Who needs the hotspot? The effect of temperature on the fish host immune response to *Tetracapsuloides Bryosalmonae* the causative agent of Proliferative Kidney Disease

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ABSTRACT

Proliferative kidney disease (PKD) of salmonids, caused by *Tetracapsuloides bryosalmonae* may lead to high mortalities at elevated water temperatures. However, it has not yet been investigated how temperature affects the fish host immune response to *T. bryosalmonae*. We exposed YOY (young of the year) rainbow trout (*Oncorhynchus mykiss*) to *T. bryosalmonae* at two temperatures (12 °C and 15 °C) that reflect a realistic environmental scenario and could occur in the natural habitat of salmonids. We followed the development of the parasite, host pathology and immune response over seven weeks. We evaluated the composition and kinetics of the leukocytes and their major subgroups in the anterior and posterior kidney. We measured immune gene expression profiles associated with cell lineages and functional pathways in the anterior and posterior kidney. At 12 °C, both infection prevalence and pathogen load were markedly lower. While the immune response was characterized by subtle changes, mainly an increased amount of lymphocytes present in the kidney, elevated expression of Th1-like signature cytokines and strong upregulation of the natural killer cell enhancement factor, NKEF at week 6 P.E. At 15 °C the infection prevalence and pathogen burden were ominously greater. While the immune response as the disease progressed was associated with a Th2-like switch at week 6 P.E and a prominent B cell response, evidenced at the tissue, cell and transcript level. Our results highlight how a subtle, environmentally relevant difference in temperature resulted in diverse outcomes in terms of the immune response strategy, altering the type of interaction between a host and a parasite.

Key words: Rainbow trout; Proliferative kidney disease; PKD; *Tetracapsuloides bryosalmonae*; temperature; immune response; resistance; tolerance; Th1, Th2

Highlights

Temperature modulated pathogen burden, pathology and immune response

Th1-like genes were upregulated at cooler temperature

Th2-like genes and B cell response were prominent at warmer temperature

The immune response strategy at cooler temperature appears to tolerate the parasite

The immune response strategy at warmer temperature appears to resist the parasite

1. Introduction

PKD (Proliferative Kidney Disease) of salmonids is an emerging disease of economic and environmental concern [1; 2]. PKD is caused by a myxozoan parasite, *Tetracapsuloides bryosalmonae*. *T. bryosalmonae* spores are released from bryozoans, the invertebrate host of the parasite, into the water and then infect the fish host primarily through the gills and/or skin, and are then transported by the vascular system to the target organ, the PK (posterior kidney). Once in the PK, *T. bryosalmonae* penetrates the interstitial tissue, multiplies and differentiates from extrasporogonic to sporogenic stages [3]. Depending on environmental conditions *T. bryosalmonae* may induce clinical PKD and mortalities [2].

The fish host immune response to *T. bryosalmonae*, when originally described via histology, led to macrophages being propositioned as the major immune cell present in infected tissue [4]. However, a more recent cellular study using the higher accuracy of flow cytometry demonstrated the dominance of lymphocytes in infected tissue [5]. The most comprehensive study on the immune response of rainbow trout (*Oncorhynchus mykiss*) to PKD thus far was based solely on the transcript level and investigated genes from a broad panel of innate and adaptive immune pathways. The study related immune gene transcripts to kidney swelling grade and parasite burden. Based on this analysis it was suggested that PKD pathogenesis is shaped by a profound B cell/antibody response and potentially dysregulated Th cell activity [6]. All things considered there is not one collective study integrating pathological, cellular and molecular approaches to investigate the nature of the fish host immune response to PKD. Such a study compromising this plethora could refine the subtleties underlying the immune response.

Temperature may be an essential mechanism modulating PKD-induced clinical signs and mortality [7]. For instance, clinical signs and PKD mortality increase at water temperatures above 15 °C, whereas below 15 °C lower mortality rates are reported [8; 9; 10]. Likewise, temperature can be a major influence on the immune response of fish [11; 12; 13]. Nevertheless, there may be some ambiguity in such reports as studies select temperatures at the opposite ends of the physiological spectrum of the host, which may result in stress, impacting immunocompetence. Rainbow trout are fish from cold temperate waters with a physiological optimum temperature reported in the range of 12 °C -16 °C [14]. Hence it may be envisaged that an exploration of a subtler difference in temperature would not only correspond

to the physiological optimum of the fish host but to changes of temperature regimes as they occur in the natural habitats of salmonids. Though any change in temperature will also influence the parasite proliferation within the host, possibly indirectly modulating the host immune response, altering the outcome of the disease. Taking all this into account prompted us to investigate how a slight difference in temperature, one mirroring a realistic environmental change that could occur in the natural habitat of salmonids, influences the fish host immune response to *T. bryosalmonae*.

PKD studies have demonstrated that at 12 °C, the host develops a version of the disease with fewer clinical signs and lower mortality [15]. This may represent a stable co-evolved host-parasite system. However, as hosts and their parasites have an intimate and antagonistic relationship, the effect of temperature may influence the host-pathogen relationship. The outcome of this relationship may derive from two noted strategies: tolerance which is defined as the ability to limit the health impact of a given pathogen burden, or resistance which is defined as the ability to limit pathogen burden [16]. Whether the immune responses of salmonids could be assigned to one of the two strategies, and whether this changes with temperature, has not yet been elucidated.

In the present study, we established the following aims 1) To describe the host immune response at 12 °C after exposure to *T. bryosalmonae* and 2) To describe the host immune response at 15 °C after exposure to *T. bryosalmonae*, and 3) to characterise what differences occur at the warmer temperature in comparison to the cooler temperature, and, while we pay a great deal of attention to the cellular and molecular immune repertoire the fish host will use to encounter the invading pathogen, we will also take an evolutionary perspective asking whether the host at either temperature uses a noted strategy to increase its fitness. Granting this, it cannot be ruled out that the immune response is dynamic and not static and may be temporally dependant. Hence, the immune response at the warmer temperature may be faster than the cooler temperature, and a comparison of the immune response at a sequential time point may trigger false conclusions. In addition, the host immune response may be influenced via an indirect effect of temperature on parasite intensity. Therefore, to bring a sense of clarity to the study, we will attempt to elucidate such potential confounders.

In this study, we exposed YOY (young of the year) rainbow trout (*Oncorhynchus mykiss*) to *T. bryosalmonae* at two different temperatures (12 °C and 15 °C) and followed the development of the parasite, host pathology and immune response for 7 weeks. To investigate the immune response in the AK (anterior kidney) and PK we used i) flow cytometry and immunofluorescence techniques to identify leukocyte populations and subgroups, ii) total leukocyte counts in the AK, PK and blood and iii) measurement of immune gene expression profiles associated with cell lineages and functional pathways.

2. Methods

2.1. Fish specimens and exposure

Female rainbow trout, weighing 8-10g, were obtained from a commercial hatchery in western Switzerland (L'Isle, Switzerland) with no history of PKD. Fish were transported and acclimated for 2 weeks in the aquarium facilities at the FIWI (Centre for Fish and Wildlife Health), University of Bern, Bern, Switzerland). Fish were separated into respective temperature groups 12 °C and 15 °C, and a control and infection group established for each temperature. Experiments used 130 l flow-through glass tanks supplied with tap-water (approx. 1 l/m), constant aeration and artificial light (12 h light to 12 h dark).

Bryozoa (*Fredericella sultana*) were collected from Swiss rivers known to be endemic for *T. bryosalmonae*, transported to the FIWI and screened for infective sacs by dissection of the bryozoan zooids under a binocular. To release the *T. bryosalmonae* spores from the sacs, bryozoan zooid tissue was disrupted by grinding, and the homogenate kept at 4 °C for up to twenty minutes until the fish were ready for exposure. For exposure, water levels were reduced to 1/4 of the tank capacity (~30 l), water flow was stopped and aeration was increased. Equal volumes of aliquots from the parasite homogenate were then distributed to 12 °C and 15 °C infection groups. Conditions were maintained for 1 h before restoring water flow. Procedures were performed simultaneously for controls without the addition of parasites.

2.2. Fish selection and sampling

Independent fish (n = 6) were sampled for cellular, histological and molecular procedures per time point per group. Two fish were pooled for cellular procedures.

Fish were euthanized by immersion into a solution of MS-222 (100 µg/l buffered 3-aminobenzoic acid ethyl ester (MS 222®, Argent Chemical Laboratories). Following blood collection, the entire kidney was removed and separated into AK(the kidney tissue located cranially) and PK (the kidney tissue located past the narrow site below the neck until the caudal end) and weighed individually. Cellular procedures consisted of flow cytometry and leukocyte counts of the kidney and in the blood. Histology procedures consisted of histology and immunofluorescence. Molecular procedures consisted of qPCR for parasite intensity and RT- qPCR for immune gene expression analysis. AK and PK were sampled for all molecular and cellular procedures. A DNA sample was taken from the PK of all fish to confirm infection status. The data from the uninfected exposed fish was then excluded from the subsequent analysis of all parameters, pathological, cellular or molecular.

Before exposure, ten sampled fish were tested for the presence of *T. bryosalmonae* by qPCR. Then at week one P.E (post-exposure) ten sampled fish from each group were again investigated for the presence of *T. bryosalmonae* using qPCR, thus confirming successful infection of 12 °C and 15 °C infection groups. Samples were then taken at week 2, 4, 6 and 7 P.E. All groups (12 °C control, 12 °C infection, 15 °C control and 15 °C infection) were sampled at each time point. Samplings were always performed in the same manner, by the same two personnel. Molecular and cellular samplings were performed simultaneously. One person was responsible for molecular samples and one for cellular samples. As cellular procedures (flow cytometric analysis and counts) had to be performed on the day, the individual responsible for molecular procedures then took the samples for histology.

The extracted tissues for DNA/RNA isolation were placed on RNase and DNase free petri dishes and sliced using scapula and forceps into smaller pieces before being transferred to RNeasy (Qiagen, Basel, Switzerland) on ice for 24h before storage at -20 °C. Cellular procedures were performed on day of sampling. Histology samples were fixed in Histochoice (Amresco, Dietikon, Switzerland) for 3 h at RT (room temperature) and subsequently transferred to graded EtOH (ethanol) (≥ 99.8 %-70). All procedure was carried out according to the Swiss legislation for animal experimentation guidelines.

2.3. Total cell and differential leukocyte counts in the peripheral blood

Immediately after euthanizing fish, blood samples were collected from the caudal vein via a heparinised syringe. Blood yields ranged from 5 µl to 10 µl. Blood smears were prepared on SuperFrost® Plus positively charged glass slides (Menzel-Gläser, Braunschweig, Germany). Blood smears were left to air-dry overnight at RT and then fixed in absolute EtOH (≥99.8 %). All blood smears were stained with a modified May–Grunewald–Giemsa stain. One slide was analysed per fish. Blood smears were examined according to a list of randomized microscope coordinates. If a selected set of coordinates produced a field of view that resulted in a multilayer of cells, or a stain precipitation which prohibited counting, we moved on to the next field of view. Cells were identified via morphological criteria. The number of leukocytes (lymphocytes, thrombocytes, neutrophils and monocytes) was compared against the number of erythrocytes and deemed total cell counts. The number of lymphocytes was also compared against the amount of larger granular myeloid cells (neutrophils and monocytes/macrophages) and deemed differential leukocyte counts. 100 cells were counted for total cell counts and 100 leukocytes for differential leukocyte counts.

2.4. Posterior kidney somatic index

The PK of each fish was removed and weighed after blood was collected. The PKSI (Posterior kidney somatic index) was calculated as: $\text{PKSI} = \frac{\text{posterior kidney weights (g)}}{\text{body weights (g)}} \times 100$ and compared with values obtained from all other experimental groups.

2.5. DNA extraction and qPCR for determination of infection prevalence and parasite intensity

Approximately 25 mg of tissue was used for DNA extraction. DNA was extracted from the PK using the DNeasy blood and tissue kit following the manufacturer's instructions (Qiagen, Basel, Switzerland). The amount and purity of isolated DNA was determined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington DE, USA). Samples were stored at -20 °C until use for the qPCR assay. qPCR was performed using TaqMan master mix, (Applied Biosystems, Foster City, CA, USA) PKD primers and probe according to Bettge and colleagues (2009b) (Table 1). qPCR was performed on an Applied

Biosystems 7500 analyzer (Applied Biosystems, Foster City, CA, USA). The data were analyzed with the 7500-system sequence detection software version 1.3.1 of Applied Biosystems.

2.6. Histology

Following routine processing and paraffin embedding, PK sections of 3-5 μm thickness were prepared on SuperFrost® Plus positively charged glass slides and dried for 24 h at 40 °C. After deparaffinization in Histo-Clear (Agar Scientific Ltd, Essex, UK) and rehydration in graded series EtOH concentrations ($\geq 99.8\%$ -50 %) the slides for routine histology were stained with H&E (haematoxylin and eosin).

2.7. Preparation of AK and PK leukocytes

The AK and PK tissue was removed and passed through a 250 μm mesh filter with L-15 medium (Thermofisher, Reinach, Switzerland). Cell suspensions were layered onto an isotonic Ficoll gradient (Biochrom AG, Berlin, Germany) ($\rho=1.077\text{ g/ml}$) and centrifuged at 1300 RPM for 40 min at 4 °C. Cells at the Ficoll/medium interphase were collected, washed in medium and resuspended in 1 ml of medium. Red blood cells were then lysed using 9 ml cold dH₂O and the cell suspension was centrifuged at 1300 RPM for 1 min at 4 °C, resuspended in 1 ml L-15 medium and kept on ice.

2.8. Total leukocyte counts in the AK and PK

10 μl of the cell suspension was taken from the leukocyte preparation, diluted 1:10 with medium and mixed with trypan blue in a 1:1 ratio. 10 μl of the mixture was loaded into a counting cell chamber and counted by the Countess™ II - an automated cell counter (Thermofisher, Reinach, Switzerland). The obtained values of total leukocyte count of each fish were then divided by the corresponding tissue mass to back calculate the total leukocyte counts per gram of AK or PK tissue, per fish.

2.9. Flow cytometric analysis of AK and PK leukocytes

Light scattering properties FSC (size) and SSC (granularity) were used to differentiate between lymphocytes and larger granular cells of the myeloid lineage (neutrophils and monocytes/macrophages). MAbs (monoclonal antibodies) staining

the leukocyte subsets, IgM+ B cells, CD8- T cells and pan-myeloid cells, were used as previously described [17]. MABs were a kind gift from Dr. Bernd Köllner. MAb1.14 and MAbN2 were combined allowing for a multiple epitope targeting approach recognising IgM+ B cells as previously described [18; 19]. CD8- T cells were defined as all T cells except CD8+ T cells. Pan-myeloid cells were defined as such to allow discrimination between those cells gated by size and granularity (larger granular cells; neutrophils and monocytes/macrophages) and those stained by the MAb. As the MAb stained additional cells of the myeloid lineage as well as the larger granular cells. The antibody characteristics are further described in Table 2. A control for each sample was treated only with secondary antibody conjugates. The samples were measured on a BD LSR II (San Jose, CA, USA) to a total count of 10^4 events. Stained cells were evaluated using the appropriate fluorescence channel for each MAb in comparison to unstained cells and analysed by Flow Jo version 7.5.5 software. The gating strategy used to evaluate MABs is available in the supplementary figures (Fig.S2.5)

2.10. Immunofluorescence

Following histological processing steps as described (subsection 2.6.) consecutive slides for immunofluorescence were incubated in 3 % H_2O_2 in PBS-T (0.1 % Tween-20 in PBS) for 10 min to quench endogenous peroxidase activity. Slides to be treated with MAb21 required demasking by heat induced epitope retrieval with citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0). To block non-specific binding the slides were incubated in Superblock® with PBS-T (Thermofisher, Reinach, Switzerland) at RT for 15 min. Slides were then stained with MABs, diluted in 1 % Superblock® in PBS-T and incubated overnight at 4 °C. Primary antibodies MAb1.14 and MAbN2 were combined allowing for a multiple epitope targeting approach recognising IgM+ B cells as per section 2.9 [18; 19]. Whereas slides stained with MAb21 were recognized as pan myeloid cells. Thereafter, the slides were rinsed in deionised H_2O . Finally, bound MAb was visualized with a secondary Texas red-coupled anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Negative controls were incubated with PBS-T instead of antibodies.

2.11. RNA extraction

Approximately 25 mg of tissue was used for RNA extraction. RNA was extracted from the AK and PK using a Direct-zol™ RNA MiniPrep w/ TRI-Reagent® kit following the manufacturer's instructions (Zymo, Freiburg im Breisgau, Germany). The amount and purity of isolated RNA was determined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington DE, USA). The quality of some random selected samples was tested by the Agilent RNA 6000 Nano Kit, in combination with the Agilent 2100 Bioanalyzer System (Agilent Technologies, Waldbronn, Germany). Potential traces of genomic DNA contamination were removed with an on column DNase treatment provided in the kit.

2.12. Primer design and testing

24 primer sets were considered as immune response markers and assigned to a specific group of cytokines or to an immune cellular lineage, as well as the reference gene EF-1 α (elongation factor-1 α). Full primers sets as well as mean Ct values from untreated AK and PK samples of both the reference genes and gene of interest are shown in Supplementary Table S1.

Several reference genes were tested with Ef-1 α being identified as the most stably expressed in the AK and PK and thus taken as reference to which all genes were related (Supplementary Fig.S1). Primers were designed based on Primer3 algorithms and adjusted manually if necessary [23; 24]. The oligonucleotides were purchased (Microsynth, Basel, Switzerland) and rainbow trout cDNA from AK and PK were used to test the designed primers. The amplification efficacy was tested for each primer pair using different cDNA concentrations.

2.13. cDNA synthesis and RT-qPCR immune gene expression analysis

cDNA was synthesized using The GoScript™ Reverse Transcription System following the manufacturer's instructions (Promega, Madison, Wisconsin, USA). For each sample 500 ng of DNA-free RNA was used. The total volume of the cDNA syntheses was 20 μ l and cDNA was stored at -20 °C.

RT-qPCR was performed on an Applied Biosystems 7500 analyzer (Applied Biosystems, Foster City, CA, USA). The total reaction volume was 12.5 μ l, containing 6.25 μ l GoTaq® qPCR Master Mix, 1.25 μ l of 10 μ M primer stocks (final concentration 500 nM), 5 μ l Nuclease-Free water (Promega, Madison, Wisconsin,

USA) containing 0.4 µl of the cDNA synthesis mix. Result reliability was evaluated by the criteria previously explained for qPCR.

RT-qPCR was performed using fast cycle conditions with the following settings: 5 min 95°C, followed by 45 cycles of amplification. Each cycle consisted of 3 s of denaturation at 95°C, annealing and elongation at 60°C for 30 s. The PCR was always terminated with a melting curve analysis starting with a denaturation step of 95°C followed by the start ramping temperature of 60°C for 30 s. The data were analyzed with the 7500-system sequence detection software version 1.3.1 of Applied Biosystems. Data was evaluated according to the $2^{-\Delta\Delta C_t}$ method [25].

2.14. Statistics

The differences between respective control and infection groups and between infection groups were tested for significant differences using a t-test. The differences within infection groups at day P.E was tested for, using a one-way ANOVA and significant differences revealed with the post hoc test of Dunnett's. Data failing normality tests and displaying heterogeneity of variance was tested statistically applying the non-parametric Kruskal–Wallis ANOVA on ranks, and Dunn's non-parametric multiple comparison tests to reveal differences. Data were statistically evaluated with SigmaPlot 12.0 (Systat Software, San Jose, CA) and graphically presented with GraphPad Prism 5 (GraphPad Software, Inc. San Diego, CA) or SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY). Significance was set at $p \leq 0.05$.

3. Results

3.1. PKD infection prevalence and parasite intensity

Exposure of rainbow trout to *T. bryosalmonae* at 12 °C resulted in an infection prevalence ranging from 67%-83% (7/12 fish sampled) over the course of the experiment. Exposure at 15 °C ensued in a 100% prevalence (12/12 fish sampled) (Fig. 1.1). The pathogen burden of the 15 °C infection group was significantly elevated at every time point when compared to the 12 °C infection group (Fig 1.2). Furthermore, at 15 °C, at week 7 P. E, the parasite intensity was significantly increased in comparison to the other time points (Fig.1.2). Control samples measured at each temperature per time point were negative for *T. bryosalmonae*.

3.2. Pathological response

At 12 °C, albeit at week 7 P.E, there were signs of parasite induced alterations microscopically visible in the PK. (Fig.2.1). These included small focal areas of enlargement of the interstitial space due to increased cell numbers. Only rarely were parasites seen and if so their appearance was degenerative, characterized by a more eosinophilic staining than the parasites seen at 15 °C (Fig.2.1). At 12 °C there were no significant increases of the PKSI in comparison to the control or 15 °C infection group (Fig.3.1). Although there was a significant difference within group at week 4 P.E.

In stark contrast to this, at 15 °C, macroscopic enlargement of the PK was visible at week 2 P.E. By week 6 and 7 P.E the PK had become grossly enlarged, consistent with the classical hallmark of PKD pathology. Microscopically the most prominent histopathological lesions were seen in the interstitial haematopoietic tissue (characterized by an increase in cell numbers in this part (Fig.2.2). In the affected renal areas, moderate to high amounts of parasites were observed (Fig.2.2). The microscopic observations were reinforced by the PKSI. The PKSI was significantly increased compared to the control and to the 12 °C infection group at week 4, 6, and 7 P.E. Furthermore, at week 7 P.E, the PKSI was significantly elevated compared to the other time points (Fig.3.2).

3.3. Leukocyte kinetics and composition

3.3.1. Total leukocyte counts in the kidney

To elucidate, if the pathological reaction as a tissue response to PKD was associated with an increased amount of immune cells, we quantified the total leukocyte number per gram of tissue in both the AK and PK. However, no significant differences were found between or among any of the experimental groups.

3.3.2. Flow cytometric analysis of AK and PK leukocytes

Using light scattering properties FSC (size) and SSC (granularity) we distinguished between the major types of leukocytes. The results of the infection groups portrayed in the PK a general picture of increasing lymphocytes and decreasing larger granular cells of the myeloid origin (Fig.4, Fig.5). But there were huge differences in terms of the magnitude of these patterns. At 12 °C, lymphocyte

increases were significant relative to the control at all weeks in the AK, but only at week 6 and 7 P.E in the PK (Fig.4.1-2). In addition, significant decreases of larger granular myeloid cells occurred in the AK at week 2, 6, and 7 P.E and in the PK at week 6 and 7 P.E, in comparison to the control (Fig.4.1-2). At 15 °C there were significant increases in lymphocytes relative to the control and the 12 °C infection group at every time point in both AK and PK, except at week 2 P.E (Fig.4.3-4). There were also significant decreases in larger granular myeloid cells at week 2 and 6 P.E in the AK and week 4, 6 and 7 P.E in the PK in comparison to the control (Fig.4.3-4).

3.3.3. Leukocyte analysis in the peripheral blood

To explore the leukocyte composition in the blood, cellular profiles were evaluated from smears. At 12 °C there was a significant increase in leukocytes within the group at week 7 P.E (Fig.6). At 15 °C there was a significant elevation in the number of leukocytes when compared to that of the control at week 4, 6 and 7 P.E. While in comparison to the 12 °C infection group, there were significant increases in number of leukocytes at week 2, 4 and 6 and 7 P.E (Fig.6). In addition, there was also a significant increase in leukocytes within the 15 °C infection group at week 6 and 7 P.E.

3.4. Analysis of leukocyte subpopulations in the AK and PK

For a further characterization of the cellular composition, subgroups of leukocytes were identified using MAbs staining IgM+ B cells, CD8- T cells and pan myeloid cells. Relative to this a flow cytometric analysis was performed in AK and PK leukocytes using these MAbs. At 12 °C there was an early significant increase at week 2 P.E in the number of pan myeloid cells in comparison to control (AK) and 15 °C infection group (PK). However, the more prominent significant differences occurred later in the experiment at week 6 and 7 P.E in the AK and PK of the 15 °C infection group. Here, the amount of pan myeloid cells significantly decreased in the AK (week 6 P.E) in comparison to the control and in the PK in comparison to the control (week 6 and 7 P.E) and relative to the 12 °C infection group (week 6 P.E) (Fig. 7.1-2). Moreover, in the PK, this was correlated by a significant increase of IgM+ B cells at week 6 P.E relative to the control and at week 7 P.E relative to the 12 °C infection group (Fig.8.1-2).

We assessed the leukocyte subpopulations of IgM+ B cells and pan myeloid cells using immunofluorescence microscopy in the PK. In the 12 °C infection group there were no clear differences from tagging in the PK in comparison to the control for either MAb. At week 6 P.E in the 15 °C infection group there were decreases in the amount of pan myeloid cells in the PK, relative to the control (Fig.7.3-4) and increases in the amount of IgM+ B cells, relative to the control (Fig.8.3-4). Furthermore, at 15 °C infected fish occasionally exhibited an alteration in the staining of IgM+ B cells. Positively stained cells displayed a gold-yellowish colour, in contrast to the usual bright red colour which may be due to an increased fluorescence intensity, implying more cell surface expression of IgM (Fig. 8.4). Negative controls, using PBS-T, did not show a positive tagging, confirming the positive staining's with these MAbs. Although on occasion some staining could be occasionally be observed in tubules of the PK. For a complete outline of all results from the analysis of leukocytes and their subsets refer to supplementary figures (S2, S3).

3.5. Immune gene analysis

We measured immune genes that could be grouped to either a functional cytokine pathway or cellular lineage. Here is a summary of the significant RT-qPCR results. For a complete outline of the RT-qPCR results as well as additional figures refer to supplementary figure (S5 and S6) and supplementary table 2 (A-D).

3.5.1. 12 °C Cytokines profiles

We measured pro/anti-inflammatory, Th1, Th2 and Th17-like signature cytokine profiles as well as the Treg-like master transcription factor Foxp3a. At 12°C, there was significant elevation of the archetypal pro-inflammatory cytokine IL-1 β in the AK at week 2 P.E relative to the control. At week 2 P.E, the expression profiles of Th1-like molecules (Tbet, IL-2 IFN γ , and TNF α 1+2) were varied. In the AK, IL-2 and TNF α 1+2 were significantly downregulated in comparison to the control. While, in the PK, Tbet was significantly downregulated and IFN γ and TNF α 1+2, significantly upregulated relative to the control. Whereas, at week 6 and 7 P.E in the AK and PK there was a Th1-like switch as all Th1-like molecules were upregulated and many significant (Fig.9).

Th2-like molecules (GATA3, IL4/13A and IL-10) were downregulated or transiently expressed in the AK and PK. Although, IL-10, a pleiotropic gene

associated with various immune functions in addition to its role as a Th2-like player, was significantly elevated at week 7 P.E in the AK (Fig.10.).

To gain an understanding of the Th17 and Treg-like response, IL-17C1 and the Treg master transcription factor Foxp3a were measured. IL-17C1 was significantly downregulated in the PK relative to the control at week 2 P.E, but became elevated at week 7 P.E in both AK and PK. Foxp3a was significantly downregulated at week 4 P.E in the PK relative to the control.

3.5.2. 12 °C Cellular lineage profiles

Concerning teleosts', MCSF is a mediator involved in regulating the numbers and functions of cells from the myeloid lineage and may contribute to myeloid heterogeneity. Hence, for the selection of genes for cells of the myeloid lineage we used MCSF and its receptor MCSF-R. In the present study, at 12 °C in the AK and PK, MCSF and MCSF-R were almost entirely unresponsive.

At 12 °C the expression of T cell signature molecules (CD4, CD8 α , CD β and TCR β) was highlighted by an early significant upregulation (week 2 P.E) of TCR β in the PK relative to the control. There was also significant elevation of CD8 β at week 7 P.E in the AK relative to the control. Further concerning lymphocyte subgroups, we measured B cell transcripts of IgM sec (secretory form of IgM), IgT mem (membrane bound form of IgT), Pax5, and Blimp1, however there was only one significant elevation concerning these molecules; IgM sec at week 2 P.E in the AK (Fig.11). In addition, MHC II, a molecule occurring on APCs (antigen presenting cells), was significantly downregulated at week 2 P.E in comparison to the control. Lastly, we examined the mRNA levels of NKEF, the natural killer cell enhancement factor, which exhibited marked differences in expression patterns between the temperatures, demonstrated by the >100-fold expression at week 6 P.E in the AK and significant upregulation at week 7 P.E relative to the control and to the 15 °C infection group (Fig.12).

3.5.3. 15 °C Cytokines profiles

At 15 °C, week 2 P.E, pro-inflammatory cytokines IL-1 β and TNF α 1+2 were significantly elevated. Despite this, their activity was modest for the remainder of the experiment. We wished to examine if there was any crosstalk or a skewed reaction differentiating into a Th1 or Th2-like phenotype, thus we completed an in-depth

analysis of cytokines associated with these functional pathways. At week 2 P.E, all Th1-like molecules were upregulated and predominantly significant in the AK and PK. At week 6 P.E, decreases in fold expression or downregulation occurred for all Th1-like molecules (Fig. 9). While at week 7 P.E, TNF α 1+2 was significantly downregulated relative to the control in the AK, but all Th1-like molecules were again upregulated in the PK with significant upregulation of Tbet relative to the control.

Concerning the expression patterns of Th2-like cytokines, all were elevated at week 2 P.E in both the AK and PK, with IL4/13A and IL-10 significantly expressed relative to the control and the 12 °C infection group. At week 4 P.E in the AK, GATA3, the Th2 master transcription factor, and IL-10 were significantly upregulated, as was IL4/13A in the PK in comparison to the 12 °C infection group. Strikingly, at week 6 P.E, IL-10 expression was significantly upregulated in the AK in comparison to the control, within group and in the PK in comparison to the control, 12 °C infection group and within group, with relative fold change in both AK and PK being >100 (Fig.10).

While Treg and Th17 cells are fundamentally different, they display many common features and we analysed them closely to explore this relationship. Both genes were responsive early in the study. IL-17C1 had an elevated presence, with significant upregulation shown in both AK (relative to 12 °C infection group) and PK (relative to control, 12 °C infection group and within group) at week 2 P.E (Fig .13). Foxp3A was expressed in both the AK and PK at all time points and significant at week 2 P.E in the PK and at week 7 P.E in the PK (Fig.13).

3.5.4. 15 °C Cellular lineage profiles

As detailed for the selection of markers for cells from the myeloid lineage we measured the mRNA levels of MCSF, which influences differentiation into macrophages and MCSF-R its receptor. Both MCSF and MCSF-R were slightly responsive at the first sampling, week 2 P.E, resulting in significant elevation of MCSF-R in the AK in comparison to the 12 °C infection group and of both MCSF and MCSF-R in the PK in comparison to the 12 °C infection group. But, this did not last and for the remainder of the experiment, both genes in the AK and PK were almost undetectable and significantly downregulated in comparison to the control (MCSF, AK week 6 and 7 P.E) and relative to the 12 °C infection group (MCSF-R, week 7 P.E).

All T cell CD phenotypes and associated molecules CD4, CD8 α , CD β and TCR β were mildly upregulated in both the AK and PK at week 2 P.E, with TCR β expression also being significant, relative to the control. Although, this was the peak of action for these molecules, except for that of CD4 in the PK which was significant at week 6 P.E. Generally, the activity of any T cell marker was completely overshadowed by the response of B cell signature genes in both the AK and PK, which amplified over the course of the experiment. At week 6 P.E all B cell signature genes in both the AK and PK were strongly upregulated (Fig. 11). The impact of these B cell expression signatures and their paramount contribution to the immune repertoire can be highlighted by the >100-fold expression recorded at week 4 P.E in the PK of IgM sec and week 6 P.E of Blimp1, the B cell differentiation factor, in both the AK and PK and, yet again of Blimp1 in the PK at week 7 P.E. Further, concentrating on molecules considered part of the adaptive immune response, MHC II was upregulated at all time points in both the AK and PK, which was significant at week 2 (PK) and 6 P.E (AK) relative to the 12 °C infection group. NKEF activity was for the most part unresponsive in both the AK and PK, significantly downregulated at week 7 P.E when compared to both control and the 12 °C infection group (Fig. 12).

4.0 Discussion

The aim of this study was to investigate the role of temperature on the immune response of rainbow trout to *T. bryosalmonae*. We selected the temperatures based on host-pathogen-specific knowledge and physiology. The temperature difference of 3°C reflects a realistic environmental change that could occur in the natural habitat of salmonids and influence their immune response to an invading pathogen. In contrast to previous immune studies on PKD, we characterised the time course of the disease from the initial response onwards. Different temperatures resulted in diverse outcomes in terms of the host immune response and in terms of parasite intensity.

4.1 At 12 °C the host immune response

At 12 °C we observed an asymptomatic infection characterized by minor pathological reaction in the tissue, minor to absent kidney swelling and no mortalities. Flow cytometric analysis indicated that there was an increase in the number of lymphocytes and a decrease in the amount of larger granular myeloid cells in the AK and PK. However, owing to the lower infection prevalence at 12 °C, the cellular

analysis was at times limited by the number of infected fish. We clustered genes into groupings of their functional pathways. Representative of this approach was Th1, (Tbet, IL-2, TNF α 1+2 and IFN γ) Th2, (GATA3, IL4/13A and IL-10) Treg (Foxp3a) and Th17 (IL-17C1). Although it is not conclusively demonstrated that Th-like functionality exists in fish, there is increasing evidence suggesting that these markers may have homologous physiological functions in fish to those they have in mammals [26]. Here we imply Th1 phenotypes may have a role in cell mediated cytotoxicity and Th2 phenotypes may have a role in activating B cells [27]. Touching on the Th1-like response at 12 °C, we observed upregulation of all Th1-like transcripts at week 6 and 7 P.E in both the AK and PK. In addition, expressed at these time points was NKEF, a member of the peroxidan family that enhances cytotoxic cell activity [28]. Furthermore, there was mild upregulation of cytotoxic T cell marker sub units, CD8 α and CD8 β , indicating a response of innate lymphocytic cytotoxic activity. These data correspond with the cellular composition in the tissue at these time points which had shown an increase in lymphocytes. In line with this, the mRNA levels of Tbet, IFN γ and TNF α 1+2 were elevated, which all are cytokines produced by group 1 ILCs (innate lymphoid cells). In addition, group 1 ILCs include natural killer cells and as NKEF was upregulated here (see above in this sub section), the results are suggestive of ILCs, although it is difficult to place these findings as ILCs have yet to be discovered in fish [29].

The expression levels of molecules (GATA3, IL4/13A and IL-10) did not indicate a Th2-like response. Although, IL-10, a pleiotropic gene associated with various immune functions in addition to its role as a Th2 player, was strongly upregulated at weeks 6 and 7 P.E in the AK. En masse the mRNA levels of Th1/Th2-like transcripts at 12 °C indicate a Th1-like response at week 6 P.E. Consequently, in a severe PKD infection crosstalk of Th1/Th2 has been described [6]. Granting this, skewed Th1-like switches have been suggested for carp infected with SVCV virus [30] and goldfish infected with the extracellular parasite *Trypanosoma carassii* [31]. While, as far as we are aware, a completely “Th2-skewed” response has not yet been shown in fish [32]. The Th2 bias in mammals is often propositioned when the immune response is initiated by extracellular parasites in the gut, but this correlates with antibody class switching to IgE, which is non-existent in fish [33]. A mammalian extracellular parasite driven class switch to IgE occurs as the parasites are too big to

be phagocytised and require recruitment, expansion, and differentiation of eosinophils and mast cells [34].

Concerning IL-10, discrepancies exist as to whether it should be considered a Treg/Th2 or anti-inflammatory cytokine. While we have grouped it as a Th2 cytokine, in the context of our results we discuss its functional purposes. IL-10 can suppress innate immune responses; promote T cell proliferation, memory B cells and IgM production [35; 36; 37]. In the present study, IL-10 was strongly upregulated in the AK at week 6 and 7 P.E, but not in the PK and there was no parallel elevation of T or B cell transcripts. Much mammalian literature focuses on IL-10 as an immunoregulator, in terms of controlling the adaptive response. For instance, studies of *Trichuris muris* and *Trypanosoma cruzi* demonstrated a key role for IL-10 in preventing a lethal T cell response [34]. Here, the lack of evidence for a Th2-like and/or adaptive response may be connected to IL-10 production. In addition, IL-10 may have played a role in the regulated inflammation in the tissue, allowing the parasite to persist in the absence of tissue damage in a host protective manner.

4.2. At 15 °C the host immune response

Infection prevalence and parasite intensity were ominously greater at 15 °C than at 12 °C. At 15 °C there was upregulation of many Th1/Th2-like cytokines at week 2 P.E. However, at week 6 P.E there was a switch, particularly in the PK, to a Th2-like/adaptive phenotype. This was suggested from the upregulation of GATA3, IL4/13A and a strong increase in mRNA levels (>100 fold) of IL-10 in AK and PK, an increase of lymphocytes and strong elevation of B cell expression signatures. These greater transcript levels of IL-10 may be a consequence of the parasite burden, as it has been reported in mammals during virulent infection with *Leishmania major* where high pathogen loads drive excessive Th1 responses and these, in turn, promote the development of self-limiting adaptive IL-10-producing T cells. These T cells dampen down Th1 responses, establishing a positive feedback loop whereby T cell-derived IL-10 inhibits pathogen clearance, allowing fulminant infections to develop [38; 39; 40]. Here, when IL-10 production decreased at week 7 P.E there was a return of a Th1-like response in the PK which might indicate a feedback loop.

IL-17C1 was upregulated in both the AK and PK at all time points apart from week 6 P.E, when it was downregulated in PK. So far, four IL-17 family members

have been discovered in rainbow trout, IL-17A/F2a, IL-17C1/C2, and IL-17D; however, their function in fish remains unclear [41]. Foxp3a the master transcription factor of the regulatory pathway was upregulated in the PK at every time point. Foxp3 in fish may mediate immune regulation like in mammals [42]. In mammals, the Th17 lineage has a close relationship with Foxp3+ Tregs, for example Th17 cells may exhibit both instability (not expressing their signature cytokine) and plasticity (expressing cytokines typical of other lineages) in response to a challenge, culminating in a transdifferentiation of Th17 cells into Tregs [43; 44]. Evidence of a close relationship between Foxp3+ Tregs and Th17 has also been reported in fish, for example a cloned zebrafish orthologue for mammalian Foxp3 (zFoxp3) induced a regulatory phenotype on mouse T cells and controlled IL-17 production in zebrafish embryos [42]. Accordingly, the expression patterns of Foxp3a and IL-17C1 in our study, given their functional capabilities, may demonstrate a counterbalance attempt to maintain tolerance, given the immune landscape. However, there may also be much broader implications for Foxp3, as demonstrated by a recent Nile tilapia study, which found Foxp3 protein expression present even in non-hematopoietic tissues in non-lymphoid organs [45].

As parasite intensity manifested, flow cytometric analysis showed a shift to a lymphoid response. This was further augmented by significant increases of IgM+ B cells and elevated mRNA levels of all B cell genes. In particular, Blimp1 was vigorously expressed (>50 relative fold change) in the AK and PK at week 6 and 7 P.E. Blimp1, an anti-proliferative and pro-differentiation regulatory factor, is normally induced upon B cell activation [46]. In the present study, combinatorial gene signatures of IgM sec, Blimp1 and Pax5 would suggest an inclination for the presence of plasma blasts and plasma cells, to that of pre/pro and mature B cell stages. Plasma blasts and plasma cells secrete Ig, express Blimp1 which in turn represses Pax5 [47]. Pax5 is expressed throughout B cell development from the pro-B to the mature B cell stage and is then repressed during terminal plasma cell differentiation [48]. Here, we had counter expression signatures of Pax5 and Blimp1 in the PK. Pax5 expression peaked at week 4 P.E in the PK, when Blimp1 expression was at its lowest, while Blimp1 expression was hyper upregulated at week 6 and 7 P.E, when Pax5 was at its lowest. This intense expression of Blimp1 mRNA at 15 °C may be indicative of some sort of disturbance in B cell differentiation and a disbalance among the various B cell maturation stages.

4.3 What are drivers of the difference in host immune response at 12 °C and 15 °C?

We have clearly shown the effect of temperature as a factor influencing the immune response in our study. The immune response of the fish host to the parasite may be directly modulated by the temperature difference between 12 °C and 15 °C, but also indirectly through the temperature effect on the parasite intensity. As recently reviewed by Markinos & Bowden (2016), it has been proposed that at low temperatures fish may rely more on innate immune responses, while at higher temperatures fish may rely more on adaptive immune responses [49]. Coincidentally, our results somewhat follow this stereotype, as at 12 °C infected fish relied more on innate type immune responses and at 15 °C infected fish relied more upon adaptive type immune responses. In the present study, we selected two temperatures that were in the physiological optimum temperature range of the host and that might reflect changes that occur in natural habitats of salmonids. Our results clearly show a correlation between severity of disease, host immune response and pathogen burden at the two temperatures.

We have also clearly shown the influence of temperature on parasite intensity. This greater parasite intensity at 15 °C could be a factor contributing to the different immune response at 15 °C, in particular since the parasite intensity at 15 °C compared to that at 12 °C was clearly higher than to be expected from the Q10 coefficient values. Still, different parasite intensities and accelerated metabolic rates at the 15 °C compared to 12 °C are not sufficient as explanation, mainly because the differences of the immune responses between the two temperature groups is not simply a quantitative one but as evident from figures 9 , 10 and 11 a qualitative one. The fish changes its immune defense strategy to deal with the parasite: while at 12 °C the fish immune strategy appears to tolerate the parasite and limit the harm caused by a given burden, at 15 °C it seems to mount a resistance response to limit the parasite burden. The tolerance strategy at 12 °C is evidenced here by the subtle fine-tuning of the immune response, such as the influx of lymphocytes, and expression of Th1 signature cytokines, NKEF and IL-10 in the AK and their ensuing effect on a stable parasite load. Granting this, as the parasite could not achieve 100% prevalence; equal consideration must be paid to the effect of temperature on virulence and on pathogenicity and how this related to pathogen burden, as well as the capabilities of the immune response which equally contribute to the stable, yet

tolerable system we have observed at the cooler temperature. The resistance strategy at 15 °C is evidenced by the over active immune response trying to combat the growing pathogen burden. But as parasite intensity increases the immune response must as well, which may provoke more pathogenic traits from the parasite ending in a severe infection. The high expression levels of genes in this system seem to suggest some sort of molecular exchange between the host and parasite. Furthermore, the grossly inflamed tissue at 15 °C experienced by hosts may not only result from the direct effects of parasite development but also from a chronic PKD immunopathology.

The differences we observed in parasite intensity did not clearly explain the differences we observed in the immune response. To substantiate this, we compared the immune response at time points when the parasite intensity was similar in the infection groups, such as at 12 °C weeks 7 P.E (35442) and at 15 °C week 4 P.E (48285). From this analysis, we can clearly show differences in the immune response when the parasite intensity is similar. For instance, at 15 °C there was a more pronounced pathological reaction in the tissue, increased amount of lymphocytes in the PK and increased mRNA levels of IgM sec (110.4 vs 2.8) and Pax5 (13.8 vs 3.9). This data demonstrates that the immune response is not just an indirect effect of parasite intensity but also temperature. Furthermore, the differences we observed were not temporally dependant, as there was not a faster immune response at the warmer temperature or a slower response at the cooler temperature. To illustrate this, we adhere to the immune response of 12 °C infection fish at week 7 P.E against the immune response of 15 °C infection fish at week 2 P.E. While there were parallels such as the upregulation of Th1-like cytokines, there were still major divergences. These included elevated expression patterns of Th2-like cytokines and of B and T cell markers, which were all expressed at 15 °C but not at 12 °C. We may even stress that the reason there was any common ground is due to the upregulation of almost every cytokine measured in the AK or PK at week 2 P.E in 15 °C infection fish.

5.0 Conclusion

We have detailed how an increase of only 3 °C appears to have an impact on parasite proliferation and on the biological and biochemical processes of the host, upsetting the delicate co-evolved host-pathogen system. Whereas at 12 °C the hosts

immunocompetence appears not to be negatively impacted and the host even tolerates a limited pathogen burden. Consequently, when contemplating who needs the hot-spot - it's not the fish.

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Figure legends

Figure.1. PKD disease dynamics (1) Prevalence and (2) Parasite intensity (Mean $\pm$ SE) at week P.E (post exposure) of the 12 °C infection group (white bars) and the 15 °C infection group (black bars). Parasite density was determined using copy numbers of parasite DNA as proxy. Different letters denote significant differences at $p \leq 0.05$; A indicates differences relative to the control (T-Test) B indicates differences between infection groups (T-Test) and C indicates within group differences when significant to all other time points (ANOVA). Asterisk indicates level of significance, $P < 0.005^*$, $P < 0.001^{**}$ and $P < 0.0001^{***}$.

Figure.2. Histological images of pathological changes in the posterior kidney taken at week 7 P.E. (1) At 12 °C, there are small focal areas of enlargement of the interstitial tissue (star). Additionally, there was an increased appearance of tubuloneogenesis (closed arrowhead), characterized by hyperbasophilia of tubular epithelial cells, large round nuclei and increased numbers of mitotic figures (arrows), bar = 100um, inlet = 50 um. (2) At 15 °C, interstitial tissue is multifocally to diffusely enlarged due to infiltration with immune cells and multiple parasites (star), scale bar = 100 um, inlet: *T. bryosalmonae* in a small capillary (closed arrowheads), surrounded by macrophages, scale bar = 50 um. All pictures are taken from slides stained with H&E.

Figure.3. (1) Posterior kidney somatic index of 12 °C infection group (triangles) and (2) 15 °C infection group (circles). Different letters denote significant differences at $p \leq 0.05$; A indicates differences relative to the control (T-Test) B indicates differences between infection groups (T-Test) and C indicates within group differences when significant to all other time points (ANOVA). Asterisk indicates level

of significance, $P < 0.005^*$, $P < 0.001^{**}$ and $P < 0.0001^{***}$. Uninfected exposed fish were omitted from analysis, resulting in the following number of samples for the 12 °C infection group, N=7 week 2, N=7 week 4, N=8 week 6 and N=8 week 7 P.E and for 15 °C infection group N=9 per time point

Figure. 4. Flow cytometric analysis of AK (anterior kidney) and PK (posterior kidney) lymphocytes (white bars) and myeloid cells (black bars) at week post-exposure (Week P.E) in infection groups. Data is presented as percentage of control (Mean $\pm$ SE) in the following order (1) 12 °C AK, (2) 12 °C PK, (3) 15 °C AK and (4) 15 °C PK. Different letters denote significant differences at $p \leq 0.05$; A indicates differences relative to the control (T-Test), B indicates differences between infection groups (T-Test) and C indicates within group differences when significant to all other time points (ANOVA). Asterisk indicates level of significance, $P < 0.005^*$, $P < 0.001^{**}$ and $P < 0.0001^{***}$. Two fish were pooled for flow cytometry and uninfected exposed fish were omitted from analysis, resulting in the following number of samples for the 12 °C infection group, N=3 week 2, N=2 week 4, N=2 week 6 and N=3 week 7 P.E and for the 15 °C infection group N=3 per time point.

Figure. 5. Representative dot plot of the FSC (size) and SSC (granularity) gating strategy and of the cellular kinetics seen at week P.E. in the 15 °C PK.

Figure. 6. Percentage of leukocytes (Mean $\pm$ SE) in peripheral blood of the 12 °C infection group (white bars) and the 15 °C infection group (black bars) at week (P.E) post-exposure. Different letters denote significant differences at $p \leq 0.05$; A indicates differences relative to the control (T-Test), B indicates differences between infection groups (T-Test) and C indicates within group differences when significant to all other time points (ANOVA). Asterisk indicates level of significance, $P < 0.005^*$, $P < 0.001^{**}$ and $P < 0.0001^{***}$. Uninfected exposed fish were omitted from analysis, resulting in the following number of samples for the 12 °C infection group, N=3 week 2, N=2 week 4, N=2 week 6 and N=3 week 7 P.E and for the 15 °C infection group N=3 per time point.

Figure. 7. Immunostaining of pan myeloid cells in both the AK (anterior kidney) and PK (posterior kidney). Box plot data is representative of flow cytometric analysis at specific week P.E (post exposure) and presented as percentage of control (Mean $\pm$ SE) for 12 °C and 15 °C infection groups in the following order (1) Pan

884 myeloid cells in the PK week 6 P.E (2) Pan myeloid cells in the PK at week 7 P.E.
 885 Two fish were pooled for flow cytometry and uninfected exposed fish were omitted
 886 from analysis, resulting in the following number of samples for the 12 °C infection
 887 group, N=3 week 2, N=2 week 4, N=2 week 6 and N=3 week 7 P.E and for the 15 °C
 888 infection group N=3 per time point. Immunofluorescence images from week 6 of (3)
 889 Vast amounts of tightly packed pan myeloid cells in the PK of a 15 °C control fish, in
 890 which the tissue structure remains intact in comparison to (4) Pan myeloid cells in the
 891 PK of a 15 °C infection fish, in which there is a decrease in the amount of pan
 892 myeloid cells in comparison to the control and infection induced changes in the tissue
 893 structure. Positive tagging appears bright red. Scale bar = 100 µm.

894 **Figure. 8.** Immunostaining of IgM+ B cells in both the AK (anterior kidney) and
 895 PK (posterior kidney). Box plot data is representative of flow cytometric analysis at
 896 specific week P.E (post exposure) and presented as percentage of control (Mean ±
 897 SE) for 12 °C and 15 °C infection groups in the following order. (1) IgM+ B cells in the
 898 PK at week 6 P.E and (2) IgM+ B cells in the PK at week 7 P.E. Two fish were pooled
 899 for flow cytometry and uninfected exposed fish were omitted from analysis, resulting
 900 in the following number of samples for the 12 °C infection group, N=3 week 2, N=2
 901 week 4, N=2 week 6 and N=3 week 7 P.E and for the 15 °C infection group N=3 per
 902 time point. Immunofluorescence images from week 6 of (3) Dispersed IgM+ B cells in
 903 the PK of 15 °C control fish and (4) IgM+ B cells scattered in the PK of a 15 °C
 904 infected fish. White circle indicates increase of fluorescence intensity which may
 905 imply overexpression of surface IgM. Positive tagging appears bright red. (4) Shows
 906 signs of interstitial and granuloma inflammation in comparison to the fine capillary
 907 networks and greater presence of tubuli seen in (3). Scale bar = 100 µm.

908 **Figure. 9.** Gene expression analysis of Th1-like cytokines measured in the
 909 AK (anterior kidney) and PK (posterior kidney) of the 12 °C infection group (white
 910 bars) and the 15 °C infection group (black bars) at week 6 P.E (post-exposure).
 911 Relative fold change was initially normalized to rainbow trout EF-1α and
 912 subsequently expressed as fold change relative to expression levels of control fish
 913 (Mean ± SE). Different letters denote significant differences at $p \leq 0.05$; A indicates
 914 differences relative to the control (T-Test), B indicates differences between infection
 915 groups (T-Test) and C indicates within group differences when significant to all other
 916 time points (ANOVA). Asterisk indicates level of significance, $P < 0.005^*$, $P < 0.001^{**}$

and $P < 0.0001^{***}$. Uninfected exposed fish were omitted from analysis, resulting in the following number of samples for 12 °C infection group, N=4 week 2, N=5 week 4, N=6 week 6 and N=5 week 7 post-exposure. For 15 °C infection group, N=6. At 12 °C, IL-2 was undetected at week 6 P.E in AK and PK of control samples; therefore it was omitted from analysis of all groups at this timepoint.

Figure. 10. Gene expression analysis of Th2-like cytokines measured in the AK (anterior kidney) and PK (posterior kidney) of the 12 °C infection group (white bars) and the 15 °C infection group (black bars) at week 6 P.E (post-exposure). Relative fold change was initially normalized to rainbow trout EF-1 α and subsequently expressed as fold change relative to expression levels of control fish (Mean $\pm$ SE). Different letters denote significant differences at $p \leq 0.05$; A indicates differences relative to the control (T-Test), B indicates differences between infection groups (T-Test) and C indicates within group differences when significant to all other time points (ANOVA). Asterisk indicates level of significance, $P < 0.005^*$, $P < 0.001^{**}$ and $P < 0.0001^{***}$. Uninfected exposed fish were omitted from analysis, resulting in the following number of samples for 12 °C infection group, N=4 week 2, N=5 week 4, N=6 week 6 and N=5 week 7 post-exposure. For 15 °C infection group, N=6 per time point.

Figure. 11. Gene expression analysis of B cell markers measured in the AK (anterior kidney) and PK (posterior kidney) of the 12 °C infection group (white bars) and the 15 °C infection group (black bars) at week 6 P.E (post-exposure). Relative fold change was initially normalized to rainbow trout EF-1 α and subsequently expressed as fold change relative to expression levels of control fish (Mean $\pm$ SE). Different letters denote significant differences at $p \leq 0.05$; A indicates differences relative to the control (T-Test), B indicates differences between infection groups (T-Test) and C indicates within group differences when significant to all other time points (ANOVA). Asterisk indicates level of significance, $P < 0.005^*$, $P < 0.001^{**}$ and $P < 0.0001^{***}$. Uninfected exposed fish were omitted from analysis, resulting in the following number of samples. For 12 °C infection group, N=4 week 2, N=5 week 4, N=6 week 6 and N=5 week 7 post-exposure. For 15 °C infection group, N=6 per time point.

Figure. 12. Gene expression analysis of NKEF measured in the AK (anterior kidney) and PK (posterior kidney) of the 12 °C infection group and the 15 °C infection group at week 6 P.E (post-exposure). Relative fold change was initially normalized to rainbow trout EF-1 α and subsequently expressed as fold change relative to expression levels of control fish (Mean $\pm$ SE). Different letters denote significant differences at $p \leq 0.05$; A indicates differences relative to the control (T-Test), B indicates differences between infection groups (T-Test) and C indicates within group differences when significant to all other time points (ANOVA). Asterisk indicates level of significance, $P < 0.005^*$, $P < 0.001^{**}$ and $P < 0.0001^{***}$. Uninfected exposed fish were omitted from analysis, resulting in the following number of samples. For 12 °C infection group, N=4 week 2, N=5 week 4, N=6 week 6 and N=5 week 7 post-exposure. For 15 °C infection group, N=6 per time point.

Figure. 13. Gene expression analysis of FoxP3a and IL-17C1 in the AK (anterior kidney) and PK (posterior kidney) of 15 °C infection fish at week P.E (post-exposure). Relative fold change was initially normalized to rainbow trout EF-1 α and subsequently expressed as fold change relative to expression levels of control fish (Mean $\pm$ SE). Different letters denote significant differences at $p \leq 0.05$; A indicates differences relative to the control (T-Test), B indicates differences between infection groups (T-Test) and C indicates within group differences when significant to all other time points (ANOVA). Asterisk indicates level of significance, $P < 0.005^*$, $P < 0.001^{**}$ and $P < 0.0001^{***}$. N=6 per time point.

Table 1. *Tetracapsuloides bryosalmonae* primers and probe. Sequences used for qPCR to quantify parasite intensity.

Gene	Acc. number	FWD primer (5'-3')	REV primer (5'-3')	Probe	BP	Ref.
<i>T. bryo</i> 18S	KF805631	GCGAGATTTGTTGCATT TAAAAAG	GCACATGCAGTGTCCAA TCG	CAAATTGTGGAACCGTCCGAC TACGA	73	15

Table 2. The specificity of monoclonal antibodies used in the study table is adapted from [17].

Name	Fluorochrome	Specificity	Stained cells	Ref.
MAbN2	RPE	Igc light chain	Igc+ light chain positive B cells	17
MAb1.14	RPE	IgM	IgM+ B cells	20
MAb21	RPE	ND	Pan myeloid cells	21
MAbD30/D11	Dylight 405	ND	CD8- T cells	22

Figure 1

Figure 1 consists of two panels, (1) and (2), showing histological sections of the testis. Panel (1) is a low magnification view of a testis section, showing seminiferous tubules and interstitial cells. A scale bar is present in the bottom right. An inset in the top left shows a higher magnification of the interstitial space, with arrows pointing to specific cells. Panel (2) is a high magnification view of the interstitial space, showing Leydig cells and blood vessels. A scale bar is present in the bottom right. An inset in the top left shows a higher magnification of the interstitial space, with arrows pointing to specific cells.

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

Figure 12

Figure 13