Received: 10 April 2024 | Revised: 28 June 2024 | Accepted: 5 July 2024

DOI: 10.1111/jfd.14001

RESEARCH ARTICLE

Journal of Fish Diseases WILEY

# Inflammatory responses in Atlantic lumpfish (*Cyclopterus lumpus* L.) after intraperitoneal injection of a vaccine against *Aeromonas salmonicida* and *Vibrio salmonicida* at different water temperatures

**Ingrid Svihus Knutsen** | **Toni Erkinharju** | **Jarl Bøgwald** | **Roy A. Dalmo** | **Tore Seternes**

Faculty of Biosciences, Fisheries and Economics, Norwegian College of Fishery Science, UiT the Arctic University of Norway, Tromsø, Norway

**Correspondence**
Tore Seternes, Faculty of Biosciences, Fisheries and Economics, Norwegian College of Fishery Science, UiT the Arctic University of Norway, Tromsø, Norway.
Email: tore.seternes@uit.no

**Funding information**
University of Tromsø, Grant/Award Number: 301401 and 325159

## Abstract

Studying inflammatory responses induced by vaccination can contribute to a more detailed understanding of underlying immune mechanisms in lumpfish (*Cyclopterus lumpus*). Tissue samples from lumpfish intraperitoneally immunized with a divalent oil-adjuvanted vaccine (*Aeromonas salmonicida* and *Vibrio salmonicida*) at water temperatures of 5, 10, and 15°C were collected at 630 day degrees and 18 weeks post injection. The relative amount of secretory and membrane-bound immunoglobulin M (IgM) gene transcripts in the head kidney was determined by qPCR. Vaccine-induced inflammatory lesions were assessed on histological sections of abdominal pancreatic/intestinal tissue from vaccinated fish in all three temperature groups. Inflammatory cells forming dense aggregations in lesions showed proliferative activity, many of which were identified as eosinophilic-granulocyte-like cells. IgM+ cells were scattered in inflammatory tissue dominated by connective tissue, showing no difference in numbers between lesions from fish vaccinated at 5, 10, and 15°C. Relative gene expression analysis of secretory and membrane-bound IgM revealed low overall expression in the head kidney of vaccinated fish at both 630 day-degrees and 18 weeks post injection. The results of this study indicate that the vaccine stimulated prolonged local inflammatory responses at the injection site, which were not influenced by temperature.

KEYWORDS
*Aeromonas salmonicida*, Atlantic lumpfish, immune response, temperature, vaccine

## 1 | INTRODUCTION

Sea lice infestation on salmonids is a major challenge in the aquaculture industry resulting in substantial economic losses due to associated fish mortalities and degraded product (Elghafghuf et al., 2020; Johnson et al., 2004). Various strategies have been implemented to deal with the issue including the use of lumpfish and various species of wrasse as cleaner fish. Biological control of sea lice has proven to be effective as several cleaner fish species actively feed on lice (Imsland et al., 2018; Skiftesvik et al., 2013; Treasurer, 2002).

Lumpfish (*Cyclopterus lumpus* L.) is a semi-pelagic marine teleostean species inhabiting northernly on both sides of the Atlantic Ocean. In Norway, the species is distributed along the entire coastline and can migrate far distances for search for food in deep waters (Erkinharju et al., 2020). The demand for lumpfish has led to establishment of several production farms. Today, the majority of those deployed in sea cages in Norway originate from farmed broodstock (Erkinharju et al., 2020). Farmed lumpfish enable the fish to be deployed in sea cages when they have reached optimal sizes. This reduces the dependency on wild populations (Imsland et al., 2020). The increased demand for lumpfish can be attributed to the species´ adaptility for low water temperatures, where they can maintain efficient sea lice grazing as opposed to the temperate wrasse species (Imsland et al., 2014; Powell et al., 2018). Lumpfish are thus considered as the better cleaner fish option at salmon farms located in northern regions (Imsland et al., 2014).

Lumpfish are susceptible to various *Vibrio* species, typical and atypical *Aeromonas salmonicida*, *Pasteurella* sp., *Pseudomonas anguilliseptica*, *Moritella viscosa* and *Tenacibaculum* spp. (Sommerset et al., 2020). However, disease outbreaks, often followed by substantial mortalities, are associated with atypical strains of *A. salmonicida* causing atypical furunculosis (Rønneseth et al., 2017).

The high prevalence of bacterial infections among lumpfish substantiates the need for efficient prophylactic measures. This refers to vaccination or unspecific stimulation of the immune system amplifying adaptive or innate immune mechanisms (Rønneseth et al., 2017). Oil adjuvants are typically included in injection vaccines to increase efficiency and produce strong local inflammatory response (Evensen et al., 2005). There is a risk of oil adjuvants, especially mineral oils, producing severe side effects following vaccination. Such side effects include extensive adhesions between internal organs and the abdominal wall and intra-abdominal lesions, melanization of tissue, growth reduction, and systemic autoimmunity (Koppang et al., 2005, 2008). There are five commercially available vaccines for lumpfish (Haugland et al., 2018). In addition, certified vaccine production companies offer autogenous vaccines. Most commercial vaccines combine strains of atypical *A. salmonicida* and *V. anguillarum* and are administered by immersion or injection depending on fish size. Injection vaccines are formulated with oil-adjuvant and there have been some reports of development of adhesions, though not severe. Lumpfish are typically immunized with injection vaccines when above 10 g (Haugland et al., 2018). Despite the currently available vaccines, researchers have emphasized the necessity for developing more efficient immune prophylactic measures (Erkinharju et al., 2020; Powell et al., 2018). Studies have shown that differences in environmental temperatures can affect the immune system and response after immunisation. For lumpfish, we have previously shown significant temperature dependency of specific antibody levels after vaccination at different water temperature levels (Erkinharju et al., 2018). After vaccination of lumpfish at 5°C, there was no specific antibodies in serum against *Aeromonas salmonicida* for up to 18 weeks post immunization.

This study aimed at investigating inflammatory responses due to vaccination of lumpfish by histological techniques, immunohistochemistry, and gene expression analysis. Samples from fish intraperitoneally injected with a divalent oil-adjuvanted vaccine were derived from a previous experiment, demonstrating different humoral responses of fish vaccinated at 5, 10, and 15°C (Erkinharju et al., 2018). The focus of this study was characterization of any differences in inflammatory responses among vaccinated lumpfish kept in different water temperatures, and in addition, to evaluate the influence of vaccine on growth rate and body condition of fish.

## 2 | MATERIALS AND METHODS

Atlantic lumpfish (*Cyclopterus lumpus* L.) were kindly donated by Akvaplan-niva AS (Tromsø). The fish were kept in the rearing facilities at the Aquaculture Station in Tromsø, in circular 140 L tanks with continuous flow of sea water as described in Erkinharju et al. (2018). The experiment was approved by the Norwegian Animal Research Authority (NARA), with ID number 6843.

Lumpfish were divided into three water temperature groups of 5, 10, and 15°C and acclimatized for 14 days. The fish received an intraperitoneal injection with a vegetable oil-adjuvanted vaccine containing formalin-inactivated bacterial antigens of *Aeromonas salmonicida* and *Vibrio salmonicida and sampling were done as described in* Erkinharju et al. (2018). In the present study, we selected tissue samples from a total of 20 fish (10 vaccinated and 10 control fish) in each temperature group sampled at 630 days degrees post injection (ddpi) and 18 weeks post injection (wpi) for all temperature groups for histological examination. In addition, head kidney samples from six high antibody responders from each temperature group as identified by Erkinharju et al. (2018) were included for gene expression analysis.

### 2.1 | Histology

#### 2.1.1 | Tissue processing

Formalin-fixed tissue samples of kidney, heart, spleen, liver, and pyloric caeca, including pancreatic and abdominal adipose tissue, were prepared for histological and immunohistochemical staining using standard procedures. The tissue samples were stored in 70% ethanol, dehydrated and embedded in paraffin wax (Leica EG 1150H, Leica Biosystems). The paraffin blocks were sectioned at 5 µm thickness (Leica RM2235, Leica Biosystems) and mounted on either regular or poly-L-lysine-coated glass slides (Thermo Scientific). Haematoxylin and eosin (HE) were used as staining dyes for the standard histological examination (Fischer et al., 2008). Two special histochemical staining techniques were performed on selected tissue sections from lumpfish, namely, May-Grünwald-Giemsa and van Gieson. The polychromatic staining technique May-Grünwald-Giemsa (MGG) was used to distinguish different leukocytes present in abdominal inflammatory lesions (Dey, 2018). The van Gieson staining technique was used for detection of connective tissue in abdominal inflammatory lesions (Bruce-Gregorius, 1974).

### 2.1.2 | Immunohistochemistry

Immunohistochemistry (IHC) was performed on tissue sections from vaccinated fish in which inflammation had been detected on HE-stained sections. Three primary antibodies were used for IHC, namely polyclonal rabbit anti-*Aeromonas salmonicida* LPS (Roy A. Dalmo and Jarl Bøgwald, UiT), polyclonal rabbit anti-lumpfish IgM (a gift from Ivar Hordvik, produced according to Bilal et al., 2016) and monoclonal mouse anti-proliferating cell nuclear antigen (anti-PCNA; Dako).

Immunohistochemistry was done as described by Erkinharju et al. (2019) with the following changes; For immunostaining, anti-*A. salmonicida* LPS, anti-IgM and anti-PCNA were diluted to 1:5000, 1:6000 and 1:3200, respectively. Normal horse serum (2.5%) was used as blocking agent. PIER (proteolytic-induced epitope retrieval) was used for retrieving *A. salmonicida* antigens in the tissue. For anti-*A. salmonicida* LPS and anti-PCNA, the tissue sections were incubated for 1 h at room temperature or overnight at 4°C. Polymer-based chromogenic detection systems with alkaline phosphatase were used for antigen signal amplification (ImmPRESS-AP Horse Anti-Rabbit IgG Polymer Reagent and ImmPRESS-AP Horse Anti-Mouse IgG Polymer Reagent, Vector Laboratories, Inc.).

The number of IgM+ cells in kidney, spleen, and inflammatory tissue was found by counting of stained cells on micrographs taken of the sections following IHC with anti-IgM. Eight and four micrographs of the same kidney sections from ten fish were analysed. The total number of positive stained cells in eight and four photographs was then divided by the number of photographs taken.

## 2.2 | RNA isolation, cDNA synthesis and quantitative PCR (qPCR)

RNAlater-preserved head kidney samples from vaccinated and control lumpfish in temperature groups 5, 10, and 15°C sampled at 630 ddpi and 18 wpi were used for relative gene expression analysis of secretory IgM (sIgM) and membrane-bound IgM (mIgM) (Kuang et al., 2018). The six samples from vaccinated fish were selected based on earlier collected data on specific antibody responses against *A. salmonicida* (Erkinharju et al., 2018).

Extraction of RNA was performed using the RNeasy Mini Kit from Qiagen. The samples were trimmed into small pieces (ca 30 mg) and placed in RLT lysis buffer (Qiagen) containing 20 µL 2 M DDT and steel beads (4.5 mm, Action Sport Games A/S). The tubes were inserted into TissueLyser II (Qiagen). RNA concentration and purity were assessed by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The RNA samples were diluted with RNase-free water to a concentration of 50 ng/µL prior to cDNA synthesis by which the QuantiTect Reverse Transcription Kit from Qiagen was used. Two µl of gDNA wipeout buffer was added to a sample volume of 12 µL, including 10 µL of diluted RNA and 2 µL of RNase-free water, to eliminate genomic DNA following incubation at 42°C for 2 min. After incubation, 6 µL of a master mix with the reagents Quantiscript Reverse Transcriptase (RT), Quantiscript RT buffer and RT Primer Mix were added to the samples, according to the manufactures protocol, for a final reaction volume of 20 µL. SYBR-Green-based qPCR was used for detection of elongation factor 1 alpha (EF1-α), sIgM, and mIgM transcripts in cDNA samples. EF1-α was used as reference gene. Primers for amplification of target sequences (Table 1) were derived from previous work (Erkinharju et al., 2019). The concentration of the primer solutions was 5 µM.

The cDNA sample duplicates were set up on a 96-well plate (MicroAmp® EnduraPlate™ Optical 96-Well Fast Clear Reaction Plate, Applied Biosystems) for each gene. The total reaction volume in each well was 20 µL constituting 5 µL of diluted cDNA and 15 µL of a master mix. For each sample, the master mix included 10 µL of SYBR master (Fast SYBR™ Green Master Mix, Applied Biosystems), 4 µL of ultrapure water and 1 µL of primer solution corresponding to a final concentration of 0.25 µM. Wells with ultrapure water instead of cDNA and ÷RT samples were included on each plate. The samples were run in a 7500 Fast Real-Time PCR System (Applied Biosystems). Annealing temperature was 60°C, for 30 s and 40 cycles. Melting curve analysis was performed after the qPCR run to confirm amplification of specific products.

## 2.3 | Welfare indicators

Specific growth rate (SGR) and condition factor (*K*) were calculated to assess the influence of vaccination on growth and body condition of lumpfish. The mean specific growth rates (SGR) from 0 to 126 days post vaccination and control lumpfish in temperature groups 5, 10 and 15°C were calculated from available weight data using equation I: $SGR = (Ln(wt) - Ln(wi))/t \times 100$ where Ln(wt) is the natural logarithm of the weight at time *t* (day 126) and Ln(wi) is the natural logarithm of the initial weight (day 0). SGR expresses percentage increase in growth per day (Hopkins, 1992). Fulton's condition factor (*K*) of individual lumpfish at 126 days post injection was calculated from available weight and length data using equation II:

$K = W/L3 \times 100$ (II) where *W* is the weight (g) and *L* is the length (cm) of the fish (Froese, 2006).

**TABLE 1** Primer sequences and efficiencies used for fast SYBR-Green qPCR, from Erkinharju et al. (2019).

| Gene | Forward primer | Reverse primer | Efficiency |
|---|---|---|---|
| EF1-α | GGCCAGATCAATGCCGGATA | CTCCACAACCATGGGCTTCT | 2.05 |
| sIgM | AGAACCAGTATGGGACGGGA | ACACTGACGGTCGTTGAGTC | 1.99 |
| mIgM | ACGAATGGAACAAGGGGACA | AGCAGTGGTTCCAATGGTGA | 1.94 |

### 2.4 | Data analysis

The expression of mIgM and sIgM in head kidney samples of lumpfish was analysed using relative quantification. The generated Cq-values from the qPCR assays were used for calculating changes in target gene expression in samples relative to a control sample, reported as fold-change or expression ratio. The control sample included the average of Cq values of control fish samples in each temperature and sampling time point group. The Cq-values of target genes were normalized to the reference gene EF1-α to account for variations between samples such as different starting material. Relative gene expression (ratio) was calculated with correction for different amplification efficiencies using the Pfaffl method (Pfaffl, 2001).

### 2.5 | Statistical analysis and graphics

Data processing including calculations of SGR, *K*, and relative gene expression was performed in Microsoft Excel 365 (Microsoft Corp.). Statistical analysis and graphical representation of data were performed in SPSS Statistics for Windows (IBM Corp.) and GraphPad Prism 9 (GraphPad Software Inc.). Normality distribution of data was assessed using the Shapiro–Wilk test, while Levene's test was used to check for homogeneity of variances. Two-way ANOVA was used for estimation of differences in means between groups, followed by Tukey's post hoc test for pairwise comparisons. Data was log-transformed to meet assumptions of normality. For data with unequal variances, Welch's *T*-test was used to assess differences in group means. A statistical significance level of 5% ($p < .05$) was set for all tests.

## 3 | RESULTS

### 3.1 | Histology

HE-staining was performed on tissue samples from kidney, heart, spleen, liver, pyloric caeca, and pancreatic and abdominal adipose tissue.

#### 3.1.1 | Pancreatic and adipose tissue

Observations of inflammatory tissue were noted in several of the HE-stained abdominal tissue sections derived from vaccinated fish (Figure 1).

#### 3.1.2 | Intra-abdominal inflammatory tissue

Intra-abdominal inflammatory tissue was more frequently detected in samples from vaccinated lumpfish in temperature groups 10 and 15°C compared to temperature group 5°C (Table 2). The occurrence of lesions did not differ markedly between samples taken from vaccinated fish in the 10 and 15°C groups at 18 wpi and 630 ddpi. Tissue resembling the inflammatory lesions of vaccinated fish was detected in samples from six control lumpfish in temperature group 15°C (three fish at each sampling time point, 18 wpi and 630 ddpi).

The lesions were located periviscerally in between the pancreatic and adipose tissues (Figure 2a,c,e) or localized in the connective tissue capsule of the spleen (not shown). Aggregations of inflammatory cells were, in most cases, centred around vaccine oil droplets (Figure 2a–f). Some of the cells constituting clusters within lesions were easily distinguished due to bright pink cytoplasmic staining. In sections from fish in temperature groups 15 and 10°C, at both sampling time points (18 wpi and 630 ddpi), such clusters were often seen and tended to surround oil droplets (Figure 2d,f). Pyknotic cell nuclei were seen in cell clusters, indicating cellular degradation or necrosis (Figure 2f). Such cell clusters were not observed in sections from fish in the 5°C group. The inflammatory tissue was merely as dispersed inflammatory cells throughout the abdominal connective tissue (Figure 2a), where cells exhibited similar staining pattern as those in clusters (bright pink; Figure 2b).

#### 3.1.3 | Special staining

*May-Grünwald-Giemsa (MGG)*

Leukocytes with eosinophilic cytoplasmic granules (granulocyte-like cells) and basophilic staining of the nucleus (other leucocytes) were detected in lesions on all examined sections. In lesions of vaccinated

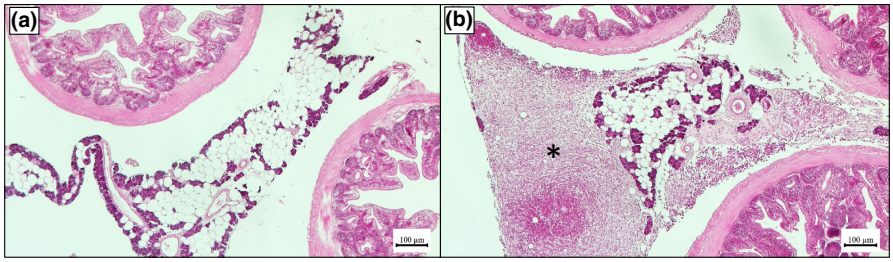


FIGURE 1 HE staining of exocrine pancreas (ep) and adipose tissue (ad) located between pyloric caeca (py) from control (a) and vaccinated (b) lumpfish. (a) shows normal tissue histology, whereas (b) shows inclusion of perivisceral inflammatory tissue indicated by an asterisk (*). Magnification is 5× and scale bar is 100 µm.

fish in all the three temperature groups, such cells were quite many. Often, the cells were scattered in loosely arranged inflammatory tissue (Figure 3a–d) and surrounded by oil droplets (Figure 3e,f). The photographed section in e and f (Figure 3) illustrates an instance in which the eosinophilic granulated cells were found in high numbers around oil droplets. It should be noted that cells showing basophilic staining were also detected in areas of such granulocyte clustering, but these were not identified as granulocytes. Similar findings were noted in lesions from vaccinated fish sampled at 630 ddpi (not shown).

*Van Gieson's staining*

For all sections examined, collagen was detected. Collagen was extensively distributed within the loosely arranged inflammatory tissue and notably surrounded aggregations of inflammatory cells centred around oil droplets (Figure 4a–f). This general pattern was also applied to inflammatory lesions from fish sampled at 630 ddpi (not shown).

**TABLE 2** Number of vaccinated (VAX) and control (PBS) lumpfish in which inflammatory tissue was detected on HE-stained sections of abdominal tissue sampled at 18 wpi and 630 ddpi (italics).

| | 5°C | | 10°C | | 15°C | |
|---|---|---|---|---|---|---|
| Wpi | VAX | PBS | VAX | PBS | VAX | PBS |
| 6 | – | – | – | – | *9/10* | *3/10* |
| 9 | – | – | *7/10* | *0/10* | – | – |
| 18 | *4/10* | *0/10* | 7/10 | 0/10 | 8/10 | 3/10 |

*Note*: 630 ddpi corresponds to 6, 9, and 18 wpi for temperature groups 15, 10, and 5°C, respectively. $n = 10$ for VAX and PBS-injected fish in all three temperature groups at all sampling time points. Dash (–) indicates information not available.

### 3.1.4 | Immunohistochemistry

Immunohistochemistry (IHC) was performed on tissue sections from fish in which inflammation had been observed following HE-staining.

*Vaccine antigen*

Positive staining of *A. salmonicida* antigen was clear in all the intraabdominal inflammatory lesions from vaccinated fish in temperature groups 5, 10, and 15°C at both sampling time points (18 wpi and 630 ddpi). At 630 ddpi the vaccine antigen was found in the periphery of oil droplets and was scattered throughout the inflammatory tissue at varying degree (Figure 5). In sections of abdominal tissue from control fish, presence of *A. salmonicida* LPS was not detected. At 18 wpi the staining pattern of *A. salmonicida* LPS was similar to that observed in lesions of vaccinated fish at 630 ddpi including intense staining along the rim of oil droplets (not shown). Mild staining of *A. salmonicida* was observed in sections of the kidney, heart, and spleen of vaccinated and some control fish.

*PCNA*

Proliferating cells (PCNA+) were detected in high numbers in most of the intraabdominal inflammatory lesions in samples from vaccinated fish in all three temperature groups (Figure 6a–f). The PCNA+

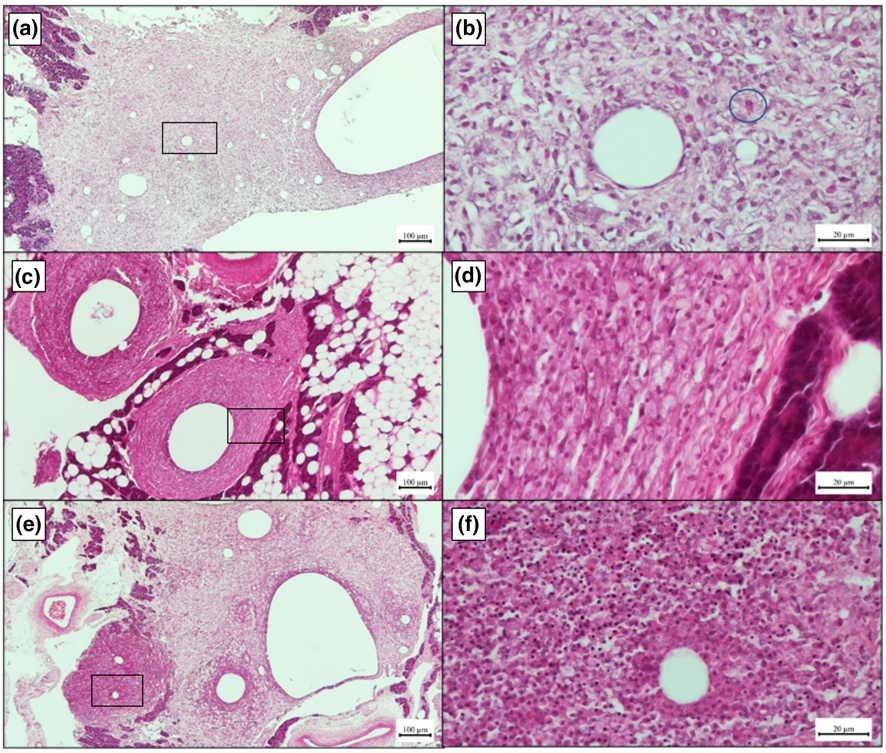


**FIGURE 2** HE staining of inflammatory lesion sections of vaccinated lumpfish in temperature groups 5°C (a, b), 10°C (c, d), and 15°C (e, f) at 18 wpi. The inflammatory lesion sections are displayed at magnification 5× to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 40× to the right in the figure (b, d, f). The circle in (b) indicates a single cell with a similar staining pattern and morphology as those seen around oil droplets in (d) and (f). Scale bar is 100 μm for a, c, e (5×) and 20 μm for b, d, f (40×).

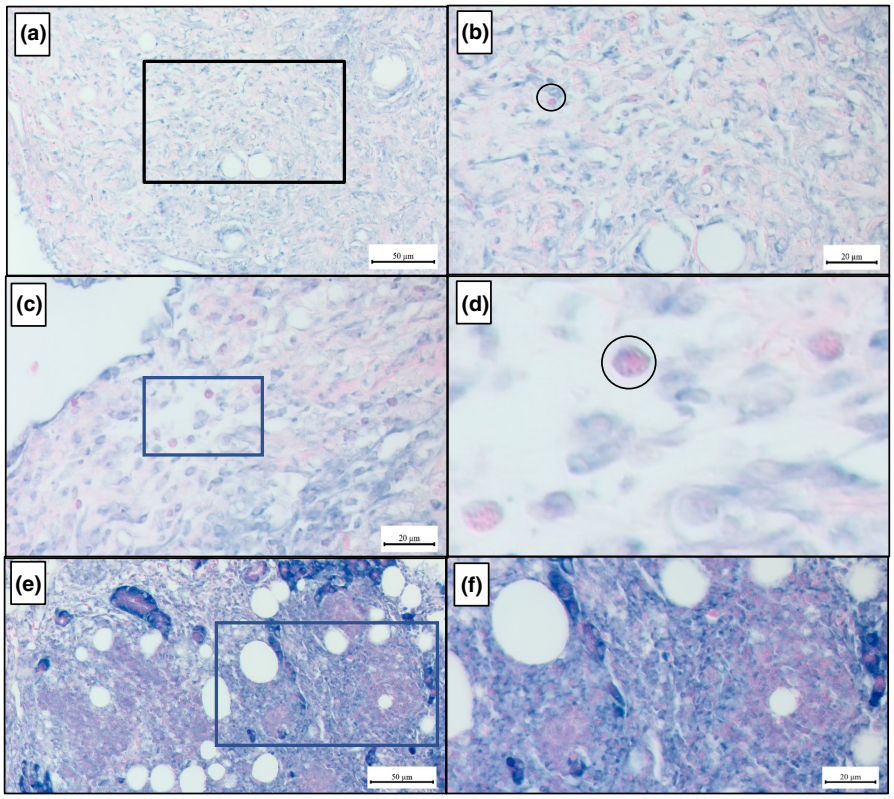


FIGURE 3 MGG staining of inflammatory lesion sections of vaccinated lumpfish in temperature groups 5°C (a, b), 10°C (c, d), and 15°C (e, f) at 18 wpi. Note the presence of cells with eosinophilic granules. The inflammatory lesion sections are displayed at magnification 20× (a, e) and 40× (c) to the left in the figure. The black squares indicate the areas displayed at magnification 40× (b, f) and (d) (80× digital magnification) to the right in the figure. The circles in (b) and (d) indicate single cells with eosinophilic granules. Scale bar is 50 µm for a, e (20×) and 20 µm for b, c, f (40×).

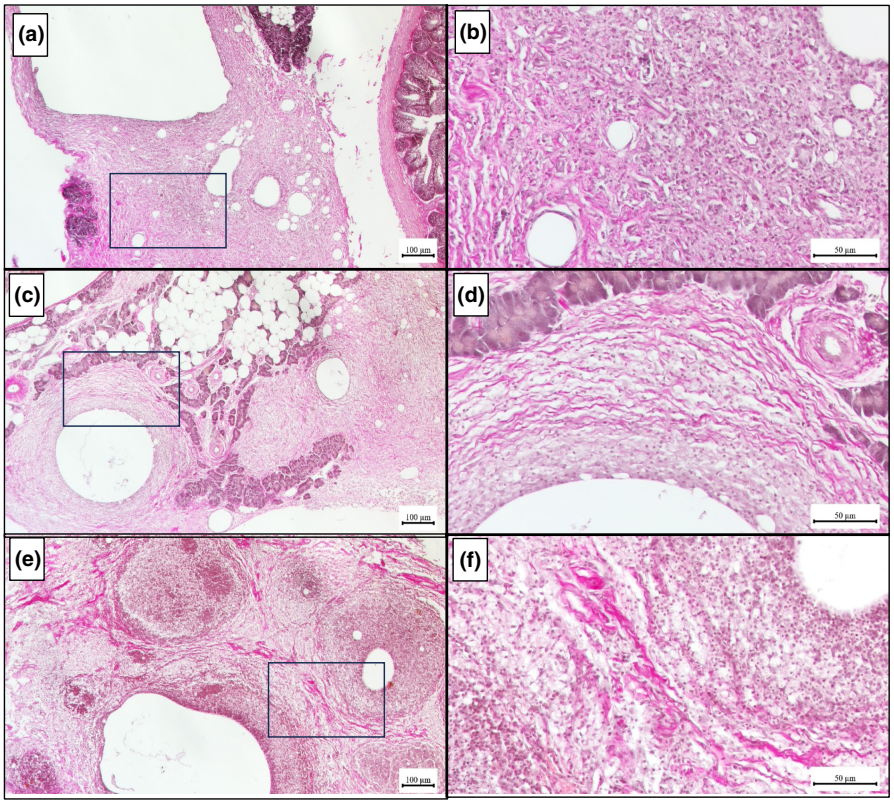


FIGURE 4 VG staining of inflammatory lesion sections of vaccinated lumpfish in temperature groups 5°C (a, b), 10°C (c, d), and 15°C (e, f) at 18 wpi. Note the presence of collagen indicated by bright pink staining. The inflammatory lesion sections are displayed at magnification 5× to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 20× to the right in the figure (b, d, f). Scale bar is 100 µm for a, c, e (5×) and 50 µm for b, d, f (20×).

cells were scattered in the loosely organized inflammatory tissue (Figure 6c–f) and cells in dense aggregations around oil droplets (Figure 6e,f). The tissue in samples from corresponding area from control fish did not show positive staining by PCNA. The number and distribution of PCNA+ cells in lesions from vaccinated fish at 630 ddpi were similar to those noted in lesions derived from fish sampled at 18 wpi (not shown). There were no clear differences in PCNA staining intensity in kidney and spleen sections from fish in the 5, 10, and 15°C temperature groups at 18 wpi, in either vaccinated or control fish.

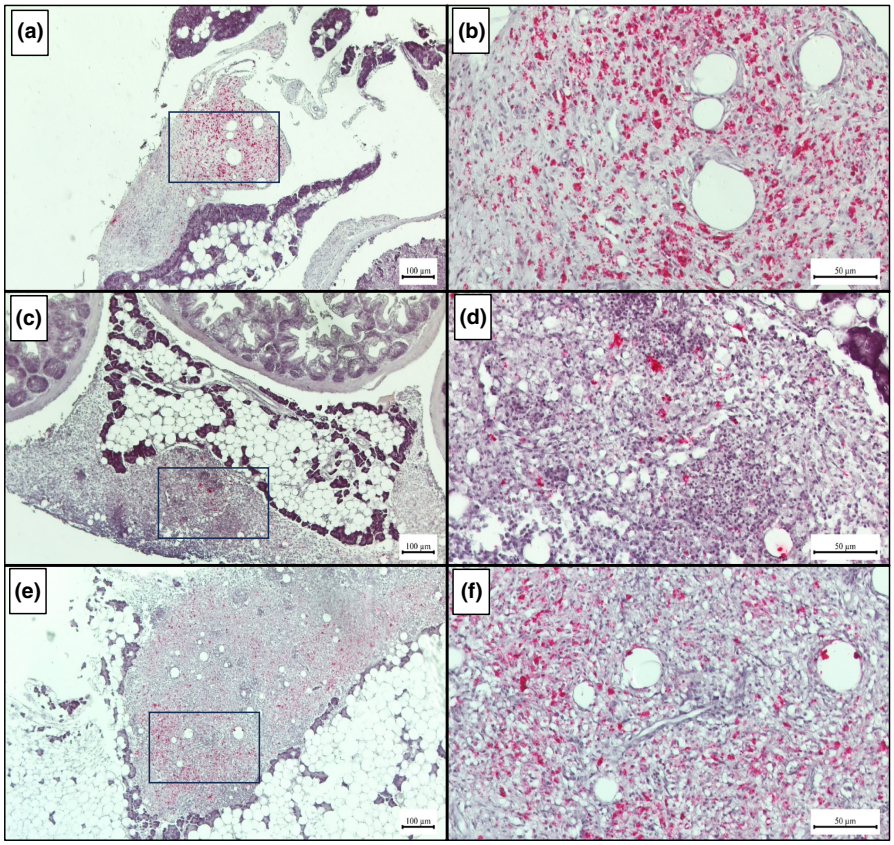


**FIGURE 5** Immunohistochemical staining of *Aeromonas salmonicida* LPS in inflammatory lesion sections from vaccinated lumpfish in temperature groups 5°C (a, b), 10°C (c, d), and 15°C (e, f) at 630 ddpi. Red colouration indicates presence of vaccine antigen. The inflammatory lesion sections are displayed at magnification 5× to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 20× to the right in the figure (b, d, f). Scale bar is 100 µm for a, c, e (5×) and 50 µm for b, d, f (20×).

*IgM*

Sections of the kidney, spleen, and intra-abdominal inflammatory tissue were used for immunohistochemical detection of IgM.

*Kidney*

The average number of IgM+ cells in kidney sections of vaccinated and control fish was similar across the different temperature groups of 5, 10, and 15°C. The IgM+ cells were scattered in the tissue (Figure 7a–f) and, in some instances, clustered around tubules or glomeruli in kidney sections from both vaccinated and control fish (Figure 7b,f). Diffuse staining was also seen among the haematopoietic kidney tissue in all the examined sections. Similar staining pattern of IgM was observed in kidney sections of vaccinated and control fish in the 5, 10, and 15°C temperature groups at 630 ddpi (not shown).

*Spleen*

There were some notably large individual differences in the number of IgM+ cells in spleen sections of vaccinated and control fish both within and across temperature groups. On a temperature group level, the highest number of IgM+ cells were detected in spleen sections from fish in the 5°C group. Overall, vaccinated and control fish did not show any large difference in the number of IgM+ cells in the spleen in any of the temperature groups. The IgM+ cells were scattered in the spleen of vaccinated and control fish in all three temperature groups (Figure 8a–f). The staining intensity of cells differed among the sections examined. In a few instances, clustering of IgM+ cells was seen around blood vessels (Figure 8b).

*Intra-abdominal inflammatory tissue*

Staining revealed presence of IgM+ cells in lesions of vaccinated fish in the 5, 10, and 15°C temperature groups at both sampling time points (630 ddpi and 18 wpi). There was no general trend in the abundance of IgM+ cells in lesions of vaccinated fish of either group. IgM+ cells were detected in lesions in three out of four vaccinated fish in the 5°C group. For vaccinated fish in the 10°C group at 18 wpi IgM+ cells were detected in the lesions from six out of seven. All lesions from fish in the 15°C group stained positive for IgM at 18 wpi. At 630 ddpi, positive staining of IgM appeared in lesions from six out of seven fish in the 10°C group. IgM+ cells were detected in the lesions from all nine fish in the 15°C group.

At 18 wpi, IgM+ cells tended to be distributed in loosely arranged inflammatory tissue of vaccinated fish in all three temperature groups (Figure 9a–f). This was especially noticeable in lesions derived from vaccinated fish in the 10 and 15 °C temperature groups which also included areas of more densely arranged cells (Figure 9c,e). With one exception, the presence of IgM+ cells was confined to the areas of loose inflammatory tissue surrounding dense aggregations of cells in sections from fish vaccinated at 10 and 15°C (Figure 9d,f). Positive staining of IgM was also detected in some of the sections from control fish; two in the 5 and 10°C groups and three in the 15°C group. Diffuse staining was seen in some arteries, while a couple of positive cells were found

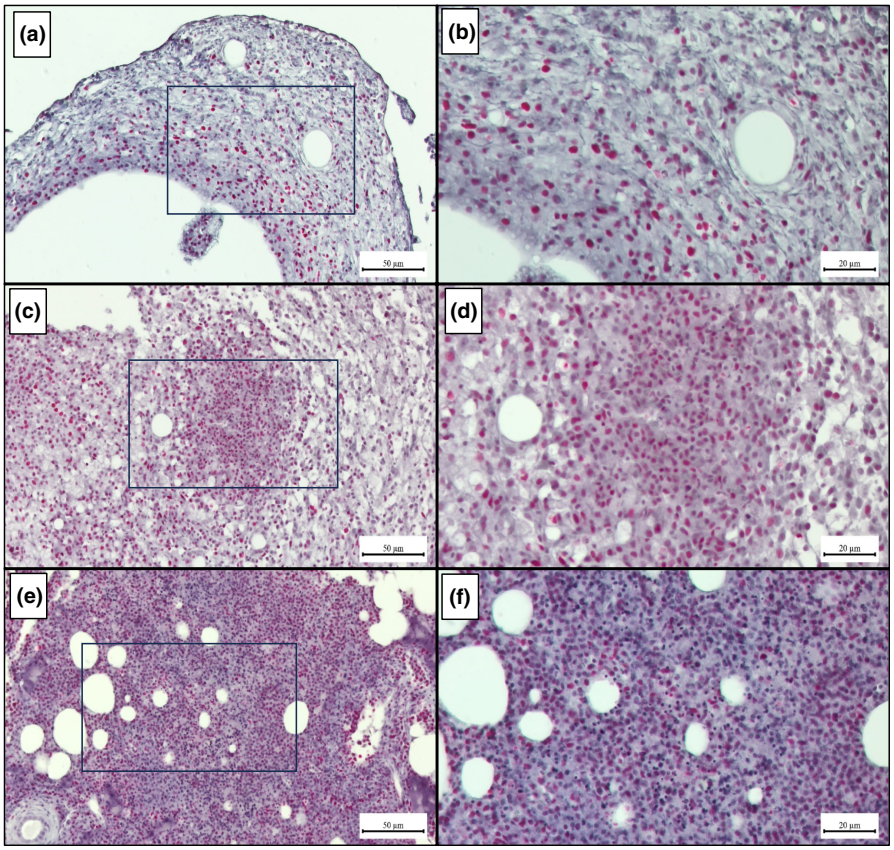


**FIGURE 6** Immunohistochemical staining of PCNA in inflammatory lesion sections of vaccinated (VAX) lumpfish in temperature groups 5°C (a, b), 10°C (c, d), and 15°C (e, f) at 18 wpi. Red colouration indicates presence of PCNA. The inflammatory lesion sections are displayed at magnification 20× to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 40× to the right in the figure (b, d, f). Note the presence of proliferating cells around oil droplets. Scale bar is 50 μm for a, c, e (20×) and 20 μm for b, d, f (40×).

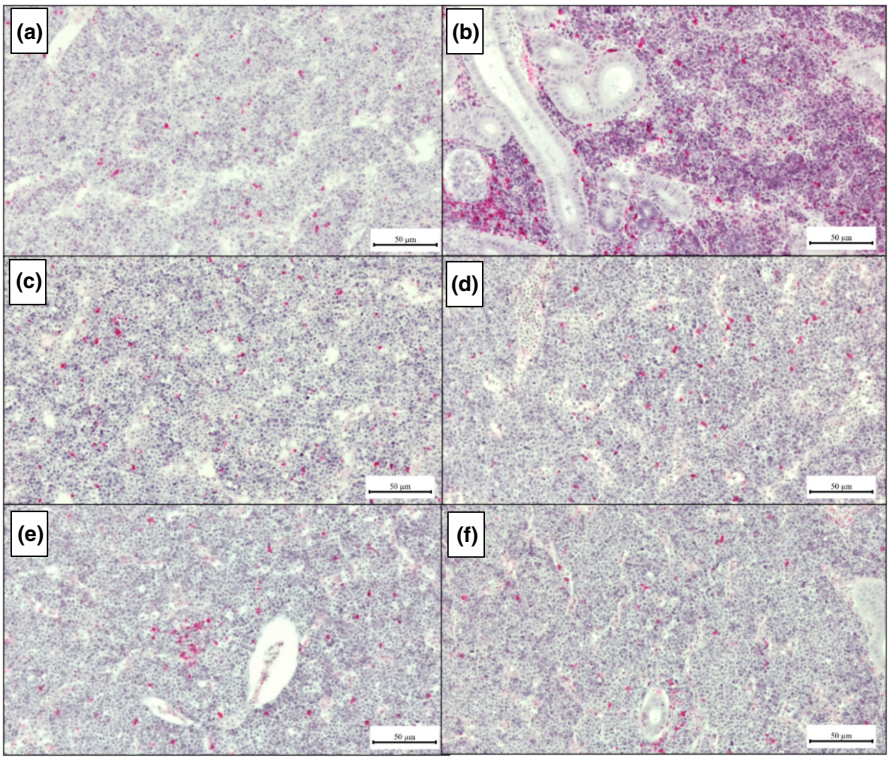


**FIGURE 7** Immunohistochemical staining of IgM in kidney sections of vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5, 10, and 15°C at 18 wpi. Red colouration indicates presence of IgM. Kidney sections of VAX and PBS fish are displayed to the left and right in the figure, respectively (a, b: 5°C, c, d: 10°C, e, f: 15°C). Magnification is 20× and scale bar is 50 μm.

around arteries or in the pyloric caeca in the sections from control fish. At 630 ddpi, the staining pattern of IgM was similar to that observed in lesions from tissue samples taken at 18 wpi. IgM+ cells were scattered in the loosely arranged inflammatory tissue (Figure 10a–d) and were only detected among dense aggregations of cells in two lesions derived from different fish in the 15°C group (Figure 10f).

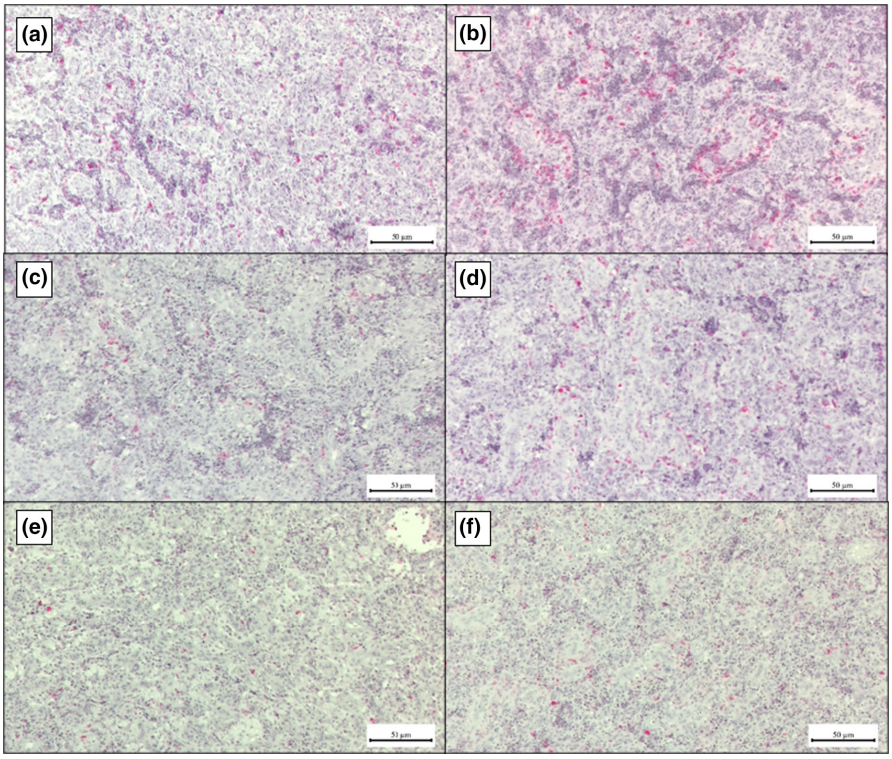


**FIGURE 8** Immunohistochemical staining of IgM in spleen sections of vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5, 10, and 15°C at 18 wpi. Red colouration indicates presence of IgM. Spleen sections of VAX and PBS fish are displayed to the left and right in the figure, respectively (a, b: 5°C, c, d: 10°C, e, f: 15°C). Magnification is 20× and scale bar is 50 μm.

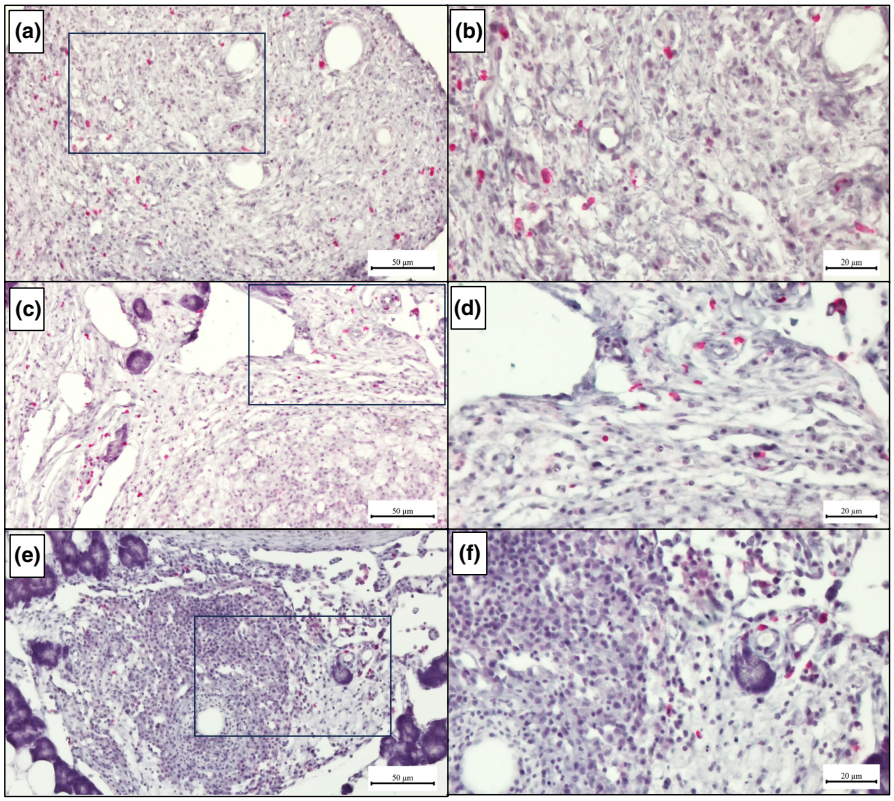


**FIGURE 9** Immunohistochemical staining of IgM in inflammatory lesion sections of vaccinated (VAX) lumpfish in temperature groups 5°C (a, b), 10°C (c, d), and 15°C (e, f) at 18 wpi. Red colouration indicates presence of IgM. The inflammatory lesion sections are displayed at magnification 20× to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 40× to the right in the figure (b, d, f). Note the presence of cell-associated IgM in the loose inflammatory tissue. Scale bar is 50 μm for a, c, e (20×) and 20 μm for b, d, f (40×).

## 3.2 | qPCR

The relative expression ratios or fold changes of mIgM in head kidney samples of lumpfish at 18 wpi are presented in Figure 11. The results showed a slight increase in the mean mIgM expression for vaccinated fish in temperature groups 5°C of 0.20-fold [−0.15, 0.55] and 10°C of 0.71-fold [0.21, 1.21] relative to their respective control groups, while a decrease for fish vaccinated at 15°C of −0.52-fold [−1.11, 0.06]. There was a significant mean difference of 1.23-fold [0.38, 2.08] in the relative gene expression of mIgM between vaccinated fish at 10 and 15°C ($p = .0016$). Differences in mIgM expression between other groups of vaccinated and control

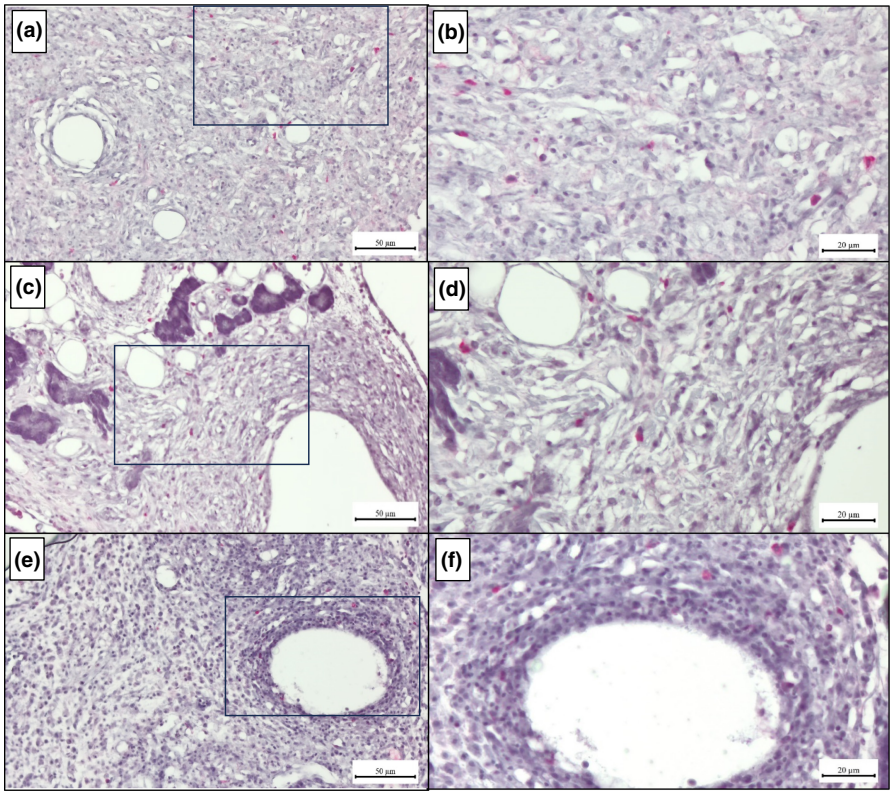


FIGURE 10 Immunohistochemical staining of IgM in inflammatory lesion sections of vaccinated (VAX) lumpfish in temperature groups 5°C (a, b), 10°C (c, d), and 15°C (e, f) at 630 ddpi. Red colouration indicates presence of IgM. The inflammatory lesion sections are displayed at magnification 20× to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 40× to the right in the figure (b, d, f). Note the presence of cell-associated IgM in the loose inflammatory tissue. Scale bar is 50 μm for a, c, e (20×) and 20 μm for b, d, f (40×).

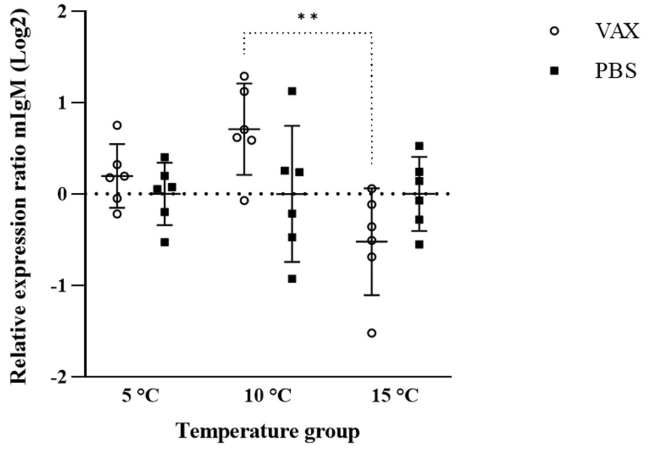


FIGURE 11 Relative gene expression ratio of mIgM in head kidney of vaccinated (VAX) and control (PBS) lumpfish ($n = 6$) at 18 wpi. Changes in gene expression are relative to average Cq values of control lumpfish in respective temperature groups. Error bars indicate ±95% confidence intervals of the average expression ratio in each group. Statistically significant differences are indicated with asterisks (**: $p \leq .005$).

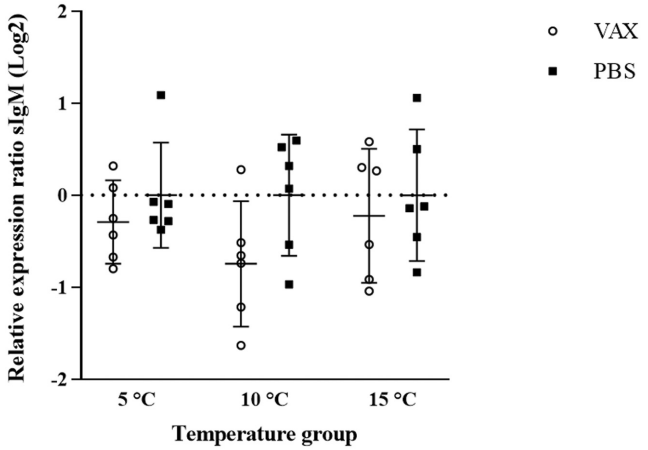


FIGURE 12 Relative gene expression ratio of sIgM in head kidney of vaccinated (VAX) and control (PBS) lumpfish ($n = 6$) at 18 wpi. Changes in gene expression are relative to average Cq values of control lumpfish in respective temperature groups. Error bars indicate ±95% confidence intervals of the average expression ratio in each group.

fish were insignificant. There was a significant interaction effect of temperature and vaccine group on the relative expression ratio of mIgM in the head kidney of lumpfish at 18 wpi (two-way ANOVA, $p = .0144$).

The relative expression ratios or fold changes of sIgM in head kidney samples of lumpfish at 18 wpi are presented in Figure 12. A mean decrease in the relative expression of sIgM for vaccinated fish in all three temperature groups, 5, 10, and 15°C was seen – compared to control fish. The differences in gene expression levels between vaccinated and control fish were insignificant, both within and between each temperature group. Vaccinated fish in the 10°C group showed the highest decrease in mean relative gene expression of −0.75-fold [−1.43 −0.064], in which five out of six individuals showed downregulation. In general, the relative expression of sIgM showed high individual variability within every temperature group, with some individuals showing upregulation and others downregulation. Fish vaccinated at 15°C, for instance, showed high individual variability as the spread in relative sIgM gene expression was from 0.58- to 1.04-fold.

The relative expression ratios or fold changes of mIgM in head kidney samples of lumpfish at 630 ddpi are presented in Figure 13. The results illustrated in Figure 13 show a general trend of increased mean mIgM expression in the head kidney of vaccinated fish in all three temperature groups, compared to control fish at 630 ddpi. Relative expression of mIgM was highest among vaccinated fish in the 10°C group compared to all other groups. The mean increase in mIgM expression of fish vaccinated at 10°C was 1.88-fold [0.82, 2.95] relative to the control group ($p = .0001$). Vaccinated and control fish subjected to water temperatures of 5 and 15°C showed no significant differences in mean relative mIgM expression. There was a significant interaction effect of temperature and vaccination group on the relative expression ratio of mIgM in the head kidney of lumpfish at 630 ddpi (two-way ANOVA, $p = .004$).

The relative expression ratios or fold changes of sIgM in head kidney samples of lumpfish at 630 ddpi are presented in Figure 14. The calculation showed that the mean relative expression ratio of sIgM was highest for vaccinated lumpfish in the 10°C group at 630 ddpi. For these fish, the mean increase in sIgM expression was 1.75-fold [1.35, 2.16] compared to the control group ($p < .0001$). The individual fold-changes in sIgM expression of fish vaccinated at 15°C were highly variable in contrast to fish vaccinated at 10 and 5°C. As example, one vaccinated individual in the 15°C group showed an increase in the relative expression of sIgM at a level like vaccinated fish in the 10°C group. There were no significant differences in mean relative expression of sIgM between vaccinated and control fish in the 5 and 15°C groups.

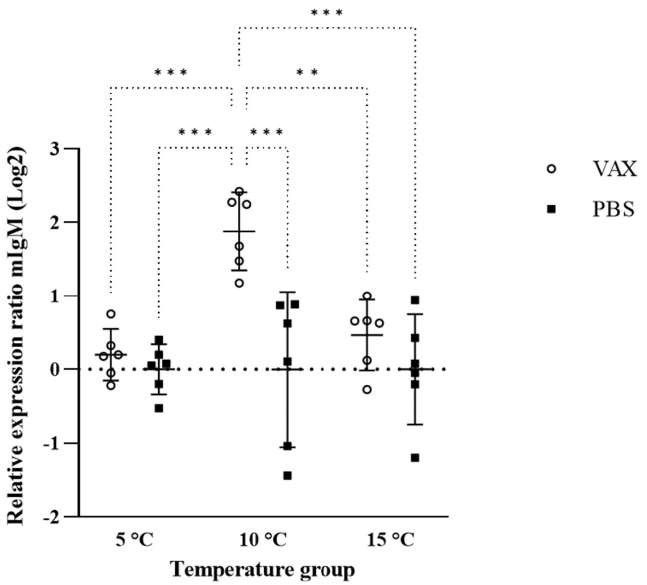


FIGURE 13 Relative gene expression ratios of mIgM in head kidney of vaccinated (VAX) and control (PBS) lumpfish ($n = 6$) at 630 ddpi. Changes in gene expression are relative to average Cq values of control lumpfish in respective temperature groups. Error bars indicate ±95% confidence intervals of the average expression ratio in each group. Statistically significant differences are indicated with asterisks (**: $p \leq .005$, ***: $p \leq .0005$).

## 3.3 | Welfare indicators

### 3.3.1 | Specific growth rate

The mean specific growth rates for both vaccinated and control fish were higher in the 10 and 15°C groups compared to the 5°C group. Differences in mean specific growth rates between fish subjected to water temperatures of 10°C versus 15°C were negligible. In these two temperature groups, however, control fish showed higher mean specific growth rates than vaccinated fish in contrast to fish in the 5°C group (data not shown).

### 3.3.2 | Condition factor

The results illustrated in Figure 15 show that lumpfish in the 15°C group had significantly higher condition factors on average compared to those in the 10 and 5°C groups ($p < .001$). The mean condition factors of lumpfish were 4.3 (±0.54), 3.8 (±0.46), and 4.9 (±0.78) in the 5, 10, and 15 groups, respectively. There were no significant differences in mean condition factor between vaccinated and control fish.

## 4 | DISCUSSION

In this study, inflammatory responses in lumpfish vaccinated at different water temperatures of 5, 10, and 15°C were assessed. Samples included for analysis originated from an earlier experiment in which antibody responses and vaccine side effects following intraperitoneal vaccination were studied (Erkinharju et al., 2018).

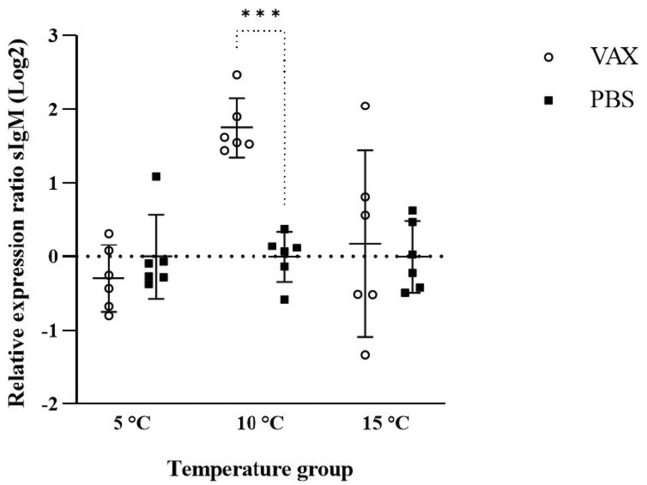


FIGURE 14 Relative gene expression ratios of sIgM in head kidney of vaccinated (VAX) and control (PBS) lumpfish ($n = 6$) at 630 ddpi. Changes in gene expression are relative to average Cq values of control lumpfish in respective temperature groups. Error bars indicate ±95% confidence intervals of the average expression ratio in each group. Statistically significant differences are indicated with asterisks (***: $p \leq .0005$).

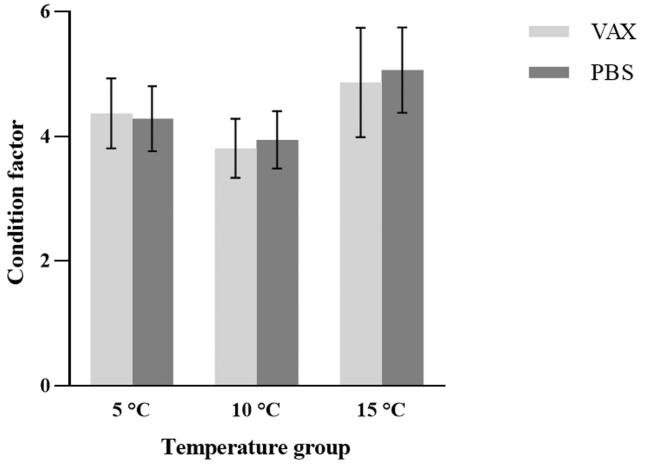


**FIGURE 15** Condition factor of vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5, 10, and 15°C at 126 days post injection. Error bars indicate ± standard deviation of mean condition factor. $n = 30$ for PBS and VAX fish in the 5°C group, $n = 28$ for VAX fish in the 10°C group, $n = 29$ for PBS fish in the 10°C group, $n = 23$ for VAX fish in the 15°C group, $n = 27$ for PBS fish in the 15°C group.

Intra-abdominal lesions were detected on haematoxylin and eosin (HE)-stained sections, which showed positive staining of all three immunohistochemistry (IHC)-targeted antigens (*A. salmonicida* LPS, PCNA, and IgM). No overall difference in the abundance of IgM+ cells was detected within lesions at different water temperatures and time points post-vaccination. Furthermore, kidney and spleen sections of vaccinated and control fish showed no differences in staining pattern of cellular-associated IgM. A low relative gene expression of mIgM and sIgM in the head kidney of vaccinated fish sampled at 18 wpi was observed.

Development of intra-abdominal lesions of varying severity is a well-recognized side effect of oil-adjuvanted vaccines administered with intraperitoneal injection (Poppe & Koppang, 2014). Histological examination revealed presence of perivisceral lesions that were positive for *A. salmonicida* and included negative imprints of oil droplets, which showed that these were vaccine-induced. The lesions were found between the pancreas, pyloric caeca, and spleen, which have been commonly reported for other species including Atlantic salmon (*Salmo salar*; Mutoloki et al., 2004), Atlantic cod (*Gadus morhua*; Mutoloki et al., 2008), and sea bass (*Dicentrarchus labrax*; Afonso et al., 2005). The observed lesions were characterized by both loose inflammatory tissue and (in fish vaccinated at 10 and 15°C) and dense aggregations of cells were detected within the lesions notably surrounding the vaccine oil droplets. The cell aggregations resembled granulomas (Petersen & Smith, 2013). Accumulation of collagen, as detected by van Gieson staining, supports the presence of granulomatous inflammatory lesions (Wynn & Ramalingam, 2012). Fibroblasts and connective tissue are commonly abundant in older, more mature granulomas (Poppe, 2002), which are consistent with the present findings. Some of the granulomas showed signs of necrosis with pyknotic nuclei in the centre of the granuloma. Also, the severity of lesions typically increased with time, which also has been shown for sea bass (Afonso et al., 2005) and Atlantic salmon (Midtlyng, 1996). However, the examined samples were derived from lumpfish immunized with a vegetable oil adjuvanted vaccine. Expectedly, non-mineral metabolizable oils lead to lower degree of side effects and only induce weak inflammatory responses (Spickler & Roth, 2003). This was not, however the case in the current study as many of the lesions were quite extensive and had progressed into a chronic phase. Additionally, adhesions between visceral organs and the abdominal wall were seen following macroscopic examination (Erkinharju et al., 2018). An earlier study on lumpfish did report more severe side effects in the form of adhesions following injection with a vegetable oil-adjuvanted vaccine when compared to a mineral oil-adjuvanted vaccine. This injection vaccine, however, had four bacterial antigens (Erkinharju et al., 2017). Increased levels of macrophages in lesions have been used as an indicator for the establishment of chronic inflammation in sea bass (Afonso et al., 2005; Petersen & Smith, 2013).

The vaccination did not significantly affect the condition factor of vaccinated fish at any temperature. However, we previously found a significant reduction in mean weight at 15- and 18-week post immunization for lumpfish vaccinated at 10°C (Erkinharju et al., 2018). In this study, the control fish showed a higher mean specific growth rate than vaccinated fish at 10 and 15°C in contrast to 5°C. The exact cause of these difference is unknown. Earlier studies have shown both increased, decreased, and no effect on growth after vaccination in lumpfish (Imsland et al., 2016; Sæbjørnsen, 2017).

Few studies have tried to characterize lumpfish leukocytes within histological sections, but many cell types have been isolated from tissues. These include lymphocytes, monocytes, macrophages, and granulocytes (Haugland et al., 2018). Some leukocytes in lumpfish have different morphology than those in Atlantic salmon, making identification challenging (Erkinharju et al., 2019; Haugland et al., 2018). Applying different specific cell markers or stains could have enabled identification of inflammatory cells. For instance, isolated macrophages from the blood, spleen, and head kidney of lumpfish have been shown to stain positive for acid phosphatase, which is an enzyme taking part in the elimination of phagocytosed pathogens. In the same study, neutrophils were reported to stain positive for myeloperoxidase involved in respiratory burst activity (Rønneseth et al., 2015). However, Erkinharju et al. (2019) reported uninterpretable results when using the same stains on histological sections with lesions derived from vaccinated lumpfish. These results were probably due to incompatibility of the stains with formalin-fixed histology samples, as they were developed for use on fresh cytological preparations (T Erkinharju 2024, pers. comm).

Positive staining of *A. salmonicida* LPS was detected in some of the kidney and spleen sections. These organs are known to function in antigen trapping and clearing exogenous substances from the circulation (Espenes et al., 1996; Press & Evensen, 1999). In Atlantic salmon, vaccine antigen (*A. salmonicida*) detected by IHC was shown to be present in macrophages and epithelial cells in the head kidney and spleen at three- and six-months post vaccination (Mutoloki

et al., 2004). Similarly, macrophages holding vaccine components have been detected in the head kidney and spleen of turbot following intraperitoneal injection (Noia et al., 2014).

PCNA is an established marker for active phases of the cell cycle and DNA repair (Maga & Hubscher, 2003). Presence of proliferating cells has been linked with inflammatory responses in fish. For instance, an increase in number of PCNA+ cells in the intestines of trout was associated with inflammation caused by helminth infection (Dezfuli et al., 2012). Also, strong staining of PCNA has been observed in diseased salmon hearts following viral infections and was linked to tissue regeneration of tissue (Yousaf et al., 2013). Immunostaining of PCNA indicated proliferative cell activity in the inflammatory lesions of lumpfish Erkinharju et al. (2019). In the current study, the PCNA+ cells were scattered throughout the lesions, but staining was especially pronounced in cell aggregations surrounding oil droplets. Many of these aggregated cells were found to have pink/eosinophilic granules following MGG staining. Thus, it is possible that some of the proliferating cells were granulocytes.

In teleost fish, the kidney and spleen are considered as secondary lymphoid organs where immune responses are initiated (Soulliere & Dixon, 2017). IHC revealed presence of IgM+ cells in the kidney and spleen of vaccinated and control lumpfish in all three temperature groups and at both sampling time points (note that the spleen was not sampled at 630 ddpi). Numerical increases of B cells in the (head) kidney and spleen have been associated with the vital role of these organs in initiating humoral responses including B cell response (Bermúdez et al., 2006). Interestingly, similar observations were not noted in the kidney and spleen sections of vaccinated lumpfish in neither of the temperature groups (5, 10, and 15°C) at both sampling time points. The IgM+ cells in the kidney and spleen were mostly scattered in the tissue exhibiting no signs of distinct distribution patterns such as grouping in clusters and the abundance of the B cells was not affected by immunization. Similar results were reported previously for lumpfish immunized intramuscularly (Erkinharju et al., 2019). This is not in line with a study in juvenile sea bass, where a vaccination against *Listonella anguillarum* was shown to increase the density of IgM+ cells over time in both the head kidney and spleen (Galeotti et al., 2013). Similarly, in rainbow trout (*Oncorhynchus mykiss*), the level of IgM+ cells significantly increased in the spleen after antigenic stimulation (Martín-Martín et al., 2020). With regards to a temperature effect, an increase of IgM+ B cells in the spleen of rainbow trout has been shown to be less for fish reared at lower (11°C) as opposed to higher temperature (16°C) following infection with *A. salmonicida* (Köllner & Kotterba, 2002). In our study, the possibility of an increase in number of IgM+ B cells occurring at earlier timepoints than 660 ddpi cannot be dismissed. In general low water temperatures slow down immune responses in teleosts, especially those related to the adaptive arm of immunity (Abram et al., 2017). In this study, it was shown that the vaccine induced local inflammatory responses in lumpfish even in the 5°C group, while in another study no significant humoral responses were demonstrated in fish reared at 5°C (Erkinharju et al., 2018).

Presence of IgM+ B cells in the peritoneum following antigenic stimulation has been reported previously in Atlantic salmon (Haugarvoll et al., 2010) and rainbow trout (Castro et al., 2017). IgM+ B cells are thus considered central participants in an inflammatory responses in the abdominal cavity of teleost fish (Korytář et al., 2013). Furthermore, administration of a virus vaccine (IPNV particles) in rainbow trout led to mobilization of IgM+ cells to the peritoneum (Martinez-Alonso et al., 2012). In another study in rainbow trout, it was shown that IgM+ cells were the dominating cell population in the peritoneum following injection with *Escherichia coli* and viral haemorrhagic septicaemia virus (VHSV). Furthermore, it was established that a large fraction of these IgM+ B cells was differentiating from antigen-secreting cells and were involved in antigen presentation through phagocytosis of antigen (Castro et al., 2017). As for other fish species, phagocytic capabilities of B cells have also been demonstrated in lumpfish. But it is not known whether lumpfish B cells possess MHC class II molecules needed for presentation of antigen (Rønneseth et al., 2017). The peritoneal cavity has been proposed to be an important immunological site in Atlantic salmon. This was supported by observations of increased frequency of IgM+ cells and prolonged antibody-secreting cell responses with no corresponding activity in internal lymphoid organs (head kidney and spleen) following challenge with salmonid alphavirus subtype 3 (SAV3). However, intraperitoneal injection with inactivated SAV3 virus did not produce local responses to the same extent (Tiruneh, 2019). One may hypothesize that the significant increase in antigen-specific antibodies in serum of fish vaccinated at 10 and 15°C at 18 wpi (Erkinharju et al., 2018) may be a result from local intraperitoneal production by B cells at the injection site, supported by qPCR results, revealing low abundance of mIgM and sIgM in the head kidney of vaccinated fish.

The relative gene expression analysis revealed individual variability between fish in the same treatment group, such as those vaccinated at 15°C. Normally, the response to vaccination is not uniform among fish unless domestication/breeding has reduced individual variability. Often vaccine trials reveal high and low responders within a vaccine group (R Dalmo 2021, pers. comm., 16 April). Variable responses may be reflected in variable relative gene expression values of replicate fish (Thim et al., 2014). However, in this case, head kidney samples included for qPCR analysis were derived from (only) six vaccinated fish (from the previous study) in each temperature group showing the highest concentration of specific IgM antibodies in response to the vaccine (*A. salmonicida* specific). Therefore, non-responders were not included, but the spread in gene expression ratios still reflects individual differences typically observed in experiments on fish.

Antigenic challenges, including vaccination, have been reported to induce upregulation of immune-related genes in the head kidney and spleen of teleost fish (Bjørgen & Koppang, 2021). In this study, insignificant differences in head kidney expression level of mIgM and sIgM between vaccinated and control fish were detected. There was one exception to this general trend. At 630 ddpi, the relative expression of both mIgM and sIgM was significantly upregulated in

the head kidney of vaccinated fish in the 10°C group. This suggests that response in the head kidney was induced by the vaccine for fish at the 10°C at 630 ddpi. This finding is contradictory to detected levels of specific IgM antibodies in serum, which were reported to be low and not significantly different from the control group (Erkinharju et al., 2018). The reason for increased expression of sIgM and mIgM in the head kidney of vaccinated fish in the 10°C group ay 630 ddpi is not clear.

## 5 | CONCLUSIONS

Local inflammatory responses were induced by intraperitoneal vaccination in lumpfish subjected to different water temperatures of 5, 10, and 15 °C. Some of the intra-abdominal lesions identified in histological sections were characterized by chronically inflamed tissue, with formation of granulomas and deposition of connective tissue. IHC indicated that *A. salmonicida* vaccine antigen persisted at the injection site stimulating a prolonged inflammatory responses. MGG staining revealed participation of eosinophilic-granulocyte-like cells in the locally induced inflammatory responses. Interestingly, these cells tended to aggregate around vaccine oil droplets and showed active proliferative activity following immunohistochemical staining with anti-PCNA. Variable numbers of IgM+ B cells were distributed in lesions of vaccinated fish with no apparent difference between temperature groups. Furthermore, the results of this study suggest that most cells needed for the development of a specific immune response are present, at the time points and temperatures investigated, at the site of vaccination at all temperatures investigated. The lack of a specific antibody response at 5°C (Erkinharju et al., 2018) does not appear to result from a missing host response against the injected antigens. Further work should be done to investigate the effect of even lower temperatures on the immune response including adaptive immunity in lumpfish, as the environmental temperature in the northern regions can be as low as zero-degree C.

### AUTHOR CONTRIBUTIONS

**Ingrid Svihus Knutsen:** Conceptualization; investigation; writing – original draft; methodology; writing – review and editing; formal analysis; validation. **Toni Erkinharju:** Conceptualization; investigation; writing – review and editing; methodology; validation. **Jarl Bøgwald:** Writing – review and editing; conceptualization; writing – original draft; methodology; validation. **Roy A. Dalmo:** Conceptualization; funding acquisition; investigation; writing – review and editing; writing – original draft. **Tore Seternes:** Conceptualization; investigation; writing – original draft; writing – review and editing; methodology; validation; formal analysis; project administration; supervision.

### ACKNOWLEDGEMENTS

The authors thank Vaxxinova Norway AS for their advice and support during the conduct of this immunization trial. We thank Miroslava Hansen for her technical support with histology. We also want to thank the staff at Tromsø Aquaculture Station AS in Tromsø for their technical support during the set-up and collection of samples from the experiment. The study was funded by the University of Tromsø—the Arctic University of Norway and the Research Council of Norway grants no 301401 and 325159.

### CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### ORCID

*Roy A. Dalmo* https://orcid.org/0000-0002-6181-9859
*Tore Seternes* https://orcid.org/0000-0001-5010-5095

### REFERENCES

Abram, Q. H., Dixon, B., & Katzenback, B. A. (2017). Impacts of low temperature on the teleost immune system. *Biology*, *6*, 39.

Afonso, A., Gomes, S., Da Silva, J., Marques, F., & Henrique, M. (2005). Side effects in sea bass (*Dicentrarchus labrax* L.) due to intraperitoneal vaccination against vibriosis and pasteurellosis. *Fish and Shellfish Immunology*, *19*, 1–16.

Bermúdez, R., Vigliano, F., Marcaccini, A., Sitjà-Bobadilla, A., Quiroga, M. I., & Nieto, J. M. (2006). Response of Ig-positive cells to *Enteromyxum scophthalmi* (myxozoa) experimental infection in turbot, *Scophthalmus maximus* (L.): A histopathological and immunohistochemical study. *Fish and Shellfish Immunology*, *21*, 501–512.

Bilal, S., Lie, K., Karlsen, O., & Hordvik, I. (2016). Characterization of IgM in Norwegian cleaner fish (lumpfish and wrasses). *Fish & Shellfish Immunology*, *59*, 9–17.

Bjørgen, H., & Koppang, E. (2021). Anatomy of teleost fish immune structures and organs. *Immunogenetics*, *73*, 53–63.

Bruce-Gregorius, J. H. (1974). *Histopathologic Techniques*. JMC Press Inc.

Castro, R., Abós, B., González, L., Granja, A. G., & Tafalla, C. (2017). Expansion and differentiation of IgM+ B cells in the rainbow trout peritoneal cavity in response to different antigens. *Developmental and Comparative Immunology*, *70*, 119–127.

Dey, P. (2018). *Basic and advanced laboratory techniques in histopathology and cytology*. Springer Singapore.

Dezfuli, B. S., Giari, L., Lui, A., Squerzanti, S., Castaldelli, G., Shinn, A. P., Manera, M., & Lorenzoni, M. (2012). Proliferative cell nuclear antigen (PCNA) expression in the intestine of *Salmo trutta trutta* naturally infected with an acanthocephalan. *Parasites & Vectors*, *5*, 198.

Elghafghuf, A., Vanderstichel, R., Hammell, L., & Stryhn, H. (2020). Estimating sea lice infestation pressure on salmon farms: Comparing different methods using multivariate state-space models. *Epidemics*, *31*, 100394.

Erkinharju, T., Dalmo, R. A., Hansen, M., & Seternes, T. (2020). Cleaner fish in aquaculture: Review on diseases and vaccination. *Reviews in Aquaculture*, *13*, 189–237.

Erkinharju, T., Dalmo, R. A., Vågsnes, Ø., Hordvik, I., & Seternes, T. (2018). Vaccination of Atlantic lumpfish (*Cyclopterus lumpus* L.) at a low temperature leads to a low antibody response against Aeromonas salmonicida. *Journal of Fish Diseases*, *41*, 613–623.

Erkinharju, T., Lundberg, M. R., Isdal, E., Hordvik, I., Dalmo, R. A., & Seternes, T. (2017). Studies on the antibody response and side effects after intramuscular and intraperitoneal injection of Atlantic lumpfish (*Cyclopterus lumpus* L.) with different oil-based vaccines. *Journal of Fish Diseases*, *40*, 1805–1813.

Erkinharju, T., Strandskog, G., Vågnes, Ø., Hordvik, I., Dalmo, R. A., & Seternes, T. (2019). Intramuscular vaccination of Atlantic lumpfish (*Cyclopterus lumpus* L.) induces inflammatory reactions and local immunoglobulin M production at the vaccine administration site. *Journal of Fish Diseases*, *42*, 1731–1743.

Espenes, A., Press, C. M., Reitan, L. J., & Landsverk, T. (1996). The trapping of intravenously injected extracellular products from *Aeromonas salmonicida* in head kidney and spleen of vaccinated and nonvaccinated Atlantic salmon, *Salmo salar* L. *Fish and Shellfish Immunology*, *6*, 413–426.

Evensen, Ø., Brudeseth, B., & Mutoloki, S. (2005). The vaccine formulation and its role in inflammatory processes in fish - effects and adverse effects. *Developments in Biologicals*, *121*, 117–125.

Fischer, A. H., Jacobson, K. A., Rose, J., & Zeller, R. (2008). Hematoxylin and eosin staining of tissue and cell sections. *CSH Protocols*, *2008*, pdb.prot4986.

Froese, R. (2006). Cube law, condition factor and weight–length relationships: History, meta-analysis and recommendations. *Journal of Applied Ichthyology*, *22*, 241–253.

Galeotti, M., Romano, N., Volpatti, D., Bulfon, C., Brunetti, A., Tiscar, P. G., Mosca, F., Bertoni, F., Marchetti, M. G., & Abelli, L. (2013). Innovative vaccination protocol against vibriosis in *Dicentrarchus labrax* (L.) juveniles: Improvement of immune parameters and protection to challenge. *Vaccine*, *31*, 1224–1230.

Haugarvoll, E., Bjerkås, I., Szabo, N. J., Satoh, M., & Koppang, E. O. (2010). Manifestations of systemic autoimmunity in vaccinated salmon. *Vaccine*, *28*, 4961–4969.

Haugland, G. T., Rønneseth, A., & Wergeland, H. I. (2018). Immunology and vaccinology of lumpfish and wrasse. In J. Treasurer (Ed.), *Cleaner fish biology and aquaculture applications*. 5m Publishing.

Hopkins, K. (1992). Reporting fish growth: A review of the basics. *Journal of the World Aquaculture Society*, *23*, 173–179.

Imsland, A., Reynolds, P., Lorentzen, M., Eilertsen, R., Micallef, G., & Tvenning, R. (2020). Improving survival and health of lumpfish (*Cyclopterus lumpus* L.) by the use of feed blocks and operational welfare indicators (OWIs) in commercial Atlantic salmon cages. *Aquaculture*, *527*, 735476.

Imsland, A. K., Reynolds, P., Eliassen, G., Hangstad, T. A., Foss, A., Vikingstad, E., & Elvegård, T. A. (2014). The use of lumpfish (*Cyclopterus lumpus* L.) to control sea lice (*Lepeophtheirus salmonis* Krøyer) infestations in intensively farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture*, *424-425*, 18–23.

Imsland, A. K., Reynolds, P., Nytro, A. V., Eliassen, G., Hangstad, T. A., Jonsdottir, O. D. B., & Jonassen, T. M. (2016). Effects of lumpfish size on foraging behaviour and co-existence with sea lice infected Atlantic salmon in sea cages. *Aquaculture*, *465*, 19–27. https://doi.org/10.1016/j.aquaculture.2016.08.015

Imsland, A. K. D., Hanssen, A., Nytrø, A. V., Reynolds, P., Jonassen, T. M., Hangstad, T. A., Elvegård, T. A., Urskog, T. C., & Mikalsen, B. (2018). It works! Lumpfish can significantly lower sea lice infestation in large-scale salmon farming. *Biology Open*, *7*, bio036301.

Johnson, S. C., Bravo, S., Nagasawa, K., Kabata, Z., Hwang, J., Ho, J., & Shih, C. (2004). A review of the impact of parasitic copepods on marine aquaculture. *Zoological Studies*, *43*, 229–243.

Köllner, B., & Kotterba, G. (2002). Temperature dependent activation of leucocyte populations of rainbow trout, *Oncorhynchus mykiss*, after intraperitoneal immunisation with *Aeromonas salmonicida*. *Fish Shellfish Immunology*, *12*, 35–48.

Koppang, E. O., Bjerkås, I., Haugarvoll, E., Chan, E. K. L., Szabo, N. J., Ono, N., Akikusa, B., Jirillo, E., Poppe, T. T., Sveier, H., Tørud, B., & Satoh, M. (2008). Vaccination-induced systemic autoimmunity in farmed Atlantic salmon. *The Journal of Immunology*, *181*, 4807–4814.

Koppang, E. O., Haugarvoll, E., Hordvik, I., Aune, L., & Poppe, T. T. (2005). Vaccine-associated granulomatous inflammation and melanin accumulation in Atlantic salmon, *Salmo salar* L., white muscle. *Journal of Fish Diseases*, *28*, 13–22.

Korytář, T., Jaros, J., Verleih, M., Rebl, A., Kotterba, G., Kühn, C., Goldammer, T., & Köllner, B. (2013). Novel insights into the peritoneal inflammation of rainbow trout (*Oncorhynchus mykiss*). *Fish and Shellfish Immunology*, *35*, 1192–1199.

Kuang, J., Yan, X., Genders, A. J., Granata, C., & Bishop, D. J. (2018). An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research. *PLoS One*, *13*, e0196438.

Maga, G., & Hubscher, U. (2003). Proliferating cell nuclear antigen (PCNA): A dancer with many partners. *Journal of Cell Science*, *116*, 3051–3060.

Martinez-Alonso, S., Vakharia, V. N., Saint-Jean, S. R., Pérez-Prieto, S., & Tafalla, C. (2012). Immune responses elicited in rainbow trout through the administration of infectious pancreatic necrosis virus-like particles. *Developmental and Comparative Immunology*, *36*, 378–384.

Martín-Martín, A., Simón, R., Abós, B., Díaz-Rosales, P., & Tafalla, C. (2020). Rainbow trout mount a robust specific immune response upon anal administration of thymus- independent antigens. *Developmental and Comparative Immunology*, *109*, 103715.

Midtlyng, P. J. (1996). A field study on intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. *Fish and Shellfish Immunology*, *6*, 553–565.

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

Mutoloki, S., Alexandersen, S., Gravningen, K., & Evensen, Ø. (2008). Time-course study of injection site inflammatory reactions following intraperitoneal injection of Atlantic cod (*Gadus morhua* L.) with oil-adjuvanted vaccines. *Fish and Shellfish Immunology*, *24*, 386–393.

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

Petersen, H. J., & Smith, A. M. (2013). The role of the innate immune system in granulomatous disorders. *Frontiers in Immunology*, *4*, 120.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, *29*, e45.

Poppe, T. (2002). Systematisk patologi og patofysiologi. In T. Poppe (Ed.), *Fiskehelse og fiskesykdommer*. Universitetsforlaget AS.

Poppe, T. T., & Koppang, E. O. (2014). Side-effects of vaccination. In R. Gudding, A. Lillehaug, & Ø. Evensen (Eds.), *Fish Vaccinaiton Hoboken*. John Wiley & Stones, Incorporated.

Powell, A., Treasurer, J. W., Pooley, C. L., Keay, A. J., Lloyd, R., Imsland, A. K., & Garcia De Leaniz, C. (2018). Use of lumpfish for sea-lice control in salmon farming: Challenges and opportunities. *Reviews in Aquaculture*, *10*, 683–702.

Press, C. M., & Evensen, Ø. (1999). The morphology of the immune system in teleost fishes. *Fish and Shellfish Immunology*, *9*, 309–318.

Rønneseth, A., Ghebretnsae, D. B., Wergeland, H. I., & Haugland, G. T. (2015). Functional characterization of IgM+ B cells and adaptive immunity in lumpfish (*Cyclopterus lumpus* L.). *Developmental and Comparative Immunology*, *52*, 132–143.

Rønneseth, A., Haugland, G. T., Colquhoun, D. J., Brudal, E., & Wergeland, H. I. (2017). Protection and antibody reactivity following vaccination of lumpfish (*Cyclopterus lumpus* L.) against atypical *Aeromonas salmonicida*. *Fish and Shellfish Immunology*, *64*, 383–391.

Sæbjørnsen, H. (2017). *Feltoppfølging av rognkjeks vaksinert mot Aeromonas salmonicida A-lagstype V og VI*. (MSc Masters Thesis). University of Bergen.

Skiftesvik, A. B., Bjelland, R. M., Durif, C. M. F., Johansen, I. S., & Browman, H. I. (2013). Delousing of Atlantic salmon (*Salmo salar*) by cultured vs. wild ballan wrasse (*Labrus bergylta*). *Aquaculture*, *402-403*, 113–118.

Sommerset, I., Walde, C. S., Jensen, B. B., Bornø, G., Haukaas, A., & Brun, E. (Eds.). (2020). *Fiskehelserapporten 2019*. Veterinærinstituttet.

Soulliere, C., & Dixon, B. (2017). Immune system organs of bony fishes. In *Reference module in life sciences*. Elsevier.

Spickler, A. R., & Roth, J. A. (2003). Adjuvants in veterinary vaccines: Modes of action and adverse effects. *Journal of Veterinary Internal Medicine*, *17*, 273–281.

Thim, H. L., Villoing, S., Mcloughlin, M., Christie, K. E., Grove, S., Frost, P., & Jørgensen, J. B. (2014). Vaccine adjuvants in fish vaccines make a difference: comparing three adjuvants (Montanide ISA763A Oil, CpG/Poly I:C Combo and VHSV Glycoprotein) alone or in combination formulated with an inactivated whole salmonid alphavirus antigen. *Vaccine*, *2*, 228–251.

Tiruneh, S. J. (2019). *Atlantic salmon B cells - local and systemic reponses to intraperitoneally administred salmonid alphavirus Philosophiae Doctor*. UiT - The Arctic University of Norway.

Treasurer, J. W. (2002). A review of potential pathogens of sea lice and the application of cleaner fish in biological control. *Pest Management Science*, *58*, 546–558.

Wynn, T. A., & Ramalingam, T. R. (2012). Mechanisms of fibrosis: Therapeutic translation for fibrotic disease. *Nature Medicine*, *18*, 1028–1040.

Yousaf, M. N., Koppang, E. O., Skjødt, K., Hordvik, I., Zou, J., Secombes, C., & Powell, M. D. (2013). Comparative cardiac pathological changes of Atlantic salmon (*Salmo salar* L.) affected with heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and pancreas disease (PD). *Veterinary Immunology and Immunopathology*, *151*, 49–62.

**How to cite this article:** Knutsen, I. S., Erkinharju, T., Bøgwald, J., Dalmo, R. A., & Seternes, T. (2024). Inflammatory responses in Atlantic lumpfish (*Cyclopterus lumpus* L.) after intraperitoneal injection of a vaccine against *Aeromonas salmonicida* and *Vibrio salmonicida* at different water temperatures. *Journal of Fish Diseases*, *00*, e14001. https://doi.org/10.1111/jfd.14001