The importance of the Atlantic salmon peritoneal cavity B cell response: local IgM secreting cells are predominant upon *Piscirickettsia salmonis* infection.

Authors

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YvW and JJ conceived and designed research. YvW, SJ, HN, and LGT performed research and analyzed data. SJ, IJ and JK helped design experiments. YvW and JJ wrote the paper. All authors reviewed and approved the manuscript.

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YvW and JK are employees of the company Vaxxinova Research & Development GmbH which amongst others develops and commercializes vaccines for aquaculture. Most of the research was funded by Vaxxinova Research & Development GmbH. Vaxxinova had an advisory role in the study design and final report.

Abstract

The intraperitoneal route is favored for administration of inactivated and attenuated vaccines in Atlantic salmon. Nevertheless, the immune responses in the teleost peritoneal cavity (PerC) are still incompletely defined. In this study, we investigated the B cell responses after intraperitoneal *Piscirickettsia salmonis* (*P. salmonis*) challenge of Atlantic salmon, focusing on the local PerC response versus responses in the lymphatic organs: spleen and head kidney. We observed a major increase of leukocytes, total IgM antibody secreting cells (ASC), and *P. salmonis*-specific ASC in the PerC at 3- and 6-weeks post infection (wpi). The increase in ASC frequency was more prominent in the spleen and PerC compared to the head kidney during the observed 6 wpi. The serum antibody response included *P. salmonis*-specific antibodies and non-specific antibodies against the non-related bacterial

pathogen *Yersinia ruckeri* and the model antigen TNP-KLH. Finally, we present evidence that supports a putative role for the adipose tissue in the PerC immune response.

Key words

Piscirickettia salmonis, Atlantic salmon, peritoneal cavity, ELISpot, B cell, adipose tissue

Abbreviations

Ab (Abs) – antibody (antibodies)
ASC – antibody-secreting cell(s)
AT – adipose tissue
DC – dendritic cells
Dpi – days post infection
HK – head kidney
IP - intraperitoneal
NC – negative control
P. salmonis – *Piscirickettsia salmonis*
PerC – peritoneal cavity
SRS – salmonid rickettsial septicemia
TLRs – Toll-like receptors
Wpi – weeks post infection
Y. ruckeri – *Yersinia ruckeri*

1 Introduction

Although humoral responses are crucial to the protection induced by vaccines, in depth studies on B cell biology, including how the exposure to pathogens initiates B cell responses and leads to subsequent antibody (Ab) production in Atlantic salmon (*Salmo salar L*) are elusive. Teleosts lack germinal centers and follicular structures, which in mammals have roles in the proliferation and differentiation of B cells to plasma cells, and there is no class switching. Furthermore, the mechanisms and limitations of immunological memory in teleosts are still being questioned (Yamaguchi et al. 2019). One reason for this is that the teleost secondary immune response results in only slightly higher Ab responses than the primary response (Cossarini-Dunier 1986). By contrast, 100-1000 times more antibodies are produced in the mammalian secondary response. Like mammals, teleosts have non-specific or natural Abs that are present without prior specific antigen encounters (Magnadóttir 2006), but their role in immune responses after pathogen encounters remains to be elucidated. Finally, the existence of affinity maturation of Abs in teleosts has been questioned, but several recent studies have identified the occurrence of affinity maturation in bony fish (Wu et al. 2019a; Wu et al. 2019b; Ye et al. 2013). Nevertheless, the location and mechanisms of the teleost affinity maturation are still largely unknown (Magor 2015), and the affinity maturation in fishes is generally considered as much less efficient than in mammals (Kaattari 2002). The absence of true germinal centers is often provided as an explanation, resulting in an inefficient selection of high-affinity clones (Magor 2015; Muthupandian et al. 2021).

Only recently, the role of local B cell responses in the peritoneal cavity (PerC) of teleosts has gained more attention. This is remarkable because intraperitoneal injection (IP) is the most commonly used route of vaccine administration in Atlantic salmon (Plant and Lapatra 2011). Castro et al. (Castro et al. 2017) found an increase of B cell numbers in the PerC within 2-3 days after IP stimulation of rainbow trout with *Escherichia coli* or viral haemorrhagic septicaemia virus. After 6 days, they also observed a significant increase of antibody secreting cells (ASC) in the PerC. In concordance with these results, Jenberie et al. (Jenberie et al. 2020) showed a significant increase in ASC in the PerC after IP challenge with salmonid alphavirus subtype 3 (SAV3) over a period of nine weeks. In this study, the increase of ASC in the PerC was higher than in the head kidney (HK) and spleen. This raises questions on the formation, location, and migration of ASC after IP stimulation with different pathogens. Additionally, Pignatelli et al. (Pignatelli et al. 2014) suggested an immunological role for the adipose tissue (AT) in the rainbow trout PerC, where it may take part in regulating PerC immune responses. Clarifying the participation and contribution of B cell responses after IP encounter with pathogens or immunogens, is crucial to perceive how the PerC and the systemic sites interplay, and how this affects the overall humoral immune response. In the future, an extended understanding of the dynamics between different immune sites in bony fish may give clues on how to improve protective responses against diseases for which effective vaccines are currently lacking.

One such a disease is salmonid rickettsial septicemia (SRS), a severe systemic disease that mainly affects salmonid species and was first described in 1989 (Rozas and Enríquez 2014; Fryer et al. 1992). SRS is a major concern for the Chilean aquaculture industry, where it causes severe mortalities and, in extension, annual economic losses estimated at USD $700 million (Maisey et al. 2017). The facultative intracellular bacterium *Piscirickettsia salmonis* (*P. salmonis*) causes SRS (Fryer et al. 1992). The natural route of infection for *P. salmonis* is through the gills and wounds in the skin, leading to a systemic infection (Smith et al. 1999). The bacteria are targeting hepatocytes and liver associated macrophages, as well as kidney, spleen, and peripheral blood macrophages (reviewed in (Almendras and Fuentealba 1997). Over thirty vaccines containing *P. salmonis* are currently available for aquaculture in Chile. Most of these vaccines are inactivated or subunit vaccines, while a recent vaccine contains a live-attenuated strain (Maisey et al. 2017; Servicio Agrícola y Ganadero 2020). Even though initial experimental tests for the vaccines show promising protection shortly after vaccination (usually around 600 degree-days), the tested vaccines failed to protect at 1500 degree-days (Intesal Febuary 2014). The knowledge concerning the host response to this pathogen is still limited and mainly based on *in vivo* transcriptomics or *in vitro* studies (Rozas and Enríquez 2014). This incomplete understanding of the Atlantic salmon immune responses probably contributed to the inability to develop efficacious vaccines against *P. salmonis* for thirty years.

To explore how B cell responses are induced upon infection with a bacterial pathogen, we here examined the characteristics of Atlantic salmon humoral responses in various sites, including the PerC, spleen, and HK after IP challenge with *P. salmonis*. We observed a major increase of leukocytes, total IgM ASC, and *P. salmonis*-specific ASC in the PerC. Additionally, we found that the early response included an induction of non-specific Abs. To our knowledge, this is the first description in a teleost species that a bacterial infection in the PerC elicits a strong local antigen specific Ab response over the course of several weeks. This raises the question where and how local and systemic responses develop after IP immunization in salmon. Correlations between serum Abs titre and frequency of ASC in the PerC and spleen suggest that this early Ab production is mainly located in these two sites. In accordance with earlier studies (Veenstra et al. 2018; Pignatelli et al. 2014), the infection induced the upregulation of different immune genes, including markers for different B and T cell populations and professional antigen presenting cells, in the PerC AT. This indicates its role as an immune site in salmonids. Finally, we discuss how the strong immune response in the PerC could influence the predicted efficacy of vaccines.

2 Materials and methods

2.1 Bacterial strains and antigen

P. salmonis strain PM15972 (EM-90-like) was obtained from Marcos Mancilla (ADL Diagnostic Chile), cultivated for two passages on PSA agar plates at 17,5°C (as described in Henriquez et al., 2015), and harvested after 5 days. The harvested bacterial suspension had an OD600 of 0,210, corresponding to around 7 x 10^7 CFU/mL, and was kept on ice until use as challenge material. *P. salmonis* antigen for ELISpot and ELISA was generated by heat-inactivation (60°C for 15 minutes) and sonication (90 cycles for 2 minutes) of the challenge material. *Yersinia ruckeri* (*Y. ruckeri*) strain CCUG 14190, A4-53 was obtained from Lill-Heidi Johansen (NOFIMA) and cultivated on LB agar and in liquid LB medium at room temperature. *Y. ruckeri* antigen was generated by heat inactivating (65°C for 30 minutes) and sonicating (90 cycles for 2 minutes) a liquid culture at 0,8 OD_{600}.

2.2 Fish and *P. salmonis* challenge

Atlantic salmon presmolts (Aquagen standard) were produced and housed at the Tromsø Aquaculture Research Station in fresh water at 10°C and acclimatized to the tank for three weeks. Fish were kept at 24 h light and were fed commercial feed (Skretting) according to appetite. At the start of the experiment, the mean weight of the fish was 56,2 g. Fish were starved one day before challenge or sampling and anesthetized (40 µg/mL) or euthanized (80 µg/mL) using benzocaine (ACD Pharmaceuticals) before handling. The Atlantic salmon were randomly allocated to two tanks: 40 fish for infection and 24 for control. Fish were IP challenged with 0,1 mL *P. salmonis* (0,21 OD_{600}, corresponding to approximately 7 x 10^6 CFU/fish) or injected IP with 0,1 mL PBS to form the negative

control (NC) group. In addition, four fish were sampled at day 0. The animal experiment was evaluated and approved by the Norwegian Food Safety Authority (ID 21507).

2.3 Sampling and Leukocyte isolation

Blood, PerC cells, AT (see figure 3e), liver, spleen, and HK were sampled from 8 NC and 8 infected fish at 3 days post infection (dpi), 3 weeks post infection (wpi), and 6 wpi, with the exception of blood samples at 6 wpi being taken from 12 individuals. Peritoneal washes with visible blood contamination were removed. At 0 dpi, the same samples were taken from 4 non-injected fish. Blood samples (0,3 to 1 mL, depending on fish size) were stored in regular Eppendorf tubes overnight at 4°C, centrifuged at 2000xg for 10 minutes, serum was harvested, diluted 1:1 in glycerol, and stored at -20°C until analysis. AT and liver samples were stored in RNAlater (Invitrogen) at -20°C after overnight incubation at 4°C. HK and spleen samples were kept in transport medium (L-15 with 2% FBS, 0,4% heparin, 10 U/mL penicillin, and 10 µg/mL streptomycin) on ice until further processing. PerC cells were harvested as described by Jenberie et al. (Jenberie et al. 2020) by washing and gently scraping the abdominal wall of the PerC three times with 1 mL wash medium (PBS with 2% FBS and 20U/mL heparin). The PerC cell suspension was collected in 2 mL transport medium on ice until further processing.

Leukocytes were isolated from PerC, spleen, and HK as described earlier (Iliev et al. 2010; Jenberie et al. 2020). Spleen and HK tissue were dissociated using a 100 µm cell strainer (Falcon). Cells were collected in transport medium, layered on 25%/54% discontinuous Percoll (GE Healthcare) gradients, and centrifuged 400xg for 40 minutes at 4°C. Leukocytes were collected from the interface, washed twice in L-15 with 10 U/mL penicillin and 10 µg/mL streptomycin, counted (Countess II FL; Invitrogen), and kept on ice. To compare the total number of leukocytes per organ, we sampled the complete spleen and weighed the sampled HK. Due to a technical issue with only part of the HK being sampled from infected fish at 3 dpi, those numbers were normalized (see suppl. table 1).

2.4 Total IgM ASC ELISpot assay

An ELISpot assay was performed to enumerate the number of IgM-producing cells as described by Jenberie et al. (Jenberie et al. 2020). In short, MSIPS4510 plates (Merck Millipore) were activated with 35% ethanol, washed with water and PBS, and coated with 1.5 µg/well capture Ab (Anti-trout IgM/F1-18, kindly provided by Dr. Karsten Skjødt) diluted in PBS (100 µL/well) at 4°C overnight. This mAb was originally produced towards purified IgM from rainbow trout plasma but was later shown to bind both subtypes of Atlantic salmon IgM (IgM A and B) (Hedfors et al. 2012). After washing four times with PBS, the membrane was blocked with 100 µL L-15 with 10 U/mL penicillin, 10 µg/mL streptomycin, and 2% BSA per well for 90 minutes. Isolated leukocytes were seeded at 12500 cells per well and incubated for 48 hours at 15°C. This was followed by incubating with 100 µL of anti-trout IgM (F1-18), biotinylated using the EZ-Link NHS-PEG solid phase biotinylation kit (Thermo Fisher Scientific), diluted in PBS with

1% BSA and 0,1% Tween 20 per well for 90 minutes. Plates were then developed using 100 µL streptavidin-HRP conjugate (Mabtech) diluted 1:500 in PBS per well for 1 hour, and 100 µL filtrated TMB ultra substrate (Mabtech) per well for 10 minutes. Washing of the plates with PBS was performed before each of these steps. Finally, the plates were washed under tap water to stop the reaction, air-dried overnight, and spots were counted using the S6 Ultra-V analyzer and ImmunoSpot software (both from ImmunoSpot, CTL). No cell- and no biotinylated Ab- controls were included.

2.5 Specific ELISpot assay for *P. salmonis* and *Y. ruckeri*

To enumerate the number of ASC producing *P. salmonis* or *Y. ruckeri*-recognizing IgM, we established two specific ELISpot assays. The ELISpot assays were performed as described above, with the following deviations: ELISpot wells were coated with 8 µg/well *P. salmonis* or *Y. ruckeri* antigen (preparation described in 2.1) in 100 µL PBS at 4°C overnight. Leukocytes were seeded at 250000 cells per well for both the specific ELISpots. No cell- and no biotinylated Ab- controls were included. Limited leukocyte numbers and reagents led to reduced numbers of wells in the NC group for the *Y. ruckeri* ELISpot (specified in the figure caption).

2.6 ELISA

ELISAs were used to measure anti-*P. salmonis*, anti-*Y. ruckeri*, and anti-TNP-KLH Abs in serum. ELISA plates (Microlon® 200, Greiner) were coated with 2 µg/well of *P. salmonis* or *Y. ruckeri* antigen, as used for coating of specific ELISpot plates, or with 0.5 µg/well TNP-KLH (LGC Biosearch Technologies), diluted in 100 µl PBS at 4°C overnight. After washing three times, the wells were blocked with 200 µL PBS with 0,05 % Tween 20 and 5% non-fat milk for 1 hour. Serum samples were diluted 1:50 (exceptions: 1:200 for *P. salmonis* 6 wpi and all TNP-KLH samples, and 1:10 for *Y. ruckeri* samples) in PBS with 0,05 % Tween 20 and 1% non-fat milk, and 100 µL was added to each well for 1 hour after washing. After washing, 100 µL/well of the secondary Ab (LS-C63026-100, clone IPA-5F12, HRP conjugated – Bio-Rad) diluted 1:2000 in PBS with 0,05 % Tween 20 and 1% BSA was added and incubated for 1 hour. Wells were developed for 20 minutes using 100 µL/well 1-step ultra TMB-ELISA substrate (Thermo Fisher Scientific) before stopping the reaction by adding 100 µL/well 2M H_2SO_4. Optical density was read at 450 nm using a Sunrise absorbance reader (Tecan). Ab titers are presented as percentage of a positive standard for the antigens used; pooled sera from infected fish at 6 wpi for *P. salmonis* and TNP-KLH, or sera from salmon vaccinated for *Y. ruckeri* (courtesy of Vaxxinova Norway AS), by using a standard curve. This method is also described by Bailey et al. (Bailey et al. 2004).

2.7 *P. salmonis* qPCR

DNA from liver and HK samples on RNAlater or from isolated PerC leukocytes was isolated using the QIAamp cador Pathogen kit (Qiagen) as per manufacturer's instructions with lysis of organ samples using buffer ATL (Qiagen). Five µL DNA template (1:10 diluted for mortality and PerC samples,

otherwise undiluted) was mixed with 10 µL 2x KAPA SYBR FAST qPCR master mix (Kapa Biosystems), 4 µL water, and 0,5 µL of both *P. salmonis* 16S primers (table 1). These reactions were run on the CFX96 system (Bio-Rad) at 95°C for 3 minutes, followed by 40 cycles of 95°C for 3 seconds and 65°C for 20 seconds, along with a dilution series of an amplicon with known copy numbers. Melting curves (increase from 65 °C to 97 °C with standard ramp rate) were used to verify the presence of a single product.

2.8 RT-qPCR on PerC AT

Total RNA was isolated from AT using the RNeasy Mini Kit (Qiagen), and 0,5 µg RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen), both as per manufacturer's instructions. Six µL 1:10 diluted cDNA was used per 15 µL PCR reaction using 2× Fast SYBR® Green Master Mix (Applied Biosystems) with primer concentrations of 0,5 µM (see table 1 for all primers). The reactions were run under the following conditions: 95 °C for 5 minutes and 45 cycles of 95 °C for 5 seconds, 60 °C for 15 seconds, and 72 °C for 15 seconds (7500 Fast Real-Time PCR System, Applied Biosystems). Melting curves were used to verify the presence of a single product, and the used primers had previously determined efficiencies between 2,04 and 2,22 (see table 1 for references per primer). Relative expression was calculated using the delta ct method with Elf2a as a reference gene (Schmittgen and Livak 2008).

2.9 Statistical analysis

We performed statistical analyses in GraphPad Prism version 8.4.1. We used unpaired t-tests with Welch's correction to compare the leukocyte numbers, ASC numbers, specific Ab titers, and *P. salmonis* load in the PerC between the *P. salmonis* infected group with the NC group at all time-points. Since we did not deviate from the clearly predefined comparisons, we did not correct for multiple testing in these tests. The fold increase in specific Ab titers was tested using a one-sample t-test against the theoretical mean 1 (no change). We used Spearman correlation for correlations between serum IgM and ASC. Welch's ANOVA with Dunnett's T3 post hoc test was used to test gene expression differences between all NC and *P. salmonis* infected groups at time points 3 dpi, 3 wpi, and 6 wpi. The statistical significance level was set to $p < 0,05$ and is indicated with *.

Table 1. List of primers used in this study with references to original publications of the primers.

Target	FW/RV	Sequence	Published in:
CD4	FW	GTTGAAAGGGCGAAAGTGAG	(Sobhkhez et al. 2018)
	RV	GTGCCTTCGATGAGGACATT	
CD40	FW	ATGCCATGCCAAGAGGGTGAAT	(Lagos et al. 2012)
	RV	ATTTGCATGGGCTGAGGCTTGT	
CD40L	Fw	CACCAGGACCGGGCCACAAC	(Lagos et al. 2012)
	Rev	TGGGCACACCCCCAGTGAGT	
CD83	FW	GTGGCGGCATTGCTGATATT	(Iliev et al. 2013)
	RV	CTTGTGGATACTTCTTACTCCTTTGCA	
CD8a	FW	CGTCTACAGCTGTGCATCAATCAA	(Strandskog et al. 2011)
	RV	GGCTGTGGTCATTGGTGTAGTC	
elf2aB	FW	TGCCCCTCCAGGATGTCTAC	(Iliev et al. 2013)
	RV	CACGGCCCACAGGTACTG	
IFNγ	FW	AAGGACCAGCTGTTCAACGG	(Thim et al. 2014)
	RV	CACACCCTCCGCTCACTGT	
MARCO	FW	AGGACCTGCTGGTGTTAATG	(Jenberie et al. 2018)
	RV	CTGCTCTTTCACCCTTCTCTC	
MHCI	FW	GAAGAGCACTCTGATGAGGACAG	(Sobhkhez et al. 2018)
	RV	CACCATGACTCCACTGGGGT	
MHCI lga	FW	CACAAAAACCAAGGACGATGAA	(Svenning et al. 2019)
	RV	CGGTGCTTTAGTTCAAATGATCTG	
MHCII	FW	AGAAGCCTGGAACAAAGGTCCTGA	(Jenberie et al. 2018)
	RV	AACTGTCTTGTCCAGTATGGCGCT	
mIgM	FW	CCTACAAGAGGGAGACCGA	(Iliev et al. 2013)
	RV	GATGAAGGTGAAGGCTGTTTT	
Myeloperoxidase (MPO)	FW	CGAAACACGACCTTCAACAAC	(Jenberie et al. 2020)
	RV	AACTCGCTATCGTTCACTACAC	
Pax5	FW	ACGGAGATCGGATGTTCCTCTG	(Zwollo et al. 2008)
	RV	GATGCCGCGCTGTAGTAGTAC	
P. salmonis 16S	FW	AGGGAGACTGCCGGTGATA	(Karatas et al. 2008)
	RV	ACTACGAGGCGCTTTCTCA	
sIgM	FW	CTACAAGAGGGAGACCGGA	(Iliev et al. 2013)
	RV	AGGGTCACCGTATTATCACTAGTTT	
TNFα	FW	TGCTGGCAATGCAAAAGTAG	(Iliev et al. 2010)
	RV	AGCCTGGCTGTAAACGAAGA	

3 Results

3.1 *P. salmonis* infection in Atlantic salmon

The *P. salmonis* challenge resulted in an infection of the Atlantic salmon based on the following observations. We euthanized four fish according to the humane endpoints (loss of buoyancy, lack of

response to stimuli, or severe injury or ulceration) between day 36 and 41 and counted these as mortalities. Furthermore, fish weight was reduced by around 15% in infected fish at 3 wpi, and a similar, though not significant, reduction was observed at 6 wpi. Additionally, we observed discoloration and bleeding in liver and PerC of some infected fish at both 3- and 6-wpi (see suppl. table 2), corresponding to symptoms of SRS (Rozas and Enríquez 2014). *P. salmonis* DNA was demonstrated in liver and HK of all four mortalities, as well as in the liver of all infected fish at 3- and 6-wpi (see suppl. table 3), indicating that an infection was established. No *P. salmonis* DNA was detected in the liver of infected fish at 3 dpi nor in any NC samples. PerC leukocytes from infected fish were tested positive for *P. salmonis* DNA at the three time points: 3 dpi, 3 wpi, and 6 wpi (fig. 1a), with the highest signal at 3 dpi. Together, these results demonstrate that *P. salmonis* established an infection in Atlantic salmon.

3.2 Leukocyte and total IgM ASC increase is highest in infected PerC

Previous studies have shown that upon IP injection with bacterial pathogens, IgM+ B cells become the most abundant leukocyte population in the PerC (Korytář et al. 2013; Castro et al. 2017). However, these reports have focused on early time points (up to 72 hours post infection), whereas studies investigating more prolonged responses are currently lacking. Herein, the total and specific IgM ASC in PerC and systemic tissues of Atlantic salmon were monitored over a period of 6 weeks upon challenge by *P. salmonis*, aiming at understanding more about the interplay between local ASC and systemic responses during a prolonged bacterial infection.

First, the total number of leukocytes isolated per organ was determined at different time-points after infection. In the steady state, at 0 dpi, most leukocytes were present in the HK followed by the spleen, while in the PerC of non-infected fish the numbers remained very low (>20 times lower than in HK). Upon *P. salmonis* challenge, a very strong increase in leukocyte numbers was evident in the PerC of infected fish at all time-points with a peak at 3 wpi (Fig.1b). For the HK there was also a significant increase in the number of leukocytes at both 3- and 6-wpi (fig. 1b), while the number of leukocytes in the spleen of infected fish was similar to the controls.

Next, we determined the number of IgM ASC in HK, spleen, and PerC using ELISpot. The relative frequency of total IgM secreting cells per 12500 leukocytes peaked in the HK and spleen of infected fish at 3 wpi (fig. 1c). In the HK, it was still slightly elevated, though not statistically significant, at 6 wpi. In the spleen, the levels in the infected fish paralleled controls at 6 wpi. In the PerC of infected fish, the frequency of IgM producing cells was slightly reduced at 3 dpi (fig. 1c). In contrast, a significant IgM ASC increase was seen in the PerC at 3 wpi that increased even further at 6 wpi. By looking at the numbers of IgM secreting cells per organ, we see comparable amounts in the HK and in the PerC of infected fish, and the numbers in both sites were increased compared to negative controls at all time-

points (fig. 1d). The spleen, in contrast, contained lower total numbers and showed an increase only at 3 wpi.

3.3 Specific anti-*P. salmonis* ASC and non-specific anti-*Y. ruckeri* ASC are mainly increased in PerC

We established a *P. salmonis* ELISpot assay to enumerate the anti-*P. salmonis* ASC in the different organs. We optimized coating and cell seeding densities, while no cell- and no biotinylated antibody-controls showed no to very low background (suppl. fig. 2c and 2d). In the HK, there was no significant difference in the frequency of anti-*P. salmonis* ASC per 250000 leukocytes between NC and infected fish at both 3- and 6-wpi (fig. 2a). This contrasted with the frequency of total IgM ASC, which were higher in infected fish at both these time-points, though not significantly at 6 wpi (fig. 1c). Interestingly, the frequency of anti-*P. salmonis* ASC in the spleen was higher after infection at both time points, while the frequency of total IgM ASC was not increased at 6 wpi (fig. 1c and 2a).

The increase in anti-*P. salmonis* ASC frequency in the PerC was more pronounced compared to the HK and spleen at both time-points, with the highest frequency of specific ASC found at 6 wpi (fig. 2a). This agrees with earlier findings (suppl. fig. 1c) and suggests that anti-*P. salmonis* ASC were mainly induced at the site of injection and not in the systemic immune organs at these time points. The total numbers of anti-*P. salmonis* ASC per organ showed similar results (fig. 2b). For HK, a significant increase in anti-*P. salmonis* ASC per organ appeared at 6 wpi, although the increase compared to the NC was highest in the PerC. The spleen clearly contained the lowest number of anti-*P. salmonis* ASC when compared to HK and PerC. The control group (injected with PBS) did not exhibit any significant change during the study period (fig. 2).

To investigate the presence of non-specific (cross-reacting) ASC, a second specific ELISpot assay was established using antigen from the unrelated fish pathogen *Y. ruckeri.* Interestingly, we observed a significant increase of non-specific ASC frequency in infected PerC at 6 wpi, which was around 15% of the increase in anti-*P. salmonis* ASC numbers (fig. 2c). No differences in anti-*Y. ruckeri* ASC frequency were found in HK. At 3 wpi, some anti-*Y. ruckeri* ASC were found in all organs, and, although the number of observations was too low for statistical analysis, the frequencies seemed to be higher in infected Atlantic salmon (fig. 2c). The total numbers of anti-*Y. ruckeri* ASC per organ showed a similar trend as seen for anti-*P. salmonis* ASC, although they were a bit lower and lacked statistical significance (fig. 2d).

3.4 Anti-*P. salmonis* Ab titers, as well as anti-*Y. ruckeri* and anti-TNP-KLH titers, increase over time.

The serum Ab response to *P. salmonis*, as measured by ELISA, was induced at low levels in infected fish after 3 wpi and continued to increase at 6 wpi (fig. 2e). In addition, non-specific Abs recognizing *Y.*

ruckeri and TNP-KLH were detected, and their levels in the infected fish gradually increased until at 6 wpi (fig. 2f and 2g). No changes in Ab titers were detectable in the control fish compared to the titers at day 0. The level of *Y. ruckeri* Abs, given as percentage of a reference sample from vaccinated Atlantic salmon with high *Y. ruckeri* Ab titers, was only 2 % of this reference. Thus, there was a slight, but clear increase in *Y. ruckeri*-recognizing Abs in the *P. salmonis*-infected fish. For the TNP-KLH Abs, we used pooled sera from the *P. salmonis* infected salmon as a standard, and the levels in infected fish increased at 3- and 6-wpi. The use of different reference samples in the *Y. ruckeri* and TNP-KLH ELISAs does not allow direct comparison. Still, if we compare the increases in the infected groups to the NC groups, we can observe similar trends.

To enable a better comparison of the increase of serum Abs with different specificities, we calculated the relative increase of Abs per fish after infection. This increase was calculated by dividing the Ab titer for each infected fish (as presented by the individual dots in figures 2e-g) by the average of the NC group for the same time point (as presented by the white bars in figures 2e-g). An increase of 7- and 90-fold was visible for *P. salmonis* Abs on 3- and 6-wpi respectively (fig. 2h). For *Y. ruckeri*, a 2- and 3-fold increase were detected at 3- and 6-wpi, respectively, similar to TNP-KLH, which showed a 2- and 4-fold increase at those time points. A comparison of the non-specific Ab increase with the increase in specific *P. salmonis* Abs shows relatively more non-specific Abs at 3 wpi than at 6 wpi (fig. 2h). This indicates that although the numbers of non-specific Abs increased in time, the overall response became more specific at 6 wpi, as relatively more specific Abs are present at that time point.

We investigated the correlations between the serum Ab titers for *P. salmonis* and *Y. ruckeri* and the ASC frequencies using Spearman correlation. The analysis was performed for the HK, spleen, and PerC at 3- and 6-wpi. Significant correlations were found between *Y. ruckeri*-recognizing Abs in serum and total ASC in PerC at 3 wpi (rs: 0,76), and between the *Y. ruckeri*-recognizing Abs in the serum and total ASC in spleen (rs: 0,83), total ASC in PerC (rs: 0,86), and *Y. ruckeri*-recognizing ASC in PerC (rs: 0,78, see suppl. table 4). Surprisingly, correlations between *P. salmonis* Ab titers and ASC numbers in organs were weaker than those for *Y. ruckeri* Ab titers and below the significance limit. The highest correlation coefficients found for *P. salmonis* Ab titers came up with anti-*P. salmonis* ASC and total IgM ASC in the spleen and PerC at 6 wpi (rs: 0,62 to 0,71). All correlation coefficients between serum Ab and ASC in the HK were lower compared to the other two organs at 6 wpi (suppl. table 4).

3.5 Immune cell marker expression in PerC AT suggests immunological role.

A previous study has stated that rainbow trout AT contains various immune cell populations including macrophages, T-, and B cells (Pignatelli et al. 2014). To investigate the possible role of the AT in the immune responses against an IP challenge of *P. salmonis*, we evaluated the expression of several immune cell marker genes and related immune relevant genes. The expression of investigated genes

in the NC group was not significantly altered between any of the time points (fig. 3), which suggests that the PBS injection induced no change in expression and that we measured the steady state for these genes. In this steady state, B cells were clearly present based on the basal expression of *membrane bound IgM* (*mIgM*), *IgT*, and *IgD*, of which *IgM* was most highly expressed, while *IgD* expression was barely detectable (fig. 3a). Basal levels of *Pax5* in the AT were low as well. *Secreted IgM* (*sIgM*) transcripts were also expressed, indicating the presence of ASC. T cell markers, both *CD4* and *CD8*, were observed, but the low expression of *CD40L* suggests that the majority of T cells was not activated. Professional antigen presenting cells (such as B cells, tissue resident macrophages, and dendritic cells (DCs)) are characterized by their expression of *MHCII*, *CD40*, and *CD83* and were also present based on detected gene expression. Together, this indicates a presence of all cell types necessary for antigen presentation in the steady state AT.

The expression of most of the measured genes changed upon *P. salmonis* challenge. Early in the infection, at 3 dpi, an increase of *IFNγ* expression may have initiated a Th1 response since it coincided with an increase in *CD8* and *MCHI* expression (fig. 3b). Later in the infection, at 3 and 6 wpi, B cell markers (*Ig*'s, *CD40*, and *MHCII*) were increased, with *sIgM* levels being higher at 6 wpi than at 3 wpi (fig. 3a & 3c). The latter indicates a higher proportion of more differentiated B cells (plasmablasts or plasma cells) compared to the earlier time points, although the expression of *Pax5* (immature B cells) did not change significantly. The increase in *IgT* seemed to follow the general increase in *mIgM* and *sIgM*, but the *IgD* expression remained extremely low. Macrophages also seemed to be present in higher numbers at the later time points based on the *MARCO* expression increase. Nevertheless, macrophages might have been less abundant than DCs, since the increase in *CD83* expression was higher than that of *MARCO*.

The Th1 response that started at 3 dpi was more pronounced at later time points based on the increase in *IFNγ*, *CD8*, *MHCI*, and *CD40L* expression (fig. 3b). T-helper cell and cytotoxic T cell markers (*CD4* and *CD8*) were increased at 3 and 6 wpi, in addition to *CD40L*, a marker for T cell activation. *P. salmonis 16S* RNA was present in the AT of infected fish at 3- and 6-wpi, although the detected increase was not statistically significant due to large variation (fig. 3d). Interestingly, no inflammation seemed to be present in the AT based on the lack of *myeloperoxidase* expression that would signal neutrophil activity. The bacterium was possibly mainly present in infected or presenting cells, which would explain the Th1 response. Finally, the peak in expression of the pro-inflammatory cytokine *TNFα* at 3 weeks, without a clear inflammatory response in the AT, might indicate a regulation of the immune response in the PerC. Together, the basal expression levels and the induced expression of immune cell markers after *P. salmonis* infection point to an immunological role for the AT.

4 Discussion

4.1 PerC response and leukocyte migration

The main aim of our study has been to define the characteristics of the local (peritoneal) and the systemic B cell Ab responses in Atlantic salmon upon IP challenge with *P. salmonis*. Our results revealed a strong local B cell response in the PerC of the infected fish. This local response was characterized by increased numbers of leukocytes, as well as elevated frequencies of total-, specific-, and non-specific ASC at 3- and 6-wpi. At 3 dpi, an increase of leukocytes in the PerC coincided with a decrease in total IgM ACS, indicating that leukocytes other than plasmablasts or plasma cells were mainly present at that time point. This is in line with the observations by Korytář et al. (Korytář et al. 2013), where the ratio of myeloid cells in the PerC is increased after *Aeromonas salmonicida* infection at 12 hours and was still elevated at 72 hours.

In a recent study by our group (Jenberie et al. 2020), the viral pathogen SAV3 was IP administered to Atlantic salmon. They found prolonged ASC responses in the PerC, similar to what we report here. Collectively, the two studies demonstrate the presence and maintenance of ASC in the PerC, highlighting an active role of the PerC to IP challenge. However, when comparing these studies, the bacterial infection resulted in a stronger response (increase in leukocyte- and total IgM ASC numbers) compared to the viral infection. Notably, the effects of the SAV3 infection were milder (no mortalities and less observed pathology) compared to our bacterial challenge. Jenberie et al. (Jenberie et al. 2020) observed a decrease of leukocytes in the HK at 1- and 2-wpi, coinciding with an increase in the PerC. They suggested that the leukocytes might have migrated from the HK to the PerC. In contrast, we did not see a decrease in leukocyte numbers in the HK or spleen at any time point. This suggests either a fast local expansion of leukocytes in the PerC or an influx from other organs than HK or spleen. The AT might be a source of origin of the leukocytes, as well as the peripheral blood. One explanation as to why no decrease in leukocyte numbers in the HK was observed might be the observed presence of *P. salmonis* in the HK of Atlantic salmon as early as 3 dpi (Svenning et al. 2019). This is possible through transport by infected macrophages that return to the HK for antigen presentation. As a result, the presence of *P. salmonis* infected cells could attract more leukocytes to the HK. This trafficking of leukocytes between HK and PerC is in accordance with a previous study in Atlantic salmon, where IP injected fluorescent labeled ovalbumin was rapidly endocytosed by MHCII+ cells in the PerC, and subsequently transferred to the HK, where they remained present for at least two weeks (Iliev et al. 2013). At 3 dpi, the leukocyte counts for the infected HK had to be normalized due to a technical issue. Since this normalization could mask a reduction in leukocyte numbers, it cannot be completely ruled out that HK leukocyte numbers were reduced.

This strong local response in the PerC could result in an overestimation of vaccine protection in vaccination/challenge studies, especially when vaccination and challenge are both administered IP. Several *P. salmonis* vaccine efficacy trials performed the challenge not long after vaccination, e.g. after 600 degree days (around 2 months at 10°C) (Evensen 2016; Tobar et al. 2011; Wilhelm et al. 2006), probably since longer experiments are costly. The response to an early IP challenge in vaccine trials could be significantly influenced by the observed strong local response in the PerC and lead to lower mortalities and thus higher predicted protection. This could explain, at least partly, why the *P. salmonis* vaccines show protection in experimental settings but fail to do so in in the field. However, since our study was performed with a live pathogen, the outcome of vaccine trial using inactivated antigens may elicit responses that are different from reported here. This would be an interesting objective for a future study.

4.2 Non-specific versus specific B cell activation

Another possible reason for overestimation of vaccine efficiency, in this instance against heterologous strains, could be the cross-protection from non-specific Abs. Such Abs, which are present early in the immune response, can bind to a secondary pathogen and lead to cross-protection. The non-specific Abs and ASC that we found at 3- and 6- wpi show that this is probable, especially since they recognize *Y. ruckeri*, a non-related pathogen. At 6 wpi, we found less non-specific Abs relative to specific Abs compared to 3 wpi. This could indicate a reduction in cross-reactivity at later time points, as can be observed after vaccination of rainbow trout against viral haemorrhagic septicaemia (Yamaguchi et al. 2019; Lorenzen 2002). A non-specific Ab response after IP vaccination of Atlantic salmon has also been found by Lund et al. (Lund et al. 2019). An early challenge with a heterologous strain after vaccination could thus encounter more cross-protection than a challenge after a longer period. Our data indicate that careful design of vaccination experiments is of outmost importance to obtain a readout of specific protection.

This non-specific response could originate from non-specifically activated B cells. In addition to their clonally rearranged B cell receptor that responds to specific antigens, teleost B cells express a range of Toll-like receptors (TLRs) that allows them to react directly to microbial products (Abós et al. 2013; Jenberie et al. 2018; Peñaranda et al. 2019). In mice, the recognition of pathogen associated molecular patterns may lead to the production of non-specific or natural Abs that are poly-reactive and are able to react with foreign antigens that the host never met, as shown by Gunti et al. (Gunti et al. 2015). The non-specific activation of natural ASC might also explain why correlations between *Y. ruckeri* recognizing Abs and ASC were stronger than between anti-*P. salmonis* Abs and ASC. Several of the found correlations for Y. ruckeri serum Abs were significant at 6 wpi, while the best correlations for *P. salmonis* Abs were just below the significance level. While one would intuitively expect the specific

reaction to the pathogen to have a better correlation, a higher variation in the specific responses between individual fish could explain the lower correlations found. An activation through TLRs by pathogen associated molecular patterns would be much less varied than an activation through a B cell receptor that has undergone somatic hypermutations. Additionally, the repertoire of natural Abs is less varied (Bilal et al. 2021), restricting the variation of the response. Finally, we correlated serum Abs with ASC numbers. This could result in lower correlations if some ASC would produce more Ab than others, which could be the case for specific ASC.

The mechanisms of the transition to a more specific Ab response in teleosts still warrants more research. The affinity maturation that leads to specific responses in mammals is driven by somatic hypermutation and clonal selection. This is realized through mutations and antigen-dependent survival of activated B cells. In mammals, this phenomenon is linked to follicular B cells (also named B2 cells), follicular DCs, and T cell help. Although less efficient, activated teleost B cells share the ability to undergo somatic hypermutation and clonal selection with their mammalian counterparts, but germinal centers, the sites of clonal selection, together with follicular DCs, have not been found in the teleosts (Magor 2015; Steinel and Bolnick 2017; Stosik et al. 2019). Melanomacrophage centers might function as maturation sites in fish, where macrophages and follicular DC-related reticular cells could take part in retention and presentation of antigen (Magor 2015). According to their observations, melanomacrophage centers appear to contain more antigen-trapping cells than mammalian germinal centers, thus low affinity B cells have a possibility of receiving antigen signals as well. This, in turn, may result in a weaker selection of high-affinity clones. Magor (Magor 2015; Muthupandian et al. 2021) suggests that this could explain the slow and weak affinity maturation found in fish. The main increase of specific ASC in the PerC observed in our study suggests that clonal selection might be possible locally. A possible location would be the PerC AT, where melanomacrophage centers have been observed after vaccination (Villumsen et al. 2017).

4.3 ASC localization

The fact that the PerC showed the highest induction of all ASC, compared to HK and spleen, raises the question on the main location of systemic Ab production early after IP *P. salmonis* infection. Based on the ASC frequencies, this would be the PerC, followed by the spleen. The correlations between serum Abs and different ASC in the investigated organs support this (suppl. table 4). Unexpectedly, the HK showed no significant increase of specific ASC frequency at any of the included time points. This is interesting because the HK is the main hematopoietic organ and the organ considered to be the main location for ASC at later (>8 wpi) time points (Bromage et al. 2004). Bromage et al. describe antigen-specific ASC in blood, spleen, and HK of rainbow trout immunized with TNP-KLH in Freund's Complete Adjuvant. They observed an initial peak of specific ASC numbers in all three organs at 8 wpi, after which

only ASC numbers in the HK remained elevated. These HK ASC were hydroxyurea-resistant, meaning that they did not proliferate any more, and their presence coincided with elevated serum titers. This led the authors to propose a model where long-lived (hydroxyurea-resistant) ASC in the HK are mainly responsible for the increased serum Ab titers late in the response. In comparison, we found significant serum Ab titers and an increased number of specific ASC in the spleen and PerC, not the HK, as early as 3- and 6-wpi. This seems to indicate that the HK is less important for Ab production during the early response, which is in accordance with the kinetics found by Bromage et al. (Bromage et al. 2004). The observed faster and more pronounced Ab response, compared with Bromage et al. (Bromage et al. 2004), could be due to a difference in host/pathogen interactions, fish species, and/or environment. A notable difference lies in the antigens used in the two studies; a living pathogen usually leads to a more complete response than a model antigen. Based on a combination of the findings one could propose a model in which Ab secretion is initiated locally in the PerC with contribution from the spleen, while the HK facilitates long-term Ab responses.

Higher ASC frequencies or numbers do not necessarily reflect that more Abs are produced as the ASC in some organs might produce more Abs per ASC. In the present study, the spots in the ELISpot wells differed in size and intensity between the three organs (fig. 2i). This indicates a difference in the quantity of secreted Abs per specific ASC. The most pronounced difference was present in the spleen samples, where some spots were clearly bigger and more intense than in the other organs, while the HK spots were the faintest. Additionally, the (counted) average spot sizes were larger in both the spleen and PerC compared to the HK at 6 wpi (see suppl. fig. 3). The total well intensity was also lowest in the HK (data not shown). These results suggest that the spleen and PerC not only had a larger increase in specific ASC numbers, but also produced more Abs per ASC when compared to the HK.

4.4 Immunological role of the PerC AT

A logical explanation for the presence of ASC in the PerC is a local B cell activation. Teleost B cells most closely resemble mammalian innate, or B1, B cells in that they express several pattern recognition receptors, phagocytize actively, and respond to inflammatory signals (Díaz-Rosales et al. 2019; Peñaranda et al. 2019). Additionally, their expression profile of cluster of differentiation molecules corresponds best with mammalian B1 cells: CD5, CD9, CD11a, CD11c, and CD22 (Peñaranda et al. 2019). In mammals, the B1 B cells reside in the PerC and pleural cavity and can be activated in the PerC before migrating to the spleen (Baumgarth 2013). Interestingly, our gene expression analysis of immune cell markers indicates that the PerC AT may harbor the immune cells necessary for antigen presentation and B cell activation. Still, while we expect the expression of *Pax5*, a marker for immature B cells, to go down in a population of maturing B cells, it did not change significantly. A possible

explanation is the recruitment of more immature B cells, possibly coupled with an efflux of mature B cells.

In addition to the presence and increase of *IgM* transcripts during the *P. salmonis* infection, an increase in *IgT* transcripts was also evident, indicating a role of IgT^+ B cells in the AT. Pignatelli et al. (Pignatelli et al. 2014) also observed increased *IgT* expression after viral challenge of rainbow trout, as well as the presence of IgT^+ cells by flow cytometry of the AT.

The expression of TNFα without an inflammatory response (no increase in *myeloperoxidase* expression) in the AT could furthermore indicate a regulating role for the AT in the PerC immune response. Together with the possible antigen presentation, the early cellular-, and the later humoral response, this indicates an immunological response in the AT to an infection of the PerC. This is in agreement with the hypothesis that the AT is a relevant immune organ in teleosts (Pignatelli et al. 2014; Veenstra et al. 2018). Functional studies are needed to confirm the presence of all components of efficient antigen presentation in the naïve AT and thus its role as a secondary immune organ. Nevertheless, our gene expression results suggest an immune regulatory role for the AT in the PerC.

Our findings indicate that the PerC is an essential site of B cell Ab production upon IP challenge with *P. salmonis* (and probably vaccination) and suggest a more extended role of the PerC than previously assumed. Although our findings only span the first 6 wpi, it is possible that ASC in the PerC continue to produce Abs after 6 wpi. Jenberie et al. (unpublished, personal communication S. Jenberie, 03/04/2020) found this for SAV by showing the presence of SAV specific ASC in the PerC of infected fish at 13 wpi, their latest time point, supporting this view. For IP oil-based vaccines, a long-term presence of ASC in the PerC might additionally be maintained due to depot forming and gradual antigen release. Whether Abs produced in the PerC also contribute to systemic immunity, or whether ASC in spleen and HK are mainly responsible, is a pending question. If the role of the PerC in short- and long-term immunity is elucidated, new vaccines can be evaluated more accurately. Further research into the numbers of ASC in the PerC and their levels of Ab secretion during longer periods after infection or vaccination will help us to expand the current model of B cell biology and ASC localization.

5 Conclusion

The successful development of specific ELISpot assays for *P. salmonis* and *Y. ruckeri* allowed us to observe a large increase in specific ASC in the Atlantic salmon PerC after IP *P. salmonis* challenge. This strong local response, combined with a non-specific response, could possibly lead to overestimation of vaccine efficacy if fish are IP-challenged a few weeks after vaccination. Another intriguing finding is that the anti-*P. salmonis* ASC frequency in PerC, and to a lesser extent in the spleen, is higher than in the HK. This sheds a new light on the main location of Ab production in

infected salmon during the early response to *P. salmonis* infection and is a highly relevant issue related to different vaccination regimes. Finally, the expression of several immune cell markers indicates an immunological role for the AT in the PerC. Together, these findings suggest a more important role of the PerC after IP challenge and vaccination than previously held. Taking this into account could have important consequences for future vaccine development.

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Figure captions

Figure 1. ***P. salmonis*** **DNA copies, leukocytes, and total IgM ASC in infected Atlantic salmon (a)** *P. salmonis* 16S DNA copies per 1 x 10^6 peritoneal cavity leukocytes from negative control (NC) and *P. salmonis* infected Atlantic salmon. n=8 for the infected salmon at all time points. At 3 days post infection, 8 NC fish were pooled in 3 pools, and at 3- and 6-weeks post infection, 8 NC fish were pooled in 1 pool. Individual measurements shown as squares and triangles. **(b)** Total number of leukocytes from NC and *P. salmonis* infected Atlantic salmon per organ. **(c & d)** Number of total IgM ASC per 12500 leukocytes (c) or per organ (d) from NC and *P. salmonis* infected Atlantic salmon. n=8 for all time points, except for day 0 (n=4). Individual measurements shown as dots. (*) Statistically significant ($p < 0,05$) between the infected group and the NC, error bars indicate 95% confidence interval. **(e)** Representative total IgM ELISpot wells from NC and *P. salmonis* infected head kidney, spleen, and peritoneal cavity at 6 weeks post infection.

Figure 2. **Specific and non-specific ASC and antibody responses in *P. salmonis* infected Atlantic salmon. (a & b)** Number of anti-*P. salmonis* IgM ASC per 250000 leukocytes (a) or per organ (b) from negative control (NC) and *P. salmonis* infected Atlantic salmon. n=8 for all time points. **(c & d)** Number of anti-*Y. ruckeri* IgM ASC per 250000 leukocytes (c) or per organ (d) from NC and *P. salmonis* infected Atlantic salmon. n=1-3 for 3 weeks and n=8 for 6 weeks (NC: PerC n=2). **(e - g)** Relative amount of anti-*P. salmonis* (e), anti-*Y. ruckeri* (f), and anti-TNP-KLH (g) IgM in serum of NC and infected Atlantic salmon presented as a percentage of an included standard (pooled sera from infected fish at 6 weeks post infection for *P. salmonis* and TNP-KLH; serum from salmon vaccinated against *Y. ruckeri* for *Y. ruckeri*). n=8 for all time points, except 0 days (n=4, pooled in 1) and infected salmon at 6 weeks post infection (n=12). At 3 days post infection the 8 samples were pooled in 3 pools. Individual measurements shown

as dots. (*) Statistically significant ($p < 0,05$) between the infected group and the NC, error bars indicate 95% confidence interval. **(h)** Fold increase in serum IgM for anti-*P. salmonis*, anti-*Y. ruckeri*, and anti-TNP-KLH relative to the average of the NC group per time point, n=8-12. At 3 days post infection the 8 samples were pooled in 3 pools. Error bars indicate 95% confidence interval. Statistically significant difference (*) from mean 1 (no change, dotted line) based one-sample t-test. **(i)** Representative anti-*P. salmonis* ELISpot wells (96-well) from NC and *P. salmonis* infected head kidney, spleen, and peritoneal cavity at 6 weeks post infection.

Figure 3. **Relative immune gene expression levels in peritoneal adipose tissue** from negative control (NC) and *P. salmonis* infected Atlantic salmon for B cell (a), T cell (b), professional antigen presenting cell (c), and inflammation (d) related genes at 3 days, 3-, and 6-weeks post infection. Graph shows the mean expression of genes, including the bacterial 16S gene, presented relative to a control gene (ELF-2a). n=7 for NC and n=8 for infected fish at all time points. (*) Statistically significant between the infected group and the NC, error bars indicate 95% confidence interval. **(e)** Peritoneal cavity of *P. salmonis* infected Atlantic salmon. The sampled adipose tissue is indicated with an arrow.