight/day, divided over two feeds. Throughout Trials 1, 2 and 3, the fish were maintained on a photoperiod of 12 h light: 12 h dark. At the start of Trials 1 and 2, fish weighed 60.8±9.45 g, by the end of the study the average weight was 135.5±2.96 g. In Trial 3, Atlantic salmon smolts weighed 98.2 g±4.93g at the beginning of the trial and 177.0±10.1g at the conclusion of the study.

Sea Lice Culture and Infection

Gravid female sea lice (*Lepeophtheirus salmonis*) were obtained from cage cultured Atlantic salmon in New Brunswick, Canada and transported back to Atlantic Veterinary College's Aquatic Animal Facility. Egg strings were removed and maintained in aerated, 13^{o}C saltwater until the nauplii hatched and molted into copepodids (~7-10 days). Atlantic salmon were subsequently infected following a method modified from

Fast et al. (2000). During infection, water recirculation was suspended for six hours after addition of approximately 15 copepodids/fish to each tank. Oxygen levels were maintained at > 7 mg/L by heightened aeration during this period. The control tanks were subjected to the same conditions but without the addition of copepodids. Prior to restoring recirculation, 100μm mesh was placed over the inflow of the control uninfected tanks to prevent copepodid exposure at any point during the trial. Under sea cage culture conditions, fish are continually exposed to infective stages of *L. salmonis*. In order to emulate field conditions, fish were exposed to this infection procedure three times, (10 days prior to the second sampling time, and twice between the second and third sampling times (within 12 days of each other). This exposure protocol (multiple exposures of low numbers of lice) resulted in less than 15% of attached stages occurring on the gills which is a common artifactual problem observed during lab-based studies. The multiple exposure protocol results in an infection closer to what is observed in a cage culture setting.

In trial 2, the fish fed ProVale during Trial 1 were switched to a CpG ODN feed (1/2 dose; 10 g/1000 kg feed) for three weeks. Three days after commencing this new feed, replicate ProVale/CpG tanks and control infected tanks were exposed to an additional 120 copepodids/fish as described above. There were two controls utilized in Trial 2. The first infection control was composed of fish from the uninfected control group from Trial 1 and during the second study were exposed to their first experimental infection. As the second infection control was comprised of fish that were the infected control during Trial 1, consequently the exposure to sea lice during Trial 2 was a re-infection.

During Trial 3, fish were infected three times with 10-12 copepodids/fish over a two-week period. The infection process involved turning off water flow for eight hrs and adding copepodids directly to tanks. This exposure protocol (multiple exposures of low numbers of lice) resulted in less than 20% of attached stages occurring on the gills.

Feed Production

Feeds for Trials 1 and 2 were produced by Northeast Nutrition Ltd. (St. Andrews, New Brunswick, Canada). Corey Signature salmon feed (2.5mm) was used as the base feed for these studies. Fish were divided into to five groups with the infected control fish and uninfected control fish receiving the base feed (Northeast Nutrition, Truro, Nova Scotia). Three treatment groups received: ProVale™ (400 g/1000kg feed; Stirling Products, PEI, Canada), CpG ODN 1668 (20 g/1000kg feed; Sigma), and All Brew (commercial yeast extract)/Nupro (ABN- Alltech Inc.). The CpG ODN and Provale (β-glucan) components were dissolved in water and then applied to the feed as a topcoat with ethanol. These feeds were further coated with fish oil to increase palatability. However, the ABN feed was milled into the base feed directly.

Feeds for Trial 3 were produced by an industrial collaborator, who have asked for the identification/composition of these feed to be kept private at the time of this writing. In this study, fish were divided into 3 groups: control, high dose immunostimulant and low dose immunostimulant. Control fish received a base 3.0 mm feed. Fish in the high dose group were fed the control feed + 0.2% immunostimulant + nucleotide additive (i.e. A, G, C, U, T). The low dose group was given control + 0.12% immunostimulant + nucleotide additive.

Study Design

Trial 1

After completing the saltwater acclimatization period, 10 (350 L) tanks with 50 fish each were randomly assigned to five treatment groups, with each treatment fed to duplicate tanks. Prior to receiving the treated feeds, a Time (0) sampling of two fish/tank (n=20) was conducted. These measurements were used as a normalization factor for future sampling measurements. Fish were maintained on treated feed for seven weeks. Time 1 sampling (n=12/group) occurred after two and a half weeks on treated feed, and prior to sea lice exposure. Time 2 sampling (n=8/group) occurred after five weeks on feed and 10 days after the initial lice exposure. Time 3 sampling (n=8/group) occurred after seven weeks on treated feed and 22 days post infection (dpi) and 10 days post final exposure (dpfe). Time 4 (n=8/group) sampling was 29 dpi and 17 dpfe, and 1 week off of treated feed. Time 5 sampling occurred at the conclusion of the study, 37 dpfe, and was used only for final sea lice abundance and staging.

All control and treatment groups were sampled at each of the 5 time points. Feed was withheld for 24 h prior to sampling, and fish were euthanized with an overdose of tricaine methanesulfonate (250 mg/L) and bled within minutes of immobilization (UPEI-AVC, Animal Care Protocol #10-014).

At each of the sampling points post-infection (Time 2-5), the numbers of lice found on each fish were recorded. A subset of lice was also placed in 10% NBF (neutral buffered formalin) for future staging. Spleen, intestine, anterior kidney and skin were excised at sampling points T(0) - (4) and placed in dry ice. Intestine samples were taken just posterior to the pyloric caecae in an effort to standardize the sampling. Skin samples

were taken posterior to the left pectoral fin above the lateral line. All tissues were stored at -80 °C until required for gene expression analysis. Additionally, skin and intestine were placed in 10% NBF for histological analysis. Gene analysis for skin, head kidney and intestine are reported elsewhere (Covello et al., 2011 – under review) as are the histopathological data for the intestinal samples.

Trial 2

This trial was an extension of Trial 1 above. The replicate tanks previously fed ProVale were switched over to a half dose of CpG ODN for three days prior to re-exposure to 120 copepodids/fish. Uninfected control tanks from Trial 1 were exposed to the same infection procedures, as were infected controls from Trial 1. Four to six fish were sampled from each tank at seven and seventeen days post re-infection exposure. All sampling and analysis were carried out as in Trial 1.

Trial 3

Fish were maintained as above with the following exceptions. Fish were randomly assigned (40-45 per tank) to 10 (250 L) tanks. Following acclimation to the system triplicate tanks were put on a control feed, low dose nucleotide inclusion, or a high dose nucleotide inclusion. Tank 10 in the system remained on a control feed and served as the uninfected control for the rest of the experiment. The day prior to receiving differential feed treatments, 2 fish from each tank were euthanized and tissues collected to act as a reference for time 0. At all sampling times, 4 fish per tank were sampled (n=12 per infected treatment): Time (1) 17 days on feed treatment and Time (2) 28 days post feed

treatment and 7 dpi, Time (3) 64 days post feed treatment and 9-11 dpfe, Time (4) 71 days post feed and 29-31 dpfe.

On all sampling days, fish were euthanized with 250mg/L MS-222 and blood was taken from the caudal vein and centrifuged for serum collection. Portions of the head kidney (HK), spleen, intestines, and skin (at and away from sites of parasite attachment) excised and flash frozen for future molecular analysis and a further portion fixed in 10% neutral buffered formalin for histopathological analysis.

Histological Analysis (Completed for Trial 1 and 2)

During tissue collection for the gene expression studies, samples were also collected for histological analysis. Intestine and skin samples were collected from each fish as described above, and placed in 10% NBF. Additional samples were taken at the site of parasite attachment on the skin and evaluated. Samples were processed, paraffin-embedded and cut into 5µm sections before staining with hematoxylin and eosin. Stained slides were double-blinded and examined by the AVC Aquatic Health Diagnostic Fish Pathologist (Dr. David Groman) for cellular evidence of host inflammation and ulceration.

The level of inflammation was given a score based on a scale from 0-3, with 0 being normal morphology with no cellular infiltrate, 1 being mild cellular infiltrate present, 2 being moderate cellular infiltrate and 3 being marked cellular infiltrate (Jones et al., 2007). Ulceration was evaluated as absent (0) or present (1). Chi-squared analysis was carried out on the ulceration scores across the groups and Mann-Whitney test was performed on the inflammation scores across the groups.

Isolation of RNA and cDNA synthesis

RNA was isolated from spleen tissues following the TRIzol® Reagent protocol (Invitrogen, Carlsbad, CA). Total RNA was measured with a Nanodrop-1000 spectrophotometer and product integrity verified with a 1.5% agarose gel electrophoresis. RNA was treated with Ambion® TURBO DNA-free™ reagents (Applied Biosystems/Ambion, Austin, TX) to remove small amounts of contaminating DNA that may be present after RNA isolation.

Reverse transcription reactions were run following the Promega Reverse Transcription system protocol. In overview, 1µg of total RNA was added to a master mix prepared with random primers for a total 20µl reaction. First strand complementary DNA (cDNA) was synthesized under the following conditions; 10 minute room temperature incubation, 42°C/ 15 min, 95°C/5 min, 4°C/ 5 min program on a thermocycler. Samples were stored at -20°C until ready for real-time qPCR.

Real-time qPCR

The primers for RT qPCR were derived from previously published sources and are listed in Table 1 below. Primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The primer stock solutions were diluted to 100µM with nuclease-free water and stored at -80°C. Working stocks were diluted to 10µM and stored at -20°C.

Real-time quantitative polymerase chain reaction (RT qPCR) was used to amplify the reference genes and genes of interest using a real-time thermal cycler (Eppendorf,

Westbury, NY) and GoTaq® qPCR mix (Promega, Madison, WI). The reaction was prepared using 10µl of GoTaq® qPCR mix, 7µl nuclease-free H20, 1µl each of forward and reverse primers (10µM concentration), and 1 µl of cDNA for a total reaction volume of 20µl. A no template control and negative RT control were used to ensure that no genomic DNA or other contaminants were amplified. All qPCR reactions involved an initial 10-minute denaturation at 95°C, followed by 40 cycles of: denaturation (95°C for 15s), annealing (55°C for 20s) and extension (72°C for 30s). This was followed by the melt curve analysis, which was used to confirm the amplification of a single product. Gene expression was evaluated relative to three housekeeping genes: 18s, EF-1A and RPS20. The stability of these genes was evaluated using geNORM software.

Table 1. Sequences of primers used in RT-qPCR

Genes	Primers	Sequence 5'→3'	Source
18S RNA	18s - forward	CCCCGTAATTGGAATGAGTACACTTT	Olsvik et al, 2005
	18s - reverse	ACGCTATTGGAGCTGGAATTACC	
RPS20	RPS20 - for	GCAGACCTTATCCGTGGAGCTA	Olsvik et al, 2005
	RPS20 - rev	TGGTGATGCGCAGAGTCTTG	
EF1Ab	EF-1Ab - for	TGCCCCTCCAGGATGTCTAC	Olsvik et al, 2005
	EF-1Ab - rev	CACGGCCCACAGGTACTG	
IL-1β	IL-1β - for	ATGCGTCACATTGCCAAC	Fast, 2006
	IL-1β - rev	GGTCCTTGTCCTTGAACTCG	
IL-8	IL-8 - for	GAATGTCAGCCAGCCTTGTC	Fast et al, 2009
	IL-8 rev	TCCAGACAAATCTCCTGACCG	
TLR-9	TLR-9 - for	TCTATGGCTGGGATGTCTGGTA	Skjæveland et al, 2008
	TLR-9 - rev	CAGTTGTGAGTAGCCCTTGTGT	
MMP 9	MMP 9 - for	AGTCTACGGTAGCAGCAATGAAGGC	Skugor et al, 2008
	MMP 9 - rev	CGTCAAAGGTCTGGTAGGAGCGTAT	

Statistical analysis:

Statistical analysis was performed using SigmaStat 3.5 for Windows. Gene expression data for T(1) –T(4) was normalized to expression in T(0) and assessed relative to 3 housekeeping genes (18s, EF-1A and RPS20) using the $\Delta\Delta C_T$ method previously described by Pfaffl et al. (2001). Mean Normal Relative Quantitative (MNRQ) expression was calculated using the equation below:

$\Delta\Delta C_T = GOI - HKG_{AVG}$

$MNRQ = 2^{-\Delta\Delta CT}$

Where:

C_T (N) = the mean C_T value of sample from T(1) to T(4)

$C_T(T_0)$ = the average of the mean C_T values of all samples within a gene in T(0)

GOI = the expression of gene of interest normalized to T(0) = $C_T(N) - C_T(T_0)$

HKG = expression of a housekeeping gene normalized to T(0) = C_T (N) – C_T (T_0)

HKG_{AVG} = the average of 3 housekeeping genes = $[HKG_1 + HKG_2 + HKG_3] / 3$

Two –way ANOVA ($P < 0.05$) were used to detect significant differences across treatments and time between treatment and control groups. Multiple comparisons were conducted with *post hoc* Tukey tests. All data was tested for normality, all non-normal data were transformed with a natural log (ln) function. Values are expressed as mean ± SE. The sea lice infection data in Trial 3 was analyzed using a one-way ANOVA with Tukey *post hoc* tests. Tables describing the outcomes of all statistical tests are provided in the Appendix. Data on sea lice infestation was not subjected to statistical analysis, but is provided just to give an indication of relative parasite load.

RESULTS

Trial 1

Sea lice infection

The sea lice infection data was collected at T(3), T(4), and T(5) which were 10, 17 and 37 days, respectively, after the final sea lice exposure. This experimental infection resulted in mean lice numbers of 3.08, 4.00 and 2.27 lice/fish for the control group, 2.58, 2.42 and 1.13 lice/fish for the CpG ODN group, and 3.09, 2.42 and 1.67 lice/fish for the ABN group. The ProVale group had 3.42 and 4.34 lice/fish at T(3) and T(4). The ProVale treatment was discontinued after T(4), and remaining fish used in a follow up study, as the numbers of lice were greater than in the control infected group (Fig. 2). The prevalence of *L. salmonis* in all groups was 98% at T(3), 92% at T(4) and 71% at T(5) and abundances ranged from 1 to 10 lice/fish. The sea lice prevalence over the entire study was 75% in CpG treated fish and >83% in all other groups. The percent reduction in lice against the control group is shown in Figure 3, with an 11% and 24% increase in the ProVale group, a 16%, 31% and 46% reduction in the CpG ODN group and a 0%, 31% and 20% reduction in the ABN group, over the T(3), T(4), and T(5) sampling times.

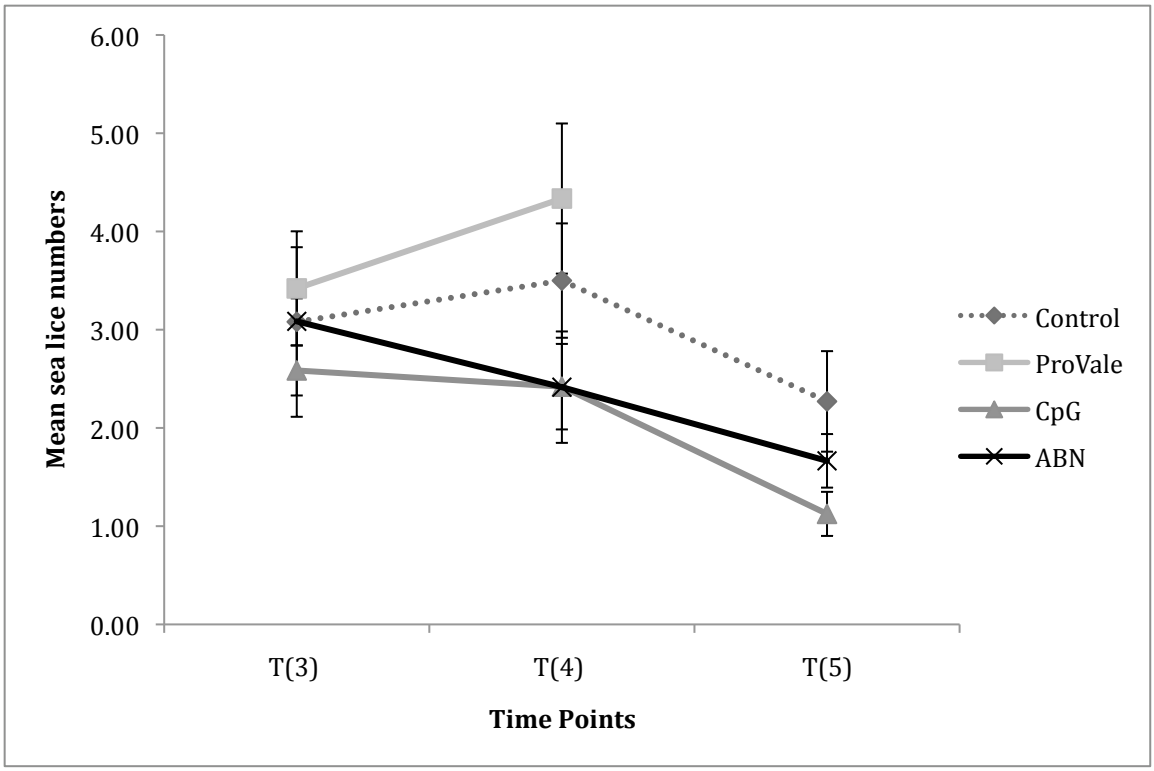

Figure 2. Mean ± SEM sea lice numbers per fish at sampling times 3 (22dpi/10 dpfe), 4 (29dpi/17dpfe), and 5 (49dpi/37 dpfe). The ProVale treatment was discontinued after T(4).

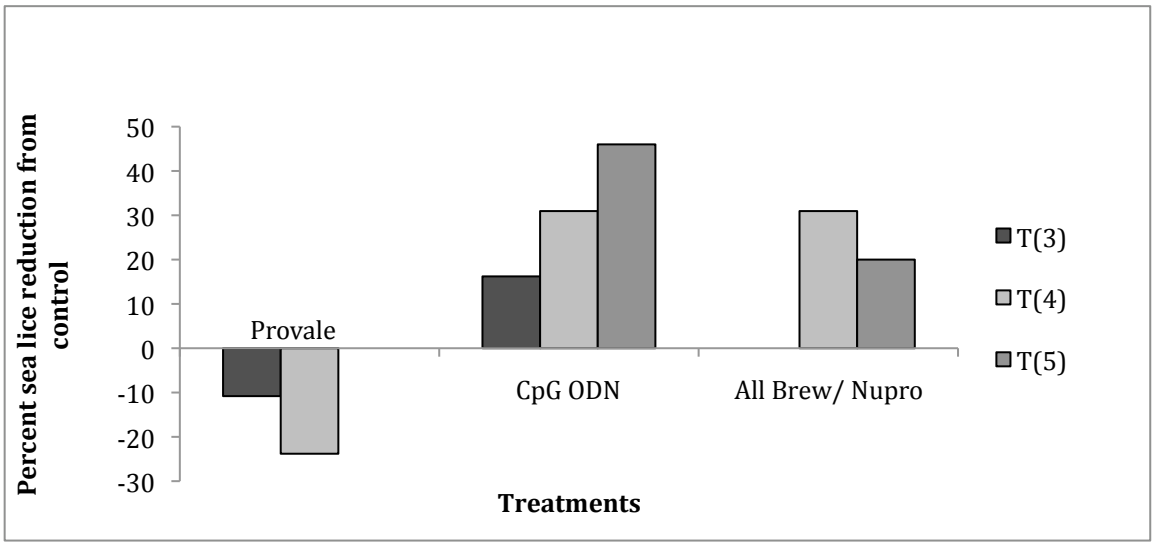

Figure 3. Percent reduction in sea lice from the control group. The sampling for the ProVale treatment was discontinued after T(4). The All Brew/Nupro at T(3) reduction was 0%.

Sea lice life stage was determined at the conclusion of Trial 1 (Time 5). Total numbers of sea lice were counted on remaining fish and female to male ratios were calculated (Fig. 4). Replicate CpG treated tanks had 17 and 10 adult lice of which 35% and 40% were adult females, respectively. The first CpG tank also had 2 pre-adult females present. The two ABN treated tanks had 22 and 14 adult lice consisting of 41% and 64% adult females. The first ABN tank had 1 pre-adult male present at the time of sampling. No other pre-adult lice were found during counting. One infected control tank was included for reference, with 25 total adult lice made up of 48% adult females lice.

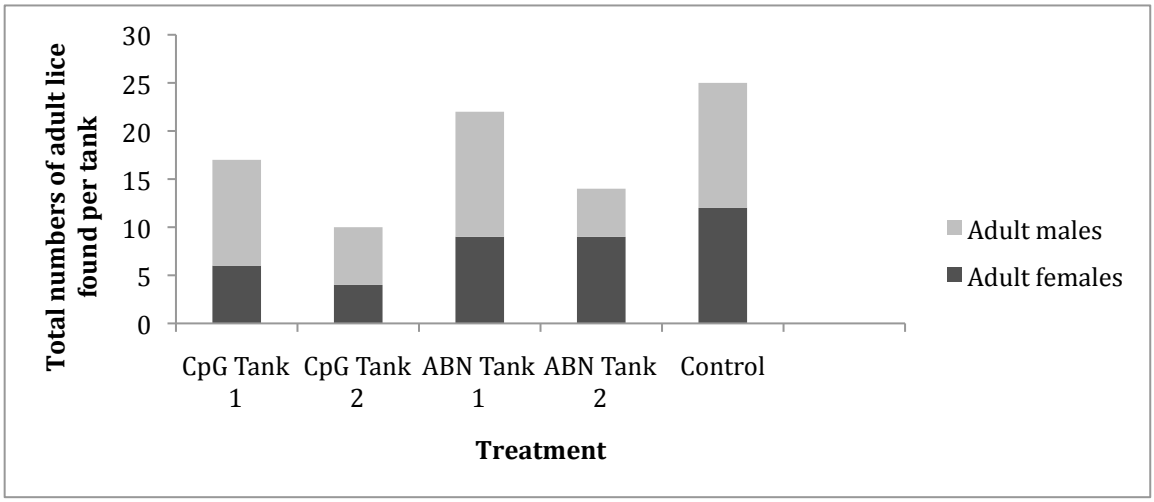

Figure 4. Adult sea lice per treatment tank, showing percentage of adult female vs. adult male sea lice

Histology:

Histological analysis of the intestine found that in all cases there was no (0) to mild (1) inflammation across all of the groups. It was concluded that none of the treated feeds had

any adverse affect on the intestines. Samples of skin, both at and away from the site of parasite attachment were also examined (Fig. 5). The results of this analysis are reported elsewhere (Covello et al., 2011a). In short, the inflammation score away from the site of attachment was subtracted from the score at the attachment site to account for any inflammation not associated with parasite attachment. Chi-squared analysis of the lesion scores showed no significant difference across the groups. There was a significant difference in the inflammation score between the control infected and CpG ODN groups (Mann-Whitney-Wilcoxon $p<0.05$). Despite the higher inflammation score in the CpG ODN group, it also had the lowest ulceration score, indicating that an inflammatory response was occurring in the absence of a necrotic lesion.

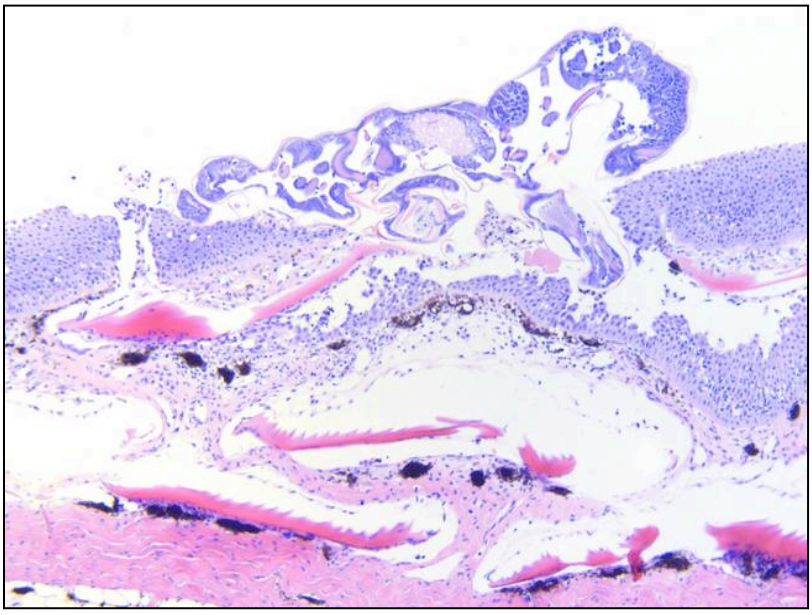

Figure 5. Chalimus II/III stage infection of *Lepeophtheirus salmonis* on Atlantic salmon (*Salmo salar*) epithelium. (10X Magnification)

Gene Expression:

IL-Interleukin 1β: Data on expression of IL-1β in spleen from fish in the Trial 1 is shown in Figure 6. Statistically significant differences were observed in IL-1β

expression among treatments (p=0.003) and the treatment by time interaction was also significant (p=0.047). When compared to the infected control, no significant differences in gene expression were detected for any of the treatments. However, ProVale and CpG ODN showed significant up-regulation in IL-1β expression from the uninfected controls across all treatments (p=0.004 and p=0.018 respectively). At T(2), the CpG ODN treatment was significantly up-regulated as compared to the uninfected control (p=0.003, Fig. 6) as well as the CpG treatment for time T(4) (p=0.043). Expression in the ABN group was significantly elevated prior to sea lice exposure at T(1) as compared to after lice exposure at T(4) (p<0.001). Within T(4), the ProVale and ABN treatments showed differential IL-1β expression when compared to each other.

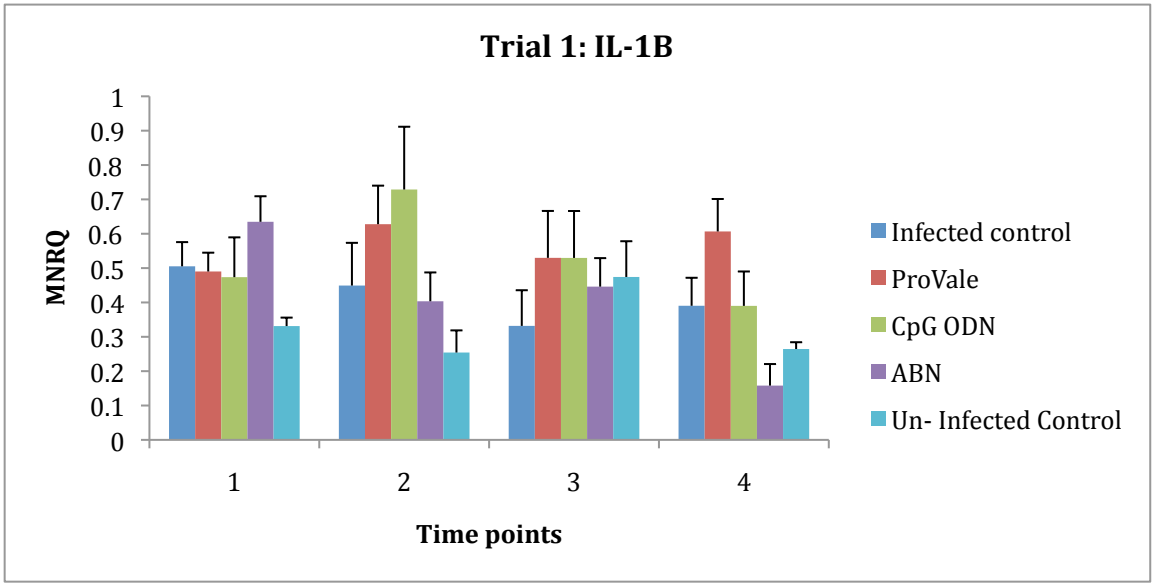

Figure 6. Mean normal relative quantitative (MNRQ) expression of IL-1β ± SE. Results of statistical comparison are given in Appendix 5.

IL-Interleukin 8: Unlike expression of IL-1β, expression of IL-8 showed a strong temporal pattern with expression levels especially elevated in Time 4. Two-way ANOVA results showed a significant difference in IL-8 by time at the level of p<0.001. The Tukey

tests revealed that IL-8 was significantly up-regulated for the uninfected control and CpG ODN at T(4) against all other time points, as well as, for ProVale at time (4) versus T(1) and ABN at Time (4) as compared to T(1) and T(2) (Fig. 7). Between the treatments, the only difference observed was between the infected and uninfected controls at Time (2) ($p=0.012$).

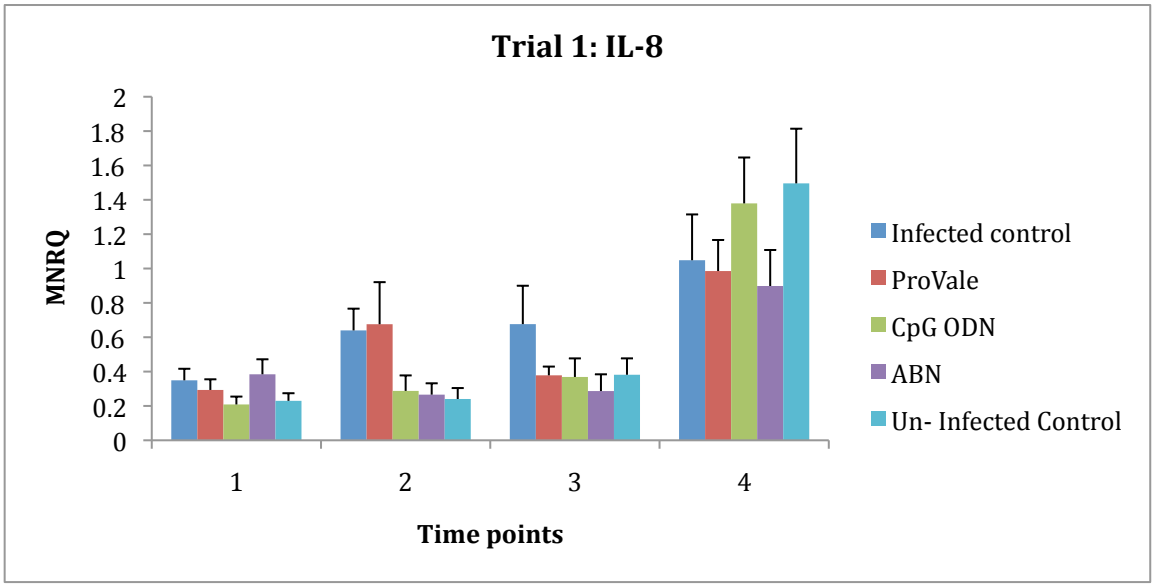

Figure 7. Mean normal relative quantitative (MNRQ) expression of IL-8 ± SE. Results of statistical comparison are given in Appendix 5.

Matrix Metalloproteinase 9: Differential expression in MMP 9 was observed at the treatment level ($p<0.001$) with most observed differences between CpG ODN and the controls. Specifically, the CpG group was up-regulated from the uninfected and infected controls at $p<0.001$ and $p=0.016$ respectively (Fig. 8). Within T(2), these difference were seen at a level of $p=0.025$ and $p=0.032$ and within T(3) only between CpG ODN and the uninfected control ($p=0.020$). The only other treatment differences seen are between ProVale and the uninfected control ($p=0.006$).

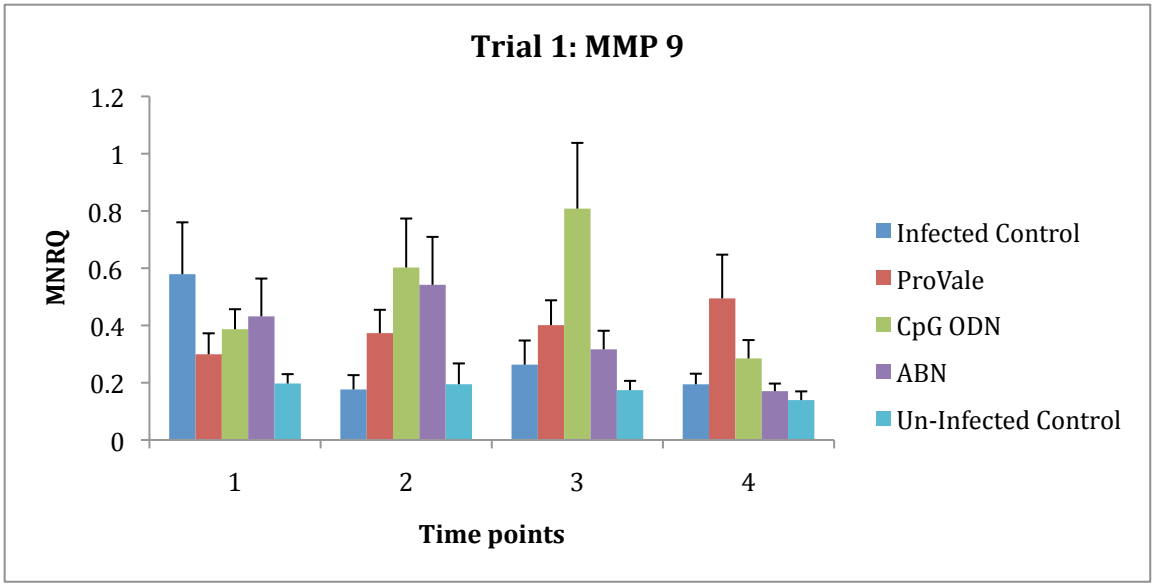

Figure 8. Mean normal relative quantitative (MNRQ) expression of MMP 9 ± SE. Results of statistical comparison are given in Appendix 5.

Toll-like Receptor 9: In general, expression of TLR 9 varied significantly over time (P=0.024). When broken down by multiple comparisons this was seen as a statistical difference between T(3) and T(1). The ProVale treatment also was up-regulated in Time (3) versus T(4). Within time (4), TLR 9 expression for the ProVale treatment was also significantly lower than the uninfected control (p=0.044) (Fig 9).

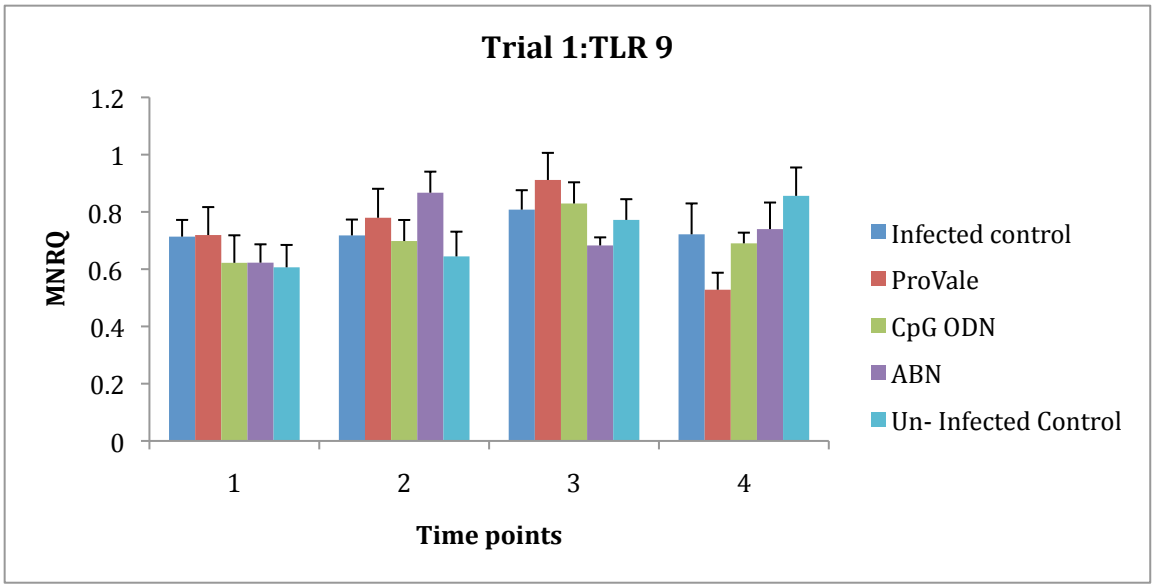

Figure 9. Mean normal relative quantitative (MNRQ) expression of TLR 9 ± SE. Results of statistical comparison are given in Appendix 5.

Trial 2

Sea lice infection

Following time T(4) of Trial 1, the ProVale treatment was discontinued and the remaining fish in that group switched to the CpG ODN treatment. The uninfected and infected controls from Trial 1, as well as the ProVale/CpG ODN group, were then infected with a heavy load of sea lice (120 lice/fish). This was a secondary re-infection for the treatment and infected control and the first infection experienced by the previous uninfected control. For this reason, the controls for Trial 2 are renamed 1st infection control and 2nd infection control.

Infection data was collected at only two time points: at 7 T(1) and 17 T(2) days, respectively, following re-infection. This experimental re-infection resulted in mean lice numbers of 60.5 and 49.19 lice/fish for the 1st infection control, 44.17 and 42.45 lice/fish for the 2nd infection control, and 31.13 and 35.80 lice/fish for the CpG ODN treatment

group (Fig 10). This represents a percent reduction in lice from the 1st infection control of 27% and 13% for the 2nd infection and 48.5% and 27% for the treatment, at times 1 and 2, respectively. The CpG treatment was 29.5% and 16% reduced from the 2nd infection control group (Fig. 11).

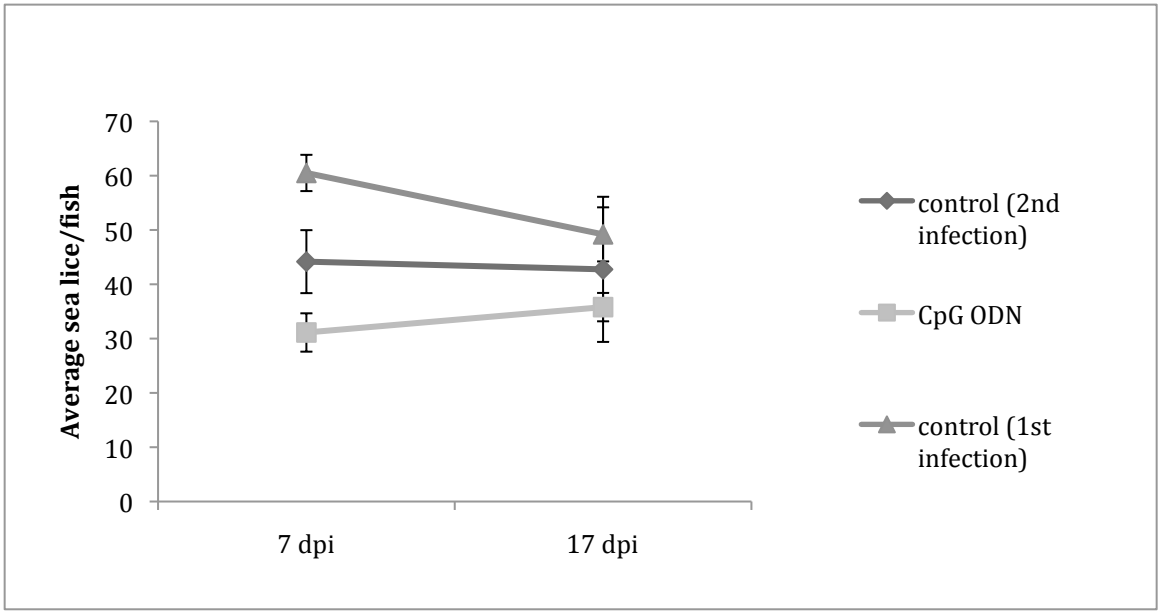

Figure 10. Trial 2: Average sea lice per fish ±SEM at sampling times 1 (7 dpi) and 2 (17 dpi).

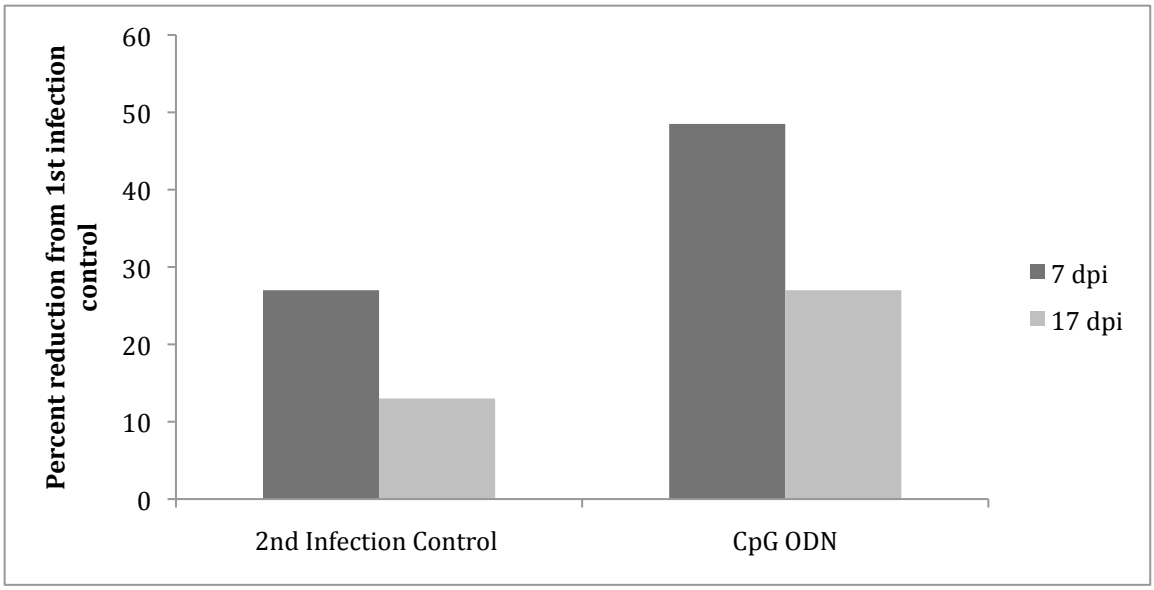

Figure 11. Trial 2: Percent reduction in sea lice from the 1st infection control

Histology

Histopathological analysis found only mild inflammation (scored 1/4) for each group (n=4). Both the 1st and 2nd infection control showed the presence of ulceration with inflammation, where as no ulceration was observed in the CpG ODN treatment group.

Gene Expression

Interleukin 1β: Gene expression results for IL-1β did not show a significant pattern relating to the sea lice reduction described above. IL-1β was significantly different at 17dpi vs. 7dpi within the 1st infection control group ($p=0.038$) (Fig. 12A). No other significant results were found within IL-1β expression between times or treatments.

Interleukin 8: Expression of IL-8 showed a strong temporal pattern and was significantly different over time at a level of $p<0.001$. Both the 1st and 2nd infection controls were up

regulated at 17dpi over the expression seen at 7dpi. The 2nd infection control differed significantly between times (p=0.011) while the 1st infection control showed up-regulation of IL-8 in at 17dpi over 7dpi (p<0.001). Within time 2, the CpG treatment showed significantly reduced expression as compared to the 1st infection control (Fig. 12B).

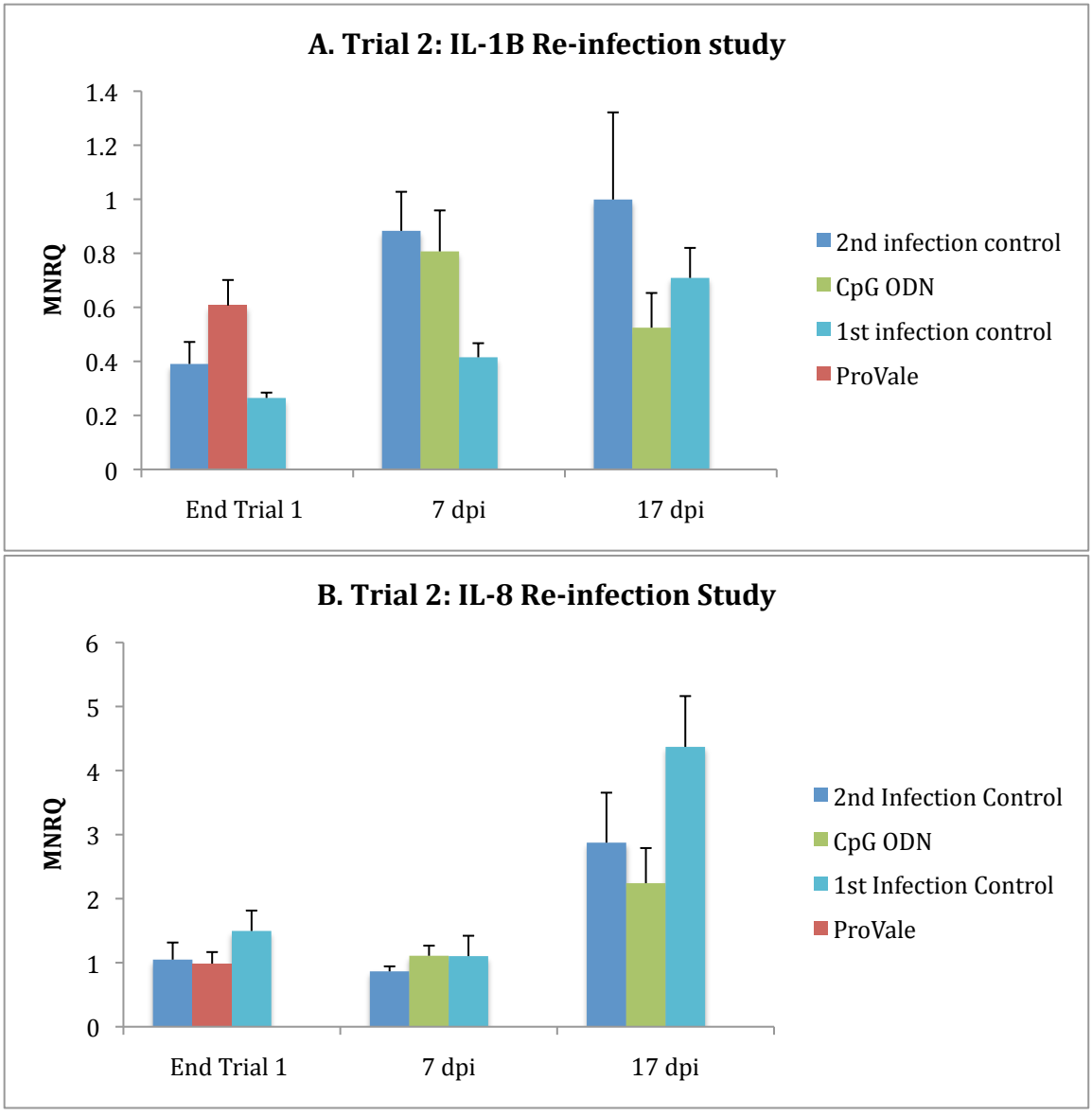

Figure 12A and B. A. Mean normal relative quantitative (MNRQ) expression of IL-1β ± SE. B. Mean normal relative quantitative (MNRQ) expression of IL-8 ± SE. Results of statistical comparison are given in Appendix 5. The data from the end of Trial 1 were include for visual reference but not included during statistical analysis.

Matrix Metalloproteinase 9: Similar to the expression of IL-8 shown above, MMP 9 also displayed a strong temporal with higher expression levels observed at 17 dpi. MMP 9 expression was up-regulated in all time 2 groups when compared to time 1 ($p<0.001$) (Fig 13A).

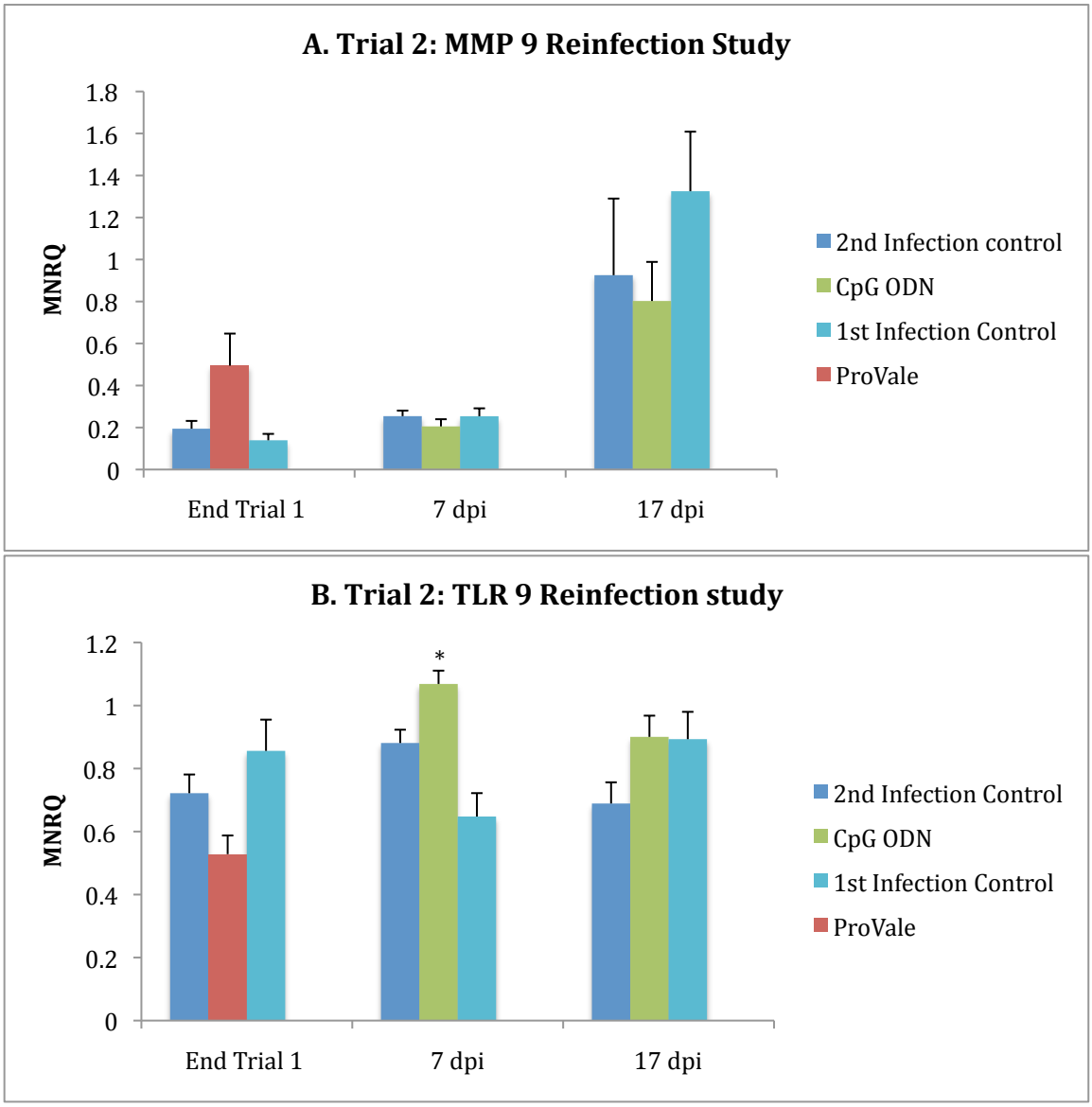

Figure 13A and B. A. Mean normal relative quantitative (MNRQ) expression of MMP 9 ± SE. B. Mean normal relative quantitative (MNRQ) expression of TLR 9 ± SE. Results of statistical comparison are given in Appendix 5. The data from the end of Trial 1 were include for visual reference but not included during statistical analysis.

Toll-like Receptor 9: In contrast to the temporal pattern observed in IL-8 and MMP 9, differences in TLR9 expression were evident between treatments. Significant differences were observed at the treatment level for TLR 9 expression ($p=0.010$) and treatment x time ($p=0.009$). The CpG ODN group showed up-regulation in TLR 9 over the 1st infection control in T(1) ($p<0.001$). Additionally, the 1st infection control was significantly lower at T(1) than at T(2) ($p=0.020$) (Fig 13B).

Trial 3

Sea lice infection

Following 7-10 weeks on treated feed and several exposures to *L. salmonis* copepodids, sea lice infection data were collected at times 2, 3 and 4, which were 7dpi, 9-11dpfe and 29-31 dpfe respectively. The experimental infection resulted in mean sea lice numbers of 3.44, 2.44 and 3.96 lice/fish in the control group, 2.83, 2.11, and 3.71 in the high dose treatment and 1.78, 1.44 and 2.16 in the low dose treatment (Fig 14). This equated to a significant reduction (50%) in total lice numbers in the low dose treatment as compared to the high dose and control feeds, as well as a significant reduction (65%) in adult female lice in the low dose treatment as compared to the high dose and control feeds (data not shown).

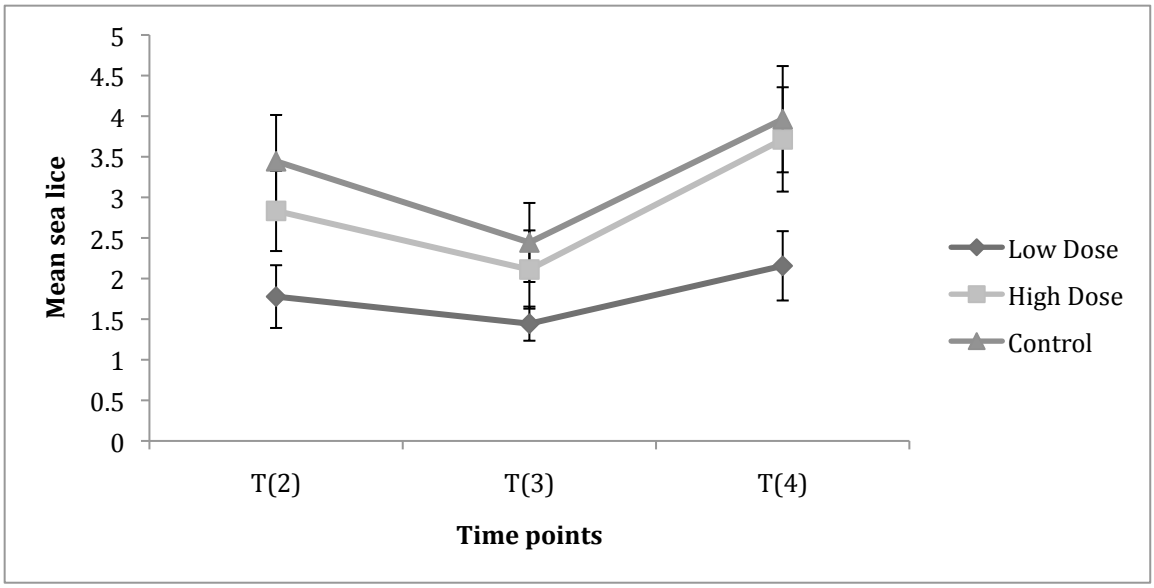

Figure 14. Trial 3: Mean ± SEM number of lice per fish at sampling times 2 (7dpi), 3 (9-11 dpfe) and 4 (29-31 dpfe).

Gene expression

Interleukin 1β: During Trial 3, no significant differences in IL-1β were observed between treatments and controls. The only significant result was the differential expression seen in the infected control between T(2) and T(4) ($p=0.011$). While not statistically significant, levels of IL-1β in the LD and HD treatments remained consistently high, while the infected control showed much more variability in expression throughout the study (Fig. 15).

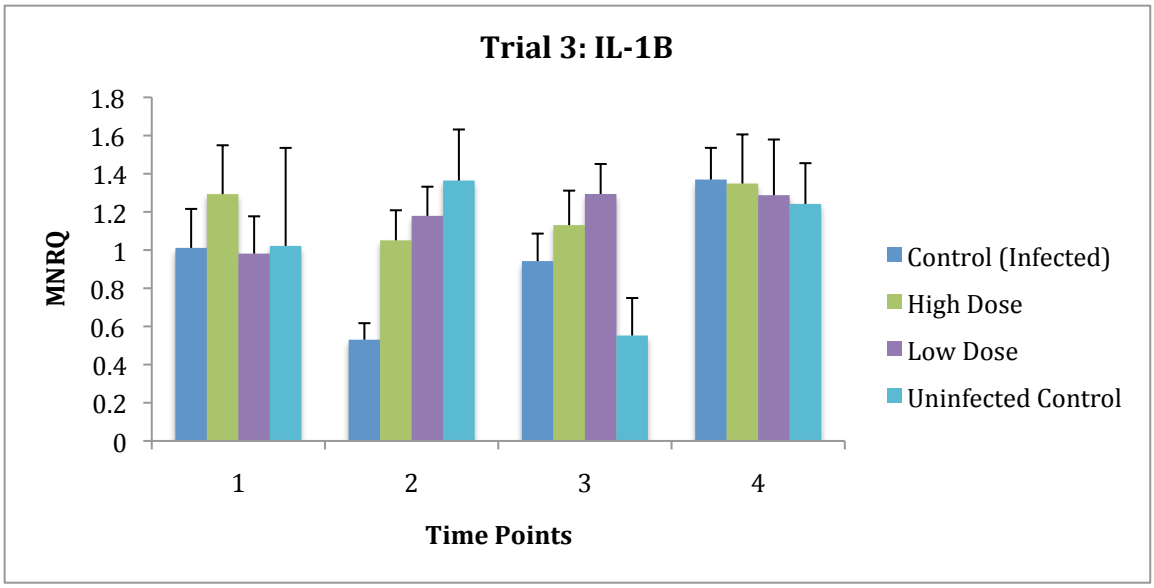

Figure 15. Mean normal relative quantitative (MNRQ) expression of IL-1β ± SE. Results of statistical comparison are given in Appendix 5.

Interleukin 8: No treatment differences were observed within sampling days, however significant variations in expression were observed between treatments ($p=0.035$) and time points ($p<0.001$). The low dose treatment showed differential expression between sampling points T(4) and all other sampling days. The infected control and high dose treatments showed significant down-regulation in IL-8 at T(4) versus times 1 and 2 (Fig16).

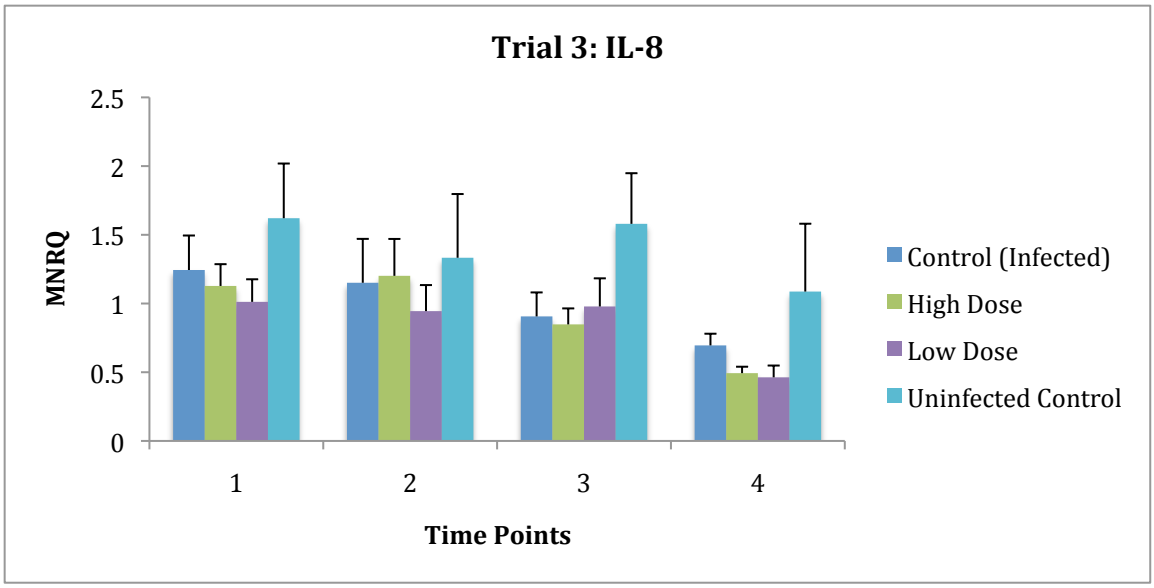

Figure 16. Mean normal relative quantitative (MNRQ) expression of IL-8 ± SE. Results of statistical comparison are given in Appendix 5.

Matrix Metalloproteinase 9: MMP 9 expression was significantly different between the T(4) high dose and low dose treatments ($p = 0.027$), with the high dose showing significant up-regulation over the low dose. No other significant results were observed for MMP 9 expression (Fig. 17).

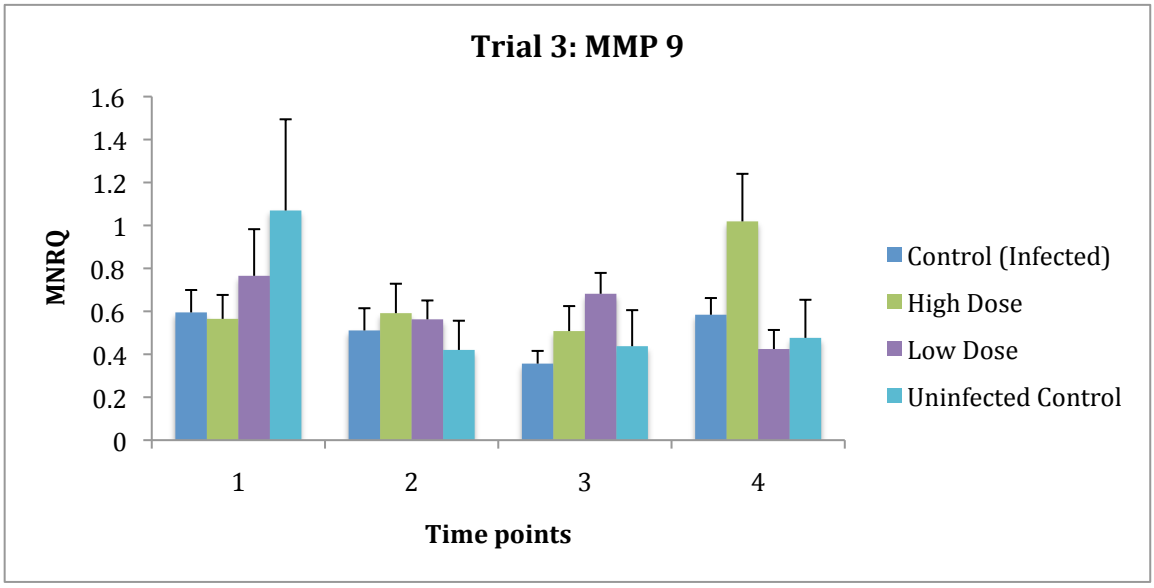

Figure 17. Mean normal relative quantitative (MNRQ) expression of MMP 9 ± SE. Results of statistical comparison are given in Appendix 5.

Toll-like Receptor 9: Significant differences were observed between the doses ($p<0.001$) and dose x time ($p<0.001$). Pairwise multiple comparisons (Tukey test) revealed that the uninfected control showed significant up-regulation of TLR 9 at T(2) over the uninfected controls at all other sampling times. Additionally, at T(2) the uninfected control showed a considerable increase over all infected groups. The high dose treatment was up-regulated at T(2) over T(4). The infected control was higher at both T(1) and T(2) over T(3). The low dose treatment showed a significant increase in IL-8 expression over the control at T(3) (Fig. 18).

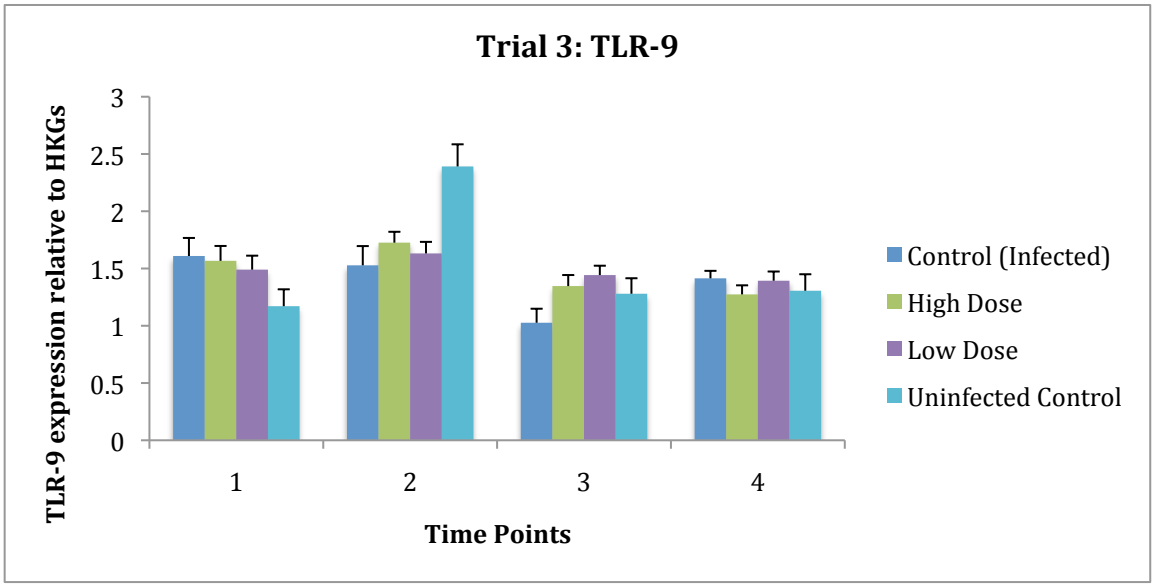

Figure 18. Mean normal relative quantitative (MNRQ) expression of TLR 9 ± SE. Results of statistical comparison are given in Appendix 5.

DISCUSSION

The goal of this project was to evaluate the efficacy of dietary additives to stimulate immune response to *L. salmonis* in Atlantic salmon. We hypothesized that enhancing the inflammatory response of susceptible hosts responses prior to sea lice exposure would be a useful mechanism to increasing resistance to *L. salmonis* infections. Additionally, we tested whether dietary immunostimulation could work at a lower dose, over a shorter time period of administration and/or increase the adaptive immune response by experimentally re-infecting salmon fed a treatment and control feed. Finally, we compared the effectiveness low and high doses of patent-pending immunostimulant diets to establish whether administration of this feed during Atlantic salmon culture could be expected to reduce the impacts of sea lice infection on farmed salmon, and whether the same pathways would be stimulated within the host.

Trial 1: Comparison of CpG ODN, ProVale and All Brew/Nupro immunostimulants

The findings from the first trial in this study indicate that oral administration of CpG ODN enhanced feeds has the potential to significantly reduce the impacts of *L. salmonis* infection on Atlantic salmon smolts. With regards to sea lice abundance, the CpG treatments showed a greater reduction in mean lice numbers over the β-glucan (ProVale) and commercial yeast extract (ABN) treated fish. This resulted in a nearly 50% reduction in final sea lice abundance from the control. CpG treated salmon also showed a decrease in the percentage of adult female lice. As the percentage reduction compared to the control increased over the course of this study, this may indicate that the CpG treated feed has an additive effect over time, or may even enhance acquired

responses to lice. A stimulated innate response could lead to cumulative lice mortality, especially following stressful periods during their life cycle such as molting and reproduction. The mean sea lice abundance for all groups was lowest in Time 5 (37dpfe) with the exception of the ProVale fed group, which was discontinued prior to time 5 sampling.

Any evaluation of the timing of responses needs to also consider the effects that sea lice have on their host's immune systems at different stages in their life cycles. For this reason, the timing of sampling in sea lice infection studies corresponds to key stages such as the molt from chalimus to pre-adult. In resistant host species, the loss of the majority of lice is observed prior to development into pre-adults (Johnson and Albright, 1992). In this study, time 3, 4 and 5 correspond to the sea lice stages of chalimus II/III, pre-adult and adult respectively. The CpG and ABN groups continued to show reductions throughout the life cycle of the sea lice, suggesting greater resistance, where as both the control and ProVale groups saw a increase in sea lice burdens between times 3 and 4 indicating that these groups showed greater susceptibility to infection.

Histological analysis of the sea lice inflicted lesions in the CpG ODN treated fish showed increased inflammation without necrosis, indicating that an enhanced inflammatory response may be responsible for the reduction in lice seen in the infection data (Covello et al., 2011a). Earlier work by Johnson and Albright (1992) has shown that resistant salmonids, such as coho salmon, display greater inflammation at the sites of attachment than susceptible species like Atlantic salmon.

IL-1β and IL-8 expression were examined to determine if an up-regulation in pro-inflammatory genes accompanied the inflammatory responses observed in the

histopathological analysis. CpG ODNs, including the group used here (Group B) have previously been shown to increase IL-1β expression in stimulated rainbow trout and Atlantic salmon when administered *in vitro* (Iliev et al., 2010; Jørgenson et al., 2001). In this study, no significant difference in IL-1β expression was observed prior to sea lice exposure indicating there was no early stimulation of expression to explain later differences in sea lice burdens. Resistant hosts have previously been shown to have constitutively higher expression of IL-1β (Jones et al., 2007). A significant up-regulation in IL-1β was observed 10 days after the initial lice exposure in the CpG ODN fed fish. While this trend of up-regulation does not continue through later sampling times, when considered in conjunction with the sea lice reduction and histological observations, it may indicate that up-regulation of IL-1β early in the infection can have lasting effects on the severity of ongoing sea lice infestations. Other studies have observed a late induction in IL-1β expression that was not detected here, even within the infected control groups (Fast et al., 2006a). However, this observation was made in Atlantic salmon experiencing a much heavier infection with an increased cortisol response, suggesting that the level of infection caused significant stress to the host. In contrast, the lower level infection employed in our study may have influenced the timing and amplitude of the inflammatory response observed here.

Control infected fish showed significant up-regulation of IL-8 over the non-infected control at 10 days post infection. Though not statistically significant, a comparable increase in expression was also observed in the ProVale treated fish at 10 dpi. This was surprising as early expression of IL-8 (but not IL-1B) has been observed in resistant pink salmon, which was linked to higher resistance (Jones et al., 2007; Wagner

et al., 2008). Significant up-regulation in IL-8 was observed at 29 dpi/17 dpfe in all groups, which does coincide with the higher percent reductions in sea lice numbers observed late in Trial 1. A considerable increase in all groups, including the uninfected control, at this later time point indicates that this up-regulation is not due to treatment or infection effects. It may be the result of changes in gene regulation as fish age, as many innate immune response genes have been shown to increase over the life of larval and juvenile fishes.

MMP 9 was chosen as an indicator of tissue remodeling and wound repair. The fish fed CpG ODN showed higher expression of this gene at 10 dpi and 22dpi/10dpfe. This is noteworthy in light of the histological and infection results, in that it may indicate that in the presence of an increased inflammatory response and reduced infection, greater healing can occur to repair epithelial disruption caused by ectoparasites, such as *L. salmonis*. This is consistent with recent work by Iliev et al. (2010), which found that CpG ODN up-regulates MMP 9 expression. Skugor et al. (2008) observed the highest expression of MMP 9 in Atlantic salmon spleen samples at 22 and 33 dpi following infection with *L. salmonis*. The earlier up-regulation seen here in the presence of CpG might point to earlier healing occurring when inflammatory responses are also activated earlier in the infection process.

TLR 9, selected as an indication of the initiation of immune signaling following CpG ODN treatment, showed no significant changes in CpG ODN over the other treatment or control groups. ProVale treatments did show significant differences within the TLR 9 gene expression, which was unanticipated given that β-glucan is not known to signal through TLR 9. While unexpected, this may indicate that previously unreported

signaling could be occurring through TLR 9. Alternatively, TLR 9 expression could be influenced by other induced signals. Current understanding of PRR binding and signal cascades in fish is very incomplete and future research is likely shed light in this field.

While not as effective as the CpG ODN treatment, the All Brew/ Nupro commercial yeast compound also showed some benefits in reducing numbers of lice on infected Atlantic salmon. This was not evident at the time 3 (22dpi/10dpfe) sampling, where the mean lice numbers per fish equaled that of the control group resulting in 0% reduction in lice over the control. However, at time 4 (29 dpi/17dpfe) the ABN group showed a reduction equal to the CPG treatment, with both at 31%. At the end of the study, the percent reduction in the ABN group fell to 20% while, in contrast, the lice reduction CpG treated fish continued to rise to 46%. Gene expression and histology data showed no significant correlation to the percent reductions observed in the ABN group. A decrease in effectiveness of ABN over time may imply that this immune stimulant has no effect on adaptive immunity or that fish may be developing a tolerance to the immunostimulant over time. Pathways associated with enhanced resistance of ABN to *L. salmonis* are still unknown.

Surprisingly, fish receiving the ProVale (β-glucan) treated feeds showed the highest number of sea lice per fish, exceeding even the control group. This indicates that the ProVale feed may have increased the susceptibility of Atlantic salmon to *L. salmonis* infection. ProVale has previously been shown to be effective in reducing the impacts of microsporidan parasites on rainbow trout gills when administered both intraperitoneally and orally (Guselle et al, 2010). Oral and IP administration of β-glucans has also been reported to aid bacterial challenges in fish, including salmonids (Dalmo and Bogwald,

2008; Guselle et al, 2010). However, in this study, no protective benefit of dietary β-glucan administration was observed. Although, β-glucans have been previously shown to enhance expression of IL-1β, this was also not observed here. This may be related to the dosage of ProVale used in this experiment. Guselle et al. (2010) looked at the effectiveness of 3 orally administered dosages of ProVale (50, 100 and 200g/1000kg of feed) at inhibiting microsporidian induced xenoma formation. They found that both 100 and 200g/1000kg dosages were effective but that 200g/1000kg dose resulted in the greatest reduction in xenoma (over 50%). However, they also noted that a dose-response effect may begin to plateau between the two upper dosages used and suggest evaluation of ProVale doses in the 300-600g/1000kg to determine whether increasing the dose results in further reductions in xenomas. Our study utilized one ProVale dose of 400g/1000kg. As discussed later in relation to the high and low dose immunostimulant study conducted in Trial 3, a higher dose not necessarily equate to a better immune response. As these compounds work by simulating infection to prime the immune response, over stimulation may lead to stress or toxicity, which can have a negative effect on actual infection outcomes.

It has been suggested that continual feeding of immunostimulants, as was employed in this study may not be the most effective means of increasing the long-term immunological response of fish (Bricknell and Dalmo, 2005). While it is possible that continual feeding may result in constant and invariable up-regulation of immune responses, previous work has shown this effect to be uncommon. Similar to other medications and parasiticide treatments, constant administration of immune system activating compounds may ultimately lead to tolerance as the host immune system

becomes desensitized to the immunostimulant's effects (Bricknell and Dalmo, 2005). Therefore, a commonly employed approach to immunostimulation is to use pulse feeding, where the immunostimulant is fed to fish constantly for 4-6 weeks, withdrawn from treated feeds for some period and then re-administered in the feed for several more weeks (Bricknell and Dalmo, 2005).

In this study, fish were maintained on treated feeds for 7 weeks. It was our goal to ensure a maximal time to allow for stimulation to occur just prior to louse settlement. While this resulted in the study carrying just beyond the recommended 4-6 week immune induction period, it is possible that toward the end of this period the effects of tolerance were being seen. It is also unknown if different immunostimulants have different time periods of effectiveness before tolerance effects are observed. Based on the sea lice burdens recorded throughout the later portion of this study, no obvious tolerance was observed in the CpG and ABN fed fish. These groups continued to show decreasing levels of infection over time, though the lower amplitude of reduction in ABN treated fish may signal the beginning of tolerance to these compounds. However, the ProVale fed fish saw an increase in lice abundance between times 3 and 4 amounting to a 20% increase over the control group. Previous studies of long-term oral administration of β-glucans have used a system of two weeks on, two weeks off treated feeds to avoid encouraging tolerance (Bagni et al., 2000). Further worker on immunostimulants should investigate the effects of long-term versus pulse administration to determine the optimal delivery schedule for maximum immunostimulation.

Trial 2: Effects of CpG ODN treatment during sea lice re-infection

Trial 2 was conducted as a continuation of Trial 1 to look at the effects of dietary CpG ODN during a heavy re-infection of sea lice. The combined data from these two studies provides a fuller depiction of the effects of immunostimulants during low-level primary infections and higher-level re-infection. In this study, both the 2nd infection control and the CpG ODN treatment group displayed evidence of an adaptive immune response during the secondary exposure to sea lice. This is evident in the percent reduction in lice over the 1st infection control. The immune stimulated CpG group showed a nearly 50% reduction in parasite load over the 1st infection control and 30% reduction over the 2nd infection control.

Though not statistically significant, both previously infected groups had higher expression in IL-1β at 7 dpi, where as a significant up-regulation was observed within the 1st infection control at 17 dpi. The early increase in IL-1β in the previously infected groups suggests that a faster response is occurring upon re-infection with *L. salmonis*, which is further indication of an adaptive response in these groups. An increased inflammatory reaction early in the infection could explain the reduction in lice observed. However, it is also possible that adult sea lice pre-infecting these fish at the start of this study had some effect on settlement of newly introduced copepodids. It has been previously postulated that adult stages may impede the survival and attachment of immature lice (Fast et al., 2006b).

All fish groups saw increases in the expression of IL-8 and MMP 9 at 17 dpi. By 17 dpi, *L. salmonis* had molted from the attached chalimus to the mobile pre-adult stage. The elevated IL-8 expression is consistent with previous studies, which have found an

increase in pro-inflammatory gene expression following the molt to the pre-adult stages (Fast et al, 2006b; Skugor et al., 2008). This may be attributable to the changes in host immune modulation by the parasite when its life style changes from attached to mobile. Earlier studies have noted that while chalimus stages are attached to one fixed feeding location for days no tissue response occurs, indicating that parasite induced immunomodulation is most effective during the attached feeding stages (Fast et al., 2007; Johnson and Albright, 1992). *Lepeophtheirus salmonis* secretions may inhibit pro-inflammatory gene expression in an effort by the lice to avoid exposure to innate and adaptive cells and their products (Fast et al, 2007).

The CpG treatment induced a significant up-regulation in TLR 9 over the 1st infection control at 7dpi. This early increase in TLR 9 was not observed during Trial 1. The fish in Trial 2 were stimulated for 3 days prior to sea lice exposure where as the fish in Trial 1 received treated CpG feeds for approximately 4 weeks prior to sea lice infection. Up-regulation of TLR 9 may occur soon after first exposure to CpG ODNs and was possibly missed in the first trial. However, the increase in TLR 9 in Trial 2 may also point to the possibility of the initiation of an adaptive response.

In addition to the reported ability of CpG ODNs to stimulate the innate immune system, evidence is growing that CpG ODNs may enhance activation of the adaptive immune response (Carrington and Secombes, 2006, Iliev et al., 2010). Following phagocytosis, antigen-presenting cells (APCs), in concert with major histocompatibility class II (MH II) molecules, introduce antigens to adaptive immune cells, such as T cells. APCs, which include dendritic cells and macrophages, are essential to the development of

an adaptive immune response. These cells are also known to express TLR 9, giving this PRR a roll in the activation of adaptive immunity.

Examination of the histopathological data showed only mild inflammation for each group. However, both control groups showed the presence of ulceration at the site of lice attachment; while no ulceration was observed in the CpG ODN treated fish. The lack of ulceration implies that increased wound healing and tissue remodeling may be occurring in the presence of CpG ODN. Lack of ulceration in CpG treated fish could also be attributed to the lower infection levels observed.

MMP 9 showed increased expression in all groups at 17dpi versus the 7dpi sampling point, not just the CpG ODN group, possibly due to increased damage being inflicted during the on going infection by larger mobile pre-adult stages. Skugor et al. (2008) correlated the up-regulation of MMP 9 with the development of chronic wounds and suggested that modulatory products of sea lice could contribute to prolonged healing time in infected fish.

While many factors point to a higher activation of the adaptive immune response by the CpG treated feed, additional studies are needed that incorporate antibody production, T-cell responses and gene expression to further test and evaluate whether this is occurring. As mentioned earlier, some of the differences between the Trial 1 and Trial 2 designs could be confounding the results seen. It is unclear what effect pre-infection with mobile adult lice has on settlement and attachment of copepodids during a re-infection event. Also, receiving the immunostimulant feed for a shorter period of time prior to infection and sampling may have also influenced the observed responses.

Trial 3: Comparison of high and low dose immunostimulant feeds on sea lice infection

Trial 3 was conducted independently of the first two trials and was designed to compare the effectiveness of high dose and low dose immunostimulant feed formulations. The fish receiving the low dose feed showed significant reductions in sea lice over the high dose and control groups. This also equated to a significant reduction (65%) in adult female lice as compared to the other two groups.

The gene expression data does not clearly show that induction of inflammatory mechanisms were responsible for the significant reduction in lice numbers observed in the low dose treatment group over the high dose. While not statistically significant, the low and high dose treatments showed very consistent expression of IL-1β across all sampling times. This is in contrast to the infected control, which showed more variability in expression throughout the study and was significantly down-regulated at T(2) (7 dpi) versus T(4) (29-31 dpfe). This may indicate that consistent expression of pro-inflammatory genes over time is important in protecting against high levels of infection. However, a related study on these same fish found significant up-regulation of IL-1β in head kidneys of fish fed the low dose immunostimulant compared to both the infected and uninfected controls throughout the study. They also observed a significantly lower IL-1β expression in infected controls compared to uninfected control fish head kidneys at 71 days post infection (T4) (Covello et al., 2011b – in preparation).

Differences between the expression of IL-1β between the spleen and head kidney samples indicate that inflammation in the spleen is not as noticeable in comparison to the head kidney. As fish lack bone marrow and lymph nodes, the head kidney and spleen function as the primary sites of blood cell formation as well as the major lymphoid

organs (Alvarez-Pellitero, 2008; Iliev et al., 2010). While both of these organs contain diverse collections of white blood cells, the spleen contains predominantly lymphoid cells while head kidneys cell cultures show rich populations of granular cells, composed of monocytes, macrophages and dendritic cells (Iliev et al., 2010). For this reason, the head kidney is often considered to be analogous to the bone marrow of higher vertebrates (Alvarez-Pellitero, 2008). While expression of pro-inflammatory genes is often similar between head kidney and spleen samples, this is not always the case. Skugor et al. (2008) observed differences in gene expression between head kidney and spleen during a microarray analysis of immunological genes during a sea lice infection in Atlantic salmon. In general, expression levels were comparable between the two organs across three sampling times, but there were instances when significant up- or down- regulation occurred in one organ and not the other or at different sampling times.

IL-8 showed no differences between treatments within sampling times. However, the infected control, low and high dose all showed significant up-regulation at the first sampling time (T1) versus 29-31 dpfe (T4). The high dose and infected control were also up-regulated at 7 dpi (T2) over 29-31 dpfe while significant up-regulation is seen in 9-11 dpfe (T3) versus 29-31dpfe (T4) in the low dose. MMP 9 showed a significant increase in expression in the high dose over the low dose treatment. This could be due to increased would repair occurring in this group in response to the higher sea lice abundance observed in the high dose group. No other significant effects were observed in MMP 9 expression. TLR 9 showed significant down-regulation in all infected groups as compared to the uninfected control at 7 dpi (T2) after the initial exposure to sea lice.

Some previous studies have looked at whether the immunostimulatory action of certain compounds exhibit dose-dependency. In a review of immunostimulants research in fish, Sakai (1999) brings up several examples where higher doses of immunostimulants showed either no effects or were inhibitory to the immune responses in fish. In this study, the high dose treatment resulted in comparable infection levels to the control group, where as the low dose treated fish exhibited a 50% reduction in sea lice abundance from the high dose and control. While it's not clear from the gene expression data what is driving the differences between the high and low dose formulations, it seems obvious that in this case a higher dose does not amount to an increase in immunostimulation. Further work is needed to determine the optimal dosage for maximum sea lice resistance in Atlantic salmon.

CONCLUSIONS AND SIGNIFICANCE

The work presented here supports the use of dietary immunostimulation as a valuable tool for combating sea lice infections in Atlantic salmon. This study shows that the use of in-feed immunostimulation may be effective in enhancing the innate and adaptive immune response of Atlantic salmon to *L. salmonis* infection. Data indicate that CpG ODN shows potential over β –glucan and yeast immunostimulants to increase the inflammatory response of Atlantic salmon, thus increasing their resistance to infection. This study also demonstrates that CpG ODNs also have potential to relieve sea lice burdens during repeated exposures, which suggests that CpG ODNs may augment development of an adaptive response beyond prior exposure alone. Additional studies are needed looking specifically at measures of adaptive immunity to evaluate whether the reductions in sea lice burdens observed in this study are due to an increase in memory response or other factors. Finally, this study also showed that the commercial application of the patent-pending formulation of nucleotides and immunostimulants, administered at a low dose, could significantly reduce effects of sea lice infection on Atlantic salmon culture. Furthermore, we illustrate that higher doses of immunostimulants do not necessarily equate to greater protection from sea lice. The data from the ABN feed and commercial immunostimulant blend suggest that further work needs to be done to identify the initiating pathways of host resistance obtained through these treatments.

Future research should look into the most efficient and effective application of dietary immunostimulants for sea lice reduction during Atlantic salmon sea cage culture. It is presently unclear whether long-term administration of immunostimulants will

provide lasting protection from ectoparasitic copepods or if a pulse treatment schedule would help avoid the development of tolerance to these compounds. Further studies looking at the most effective doses at relieving sea lice burdens are also needed. Determination of the dose-response relationship is essential to reducing the economic burdens felt by salmon growers, where both the costs of sea lice related losses and the prophylactic treatment to avoid animal loss can weigh against the profitability of commercial culture.

Immunostimulation may also prove to be a valuable component of Integrated Pest Management (IPM) strategies. IPM advocates using all available approaches to minimize pest effects while minimizing use of toxic chemicals. Incorporating immunostimulation into existing treatment protocols using IPM could minimize the negative environmental effects of aquaculture by decreasing the amount of chemical treatments discharged into the local ecosystems and limiting the reservoirs of diseases which may impact endangered wild salmonids.

Despite decades of research, sea lice infection remains the most economically significant disease impacting Atlantic salmon culture, with the costs of sea lice control estimated at nearly half a billion US dollars annually (Costello, 2009). While multiple parasiticide treatments have been employed over the years, none have provided a lasting solution to this endemic pathogen. Identification of immunostimulants, such as CpG ODN, All Brew/ Nupro and commercial immunostimulant blends that provide protection from sea lice infection has the potential to provide immediate relief from this damaging parasite. Development of new chemical parasiticides and/or vaccines could take years to reach the market as these products must undergo extensive testing and regulatory

processes. However, feed supplements, including immunostimulants, are not subject to the same delays because issues such as tissue residence times and environmental impacts are not a concern. Many immunostimulants are already approved for use in agriculture. Therefore, effective dietary immunostimulants could be implemented almost immediately to relieve the pressures of *L. salmonis* infection and minimize the need for costly and potentially environmentally hazardous chemotherapeutants. Enhancing the resistance to *L. salmonis* infections through immunostimulation affords direct benefits to the Atlantic salmon farming industry and consumers and may indirectly reduce impacts to vulnerable species and habits.

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APPENDIX 1: RNA EXTRACTION

Always use sterile, RNAse/ DNAse free pipette tips and microcentrifuge tubes. Wear disposable gloves and a lab coat and perform all work with Trizol and chloroform in a fume hood.

I. Homogenization

1. Homogenize 50-75mg of spleen tissue with 0.75ml of TRIzol® reagent (Invitrogen, Carlsbad, CA) or TRI reagent (Molecular Research Center, Cincinnati, OH) with a sterile, disposable blue pestle in a 1.5ml microcentifuge tube.

II. Phase separation

2. Incubate homogenized sample for 5 minutes at room temperature.
3. Add 0.2ml (200µl) chloroform (brand?) to each sample, shake vigorously by hand for 15 seconds and incubate at room temperature for 2-3 minutes.
4. Centrifuge samples at 10,000 x g for 15 minutes at 4°C.

III. RNA Precipitation

5. Transfer aqueous phase to a new 1.5 ml tube, add 0.5ml (500 µl) of 2-propanol (brand), and incubate at room temperature for 10 minutes.
6. Centrifuge sample tubes at 10,000 x g for 10 minutes at 4°C.

IV. RNA wash

7. Remove the supernatant and wash the RNA pellet in 1 ml of ice cold 75% ethanol (brand) and briefly vortex.
8. Centrifuge at 7,500 x g for 5 minutes at 4°C and remove the supernatant
9. Air dry the RNA pellet for 10-15 minutes.

V. Redissolving RNA pellet

10. Dissolve the pellet in 100µl of RNase-free water.
11. Incubate at 60°C for 10 minutes then store at -80°C.

APPENDIX 2: DNASE TREATMENT

Always use sterile, RNAse/ DNAse free pipette tips and microcentrifuge tubes. Wear disposable gloves and a lab coat.

1. Thaw RNA and TURBO DNA-free kit™ (Applied Biosystems/Ambion, Austin, TX) reagents on ice.
2. Add RNA sample and nuclease-free water at a concentration of 5µg RNA/44µl to a 0.5 ml microcentrifuge tube
3. Add 5µl of 10x TURBO DNase Buffer and 1µl TURBO DNase to the RNA for a final reaction volume of 50µl, and mix well.
4. Incubate for 30 minutes at 37°C.
5. Add 5µl of resuspended DNase Inactivation Reagent and mix well.
6. Incubate for 5 minutes at room temperature, mixing often as suspension settles during incubation.
7. Centrifuge the sample at 10,000 x g for 1.5 minutes.
8. Transfer the supernatant to a clean 0.5 ml tube. This should yield an RNA concentration of approximately 1µg/10ml.

APPENDIX 3: CDNA SYNTHESIS

Use RNase/DNase – free pipette tips and microcentrifuge tubes. Prepare reaction mixes in a clean, no-template hood. Wear disposable gloves and a lab coat.

1. Thaw RNA (previously treated with Turbo DNA-free kit™) and reagents from the Promega Reverse Transcription System kit (Promega, Madison, WI) on ice.
2. Transfer 10µl of RNA to new 0.5ml PCR tube and store remaining RNA at -80°C until next use.
3. Incubate at 70°C for 10 minutes, centrifuge briefly then store on ice.
4. Prepare the reverse transcription reaction master mix as follows (per reaction):

    - $MgCl_2$ 4µl
    - RT 10x Buffer 2µl
    - dNTP 2µl
    - RNase inhibitor .5µl
    - Random Primers 1µl
    - AMV .6µl

5. Transfer 10µl of master mix to 0.5ml PCR tube containing 10µl RNA.
6. Incubate reaction at room temperature for 10 minutes

7. Incubate in thermocycler under the following conditions:
    42°C for 15min
    95°C for 5
    4°C for 5
8. First strand cDNA synthesis complete.
9. Dilute samples 1:1 with nuclease-free water and store at -20°C.

APPENDIX 4: QRT-PCR

Use RNase/DNase – free pipette tips and microcentrifuge tubes. Prepare reaction mixes in a clean, no-template hood. Wear disposable gloves and a lab coat.

1. Thaw cDNA, reagents provided in the GoTaq® qPCR mix (Promega, Madison, WI), and forward and reverse primers on ice.
2. Program real-time thermal cycler (Eppendorf, Westbury, NY) as follows: 10 minute at 95°C, 40 cycles (95°C for 15 sec, 55°C for 20 sec, 72°C for 30 sec), 95°C for 15 sec, 60°C for 15 sec, followed by a melt curve analysis for 20 minutes and finishing with 95°C for 15 sec.
3. Program thermal cycler with plate layout and reaction volume.
4. Prepare 5-6 1:10 dilutions for house keeping genes by combining pooled cDNA from all samples with nuclease-free water. Prepare 5-6 1:5 dilutions for genes of interest.
5. Prepare a master mix as follows:

    - GoTaq qPCR MM 2x 10µl
    - Nuclease –free H_2O 7µl
    - Fwd Primer (10µM stock) 1µl
    - Rev Primer (10µM stock) 1µl

6. Place 96-well PCR plate on ice.
7. Add 19µl of master mix to each well of plate following predetermined template.
8. Add 1µl of cDNA, negative reverse transcription sample or nuclease-free water (no-template control) following template.
9. Seal plate with qPCR grade optical film and centrifuge for 1.5 minutes at 1000 x g.
10. Place plate in thermal cycler and press play to run assay.

APPENDIX 5: STATISTICAL DATA TABLES

Table 2: Trial 1 Statistical differences in expression of IL-1β using Two-way ANOVA and Tukey multiple comparison tests

A. ANOVA

Trial 1 Gene	Source of Variation	P
IL-1B	Treatment	0.003
IL-1B	Time	0.107
IL-1B	Treatment x Time	0.047

B. Multiple comparison Tests

Trial 1 Gene	Treatment Comparison	P	Time Comparison	P
IL-1B	Provale vs. Uninf. CNTL	0.004	Time 2 vs. Time 4	0.145
IL-1B	Provale vs. ABN	0.135	Time 2 vs. Time 3	0.961
IL-1B	Provale vs. CNTL Inf.	0.202	Time 2 vs. Time 1	1
IL-1B	Provale vs. CpG ODN	0.987	Time 1 vs. Time 4	0.115
IL-1B	CpG ODN vs. Uninf. CNTL	0.018	Time 1 vs. Time 3	0.972
IL-1B	CpG ODN vs. ABN	0.331	Time 3 vs. Time 4	0.355
IL-1B	CpG ODN vs. CNTL Inf.	0.439		
IL-1B	CNTL Inf. vs. Uninf. CNTL	0.685		
IL-1B	CNTL Inf. vs. ABN	1		
IL-1B	ABN vs. Uninf. CNTL	0.742		

C. Multiple Comparison Tests

Trial 1 Gene	Time w/in Control Infected Comparison	P	Time w/in Provale Comparison	P	Time w/in CpG ODN Comparison	P	Time w/in ABN Comparison	P	Time within Unifected Control Comparison	P
IL-1B	Time 1 vs. Time 3	0.562	Time 2 vs. Time 1	0.7	Time 2 vs. Time 4	0.043	Time 1 vs. Time 4	<0.001	Time 3 vs. Time 2	0.381
IL-1B	Time 1 vs. Time 4	0.784	Time 2 vs. Time 3	0.883	Time 2 vs. Time 1	0.144	Time 1 vs. Time 2	0.247	Time 3 vs. Time 4	0.424
IL-1B	Time 1 vs. Time 2	0.975	Time 2 vs. Time 4	0.999	Time 2 vs. Time 3	0.442	Time 1 vs. Time 3	0.393	Time 3 vs. Time 1	0.674
IL-1B	Time 2 vs. Time 3	0.86	Time 4 vs. Time 1	0.818	Time 3 vs. Time 4	0.722	Time 3 vs. Time 4	0.135	Time 1 vs. Time 2	0.93
IL-1B	Time 2 vs. Time 4	0.975	Time 4 vs. Time 3	0.946	Time 3 vs. Time 1	0.97	Time 3 vs. Time 2	0.989	Time 1 vs. Time 4	0.953
IL-1B	Time 4 vs. Time 3	0.975	Time 3 vs. Time 1	0.988	Time 1 vs. Time 4	0.898	Time 2 vs. Time 4	0.283	Time 4 vs. Time 2	1

D. Multiple Comparison Tests

Trial 1 Gene	Treatment within Time 1 Comparison	P	Treatment within Time 2 Comparison	P	Treatment within Time 3 Comparison	P	Treatment within Time 4 Comparison	P
IL-1B	ABN vs. Uninf. CNTL	0.055	CpG ODN vs. Uninf. CNTL	0.003	Provale vs. CNTL Inf.	0.614	Provale vs. ABN	0.015
IL-1B	ABN vs. CpG ODN	0.585	CpG ODN vs. ABN	0.105	Provale vs.ABN	0.967	Provale vs. Uninf. CNTL	0.119
IL-1B	ABN vs. Provale	0.701	CpG ODN vs. CNTL Inf.	0.262	Provale vs. Uninf. CNTL	0.994	Provale vs. CpG ODN	0.526
IL-1B	ABN vs. Control Infe	0.78	CpG ODN vs. Provale	0.943	Provale vs. CpG ODN	1	Provale vs. CNTL Inf.	0.528
IL-1B	CNTL Inf vs. Uninf. CNTL	0.557	Provale vs. Uninf. CNTL	0.053	CpG ODN vs. CNTL Inf.	0.641	CNTL Inf. vs. ABN	0.409
IL-1B	CNTL Inf. vs. CpG ODN	0.999	Provale vs. ABN	0.48	CpG ODN vs. ABN	0.971	CNTL Inf. vs. Uninf. CNTL	0.88
IL-1B	CNTL Inf. vs. Provale	1	Provale vs. CNTL Inf.	0.725	CpG ODN vs. Uninf. CNTL	0.995	CNTL Inf. vs. CpG ODN	1
IL-1B	Provale vs. Uninf. CNTL	0.642	CNTL Inf. vs. Uninf. CNTL	0.654	Uninf. CNTL vs. CNTL Inf.	0.859	CpG ODN vs. ABN	0.41
IL-1B	Provale vs. CpG ODN	1	CNTL Inf. vs. ABN	0.998	Uninf. CNTL vs. ABN	1	CpG ODN vs. Uninf. CNTL	0.881
IL-1B	CpG ODN vs. Uninf. CNTL	0.714	ABN vs. Uninf. CNTL	0.816	ABN vs. CNTL Inf.	0.924	Uninf. CNTL vs. ABN	0.939

Notes: **Uninf. CNTL refers to the uninfected control group, CNTL Inf. refers to the infected control, ABN refers to the All Brew/Nupro treated group**

Table A: Two-way ANOVA results, Table B: Tukey multiple comparison tests of overall treatment and overall time, Table C: Tukey multiple comparison tests of Time within treatment, Table D: Tukey multiple comparison tests of Treatment within sampling time points.

Statistical differences of $p<0.05$ are highlighted in green

Table 3: Trial 1 Statistical differences in expression of IL-8 using Two-way ANOVA and Tukey multiple comparison tests

A. ANOVA

Trial 1 Gene	Source of Variation	P
IL-8	Treatment	0.075
IL-8	Time	<0.001
IL-8	Treatment x Time	0.125

B. Multiple comparison Tests

Trial 1 Gene	Treatment Comparison	P	Time Comparison	P
IL-8	CNTL Inf. vs. ABN	0.102	Time 4 vs. Time 1	<0.001
IL-8	CNTL Inf. vs. Uninf. CNTL	0.174	Time 4 vs. Time 2	<0.001
IL-8	CNTL Inf. vs. CpG ODN	0.243	Time 4 vs. Time 3	<0.001
IL-8	CNTL Inf. vs. Provale	0.899	Time 3 vs. Time 1	0.214
IL-8	Provale vs. ABN	0.521	Time 3 vs. Time 2	0.908
IL-8	Provale vs. Uninf. CNTL	0.69	Time 2 vs. Time 1	0.602
IL-8	Provale vs. CpG ODN	0.777		
IL-8	CpG ODN vs. ABN	0.994		
IL-8	CpG ODN vs. Uninf. CNTL	1		
IL-8	Uninf. CNTL vs.ABN	0.998		

C. Multiple Comparison Tests

Trial 1 Gene	Time w/in Control Infected Comparison	P	Time w/in Provale Comparison	P	Time w/in CpG ODN Comparison	P	Time w/in ABN Comparison	P	Time within Unifected Control Comparison	P
IL-8	Time 4 vs. Time 1	0.006	Time 4 vs. Time 1	0.002	Time 4 vs. Time 1	<0.001	Time 4 vs. Time 3	0.004	Time 4 vs. Time 2	<0.001
IL-8	Time 4 vs. Time 3	0.452	Time 4 vs. Time 3	0.109	Time 4 vs. Time 2	<0.001	Time 4 vs. Time 2	0.009	Time 4 vs. Time 1	<0.001
IL-8	Time 4 vs. Time 2	0.709	Time 4 vs. Time 2	0.238	Time 4 vs. Time 3	0.004	Time 4 vs. Time 1	0.069	Time 4 vs. Time 3	0.001
IL-8	Time 2 vs. Time 1	0.154	Time 2 vs. Time 1	0.396	Time 3 vs. Time 1	0.421	Time 1 vs. Time 3	0.713	Time 3 vs. Time 2	0.412
IL-8	Time 2 vs. Time 3	0.971	Time 2 vs. Time 3	0.971	Time 3 vs. Time 2	0.742	Time 1 vs. Time 2	0.807	Time 3 vs. Time 1	0.575
IL-8	Time 3 vs. Time 1	0.413	Time 3 vs. Time 1	0.721	Time 2 vs. Time 1	0.963	Time 2 vs. Time 3	0.999	Time 1 vs. Time 2	0.981

D. Multiple Comparison Tests

Trial 1 Gene	Treatment within Time 1 Comparison	P	Treatment within Time 2 Comparison	P	Treatment within Time 3 Comparison	P	Treatment within Time 4 Comparison	P
IL-8	ABN vs. CpG ODN	0.525	CNTL Inf. vs. Uninf. CNTL	0.012	CNTL Inf. vs.ABN	0.183	Uninf. CNTL vs. ABN	0.717
IL-8	ABN vs. Uninf. CNTL	0.698	CNTL Inf. vs. CpG ODN	0.058	CNTL vs. Uninf. CNTL	0.772	Uninf. CNTL vs. CNTL Inf.	0.856
IL-8	ABN vs. Provale	0.975	CNTL Inf. vs.ABN	0.088	CNTL Inf. vs. CpG ODN	0.813	Uninf. CNTL vs. Provale	0.877
IL-8	ABN vs. CNTL Inf.	0.999	CNTL Inf. vs. Provale	0.942	CNTL Inf. vs. Provale	0.955	Uninf. CNTL vs. CpG ODN	1
IL-8	CNTL Inf. vs. CpG ODN	0.661	Provale vs. Uninf. CNTL	0.103	Provale vs.ABN	0.588	CpG ODN vs. ABN	0.779
IL-8	CNTL Inf. vs. Uninf. CNTL	0.826	Provale vs. CpG ODN	0.311	Provale vs. Uninf. CNTL	0.993	CpG ODN vs. CNTL	0.898
IL-8	CNTL Inf. vs. Provale	0.997	Provale vs. ABN	0.387	Provale vs. CpG ODN	0.996	CpG ODN vs. Provale	0.915
IL-8	Provale vs. CpG ODN	0.864	ABN vs. Uninf. CNTL	0.977	CpG ODN vs. ABN	0.823	Provale vs. ABN	0.997
IL-8	Provale vs. Uninf. CNTL	0.958	ABN vs. CpG ODN	1	CpG ODN vs. Uninf. CNTL	1	Provale vs. CNTL Inf.	1
IL-8	Uninf. CNTL vs. CpG ODN	0.998	CpG ODN vs. Uninf. CNTL	0.984	Uninf. CNTL vs. ABN	0.825	CNTL Inf. vs. ABN	0.998

Notes: **Uninf. CNTL refers to the uninfected control group, CNTL Inf. refers to the infected control, ABN refers to the All Brew/Nupro treated group**

Table A: Two-way ANOVA results, Table B: Tukey multiple comparison tests of overall treatment and overall time, Table C: Tukey multiple comparison tests of Time within treatment, Table D: Tukey multiple comparison tests of Treatment within sampling time points.

Statistical differences of p<0.05 are highlighted in green

Table 4: Trial 1 Statistical differences in expression of MMP 9 using Two-way ANOVA and Tukey multiple comparison tests

A. ANOVA

Trial 1 Gene	Source of Variation	P
MMP 9	Treatment	<0.001
MMP 9	Time	0.138
MMP 9	Treatment x Time	0.157

B. Multiple comparison Tests

Trial 1 Gene	Treatment Comparison	P	Time Comparison	P
MMP 9	CpG ODN vs. Uninf. CNTL	<0.001	Time 3 vs. Time 4	0.178
MMP 9	CpG ODN vs. CNTL Inf.	0.016	Time 3 vs. Time 2	0.968
MMP 9	CpG ODN vs. ABN	0.228	Time 3 vs. Time 1	0.998
MMP 9	CpG ODN vs. Provale	0.702	Time 1 vs. Time 4	0.158
MMP 9	Provale vs. Uninf. CNTL	0.006	Time 1 vs. Time 2	0.986
MMP 9	Provale vs. CNTL Inf.	0.366	Time 2 vs. Time 4	0.376
MMP 9	Provale vs. ABN	0.932		
MMP 9	ABN vs. Uninf. CNTL	0.062		
MMP 9	ABN vs. CNTL Inf.	0.85		
MMP 9	CNTL Inf. vs. Uninf CNTL	0.452		

C. Multiple Comparison Tests

Trial 1 Gene	Time w/in Control Infected Comparison	P	Time w/in Provale Comparison	P	Time w/in CpG ODN Comparison	P	Time w/in ABN Comparison	P	Time w/in Unifected Control Comparison	P
MMP 9	Time 1 vs. Time 2	0.062	Time 4 vs. Time 1	0.725	Time 3 vs. Time 4	0.101	Time 2 vs. Time 4	0.067	Time 1 vs. Time 4	0.815
MMP 9	Time 1 vs. Time 4	0.162	Time 4 vs. Time 2	0.968	Time 3 vs. Time 1	0.443	Time 2 vs. Time 1	0.793	Time 1 vs. Time 2	0.925
MMP 9	Time 1 vs. Time 3	0.26	Time 4 vs. Time 3	1	Time 3 vs. Time 2	0.956	Time 2 vs. Time 3	0.876	Time 1 vs. Time 3	0.983
MMP 9	Time 3 vs. Time 2	0.904	Time 3 vs. Time 1	0.784	Time 2 vs. Time 4	0.256	Time 3 vs. Time 4	0.352	Time 3 vs. Time 4	0.974
MMP 9	Time 3 vs. Time 4	0.996	Time 3 vs. Time 2	0.981	Time 2 vs. Time 1	0.76	Time 3 vs. Time 1	1	Time 3 vs. Time 2	0.997
MMP 9	Time 4 vs. Time 2	0.967	Time 2 vs. Time 1	0.944	Time 1 vs. Time 4	0.753	Time 1 vs. Time 4	0.281	Time 2 vs. Time 4	0.996

D. Multiple Comparison Tests

Trial 1 Gene	Treatment within Time 1 Comparison	P	Treatment within Time 2 Comparison	P	Treatment within Time 3 Comparison	P	Treatment within Time 4 Comparison	P
MMP 9	CNTL Inf. vs. Uninf. CNTL	0.171	CpG ODN vs. Uninf. CNTL	0.025	CpG ODN vs. Uninf. CNTL	0.02	Provale vs. Uninf. CNTL	0.108
MMP 9	CNTL Inf. vs. Provale	0.628	CpG ODN vs. CNTL Inf.	0.032	CpG ODN vs. CNTL Inf.	0.053	Provale vs.ABN	0.181
MMP 9	CNTL Inf vs. ABN	0.869	CpG ODN vs. Provale	0.711	CpG ODN vs. ABN	0.415	Provale vs. CNTL Inf.	0.439
MMP 9	CNTL Inf vs. CpG ODN	0.995	CpG ODN vs. ABN	0.983	CpG ODN vs. Provale	0.683	Provale vs. CpG ODN	0.844
MMP 9	CpG ODN vs. Uninf. CNTL	0.356	ABN vs. Uninf. CNTL	0.103	Provale vs. Uninf. CNTL	0.388	CpG ODN vs. Uninf. CNTL	0.603
MMP 9	CpG ODN vs. Provale	0.848	ABN vs. CNTL Inf.	0.128	Provale vs. CNTL Inf.	0.657	CpG ODN vs. ABN	0.749
MMP 9	CpG ODN vs.ABN	0.98	ABN vs. Provale	0.951	Provale vs.ABN	0.994	CpG ODN vs. CNTL Inf.	0.964
MMP 9	ABN vs. Uninf. CNTL	0.69	Provale vs. Uninf. CNTL	0.406	ABN vs. Uninf. CNTL	0.644	CNTL Inf. vs. Uninf. CNTL	0.932
MMP 9	ABN vs. Provale	0.989	Provale vs. CNTL Inf.	0.462	ABN vs. Control Infe	0.889	CNTL Inf. vs. ABN	0.981
MMP 9	Provale vs. Uninf. CNTL	0.939	CNTL Inf. vs. Uninf. CNTL	1	CNTL Inf. vs. Uninf. CNTL	0.984	ABN vs. Uninf. CNTL	0.999

Notes: **Uninf. CNTL refers to the uninfected control group, CNTL Inf. refers to the infected control, ABN refers to the All Brew/Nupro treated group**

Table A: Two-way ANOVA results, Table B: Tukey multiple comparison tests of overall treatment and overall time, Table C: Tukey multiple comparison tests of Time within treatment, Table D: Tukey multiple comparison tests of Treatment within sampling time points.

Statistical differences of p<0.05 are highlighted in green

Table 5: Trial 1 Statistical differences in expression of TLR 9 using Two-way ANOVA and Tukey multiple comparison tests

A. ANOVA

Trial 1 Gene	Source of Variation	P
TLR 9	Treatment	0.984
TLR 9	Time	0.024
TLR 9	Treatment x Time	0.126

B. Multiple comparison Tests

Trial 1 Gene	Treatment Comparison	P	Time Comparison	P
TLR 9	CNTL Inf. vs. CpG ODN	0.982	Time 3 vs. Time 1	0.013
TLR 9	CNTL Inf. vs. Uninf. CNTL	0.996	Time 3 vs. Time 4	0.283
TLR 9	CNTL Inf. vs. ABN	0.999	Time 3 vs. Time 2	0.662
TLR 9	CNTL Inf. vs. Provale	1	Time 2 vs. Time 1	0.269
TLR 9	Provale vs. CpG ODN	0.992	Time 2 vs. Time 4	0.912
TLR 9	Provale vs. Uninf. CNTL	0.999	Time 4 vs. Time 1	0.715
TLR 9	Provale vs. ABN	1		
TLR 9	ABN vs. CpG ODN	0.998		
TLR 9	ABN vs. Uninf. CNTL	1		
TLR 9	Uninf. CNTL vs. CpG ODN	1		

C. Multiple Comparison Tests

Trial 1 Gene	Time w/in Control Infected Comparison	P	Time w/in Provale Comparison	P	Time w/in CpG ODN Comparison	P	Time w/in ABN Comparison	P	Time w/in Unifected Control Comparison	P
TLR 9	Time 3 vs. Time 1	0.803	Time 3 vs. Time 4	0.009	Time 3 vs. Time 1	0.193	Time 2 vs. Time 1	0.089	Time 4 vs. Time 1	0.087
TLR 9	Time 3 vs. Time 2	0.86	Time 3 vs. Time 1	0.288	Time 3 vs. Time 4	0.641	Time 2 vs. Time 3	0.403	Time 4 vs. Time 2	0.249
TLR 9	Time 3 vs. Time 4	0.875	Time 3 vs. Time 2	0.679	Time 3 vs. Time 2	0.66	Time 2 vs. Time 4	0.682	Time 4 vs. Time 3	0.883
TLR 9	Time 4 vs. Time 1	1	Time 2 vs. Time 4	0.145	Time 2 vs. Time 1	0.885	Time 4 vs. Time 1	0.675	Time 3 vs. Time 1	0.402
TLR 9	Time 4 vs. Time 2	1	Time 2 vs. Time 1	0.938	Time 2 vs. Time 4	1	Time 4 vs. Time 3	0.963	Time 3 vs. Time 2	0.68
TLR 9	Time 2 vs. Time 1	1	Time 1 vs. Time 4	0.294	Time 4 vs. Time 1	0.924	Time 3 vs. Time 1	0.946	Time 2 vs. Time 1	0.984

D. Multiple Comparison Tests

Trial 1 Gene	Treatment within Time 1 Comparison	P	Treatment within Time 2 Comparison	P	Treatment within Time 3 Comparison	P	Treatment within Time 4 Comparison	P
TLR 9	Provale vs. Uninf. CNTL	0.762	ABN vs. Uninf. CNTL	0.292	Provale vs. ABN	0.332	Uninf. CNTL vs. Provale	0.044
TLR 9	Provale vs. CpG ODN	0.837	ABN vs. CpG ODN	0.577	Provale vs. Uninf. CNTL	0.763	Uninf. CNTL vs. CpG ODN	0.626
TLR 9	Provale vs.ABN	0.839	ABN vs. CNTL Inf.	0.688	Provale vs. CNTL Inf.	0.906	Uninf. CNTL vs. CNTL Inf.	0.765
TLR 9	Provale vs. CNTL Inf.	1	ABN vs. Provale	0.94	Provale vs. CpG ODN	0.958	Uninf. CNTL vs. ABN	0.848
TLR 9	CNTL Inf. vs. Uninf. CNTL	0.794	Provale vs. Uninf. CNTL	0.762	CpG ODN vs. ABN	0.728	ABN vs. Provale	0.379
TLR 9	CNTL Inf. vs. CpG ODN	0.864	Provale vs. CpG ODN	0.954	CpG ODN vs. Uninf. CNTL	0.987	ABN vs. CpG ODN	0.993
TLR 9	CNTL Inf. vs. ABN	0.867	Provale vs. CNTL Inf.	0.983	CpG ODN vs. CNTL Inf.	1	ABN vs. CNTL Inf.	1
TLR 9	ABN vs. Uninf. CNTL	1	CNTL Inf. vs. Uninf. CNTL	0.968	CNTL Inf. vs. ABN	0.828	CNTL Inf. vs. Provale	0.474
TLR 9	ABN vs. CpG ODN	1	CNTL Inf. vs. CpG ODN	1	CNTL Inf. vs. Uninf. CNTL	0.998	CNTL Inf. vs. CpG ODN	0.999
TLR 9	CpG ODN vs. Uninf. CNTL	1	CpG ODN vs. Uninf. CNTL	0.99	Uninf. CNTL vs. ABN	0.943	CpG ODN vs. Provale	0.675

Notes: Uninf. CNTL refers to the uninfected control group, CNTL Inf. refers to the infected control, ABN refers to the All Brew/Nupro treated group

Table A: Two-way ANOVA results, Table B: Tukey multiple comparison tests of overall treatment and overall time, Table C: Tukey multiple comparison tests of Time within treatment, Table D: Tukey multiple comparison tests of Treatment within sampling time points.

Statistical differences of $p<0.05$ are highlighted in green

Table 6: Trial 2 Statistical differences in expression of IL-1β and IL-8 using Two-way ANOVA and Tukey multiple comparison tests

A. ANOVA

Trial 2 Gene	Source of Variation	P
IL-1B	Treatment	0.062
IL-1B	Time	0.558
IL-1B	Treatment x Time	0.037

B. Multiple comparison Tests

Trial 2 Gene	Treatment Comparison	P	Time Comparison	P
IL-1B	2nd CNTL vs. 1st CNTL	0.054	Time 2 vs. Time 1	0.558
IL-1B	2nd CNTL vs. CpG ODN	0.151		
IL-1B	CpG ODN vs. 1st CNTL	0.84		

C. Multiple comparison Tests

Trial 2 Gene	Time w/in 2nd Control Comparison	P	Time w/in CpG ODN Comparison	P	Time w/in 1st Control Comparison	P
IL-1B	Time 2 vs. Time 1	0.619	Time 1 vs. Time 2	0.112	Time 2 vs. Time 1	0.038

D. Multiple Comparison Tests

Trial 2 Gene	Treatment within Time 1 Comparison	P	Treatment within Time 2 Comparison	P
IL-1B	2nd CNTL vs.1st CNTL	0.051	2nd CNTL vs. CpG ODN	0.082
IL-1B	2nd CNTL vs. CpG ODN	0.918	2nd CNTL vs. 1st CNTL	0.558
IL-1B	CpG ODN vs. 1st CNTL	0.085	1st CNTL vs. CpG ODN	0.278

E. ANOVA

Trial 2 Gene	Source of Variation	P
IL-8	Treatment	0.449
IL-8	Time	<0.001
IL-8	Treatment x Time	0.104

F. Multiple comparison Tests

Trial 2 Gene	Treatment Comparison	P	Time Comparison	P
IL-8	1st CNTL vs. CpG ODN	0.429	Time 2 vs. Time 1	<0.001
IL-8	1st CNTL vs. 2nd CNTL	0.74		
IL-8	2nd CNTL vs. CpG ODN	0.935		

G. Multiple comparison Tests

Trial 2 Gene	Time w/in 2nd Control Comparison	P	Time w/in CpG ODN Comparison	P	Time w/in 1st Control Comparison	P
IL-8	Time 2 vs. Time 1	0.011	Time 2 vs. Time 1	0.164	Time 2 vs. Time 1	<0.001

H. Multiple Comparison Tests

Trial 2 Gene	Treatment within Time 1 Comparison	P	Treatment within Time 2 Comparison	P
IL-8	CpG ODN vs. 2nd CNTL	0.817	1st CNTL vs. CpG ODN	0.031
IL-8	CpG ODN vs. 1st CNTL	0.816	1st CNTL vs. 2nd CNTL	0.605
IL-8	1st CNTL vs. 2nd CNTL	0.999	2nd CNTL vs. CpG ODN	0.53

Notes: **1st CNTL refers to the 1st infection control group, 2nd CNTL refers to the 2nd infection control group**

Tables A-D: IL-1β data; Table A: Two-way ANOVA results, Table B: Tukey multiple comparison tests of overall treatment and overall time, Table C: Tukey multiple comparison tests of Time within treatment, Table D: Tukey multiple comparison tests of Treatment within sampling time points.

Tables E-H :IL-8 data; Table E: Two-way ANOVA results, Table F: Tukey multiple comparison tests of overall treatment and overall time, Table G: Tukey multiple comparison tests of Time within treatment, Table H: Tukey multiple comparison tests of Treatment within sampling time points.

Statistical differences of $p<0.05$ are highlighted in green

Table 7: Trial 2 Statistical differences in expression of MMP 9 and TLR 9 using Two-way ANOVA and Tukey multiple comparison tests

A. ANOVA

Trial 2 Gene	Source of Variation	P
MMP 9	Treatment	0.28
MMP 9	Time	<0.001
MMp 9	Treatment x Time	0.692

B. Multiple comparison Tests

Trial 2 Gene	Treatment Comparison	P	Time Comparison	P
MMP 9	1st CNTL vs. CpG ODN	0.249	Time 2 vs. Time 1	<0.001
MMP 9	1st CNTL vs. 2nd CNTL	0.773		
MMp 9	2nd CNTL vs. CpG ODN	0.774		

C. Multiple comparison Tests

Trial 2 Gene	Time w/in 2nd Control Comparison	P	Time w/in CpG ODN Comparison	P	Time w/in 1st Control Comparison	P
MMP 9	Time 2 vs. Time 1	0.014	Time 2 vs. Time 1	<0.001	Time 2 vs. Time 1	<0.001

D. Multiple Comparison Tests

Trial 2 Gene	Treatment within Time 1 Comparison	P	Treatment within Time 2 Comparison	P
MMP 9	2nd CNTL vs. CpG ODN	0.715	1st CNTL vs. CpG ODN	0.244
MMP 9	2nd CNTL vs. 1st CNTL	0.993	1st CNTL vs. 2nd CNTL	0.534
MMP 9	1st CNTL vs. CpG ODN	0.744	2nd CNTL vs. CpG ODN	0.98

E. ANOVA

Trial 2 Gene	Source of Variation	P
TLR 9	Treatment	0.01
TLR 9	Time	0.567
TLR 9	Treatment x Time	0.009

F. Multiple comparison Tests

Trial 2 Gene	Treatment Comparison	P	Time Comparison	P
TLR 9	CpG ODN vs. 1st CNTL	0.013	Time 1 vs. Time 2	0.568
TLR 9	CpG ODN vs. 2nd CNTL	0.062		
TLR 9	2nd CNTL vs. 1st CNTL	0.984		

G. Multiple comparison Tests

Trial 2 Gene	Time w/in 2nd Control Comparison	P	Time w/in CpG ODN Comparison	P	Time w/in 1st Control Comparison	P
TLR 9	Time 1 vs. Time 2	0.171	Time 1 vs. Time 2	0.105	Time 2 vs. Time 1	0.02

H. Multiple Comparison Tests

Trial 2 Gene	Treatment within Time 1 Comparison	P	Treatment within Time 2 Comparison	P
TLR 9	CpG ODN vs. 1st CNTL	<0.001	CpG ODN vs. 2nd CNTL	0.226
TLR 9	CpG ODN vs. 2nd CNTL	0.246	CpG ODN vs. 1st CNTL	0.997
TLR 9	2nd CNTL vs. 1st CNTL	0.119	1st CNTL vs. 2nd CNTL	0.249

Notes: **1st CNTL refers to the 1st infection control group, 2nd CNTL refers to the 2nd infection control group**

Tables A-D: MMP 9 data; Table A: Two-way ANOVA results, Table B: Tukey multiple comparison tests of overall treatment and overall time, Table C: Tukey multiple comparison tests of Time within treatment, Table D: Tukey multiple comparison tests of Treatment within sampling time points.

Tables E-H :TLR 9 data; Table E: Two-way ANOVA results, Table F: Tukey multiple comparison tests of overall treatment and overall time, Table G: Tukey multiple comparison tests of Time within treatment, Table H: Tukey multiple comparison tests of Treatment within sampling time points.

Statistical differences of $p<0.05$ are highlighted in green

Table 8: Trial 3 Statistical differences in expression of IL-1β using Two-way ANOVA and Tukey multiple comparison tests

A. ANOVA

Trial 3 Gene	Source of Variation	P
IL-1B	Treatment	0.244
IL-1B	Time	0.124
IL-1B	Treatment x Time	0.273

B. Multiple comparison Tests

Trial 3 Gene	Treatment Comparison	P	Time Comparison	P
IL-1B	High Dose vs. CNTL Inf.	0.28	Time 4 vs. Time 3	0.121
IL-1B	High Dose vs. Uninf. CNTL	0.76	Time 4 vs. Time 2	0.205
IL-1B	High Dose vs. Low dose	0.999	Time 4 vs. Time 1	0.467
IL-1B	Low dose vs. CNTL Inf.	0.352	Time 1 vs. Time 3	0.868
IL-1B	Low dose vs. Uninf. CCNTL	0.822	Time 1 vs. Time 2	0.952
IL-1B	Uninf. CNTLl vs. CNTL Inf.	0.959	Time 2 vs. Time 3	0.996

C. Multiple Comparison Tests

Trial 3 Gene	Time w/in Low Dose Comparison	P	Time w/in High Dose Comparison	P	Time w/in Control Infected Comparison	P	Time w/in Uninfected Control Comparison	P
IL-1B	Time 3 vs. Time 1	0.798	Time 4 vs. Time 2	0.708	Time 4 vs. Time 2	0.011	Time 2 vs. Time 3	0.133
IL-1B	Time 3 vs. Time 2	0.918	Time 4 vs. Time 3	0.862	Time 4 vs. Time 3	0.393	Time 2 vs. Time 1	0.798
IL-1B	Time 3 vs. Time 4	1	Time 4 vs. Time 1	0.997	Time 4 vs. Time 1	0.531	Time 2 vs. Time 4	0.988
IL-1B	Time 4 vs. Time 1	0.81	Time 1 vs. Time 2	0.798	Time 1 vs. Time 2	0.286	Time 4 vs. Time 3	0.255
IL-1B	Time 4 vs. Time 2	0.925	Time 1 vs. Time 3	0.928	Time 1 vs. Time 3	0.994	Time 4 vs. Time 1	0.936
IL-1B	Time 2 vs. Time 1	0.996	Time 3 vs. Time 2	0.991	Time 3 vs. Time 2	0.445	Time 1 vs. Time 3	0.594

D. Multiple Comparison Tests

Trial 3 Gene	Treatment within Time 1 Comparison	P	Treatment within Time 2 Comparison	P	Treatment within Time 3 Comparison	P	Treatment within Time 4 Comparison	P
IL-1B	High Dose vs. CNTL Inf.	0.713	Uninf. CNTL vs. CNTL Inf.	0.055	Low dose vs. Uninf. CNTL	0.102	CNTL vs. Uninf. CNTL	0.979
IL-1B	High Dose vs. Uninf. CNTL	0.838	Uninf. CNTL vs. High Dose	0.769	Low dose vs. CNTL Inf.	0.565	CNTL vs. Low dose	0.99
IL-1B	High Dose vs. Low dose	0.8	Uninf. CNTL vs. Low dose	0.879	Low dose vs. High Dose	0.927	CNTL vs. High Dose	1
IL-1B	Low dose vs. CNTL Inf.	0.999	Low dose vs. CNTL Inf.	0.167	High Dose vs. Uninf CNTL	0.282	High Dose vs. Uninf. CNTL	0.989
IL-1B	Low dose vs. Uninf. CNTL	1	Low dose vs. High Dose	0.996	High Dose vs. CNTL Inf.	0.899	High Dose vs. Low dose	0.996
IL-1B	Uninf. CNTL vs. CNTL Inf.	1	High Dose vs. CNTL Inf.	0.22	CNTL vs. Uninf. CNTL	0.637	Low dose vs. Uninf. CNTL	0.999

Notes: **Uninf. CNTL refers to the uninfected control group; CNTL Inf. refers to the infected control**

Table A: Two-way ANOVA results, Table B: Tukey multiple comparison tests of overall treatment and overall time, Table C: Tukey multiple comparison tests of Time within treatment, Table D: Tukey multiple comparison tests of Treatment within sampling time points.

Statistical differences of $p<0.05$ are highlighted in green

Table 9: Trial 3 Statistical differences in expression of IL-8 using Two-way ANOVA and Tukey multiple comparison tests

A. ANOVA

Trial 3 Gene	Source of Variation	P
IL-8	Treatment	0.035
IL-8	Time	<0.001
IL-8	Treatment x Time	0.959

B. Multiple comparison Tests

Trial 3 Gene	Treatment Comparison	P	Time Comparison	P
IL-8	Uninf. CNTL vs. Low dose	0.018	Time 1 vs. Time 4	<0.001
IL-8	Uninf. CNTL vs. CNTL Inf.	0.115	Time 1 vs. Time 2	0.626
IL-8	Uninf. CNTL vs. High Dose	0.115	Time 1 vs. Time 3	0.646
IL-8	High Dose vs. Low dose	0.837	Time 3 vs. Time 4	<0.001
IL-8	High Dose vs. CNTL Inf.	1	Time 3 vs. Time 2	1
IL-8	CNTL Inf. vs. Low dose	0.837	Time 2 vs. Time 4	<0.001

C. Multiple Comparison Tests

Trial 3 Gene	Time w/in Low Dose Comparison	P	Time w/in High Dose Comparison	P	Time w/in Control Infected Comparison	P	Time w/in Uninfected Control Comparison	P
IL-8	Time 1 vs. Time 4	0.008	Time 1 vs. Time 4	0.017	Time 1 vs. Time 4	0.017	Time 3 vs. Time 4	0.345
IL-8	Time 1 vs. Time 2	0.97	Time 1 vs. Time 3	0.663	Time 1 vs. Time 3	0.663	Time 3 vs. Time 2	0.696
IL-8	Time 1 vs. Time 3	0.983	Time 1 vs. Time 2	0.981	Time 1 vs. Time 2	0.981	Time 3 vs. Time 1	1
IL-8	Time 3 vs. Time 4	0.025	Time 2 vs. Time 4	0.042	Time 2 vs. Time 4	0.042	Time 1 vs. Time 4	0.368
IL-8	Time 3 vs. Time 2	1	Time 2 vs. Time 3	0.866	Time 2 vs. Time 3	0.866	Time 1 vs. Time 2	0.721
IL-8	Time 2 vs. Time 4	0.032	Time 3 vs. Time 4	0.231	Time 3 vs. Time 4	0.231	Time 2 vs. Time 4	0.941

D. Multiple Comparison Tests

Trial 3 Gene	Treatment within Time 1 Comparison	P	Treatment within Time 2 Comparison	P	Treatment within Time 3 Comparison	P	Treatment within Time 4 Comparison	P
IL-8	Uninf. CNTL vs. Low dose	0.518	Uninf. CNTL vs. Low dose	0.946	Uninf. CNTL vs. CNTL Inf.	0.217	Uninf. CNTL vs. Low dose	0.123
IL-8	Uninf. CNTL vs. CNTL Inf.	0.799	Uninf. CNTL vs. CNTL Inf.	1	Uninf. CNTL vs. High Dose	0.217	Uninf. CNTL vs. CNTL Inf.	0.391
IL-8	Uninf. CNTL vs. High Dose	0.799	Uninf. CNTL vs. High Dose	1	Uninf. CNTL vs. Low dose	0.311	Uninf. CNTL vs. High Dose	0.391
IL-8	High Dose vs. Low dose	0.947	High Dose vs. Low dose	0.922	Low dose vs. CNTL Inf.	0.997	High Dose vs. Low dose	0.901
IL-8	High Dose vs. CNTL Inf.	1	High Dose vs. CNTL Inf.	1	Low dose vs. High Dose	0.997	High Dose vs. CNTL Inf.	1
IL-8	CNTL Inf. vs. Low dose	0.947	CNTL Inf. vs. Low dose	0.922	High Dose vs. CNTL Inf.	1	CNTL Inf. vs. Low dose	0.901

Notes: **Uninf. CNTL refers to the uninfected control group; CNTL Inf. refers to the infected control,**

Table A: Two-way ANOVA results, Table B: Tukey multiple comparison tests of overall treatment and overall time, Table C: Tukey multiple comparison tests of Time within treatment, Table D: Tukey multiple comparison tests of Treatment within sampling time points.

Statistical differences of p<0.05 are highlighted in green

Table 10: Trial 3 Statistical differences in expression of MMP 9 using Two-way ANOVA and Tukey multiple comparison tests

A. ANOVA

Trial 3 Gene	Source of Variation	P
MMP 9	Treatment	0.771
MMP 9	Time	0.269
MMP 9	Treatment x Time	0.092

B. Multiple Comparison Tests

Trial 3 Gene	Treatment Comparison	P	Time Comparison	P
MMP 9	High Dose vs. CNTL Inf.	0.767	Time 1 vs. Time 3	0.242
MMP 9	High Dose vs. Low dose	0.986	Time 1 vs. Time 2	0.426
MMP 9	High Dose vs. Uninf CNTL	1	Time 1 vs. Time 4	0.735
MMP 9	Uninf. CNTL vs. CNTL Inf.	0.855	Time 4 vs. Time 3	0.83
MMP 9	Uninf. CNTL vs. Low dose	0.993	Time 4 vs. Time 2	0.961
MMP 9	Low dose vs. CNTL Inf.	0.92	Time 2 vs. Time 3	0.984

C. Multiple Comparison Tests

Trial 3 Gene	Time w/in Low Dose Comparison	P	Time w/in High Dose Comparison	P	Time w/in Control Infected Comparison	P	Time w/in Uninfected Control Comparison	P
MMP 9	Time 3 vs. Time 4	0.189	Time 4 vs. Time 3	0.176	Time 4 vs. Time 3	0.356	Time 1 vs. Time 3	0.227
MMP 9	Time 3 vs. Time 2	0.932	Time 4 vs. Time 2	0.307	Time 4 vs. Time 2	0.833	Time 1 vs. Time 4	0.259
MMP 9	Time 3 vs. Time 1	0.981	Time 4 vs. Time 1	0.357	Time 4 vs. Time 1	0.997	Time 1 vs. Time 2	0.294
MMP 9	Time 1 vs. Time 4	0.37	Time 1 vs. Time 3	0.98	Time 1 vs. Time 3	0.456	Time 2 vs. Time 3	0.999
MMP 9	Time 1 vs. Time 2	0.996	Time 1 vs. Time 2	1	Time 1 vs. Time 2	0.916	Time 2 vs. Time 4	1
MMP 9	Time 2 vs. Time 4	0.529	Time 2 vs. Time 3	0.987	Time 2 vs. Time 3	0.831	Time 4 vs. Time 3	1

D. Multiple Comparison Tests

Trial 3 Gene	Treatment within Time 1 Comparison	P	Treatment within Time 2 Comparison	P	Treatment within Time 3 Comparison	P	Treatment within Time 4 Comparison	P
MMP 9	Uninf. CNTL vs. High Dose	0.272	Low dose vs.CNTL Inf.	0.932	Low dose vs.CNTL Inf.	0.18	High Dose vs. Low dose	0.027
MMP 9	Uninf. CNTL vs.CNTL Inf.	0.387	Low dose vs. Uninf. CNTL	0.977	Low dose vs. Uninf. CNTL	0.699	High Dose vs. Uninf. CNTL	0.35
MMP 9	Uninf. CNTL vs. Low dose	0.485	Low dose vs. High Dose	0.989	Low dose vs. High Dose	0.592	High Dose vs. CNTL Inf.	0.667
MMP 9	Low dose vs. High Dose	0.962	High Dose vs. CNTL Inf.	0.991	High Dose vs.CNTL Inf.	0.862	CNTL Inf. vs. Low dose	0.381
MMP 9	Low dose vs.CNTL Inf.	0.997	High Dose vs. Uninf. CNTL	0.999	High Dose vs. Uninf. CNTL	1	CNTL Inf. vs. Uninf. CNTL	0.9
MMP 9	Control vs. High Dose	0.991	Uninf. CNTL vs. CNTL Inf.	1	Uninf. CNTL vs. CNTL Inf.	0.924	Uninf. CNTL vs. Low dose	0.922

Notes: **Uninf. CNTL refers to the uninfected control group; CNTL Inf. refers to the infected control**

Table A: Two-way ANOVA results, Table B: Tukey multiple comparison tests of overall treatment and overall time, Table C: Tukey multiple comparison tests of Time within treatment, Table D: Tukey multiple comparison tests of Treatment within sampling time points.

Statistical differences of $p<0.05$ are highlighted in green

Table 11: Trial 3 Statistical differences in expression of TLR 9 using Two-way ANOVA and Tukey multiple comparison tests

A. ANOVA

Trial 3 Gene	Source of Variation	P
TLR 9	Treatment	0.405
TLR 9	Time	<0.001
TLR 9	Treatment x Time	<0.001

B. Multiple Comparison Tests

Trial 3 Gene	Treatment Comparison	P	Time Comparison	P
TLR 9	Uninf. CNTL vs. CNTL Inf.	0.399	Time 2 vs. Time 3	<0.001
TLR 9	Uninf. CNTL vs. High Dose	0.917	Time 2 vs. Time 4	<0.001
TLR 9	Uninf. CNTL vs. Low dose	0.952	Time 2 vs. Time 1	<0.001
TLR 9	Low dose vs. CNTL Inf.	0.588	Time 1 vs. Time 3	0.123
TLR 9	Low dose vs. High Dose	0.999	Time 1 vs. Time 4	0.528
TLR 9	High Dose vs. CNTL Inf.	0.69	Time 4 vs. Time 3	0.821

C. Multiple Comparison Tests

Trial 3 Gene	Time w/in Low Dose Comparison	P	Time w/in High Dose Comparison	P	Time w/in Control Infected Comparison	P	Time w/in Uninfected Control Comparison	P
TLR 9	Time 2 vs. Time 4	0.363	Time 2 vs. Time 4	0.011	Time 1 vs. Time 3	<0.001	Time 2 vs. Time 1	<0.001
TLR 9	Time 2 vs. Time 3	0.569	Time 2 vs. Time 3	0.065	Time 1 vs. Time 4	0.562	Time 2 vs. Time 3	<0.001
TLR 9	Time 2 vs. Time 1	0.781	Time 2 vs. Time 1	0.715	Time 1 vs. Time 2	0.948	Time 2 vs. Time 4	<0.001
TLR 9	Time 1 vs. Time 4	0.917	Time 1 vs. Time 4	0.205	Time 2 vs. Time 3	0.008	Time 4 vs. Time 1	0.915
TLR 9	Time 1 vs. Time 3	0.989	Time 1 vs. Time 3	0.496	Time 2 vs. Time 4	0.88	Time 4 vs. Time 3	0.999
TLR 9	Time 3 vs. Time 4	0.987	Time 3 vs. Time 4	0.965	Time 4 vs. Time 3	0.064	Time 3 vs. Time 1	0.953

D. Multiple Comparison Tests

Trial 3 Gene	Treatment within Time 1 Comparison	P	Treatment within Time 2 Comparison	P	Treatment within Time 3 Comparison	P	Treatment within Time 4 Comparison	P
TLR 9	CNTL Inf. vs. Uninf. CNTL	0.069	Uninf. CNTL vs. CNTL Inf.	<0.001	Low dose vs. CNTL Inf.	0.034	CNTL Inf. vs. High Dose	0.785
TLR 9	CNTL Inf. vs. Low dose	0.857	Uninf. CNTL vs. Low dose	<0.001	Low dose vs. Uninf. CNTL	0.801	CNTL Inf. vs. Uninf. CNTL	0.934
TLR 9	CNTL Inf. vs. High Dose	0.992	Uninf. CNTL vs. High Dose	0.001	Low dose vs. High Dose	0.924	CNTL Inf. vs. Low dose	0.999
TLR 9	High Dose vs. Uninf. CNTL	0.131	High Dose vs. CNTL Inf.	0.55	High Dose vs. CNTL Inf.	0.19	Low dose vs. High Dose	0.847
TLR 9	High Dose vs. Low dose	0.959	High Dose vs. Low dose	0.919	High Dose vs. Uninf. CNTL	0.984	Low dose vs. Uninf. CNTL	0.962
TLR 9	Low dose vs. Uninf. CNTL	0.297	Low dose vs.CNTL Inf.	0.9	Uninf. CNTL vs. CNTL Inf.	0.52	Uninf. CNTL vs. High Dose	0.998

Notes: Uninf. CNTL refers to the uninfected control group, CNTL Inf. refers to the infected control,

Table A: Two-way ANOVA results, Table B: Tukey multiple comparison tests of overall treatment and overall time, Table C: Tukey multiple comparison tests of Time within treatment, Table D: Tukey multiple comparison tests of Treatment within sampling time points.

Statistical differences of p<0.05 are highlighted in green