RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) ADIPOSE TISSUE UNDERGOES MAJOR CHANGES IN IMMUNE GENE EXPRESSION FOLLOWING BACTERIAL INFECTION OR STIMULATION WITH PRO-INFLAMMATORY MOLECULES

Kimberly A. Veenstra[a], Eakapol Wangkahart[a b], Tiehui Wang[a], Lincoln Tubbs[c], Juliette Ben Arous[d], Christopher J. Secombes[a *]

[a] Scottish Fish Immunology Research Centre, Institute of Biological and Environmental Sciences, University of Aberdeen, Tillydrone Avenue, Aberdeen, AB24 2TZ, UK

[b] Division of Fisheries, Department of Agricultural Technology, Faculty of Technology, Mahasarakham University, Khamriang Sub-District, Kantarawichai, Mahasarakham, 44150, Thailand

[c] Elanco Canada Ltd., Aquaculture Research and Development, P.O. Box 17, Victoria P.E., C0A 2G0, CANADA

[d] SEPPIC, 22 Terrasse Bellini, Paris La Défense, 92806 Puteaux, France

* Corresponding author at: Institute of Biological and Environmental Sciences, University of Aberdeen, Tillydrone Avenue, Aberdeen AB24 2TZ, UK. Tel: +44 (0)1224 272872; fax: +44 (0)1224 274429.

Email addresses: k.veenstra@abdn.ac.uk (K. Veenstra), r03ew13@abdn.ac.uk (E. Wangkahart) t.h.wang@abdn.ac.uk (T. Wang), tubbs_lincoln@elanco.com (L. Tubbs), juliette.benarous@airliquide.com (J. Ben Arous), c.secombes@abdn.ac.uk (C. Secombes).

Keywords:
rainbow trout
adipose tissue
gene expression
A. salmonicida infection
flagellin
cytokines

Abstract

In mammals, visceral adipose is increasingly seen as playing an important role in immune function with numerous pro-inflammatory, anti-inflammatory and immune-modulating proteins and peptides being identified in adipocytes. Adipose is also now known as a tissue that has an important role in the regulation of peritoneal immune responses. Despite this, only lately has consideration been given to visceral adipose as an important immune tissue in fish, especially in the context of intraperitoneal vaccination. The present study demonstrates that fish visceral adipose is capable of expressing a large range of immune molecules in response to stimulation with a live bacterium (*A. salmonicida*), a bacterial PAMP (*Y. ruckeri* flagellin), and the pro-inflammatory cytokines IL-1β, TNF-α3 and IFN-γ. Following infection and stimulation with flagellin and IL-1β a large upregulation of pro-inflammatory and antimicrobial molecules was seen, with a high degree of overlap. TNF-α treatment affected relatively few genes and the effects were more modest. IFN-γ had the smallest impact on adipose but IFN-γ inducible genes showed some of the largest effects. Overall, it is clear that adipose tissue should be considered an active immune site in fish, capable of participating in and influencing immune responses.

1. Introduction

In fish the major sites of lipid deposition are species dependent but tend to be located in the muscle, liver and the perivisceral cavity (Sheridan, 1988; 1989; 1994). The visceral fat deposit is located around the digestive tract and can account for 2–25 % of body weight (Weil et al., 2013) and consists almost entirely of adipocytes (Zhou et al., 1996; Fauconneau et al., 1997). While the mechanisms regarding the deposition and mobilization of lipid in relation to different external and internal factors haves been studied extensively in salmonids (Sheridan, 1988; Zhou et al., 2011; Todorčević & Hodson, 2016), the focus has primarily been on understanding lipid metabolism and its impact on flesh quality (Weil et al., 2013). However, mammalian perivisceral adipose (often referred to as the omentum) is increasingly seen as playing an important role in immune function (Lumeng & Saltiel, 2011; Ferrante, 2013; Grant & Dixit, 2015).

Immune cells and numerous pro-inflammatory, anti-inflammatory and immune-modulating proteins and peptides belonging to the cytokine, chemokine, complement and growth factor families have been identified in mammalian adipocytes (Rangel-Moreno et al., 2009; Schäffler & Schölmerich, 2010; Chandra et al., 2011). In fish, adipocytes have been shown to constitutively express pro-inflammatory cytokines and genes relating to the interferon response (Todorčević et al., 2010; Pignatelli et al., 2014), with the latter increased in response to viral hemorrhagic septicemia (VHSV) infection (Pignatelli et al., 2014). Mammalian perivisceral adipose can influence and be influenced by adjacent and embedded lymphocytes and has been found to play an important role in the regulation of the peritoneal immune responses (Walker et al.1960; Walker and Rogers, 1961; Rangel-Moreno et al. 2009). Leukocyte populations are also present in fish adipose, as seen in rainbow trout (*Oncorhynchus mykiss*) where the majority show a typical lymphocyte-like morphology and are able to transcribe Mx, IL-6, IL-8, IgM and IgT (Pignatelli et al. 2014). Furthermore, perivisceral adipose is capable of collecting bacteria and other particulates from the peritoneal cavity (Fedorko et al., 1971; Ha et al., 2006; Rangel-Moreno et al., 2009), a function that appears to be conserved in fish (Pignatelli et al., 2014).

Fish vaccination has been shown to be a very effective means to control infectious diseases in aquaculture, and most often occurs via direct injection into the peritoneal cavity. The injection of vaccines or other inflammatory agents into the peritoneal cavity of fish generates a change in composition as well as a rapid increase in the number of cells present within the cavity (Korytář et al., 2013; Noia et al., 2014). Following administration of oil-based vaccines antigen persistence at the injection site is associated with stimulation and maintenance of inflammatory reactions, as seen in Atlantic salmon (*Salmo salar*) (Mutoloki et al., 2004; Evensen et al., 2005). More recently the work of Veenstra et al. (2017) has established that a broad range of immunomodulatory genes are differentially expressed in adipose tissue after intraperitoneal (~~i.p.~~ip) injection of oil-adjuvanted bacterial vaccines. This study highlighted perivisceral adipose as a particularly sensitive and discriminatory tissue for studying adjuvant effects, and revealed a relationship between adipose tissue immune function and the development of vaccine-induced adhesions.

Such studies show that fish adipose tissue is immune reactive and responds to viruses and killed bacterial antigens when delivered into the peritoneal cavity. However, to date there have been no studies to determine the responsiveness of adipose to bacterial infection per se, or to relevant pathogen-associated molecular patterns (PAMPs) and cytokines that drive pro-inflammatory responses following infection. Thus in the present investigation we examined whether immune-related gene expression occurs within the adipose tissue of rainbow trout in response to ~~i.p.~~ip challenge with the bacterial pathogen *Aeromonas salmonicida* and in response to ~~i.p.~~ip injection of a bacterial PAMP –a recombinant flagellin of *Yersinia ruckeri*. In addition we examined immune-related gene expression within the adipose tissue in response to ~~i.p.~~ip injection of recombinant pro-inflammatory cytokines IL-1β, TNF-α3 and IFN-γ to elucidate how such immune proteins, produced in response to infection or vaccination, can influence the immune response in adipose tissue, and whether such responses are comparable with those seen in other immune tissues. We demonstrate a large modulation of immune genes occurs in rainbow trout adipose in response to bacterial infection or stimulation with flagellin/ cytokines.

2. Methodology

2.1 ~~2.1~~ Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) ~~weighing approximately 60g~~weighing 1~~22.6~~08.8 ± 4.~~1~~3.9 g (mean ± SEM) were purchased from College Mill Trout Farm (Perthshire, U.K.). ~~and They~~The fish were maintained at the University of Aberdeen aquarium facility in ~~~~400L tanks at a stocking density of <20 kg/m^3, which were ~~at the University of Aberdeen aquarium facility~~ supplied with recirculating freshwater with a ~~water~~ flow~~-~~ rate of 1.5 L/s.~~,~~ ~~They~~Fish were kept at a temperature of~~at~~ 14±1°C~~,~~ and a photoperiod of 12:12 light:dark. Water quality was continually m~~easured~~onitored ~~by a~~ via a computerised ~~monitoring and~~ control system, and assessed manually every 1-2 weeks. Fish were fed *ad libitum* daily with commercial pellets (EWOS Sigma 150) and were acclimated for at least two weeks before use. All trials were carried out in compliance with the Animals (Scientific Procedures) Act 1986 by a UK Home Office license holder and approved by the ethics committee at the University of Aberdeen.

2.2 ~~2.2~~ Injection

Fish were anaesthetised by immersion in 2-phenoxyethanol (Fluka, Gillingham, U.K.) and then given an intraperitoneal (ip~~.p.~~) injection with each formulation posterior to the pelvic girdle. Six treatment groups were examined during this study, as outlined in Table 1.

Table 1: Treatment groups examined in this study. ~~* sterile phosphate buffered saline.r : recombinant.~~

No.	Treatment	Concentration	Source
1	PBS*	0.2 mL	-
2	rYRF	10µg in 0.2 mL	Wangkahart et al., 2016
3	rIL-1β	5µg in 0.2 mL	Hong et al., 2003
4	rTNF-α3	5µg in 0.2 mL	Hong et al., 2013
5	rIFN-γ	5µg in 0.2 mL	Wang et al., 2011
6	*A. salmonicida*~~$~~	0.5 x 10^6 cfu in 0.2 mL	Hooke strain~~virulent isolate~~

~~Table 1: Treatment groups examined in this study. * sterile phosphate buffered saline. r : recombinant~~* sterile phosphate buffered saline. r : recombinant. $ A non-motile virulent isolate (Scott et al., 2013).

After injection with *A. salmonicida* the fish were maintained in 400 L tanks at the University of Aberdeen's freshwater challenge facility. The fish were observed at regular intervals and did not display any symptoms of the disease during the experiment and no mortalities occurred (but were expected from day 3 onwards in the challenged fish). For all groups, the fish were kept in an appropriate number of tanks to allow sampling at different timings without stressing remaining fish. In the case of the *A. salmonicida* challenge the fish were killed by phenoxyethanol overdose and exsanguinated by cutting the gills at 24 h post-injection (hpi) and 48 hpi, since we have shown previously that major changes in gene expression are seen in adipose tissue by 72 hpi (Veenstra et al., 2017), and hence anticipated gene modulation would be faster following injection with a live, virulent pathogen. In the case of flagellin, we have found previously that larger responses are seen in spleen, liver, gills and skin at 6 hpi compared to 24 hpi at the dose used in this study (unpublished data), and hence we sampled adipose at both these timings. Similarly with the recombinant cytokines we sampled at 6 hpi and 24 hpi, to be comparable to the above. The cytokine dose used was based on our previous studies (Holland et al., 2002; Hong et al., 2003; Zou et al., 2005; Wang et al., 2011a; Hong et al., 2013). Lastly, the PBS injected fish were sampled at appropriate timings to act as controls.

2.3 ~~2.3~~ Tissue sampling, RNA extraction and cDNA synthesis

V~~Spleen, head kidney, and v~~isceral adipose tissue ~~(~~located around the internal organs (approx. 300 mg)~~)~~ was harvested from freshly killed trout (n=6 per treatment group per time point) and~~) at 3, 14 and 28 dpv and~~ snap frozen in dry ice. ~~Spleen and head kidney (~100 mg) were then then homogenized in 1.5 mL of TRI Reagent (Sigma, Gillingham, U.K.) using a 5mm stainless steel bead (Qiagen, Manchester, U.K.) in a Qiagen Tissue Lyser II (2 min., 30 Hz). Samples were kept on ice for 5-10 min. before being homogenized a second time (2 min., 30 Hz) and then stored at -80°C until RNA extraction.~~ The a~~A~~dipose tissue ~~(~300 mg)~~ was homogenized in 0.7 mL of TRI Reagent (Sigma, Gillingham, U.K.) using a 5mm stainless steel bead (Qiagen, Manchester, U.K.) in a Qiagen Tissue Lyser II (2 min., 30 Hz) after which the samples were centrifuged (5000g, 5 min., 4°C) and the oil layer removed. A

further 0.7 mL of TRI Reagent (Sigma) was added before samples were homogenized a second time (2 min., 30 Hz) with the samples then stored at -80°C until RNA extraction. ~~Visceral adipose tissue located around the internal organs was harvested from 6 freshly killed trout per treatment group per time point and snap frozen. Adipose tissue (~300 mg) was subsequently homogenised in TRI Reagent (Sigma) and T~~total RNA was isolated following the manufacturer's guidelines. 6 µg of RNA with an A260/A280 ratio between 1.8 - 2.2 measured with a NanoDrop® ND-1000 Spectrophotometer ~~ND-1000~~ (ThermoFisher, Loughborough, U.K.) was diluted in 29.4 µL of 2mM Oligo(dT) T28VN (Eurofins, Koeln, Germany) solution. The tube was then vortexed, centrifuged (5,000g, 30 s, 4°C), incubated at 70°C for 2 min, and placed on ice. cDNA was produced using a RevertAid™ Reverse Transcriptase Kit (ThermoFisher). The 29.4 µL Oligo(dT) / RNA mixture was added to new PCR tubes containing 1.6 µL of 10mM dNTP (ThermoFisher), 8 µL of 5x Reaction Buffer (ThermoFisher) and 1 µL of reverse transcriptase (ThermoFisher) per reaction. cDNA was synthesized in an Eppendorf Thermocycler (Stevenage, U.K.) using the following amplification program: 42°C for 1 h, 45°C for 30 min, 50°C for 30 min, 80°C for 10 min. The cDNA was then diluted to 600 µL in TE Buffer (pH 6.0) and stored at -20°C until use.

~~ and as described previously (Veenstra et al., 2017). cDNA was synthesized using RevertAid reverse transcriptase (ThermoFisher, Loughborough, U.K.) with 6µg of total RNA per 40µL reaction as per the manufacturer's instructions, then diluted with 600 µL TE buffer (pH 8.0) and stored at −20°C.~~

2.4 ~~2.4~~ Real-Time Quantitative PCR (RT-qPCR)

RTqPCR was performed in a Roche LightCycler 480 using 2x SYBR Green qPCR Master Mix made with a DNA Polymerase Immolase (Bioline, London, U.K.) with 10 µL reaction mixtures in 384-well plates (Roche, West Sussex, U.K.). Data were analysed using LightCycler®480 Software 1.5.1 (Roche). The amplification program was as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 62-68ºC for 30s, 72°C for 20-30 s, followed by fluorescent acquisition. The primers used for each gene and associated information are reported in Suppl. Table 1. All primers were

designed so at least one primer crossed and intron and tested to ensure that each primer pair could not amplify genomic DNA using the RTqPCR protocols. Primer efficiency was determined to be between 95% and 105% by serial dilutions of reference DNA run along with the cDNA samples in the same plate, and was used for quantification of the cDNA concentration. A melting curve for each RTqPCR reaction was established between 72°C and 95°C to ensure only a single product had been amplified. All samples were measured in duplicate and the expression level of each gene was calculated as arbitrary units normalised to the expression of elongation factor (EF)-1α. The Cp value of EF-1α across all treatment groups in adipose tissue used in this study was found to be 11.3 ± 0.11 (mean ± SEM).

~~The expression of each gene was measured in duplicate as described previously (Hong et al., 2013; Wang et al., 2016). QPCR was performed in a Roche LightCycler 480 using 2x SYBR Green qPCR Master Mix made with an Immolase DNA Polymerase kit (Bioline) with 10 µL reaction mixtures in 384-well plates (Roche). Data were analysed using LightCycler®480 Software 1.5.1 (Roche). The primers used for each gene and associated information are reported in Suppl. Table 1. The relative expression level of each gene in tissues was expressed as arbitrary units that were calculated from the references and normalized against the expression level of the house keeping gene, elongation factor (EF)-1α as described previously (Wang et al., 2011b).~~

2.5 ~~2.5~~ Gene Expression

Fifty eight selected rainbow trout immune genes including acute phase proteins (APPs), antimicrobial peptides (AMPs), pro- and anti-inflammatory cytokines, cytokines of adaptive immunity and associated Th master transcription factors, the IL-12 family cytokines, IFN-γ induced genes and B cell markers were analysed. The APPs included cyclooxygenase (Cox)-2 (Zou et al., 1999); Serum amyloid A (SAA); Serum amyloid P (SAP)-1 and -2. The AMPs analysed included ~~Cath~~Cathelicidin~~elicidin- (CATH)-~~1 and -2 (Chang et al., 2006) and ~~Hepcidin~~Hepcidin (Douglas et al., 2003). The pro- and anti-inflammatory cytokines included the highly expressed IL-1β paralogue IL-1β1 (Husain et al., 2012); IL-6A ~~(Iliev et al., 2007)~~ and IL-6B (Iliev et al., 2007); IL-8 (Laing et al., 2002); IL-11 (Wang et al., 2005); IL-18 (Zou et al., 2004); the four tumour necrosis factor-alpha (TNF-α) paralogues (TNF-α1-3,

Hong et al., 2013; TNF-α4, ~~unpublished~~Milne et al., 2017); two IL-10 paralogues (IL-10A and IL-10B, Harun et al., 2011) and transforming growth factor (TGF)-β1B (Maehr et al., 2013). The cytokines of adaptive immunity (Wang & Secombes, 2013) included the markers for Th1 (interferon (IFN-γ)1,2 (Zou et al., 2005); Th2 (IL-4/13A; IL-4/13B1,2, Wang et al., 2016) and Th17 (the IL-17A/F paralogues IL-17A/F1a, IL-17A/F2a, IL-17A/F3 and IL-17N, (Monte et al., 2013, Wang et al., 2015); and IL-22, (Monte et al., 2011)) type responses. The subunits (IL-12 [p35A1, p40B1, p40C], IL-23 [p19], IL-27 [p28A and B, and EBI3]) (Husain et al., 2014; Jiang et al., 2015; Wang & Husain, 2014; Wang et al., 2014) of IL-12 family cytokines, that bridge innate and adaptive immunity, were also analysed. Other cytokine genes studied included IL-2 (Díaz-Rosales et al., 2009), IL-15 (Wang et al., 2007), IL-15Like (~~unpublished~~Gunimaladevi et al., 2007), the IL-17C-like paralogues IL-17C1 and C2, (Wang et al., 2010a), IL-17D, IL-20-like (Wang et al., 2010b), IL-21 (Wang et al., 2011b) and IL-34 (Wang et al., 2013). The master transcription factors analysed were T-bet, GATA3, RORγ and FOXP3a (Wang et al., 2010c,d; Monte et al., 2012). The B cells markers included membrane bound (m) and secreted (s) IgM and IgT (mIgM, mIgT, sIgM and sIgT). Lastly, the IFN-γ inducible genes studied were MX2, CXCL11, IRF-1 and SOCS1 (Wang & Secombes, 2008).

2.6 ~~2.6~~ Statistical Analysis

Relative immune gene expression values (after normalization against the housekeeping gene EF-1α) were log2 transformed prior to statistical analysis (as described in Wang et al., 2011b). Differential gene expression was assessed by fitting a linear model with treatment as explanatory variables for 6 hpi and 24 hpi. When significant treatment effects were detected, group means of rYRF, rIL-1β, rTNF-α3 and rIFN-γ were compared to the PBS group *post hoc* using HSD tests. *A. salmonicida*-induced gene expression at 24 hpi and 48 hpi were compared to the PBS group at 24 hpi. Model validation was performed by inspection of standard residual plots and significance assessed at $p<0.05$ (Venables & Ripley, 2002). All statistical analyses~~is~~ were~~as~~ performed using the R statistical environment (version R-3.2.3., R Development Core Team, 2016) with heat maps generated using the" gplots" package (Warnes et al., 2016).~~.~~

3. Results

3.1 Response to *Aeromonas salmonicida* challenge

A large number of genes were affected by the live pathogen challenge, with 44 of the 58 genes studied found to be significantly altered at 24 hpi and/or 48 hpi in adipose tissue (Fig. 1, Suppl. Tables 2-3). The largest increase was seen at 24 hpi for IL-6A, with a 1,165 fold increase. Most of the genes showing changes were statistically significant at both time points, however some were more highly upregulated at 24 hpi (over double the fold increase at 48 hpi) (e.g. IL-1β, IL-6A, IL-6B, IL-10A, IL-17D, IL-34 and sIgT) whilst others were more highly upregulated at 48 hpi (over double the fold increase at 24 hpi) (e.g. IL-4/13A, IL-17C2, IL-21, IFN-γ2, SAA, sIgM, ~~CATH~~Cathelicidin-2, Cox-2, CXCL11 and IRF-1). Two genes were only upregulated at 24 hpi (TNF-α4 and RORγ), whilst IL-2, IL-17C1, p19A, p40B1 and T-bet were only upregulated at 48 hpi. Curiously, IL-11 and IL-22 were significantly downregulated at both time points, while EBI3 was downregulated at 48 hpi. IL-10B, IL-17A/F1-3, IL-17N, p28A, SAP1 and SAP2, TGF-β1B, TNF-α3, ~~CATH~~Cathelicidin-1 and GATA3 were not significantly altered in adipose tissue in response to the pathogen. Lastly, IL-20 and p28B transcript expression was not detectable at either time point, the latter highlighting the large differences in expression that can be seen between gene paralogues in some cases.

Figure 1: Average fold change of gene expression in adipose tissue of fish administered live *A. salmonicida* (mean + SE, n=6 per treatment group per time point) at 24 hpi and 48 hpi for A. the interleukins and IL-12 family alpha chains studied, and B. the IL-12 family beta chains, other cytokines, APPs, Igs, AMPs, master transcription factors and IFN-γ inducible genes studied. All gene values were normalized against the housekeeping gene EF-1α followed by normalization against the average relative expression of control group individuals at 24 hpi. *** = p< 0.001; **= p< 0.01; * = p< 0.05. nd[†] (not detectable) = no expression in CTRL group, no expression in treatment group.

~~nd* = not determined.~~

3.2 Response to rYRF

Within the adipose tissue, the genes which exhibited statistically significant changes in expression in response to rYRF are displayed in Figure 2, with the ~~average fold change~~normalised Z-scores for all genes examined shown in a ~~heatmap~~heat map in Figures 6-7 (with summary statistics for all genes in Suppl. Tables 2-3) to allow a comparative analysis of the changes seen across the 4 recombinant proteins studied. Fewer genes were modulated by rYRF relative to *A. salmonicida* challenge, and some interesting differences in kinetics were apparent. Most of the genes modulated were increased significantly at 6 hpi (19), with 12 remaining upregulated at 24 hpi. Of these IL-1β, IL-6A and IL-6B were more highly upregulated at 6 hpi whilst IL-17C2, SAA, ~~CATH~~Cathelicidin-2 and ~~hepcidin~~Hepcidin were more highly upregulated at 24 hpi. IL-10A, IL-11, IL-17C1, IL-22, p19A, p35A1, p40B1 and ~~CATH~~Cathelicidin-1 were only upregulated at 6 hpi, with IL-17D only increased significantly at 24 hpi. In contrast, IL-17A/F2 was significantly downregulated following rYRF stimulation, at 24 hpi.

Figure 2: Average fold change of gene expression in adipose tissue of fish administered rYRF which exhibited a statistically significant change in expression (mean + SE, n=6 per treatment group per time point) at 6 hpi and/or 24 hpi. All gene values were normalized against the housekeeping gene EF-1α followed by normalization against the average relative expression of control group individuals. *** = p< 0.001; **= p< 0.01; * = p< 0.05.

3.3 ~~-~~Response to rIL-1β

The genes which exhibited statistically significant changes in expression in adipose in response to rIL-1β are displayed in Figure 3, with the normalised Z-scores for all genes examined shown in a heat map in Figures 6-7~~with the average fold change for all genes examined shown in a heatmap in Figure 6~~ (with summary statistics for all genes in Suppl. Tables 2-3). Of the three recombinant cytokines used, rIL-1β induced the largest response in adipose tissue, in many ways resembling the response to pathogen challenge. However, most gene upregulation in response to rIL-1β occurred at 6 hpi, with the largest increases being seen in pro-inflammatory genes, with IL-1β, IL-6A, IL-6B, IL-8, IL-11, IL-22, p19A, Cox-2 and ~~hepcidin~~Hepcidin showing increases in excess of 100 fold. Genes that remained elevated at 24 hpi, often at lower levels of induction, included IL-4/13B1, IL-8, IL-11, IL-18, IL-22, IFN-γ1, p19A, p40C,

~~CATH~~Cathelicidin-1, ~~CATH~~Cathelicidin-2 and ~~hepcidin~~Hepcidin. Whilst no genes were upregulated at 24 hpi only, mIgT was downregulated at this time.

Figure 3: Average fold change of gene expression in adipose tissue of fish administered rIL-1β which exhibited a statistically significant change in expression (mean + SE, n=6 per treatment group per time point) at 6 hpi and/or 24 hpi. All gene values were normalized against the housekeeping gene EF-1α followed by normalization against the average relative expression of control group individuals. *** = p< 0.001; **= p< 0.01; * = p< 0.05.

3.4 ~~-~~Response to rTNF-α3

Within the adipose tissue, fewer genes exhibited statistically significant changes in expression after rTNF-α3 treatment relative to the response to rIL-1β (Figs. 4~~, and~~ 6~~-7 and~~~~,~~ Suppl. Table~~s~~ 2~~-3~~). p19A1 was the most highly induced gene at 6 hpi (~70-fold increase), and whilst this high level expression persisted to 24 hpi it was no longer significant. Other genes that were only upregulated at 6 hpi included IL-1β, IL-6A, IL-6B and TNF-α2. Interesting a relatively large number of genes were increased significantly only at 24 hpi (in contrast to rIL-1β induced effects) as with IL-2, IL-4/13B2, IL-11, IL-15, IL-17C1, IL-17C2, IL-18, IL-22, p40B1, p40C and ~~CATH~~Cathelicidin-1. More genes were also down~~–~~regulated in adipose as a consequence of rTNF-α treatment (vs rIL-1β treatment), as with IFN-γ1, TGF-β1B, TNF-α3 and GATA3 at 6 hpi.

Figure 4: Average fold change of gene expression in adipose tissue of fish administered rTNF-α3 which exhibited a statistically significant change in expression (mean + SE, n=6 per treatment group per time point) at 6 hpi and/or 24 hpi. All gene values were normalized against the housekeeping gene EF-1α followed by normalization against the average relative expression of control group individuals. *** = p< 0.001; **= p< 0.01; * = p< 0.05.

3.5 ~~-~~Response to rIFN-γ

The fewest number of genes were modulated by rIFN-γ in adipose tissue relative to the other treatments. The genes which exhibited statistically significant changes in

expression are shown in Figure 5, with the average fold change for all genes examined shown in a ~~heatmap~~heat map in Figures 6-7 ~~6~~ (with summary statistics for all genes in Suppl. Tables 2-3). As with the rTNF-α3 treatment group, the increase in expression was typically modest, with the exception of CXCL11 that was induced >200-fold at 6 hpi, and was still 40-fold increased at 24 hpi. IL-4/13B1, IL-6B, IRF-1 and SOCS1 also exhibited a modest increase at 6 hpi, which had decreased by 24 hpi with the exception of IL-4/13B1. IL-8, IL-15, IL-18, IFN-γ1, SAP2, ~~CATH~~Cathelicidin-2 and ~~hepcidin~~Hepcidin showed induced expression at 24 hpi only, following a significant decrease in expression at 6 hpi in the case of IFN-γ1.

Figure 5: Average fold change of gene expression in adipose tissue of fish administered rIFN-γ which exhibited a statistically significant change in expression (mean + SE, n=6 per treatment group per time point) at 6 hpi and/or 24 hpi. All gene values were normalized against the housekeeping gene EF-1α followed by normalization against the average relative expression of control group individuals. *** = p< 0.001; **= p< 0.01; * = p< 0.05.

3.6 ~~Heatmap~~Heat map analysis

A comparative analysis of the changes in adipose induced by the ~~4~~ recombinant proteins used in this investigation was performed by calculating the average fold change of the 58 genes studied in a heat map for 6 hpi (Fig. 6) and 24 hpi (Fig. 7), with log2-normalisation, followed by standardisation within each treatment group by calculating the Z-score. Hierarchical clustering using Euclidean distance was then applied. This revealed a clear distinction in the gene expression patterns found in trout adipose tissue in response to the four treatments at each time point. At 6 hpi there was a large overlap in genes expressed in response to rIL-1β and rYRF. At this time point IL-8, Hepcidin, IL-1β, IL-6A, p19A, and ~~IL-1β, IL-8,~~ IL-6B ~~and hepcidin~~Hepcidin were all more highly expressed in these groups as well as in response to rTNF-α3. However, rIL-1β was the only treatment that~~showed an~~ increased ~~in~~ IL-34 and TNF α1 expression while in all other treatment groups these genes were more lowly expressed or showed little change. In contrast SAA was downregulated by rIL-1β but was upregulated by rYRF. ~~Conversely~~Another interesting response was seen with ~~,~~IL-4/13B1, that was refractory ~~in response~~ to rYRF but more highly expressed in

response to rIL-1β and rTNF-α3. Following treatment with rTNF-α3 SAP1, IL-17N, SAP2~~, IL-17N~~ and IL-4A/13A were upregulated while the other treatment groups induced a lower expression of the genes or no change. Curiously~~IL-20 in response to~~ rTNF-α3 induced a considerably lower expression of IL-20 in comparison to all other treatment groups and genes, with a similar but smaller effect on itself. In contrast, the fish administered rIFN-γ showed a high expression of CXCL11, IRF-1, p19A and IL-6B, and this was the only treatment to upregulate ~~. rIL-1β showed an increase in IL-34 and TNF-α1 while in all other treatment groups these genes were more lowly expressed or showed little change. SAA was downregulated by rIL-1β but was upregulated by rYRF. Conversely, IL-4/13B1 was refractory in response to rYRF but more highly expressed in response to rIL-1β and rTNF-α3. Lastly,~~ CXCL11, SOCS1, IL-21 and IL-17D ~~highly expressed in response to rIFN-γ but had lower expression or no change from the average in response to rTNF-α3, rYRF and IL-1β~~.

At 24 hpi ~~fish administered~~ a higher expression in the pro-inflammatory gene IL-11, and the AMPs~~IL-6A, Cox-2,~~ Hepcidin, Cathelicidin-1 and Cathelicidin-2 was found in the adipose for all treatment groups. IL-1β and TNF-α3 showed the highest degree of overlap in genes differentially expressed, and both induced expression of IL-17A/F1a and IL-22. rYRF also induced~~showed~~ higher expression of~~,~~ a unique set of genes~~primarily of pro-inflammatory genes~~, including TNF-α1, TNF-α2, IL-6B, SAA, IL-1β, ~~as well as~~ IL-17D and p28A~~, with all other treatments inducing no change or a downregulation of these genes~~. Interestingly rYRF induced no change or a decrease in expression of p19A, IL-10a, p40C~~, IL-10A~~ and, mIgM while all other treatment groups showed mainly increased expression of these genes. IL-17N was much more lowly expressed in response to this treatment in comparison with all other genes. rIFN-γ was the only treatment to induce a marked increase in expression of CXCL11, MX2, IFN-γ2, IRF-1~~,~~ and SAP2~~, IFN-γ2 and CXCL11 expression~~ at this time, and induced the lowest relative expression of T-bet.

~~A comparative analysis of the changes in adipose induced by the 4 recombinant proteins used in this investigation was performed by calculating the average fold change of the 58 genes studied in a heat map for 6 hpi (Fig. 6) and 24 hpi (Fig. 7), with log2-normalisation, followed by standardisation within each treatment group by calculating Z-score. Hierarchical clustering using Euclidean distance was then applied. This revealed a clear distinction in the gene expression patterns found in trout adipose~~

~~tissue in response to the four treatments at each time points. At 6 hpi the highest number of genes upregulated was in response to rIL-1β and the least number of genes upregulated was in response to rIFN-γ. At this time point there was a large overlap in genes upregulated in response to rIL-1β and rYRF. IL-6A, p19A, IL-22, IL-1β, IL-8, IL-6B and hepcidin~~Hepcidin ~~were all strongly upregulated in these groups as well as in response to rTNF-α3. In contrast, the fish administered rIFN-γ only showed clear upregulation of p19A and IL-6B. rIL-1β showed an increase in IFN-γ2, IL-17A/F1 and p40C while in all other treatment groups these genes were downregulated or showed little change. Interestingly, SAP1, SAP2, IL-17N and IL-17A/F2a were more highly upregulated in response to rTNF-α3 while rIL-1β induced almost no change, and rYRF induced a downregulation. SAA was downregulated by rIL-1β but was upregulated by rYRF. Conversely, IL-4/13B1 was refractory in response to rYRF but upregulated in response to rIL-1β and rTNF-α3. Lastly, IRF1 and SOCS1 were upregulated in response to rIL-1β and rIFN-γ but downregulated in response to rTNF-α3 and rYRF.~~

~~At 24 hpi fish administered rYRF showed the largest degree of upregulation, primarily of pro-inflammatory genes including TNF-α1, TNF-α2, IL-6B, IL-17C2, IL-1β as well as SAA, with all other treatments inducing no change or a downregulation of these genes. An upregulation in the pro-inflammatory genes IL-11, IL-6A, Cox-2 and IL-8 was found in the adipose from the rYRF, rIL-1β and rTNF-α3 treatment groups, but not the rIFN-γ treatment group. Interestingly rYRF induced no change or a downregulation of IL-15, IL-21, IL-10A and p19A, while all other treatment groups showed mainly upregulation of these genes. rIFN-γ was the only treatment to induce a marked upregulation of MX2, IRF-1, SAP2, IFN-γ1/2 and CXCL11 expression at this time.~~

Figure 6: Expression data from 58 genes examined at 6 hpi were normalized against the housekeeping gene EF-1α, followed by normalization against the average relative expression of control group individuals. Values were log2 normalised and Z-scores calculated within each treatment group (rIFN-γ, rIL-1β, rTNF-α3, and rYRF). The dendrograms visualise hierarchical clustering using Euclidean distance. ~~Average fold change of the 58 genes studied in fish administered recombinant proteins (rIFN-γ, rIL-1β, rTNF-α3, and rYRF) relative to control samples at 6 hpi (Fig. 6A) and 24 hpi (Fig. 6B). Each column displays the average fold change (n=6) and each row corresponds to the individual genes. Expression levels are colour coded relative to the control group: blue for downregulation, red for upregulation and white for no difference~~

~~compared to the control. The dendrogram on the left illustrates the final clustering tree resulting from hierarchical clustering of gene values.~~

Figure 7: Expression data from 58 genes examined at 24 hpi were normalized against the housekeeping gene EF-1α, followed by normalization against the average relative expression of control group individuals. Values were log2 normalised and Z-scores calculated within each treatment group (rIFN-γ, rIL-1β, rTNF-α3, and rYRF). The dendrograms visualise hierarchical clustering using Euclidean distance.

4. Discussion

Adipose is no longer considered to be an inert tissue functioning solely as an energy store but ~~is~~ has emerged~~ing~~ as an important site for the regulation of many pathological processes (Matarese & La Cava, 2004; Tilg & Moschen, 2006; Grant & Dixit, 2015). In addition to containing immune cells, in mammals the adipocytes themselves can express pro-inflammatory, anti-inflammatory and immune-modulating proteins and peptides (Miner, 2004; Rangel-Moreno et al., 2009). Indeed, high levels of pro- and anti-inflammatory cytokines are present under normal physiological conditions in human omentum and under pathological conditions a significant induction of these molecules can occur (Chandra et al. 2011). Human adipocytes respond strongly to lipopolysaccharides (LPS) and it has been suggested that the omentum is an important site of antibody formation when the route of administration is via ~~i.p.~~ip injection (Walker and Rogers, 1960; Rangel-Moreno et al., 2009). Although much attention has been given to adipose tissue with regard to obesity in mammals, it is a largely unexplored immune site in other animal models or farmed species. Interestingly in farmed trout selection for rapid growth is associated with increased amounts of fat used for storage, with visceral fat a moderately heritable trait (Kause et al., 2002). Thus the potential immune modulatory role of adipose in this species is important to establish.

In fish Ig (IgM and IgT) reactivity was identified recently in the interstitial space between adipocytes in rainbow trout (Pignatelli et al., 2014) and the transcription levels of both IgM and IgT increased in adipose in response to oral vaccination (Ballesteros et al. 2013). The leukocytes present in trout adipose were mostly lymphocyte-like, with

~80% expressing MHC-II on their surface (Pignatelli et al., 2014). Interestingly, following ~~i.p.~~ip viral challenge with VHSV the virus was taken up by the adipocytes and could replicate, subsequently inducing upregulation of a variety of antiviral genes (eg Mx, IFN-γ) and secretory IgM (sIgM) (Pignatelli et al., 2014). To date no studies have addressed the response of fish adipose tissue to bacterial infection. However, our previous study of trout vaccinated with *Aeromonas salmonicida* oil-adjuvanted vaccines showed that a large number of genes were modulated in adipose tissue, especially the pro-inflammatory genes which were strongly upregulated (Veenstra et al., 2017). Hence in the present study we extended these findings and initially examined the impact of *A. salmonicida* infection to modulate immune gene expression in trout adipose tissue, at 24 h and 48 h post-challenge.

Following bacterial infection a large number of immune genes (~75% of those examined) were highly upregulated in response to the pathogen, with the majority upregulated at both time points. The most highly upregulated (>100-fold) genes were pro-inflammatory cytokines (IL-1β, IL-6A, IL-6B), antimicrobial peptides (~~CATH~~Cathelicidin-2, ~~hepcidin~~Hepcidin), and other genes involved in pro-inflammatory responses such as COX-2 and SAA, with the cytokines being higher at 24 h vs 48 h in contrast to most of the other molecules. Curiously IRF1 was also highly upregulated at 48 h post-challenge, perhaps also linked to IL-6 release, or the more modest but highly significant upregulation of IFN-γ at this time. LPS is a major cell wall component of Gram negative bacteria such as *A. salmonicida* and studies in humans have shown it can induce pro-inflammatory cytokines (eg IL-1β, IL-2, IL-4, IL-8, IL-10 and TNF-α) in omentum (Chandra et al., 2011). Similarly, during LPS-induced systemic inflammation in mice adipose was found to be the major source of IL-6 (Starr et al., 2009). In trout IL-6 is known to induce AMPs such as ~~CATH~~Cathelicidin-2 and ~~hepcidin~~Hepcidin but not ~~CATH~~Cathelicidin-1 (Costa et al., 2011), perhaps suggesting these effects in trout adipose are IL-6 induced. TNF-α1 and -α2 were also increased to a relatively high degree post *A. salmonicida* infection, in contrast to TNF-α3/-α4. Curiously the opposite is seen in adipose tissue post-vaccination where TNF-α3 is more highly upregulated in fish that are expected to establish protective immunity to furunculosis (Veenstra et al., 2017). These isoforms represent the two types of TNF-α present in teleosts, as a consequence of a teleost whole genome duplication (WGD) event, with further duplication from a salmonid specific WGD (Zou et al., 2003; Laing

et al., 2001; Hong et al., 2013). Classical cytokines involved in Th17 type responses (IL-17A/F1-3 and IL-22) considered crucial for protection against extracellular pathogens, showed no change post infection or were downregulated (IL-22), perhaps because adipocytes cannot express these genes or the pathogen can suppress their expression. However, a modest increase in IL-17C (especially IL-17C2) and IL-17D was apparent. Moderate upregulation of p19 and p35 (alpha chains for IL-23 and IL-12/IL-35 respectively) was also seen~~, alpha chains for IL-23 and IL-12/IL-35 respectively~~, but no significant changes in p28 (alpha chain for IL-27) were found~~seen~~.~~, which is the alpha chain for IL-27 (with EBI3) (Vignali and Kuchroo, 2012).~~ Indeed, that EBI3 (beta~~alpha~~ chain for IL-27 and IL-35) was downregulated at 48h post-infection suggests that IL-35 was also not produced. Lastly, sIgM and sIgT were upregulated in response to live *A. salmonicida*, as early as 24 hpi, in contrast to challenge with live virus (VHSV) where this was not seen until 5 days post injection (Pignatelli et al., 2014). The mechanisms of antigen presentation/ lymphocyte activation in adipose should be investigated further, as a significant increase in the sIgM transcript was also seen in trout adipose post-vaccination (Veenstra et al., 2017).

We next studied the effect of flagellin on adipose gene expression, since it is a powerful bacterial PAMP able to increase pro-inflammatory cytokine expression in a range of immune tissues in salmon (Hynes et al., 2011) and can induce non-specific protection to bacterial infection in trout (Scott et al., 2013). Overlap in genes induced following stimulation with *A. salmonicida* and rYRF was apparent, with genes such as IL-1β, IL-6, IL-8, SAA,~~ CATH~~ Cathelicidin-2, Cox-2 and ~~hepcidin~~Hepcidin highly induced, with TNF-α1 and -α2 more modestly induced. Some differences in kinetics were apparent, with IL-1β and IL-6 being much higher at 6 hpi, whereas SAA, ~~CATH~~Cathelicidin-2 and ~~hepcidin~~Hepcidin were higher at 24 hpi. Interestingly several genes not induced by infection were notably induced by YRF, including IL-11, IL-22, and ~~CATH~~Cathelicidin-1, and p19A was induced to a much higher level. This may imply that different antimicrobial pathways are being stimulated, although again there was no upregulation of IL-17A/F expression seen. Lastly, in contrast to infection no change was seen in Ig or IFN-γ inducible gene transcript levels. Previous work using YRF for *in vitro* stimulation found that most pro-inflammatory cytokines and IL-12 family cytokines, together with APPs and AMPs were highly induced in a trout (RTS-11) macrophage/monocyte cell line (Wangkahart et al., 2016). However, the study of

Chettri et al. (2011) using trout HK leukocytes stimulated with flagellin induced only moderate upregulation of pro-inflammatory cytokines and a down-regulation of IL-6.

To understand further the reactivity of trout adipose to pro-inflammatory factors, we studied the impact of ~~i.p.~~ip injection of the pro-inflammatory cytokines IL-1β, TNF-α3 and IFN-γ. IL-1β is considered a key cytokine for the early response following immunostimulation and essential for resistance to pathogens. Previously, trout rIL-1β was shown to increase IL-1β, Cox-2, TNF-α and MHC II (β chain) transcription in RTS-11 cells (Hong et al., 2001; Laing et al., 2001) and when administered ~~i.p.~~ip increased head kidney IL-1β and Cox-2 expression (Hong et al., 2001). In this study IL-1β was shown to induce the expression of many immune genes in adipose, largely overlapping with the responses to YRF and *A. salmonicida* infection. A large increase (>200-fold change) was seen of itself, IL-6(A,B), IL-8, IL-11, IL-22, Cox-2 and ~~hepcidin~~Hepcidin, with higher expression seen at 6 hpi than 24 hpi. Several of the IL-12 family α/β subunits were again upregulated, especially p19A. More modest upregulation was seen for TNF-α1 and –α2 at 6 hpi, and for ~~CATH~~ Cathelicidin-1/2, which persisted to 24 hpi. Moderate upregulation of IL-4/13B1 was also seen, considered a type-2 cytokine in fish (Wang et al., 2016) but was not associated with any change in Ig transcript levels. Curiously SAA was not upregulated by IL-1β in adipose. It is highly and selectively expressed in human adipocytes and can be modulated by stimulation with inflammatory cytokines (Yang et al., 2006) but in fish it appears to be more responsive to PAMPs. Lastly, there was again no impact on IL-17A/F expression (although IL-17C1 and IL-17D were upregulated ~10-fold at 6 hpi) or Th associated transcription factors.

TNF-α3 is a type II TNF- α in teleosts, and has been shown to elicit a range of biological activities in trout (Hong et al., 2013), similar to the effects seen with TNF-α1/2 (Zou et al., 2003). In primary HK macrophages rTNF-α3 stimulation leads to upregulation of IL-1β, IL-6, IL-8, IL-17C1, TGF-β1B, Cox-2, TNF-α2 and IL-34 at 4 h post-stimulation (hps); TNF-α1, ~~CATH~~Cathelicidin-1 and ~~hepcidin~~Hepcidin at 8 hps; and TNF-α3, IL-17C2 and SOCS1 at 24 hps (Hong et al., 2013). In mammals TNF-α expression can increase in adipose tissue and is thought to play a crucial role in metabolic diseases such as obesity, insulin resistance and type 2 diabetes (Tilg & Moschen, 2006; Ouchi et al., 2011). The results of our study found that *in vivo*

stimulation of trout adipose by ~~i.p.~~ip injection of rTNF-α3 resulted in relatively few changes in gene expression, with modest increases (5-20-fold) seen, as with IL-4/13B1, IL-6 (A,B), p40C and ~~CATH~~Cathelicidin-2 expression at 6 and/or 24 hpi. p19A was an exception and showed the highest upregulation (71-fold) at 6 hpi. At 24 hpi upregulation of p40B1 and p40C was also seen, suggesting again that IL-23 is likely produced in adipose tissue. Contrary to the results found by Hong et al. (2013) with HK macrophages, rTNF-α3 induced a significant downregulation of IFN-γ1, TGF-β1B and TNF-α3 in adipose at 6 hpi, potentially as a negative feedback on the latter.

IFN-γ is mainly synthesized and secreted by Th1 cells and NK cells, and acts on many types of target cell to mediate leukocyte trafficking, enhance antigen presentation and exert antiviral and antimicrobial properties via the JAK/STAT signalling pathway (Schroder et al., 2004; Robertsen, 2006; Sun et al., 2011). In fish it modulates cytokine and chemokine expression with induction of IL-1β, IL-6, IL-12, TNF-α and CXCL11 in macrophages/ phagocytes (Zou et al., 2005; Grayfer et al., 2010, Arts et al., 2010). However, IFN-γ affected the fewest number of genes in adipose tissue of the stimulants used in this study, with just 12 genes modulated. Of these the IFN-γ inducible genes CXCL11, IRF1 and SOCS1 showed some of the largest effects, confirming the rIFN-γ was active. In mammals IFN-γ is known to increase CXCL11 expression in adipocytes (Rocha et al. 2008). Interestingly, rIFN-γ treatment ~~down-regulated~~downregulated IFN-γ expression at 6 hpi in adipose, again possibly as a negative feedback effect in the short term, with subsequent upregulation by 24 hpi possibly linked with upregulation of IL-15 and IL-18 that are known to induce IFN-γ (Okamura et al., 1995; Wang et al., 2007). ~~-~~IFN-γ was the only treatment that had no effect on IL-12 family genes, most pro-inflammatory genes, AMPs and APPs, although uniquely SAP2 was upregulated. The upregulation of IL-4/13B1 is interesting, and is one of three IL-4/13 paralogues identified in trout with overlapping yet distinct functionalities (Wang et al., 2016). The constitutive expression of IL-4/13B1 and B2 isoforms in most tissues is lower compared to IL-4/13A, but they are more highly induced by infection and PAMP stimulation. In response to rIFN-γ no significant modulation of IL-4/13 occurred in primary rainbow trout HK macrophages, so the effect here could be part of the antagonistic actions between IL-4 and IFN-γ that occur due to their roles in Th1 and Th2 functional polarity (Boehm et al. 1997).

In conclusion, this study has demonstrated that fish adipose is capable of expressing a large range of immune molecules in response to stimulation with a live bacterium, a bacterial PAMP (*Y. ruckeri* flagellin), and the pro-inflammatory cytokines IL-1β, TNF-α3 and IFN-γ. Following infection and stimulation with flagellin and IL-1β a large upregulation of transcripts encoding pro-inflammatory and antimicrobial molecules was seen, with a high degree of overlap. TNF-α treatment affected relatively few genes and the effects were more modest (ie increases of 5-20-fold). IFN-γ had the smallest impact on adipose but IFN-γ inducible genes showed some of the largest effects. Overall, it is clear that adipose tissue should be considered an active immune site in fish, capable of participating in and influencing immune responses through the release of many immunomodulatory molecules.

Acknowledgements

KAV was supported by ELANCO and SEPPIC to undertake a PhD program at the University of Aberdeen. EW was supported by a PhD studentship from the Ministry of Science and Technology of Thailand and Mahasarakham University. TH received funding from the MASTS pooling initiative (The Marine Alliance for Science and Technology for Scotland). MASTS is funded by the Scottish Funding Council (grant reference HR09011).

Conflict of interest statement: Authors have no conflict of interest.

References

Arts, J. A. J., Tijhaar, E. J., Chadzinska, M., Savelkoul, H. F. J., & Verburg-van Kemenade, B. M. L. (2010). Functional analysis of carp interferon-γ: Evolutionary conservation of classical phagocyte activation. *Fish & Shellfish Immunology*, *29*(5), 793–802. https://doi.org/10.1016/j.fsi.2010.07.010

Ballesteros, N. A., Castro, R., Abos, B., Saint-Jean, S. S. R., Pérez-Prieto, S. I., & Tafalla, C. (2013). The pPyloric cCaeca aArea iIs a mMajor sSite for IgM+ and IgT+ B cCell rRecruitment in rResponse to oOral vVaccination in rRainbow tTrout. *PLOS ONE*, *8*(6), e66118. https://doi.org/10.1371/journal.pone.0066118

Boehm, U., Klamp, T., Groot, M., & Howard, J. C. (1997). Cellular responses to interferon-gamma. *Annual Review of Immunology; Palo Alto*, *15*, 749–95.

Chandra, A., Srivastava, R. K., Kashyap, M. P., Kumar, R., Srivastava, R. N., & Pant, A. B. (2011). The aAnti-iInflammatory and aAntibacterial bBasis of hHuman oOmental dDefense: Selective eExpression of cCytokines and aAntimicrobial pPeptides. *PLOS ONE*, *6*(5), e20446. https://doi.org/10.1371/journal.pone.0020446

Chang, C.-I., Zhang, Y.-A., Zou, J., Nie, P., & Secombes, C. J. (2006). Two cCathelicidin gGenes aAre pPresent in both rRainbow tTrout (*Oncorhynchus mykiss*) and Atlantic sSalmon (*Salmo salar*). *Antimicrobial Agents and Chemotherapy*, *50*(1), 185–195. https://doi.org/10.1128/AAC.50.1.185-195.2006

Chettri, J. K., Raida, M. K., Holten-Andersen, L., Kania, P. W., & Buchmann, K. (2011). PAMP induced expression of immune relevant genes in head kidney leukocytes of rainbow trout (*Oncorhynchus mykiss*). *Developmental & Comparative Immunology*, *35*(4), 476–482. https://doi.org/10.1016/j.dci.2010.12.001

Costa, M. M., Maehr, T., Diaz-Rosales, P., Secombes, C. J., Wang, T. (2011). Bioactivity studies of rainbow trout (*Oncorhynchus mykiss*) interleukin-6: Effects on macrophage growth and antimicrobial peptide gene expression. *Molecular Immunology*, *48*(15–16), 1903-1916. https://doi.org/10.1016/j.molimm.2011.05.027

Díaz-Rosales, P., Bird, S., Wang, T. H., Fujiki, K., Davidson, W. S., Zou, J., & Secombes, C. J. (2009). Rainbow trout interleukin-2: Cloning, expression and bioactivity analysis. *Fish & Shellfish Immunology*, *27*(3), 414–422. https://doi.org/10.1016/j.fsi.2009.06.008

Douglas, S. E., Gallant, J. W., Liebscher, R. S., Dacanay, A., & Tsoi, S. C. M. (2003). Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish. *Developmental & Comparative Immunology*, *27*(6–7), 589–601. https://doi.org/10.1016/S0145-305X(03)00036-3

Evensen, Ø., Brudeseth, B. E., & Mutoloki, S. (2005). The vaccine formulation and its role in inflammatory processes in fish--effects and adverse effects. *Developments in Biologicals*, *121*, 117–25.

Fauconneau, B., Andre, S., Chmaitilly, J., Le Bail, P.-Y., Krieg, F., & Kaushik, S. J. (1997). Control of skeletal muscle fibres and adipose cells size in the flesh of rainbow trout. *Journal of Fish Biology*, *50*(2), 296–314. https://doi.org/10.1111/j.1095-8649.1997.tb01360.x

Fedorko, M. E., Hirsch, J. G., & Fried, B. (1971). Studies on transport of macromolecules and small particles across mesothelial cells of the mouse omentum. *Experimental Cell Research*, *69*(2), 313–323. https://doi.org/10.1016/0014-4827(71)90230-8

Ferrante, A. W., (2013). The immune cells in adipose tissue*. Diabetes & Obesity Metabolism*., *15*(Suppl 3), 34-8. doi: 10.1111/dom.12154.

Grant, R. W., & Dixit, V. D. (2015). Adipose tissue as an immunological organ. *Obesity (Silver Spring, Md.)*, *23*(3), 512–518. https://doi.org/10.1002/oby.21003

Grayfer, L., & Belosevic, M. (2009). Molecular characterization, expression and functional analysis of goldfish (*Carassius aurutus* L.) interferon gamma. *Developmental & Comparative Immunology*, *33*(2), 235–246. https://doi.org/10.1016/j.dci.2008.09.001

Gunimaladevi, I., Savan, R., Sato, K., Yamaguchi, R., & Sakai, M. (2007). Characterization of an interleukin-15 like (IL-15L) gene from zebrafish Danio rerio). *Fish & Shellfish Immunology, 22*(4), 351-362.

Ha, S., Tsuji, M., Suzuki, K., Meek, B., Yasuda, N., Kaisho, T., & Fagarasan, S. (2006). Regulation of B1 cell migration by signals through Toll-like receptors. *Journal of Experimental Medicine*, *203*(11), 2541–2550. https://doi.org/10.1084/jem.20061041

Harun, N. O., Costa, M. M., Secombes, C. J., & Wang, T. (2011a). Sequencing of a second interleukin-10 gene in rainbow trout *Oncorhynchus mykiss* and comparative investigation of

the expression and modulation of the paralogues in vitro and in vivo. *Fish & Shellfish Immunology*, *31*(1), 107–117. https://doi.org/10.1016/j.fsi.2011.04.010

Harun, N. O., Wang, T., & Secombes, C. J. (2011b). Gene expression profiling in naïve and vaccinated rainbow trout after *Yersinia ruckeri* infection: Insights into the mechanisms of protection seen in vaccinated fish. *Vaccine*, *29*(26), 4388–4399. https://doi.org/10.1016/j.vaccine.2011.04.003

Holland, J.W., Pottinger, T.G. & Secombes, C.J. (2002). Recombinant interleukin-1β activates the hypothalamic-pituitary-interrenal axis in rainbow trout, *Oncorhynchus mykiss*. *Journal of Endocrinology 175*, 261-267.

Hong, S., Zou, J., Crampe, M., Peddie, S., Scapigliati, G., Bols, N., Cunningham, C., & Secombes, C.J. (2001). The production and bioactivity of rainbow trout (*Oncorhynchus mykiss*) recombinant IL-1β. *Veterinary Immunology and Immunopathology*, *81*, 1–14. https://doi.org/10.1016/S0165-2427(01)00328-2

Hong, S., Peddie, S., Campos-Perez, J.J., Zou, J. & Secombes, C.J. (2003). The effect of intraperitoneally administered recombinant IL-1β on immune parameters and resistance to Aeromonas salmonicida in the rainbow trout (*Oncorhynchus mykiss*). *Developmental & Comparative Immunology*, *27*, 801-812.

Hong, S., Li, R., Xu, Q., Secombes, C. J., & Wang, T. (2013). Two types of TNF-α exist in teleost fish: Phylogeny, expression, and bioactivity analysis of type-II TNF-α3 in rainbow trout *Oncorhynchus mykiss*. *The Journal of Immunology*, *191*(12), 5959–5972. https://doi.org/10.4049/jimmunol.1301584

Husain, M., Bird, S., Zwieten, R. van, Secombes, C. J., & Wang, T. (2012). Cloning of the IL-1β3 gene and IL-1β4 pseudogene in salmonids uncovers a second type of IL-1β gene in teleost fish. *Developmental & Comparative Immunology*, *38*(3), 431–446. https://doi.org/10.1016/j.dci.2012.07.010

Husain, M., Martin, S. A. M., & Wang, T. (2014). Identification and characterisation of the IL-27 p28 subunits in fish: Cloning and comparative expression analysis of two p28 paralogues in Atlantic salmon *Salmo salar*. *Fish & Shellfish Immunology*, *41*(1), 102–112. https://doi.org/10.1016/j.fsi.2014.06.024

Hynes, N. A., Furnes, C., Fredriksen, B. N., Winther, T., Bøgwald, J., Larsen, A. N., & Dalmo, R. A. (2011). Immune response of Atlantic salmon to recombinant flagellin. *Vaccine*, *29*(44), 7678–7687. https://doi.org/10.1016/j.vaccine.2011.07.138

Iliev, D. B., Castellana, B., MacKenzie, S., Planas, J. V., & Goetz, F. W. (2007). Cloning and expression analysis of an IL-6 homolog in rainbow trout (*Oncorhynchus mykiss*). *Molecular Immunology*, *44*(7), 1803–1807. https://doi.org/10.1016/j.molimm.2006.07.297

Jiang, Y., Husain, M., Qi, Z., Bird, S., & Wang, T. (2015). Identification and expression analysis of two interleukin-23α (p19) isoforms, in rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar*. *Molecular Immunology*, *66*(2), 216–228. https://doi.org/10.1016/j.molimm.2015.03.014

Kause, A., Ritola, O., Paananen, T., Mäntysaari, E., & Eskelinen, U. (2002). Coupling body weight and its composition: A qualitative genetic analysis in rainbow trout. Aquaculture, 211(1-4), 65-79.

Korytář, T., Jaros, J., Verleih, M., Rebl, A., Kotterba, G., Kühn, C., … Köllner, B. (2013). Novel insights into the peritoneal inflammation of rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology*, *35*(4), 1192–1199. https://doi.org/10.1016/j.fsi.2013.07.032

Laing, K. J., Zou, J. J., Wang, T., Bols, N., Hirono, I., Aoki, T., & Secombes, C. J. (2002). Identification and analysis of an interleukin 8-like molecule in rainbow trout *Oncorhynchus mykiss*. *Developmental & Comparative Immunology*, *26*(5), 433–444. https://doi.org/10.1016/S0145-305X(01)00092-1

Lumeng, C. M., & Saltiel, A. R. (2011). Inflammatory links between obesity and metabolic disease. *J Clin InvestJournal of Clinical Investigation*, *121*(6), 2111–2117. doi: 10.1172/JCI57132

Maehr, T., Costa, M. M., González Vecino, J. L., Wadsworth, S., Martin, S. A. M., Wang, T., & Secombes, C. J. (2013). Transforming growth factor-β1b: A second TGF-β1 paralogue in the rainbow trout (*Oncorhynchus mykiss*) that has a lower constitutive expression but is more responsive to immune stimulation. *Fish & Shellfish Immunology*, *34*(2), 420–432. https://doi.org/10.1016/j.fsi.2012.11.011

Matarese, G., & La Cava, A. (2004). The intricate interface between immune system and metabolism. *Trends in Immunology, 25*(4), 193-200. DOI: 10.1016/j.it.2004.02.009

Miner, J. L. (2004). The adipocyte as an endocrine cell. *Journal of Animal Science*, *82*(3), 935–941.

Milne, D.J., Campoverde, C., Andree, K., Zou, J. & Secombes C.J. (2017). Two types of TNFα in Meagre *(Argyrosomus regius*): Discovery, distribution and expression modulation. Molecular Immunology, 92, 136-145.

Monte, M. M., Zou, J., Wang, T., Carrington, A., & Secombes, C. J. (2011). Cloning, expression analysis and bioactivity studies of rainbow trout (*Oncorhynchus mykiss*) interleukin-22. *Cytokine*, *55*(1), 62–73. https://doi.org/10.1016/j.cyto.2011.03.015

Monte, M.M., Wang, T., Costa, M.M., Harun, N.O., & Secombes, C.J. (2012). Cloning and expression analysis of two ROR-γ homologues (ROR-γa1 and ROR-γa2) in rainbow trout *Oncorhynchus mykiss*. *Fish & Shellfish Immunology*, *33*, 365-374.

Monte, M. M., Wang, T., Holland, J. W., Zou, J., & Secombes, C. J. (2013). Cloning and characterization of rainbow trout interleukin-17A/F2 (IL-17A/F2) and IL-17 receptor A: expression during infection and bioactivity of recombinant IL-17A/F2. *Infection and Immunity*, *81*(1), 340–353. https://doi.org/10.1128/IAI.00599-12

Mutoloki, S., Alexandersen, S., & Evensen, Ø. (2004). Sequential study of antigen persistence and concomitant inflammatory reactions relative to side-effects and growth of Atlantic salmon (*Salmo salar* L.) following intraperitoneal injection with oil-adjuvanted vaccines. *Fish & Shellfish Immunology*, *16*(5), 633–644. https://doi.org/10.1016/j.fsi.2003.10.002

Noia, M., Domínguez, B., Leiro, J., Blanco-Méndez, J., Luzardo-Álvarez, A., & Lamas, J. (2014). Inflammatory responses and side effects generated by several adjuvant-containing vaccines in turbot. *Fish & Shellfish Immunology*, *38*(1), 244–254. https://doi.org/10.1016/j.fsi.2014.03.020

Okamura, H., Nagata, K., Komatsu, T., Tanimoto, T., Nukata, Y., Tanabe, F., Akita, K., Torigoe, K., Okura, T., Fukuda, S. & Kurimoto, M. (1995). A novel costimulatory factor for gamma interferon induction found in the livers of mice causes endotoxic shock. *Infection and Immunity*, *63,* 3966–3972.

Ouchi, N., Parker, J. L., Lugus, J. J., & Walsh, K. (2011). Adipokines in inflammation and metabolic disease. *Nature Reviews Immunology*, *11*(2), 85–97. https://doi.org/10.1038/nri2921

Pignatelli, J., Castro, R., González Granja, A., Abós, B., González, L., Jensen, L. B., & Tafalla, C. (2014). Immunological characterization of the teleost adipose tissue and its modulation in response to viral infection and fat-content in the diet. *PLoS ONE*, *9*(10), e110920. https://doi.org/10.1371/journal.pone.0110920

R Development Core Team. (2016). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.

Rangel-Moreno, J., Moyron-Quiroz, J. E., Carragher, D. M., Kusser, K., Hartson, L., Moquin, A., & Randall, T. D. (2009b). Omental milky spots develop in the absence of lymphoid tissue-inducer cells and support B and T cell responses to peritoneal antigens. *Immunity*, *30*(5), 731–743. https://doi.org/10.1016/j.immuni.2009.03.014

Robertsen, B. (2006). The interferon system of teleost fish. *Fish & Shellfish Immunology*, *20*(2), 172–191. https://doi.org/10.1016/j.fsi.2005.01.010

Rocha, V. Z., Folco, E. J., Sukhova, G., Shimizu, K., Gotsman, I., Vernon, A. H., & Libby, P. (2008). Interferon-γ, a Th1 cytokine, regulates fat inflammation. *Circulation Research*, *103*(5), 467–476. https://doi.org/10.1161/CIRCRESAHA.108.177105

Schäffler, A., & Schölmerich, J. (2010). Innate immunity and adipose tissue biology. *Trends in Immunology*, *31*(6), 228–235. https://doi.org/10.1016/j.it.2010.03.001

Schijns, V. E. J. C. (2003). Immunopotentiators in modern vaccines. *Vaccine*, *21*(9–10), 827. https://doi.org/10.1016/S0264-410X(02)00526-1

Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004). Interferon-γ: an overview of signals, mechanisms and functions. *Journal of Leukocyte Biology*, *75*(2), 163–189. https://doi.org/10.1189/jlb.0603252

Scott, C. J. W., Austin, B., Austin, D. A., & Morris, P. C. (2013). Non-adjuvanted flagellin elicits a non-specific protective immune response in rainbow trout (*Oncorhynchus mykiss*, Walbaum) towards bacterial infections. *Vaccine*, *31*(32), 3262–3267. https://doi.org/10.1016/j.vaccine.2013.05.025

Sheridan, M. A. (1988). Lipid dynamics in fish: aspects of absorption, transportation, deposition and mobilization. *Comparative Biochemistry & Physiology B., 90*(4), 679-90.

Sheridan, M. A. (1989). Alterations in lipid metabolism accompanying smoltification and seawater adaptation of salmonid fish. *Aquaculture, 82*(1–4), 191-203.

Sheridan, M. A. (1994). Regulation of lipid metabolism in poikilothermic vertebrates. *Comparative Biochemistry & Physiology B Comp. Biochem., 107*(4), 495-508. https://doi.org/10.1016/0305-0491(94)90176-7

Starr, M. E., Evers, B. M., & Saito, H. (2009). Age-associated increase in cytokine production during systemic inflammation: Adipose tissue as a major source of IL-6. *The Journals of Gerontology: Series A*, *64A*(7), 723–730. https://doi.org/10.1093/gerona/glp046

Sun, B., Skjæveland, I., Svingerud, T., Zou, J., Jørgensen, J., & Robertsen, B. (2011). Antiviral activity of salmonid gamma interferon against infectious pancreatic necrosis virus and salmonid alphavirus and its dependency on type I interferon. *Journal of Virology*, *85*(17), 9188–9198. https://doi.org/10.1128/JVI.00319-11

Tilg, H., & Moschen, A. R. (2006). Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nature Reviews Immunology*, *6*(10), 772–783. https://doi.org/10.1038/nri1937

Todorčević, M., Škugor, S., Krasnov, A., & Ruyter, B. (2010). Gene expression profiles in Atlantic salmon adipose-derived stromo-vascular fraction during differentiation into adipocytes. BMC Genomics, 11(1), 39.

Todorčević, M., & Hodson, L. (2016). The effect of marine derived *n*-3 fatty acids on adipose tissue metabolism and function. *Journal of Clinical Medicine*, *5*(1), 3.

Veenstra, K. A., Wang, T., Alnabulsi, A., Douglas, A., Russell, K. S., Tubbs, L., Ben Arous, J., & Secombes, C. J. (2017). Analysis of adipose tissue immune gene expression after vaccination of rainbow trout with adjuvanted bacterins reveals an association with side effects. Molecular Immunology, 88, 89-98.

Venables, W. N., & Ripley, B. D. (2002). *Modern Applied Statistics with S* (Fourth). New York: Springer. Retrieved from http://www.stats.ox.ac.uk/pub/MASS4

Vignali, D. A., & Kuchroo, V. K. (2012). IL-12 family cytokines: immunological playmakers. *Nature Immunology*, *13*(8), 722-8. doi: 10.1038/ni.2366.

Walker, F.C., Thompson, J.D., & Gray, J. G. (1960). Antibody formation by the greater omentum. *British Journal of Surgery*, *48*, 89-96.

Walker, F. C., & Rogers, A. W. (1961). The greater omentum as a site of antibody synthesis. *Br British Journal of Experimental Pathology*, *42*, 222-31.

Wang, T., & Husain, M. (2014). The expanding repertoire of the IL-12 cytokine family in teleost fish: Identification of three paralogues each of the p35 and p40 genes in salmonids, and comparative analysis of their expression and modulation in Atlantic salmon *Salmo salar*. *Developmental & Comparative Immunology*, *46*(2), 194–207.

Wang, T., & Secombes, C. J. (2008). Rainbow trout suppressor of cytokine signalling (SOCS)-1, 2 and 3: Molecular identification, expression and modulation. *Molecular Immunology*, *45*(5), 1449–1457. https://doi.org/10.1016/j.molimm.2007.08.016

Wang, T., & Secombes, C. J. (2013). The cytokine networks of adaptive immunity in fish. *Fish & Shellfish Immunology*, *35*(6), 1703–1718. https://doi.org/10.1016/j.fsi.2013.08.030.

Wang, T., Holland, J. W., Bols, N., & Secombes, C. J. (2005). Cloning and expression of the first nonmammalian interleukin-11 gene in rainbow trout *Oncorhynchus mykiss*. *FEBS Journal*, *272*(5), 1136–1147. https://doi.org/10.1111/j.1742-4658.2005.04544.x

Wang, T., Holland, J. W., Carrington, A., Zou, J., & Secombes, C. J. (2007). Molecular and functional characterization of IL-15 in rainbow trout *Oncorhynchus mykiss*: A potent inducer of IFN-γ expression in spleen leukocytes. *The Journal of Immunology*, *179*(3), 1475–1488.

Wang, T., Martin, S. A. M., & Secombes, C. J. (2010a). Two interleukin-17C-like genes exist in rainbow trout *Oncorhynchus mykiss* that are differentially expressed and modulated. *Developmental & Comparative Immunology*, *34*(5), 491–500. https://doi.org/10.1016/j.dci.2009.11.011

Wang, T., Díaz-Rosales, P., Martin, S. A. M., & Secombes, C. J. (2010b). Cloning of a novel interleukin (IL)-20-like gene in rainbow trout *Oncorhynchus mykiss* gives an insight into the evolution of the IL-10 family. *Developmental & Comparative Immunology*, *34*(2), 158–167. https://doi.org/10.1016/j.dci.2009.09.003

Wang, T., Holland, J. W., Martin, S. A. M., & Secombes, C. J. (2010c). Sequence and expression analysis of two T helper master transcription factors, T-bet and GATA3, in rainbow trout *Oncorhynchus mykiss* and analysis of their expression during bacterial and parasitic infection. *Fish & Shellfish Immunology*, *29*(5), 705–715. https://doi.org/10.1016/j.fsi.2010.06.016

Wang, T., Monte, M.M., Huang, W, Boudinot, P., Martin, S.A.M., & Secombes, C.J. (2010d). Identification of two FoxP3 genes in rainbow trout (*Oncorhynchus mykiss*) with differential induction patterns. Molecular Immunology, 47, 2563-2574.

Wang T, Huang W, Costa MM, Martin SA, Secombes CJ. (2011a). Two copies of the genes encoding the subunits of putative interleukin (IL)-4/IL-13 receptors, IL-4Rα, IL-13Rα1 and IL-13Rα2,

have been identified in rainbow trout (*Oncorhynchus mykiss*) and have complex patterns of expression and modulation. *Immunogenetics, 63*(4)~~.~~: 235-53.

Wang, T., Diaz-Rosales, P., Costa, M. M., Campbell, S., Snow, M., Collet, B., … Secombes, C. J. (2011b). Functional characterization of a non-mammalian IL-21: R~~r~~ainbow trout *Oncorhynchus mykiss* IL-21 upregulates the expression of the Th cell signature cytokines IFN-gamma, IL-10, and IL-22. *Journal of Immunology (Baltimore, Md.: 1950)*, *186*(2), 708–721. https://doi.org/10.4049/jimmunol.1001203

Wang, T., Kono, T., Monte, M. M., Kuse, H., Costa, M. M., Korenaga, H., … Secombes, C. J. (2013). Identification of IL-34 in teleost fish: Differential expression of rainbow trout IL-34, MCSF1 and MCSF2, ligands of the MCSF receptor. *Molecular Immunology*, *53*(4), 398–409. https://doi.org/10.1016/j.molimm.2012.09.008

Wang, T., Husain, M., Hong, S., & Holland, J. W. (2014). Differential expression, modulation and bioactivity of distinct fish IL-12 isoforms: Implication towards the evolution of Th1-like immune responses. *European Journal of Immunology*, *44*(5), 1541–1551. https://doi.org/10.1002/eji.201344273

Wang, T., Jiang, Y., Wang, A., Husain, M., Xu, Q., & Secombes, C. J. (2015). Identification of the salmonid IL-17A/F1a/b, IL-17A/F2b, IL-17A/F3 and IL-17N genes and analysis of their expression following in vitro stimulation and infection. *Immunogenetics*, *67*(7), 395–412. https://doi.org/10.1007/s00251-015-0838-1

Wang, T., Johansson, P., Abós, B., Holt, A., Tafalla, C., Jiang, Y., … Secombes, C. J. (2016). First in-depth analysis of the novel Th2-type cytokines in salmonid fish reveals distinct patterns of expression and modulation but overlapping bioactivities. *Oncotarget*, *7*(10), 10917–10946. https://doi.org/10.18632/oncotarget.7295

Wangkahart, E., Scott, C., Secombes, C. J., & Wang, T. (2016). Re-examination of the rainbow trout (*Oncorhynchus mykiss*) immune response to flagellin: *Yersinia ruckeri* flagellin is a potent activator of acute phase proteins, anti-microbial peptides and pro-inflammatory cytokines in vitro. *Developmental & Comparative Immunology*, *57*, 75–87. https://doi.org/10.1016/j.dci.2015.12.017

Warnes, G. R., Bolker, B., Bonebh~~h~~akker, L., Gentleman, R., Huber, W., Liaw, A., Lumley, T., Maechler, M., Magnusson, A., Moeller, S., Schwartz, M., & Venables, W. (2016). *Gplots: various R Programming Tools for Plotting Data*. R package version 3.0.1. https://CRAN.R-project.org/package=gplots

Weil, C., Lefèvre, F., & Bugeon, J. (2013). Characteristics and metabolism of different adipose tissues in fish. *Reviews in Fish Biology and Fisheries*, *23*(2), 157–173. https://doi.org/10.1007/s11160-012-9288-0

Yang, R.-Z., Lee, M.-J., Hu, H., Pollin, T. I., Ryan, A. S., Nicklas, B. J., … Gong, D.-W. (2006). Acute-p~~P~~hase s~~S~~erum a~~A~~myloid A: An i~~I~~nflammatory a~~A~~dipokine and p~~P~~otential l~~L~~ink between o~~O~~besity and i~~I~~ts m~~M~~etabolic c~~C~~omplications. *PLOS Medicine*, *3*(6), e287. https://doi.org/10.1371/journal.pmed.0030287

Zhou, S., Ackman, R. G., & Morrison, C. M. (1996). Adipocytes and lipid distribution in the muscle tissue of Atlantic salmon (*Salmo salar*). *Can. J. Fish. Aquat. Sci.*, *53*, 326–332.

Zhou, J., Ackman, R.G., Morison, C. (2011). Adipocytes and lipid distribution in tissue of Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries & Aquatic Sciences~~.~~, 53*(2), 326-332. doi:10.1139/cjfas-53-2-326

Zou, J, Neumann, N. F., Holland, J. W., Belosevic, M., Cunningham, C., Secombes, C. J., & Rowley, A. F. (1999). Fish macrophages express a cyclo-oxygenase-2 homologue after activation. *Biochemical Journal*, *340*(Pt 1), 153–159.

Zou, J., Peddie, S., Scapigliati, G., Zhang, Y., Bols, N. C., Ellis, A. E., & Secombes, C. J. (2003). Functional characterisation of the recombinant tumor necrosis factors in rainbow trout, *Oncorhynchus mykiss*. *Developmental & Comparative Immunology*, *27*(9), 813–822. https://doi.org/10.1016/S0145-305X(03)00077-6

Zou, J., Bird, S., Truckle, J., Bols, N., Horne, M., & Secombes, C. (2004). Identification and expression analysis of an IL-18 homologue and its alternatively spliced form in rainbow trout (*Oncorhynchus mykiss*). *European Journal of Biochemistry*, *271*(10), 1913–1923. https://doi.org/10.1111/j.1432-1033.2004.04101.x

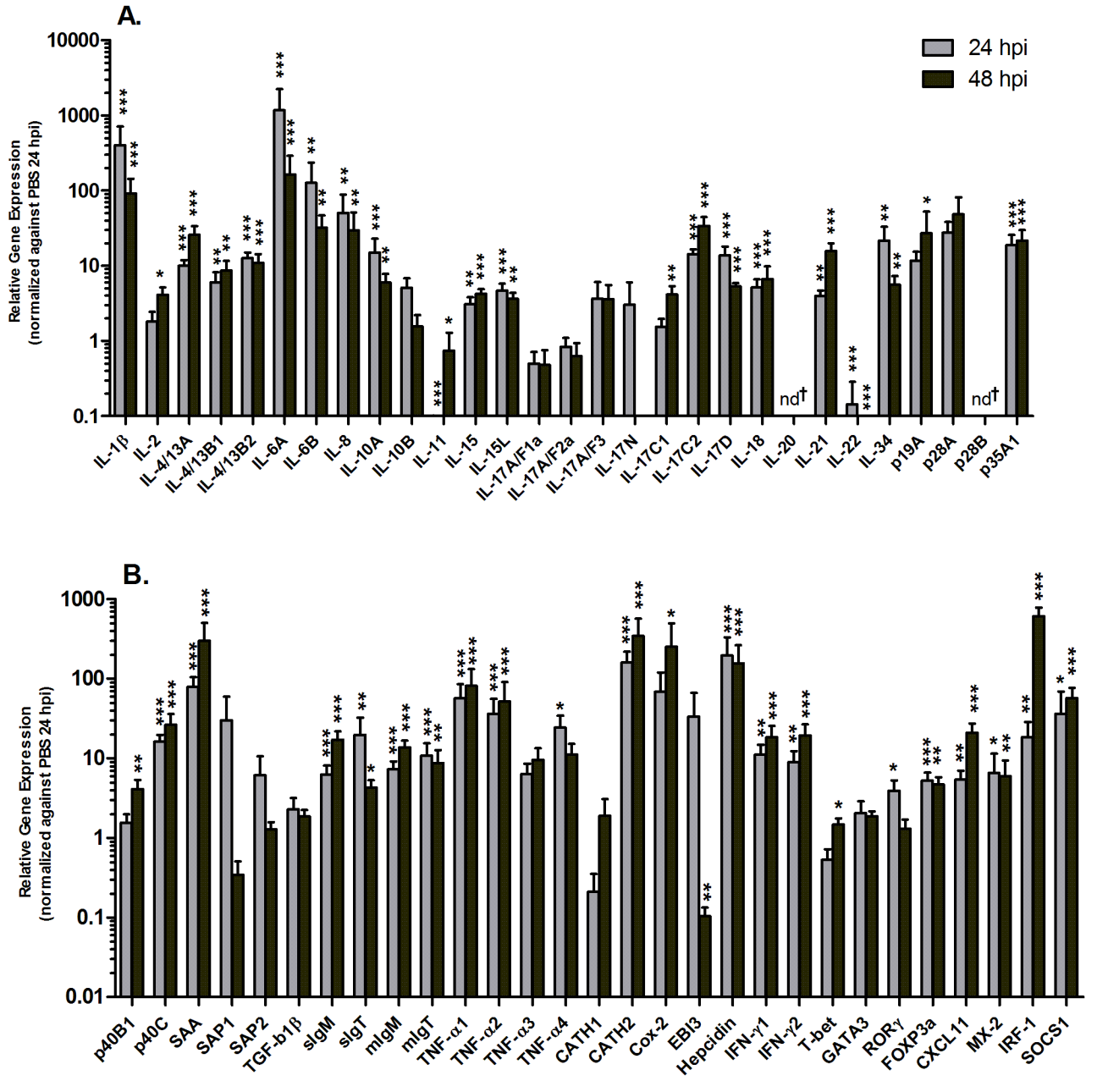
Zou, J., Carrington, A., Collet, B., Dijkstra, J. M., Yoshiura, Y., Bols, N., & Secombes, C. (2005). Identification and b~~B~~ioactivities of IFN-γ in r~~R~~ainbow t~~T~~rout *Oncorhynchus mykiss*: The f~~F~~irst Th1-t~~T~~ype c~~C~~ytokine c~~C~~haracterized f~~F~~unctionally in f~~F~~ish. ~~*The*~~ *Journal of Immunology*, *175*(4), 2484–2494. https://doi.org/10.4049/jimmunol.175.4.2484

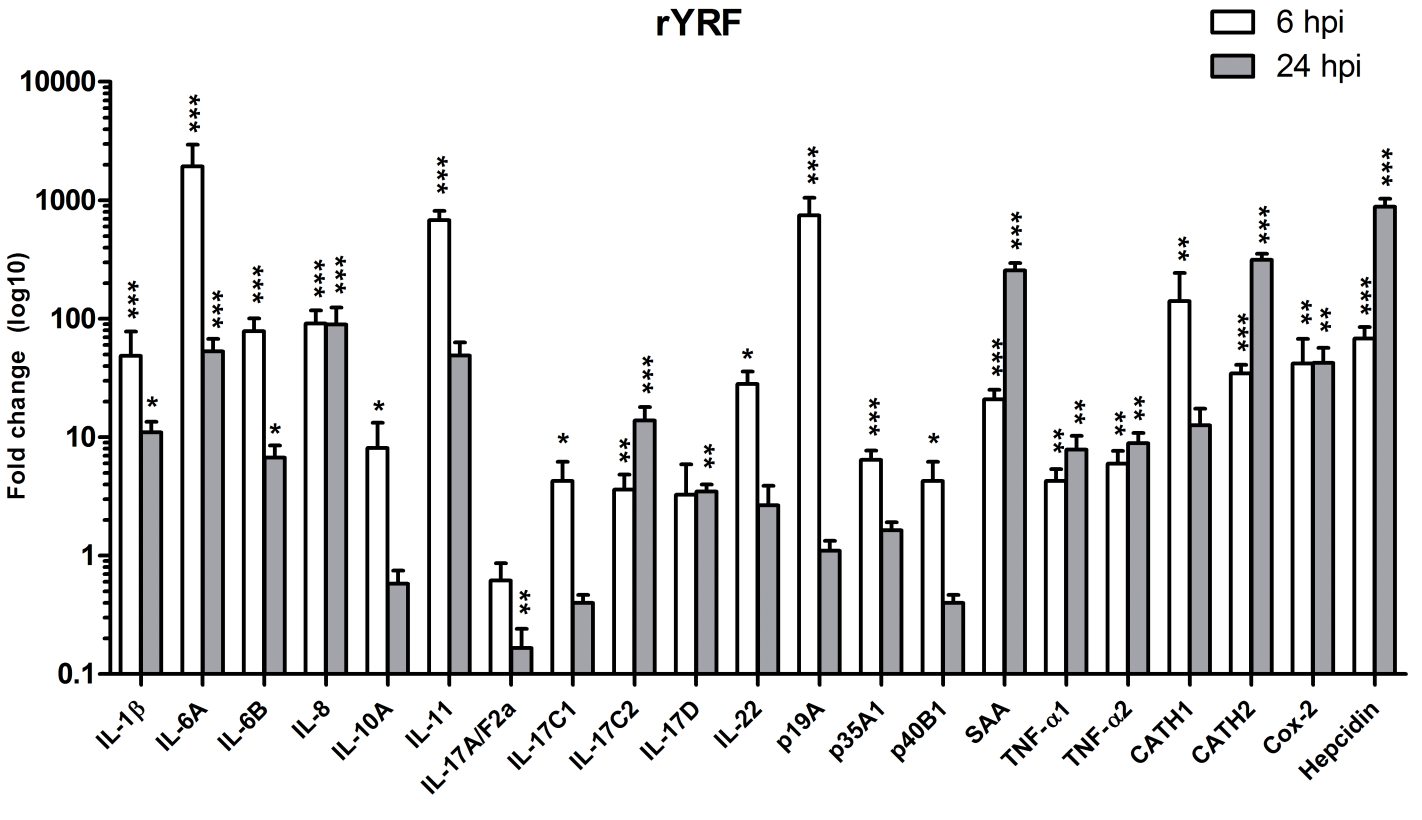
Appendix A. Supplementary Data

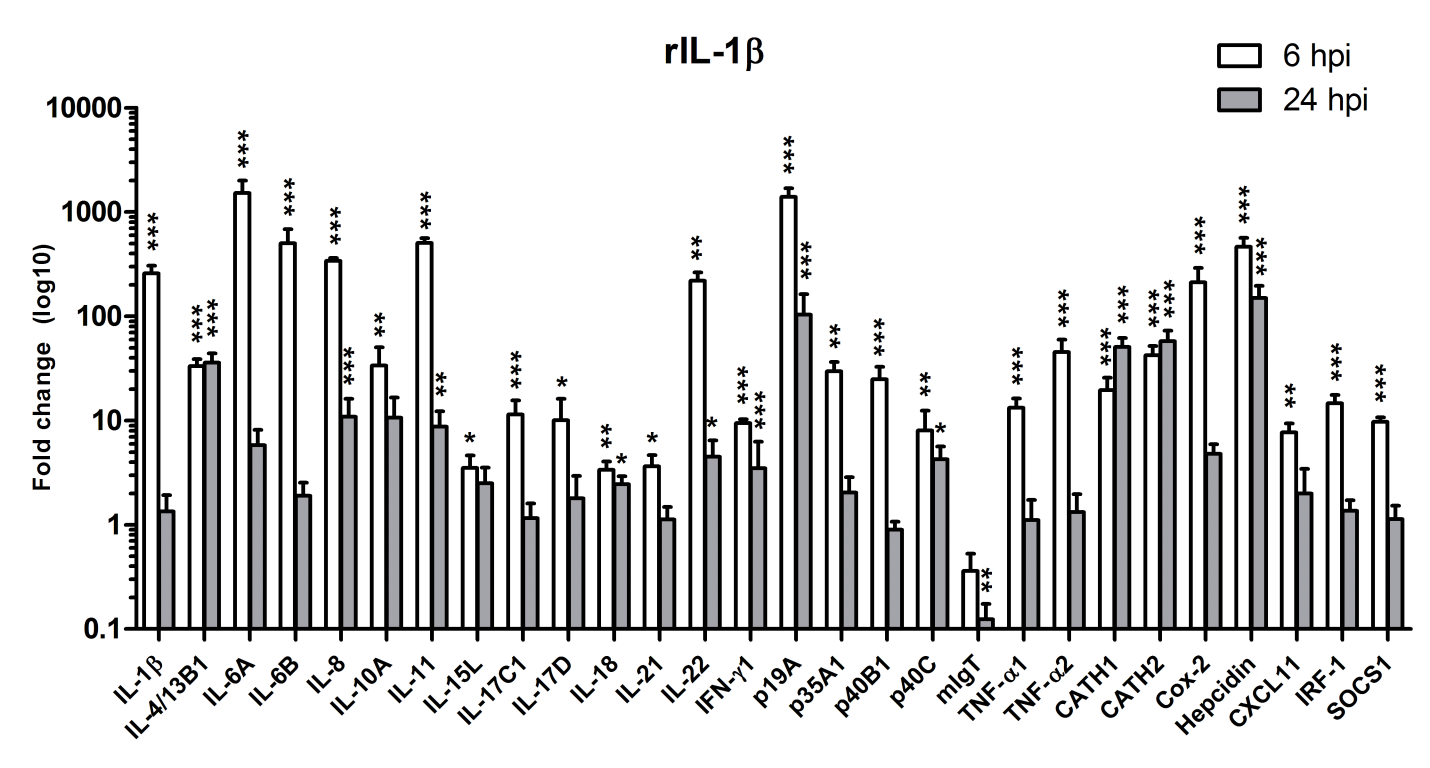
Supplementary Table 1: Gene primer sequences used for RTqPCR expression analysis.

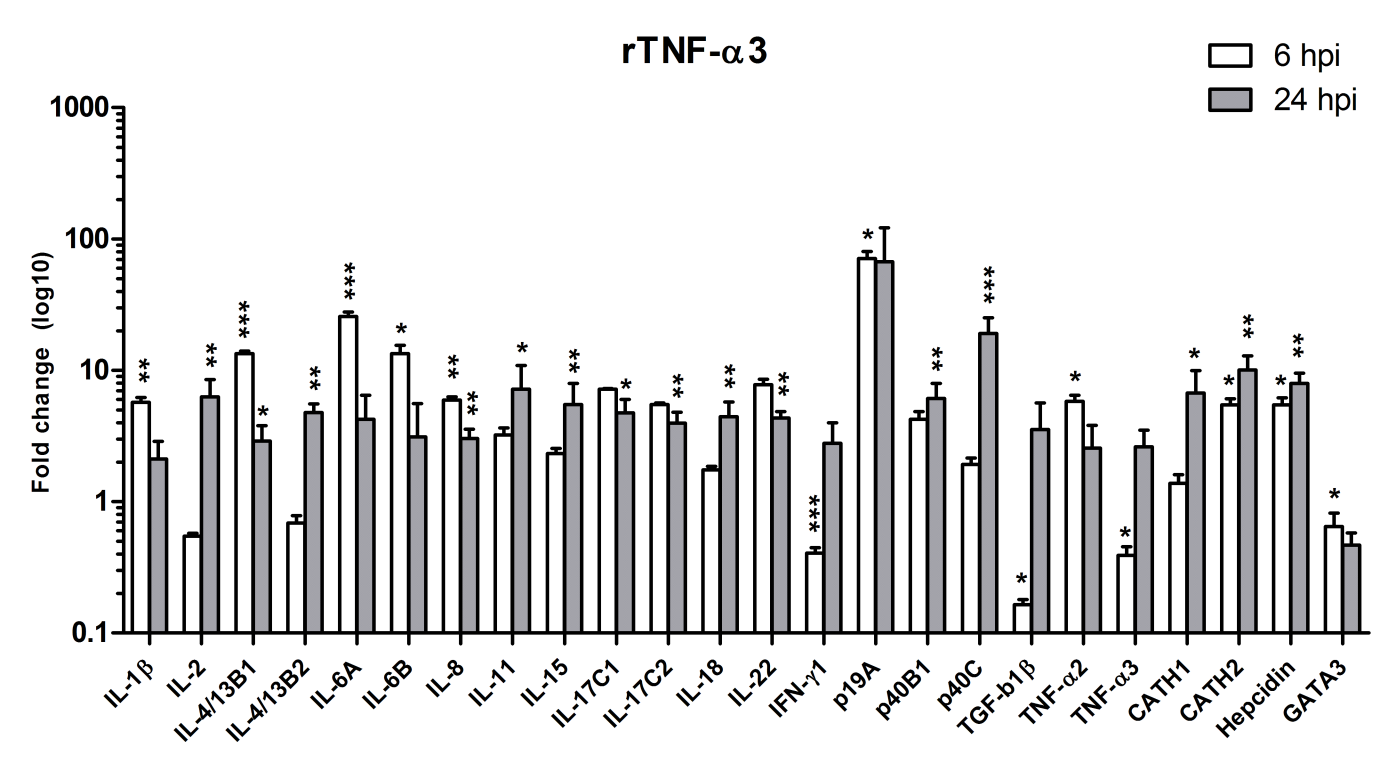
Supplementary Table 2: Summary of fold change and statistical outputs for IFNγ,~~all~~ IL-1β, TNFα3 and YRF treatment groups at 6 hpi.

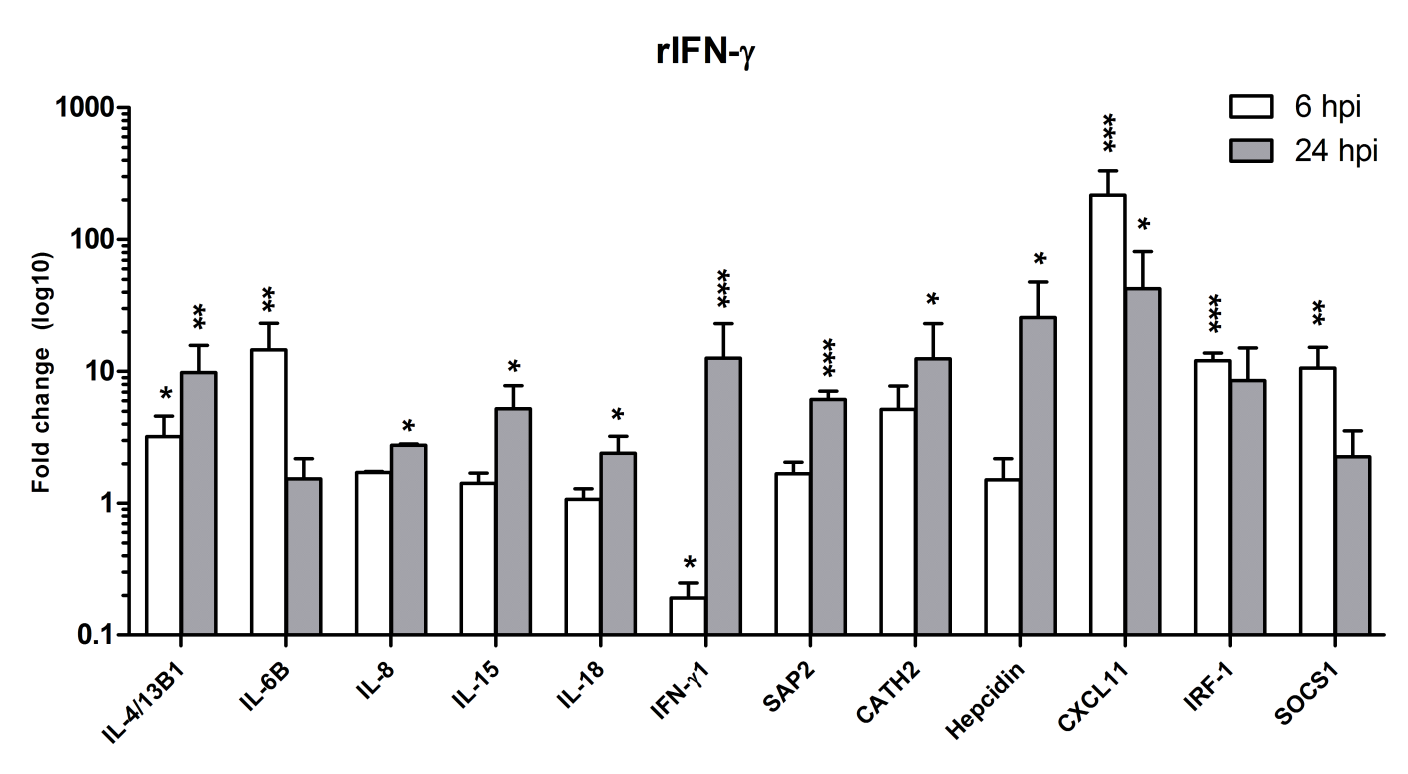
Supplementary Table 3: Summary of fold change and statistical outputs for IFNγ, IL-1β, TNFα3, YRF and *A. salmonicida*~~*all*~~ treatment groups at 24 hpi and *A. salmonicida* treatment group at 48 hpi.~~.~~

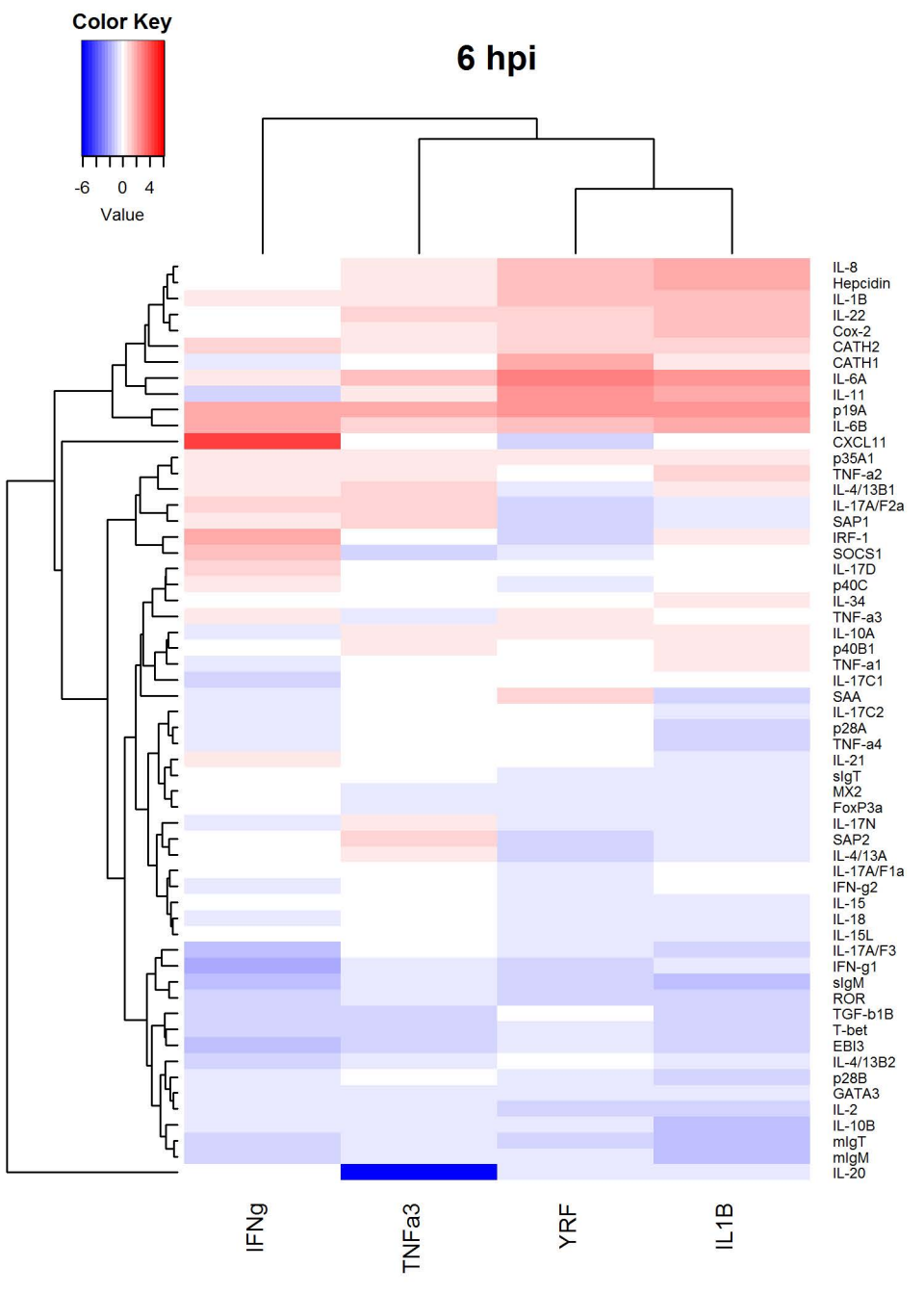

A.
24 hpi
48 hpi
Relative Gene Expression
(normalized against PBS 24 hpi)
10000
1000
100
10
1
0.1
IL-1β
IL-2
IL-4/13A
IL-4/13B1
IL-4/13B2
IL-6A
IL-6B
IL-8
IL-10A
IL-10B
IL-11
IL-15
IL-15L
IL-17A/F1a
IL-17A/F2a
IL-17A/F3
IL-17N
IL-17C1
IL-17C2
IL-17D
IL-18
IL-20
IL-21
IL-22
IL-34
p19A
p28A
p28B
p35A1
ndǂ
ndǂ
B.
Relative Gene Expression
(normalized against PBS 24 hpi)
1000
100
10
1
0.1
0.01
p40B1
p40C
SAA
SAP1
SAP2
TGF-b1β
sIgM
sIgT
mIgM
mIgT
TNF-α1
TNF-α2
TNF-α3
TNF-α4
CATH1
CATH2
Cox-2
EBI3
Hepcidin
IFN-γ1
IFN-γ2
T-bet
GATA3
RORγ
FOXP3a
CXCL11
MX-2
IRF-1
SOCS1

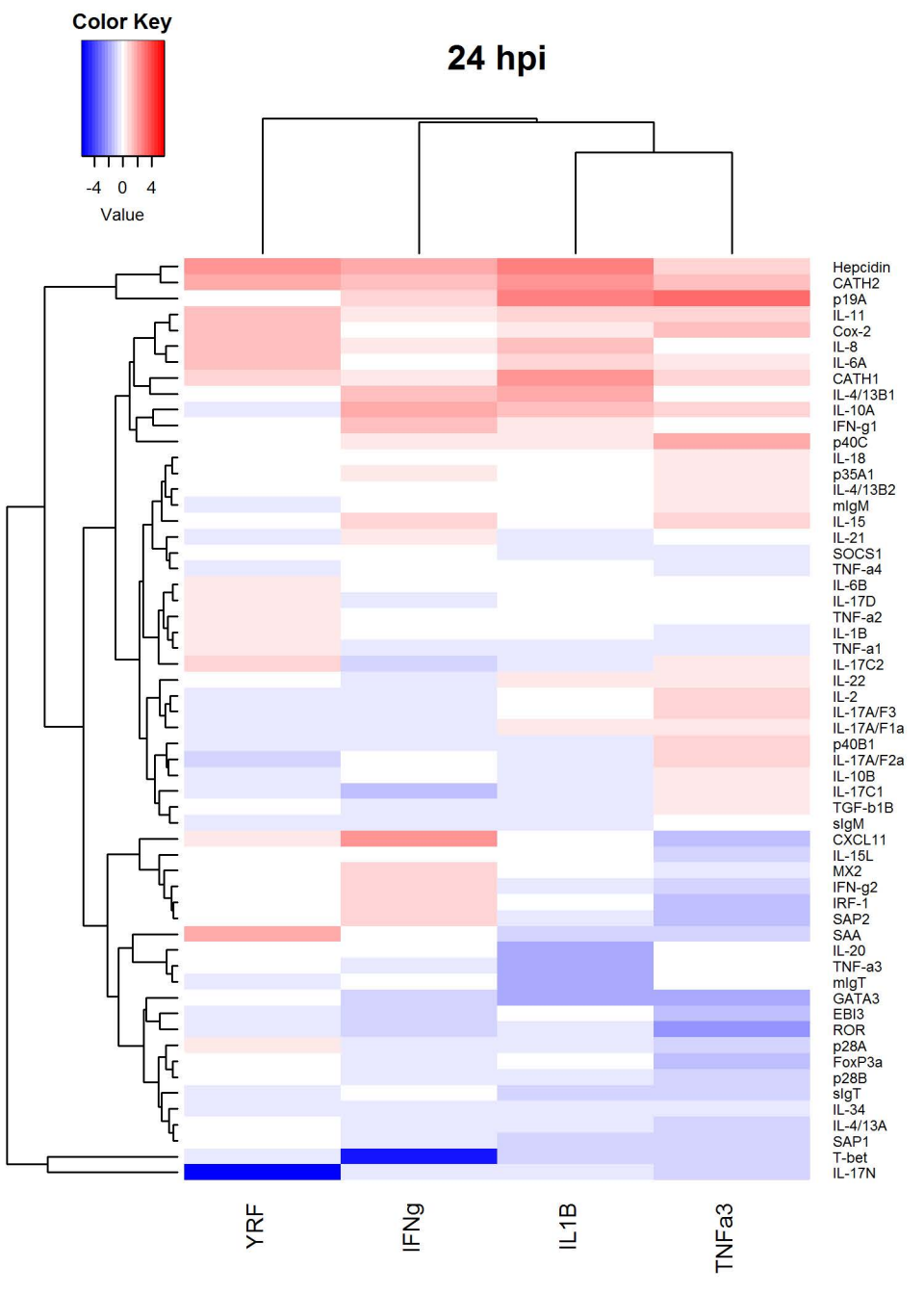

rYRF
6 hpi
24 hpi
Fold change (log10)
10000
1000
100
10
1
0.1
IL-1β
IL-6A
IL-6B
IL-8
IL-10A
IL-11
IL-17A/F2a
IL-17C1
IL-17C2
IL-17D
IL-22
p19A
p35A1
p40B1
SAA
TNF-α1
TNF-α2
CATH1
CATH2
Cox-2
Hepcidin


rIL-1β
6 hpi
24 hpi
Fold change (log10)
10000
1000
100
10
1
0.1
IL-1β
IL-4/13B1
IL-6A
IL-6B
IL-8
IL-10A
IL-11
IL-15L
IL-17C1
IL-17D
IL-18
IL-21
IL-22
IFN-γ1
p19A
p35A1
p40B1
p40C
mIgT
TNF-α1
TNF-α2
CATH1
CATH2
Cox-2
Hepcidin
CXCL11
IRF-1
SOCS1


rTNF-α3
6 hpi
24 hpi
Fold change (log10)
1000
100
10
1
0.1
IL-1β
IL-2
IL-4/13B1
IL-4/13B2
IL-6A
IL-6B
IL-8
IL-11
IL-15
IL-17C1
IL-17C2
IL-18
IL-22
IFN-γ1
p19A
p40B1
p40C
TGF-b1β
TNF-α2
TNF-α3
CATH1
CATH2
Hepcidin
GATA3


rIFN-γ
6 hpi
24 hpi
Fold change (log10)
1000
100
10
1
0.1
IL-4/13B1
IL-6B
IL-8
IL-15
IL-18
IFN-γ1
SAP2
CATH2
Hepcidin
CXCL11
IRF-1
SOCS1


Color Key
-6
0
4
Value
6 hpi
IL-8
Hepcidin
IL-1B
IL-22
Cox-2
CATH2
CATH1
IL-6A
IL-11
p19A
IL-6B
CXCL11
p35A1
TNF-a2
IL-4/13B1
IL-17A/F2a
SAP1
IRF-1
SOCS1
IL-17D
p40C
IL-34
TNF-a3
IL-10A
p40B1
TNF-a1
IL-17C1
SAA
IL-17C2
p28A
TNF-a4
IL-21
sIgT
MX2
FoxP3a
IL-17N
SAP2
IL-4/13A
IL-17A/F1a
IFN-g2
IL-15
IL-18
IL-15L
IL-17A/F3
IFN-g1
sIgM
ROR
TGF-b1B
T-bet
EBI3
IL-4/13B2
p28B
GATA3
IL-2
IL-10B
mIgT
mIgM
IL-20
IFNg
TNFa3
YRF
IL1B


Color Key
-4 0 4
Value
24 hpi
Hepcidin
CATH2
p19A
IL-11
Cox-2
IL-8
IL-6A
CATH1
IL-4/13B1
IL-10A
IFN-g1
p40C
IL-18
p35A1
IL-4/13B2
mIgM
IL-15
IL-21
SOCS1
TNF-a4
IL-6B
IL-17D
TNF-a2
IL-1B
TNF-a1
IL-17C2
IL-22
IL-2
IL-17A/F3
IL-17A/F1a
p40B1
IL-17A/F2a
IL-10B
IL-17C1
TGF-b1B
sIgM
CXCL11
IL-15L
MX2
IFN-g2
IRF-1
SAP2
SAA
IL-20
TNF-a3
mIgT
GATA3
EBI3
ROR
p28A
FoxP3a
p28B
sIgT
IL-34
IL-4/13A
SAP1
T-bet
IL-17N
YRF
IFNg
IL1B
TNFa3