Saprolegnia infection after vaccination in Scottish Salmon Aquaculture is associated with differential expression of stress and immune genes in the host

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ABSTRACT

This study assessed the impact of routine vaccination of Atlantic salmon pre-smolts on gene expression and the possible link to saprolegniosis on Scottish fish farms.

Fish were in 4 different groups 1) 'control' – fish without handling or vaccination 2) 'vaccinated' - fish undergoing full vaccination procedure 3) 'non vaccinated' - fish undergoing full vaccination procedure but not vaccinated and 4) 'vaccinated-MH' – fish undergoing vaccination, but procedure involved minimal handling. A strong increase in cortisol and glucose levels was observed after 1 h in all groups relative to the control group. Only in the non-vaccinated group did the level decrease to near control levels by 4 h. Expression levels of six stress marker genes in general for all groups showed down regulation over a 9-day sampling period. In contrast, expression levels for immune response genes in the head kidney showed significant up-regulation for all eight genes tested for both vaccinated groups whereas the non-vaccinated group showed up-regulation for only *MHC-II* and *IL*-6b in comparison to the control.

Both the vaccination procedure and the administration of the vaccine itself were factors mediating changes in gene expression consistent with fish being susceptible to natural occurring saprolegniosis following vaccination.

Key Words: Atlantic salmon pre-smolts, *Saprolegnia*, cortisol, stress, vaccination, aquaculture

1. INTRODUCTION

Long term exposure to stress is generally associated with suppressive effects on the immune system and disease resistance. However, some studies have shown that even short-term exposure to certain stress factors can affect the immune system, for example, following short exposure to handling of Atlantic salmon and rainbow trout [1, 2]. Practices at fish farms such as confinement, high density, handling and transport, have all been shown to result in stress in fish (e.g. [3]), and can increase disease susceptibility, as seen in carp subjected to daily handling stress that are more susceptible to *Trypanoplasma borreli* infection than control fish [4]. Caipang *et al.* [5] demonstrated that exposure to short term overcrowding in Atlantic cod results in a transient elevation of plasma cortisol and a concomitant increase in the expression levels of genes related to the stress response, inflammation and anti-bacterial activity. Stress hormones such as cortisol and adrenaline, or stress related proteins like heat shock proteins, plasma glucose levels, as well as innate and adaptive immune parameters and their effects on disease resistance have been extensively studied in salmonids [1, 2, 5-7]. The study of stress in farmed fish is ongoing, however, it should be acknowledged that considerable scientific progress has been achieved in farmed fish welfare at all procedural stages: farming; transportation; various pre-slaughter manipulations and stunning/killing procedures [8].

Nevertheless, to prevent diseases mediated by viral and bacterial infections, fish are routinely vaccinated, prior to seawater transfer in the case of Atlantic salmon. Atlantic salmon farmers often report the incidence of *Saprolegnia* infections following vaccination. *Saprolegnia* is a eukaryotic pathogen of fish, belonging to the oomycetes, and is endemic to all freshwater habitats around the world. Due to their filamentous hyphal structure oomycetes are often described as fungal-like microorganisms, but

they are distinct from true fungi. *Saprolegnia parasitica* is the most virulent causative agent of the disease saprolegniosis, which causes devastating infections of freshwater fish [9, 10]. The occurrence of saprolegniosis after vaccination against diseases such as Infectious Pancreatic Necrosis (IPN) and Furunculosis has in recent years caused considerable economic losses (personal communication Scottish Salmon Producers Organisation).

Studies on host immune gene expression have shown that salmon are able to produce a strong acute phase and inflammatory immune response (i.e., induction of *IL*-1β, *IL*-6 and *TNF*α) during *Saprolegnia* infections but they appear unable to generate a sufficient adaptive immune response. Indeed, it has been demonstrated that *Saprolegnia parasitica* can down-regulate T-helper cell associated cytokines resulting in the active suppression of genes associated with adaptive immunity in fish [11, 12].

During an immune and inflammatory response, depending on the duration and strength of the stressor, stress hormones can potentially suppress innate responses in addition to T helper 1(Th1)/pro-inflammatory responses, a Th2 shift may also be potentiated. *IL*-1, *IL*-6 and *TNF*α in particular play a crucial role in the cross talk between the immune and the endocrine system [13, 14], as also seen in salmonids [15, 16].

In this study we have investigated the immune modulatory effect of routine vaccination as performed in Atlantic salmon on farms in Scotland. We identified stress, due to the vaccination handling procedure, as well as the administration of and/or the vaccine itself, as the two main factors to significantly impact the immune and endocrine system. This study contributes to our understanding of the endocrine and immunological processes that are affected up to 9 days post vaccination, a period when fish farms report greatest losses due to *S. parasitica* infections.

2. MATERIAL AND METHODS

2.1 Assessment of *Saprolegnia* natural infection at fish farm during vaccination – Study A

Atlantic salmon (*Salmo salar*) pre-smolts (Salmobreed QTL PD) with an average weight of 65g (n=121) were studied in the field for the incidence of *Saprolegnia* during routine injection vaccination against common fish diseases. During this study the routine preventative treatments with bronopol and formalin were withheld after vaccination. Fish were starved 48 h before vaccination. Study fish were divided into four groups: 1) 'control' (n=29) fish caught by net from production pen and anaesthetised. 2) 'non-vaccinated' (n=30) fish that had undergone the full process of vaccination (i.e. crowded, automatically transported, graded, anaesthetised) but were collected from the vaccination table before injection of the vaccine. 3) 'vaccinated' (n=30) fish that underwent the full vaccination process and vaccinated. 4) 'vaccinated-MH' (n=32) fish that were vaccinated with minimal handling – fish caught by net in 3 batches from production pen (not automatically transported), anaesthetised and vaccinated.

To distinguish each group by specific markings, whilst the fish were anaesthetised using buffered MS-222, fish were marked with Alcian blue dye using a Panjet.

The vaccinated groups were injected by professional vaccinators with 0.05 mL of vaccine A into the peritoneal cavity (a commercial adjuvanted vaccine composed of inactivated *Aeromonas salmonicida*, inactivated *Moritella viscosa*, and inactivated infectious pancreatic necrosis virus (IPNV) serotype A_2 with a mineral oil adjuvant).

Post vaccination, fish were transferred to a holding tank according to their group and observed daily and food was slowly reintroduced *ad libitum* the next day. Any dead

fish were removed and stored at 4ºC for next day examination: (visible signs of saprolegniosis and/or skin swab for pathogen identification). After 22 days the study was terminated, and all remaining fish were examined thoroughly for saprolegniosis after terminal anaesthesia.

2.1.1 Identification and isolation of *Saprolegnia*

At the end of the study, tissue samples were collected to confirm whether *Saprolegnia* propagules were present on any of the remaining fish. Throughout the study samples were collected from the morts to confirm *Saprolegnia* infection.

In brief, the pectoral fins were cut off and placed on Potato dextrose agar plates (PDA, 24 g/L potato dextrose broth, 15 g/L micro agar) supplemented with ampicillin (500mg/L), vancomycin (100mg/L) to reduce microbial contamination and pimaricin (20mg/L) to inhibit most true fungal growth. Plates were sealed on site then incubated at 12°C upon arrival in the laboratory. Once samples showed clear mycelial growth a small plaque was excised and re-inoculated onto PDA-VAP plates until axenic cultures were obtained. Genomic DNA from each axenic culture was extracted using a phenol-chloroform-isoamyl alcohol protocol [17]. Amplification of the Internal Transcribed Spacer (ITS) region comprising the genes ITS1-5.8S-ITS2 using the ITS4/ITS5 primers [18] was performed to identify the isolates through homology with sequences deposited at GenBank using the Basic Local Alignment Search Tool (BLAST) analysis (usually >98%).

2.2 The immune modulatory effect of stress during vaccination on fish farms – Study B.

Atlantic salmon (*Salmo salar*) pre-smolts (Salmobreed QTL PD) with an average weight of 32 g (n=160) were studied during routine injection vaccination to determine the impact of the vaccination procedure and the impact of vaccination on the stress response and immune system. Fish were starved 48 h before vaccination. Study fish were divided into four groups: 1) control - fish caught by net from an uncrowded holding tank (n=21). 2) 'vaccinated' – fish that had undergone the whole process of vaccination including being pumped into crowded holding tank, and vaccinated (n=42) 3) 'non- vaccinated' - fish which underwent the whole process of vaccination including being pumped into crowded holding tank, but were collected from the vaccination table before injection of the vaccine (n=42) 4) 'vaccinated-MH' - fish that were vaccinated with minimal handling; caught by net from uncrowded holding tank, anaesthetised and vaccinated (n=42).

Groups 2 – 4 were anaesthetised using buffered MS-222 before the vaccination procedure. The vaccinated groups were injected by professional vaccinators with 0.1 mL of vaccine B into the peritoneal cavity. Vaccine B was a commercial combination vaccine composed of inactivated: *A. salmonicida*, *M. viscosa*, *V. anguillarum* O1 and O2 serotypes and IPNV with a mineral oil adjuvant. Food was slowly reintroduced *ad libitum* the day following vaccination.

Fish were transferred to 3 holding tanks (9 m^3/tank) according to their group designation. The tanks had a constant flow of fresh water. In each tank 7 x 40L-prawn-baskets were suspended in order to be stocked with 6 fish from each group. This separated the fish for different time points to prevent disturbing the remaining fish by netting. Control fish were transferred two days before the start of the experiment into two prawn-baskets and were sampled on the morning of the start of the experiment. At time points 1h, 4h, 6h, 1d, 3d, 5d and 9d post processing, one prawn-basket from

each group was lifted from the tank and transferred into an anaesthesia bath for terminal anaesthesia and killing.

2.2.1 Tissue and blood collection

Within 10 min of the onset of the anaesthesia, blood samples from 6 fish per group were taken from the posterior caudal vein. EDTA solution was used as an anticoagulant (~1.5mg/mL blood depending on the volume of blood that was collected). The blood was kept on ice until centrifugation for 5 min at 5,000g, to obtain the blood plasma. All plasma samples were kept and transported on dry ice and stored at -80°C upon arrival in the laboratory.

Head kidney and gill tissue samples were collected from 6 biological replicates within 25 min from initiation of terminal anaesthesia, washed in PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4, pH 7.4) and stored in RNAlater® (Sigma).

2.2.2 RNA extraction

Total RNA extraction and purification was performed using the acid-guanidinium-thiocyanate-phenol-chloroform method (TriReagent®, Sigma Aldrich) following standard procedures. Traces of genomic DNA contamination were digested with DNase 1 (TURBO DNA-free™, Ambion). The quantity and quality of the RNA was checked spectroscopically (NanoDrop®) and by gel electrophoresis respectively.

2.2.3 cDNA synthesis and real-time PCR

cDNA was synthesized using a First Strand cDNA Synthesis Kit (Revert Aid™; Fermentas) according to the manufacturer's instructions, using 15µg of total RNA, in a 42µL reaction and oligo-(dt) primer. Real-time PCR was performed with a

LightCycler® 480 (Roche) using the GoTaq® qPCR Master Mix (Promega) in 10µL reactions and 384-well plates with 45 cycles (95°C for 15s, 60°C for 60s, 72°C for 30s). The primers used for each gene are given in Supplementary data - S1. Cp levels were determined using the LightCycler® Software (Roche) with the second derivative max method. 'Fold differences' were calculated relative to untreated controls and normalized to endogenous reference of elongation factor 1α (Ef-1α). Fold changes were calculated according to Pfaffl [19]. Efficiency of the amplification was determined for each primer pair as well as each tissue using serial 3-fold dilutions of pooled cDNA.

2.2.4 Blood Plasma analysis

Glucose concentrations were determined colorimetrically with a kit according to manufacturer's specifications (Glucose-GO-kit, Sigma).

Cortisol was determined by radio-immuno-assay according to the protocol described by Pottinger and Carrick [20]. Total steroids from 50-100µL aliquots of plasma were extracted with 500µL ethyl acetate. Aliquots of the resulting organic supernatant were spiked with 18,000 dpm ([1,2,6,7-'H3]-cortisol, PerkinElmer) to be measured in parallel to a range of standard tubes containing 0-800 pg of inert cortisol per tube as well as 18,000 dpm [3^{H}]-cortisol.

2.3 Data processing and statistical analysis

The statistical analysis of the data was performed with IBM SPSS statistics. Students two sided t-test for independent samples was used to check for significant differences between individual time points and the control. When necessary data is displayed on a log10 y-axis.

3. RESULTS

3.1 Incidence of *Saprolegnia* natural infection – Study A

The natural infection study (Study A) found that eight out of a total of 121 fish had visible *Saprolegnia* infections. The eight fish belonged to the vaccinated groups, with four fish from each group (vaccinated and vaccinated-MH) showing saprolegniosis (**Figure 1**). The first experimental fish visibly showing signs of saprolegniosis were observed seven days into the experiment. The number of infected fish rose steadily from that moment onwards. There was one dead fish in the vaccinated group and two dead fish in the vaccinated-MH group prior to the end of the study at 22 days. For the control and non-vaccinated groups, no fish were identified with visible signs of saprolegniosis. All fish belonging to these groups appeared visually free of any lesions and seemed generally in good health.

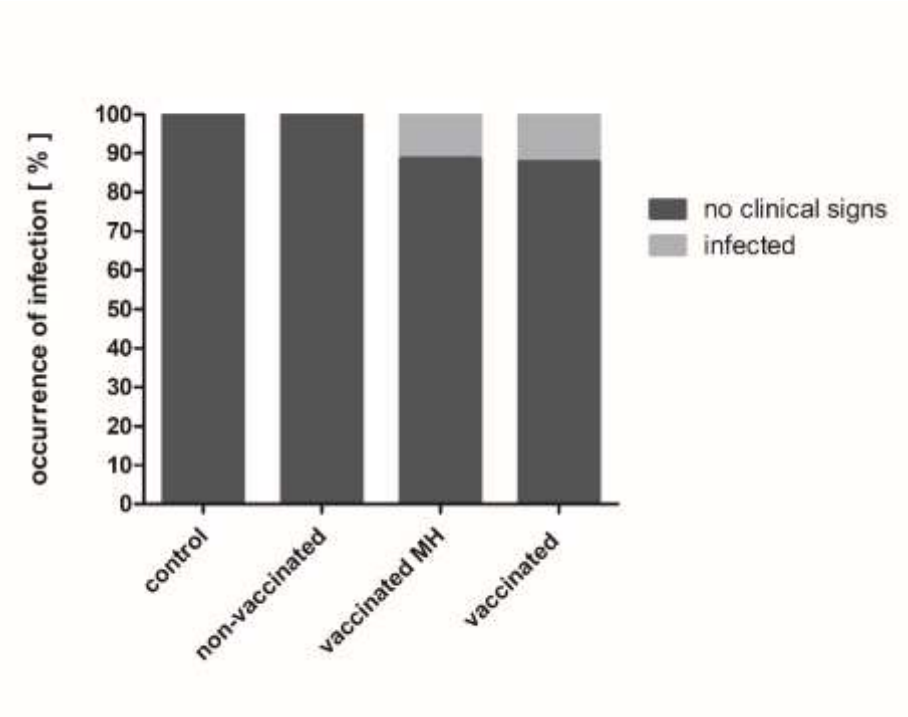

Figure 1: Occurrence of *Saprolegnia* infection in fish challenged naturally for 22 days. 'Control' fish (n=29) were anaesthetized and not vaccinated. The 'non-

vaccinated' group (n=30) were fish that had gone through the whole process of vaccination but were not injected with the vaccine. The 'vaccinated-MH' group (n=32) were caught by net and vaccinated with commercial vaccine A without going through the routine vaccination process. The 'vaccinated' group (n=30) were conventionally vaccinated with commercial vaccine A.

The analysis of mycelium from infected fish identified *S. parasitica* as the causative agent of the infection.

3.2 Evaluation of stress response – Study B

3.2.1 Plasma cortisol and glucose concentrations – Study B

To evaluate and quantitate the stress response, blood glucose and cortisol concentrations were determined. Vaccination as well as the handling of pre-smolt salmon triggered a measurable stress response as shown by increased cortisol and glucose levels (**Figure 2**). Control fish at the hatchery had an average cortisol concentration of 16.4 ± 1.7ng/mL. One hour after treatment the plasma cortisol level for all three treatment groups increased significantly: non-vaccinated group (142.7 ± 13.7ng/mL); vaccinated group (109.2 ± 17.63ng/mL); and vaccinated-MH group (93.7 ± 18.39ng/mL).

At the subsequent 4h time point, cortisol concentrations for all three treatment groups decreased, with the greatest decrease observed in the non-vaccinated group which also showed close to control levels of cortisol from 4h onwards. The vaccinated-MH group had near control values for the later timepoints, whereas the vaccinated group was more varied with values close to control levels at day 1 and 3 only.

Fish from all three treatment groups had significantly elevated glucose concentrations at 1h post treatment, which was still elevated at 1day post treatment, although the

non-vaccinated group showed a steady decrease from 1h to 3 days. After three days, glucose had decreased to near control levels in all treatment groups.

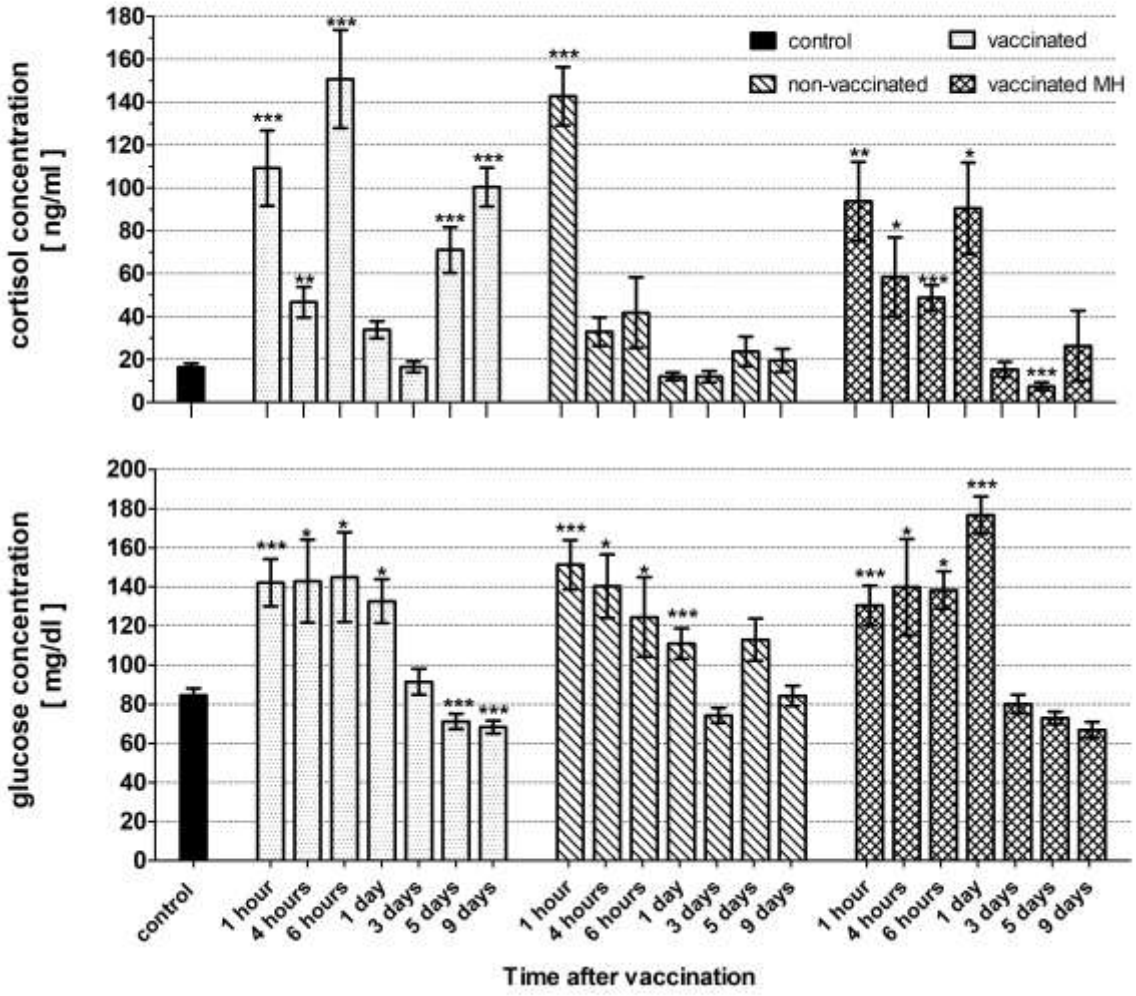

Figure 2: Cortisol and glucose plasma concentrations for Atlantic salmon pre-smolts. Displayed is the average (n=6) ± SEM for the three groups at all time points. Significant differences to the untreated control are indicated with * (P<0.05), ** (P<0.01) and *** (P<0.001). 'Control' fish were anaesthetized and not vaccinated. The 'non-vaccinated' group were fish that had gone through the whole process of vaccination but were not injected with the vaccine. The 'vaccinated-MH' group were caught by net and vaccinated with commercial vaccine B without going through the routine vaccination process. The 'vaccinated' group were conventionally vaccinated with commercial vaccine B.

3.2.2 Expression of stress-related genes – Study B

We determined expression levels of genes that are associated with stress in fish (**Figure 3**). These included genes coding for: key enzymes for cortisol production (*StAR* and *P450scc*); shuttling of the cortisol-receptor complex into the nucleus (*GR*); transporting glucose into cells (*glut1b*) and general stress response genes (*HSP70* and *HSP90*).

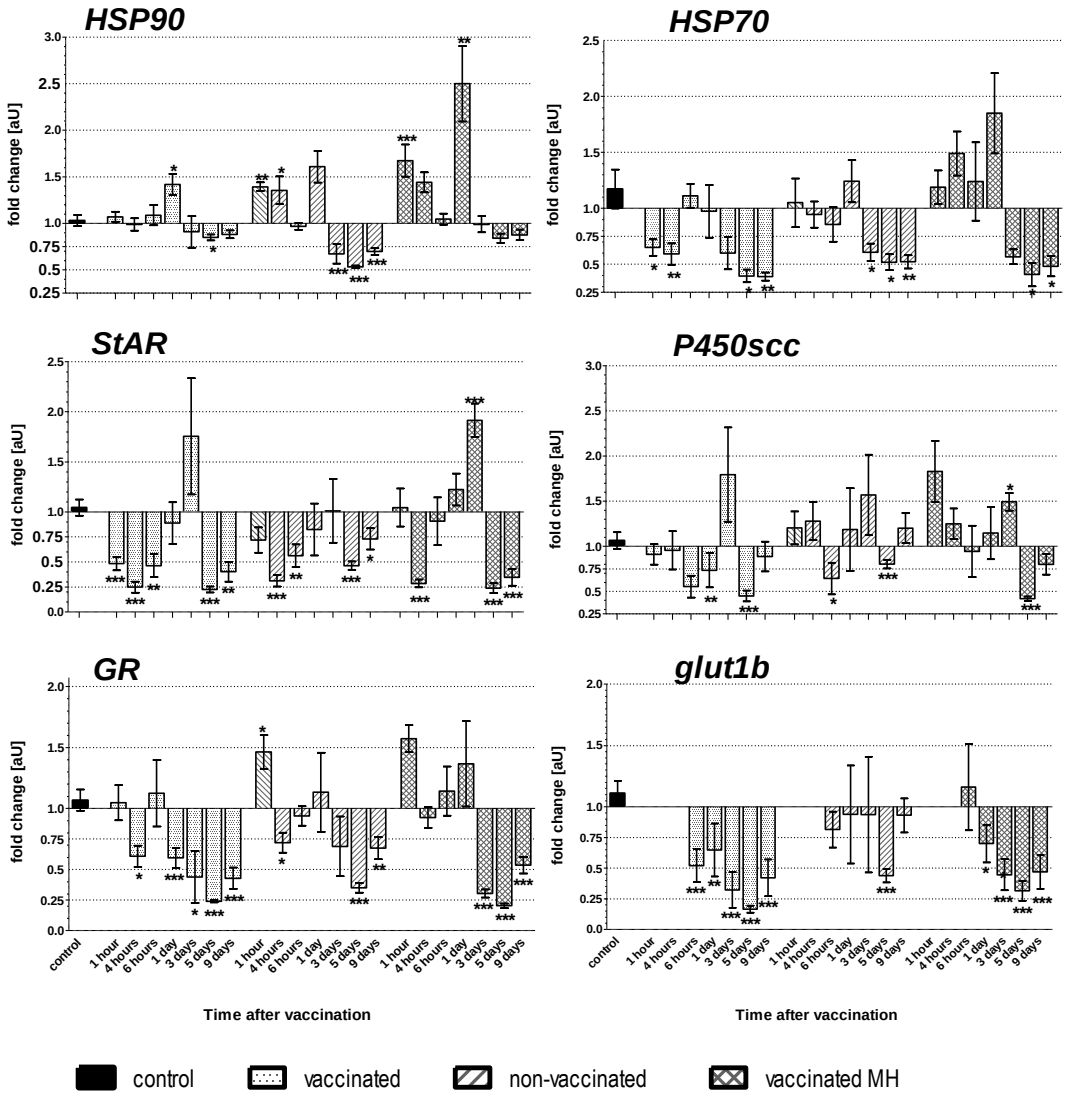

Figure 3: Stress gene expression in the head kidney of Atlantic salmon pre-smolts, presented as fold change relative to untreated controls. Displayed is the average (n=6) ± SEM for the three groups at all time points. Significant differences

compared to the untreated control are indicated with * (P<0.05), ** (P<0.01) and *** (P<0.001). 'Control' fish were anaesthetized and not vaccinated. The 'non-vaccinated' group were fish that had gone through the whole process of vaccination but were not injected with the vaccine. The 'vaccinated-MH' group were caught by net and vaccinated with commercial vaccine B without going through the routine vaccination process. The 'vaccinated' group were conventionally vaccinated with commercial vaccine B.

The expression levels of all six genes in the head kidney were significantly altered following handling/vaccination when compared to the control fish. The *StAR*, *GR* and *glut1b* genes showed the strongest overall down regulation for all 3 groups (vaccinated, vaccinated-MH and non-vaccinated fish), with expression decreasing to as low as 25% of the control. Down-regulated gene transcription could be evidence for compensatory mechanisms leading to the reduction of the cortisol response and a steady return to a state of homeostasis [21, 22]. *HSP70* also showed a similar down regulation and akin to the *GR* expression profile, expression was elevated for the initial 24h in the fish vaccinated-MH prior to the transcript levels dropping significantly. *HSP90* expression in all 3 groups was generally upregulated at the early time points following handling/vaccination, and after 3 days there was a significant down regulation in the non-vaccinated group.

3.3 Expression of immune genes – Study B

The expression profiles for selected genes associated with the acute and inflammatory response were measured in the head kidney in response to the three treatments (**Figure 4**). The genes were: Serum amyloid A (*SAA*) and Hepcidin, *Cox-2a*, *MHC-II*, *TNF*α1, *IL*-1β, *IL*-6a and *IL*-6b.

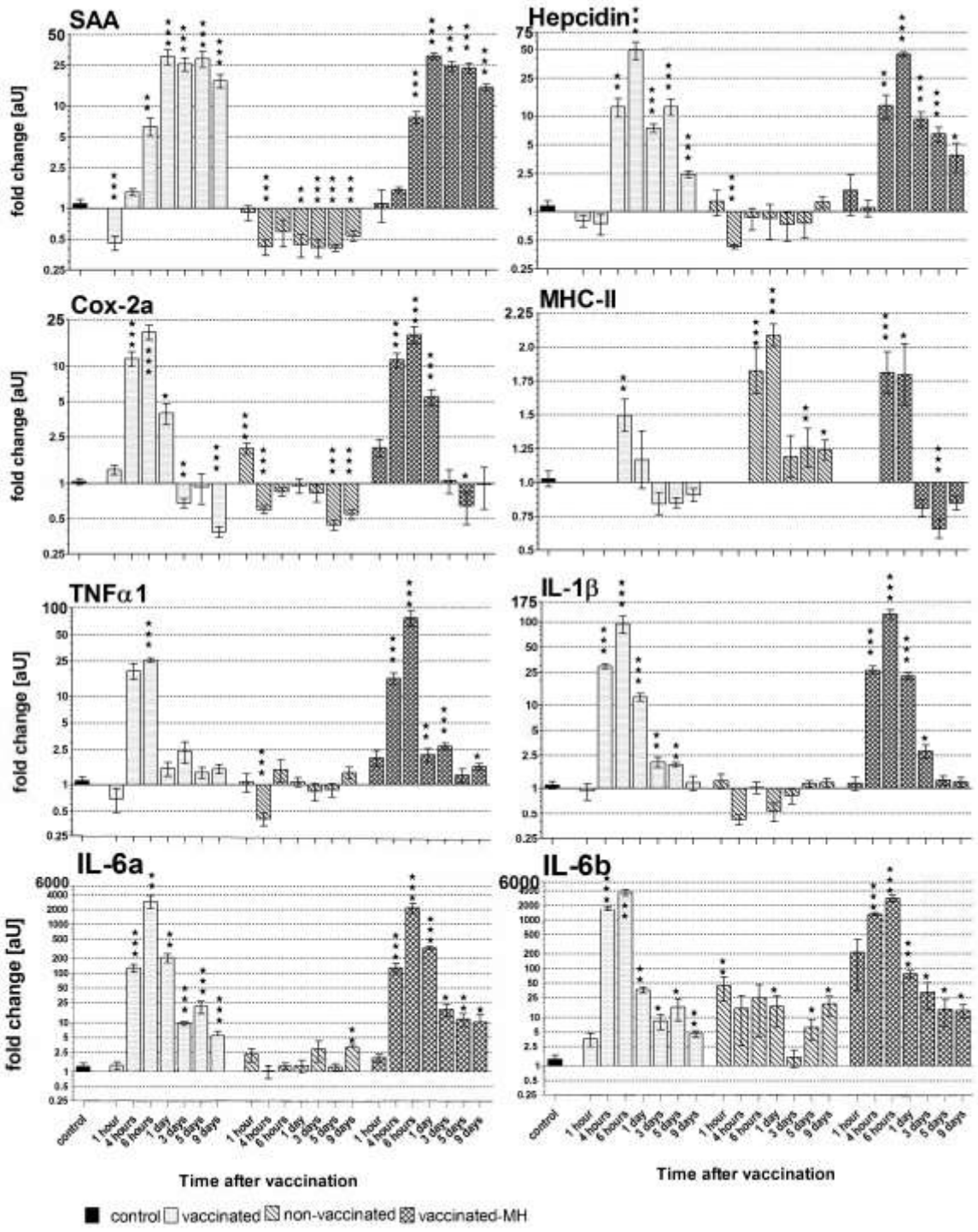

Figure 4: Acute and pro-inflammatory gene expression in the head kidney of Atlantic salmon pre-smolts, presented as fold change relative to untreated controls. Displayed is the average (n=6) ± SEM for the three groups at all time points. Significant differences to the untreated control are indicated with * (P<0.05), ** (P<0.01) and *** (P<0.001). 'Control' fish were anaesthetized and not vaccinated. The

‘non-vaccinated’ group were fish that had gone through the whole process of vaccination but were not injected with the vaccine. The ‘vaccinated-MH’ group were caught by net and vaccinated with commercial vaccine B without going through the routine vaccination process. The ‘vaccinated’ group were conventionally vaccinated with commercial vaccine B.

For both vaccinated groups all genes tested were elevated relative to the control, with the extent and pattern of elevation differing between genes and treatment. For the non-vaccinated group, only *MHC-II* and *IL*-6b were consistently elevated in comparison to the control. *SAA* gene expression was strongly stimulated 6 h following treatment in both the vaccinated and vaccinated-MH groups, and this 20 – 25-fold stimulation was still observed after nine days. In contrast, decreased expression levels for *SAA* were observed for the non-vaccinated group. The expression levels for Hepcidin followed a similar trend to *SAA* for the 3 groups, although expression levels were lower by day 9, but again no increased expression of Hepcidin was observed in the non-vaccinated group. Overall, the extent of increase in expression of *IL*6-a and *IL*-6b genes greatly exceeded the expression increase observed for all other genes studied, as seen *in vitro* in studies using flagellin stimulated trout cells [23]. This is particularly evident for the vaccinated/vaccinated-MH groups with a 2000-fold increase at 6h. Similarly, the increase in gene expression observed after 6h for *TNF*α1 was 25-fold and 50-fold for vaccinated and vaccinated-MH groups respectively. For *IL*-1β at 6h mRNA levels were 95-fold and 124-fold up-regulated for the vaccinated and vaccinated-MH groups respectively compared to the control group.

The expression profiles for *SAA*, Hepcidin, *TNF*α1, *Cox-2a* and *IL*-1β, associated with the acute and inflammatory response, as well as *MHC-II* were also measured in gill tissue (**Figure 5**). The expression profiles for *SAA*, Hepcidin, *TNF*α1 and *IL*-1β in the

gill tissue were similar to those measured in the head kidney for all 3 groups with the vaccination/vaccination-MH groups showing increased expression relative to the control but not the non-vaccinated group. Overall, the changes in gene expression were not as high in the gills as observed in the head kidney. For *Cox-2a*, the gene expression profile in the gill was different to that observed in the head kidney. In the gill expression was relatively similar for all 3 groups with all groups showing an upregulation that was highest at day 9. Whereas *Cox-2a* expression in the head kidney for the non-vaccinated group was up-regulated only at 1 h then down-regulated at the remaining time points.

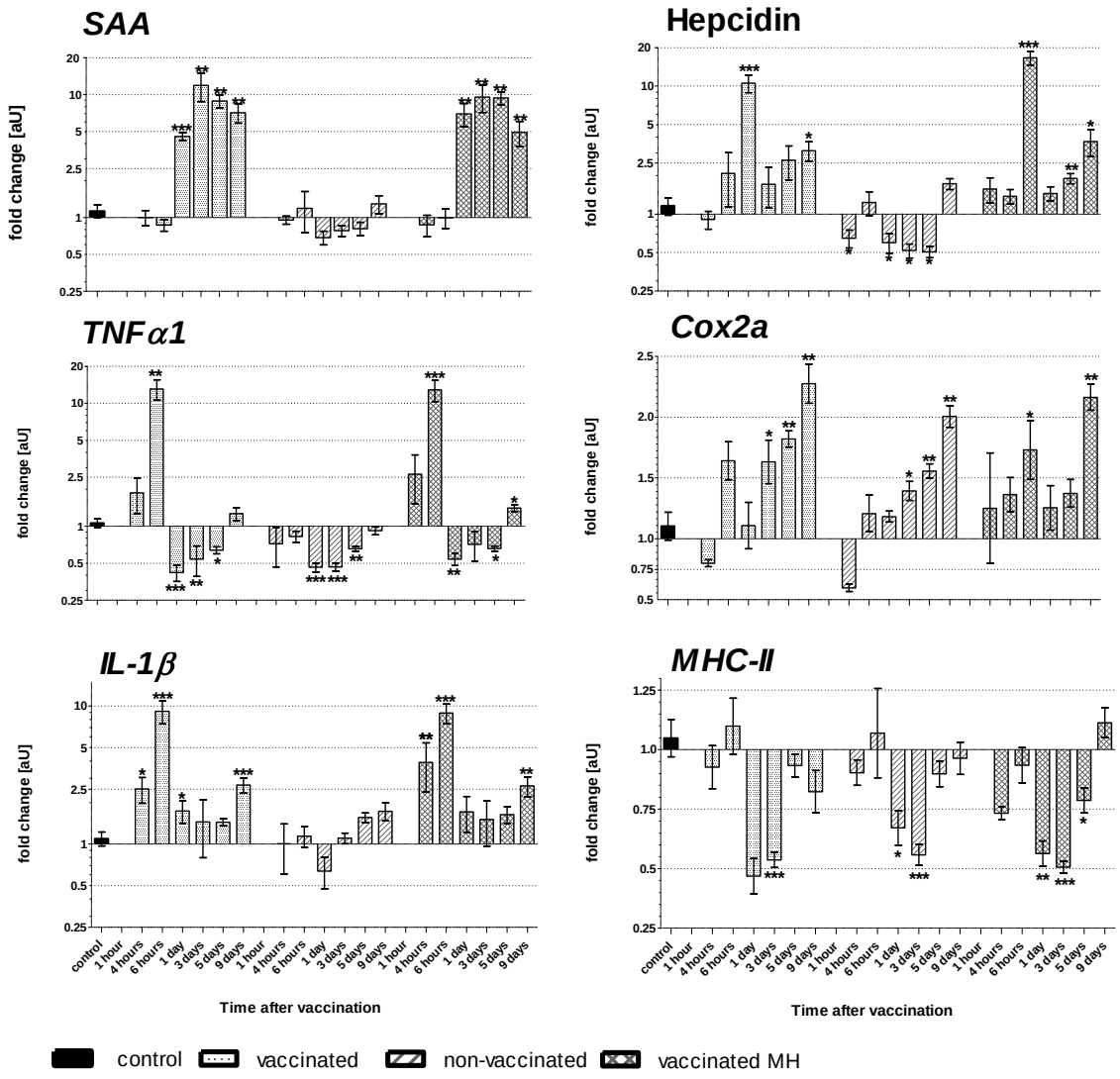

Figure 5: Acute and pro-inflammatory gene expression in the gills of Atlantic salmon pre-smolts, presented as fold change relative to untreated controls. Displayed is the average (n=6) ± SEM for the three groups at all time points. Significant differences to the untreated control are indicated with * (P<0.05), ** (P<0.01) and *** (P<0.001). 'Control' fish were anaesthetized and not vaccinated. The 'non-vaccinated' group were fish that had gone through the whole process of vaccination but were not injected with the vaccine. The 'vaccinated-MH' group were caught by net and vaccinated with commercial vaccine B without going through the routine vaccination process. The 'vaccinated' group were conventionally vaccinated with commercial vaccine B.

The gene expression profile of three transcription factors: *T-bet*, *GATA*-3, and *RoRγ* associated with driving the differentiation of the three main Th subsets in mammals (i.e. Th1, Th2 and Th17, respectively) were analysed in the head kidney (**Figure 6**).

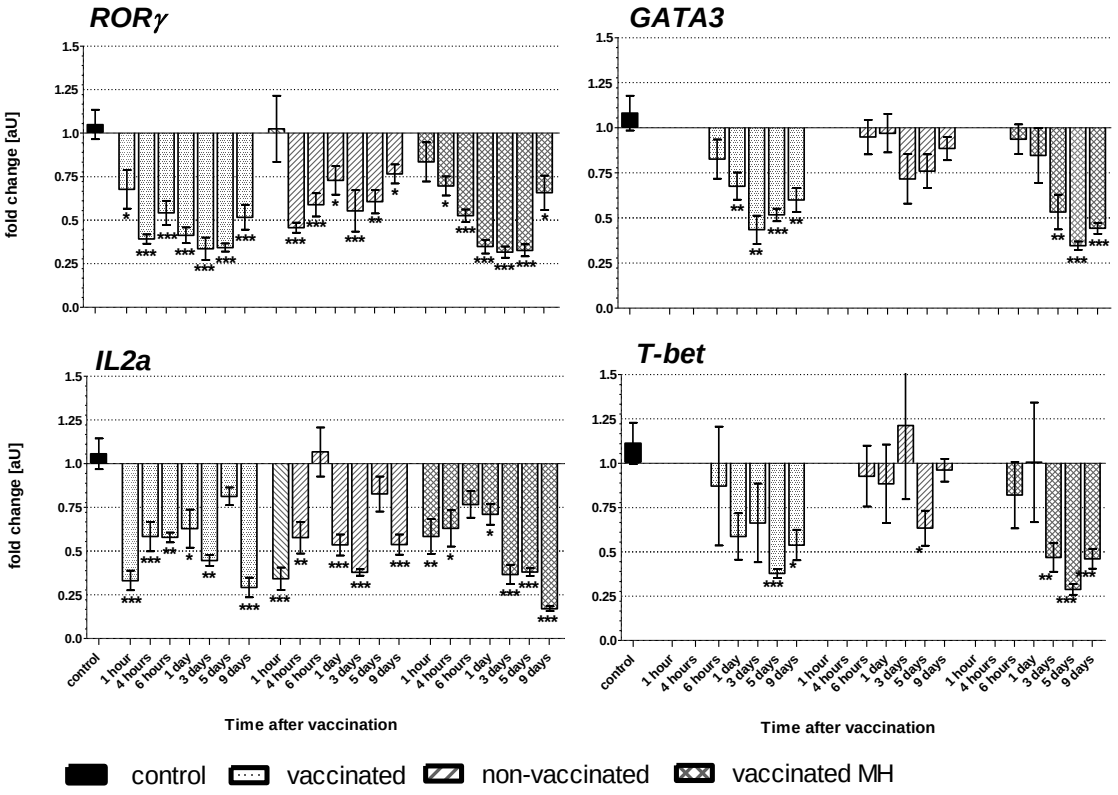

Figure 6: Transcription factors gene expression in the head kidney of Atlantic salmon pre-smolts, presented as fold change relative to untreated controls. Displayed is the average (n=6) ± SEM for the three groups at all time points. Significant differences to the untreated control are indicated with * (P<0.05), ** (P<0.01) and *** (P<0.001). 'Control' fish were anaesthetized and not vaccinated. The 'non-vaccinated' group were fish that had gone through the whole process of vaccination but were not injected with the vaccine. The 'vaccinated-MH' group were caught by net and vaccinated with commercial vaccine B without going through the routine vaccination process. The 'vaccinated' group were conventionally vaccinated with commercial vaccine B.

The transcript level for all three groups was significantly reduced overall in comparison to the control group. The down regulation was in general greater for the vaccinated/vaccinated-MH groups. *IL*-2a gene expression was also studied, as an important lymphocyte growth factor, and was significantly down-regulated in all groups.

4. DISCUSSION

Previous field observations that vaccinated salmon are more likely to get infected with *Saprolegnia* than non-vaccinated salmon were supported by the natural infection experiment (Study A). We have routinely observed that salmon pre-smolts are at risk of *Saprolegnia* infections immediately after routine vaccination against viral and bacterial diseases. In this study we found that only the vaccinated fish became infected with *Saprolegnia* over the course of sampling.

Fish vaccinations are challenging situations that require the well-tuned communication between the immune and endocrine systems to maintain a balance between the stress response and the immunological reaction to the vaccine [24-27]. The head kidney plays an important role in the immune-endocrine interaction as it combines key features of both systems: haematopoiesis, antibody production, and cortisol and catecholamine production. The induction of stress and the amplitude of a stress response are commonly measured by the concentration of cortisol in the blood plasma as a marker of hypothalamic-pituitary-interrenal (HPI)-axis activation in fish. Cortisol levels were monitored during the first 9 days post vaccination and clearly showed the induction of a stress response in all three groups. Control fish, considered unstressed, had an average cortisol level of 16.4 ± 1.72 ng/mL while basal levels of plasma cortisol in unstressed salmonid fish are normally in the range of 0-5 ng/ml [28]. Basal stress levels thus appeared slightly elevated at the hatchery. There are many environmental and developmental factors such as temperature, nutrition or time of day that have been shown to influence resting levels of cortisol in fish [29]. It is also possible that the period of confinement/crowding resulting from fish being transferred into the pens two days prior to sampling, could also explain the slightly raised cortisol level for the control fish. Pickering and Pottinger [28] reported that in salmonid fish chronic stress such as

prolonged periods of confinement or crowding resulted in elevated plasma cortisol levels to approximately 10 ng/mL. Blood cortisol levels remained elevated for periods of up to 4 weeks before acclimation finally occurred. Thus, it is possible that hatchery fish experience stressful events more regularly and thus show characteristic cortisol levels usually found in chronically stressed fish. Post the vaccination procedure, cortisol concentrations were immediately increased for all 3 groups reaching 94 – 143ng/mL at 1 h. These values are in line with cortisol concentrations reported after the occurrence of an acute stress which has been reported to range between 40 to 200ng/mL after handling or 1h confinement in both brown trout, *Salmo trutta*, and rainbow trout, *Oncorhynchus mykiss* [28]. In the current study, the levels of cortisol returned to near basal levels after 4h for the non-vaccinated group, whereas the vaccinated groups displayed more elevated cortisol levels at the later time points. The vaccinated-MH group had high cortisol levels over 24h but did not have high cortisol levels at 3 – 9 days and had lower peak cortisol levels compared to the vaccinated group and the non-vaccinated group at 1 hr. Overall the fish that went through the full vaccination procedure but were not vaccinated had the quickest return to basal cortisol levels. The released cortisol, in turn activates secondary and tertiary stress responses. The secondary phase often includes an increase in plasma glucose as well as increased gluconeogenesis in the liver [30]. Glucose profiles of the 3 groups supported the observation that there seemed to be a more sustained induction of stress in both vaccinated groups. After an initial rise the average glucose concentration decreased steadily for the non-vaccinated group whereas the vaccinated groups displayed more constant high glucose levels over 24h.

In addition to changes in metabolite levels, changes in stress protein levels are also included in secondary responses which relate to a myriad of physiological

adjustments. The gene expression profiles for *HSP90*, *HSP70*, *StAR*, *P450scc*, *GR* and *glut1b* were differentially regulated in response to the vaccination procedure. A general down regulation was observed for the vaccinated and the non-vaccinated groups. A greater difference was seen between the vaccinated groups: for the vaccinated-MH group the trend was generally initial up-regulation for *HSP90*, *HSP70*, *GR* and *P450scc* followed by subsequent down regulation at 3 – 5 days, indicative perhaps of a compensatory phase returning an organism to a state of homeostasis [21, 22]. In contrast, for the vaccinated group the initial up-regulation was in general not observed. The differential regulation of these stress genes in response to the vaccination procedure could indicate that the acute stress triggered by the vaccination procedure acts in combination with pre-vaccination stress leading to a more chronically stressed status.

Some studies have shown that acutely stressed fish show higher numbers of activated macrophages in skin, increased T-cell activation and enhanced recruitment of surveillance T cells in the skin. Similarly, acute stress administered for 2h prior to an antigenic challenge significantly enhanced skin cell-mediated immunity [31, 32]. In contrast, suppression of the skin immune response was recorded when chronic stress exposure began some weeks before the occurrence of an acute stressor. *Saprolegnia* needs to overcome the mucosal immune defence first to establish a successful infection of the skin or gills; it is therefore interesting that studies looking into the host responses to presence of the oomycete report a down regulation of *MHC-II* gene expression [11, 33].

Stimulation of inflammatory gene expression is a common response in fish to vaccination with bacterial antigens as well as to infection with bacteria, viruses and

parasites [34-36]. Gene expression profiles were determined for the acute phase and inflammatory genes (*SAA*, Hepcidin, *TNF*α1, *Cox-2a*, *IL*-1β, *IL*-6a and *IL*-6b and *MHC-II*) and a significant stimulation of those genes was demonstrated within 6h for both vaccinated groups. Similarly, in 2007 Fast *et al* [37] reported rapid up-regulation of innate inflammatory factors in the head kidney over the first 24h after vaccination. In contrast, for the non-vaccinated fish group in this study, *SAA* expression showed a clear down regulation, which was also the general trend for Hepcidin, *Cox-2a*, *TNF*α1, *IL*-1β and *IL*-6a. Only *MHC-II* and *IL*-6b showed up-regulation in all 3 groups.

Tissue specific gene expression differences were found when comparing the expression levels of these genes in the head kidney and gills. No upregulation in *MHC-II* expression was observed in the gills and for *Cox-2a* expression, upregulation was observed in all 3 groups. The head kidney in teleost fish is equivalent to the bone marrow in higher vertebrates and the main haematopoietic site. The gill is considered a secondary lymphoid tissue forming part of the tegument and is in direct contact with the environment and potential pathogens. Both innate and adaptive immune cells are present in the gill associated lymphoid tissue (GIALT) [38, 39]. Overall expression profiles for *SAA*, Hepcidin, *TNF*α1 and *IL*-1β followed the same trend for each of the 3 groups in both the head kidney and gills, but the magnitude of expression observed in the gills was lower. The clear down-regulation of *MHC-II* in all 3 groups in the gills but up-regulation in all 3 groups in the head kidney may indicate a recruitment of immune cells from peripheral tissues into the peritoneal cavity, which could result from vaccination by injection into the peritoneal cavity.

A large number of cytokines are known to interact with the HPI-axis in the bi-directional communication between the immune system and the HPI-axis with *IL*-1, *TNF*-α, and

IL-6 considered to be main players [40]. Functions of *IL*-1 largely overlap with those of *TNF*. Both are primary cytokines and often work synergistically. *IL*-6 is a secondary cytokine in the inflammatory response and exhibits pro-inflammatory as well as anti-inflammatory properties and is a major mediator of the acute phase reaction. The expression of *IL*-6 is up-regulated by *IL*-1 as well as *TNF*, and in turn *IL*-6 inhibits the production and secretion of *IL*-1 and *TNF* [40].

GATA3, *T-bet*, *RoRγ* (transcription factors driving the differentiation of the three main Th subsets) and *IL*-2 showed strong reductions in expression. It stands to reason that these expression reductions only occur in the short term as a functioning adaptive immune system provides salmon immunity against the bacteria and viruses that were injected, as intended through the vaccination. The short-term consequences of the down regulation of adaptive immune genes and possible associations with *Saprolegnia* infections are unknown at present.

In the current study, the salmon were sampled during routine vaccination at a fish farm. Exposure to multiple stressors may lead to unexpected synergistic or antagonistic effects [41], thus the individual data points should not be over interpreted but the analysis should focus on the direct comparison between the vaccinated and non-vaccinated groups and between the two vaccinated groups with different handling. We showed in Study A that naïve unvaccinated fish remained unaffected by saprolegniosis while vaccinated fish showed clinical signs of *Saprolegnia* infection. We subsequently demonstrated in Study B the induction of a complex stress mounted by salmon pre-smolts after handling / vaccination and show unique changes in stress and inflammatory gene expression over a nine-day time period when young salmon are susceptible to *Saprolegnia* infections. Our results suggest that both the vaccination

and the vaccination process play important roles in changes in gene expression in the fish that were previously demonstrated to be associated with increased susceptibility to saprolegniosis in salmon [12, 42]. Indeed, it is well-known that *Saprolegnia* usually enters the fish via the skin and gill tissue and therefore it needs to overcome the mucosal immune defence first in order to successfully establish an infection. However, recruitment of immune cells away from these peripheral tissues, due to vaccination, could result in leaving the skin and gills less protected. This process is likely to be a contributing factor that leads to increased susceptibility towards *Saprolegnia* following vaccination.

In conclusion, our data suggest industry opportunities for optimisation of vaccination to reduce associated stress, which would help reduce losses of fish due to saprolegniosis following vaccination.

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Supplementary data – S1

Table 1: Sequence from all primers used in this study.

genes		accession number	sequence 5′-3′	product size (bp)	reference
immune genes					
SAA	forward	AM422447	GGTGAAGCTGCTCAAGGTGCTAAAG	162	
	reverse		GCCATTACTGATGACTGTTGCTGC		
Hepcidin	forward	CA369786	GCTGTTCCTTTCTCCGAGGTGC	165	[43]
	reverse		GTGACAGCAGTTGCAGCACCA		
*TNF*α1	forward		ACTGGCAACGATGCAGGACAA	144	
	reverse		GCGGTAAGATTAGGATTGTATTCACCCTCT		
Cox-2a	forward	AJ238307	CCAGTACCAGAACCGTATCGCAG	200	[44]
	reverse		GTCCACCAGCCACCCTTCC		
IL-1β	forward	AJ278242	CCTGGAGCATCATGGCGTG	147	[45]
	reverse		GCTGGAGAGTGCTGTGGAAGAACATATAG		
IL-6a	forward	KJ425513	GAAGTGGGAGCAAATTATCAAGATGC	185	Not published salmon seq
	reverse		GCAGACATGCCTCCTTGTTGGT		
IL-6b	forward	KJ425514	ATGGGAGAACGTGATCAAGGTGA	198	Not published salmon seq
	reverse		TGCAGACATGCCTCCTTGTTGTA		
*GATA*3	forward	FM863826	CCAAAAACAAGGTCATGT TCAGAAGG	313	[46]
	reverse		TGGTGAGAGGTCGGTTGATATTGTG		
T-Bet	forward	FM863825	GGTAACATGCCAGGGAACAGGA	317	[46]
	reverse		TGGTCTATTTTTAGCTGGGTGATGTCTG		
IL-2a	forward	AM422779	TGATGTAGAGGATAGTTGCATTGTTGC	180	
	reverse		GAAGTGTCCGTTGTGCTGTTCTC		
MHC-II	forward	X70166.1	AGATTCAACAGCACTGTGGGGAA	187	[42]
	reverse		GTCTGACATGGGGCTCAACTGTCT		
RORγ	forward	FM883712, FM883713	ACAGACCTTCAAAGCTCTTGGTTGTG	262	[47]
	reverse		GGGAAGCTTGGACACCATCTTTG		
other genes					
Glut1b	forward		GCTGTTCCTTTCTCCGAGGTGC	186	
	reverse		GTGACAGCAGTTGCAGCACCA		

Housekeeping gene					
Elongation factor 1α	forward	AF49832	CAAGGATATCCGTCGTGGCA	317	
	reverse		ACAGCGAAACGACCAAGAGG		
Stress genes					
StAR	forward	DQ415678.1	GGTCAAGATCCTCCAGAAGATAG	147	
	reverse		ATGCCGGCAAGGAAGCACGT		
P450scc	forward		CATCTTGTTCAAGGCAGAAGGAC	139	
	reverse		CAGAATCACCCTGTTGGACCG		
HSP90	forward	NM_001146473.1	GGTCGACAGTGGGGAGCC	88	
	reverse		CCTCTTCTCCTCAACATACTCAG		
HSP70	forward	AJ632154	CCAATGACAAAGGACGTCTGACC	102	
	reverse		CTGTGACCTTCTCCTTCTGAGC		
GR	forward	GQ179974.1	CAGTGAGTCTACAGCAGGATCAG	140	
	reverse		GCTGAAGCCGACAGGAAAAGATG		
Phylogenetic					
ITS5	forward		GGAAGTAAAAGTCGTAACAAGG		[20]
ITS4	reverse		TCCTCCGCTTATTGATATGC		