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Inflammatory responses in lumpfish (*Cyclopterus lumpus* L.) vaccinated at different water temperatures with intraperitoneal injection

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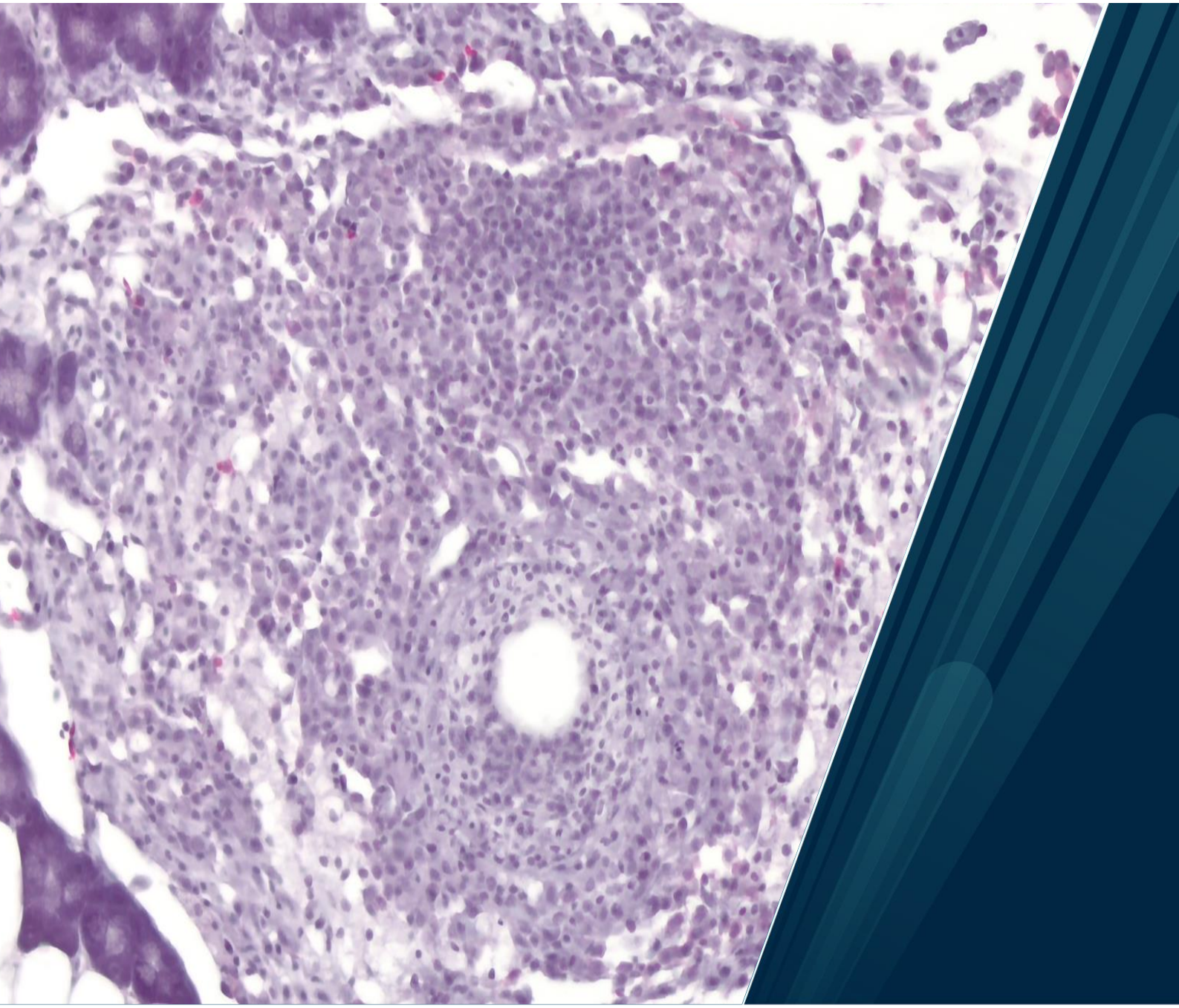

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Master of Science in Biology – Marine Ecology and Resource Biology: Fish Biology and Aquaculture

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Supervisors

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Toni Erkinharju: Norwegian Veterinary Institute in Harstad

Front page: Vaccine-induced inflammatory lesion with presence of IgM^+ cells (red stain).

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Abstract

The use of lumpfish (*Cyclopterus lumpus* L.) as cleaner fish in salmonid aquaculture for the biological control of sea lice infestation levels has increased in recent years. The species is relatively new to aquaculture and susceptible to various infectious agents. The health situation for lumpfish is currently considered poor, indicating the necessity for developing more efficient vaccines. Studying inflammatory responses induced by vaccination can contribute to a more detailed understanding of underlying immune mechanisms in lumpfish, which is important in the context of improving vaccine designs and vaccination procedures. Tissue samples from lumpfish intraperitoneally immunized with a divalent oil-adjuvanted injection vaccine at different water temperatures of 5 °C, 10 °C, and 15 °C, collected at 630 day degrees and 18 weeks post injection, were used for histological and immunohistochemical analyses. The relative amount of secretory and membrane-bound immunoglobulin M (IgM) gene transcripts in the head kidney of vaccinated fish was determined by quantitative PCR. Vaccine-induced inflammatory lesions were observed on histological sections of abdominal pancreatic/intestinal tissue from vaccinated fish in all three temperature groups. Immunohistochemical staining of *Aeromonas salmonicida* vaccine antigen was pronounced along the rim of oil droplets in lesions. 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 more loosely arranged inflammatory tissue dominated by connective tissue, showing no apparent difference in numbers between lesions from fish vaccinated at 5 °C, 10 °C, and 15 °C. The observed staining patterns of IgM^+ cells on kidney and spleen sections from vaccinated fish were no different from control fish. 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 was not greatly influenced by temperature. In addition, the results suggest local mechanisms in IgM responses with no corresponding immunoreactivity in the kidney, but further analyses, especially with regard to how antigens are presented and handled by the lumpfish immune system at the local level, are needed for confirmation.

Sammendrag

For biologisk bekjempelse av infestasjoner med lakselus i lakseoppdrett, har bruken av rognkjeks (*Cyclopterus lumpus* L.) som rensefisk økt de siste årene. Arten er relativt ny i oppdrett og utsatt for en rekke ulike infeksiøse agens. Helsesituasjonen til rognkjeks anses for tiden som dårlig, og det er behov for utvikling av mer effektive vaksiner. Studier av inflammatoriske responser frembrakt av vaksinasjon kan bidra til en mer detaljert forståelse av underliggende immunmekanismer hos rognkjeks, noe som er viktig for å forbedre vaksinedesign og vaksinasjonsprosedyrer. Histologiske og immunhistokjemiske analyser ble utført på vevsprøver fra rognkjeks immunisert intraperitonealt med en divalent oljebasert injeksjonsvaksine ved ulike vanntemperaturer på 5 °C, 10 °C og 15 °C. Prøver til analyse var tatt 630 døgngrader og 18 uker etter injeksjon. Den relative mengden gentranskripter av sekretorisk og membranbundet immunglobulin M (IgM) i hodenyre til vaksinert fisk ble bestemt ved bruk av kvantitativ PCR. Vaksineinduserte inflammatoriske lesjoner ble observert på histologiske snitt av pankreas og tarmvev fra vaksinert fisk i alle de tre temperaturgruppene. Immunhistokjemisk farging av *Aeromonas salmonicida* vaksineantigen var fremtredende langs kanten av oljedråper i lesjoner. Proliferasjon ble påvist blant inflammatoriske celler som dannet tette aggregeringer i lesjoner, hvorav mange ble identifisert som eosinofile granulocyttlignende celler. IgM^+ celler ble observert i mer løst ordnet inflammatorisk vev som var dominert av bindevev, og det var ingen tilsynelatende forskjeller i antall positive celler mellom lesjoner fra fisk vaksinert ved 5 ° C, 10 ° C og 15 ° C. Antallet og lokaliseringen av IgM^+ celler på nyre- og miltsnitt fra vaksinert fisk var heller ikke forskjellig fra kontrollfisk. Relativ genuttrykksanalyse av sekretorisk og membranbundet IgM viste totalt sett lavt genuttrykk i hodenyre til vaksinert fisk både ved 630 døgngrader og 18 uker etter injeksjon. Resultatene i denne studien indikerer at vaksinen stimulerte langvarige inflammatoriske responser ved injeksjonsstedet, noe som ikke var påvirket av temperatur i særlig grad. I tillegg antyder resultatene lokale mekanismer i IgM responser uten tilsvarende immunologisk aktivitet i nyre. Likevel er ytterligere analyser nødvendig for å kunne bekrefte resultatene, spesielt med hensyn til hvordan antigen presenteres og håndteres av rognkjeksens immunsystem på et lokalt nivå.

Abbreviations

ANOVA	Analysis of variance
BSA	Bovine serum albumin
cDNA	Complementary DNA
Cq	Quantification cycle
ddpi.	Day degrees post injection
EF1-α	Elongation factor 1 alpha
HE	Haematoxylin and eosin
HIER	Heat induced epitope retrieval
IgM	Immunoglobulin M
IHC	Immunohistochemistry
K	Condition factor
MGG	May-Grünwald-Giemsa
mIgM	Membrane-bound IgM
PBS	Control fish injected with phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PIER	Proteolytic induced epitope retrieval
qPCR	Quantitative polymerase chain reaction
RT	Reverse transcriptase
RT-qPCR	Reverse transcription-qPCR
SGR	Specific growth rate
sIgM	Secretory IgM
TBS	Tris-buffered saline
VAX	Vaccinated fish
VG	van Gieson
wpi.	Weeks post injection

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1 Introduction

1.1 Biology of lumpfish

Lumpfish (*Cyclopterus lumpus* L.) is a semi-pelagic marine teleostean species inhabiting northern waters on both sides of the Atlantic Ocean. In Norway, the species is distributed along the entire coastline with the largest populations residing off the coast of Nordland, Troms and Finnmark county. Lumpfish can migrate far distances and search for food in deep waters (Erkinharju et al. 2020). They lack a swim bladder, but have a gelatinous subcutaneous layer providing buoyancy when residing in the open ocean. In spring and summertime, lumpfish return to shallow waters to spawn. Females lay demersal eggs which are formed into clumps for the males to guard and tend. Seaweed beds are nursery grounds for hatchlings and juveniles (Powell et al. 2018a). Both adult and juvenile lumpfish feed on a wide variety of organisms, including crustaceans, and are regarded as opportunistic feeders (Ingólfsson and Kristjánsson 2002, Powell et al. 2018b). Another feature of lumpfish is their distinct morphology (Figure 1). The body is short and compressed with longitudinal ridges and tubercles along its length, and the skin has a rubbery and tough texture with no scales. A sucking disc used for attachment to substrates is present ventrally and constitutes a modification of the pelvic fins (Powell et al. 2018a). Adults show sexual dimorphism in coloration and size. During the spawning season, males have orange to purple body colorations and are smaller than the grey and blue-green females (Davenport 1985).

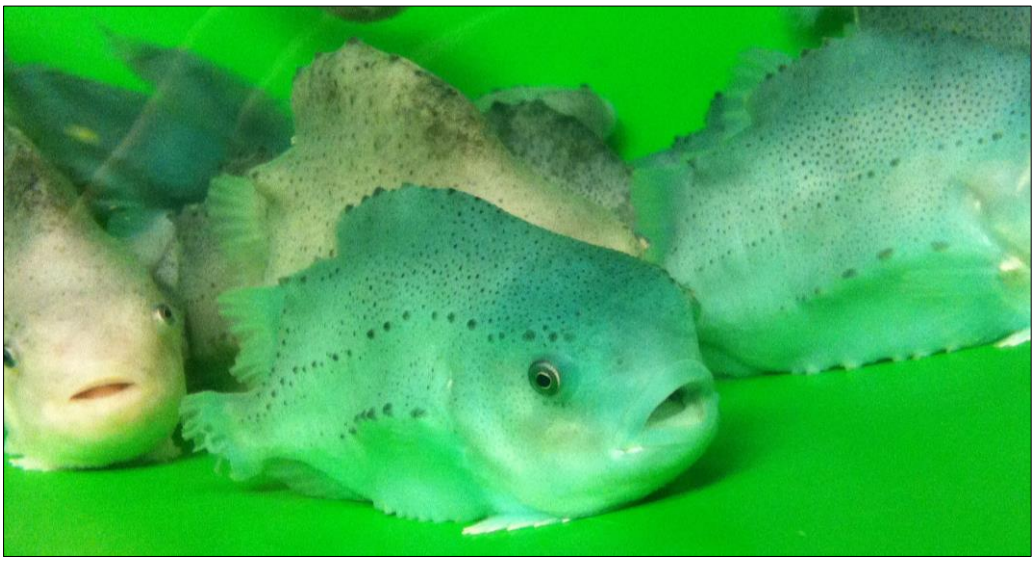

Figure 1: Lumpfish (Cyclopterus lumpus). Photo: Toni Erkinharju, Veterinary Institute, Harstad.

1.2 Lumpfish in aquaculture

Sea lice infestations on salmonids is a major challenge in the aquaculture industry resulting in substantial economic losses to the farmers every year 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 different species of wrasse as cleaner fish. Biological control of sea lice has proven to be effective as several cleaner fish species actively eat lice (Skiftesvik et al. 2013, Imsland et al. 2018b, Treasurer 2018). Importantly, use of cleaner fish limits environmental issues related to delousing by chemotherapeutants as well as being less stressful for farmed salmonids compared to the chemical and mechanical delouse treatments (Haugland et al. 2020, Treasurer 2002). As such, deployment of cleaner fish in sea cages together with salmonids has escalated through the years at farms in both Europe and Canada (Powell et al. 2018b). In Norway, the total number of cleaner fish deployed in 2020 was estimated to 59.6 million, of which the majority was lumpfish (Sommerset et al. 2021).

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 constituting 38.9 million in 2020 (Erkinharju et al. 2020, Sommerset et al. 2021). Use of farmed lumpfish increases biosecurity due to vaccination and screening programs. In addition, farming enables lumpfish to be deployed in sea cages when they have reached optimal sizes and reduces the dependency on wild populations (Imsland et al. 2020). The increased demand for lumpfish can be attributed to the species toleration of low water temperatures, in which it can maintain relatively efficient sea lice grazing as opposed to the temperate wrasse species (Imsland et al. 2014, Powell et al. 2018b). Lumpfish are thus considered as the better cleaner fish option at salmon farms located in northern regions (Imsland et al. 2014). Another important factor is the high growth potential of lumpfish in captivity as they can be ready for sea cage deployment in 4-7 months (Powell et al. 2018b, Brooker et al. 2018). Selective breeding programs are lacking for lumpfish as it is a relatively new aquaculture species (Powell et al. 2018b). Healthy and robust populations of lumpfish are needed to sustain effective sea lice grazing (Imsland et al. 2020). Thus, research focusses on optimizing husbandry practices including health management as lumpfish are known to suffer from various diseases impairing their welfare (Brooker et al. 2018).

1.2.1 Welfare and bacterial disease

Many reports have raised concerns regarding the health and welfare of cleaner fish in general (Nilsen et al. 2014, Mattilsynet 2019, Sommerset et al. 2020). According to the Norwegian Food Safety Authority (2018), frequent disease outbreaks and high mortality rates are compromising the ability of cleaner fish to perform their function, i.e. delousing. Mortalities occur for various reasons including handling and mechanical damage, predation, nutritional deficiencies, and infectious diseases caused by bacteria, viruses, and parasites (Nilsen et al. 2014). The reader is referred to a comprehensive review published recently for detailed information about the current disease situation among cleaner fish (Erkinharju et al. 2020).

Lumpfish are especially susceptible to various pathogens with bacterial infections constituting the biggest challenge (Sommerset et al. 2021). Poor husbandry practices, water quality and nutrition including handling and stress are listed as causes of secondary bacterial infections affecting lumpfish (Brooker et al. 2018). Typically, signs of septicemia, such as skin lesions, gill haemorrhages and aggregations of bacteria in the heart and spleen, can be observed in lumpfish derived from aquaculture facilities (Powell et al. 2018b). Lumpfish are infected by various *Vibrio* species, typical and atypical *Aeromonas salmonicida*, *Pasteurella* sp. *Pseudomonas anguilliseptica*, *Mortiella viscosa* and *Tenacibaculum maritimum* spp. (Sommerset et al. 2021). However, disease outbreaks, often followed by substantial mortalities, are mainly associated with atypical strains of *A. salmonicida* causing atypical furunculosis (Rønneseth et al. 2017). Last year, atypical *A. salmonicida* was detected at 51 localities (Sommerset et al. 2021). Atypical furunculosis leads to development of ulcers in the skin and granulomatous inflammation in internal organs such as the spleen, kidney, and liver (Nilsen et al. 2014). Bacterial aggregates are typically observed on histological sections derived from infected fish (Figure 2) (Erkinharju et al. 2020). 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).

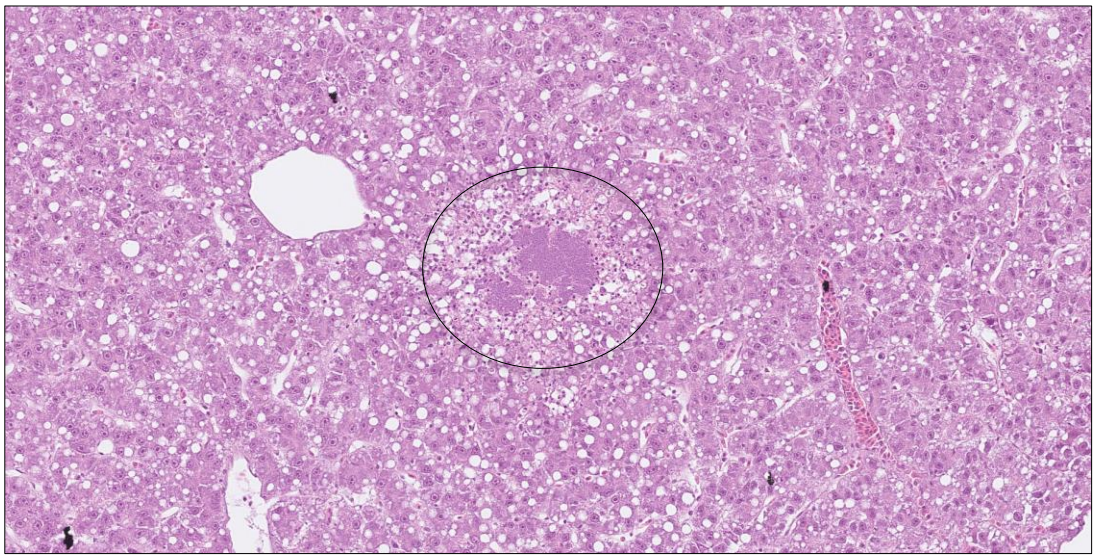

Figure 2: Liver section from lumpfish infected by atypical A. salmonicida. A bacterial microcolony (encircled) can be observed in the center surrounded by necrotic tissue. Intracellular lipid vacuoles are fewer/smaller than normal due to emaciation. Photo: Toni Erkinharju, Veterinary Institute, Harstad.

1.3 Brief overview of the immune system

The immune system of vertebrates is divided into the innate and adaptive immune systems, each with defined characteristics. Components of the innate immune system are the first to recognize and battle infection, and do this in an unspecific manner (Turvey and Broide 2010). Specialized adaptive immune mechanisms, however, need to be primed by innate immune components to become activated (Abbas et al. 2020). The adaptive immune system is responsible for generating immunological memory in the sense that it "remembers" previous encounters with pathogens, thus enabling faster and stronger responses to be initiated during recurrent infections (Bonilla and Oettgen 2010). This feature of adaptive immunity forms the basis for vaccination (Sallusto et al. 2010).

Both the innate and adaptive immune systems have humoral and cellular branches specialized in combating different types of infections. Soluble macromolecules released by cells into extracellular fluids mediate humoral immunity (Smith et al. 2019). Leukocytes function as the principle immune cells and are derived from myeloid or lymphoid linages (Janeway et al. 2001). Broadly, leukocytes can be categorized into monocytes/macrophages, granulocytes (neutrophilic, eosinophilic, and basophilic), and lymphocytes (natural-killer (NK) cells,

dendritic cells, B and T lymphocytes). Monocytes/macrophages, granulocytes, NK-cells and dendritic cells are cellular components of the innate immune system, whereas B and T lymphocytes (or B and T cells) have functions in adaptive immunity and generate memory (Turvey and Broide 2010).

In mammals, leukocytes arise within the bone marrow (primary lymphoid organ). The bone marrow is also the site for B cell maturation, whereas T cells mature and undergo selection processes in the thymus. Naïve (mature) B and T cells continuously circulate through secondary lymphoid organs (lymph nodes and spleen) in which they are activated by antigens presented to them by innate immune cells, such as dendritic cells. Activation results in clonal proliferation of cells with specific antigen receptors and further differentiation to effector cells with functions targeted to combat the infection (Janeway et al. 2001). Effector B cells (plasma cells) are major players in humoral immunity through secretion of antibodies neutralizing extracellular pathogens, whereas effector T cells mediate cellular immunity by direct killing of infected, stressed or tumour cells (cytotoxic T lymphocytes; CTLs) or by improving responses of other immune cells, such as B cells and macrophages (T helper cells; Th cells). Th cells have subgroups with distinct cytokine signatures stimulating immune responses towards different kinds of pathogens (Abbas et al. 2020).

1.3.1 The immune system of teleost fish

The immune system of teleost fish shares many similarities with the mammalian system with functional innate and adaptive immune mechanisms, but there are some distinct differences (Evensen 2020). For instance, the anatomy of lymphoid tissues differs, and neither bone marrow nor lymph nodes are present in fish (Press and Evensen 1999). Furthermore, it is generally accepted that fish rely more on nonspecific immune mechanisms in protection against infectious agents, as their immune system is less complex than that of mammals (Kordon et al. 2019). Studies have identified various immunological components in bony fish structurally analogue to those found in mammals. However, many functional pathways remain to be elucidated (Jørgensen 2014, Mutoloki et al. 2014). Knowledge of the immune system of bony fish is mainly based on experiments conducted on salmonids, cyprinids, and catfish (Bilal et al. 2016). Importantly, the inner body temperature of fish is regulated by changes in ambient water temperature. Their poikilothermic nature influences vital physiological functions including those of the immune system (Abram et al. 2017).

Immunological organs

Organs and tissues central to immune defense in bony fish include the kidney, thymus, spleen, and unorganized mucosa-associated lymphoid tissue (MALT) (Press and Evensen 1999). The fish kidney is a multifunctional organ consisting of excretory, endocrine, and immunological tissues (Rauta et al. 2012). Immunological tissue is distributed throughout the kidney with the anterior part (head kidney) being the primary site of haematopoiesis (the production of all cellular components of blood). The head kidney is thus regarded as equivalent to the mammalian bone marrow (Mutoloki et al. 2014). Also, the reticuloendothelial system, consisting of macrophages and endothelial cells clearing foreign and waste molecules from the blood circulation, is localized in the kidney of fish (Seternes et al. 2002). The thymus is the most evolutionary conserved organ and is the site for T cell development and maturation (Boehm et al. 2012). Aggregates of T cells are also present in interbranchial (in between gill arches) lymphoid tissue, which was discovered in Atlantic salmon (*Salmo salar*) (Koppang et al. 2010). A study by Løken et al. (2020) revealed presence of a lymphoepithelial structure in the cloacal region of Atlantic salmon resembling the bursa of Fabricius in birds. The spleen is a secondary lymphoid organ housing B and T cells and macrophages. Like the head kidney, the spleen functions in trapping of antigens (Rauta et al. 2012, Uribe et al. 2011). MALT cover mucosal surfaces in fish such as the gills, skin and gut, consisting of both innate and adaptive immune components including granulocytes, macrophages, T and B cells (Salinas 2015, Smith et al. 2019).

Innate immunity

The innate immune system forms the basis of protection in fish, responds rapid to infection and plays an important role in activating the adaptive immune system (Jørgensen 2014). It can be divided into physical, cellular, and humoral components (Uribe et al. 2011). Physical barriers constitute scales, skin, and mucosa which serve as the first line of defense against invading pathogens (Magnadóttir 2006). If such barriers are breached, innate immune cells manage to detect pathological agents by pattern recognition receptors. Innate immune cells in fish include monocytes/macrophages, eosinophilic granule cells, neutrophils, dendritic cells and NK-like cells (Smith et al. 2019). Natural antibodies, antimicrobial peptides, lytic enzymes, protease inhibitors, lectins, complement proteins, and cytokines are representatives of humoral factors (Uribe et al. 2011). Many can be found in the serum and mucus of teleost fish. As in mammals, humoral factors in fish function in neutralizing toxic effects of pathogens by, for instance, lysing bacterial cell walls or opsonizing pathogens facilitating phagocytosis by immune cells

(Smith et al. 2019). Cytokines serve as communicator molecules between immune cells and are important for recruitment of cells to the site of infection (Abbas et al. 2020). Important pro-inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour-necrosis factor alpha (TNF- α), have been detected in fish (Uribe et al. 2011) including lumpfish (Eggestøl et al. 2020b, Eggestøl et al. 2020a).

Pattern recognition receptors and innate immune cells

Pattern recognition receptors (PRRs) detect common structures shared by different classes of pathogens called pathogen-associated molecular patterns (PAMPs). Thus, recognition of antigens by innate immune cells is considered unspecific (Abbas et al. 2020). Intracellular signalling from PRRs activates transcription factors regulating expression of genes encoding molecules involved in immune defense (Jørgensen 2014). PRRs also recognize various molecules released from cells that are stressed or injured (Magnadóttir 2006). The PRRs of innate immune cells can be classified into three groups, namely toll-like receptors (TLRs), retinoic acid-like receptors (RLRs), and nod-like receptors (NLRs), all of which have been discovered in fish (Evensen 2020). The most studied PRRs in fish are the TLRs, which they possess a broad repertoire of in both cell membranes and endosomal compartments. About 17 TLRs have been characterized in fish as opposed to ten in humans, and many of the genes encoding these receptors are situated on different chromosomes (Jørgensen 2014). The TLRs in fish can distinguish between host and foreign nucleic acids. Some have been shown to induce signalling pathways leading to antiviral responses in leukocytes after stimulation with synthetic agonists such as CpG oligonucleotides and poly 1:C (Jørgensen et al. 2003, Zhou et al. 2014).

Phagocytes are the most widely studied innate immune cells in fish constituting mononuclear monocytes/macrophages and polynuclear neutrophils. Following recognition of PAMPs by PRRs, phagocytes engulf pathological agents into phagosomes. These fuse with lysosomes to form so-called phagolysosomes in which lytic enzymes, reactive oxygen species and nitric oxide work in concert eliminating the pathological agent (Jørgensen 2014). Upon bacterial challenge, fish neutrophils have been described to release neutrophil extracellular traps (NETs) which entangle and destroy extracellular pathogens by lytic enzymes embedded in the nets (Palić et al. 2007). In the event of inflammation, caused by either infection or vaccination, neutrophils are often the first cells to be recruited (Reite and Evensen 2006). Macrophages becoming activated at the site of inflammation are either local tissue residents or derived from monocytes in the blood (Jørgensen 2014). Like the classically activated macrophages (M1

subtype) in mammals, fish macrophages release pro-inflammatory cytokines and chemokines. The other macrophage subtype (M2) involved in tissue repair is not as well characterized in fish, but supposedly exists (Smith et al. 2019). Innate immune cells resembling NK-cells in mammals involved in combating intracellular infections, e.g. viruses, have been discovered in catfish. These include NK-like cells and non-specific cytotoxic cells exhibiting spontaneous killing of foreign cells (Shen et al. 2002).

The bridge between innate and adaptive immunity

As mentioned, components in the innate immune system are important for activation of the adaptive immune system when help in clearance of pathogens is needed. Antigens must be presented to B and T cells to initiate clonal proliferation and activate their adaptive effector functions. In mammals, macrophages serve as antigen presenting cells (APCs) to T cells and play key roles in driving the differentiation of activated Th cells to different subsets with specialized functions (Abbas et al. 2020). There is some evidence of such antigen presenting capabilities in fish macrophages as they possess major histocompatibility complex (MHC) class II molecules necessary for T cell antigen presentation (Mutoloki et al. 2014). The main cells forming the bridge between innate and adaptive immunity are the dendritic cells (Bassity and Clark 2012). Dendritic cells become specialized APCs after they recognize antigen by TLRs, increasing their expression of MHC class II and costimulatory molecules involved in activation of T cells in the lymph nodes of mammals (Abbas et al. 2020). Dendritic-like cells have been derived from haematopoietic kidney tissue of rainbow trout (*Oncorhynchus mykiss*). Bassity and Clark (2012) reported culturing of motile cells with higher surface expression of MHC class II than macrophages and B cells (other APCs) including morphology (membrane processes) resembling the mammalian dendritic cell. In addition, these cells stimulated proliferation of leukocytes to a greater degree than macrophages and B cells in mixed leucocyte reaction assays. However, knowledge on antigen presentation in fish is still quite limited. Questions about how and where antigen presentation occur remain unanswered (Mutoloki et al. 2014).

Adaptive immunity

The adaptive immune system responds to infection by specific recognition of antigens and can generate memory cells able to combat recurrent infections (Mashoof and Criscitiello 2016). It takes longer to develop adaptive immunity and the system needs to be primed by components of the innate immune system, e.g. APCs. The specificity and memory of the adaptive immune

system is contributed by B and T cells with highly diverse antigen receptors. The antigen receptor of B cells, immunoglobulin or antibody, is membrane-bound or exists freely in the circulation. The latter form is responsible for humoral immunity neutralizing extracellular pathogens. T cells have membrane-bound T cell receptors (TCR) and mediate cellular immunity through helper or cytotoxic functions. The basis for generation of antigen specific receptors are the RAG 1 and 2 proteins initiating recombination of gene segments in the loci encoding components of the TCR and immunoglobulin receptor. RAG 1 and 2 genes are conserved among vertebrates and are expressed in the head kidney and thymus of fish (Mutoloki et al. 2014).

B cells and antibodies

The distribution and maturation stages of B cells have been studied in rainbow trout (Zwollo et al. 2005). Naïve B cells which have developed in the head kidney may either stay there or become distributed through the blood to the posterior kidney, spleen, or peripheral tissues. Upon encountering antigen, the naïve B cells turn into plasma blasts and further differentiate to plasma cells characterized by high antibody secreting capabilities and no membrane-bound Ig. After reaching this terminate B cell stage, the plasma cells seem to migrate back to the head kidney to settle becoming long-lived cells secreting specific antibodies into the systemic circulation (Zwollo et al. 2005, Ye et al. 2011). Studies of B cells from different teleost species, including rainbow trout, catfish, cod, and salmon, have revealed phagocytic and bactericidal capacities similar to the B-1 subset in mammals (Li et al. 2006, Øverland et al. 2010). This suggests that fish B cells have roles in innate immunity as well (Rønneseth et al. 2015). In response to bacteria and pathogens, phagocytic B cells upregulate gene expression of molecules involved in antigen presentation (MHC class II and costimulatory molecules). Therefore, phagocytic fish B cells might also function as APCs priming T helper cell responses during infections (Wu et al. 2020).

The antibody repertoire in fish is limited compared to that in mammals (Mashoof and Criscitiello 2016). The isotype of antibodies is defined by their heavy chain providing different effector functions (Abbas et al. 2020). Three different antibody isotypes are known to be produced by fish B cells, namely IgM, IgD, and IgT (Mashoof and Criscitiello 2016). IgM is the principle systemic antibody produced by plasma cells and plasma blasts in the head kidney (Zwollo et al. 2005) but is also present in mucus (Ángeles Esteban 2012). IgM facilitates clearance of extracellular pathogens by neutralization and opsonization including activation of

complement (Bilal et al. 2016). Increased levels of IgM in serum have been shown to correlate with protection against bacterial diseases. Therefore, IgM is used as an indicator for development of humoral immunity in fish (Mutoloki et al. 2014). The functions of IgD remain unclear and this antibody has only been characterized at the protein level in channel catfish (Jørgensen 2014). IgT might function similarly to IgA in mammals being the most prominent antibody in mucosal tissues (Salinas 2015).

Heavy chain isotype switching of fish antibodies is not observed, and different subsets of B cells have been distinguished according to which antibodies they produce. These include B cells with IgM, IgD or IgT expression and B cells with co-expression of IgM and IgD (Sunyer 2012, Mutoloki et al. 2014). Affinity maturation is a process by which the affinity of antibodies towards an antigen increases with repeated exposure. This is due to point mutations occurring within the variable regions of antibodies which are responsible for antigen recognition (Abbas et al. 2020). Affinity maturation is reported to be much weaker in fish compared to mammals, possibly due to lack of germinal centres where selection of the highest affinity antibodies takes place in the lymph nodes of mammals (Warr 1997, in Morrison and Nowak 2002). However, the molecular machinery facilitating affinity maturation is present in fish (Smith et al. 2019). It is debated whether a secondary humoral response occurs in fish (Sommerset et al. 2005), which is characterized by the rapid activation of memory B cells following subsequent exposure to antigen (Ademokun and Dunn-Walters 2010). The authors of a study using rainbow trout as fish model reported a slight increase in the level of specific antibodies and prolonged response after reinfecting fish, suggesting that the secondary response in fish is modest compared to that in mammals (Ma et al. 2013). Generally, antigen specific responses take longer to develop in fish than mammals and are dependent on temperature (Sunyer 2012).

T cells

Fish T cells seem to function similarly to mammalian T cells in mediating cellular immune responses (Mutoloki et al. 2014). Indication of T cell mediated responses in fish was first discovered in goldfish where rejection of transplanted scales was monitored. The authors of the study hypothesized that T cells were responsible for the immune response rejecting the scales. To test their hypothesis, fish were irradiated with γ-rays, but the thymus was shielded to avoid affecting T cells. The discovery was that the transplanted scales were still rejected, indicating cell-mediated destruction of tissue by T cells (Desvaux and Charlemagne 1983).

T cells with CD8 and CD4 coreceptors have been found in fish representing CTLs and Th cells, respectively (Fischer et al. 2013). Isolated cells with expression of TCR and CD8 coreceptor have demonstrated cell mediated cytotoxicity towards infected syngeneic cells, which is a feature of mammalian CTLs (Somamoto et al. 2009). Furthermore, $CD8^+$ cells have been shown to kill targets by release of perforins and granzymes contributing to additional evidence for the presence of CTLs in fish (Fischer et al. 2006). There are three main subgroups of Th cells: Th1, Th2, and Th17 cells (Nakanishi et al. 2015). Genes encoding key cytokines (e.g. interferon- γ; INF- γ) and transcription factors for Th1 cells are identified in several fish species. Fish recombinant INF-γ induces higher phagocytic capacity in macrophages, release of pro-inflammatory cytokines and upregulation of anti-viral gene expression. There are few functional studies of Th2 and Th17 cells which are involved in anti-parasitic immunity and leukocyte recruitment, respectively. However, some important cytokines and transcription factors linked to Th2 and Th17 cells have also been identified in fish (Wang and Secombes 2013).

Somamoto et al. (2014) conducted a functional study of $CD4^+$ cells in ginbuna carp (*Carassius auratus langsdorfii*). $CD4^+$ cells and $CD4^+$ depleted cells from donor fish infected with Crucian carp haematopoietic necrosis virus (CHNV) were transferred to recipient fish. The recipient fish were then exposed to CHNV challenge and blood samples were collected for ELISA at different time intervals. Specific antibodies were produced by both recipients, but the amount was much higher for fish receiving $CD4^+$ cells at 40 days post infection. This led to the conclusion that $CD4^+$ cells were necessary for activation of B cells inducing rapid and strong antibody responses following CHNV infection.

Unique for teleost fish is the possession of two genes for CD4 coreceptor. The CD4-1 coreceptor has four extracellular domains (like mammalian CD4), whereas CD4-2 is shorter and only has two domains. Three populations of cells expressing CD4 have been distinguished, specifically cells with only CD4-1 or CD4-2 surface expression and cells co-expressing CD4-1 and CD4-2. Cells with double expression and only CD4-2 expression have been identified as lymphocytes morphologically and proliferate in response to a known activator of T cells (PHA). CD4-1 cells, on the other hand, are considered as myeloid cells with phagocytic capacity (Takizawa et al. 2016).

The immune system of lumpfish

Various leukocytes have been isolated from lumpfish including lymphocytes, monocytes, macrophages, granulocytes, and dendritic cells (Haugland et al. 2018). The innate immune system of lumpfish is considered efficient with notably high phagocytic capability among macrophages, neutrophils, B cells (IgM^+) and unnamed small non-granular cells (Rønneseth et al. 2015). Bactericidal actions of phagocytes have also been demonstrated (Haugland et al. 2012). Genes encoding T cell markers are present in the transcriptome of lumpfish but, other than that, not much is known about T cells within the species (Haugland et al. 2018). The transcriptome profile of lumpfish also includes complement protein and TLR genes, which become upregulated in leukocytes during early immune responses against bacteria (*V. anguillarium* O1) (Eggestøl et al. 2018).

Bilal et al. (2016) characterized lumpfish IgM and determined a total serum concentration of 1–2.6 mg/ml. Immunization trials with *A. salmonicida* have revealed development of specific antibodies with increased levels in the circulation inducing protection after bacterial challenge. This suggests that vaccination can confer protection against this bacterium in lumpfish through stimulation of the adaptive immune system (Rønneseth et al. 2017). Generation of specific antibodies against *V. anguillarum* serotype O1 and *M. viscosa* have also been successful, but circulating levels were lower compared to those accomplished by immunization with *A. salmonicida* (Erkinharju et al. 2017). Ellul et al. (2019) immunized lumpfish with *Pasteurella* sp. but reported low antibody response against this bacterial species. The humoral response of lumpfish has been reported to be lower following vaccination at 5 °C compared to higher temperatures of 10 °C and 15 °C (Erkinharju et al. 2018). Additionally, local immune responses have been shown to be induced by intramuscular injection with a multivalent vaccine containing *A. salmonicida* (type V and VI), *V. anguillarum* serotype O1, and *M. viscosa* (Erkinharju et al. 2019).

Vaccination

Adaptive or specific immunity protects against diseases caused by pathogens from previous exposures through generation of memory B and T cells and forms the basis of vaccination. Vaccination is recognized as the uttermost important measure in regulating infections disease outbreaks at fish farms (Lillehaug et al. 2002). The clear positive effects of vaccination include increased fish welfare and reduction of fish mortalities benefitting the farmers economically (Gudding et al. 1999). Also, consumptions of chemotherapeutants are significantly reduced as

disease outbreaks are kept in check contributing to a more environmentally sustainable production. Vaccine strategies are implemented in many fish farming countries with millions of fish being vaccinated every year (Ma et al. 2019).

Induction of long-term protection against infectious diseases is the principle goal of vaccination (Sommerset et al. 2005). The efficiency of the vaccine depends on several factors including composition and administration method (Lillehaug et al. 2002). The most used vaccines for fish contain inactivated whole bacteria or virus (Evensen 2020). Components or products of pathogens which are important virulence factors including surface antigens, enzymes and toxins are also used in vaccine design (sub-unit vaccines). Live vaccines contain attenuated strains of pathogens lacking the ability to cause disease, but do induce infection. Gene technology has introduced DNA-vaccines, enabling the production and expression of DNA encoded antigens to immunocompetent cells (Lillehaug et al. 2002).

Many vaccines, especially inactivated and sub-unit vaccines, are formulated with adjuvants to increase the immune response in fish, as the vaccine antigens are not capable of inducing a proper immune response by themselves. The adjuvant must stimulate components of the innate immune system for adaptive immune mechanisms to be elicited. Typically, the adjuvant forms an antigen depot at the vaccine injection site facilitating long time periods of antigen exposure to immune cells (Evensen et al. 2005). Aluminium salt and oils (mineral and vegetable) are commonly used as adjuvants in fish vaccines. Oils exert the greatest effects on the immune response and vaccines with oil-adjuvants have been reported to provide longer protection than those without (Lillehaug et al. 2002).

There are three methods for vaccinating fish. Vaccines can be given orally through food, by injection or immersion (Sommerset et al. 2005). The method of vaccine delivery influences the protection level and side effects induced in fish. Vaccine injections are preferred as protection is induced for longer durations and because it is easier to control for the correct dosage. Another advantage is that the formulations of injection vaccines can contain multiple antigens which sometimes induce better protection than monovalent vaccines. An example is vaccines with *V. anguillarum*, *V. salmonicida* and *A. salmonicida* antigens (Gudding et al. 1999). Today, most of the commercial vaccines used in salmonid farming in Norway are multivalent (Statens Legemiddelverk 2020). Oil adjuvants are typically included in injection vaccines to increase efficiency and produce strong local inflammatory reactions (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, intra-abdominal lesions, melanization of tissue, growth reduction, and systemic autoimmunity (Koppang et al. 2005, Koppang et al. 2008). However, side effects induced by vaccination decrease with increased fish size (Berg et al. 2007).

There are five commercially available vaccines for lumpfish (Haugland et al. 2018). In addition, certified vaccine production companies offer autogenous vaccines. These vaccines are custom made for fish at a particular locality containing bacterial isolates from infected fish (Vaxxinova Norway AS , Statens Legemiddelverk 2018). Most commercial vaccines combine strains of atypical *A. salmonicida* and *V. anguillarium* 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 > 10 g (Haugland et al. 2018). Despite the currently available vaccines, researcher have emphasized the necessity for developing more efficient immune prophylactic measures (Erkinharju et al. 2020, Powell et al. 2018b). Broadening the knowledge of the lumpfish immune system is central to this task (Eggestøl et al. 2018).

1.4 Aim and objectives of the study

This study aimed at investigating inflammatory responses in vaccinated 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 °C, 10 °C, and 15 °C (Erkinharju et al. 2018). In this regard, the focus of the study was characterization of possible differences in inflammatory responses among vaccinated lumpfish subjected to the different water temperatures.

Objectives:

- Evaluate vaccine influence on growth rate and body condition of fish.
- Identify components in inflammatory reactions at injection site by use of routine and special histological staining techniques.
- Assess presence and distribution of vaccine antigen, proliferating cells, and IgM in inflammatory reactions and in systemic organs (kidney and spleen) by use of immunohistochemistry.
- Compare staining pattern of IgM in kidney and spleen of vaccinated and control fish.
- Quantify relative expression of secretory and membrane-bound IgM in head kidney of vaccinated fish by use of qPCR.

2 Materials and methods

2.1 Reagents and equipment

Reagents used in this study are listed in Appendix A, whereas equipment used is mentioned in the associated method sections.

2.2 Sample material

The sample material and weight/length data from lumpfish examined in this research project originated from a previous experiment conducted by Erkinharju et al. (2018). In brief, lumpfish divided into three different water temperature groups of 5 °C, 10 °C, and 15 °C received an intraperitoneal injection with a vegetable oil-adjuvanted vaccine containing formalin-inactivated bacterial antigens of *Aeromonas salmonicida* and *Vibrio salmonicida*. Lumpfish serving as control fish in each temperature group were injected with phosphate buffered saline. Organ samples were sampled at different time points throughout the experiment and the weight and length of selected fish were measured at 126 days/18 weeks post injection (wpi.). In this study, selected organ samples from a total of 20 fish (10 vaccinated and 10 control fish) in each temperature group sampled at 630 day degrees post injection (ddpi.) and 18 wpi. were used as sample material for histological examination. The organ samples included kidney, heart, spleen, liver, pancreas, adipose tissue, and pyloric caeca. The heart, spleen and liver were not sampled from all fish. In addition, head kidney samples collected at the same sampling time points were included for gene expression analysis.

2.3 Welfare indicators

Specific growth rate (SGR) and condition factor (K) were calculated to assess vaccine influence on growth and body condition of lumpfish.

2.3.1 Specific growth rate

The mean specific growth rates (SGR) from 0-126 days post injection of vaccinated and control lumpfish in temperature groups 5 °C, 10 °C and 15 °C were calculated from available weight data using equation I:

$$SGR = (Ln(w_t) - Ln(w_i)) / t \times 100 \quad \text{I}$$

Where $Ln(w_t)$ is the natural logarithm of the weight at time t (day 126) and $Ln(w_i)$ is the natural logarithm of the initial weight (day 0). SGR expresses percentage increase in growth per day (Hopkins 1992).

2.3.2 Condition factor

Fulton's condition factor (K) of individual lumpfish at 126 days post injection was calculated from available weight and length data using equation II:

$$K = W / L^3 \times 100 \qquad \text{II}$$

Where W is the weight (g) and L is the length (cm) of the fish (Froese 2006).

2.4 Histology

2.4.1 Tissue processing

Formalin fixated organ samples of the kidney, heart, spleen, liver, and pyloric caeca, including pancreatic and abdominal adipose tissue, were prepared for histological, a few histochemical and immunohistochemical staining. Prior to tissue processing, the samples were trimmed, placed in histology cassettes, and stored in 70 % ethanol. The tissue processing steps, including dehydration, clearing and infiltration with paraffin wax, were performed overnight in the automated Shandon Citadel 2000 tissue processor (Thermo Fisher Scientific) according to Table 1. The following day, the samples were embedded in paraffin wax (Leica EG 1150H, Leica Biosystems). Tissue sectioning was performed manually by use of the microtome Leica RM2235 (Leica Biosystems). The paraffin blocks were cooled on ice and sectioned at 5 µm thickness size. Resulting tissue sections were transferred to a flotation water bath of 42 °C to allow for mounting on either regular or charged glass slides (Thermo Scientific). The charged slides were used to increase the adhesion of tissue, making them better suited for immunohistochemical analysis.

Table 1: Tissue processing steps performed in Citadel 2000 tissue processor.

Solution	Time
Ethanol 96 %	2 h
Ethanol 96 %	2 h
Ethanol 100 %	2 h
Ethanol 100 %	2 h
Ethanol 100 % and Histo-Clear (1:1)	1 h
Histo-Clear	1 h
Histo-Clear	1 h
Histo-Clear	1 h
Histo-Clear and paraffin wax (1:1)	1 h
Paraffin wax	5 h

2.4.2 Haematoxylin and eosin staining

Haematoxylin and eosin (HE) were used as staining dyes for the standard histological examination of tissue components (Table 2). Tissue components are easily distinguished from one another following HE-staining. Negatively charged molecules, such as chromatin, stain purple/blue in haematoxylin, whereas positively charged tissue components, such as cellular cytoplasm, stain different shades of pink in eosin (Fischer et al. 2008). Prior to the staining procedure, the tissue sections were incubated at 64 °C for 20 minutes to increase adherence to slides. Paraffin wax surrounding and infiltrating the tissues was dissolved in Histo-Clear. Following deparaffinization, the tissue sections were treated with ethanol and water. The rehydrated tissue sections were stained in haematoxylin and tap water was used as bluing agent. After counterstaining in eosin, the tissue sections were dehydrated in an ascending alcohol series and rinsed in Histo-Clear. Permanent mounting medium (Eukitt®, Sigma Aldrich) was used to adhere coverslips to slides. The procedure was conducted manually.

Table 2: HE-staining protocol.

Solution	Time
Histo-Clear	6 min
Ethanol 100 %	1.5 min
Ethanol 96 %	1.5 min
Ethanol 96 %	1.5 min
Running tap water	1.5 min
Gill's haematoxylin III	2.5 min
Running tap water	3 min
Eosin Y	1.5 min
Ethanol 96 %	1.5 min
Ethanol 96 %	1.5 min
Ethanol 100 %	1.5 min
Ethanol 100 %	1.5 min
Histo-Clear	1.5 min

2.4.3 Special staining

Three special histochemical staining techniques were performed on selected tissue sections from lumpfish, namely von Kossa, May-Grünwald-Giemsa and van Gieson. The two latter techniques were conducted at the Veterinary Institute in Harstad. The results from staining with von Kossa were not associated with inflammatory responses and are therefore presented in Appendix D.

von Kossa

Presence of suspected calcification in heart tissue was tested using the von Kossa staining technique which replaces calcium ions with silver ions. The deposited silver ions become visible after light treatment producing a brown-black stain. A kidney sample derived from Atlantic salmon with confirmed nephrocalcinosis was used as positive control (Veterinary Institute, Harstad). Staining was performed by using the silver plating kit acc. to von Kossa from Merck (Table 3). The sections were deparaffinized in Histo-Clear and rehydrated through a descending alcohol series, before being stained in a silver nitrate solution while exposed to light of 18 LED from above. Staining was followed by rinsing in water and treatment in sodium thiosulphate solution to remove un-reacted silver. The sections were counterstained in Nuclear

fast red-aluminium sulphate solution, dehydrated, cleared and coverslipped (Eukitt®, Sigma Aldrich).

Table 3: von Kossa staining protocol.

Solution	Time
Histo-Clear	10 min
Histo-Clear	10 min
Ethanol 100 %	5 min
Ethanol 100 %	5 min
Ethanol 90 %	2 min
Ethanol 70 %	2 min
Ethanol 50 %	2 min
MilliQ water	1 min
Silver nitrate solution with light exposure	20 min to 1 h
Running tap water	3 min
Sodium thiosulphate solution 5 %	5 min
Running tap water	1 min
Nuclear fast red-aluminium sulphate solution 0.1 %	2 min
MilliQ water	1 min
Ethanol 70 %	1 min
Ethanol 96 %	1 min
Ethanol 100 %	1 min
Ethanol 100 %	1 min
Histo-Clear	5 min
Histo-Clear	5 min

May-Grünwald-Giemsa

The polychromatic staining technique May-Grünwald-Giemsa (MGG) was used to distinguish different leukocytes present in abdominal inflammatory lesions on sections derived from vaccinated lumpfish (Table 4). MGG combines different oxidised methylene blue dyes and eosin producing different staining patterns of leukocytes. The granules of neutrophils, eosinophils, and basophils stain pink, red and blue, respectively. Red blood cells stain pink, whereas nuclei stain blue. After deparaffinization in xylene, the sections were placed in ethanol

followed by methanol treatment. The sections were stained in May-Grünwald solution and then directly transferred to Giemsa solution before being differentiated in diluted acetic acid. Treatment in ethanol and xylene was carried out prior to coverslipping. Staining was manually conducted whereas the other steps were automated in the slide stainer Tissue Tek Prisma Plus (Sakura).

Table 4: May-Grünwald-Giemsa staining protocol.

Solution	**Time**
Xylene	2 min
Xylene	6 min
Xylene	7 min
Ethanol 100 %	2 min
Ethanol 100 %	2 min
Methanol	5 min
May-Grünwald solution	5 min
Giemsa solution 5 %	15 min
Acetic acid 0.5 %	< 30 s
Ethanol 100 %	15 s
Xylene	15 s
Xylene	15 s

van Gieson

The van Gieson staining technique was used for detection of connective tissue in abdominal inflammatory lesions on sections from vaccinated lumpfish (Table 5). Staining in iron-haematoxylin and acidic van Gieson solution turns nuclei brown-black, musculature yellow, and connective tissue (collagen) bright pink/red. After deparaffinization in xylene and dehydration through a descending alcohol series, the sections were placed in water. Weigert's haematoxylin (iron salt-mordant) was added to the sections followed by rinsing in water and counterstaining in van Gieson solution. The sections were rinsed in water, dehydrated in an ascending alcohol series, and cleared in xylene before mounting. Staining was manually conducted whereas the other steps were automated in the slide stainer Tissue Tek Prisma Plus (Sakura).

Table 5: van Gieson staining protocol.

Solution	Time
Xylene	2 min
Xylene	6 min
Xylene	7 min
Ethanol 100 %	2 min
Ethanol 100 %	3 min
Ethanol 96 %	15 s
Ethanol 70 %	15 s
Tap water	1-2 min
Weigert's haematoxylin	20 min
Running tap water	2 min
van Gieson solution	5 min
Running tap water	< 30 s
Ethanol 70 %	15 s
Ethanol 96 %	15 s
Ethanol 100 %	15 s
Xylene	15 s
Xylene	15 s

2.4.4 Immunohistochemistry

Immunohistochemistry (IHC) is a technique that visualizes molecules in biological tissues through specific antigen-antibody interactions. Primary antibodies that specifically bind to the molecules (antigens) of interest are applied to the tissue sections followed by incubation with labelled secondary antibodies directed against the primary antibodies. The labels of the secondary antibodies are either fluorescent or enzymatic allowing for visualization of antigen-antibody complexes formed in the tissue (Aikawa 2011). IHC was performed on tissue sections from vaccinated fish in which inflammation had been detected on HE-stained sections for the detection of *A. salmonicida* vaccine antigen, proliferating cells, and IgM. For each temperature group at both sampling time points (18 wpi. and 630 ddpi.), sections from an equal number of control fish were included.

Primary antibodies

Three primary antibodies were used for IHC, namely polyclonal rabbit anti-*Aeromonas salmonicida* LPS (a gift from Roy Dalmo and Jarl Bøgwald, UiT), polyclonal rabbit anti-lumpfish IgM (produced according to Bilal et al. 2016) and monoclonal mouse anti-proliferating cell nuclear antigen (anti-PCNA) (Dako). The two latter antibodies have been used in previous work with lumpfish tissue (Erkinharju et al. 2019). The antibodies were diluted in 1.5 % (Anti-*A. salmonicida* LPS) and 2.5 % (anti-IgM, anti-PCNA) bovine serum albumin (BSA)-tris buffered saline solution. Dilution series were tested to determine adequate antibody concentrations to be used in which non-specific background stain was reduced. For immunostaining, Anti-*A. salmonicida* LPS, anti-IgM and anti-PCNA were diluted to 1:5000, 1:6000 and 1:3200, respectively.

Deparaffinization and rehydration

The tissue sections were incubated at 60 °C for 20 minutes to increase adherence to charged slides prior to deparaffinization with two treatments of Histo-Clear, followed by rehydration in a descending alcohol series and water (Table 6).

Table 6: Deparaffinization and rehydration protocol for IHC.

Solution	Time
Histo-Clear	10 min
Histo-Clear	10 min
Ethanol 100 %	5 min
Ethanol 100 %	5 min
Ethanol 90 %	2 min
Ethanol 70 %	2 min
Ethanol 50 %	2 min
Running tap water	5 min

Antigen retrieval and blocking

Antigen (or epitope) retrieval was performed on tissue sections to break the protein crosslinks formed during fixation in formalin, thereby unmasking the antigen binding sites (epitopes) for primary antibodies. Two retrieval methods were performed depending on the antigen to be detected, specifically proteolytic-induced epitope retrieval (PIER) for *A. salmonicida*, and heat-

induced epitope retrieval (HIER) for IgM and PCNA. PIER included incubation of tissue sections with 0.05 % trypsin solution for 15 minutes within a humidification chamber at room temperature. For HIER treatment, the tissue sections were immersed in 95 °C citrate buffer (pH: 6.0) for 20 minutes, by utilizing a water bath as heating apparatus. Following HIER, the tissue sections were cooled for 20 minutes. After restoring antigenicity, the tissue sections were rinsed for 2 x 5 minutes in tris-buffered saline (TBS) and 5 minutes in tris-buffered saline with Tween detergent (TBST). ImmunoPen (Merck) was used to encircle the tissue specimens to create a hydrophobic barrier minimizing waste of reagents. Normal horse serum (2.5 %) was added to tissue sections for blocking of potential non-specific binding sites of primary antibodies. Excess serum was removed after an incubation period of 20 minutes before proceeding with immunostaining.

Immunohistochemical staining

The tissue sections were incubated with ca. 200 µl of primary antibody dilution. For anti-*A. salmonicida* LPS and anti-PCNA, the tissue sections were incubated for 1 hour at room temperature or overnight (14-15 h) at 4 °C. Overnight incubation with anti-IgM was excluded due to issues with increased background staining and the tissue sections were incubated for 1 hour at room temperature. The tissue sections were rinsed in TBS (2 x 5 min) and TBST (5 min) prior to incubation with labelled secondary antibody (30 min) raised against the primary antibody species. 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.). After secondary antibody incubation and subsequent rinsing in TBS (2 x 5 min) and TBST (5 min), the tissue sections were incubated with alkaline phosphatase substrate (ImmPACT Vector Red, Vector Laboratories, Inc.) for 7-10 minutes and rinsed in TBS afterwards (5 min). All incubation steps were carried out within a humidification chamber.

Counterstaining

The tissue sections were rinsed in water to prepare for counterstaining in haematoxylin (Table 7). Staining was followed by bluing in tap water and dehydration through increasing alcohol concentrations. Permanent mounting medium (Eukitt®, Sigma Aldrich) was used to adhere coverslips to slides after clearing of the tissue sections in Histo-Clear.

Table 7: Counterstaining protocol for IHC.

Solution	Time
Running tap water	5 min
Gill's Haematoxylin III	3 min
Running tap water	3 min
Ethanol 70 %	15 s
Ethanol 96 %	15 s
Ethanol 100 %	30 s
Histo-Clear	15 s -

2.4.5 Microscopy

The histological tissue sections were analysed with Axio Lab A1 light microscope (ZEISS). Photographs of selected tissue sections were taken by Axiocam 208 Colour (ZEISS) connected to the microscope and viewed in Zen core v3.1 software. Photographs were edited and assembled in Microsoft Word 365 (Microsoft Corp.).

Semi-quantitative analysis of IgM^+ cells

The number of IgM^+ cells in kidney, spleen, and inflammatory tissue was determined by counting of stained cells on photographs taken of the sections following IHC with anti-IgM. Eight and four photographs of the same kidney sections from ten fish were taken. The total number of positive stained cells in eight and four photographs were then divided by the number of photographs taken. A paired T-test was run with these numbers to determine whether to count cells on eight or four photographs. Four pictures were chosen due to an insignificant T-test p-value. For the spleen sections, cells were also counted on four photographs. Positive stained cells in inflammatory lesions were counted while using the light microscope.

2.5 Reverse transcription quantitative polymerase chain reaction

RNAlater fixated head kidney samples from vaccinated and control lumpfish in temperature groups 5 °C, 10 °C, and 15 °C sampled at 18 wpi. and 630 ddpi. were used for relative gene expression analysis of secretory IgM (sIgM) and membrane-bound IgM (mIgM). In reverse transcription quantitative polymerase chain reaction (RT-qPCR), RNA is extracted from samples and reverse transcribed into complementary DNA (cDNA) which is used as template in the qPCR reaction. The qPCR reaction consists of cDNA, a thermostable DNA polymerase,

nucleotides, sequence specific primers, and a fluorescent reporter molecule. DNA polymerase driven amplification (i.e. PCR) of target sequences contained within the cDNA is monitored in real-time. The reaction takes place in a thermal cycler involving the repeated steps: denaturation (separation of strands), annealing (binding of specific primers) and extension (synthesis of new strand) (Kuang et al. 2018). After each cycle, fluorescence emitted by the reporter molecule binding to synthesized double-stranded DNA (amplicons) is measured by an instrument incorporated in the thermal cycler. The fluorescence intensity is proportional to the number amplicons generated. In the first cycles, only small changes in fluorescence is detected. The cycle number at which fluorescence intensity increases significantly above this background level, quantification cycle (Cq), is used to quantify the initial amount of target sequences in the samples. There is an inverse relationship between the Cq-value and the initial amount of target DNA. Fewer cycles are needed for the fluorescence intensity to rise above the background level when the amount of target DNA is high in samples (Thermo Fisher Scientific 2016).

RT-qPCR was performed on six samples from both vaccinated and control fish in all three temperature groups. The six samples from vaccinated fish were selected based on previous collected data on specific antibody responses against *A. salmonicida* (Erkinharju et al. 2018). Head kidney samples from fish that showed the highest concentration of specific antibodies in serum were used for RT-qPCR. RNA was first extracted from the head kidney samples and then reverse transcribed into cDNA for use in qPCR, known as two step RT-qPCR. Amplification of specific transcripts in real-time was monitored by use of SYBR-Green dye emitting fluorescence when bound to double-stranded DNA.

2.5.1 RNA extraction

Extraction of RNA from head kidney samples, stored in RNAlater at - 20 °C, was performed using the RNeasy Mini Kit from Qiagen. The samples were trimmed to small pieces, of no more than 30 mg, and placed in sample tubes SB (Qiagen) with 750 µl RLT lysis buffer (Qiagen) containing 20 µl 2M DDT/mL and steel beads (4.5 mm, Action Sport Games A/S). The tubes were inserted into TissueLyser II (Qiagen) for disruption and homogenization of the tissue samples following shaking at high speed (25 Hz) for 2 minutes. After homogenization, the lysates were centrifuged at maximum speed (16000 x g) for 3 minutes and 700 µl of the supernatants were transferred to collection tubes containing 700 µl ethanol (70 %). The supernatants were mixed carefully with the ethanol by pipetting. Then, 700 µl of the samples were transferred to RNeasy spin columns and centrifuged at 8000 x g for 30 seconds. The

collection tubes with flow-through were discarded. This step was repeated with the remainder of the samples (700 µl). Washing steps included the addition of, first, 700 µl RW1 buffer and, second, 500 µl RPE buffer to the spin columns. Centrifugation speed was 8000 x g for 30 seconds in each step. In the third and last washing step, additional 500 µl RPE buffer was added and the spin columns were centrifuged at 8000 x g for 2 minutes. Flow-through was discarded in between the washing steps, and collection tube was changed. After washing, the spin columns were centrifuged at maximum speed to dry the membranes. For the elution of RNA, 40 µl of RNase-free water was added to the spin column membranes prior to centrifugation at 8000 x g for 1 minute. The RNA samples were transferred to Eppendorf tubes and stored at -80 °C until cDNA synthesis. Before proceeding with cDNA synthesis, the RNA concentration and purity of thawed samples were assessed by Nano Drop ND-1000 spectrophotometer (Thermo Fisher Scientific).

2.5.2 cDNA synthesis

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. 2 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 minutes. After incubation, 6 µl of a master mix with the reagents Quantiscript Reverse Transcriptase (RT), Quantiscript RT buffer and RT Primer Mix was added to the samples, according to the manufactures protocol, for a final reaction volume of 20 µl. Two selected samples in each cDNA synthesis round also served as negative RT controls (÷RT) and, thus, received RNase-free water instead of Quantiscript RT. The samples were incubated at 42 °C for 15 minutes and 95 °C for 3 minutes to activate and inhibit the reverse transcriptase, respectively. The samples were cooled to 4 °C after the incubation and were kept at - 20 °C until qPCR was scheduled five days later.

2.5.3 qPCR

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 8) were derived from previous work conducted by Erkinharju et al. (2019). The concentration of the primer solutions used was 5 µM.

Table 8: 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

The cDNA samples were mixed with ultrapure water to a 1:5 dilution ratio. 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 sybermix (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. Lumpfish muscle samples that were positive for the genes tested were used as positive control. The samples were run in a 7500 Fast Real-Time PCR System (Applied Biosystems) according to Table 9. Melting curve analysis was performed after the qPCR run to confirm amplification of specific products.

Table 9: Programme for fast SYBR-Green qPCR.

Steps	Temperature (°C)	Time	Number of cycles
Denaturation	95	20 s	Hold
Denaturation	95	3 s	40
Annealing and extension	60	30 s	40
Melt curve	95	15 s	Continuous
	60	1 min	
	95	15 s	
	60	15 s	

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 (Appendix C) 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 Pfaffel method (equation III):

$$\text{Ratio} = (E_{target})^{\Delta Cq_{target}(\text{control} - \text{sample})} / (E_{ref})^{\Delta Cq_{ref}(\text{control} - \text{sample})} \quad \text{III}$$

Where E_{target} and E_{ref} are the primer efficiencies of the target and reference gene and ΔCq is the difference in Cq values of control and sample for the target and reference gene (Pfaffl 2001). In this case, the Cq value of control refers to the average of the Cq values of control fish for target and reference genes.

2.6 Statistical analysis and graphics

Data handling 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 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 % ($p < 0.05$) was set for all tests.

3 Results

3.1 Welfare indicators

3.1.1 Specific growth rate

Specific growth rates (SGR) from 0 to 126 days for vaccinated and control lumpfish reared at 5 °C, 10 °C, and 15 °C are displayed in Figure 3. Calculations of specific growth rates were based on the mean weight for each group as fish were not individually tagged during the experiment from which the data have been collected. Measurements were performed on 30 randomly selected fish at day 0, while remaining fish in each temperature group were measured at day 126 (Erkinharju et al. 2018). Statistical analysis was not performed on SGR data.

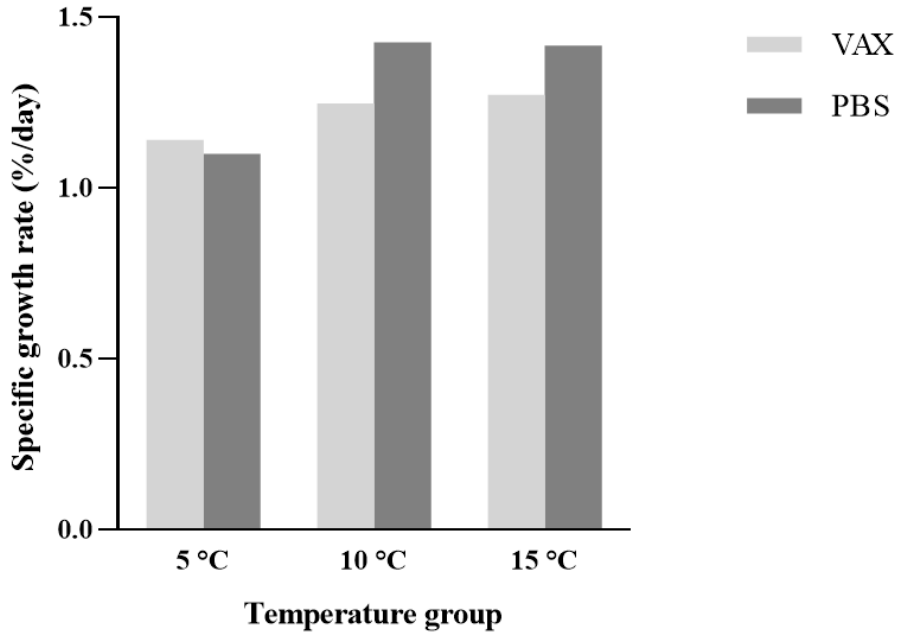

Figure 3: Mean specific growth rates of vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5 °C, 10 °C, and 15 °C from 0-126 days post injection. Day 0: n = 30 for VAX and PBS fish in all groups. Day 126: n = 30 for VAX and PBS fish in the 5 °C group, n = 28 for VAX fish in the 10 °C group, n = 30 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.

The results illustrated in Figure 3 show that the mean specific growth rates for both vaccinated and control fish were higher in the 10 °C and 15 °C group 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 close to negligible. In these two temperature groups, however, control

fish showed higher mean specific growth rates than vaccinated fish as opposed to fish in the 5 °C group. The differences in mean specific growth rates were 0.18 and 0.14 between control and vaccinated fish the 10 °C and 15 °C groups, respectively.

3.1.2 Condition factor

Fulton's condition factor was calculated (Equation II) for individual lumpfish at 126 days post injection to assess whether the given vaccine influenced the fitness of the fish. Figure 4 displays the mean condition factors of vaccinated and control lumpfish subjected to different water temperatures of 5 °C, 10 °C, and 15 °C.

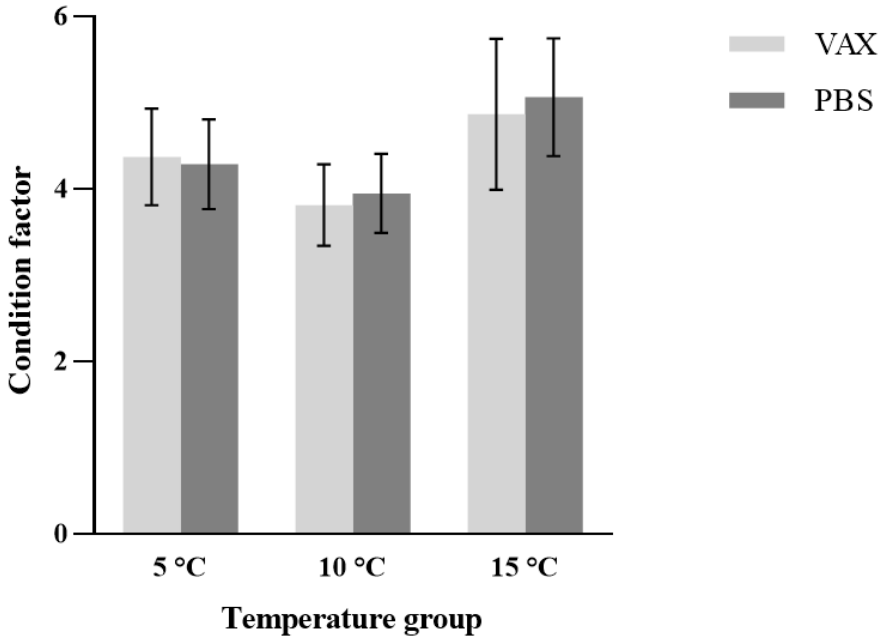

Figure 4: Condition factor of vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 126 days post injection. Error bars indicate ± standard deviation of mean condition factor. n = 30 for PBS and VAX fish in the 5 °C group, n = 28 for VAX fish in the 10 °C group, 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.

The results illustrated in Figure 4 show that lumpfish in the 15 °C group had significantly higher condition factors on average compared to those in the 10 °C and 5 °C groups ($p < 0.001$). The mean condition factors of lumpfish were 4.3 (± 0.54), 3.8 (± 0.46) and 4.9 (± 0.78) in the 5 °C, 10 °C, and 15 °C groups, respectively. There were no significant differences in mean condition factor between vaccinated and control fish.

3.2 Histology

3.2.1 Haematoxylin and eosin staining

Haematoxylin and eosin (HE) staining was used to assess the microanatomy of tissue samples from lumpfish subjected to water temperatures of 5 °C, 10 °C, and 15 °C following intraperitoneal injection of vaccine or phosphate buffered saline. HE-staining was performed on organ samples from kidney, heart, spleen, liver, pyloric caeca, pancreatic and abdominal adipose tissue.

Kidney

Figure 5 displays a photographed head kidney section (20x) from vaccinated lumpfish following HE-staining.

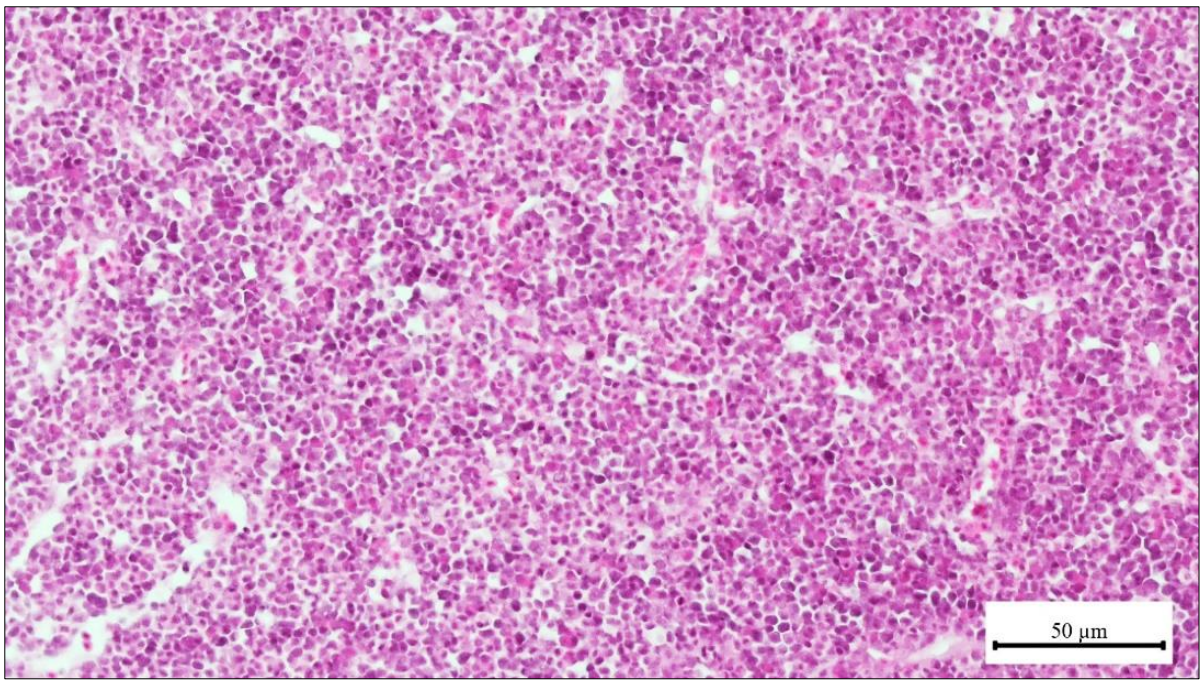

Figure 5: HE-staining of kidney section from a vaccinated lumpfish. Magnification is 20x and scale bar is 50 µm.

The normal histology of blood cell forming (haematopoietic) tissue in the kidney of lumpfish is shown in Figure 5 (HE-stain). All the examined kidney sections from vaccinated and control lumpfish in temperature groups 5 °C, 10 °C, and 15 °C showed normal structures. The examined kidney sections included both haematopoietic and excretory tissue constituting tubules and glomeruli (not shown).

Spleen

Figure 6 displays a photographed spleen section (20x) from a vaccinated lumpfish following HE-staining.

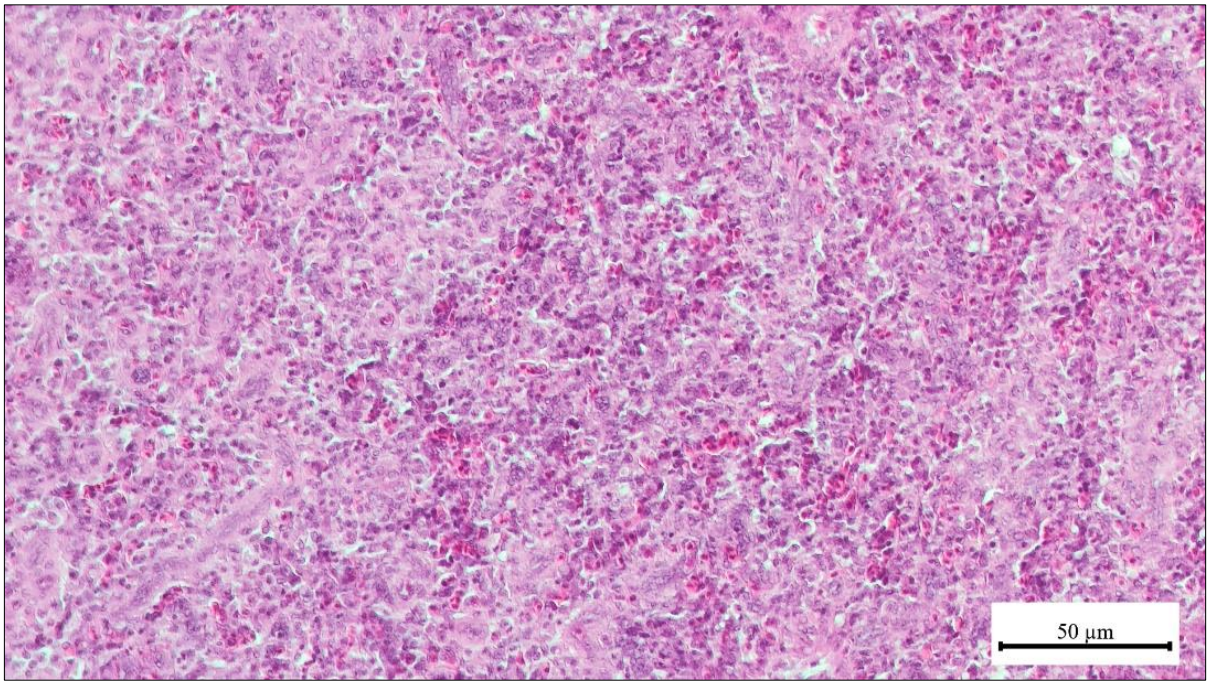

Figure 6: HE-staining of spleen section from a vaccinated lumpfish. Magnification is 20x and scale bar is 50 µm.

The normal histology of the spleen in lumpfish is shown in Figure 6 (HE-stain). Mature erythrocytes are located in between haematopoietic tissue, and a meshwork of lighter stained cells extends throughout the organ. All the examined spleen sections from vaccinated and control lumpfish in temperature groups 5 °C, 10 °C, and 15 °C showed normal structures.

Pancreatic and adipose tissue

Figure 7 displays photomicrographs (5x) of HE-stained sections of the pancreas, which is embedded in adipose tissue between the pyloric caeca. The photographed tissue sections are derived from one control and one vaccinated lumpfish illustrating absence and presence of intra-abdominal inflammatory tissue or lesion, respectively.

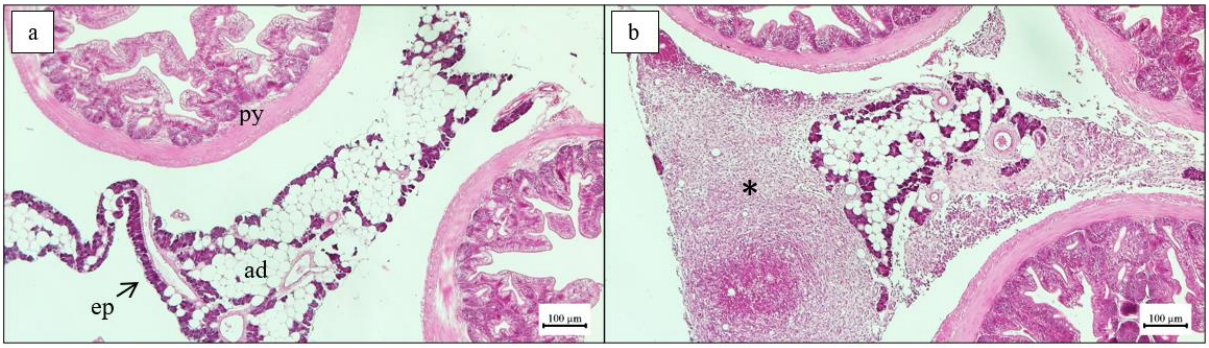

Figure 7: HE-staining of exocrine pancreas (ep) and adipose tissue (ad) located between pyloric caeca (py) from control (a) and vaccinated (b) lumpfish. a shows normal tissue histology, whereas b shows inclusion of perivisceral inflammatory tissue indicated by an asterisk (*). Magnification is 5x and scale bar is 100 µm.

Figure 7 shows histological sections of equivalent areas in the abdominal region of one control (a) and one vaccinated (b) lumpfish, including exocrine pancreas, adipose tissue, and pyloric caeca. Inflammatory tissue bordering the pancreas and pyloric caeca can be observed in the section derived from the vaccinated fish, but not in the section derived from the control fish showing normal histology of the pictured abdominal area. Observations of inflammatory tissue were noted in several of the HE-stained abdominal tissue sections derived from vaccinated fish. The following section describes these observations in more detail.

Intra-abdominal inflammatory tissue

Inflammatory lesions were observed on histological sections of abdominal tissue derived from vaccinated lumpfish subjected to water temperatures of 5 °C, 10 °C, and 15 °C at both 630 ddpi. and 18 wpi. Some of the tissue samples from control fish in the 15 °C group showed similar inflammatory tissue changes as those noted in samples from vaccinated fish. Table 10 lists the number of fish in which inflammatory tissue was detected on sections of abdominal tissue according to temperature group and sampling time point. 630 ddpi. corresponds to 6, 9, and 18 wpi. for temperature groups 15 °C, 10 °C, and 5 °C, respectively.

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

wpi.	**5 °C**		**10 °C**		**15 °C**	
	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

Intra-abdominal inflammatory tissue was more frequently detected in samples derived from vaccinated lumpfish in temperature groups 10 °C and 15 °C compared to temperature group 5 °C (Table 10). The occurrence of lesions did not differ markedly between samples taken from vaccinated fish in the 10 °C 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.).

Figure 8 displays photomicrographs of inflammatory lesions from vaccinated lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. following HE-staining. For each temperature group, one area of lesion is displayed in photomicrographs taken at two different magnifications (5x and 40x).

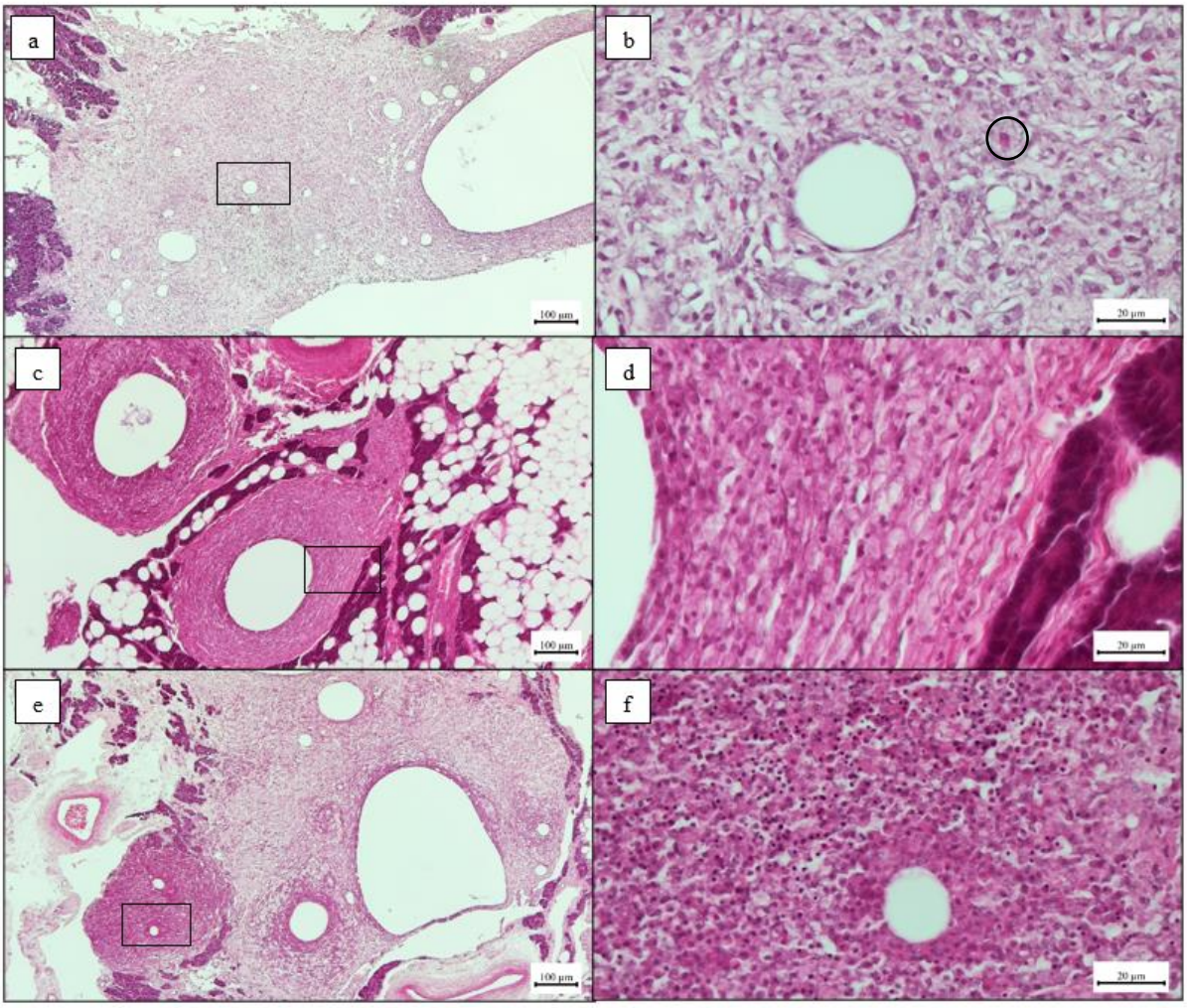

Figure 8: HE-staining of inflammatory lesion sections of vaccinated lumpfish in temperature groups 5 °C (a and b), 10 °C (c and d), and 15 °C (e and f) at 18 wpi. The inflammatory lesion sections are displayed at magnification 5x to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 40x to the right in the figure (b, d, f). The circle in b indicates a single cell with similar staining pattern and morphology as those seen around oil droplets in d and f. Scale bar is 100 µm for a, c, e (5x) and 20 µm for b, d, f (40x).

The results displayed in Figure 8 show presence of inflammatory lesions in the abdominal region of vaccinated lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. The lesions were located perivisceral in between pancreatic and adipose tissue that were among the pyloric caeca (Figure 8: a, c and e) or bordering the connective tissue capsule of the spleen (not

shown). Aggregations of inflammatory cells were, in most cases, centered around vaccine oil droplets present in the tissue (Figure 8: a-f). The appearance of the lesions ranged from loosely to densely defined arrangements of inflammatory tissue. Some of the cells constituting defined clusters within lesions were easily distinguished due to bright pink cytoplasmic staining. In sections from fish in temperature groups 15 °C and 10 °C, at both sampling time points (18 wpi. and 630 ddpi.), such clusters were frequently observed and tended to surround oil droplets if present (Figure 8: d and f). Pyknotic cell nuclei were observed in the areas of cell clustering in five of the sections, indicating cellular degradation or necrosis (Figure 8: f). Defined cell clusters were not observed in sections from fish in the 5 °C group and the inflammatory tissue was loosely arranged (Figure 8: a). Cells with similar staining pattern as those in clusters, i.e. bright pink, were scattered in the more loosely arranged areas of lesions dominated by connective tissue on sections from fish derived from all three temperature groups (Figure 8: b).

3.2.2 Special staining

May-Grünwald-Giemsa

The staining technique May-Grünwald-Giemsa (MGG) was performed on sections with inflammatory lesions derived from vaccinated lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at both sampling time points (18 wpi. and 630 ddpi.). Leukocytes with eosinophilic cytoplasmic granules were detected in lesions on all the examined sections. Figure 9 displays photomicrographs of MGG-stained lesions from fish vaccinated at 5 °C, 10 °C, and 15 °C sampled at 18 wpi. For each temperature group, one area of lesion is displayed in photomicrographs taken at two different magnifications (20x and 40x).

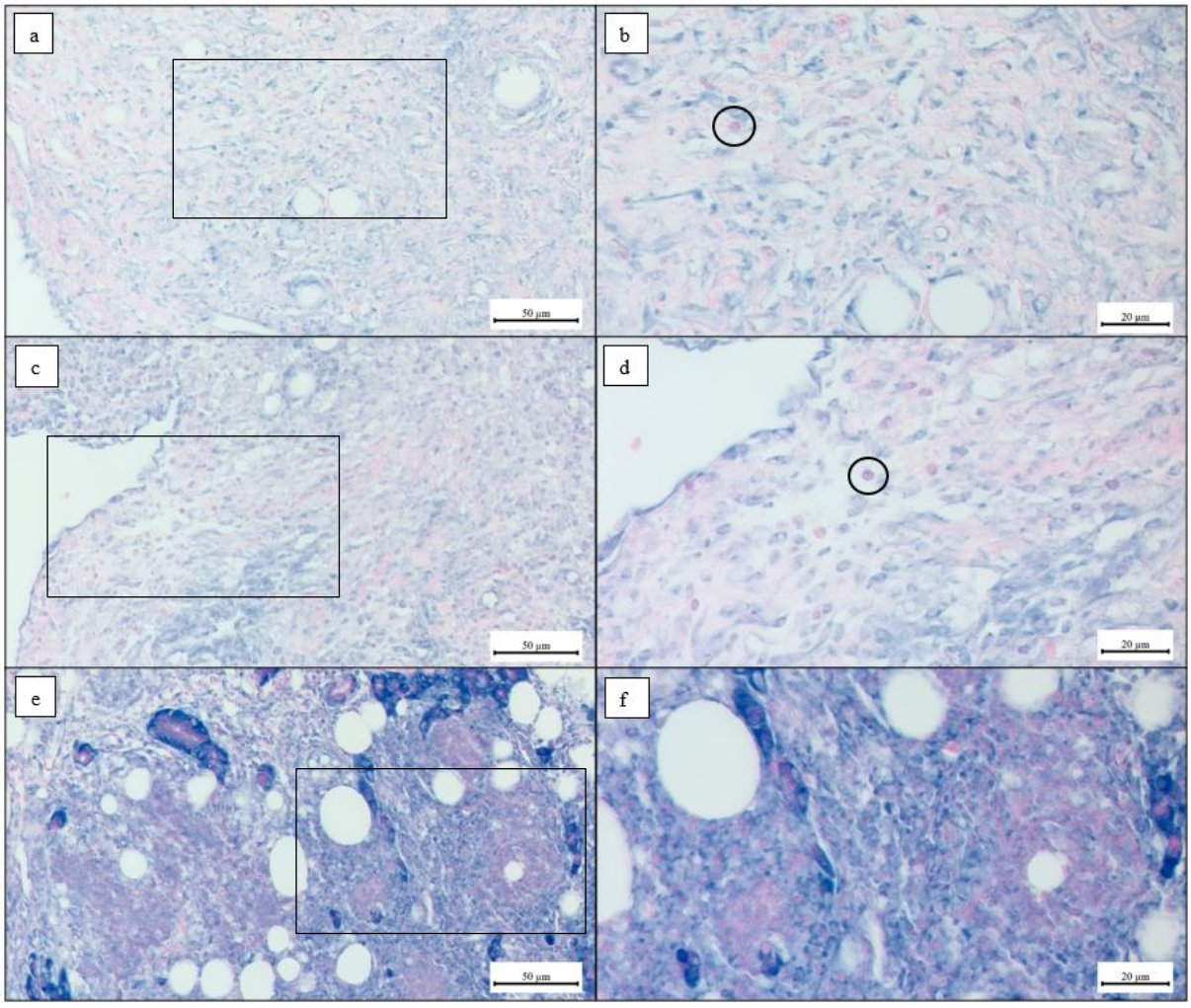

Figure 9: MGG-staining of inflammatory lesion sections of vaccinated lumpfish in temperature groups 5 °C (a and b), 10 °C (c and d), and 15 °C (e and f) at 18 wpi. Note presence of cells with eosinophilic granules. The inflammatory lesion sections are displayed at magnification 20x to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 40x to the right in the figure (b, d, f). The circles in b and c indicate single cells with eosinophilic granules. Scale bar is 50 µm for a, c, e (20x) and 20 µm for b, d, f (40x).

The results displayed in Figure 9 show presence of leukocytes with granulated cytoplasm in inflammatory lesions of vaccinated lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. following MGG-staining. The cells showed eosinophilic staining of granules and basophilic staining of the nucleus. In lesions of vaccinated fish in all the three temperature groups, the cells were quite numerous. The cells were scattered in loosely arranged inflammatory tissue (Figure 9: a-d) and surrounded oil droplets (Figure 9: e and f). The photographed section in e and f (Figure 9) illustrates an instance in which the eosinophilic granulated cells were distributed in high numbers around oil droplets. It should be noted that other cells showing basophilic staining were also detected in areas of such 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

The staining technique van Gieson (VG) was performed on sections with inflammatory lesions derived from vaccinated lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at both sampling time points (18 wpi. and 630 ddpi.). For all sections examined, collagen was detected. Figure 10 displays photomicrographs of inflammatory lesions from vaccinated fish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. following VG-staining. For each temperature group, one area of lesion is displayed in photomicrographs taken at two different magnifications (5x and 20x).

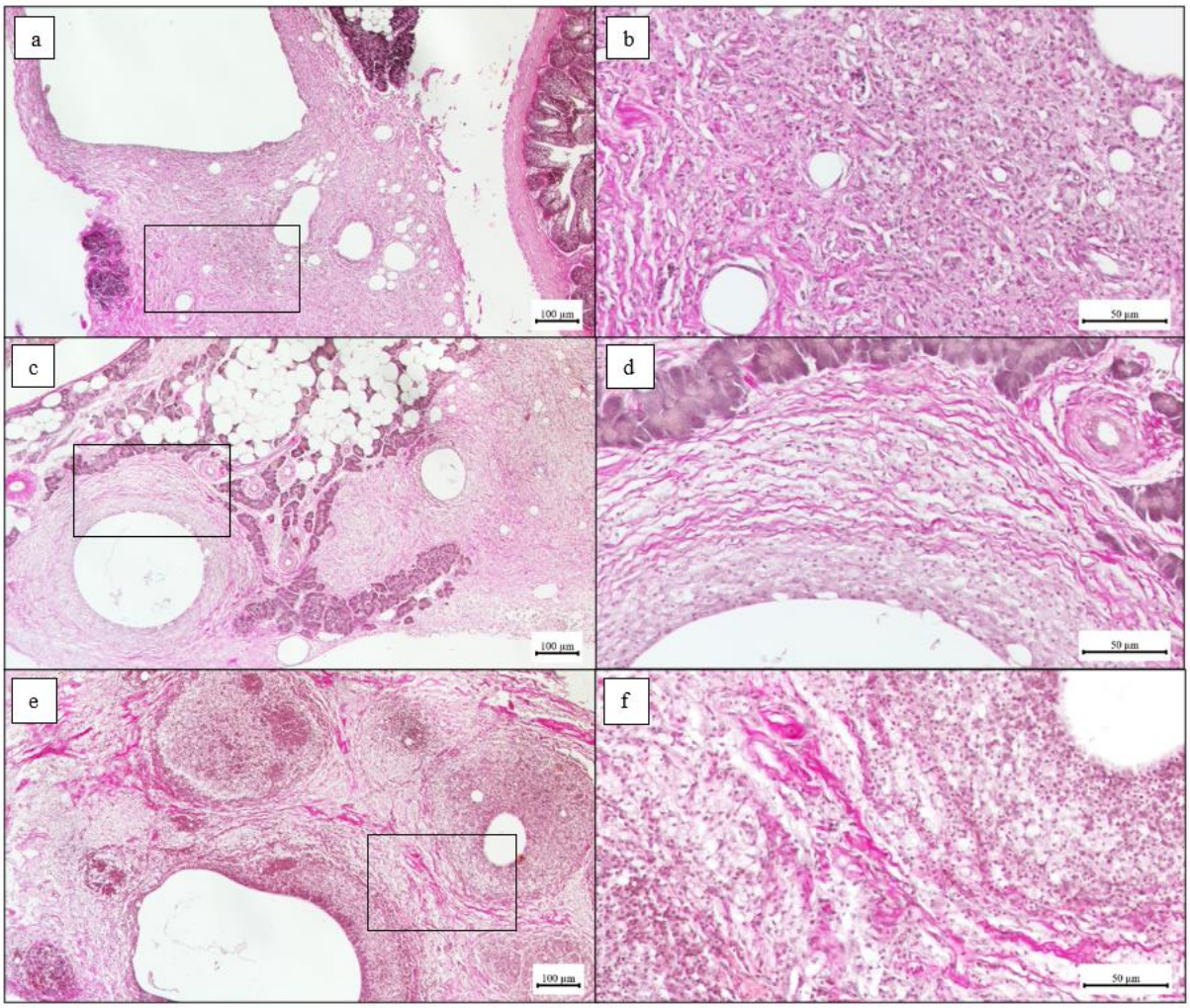

Figure 10: VG-staining of inflammatory lesion sections of vaccinated lumpfish in temperature groups 5 °C (a and b), 10 °C (c and d), and 15 °C (e and f) at 18 wpi. Note presence of collagen indicated by bright pink staining. The inflammatory lesion sections are displayed at magnification 5x to the left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

The results displayed in Figure 10 indicate collagen deposition (bright pink stain) in inflammatory lesions of vaccinated lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. after staining with van Gieson. Collagen was extensively distributed within the loosely arranged inflammatory tissue and surrounded aggregations of inflammatory cells centered around oil droplets (Figure 10: a-f). This general pattern also applied to inflammatory lesions from fish sampled at 630 ddpi. (not shown).

3.2.3 Immunohistochemistry

Immunohistochemistry (IHC) was performed on tissue sections from fish in which inflammation had been observed following HE-staining[1]. An equal number of sections from control fish was included for each temperature (5 °C, 10 °C, and 15 °C) and sampling time point group (18 wpi. and 630 ddpi.). Sections of the kidney, heart, spleen, liver, pancreatic and abdominal adipose tissue were used for IHC. For some of the sections, the heart, spleen, and liver were not included as these organs were not sampled from all fish. The target antigens in tissue sections were *A. salmonicida* LPS, PCNA and IgM.

Vaccine antigen

Sections of the kidney, heart, spleen, and inflammatory tissue were used for immunohistochemical detection of one of the bacterial vaccine antigens, *A. salmonicida*.

Kidney, heart, and spleen

Immunohistochemical staining with anti- *A. salmonicida* LPS was performed on sections of the kidney, heart, and spleen derived from vaccinated and control fish in temperature groups 5 °C, 10 °C, and 15 °C at both sampling time points (18 wpi. and 630 ddpi.). Positive staining of *A. salmonicida* was observed on sections of the kidney, heart, and spleen of vaccinated *and* control fish, but this did not apply for all sections examined.

Figure 11 displays photomicrographs of selected kidney sections from one vaccinated and one control fish in which positive staining of *A. salmonicida* was observed. Positive staining is indicated by red colouration.

[1] Except for tissue derived from one vaccinated fish in the 15 °C group at 18 wpi. and kidney tissue from one vaccinated fish in the 10 °C group at 18 wpi.

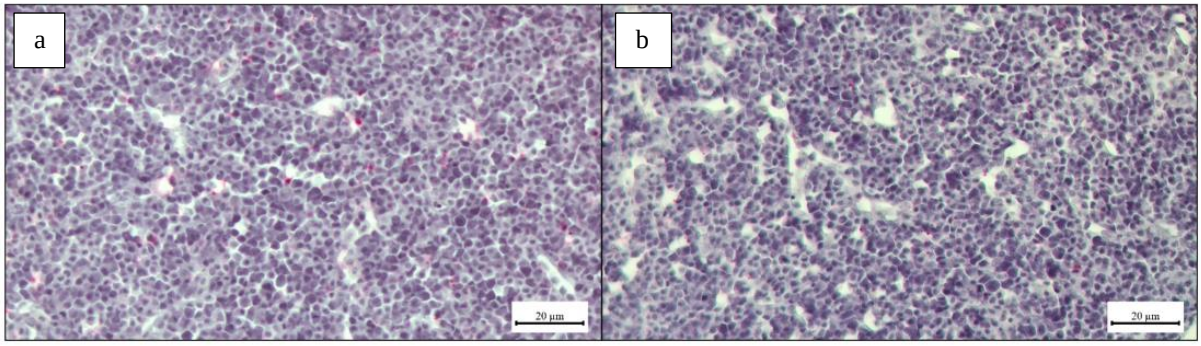

Figure 11: Immunohistochemical staining of A. salmonicida LPS in kidney sections of vaccinated (VAX) and control (PBS) lumpfish at 630 ddpi. Kidney sections of VAX and PBS fish in temperature groups 10 °C and 15 °C are displayed to the left and right in the figure, respectively (a: VAX 10 °C, b: PBS 15 °C). Magnification is 20x and scale bar is 50 µm.

The results displayed in Figure 11 show presence of *A. salmonicida* vaccine antigen in kidney sections of vaccinated (Figure 11: a) and control lumpfish (Figure 11: b). Antigen was detected in both the haematopoietic tissue and in blood vessels in kidney sections that stained positive following IHC. In heart sections, antigen was observed among blood cells in the lumen and in the myocardium (not shown). However, positive staining in the heart was relatively moderate. Kidney, heart, and spleen sections from vaccinated and control fish in the 5 ºC group were all negative for *A. salmonicida.* In the 10 ºC group at 18 wpi., kidney sections from three and heart and spleen sections from four vaccinated fish stained positive for *A. salmonicida*. None of the kidney, heart, and spleen sections from control fish in the 10 ºC group at 18 wpi. stained positive. Positive staining of *A. salmonicida* was observed in kidney sections from five, heart sections from four and a spleen section from one vaccinated fish in the 15 ºC group at 18 wpi. Control fish in the same group showed no positive staining of *A. salmonicida* in the kidney, heart, and spleen. At 630 ddpi., five vaccinated and five control fish in the 10 ºC group showed positive staining for *A. salmonicida* in kidney sections. Heart and spleen sections were not included for this group. Positive staining was observed in all kidney sections and seven heart sections from vaccinated fish in the 15 ºC group at 630 ddpi. For control fish in the same group, three kidney and two heart sections showed positive staining of *A. salmonicida*. The spleen was not sampled from fish in the 15 ºC group at 630 ddpi.

Intra-abdominal inflammatory tissue

Immunohistochemical staining with anti-*A. salmonicida* LPS was performed on sections in which inflammatory lesions had been observed (HE-stain) to detect residuals of *A. salmonicida* vaccine antigen at the injection site. Positive staining of *A. salmonicida* antigen was apparent in all the inflammatory lesions from vaccinated fish in temperature groups 5 °C, 10 °C, and 15 °C at both sampling time points (18 wpi. and 630 ddpi.). In sections of abdominal tissue from control fish, presence of *A. salmonicida* was not detected. The following results are presented according to sampling time points.

18 weeks post injection

Figure 12 displays photomicrographs of inflammatory lesions of vaccinated fish in the 5 °C, 10 °C, and 15 °C temperature groups at 18 wpi. following immunohistochemical staining with anti-*A. salmonicida* LPS. Positive staining is indicated by red colouration. For each temperature group, one area of lesion is displayed in photomicrographs taken at two different magnifications (20x and 40x).

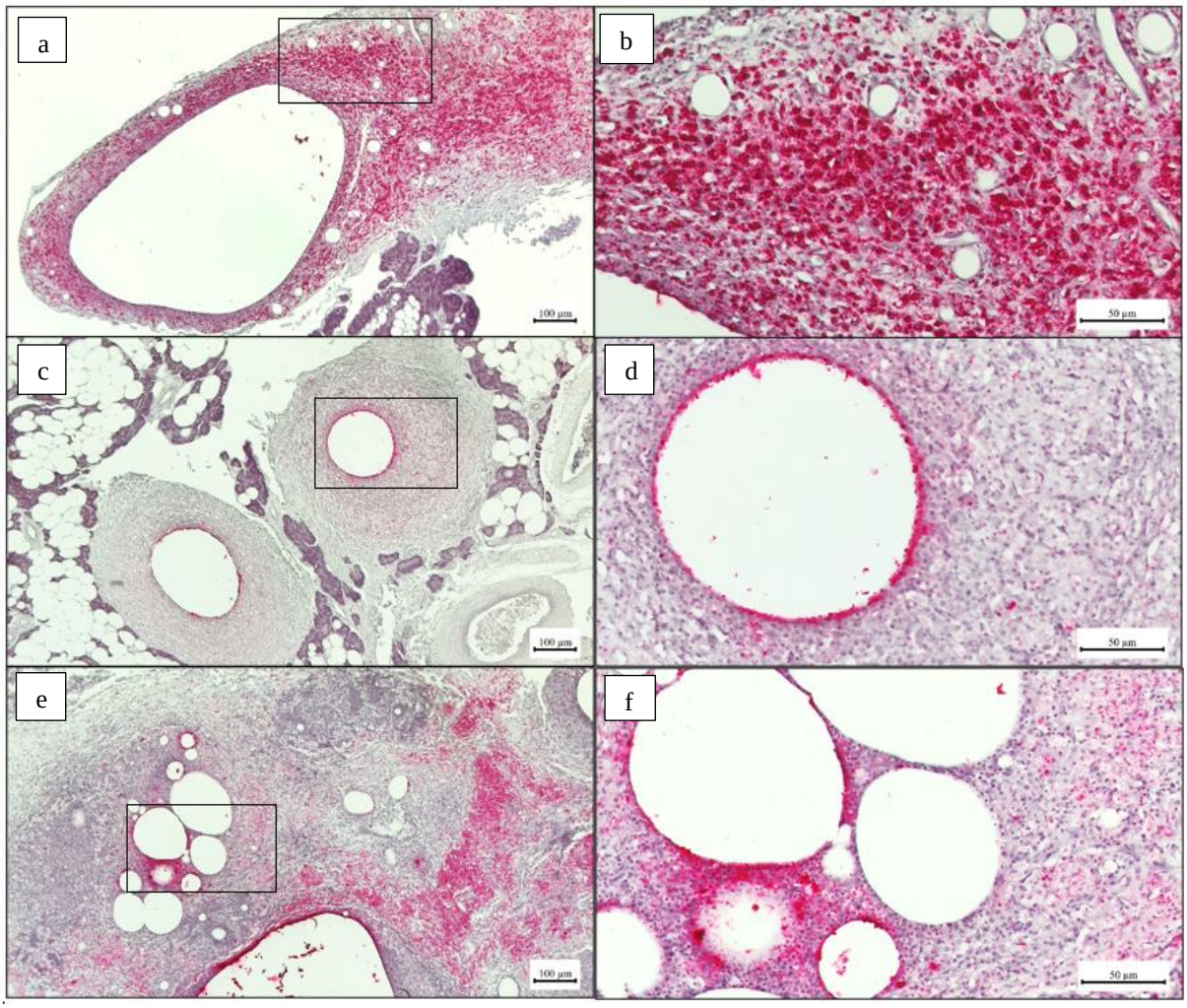

Figure 12: Immunohistochemical staining of A. salmonicida LPS in inflammatory lesion sections of vaccinated lumpfish in temperature groups 5 °C (a and b), 10 °C (c and d), and 15 °C (e and f) at 18 wpi. Red colouration indicates presence of vaccine antigen. The inflammatory lesion sections are displayed at magnification 5x to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 20x to the right in the figure (b, d, f). Scale bar is 100 µm for a, c, e (5x) and 50 µm for b, d, f (20x).

The results displayed in Figure 12 show presence of *A. salmonicida* vaccine antigen in inflammatory lesions of vaccinated lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi., which is indicated by the red stain. The vaccine antigen was located along the rim of oil droplets and was scattered throughout the inflammatory tissue of varying degree (Figure 12: a-f).

630 day degrees post injection

Figure 13 displays photomicrographs of inflammatory lesions of vaccinated fish in the 5 °C, 10 °C, and 15 °C temperature groups at 630 ddpi. following immunohistochemical staining with anti-*A. salmonicida* LPS. Positive staining is indicated by red colouration. For each temperature

group, one area of lesion is displayed in photomicrographs taken at two different magnifications (20x and 40x).

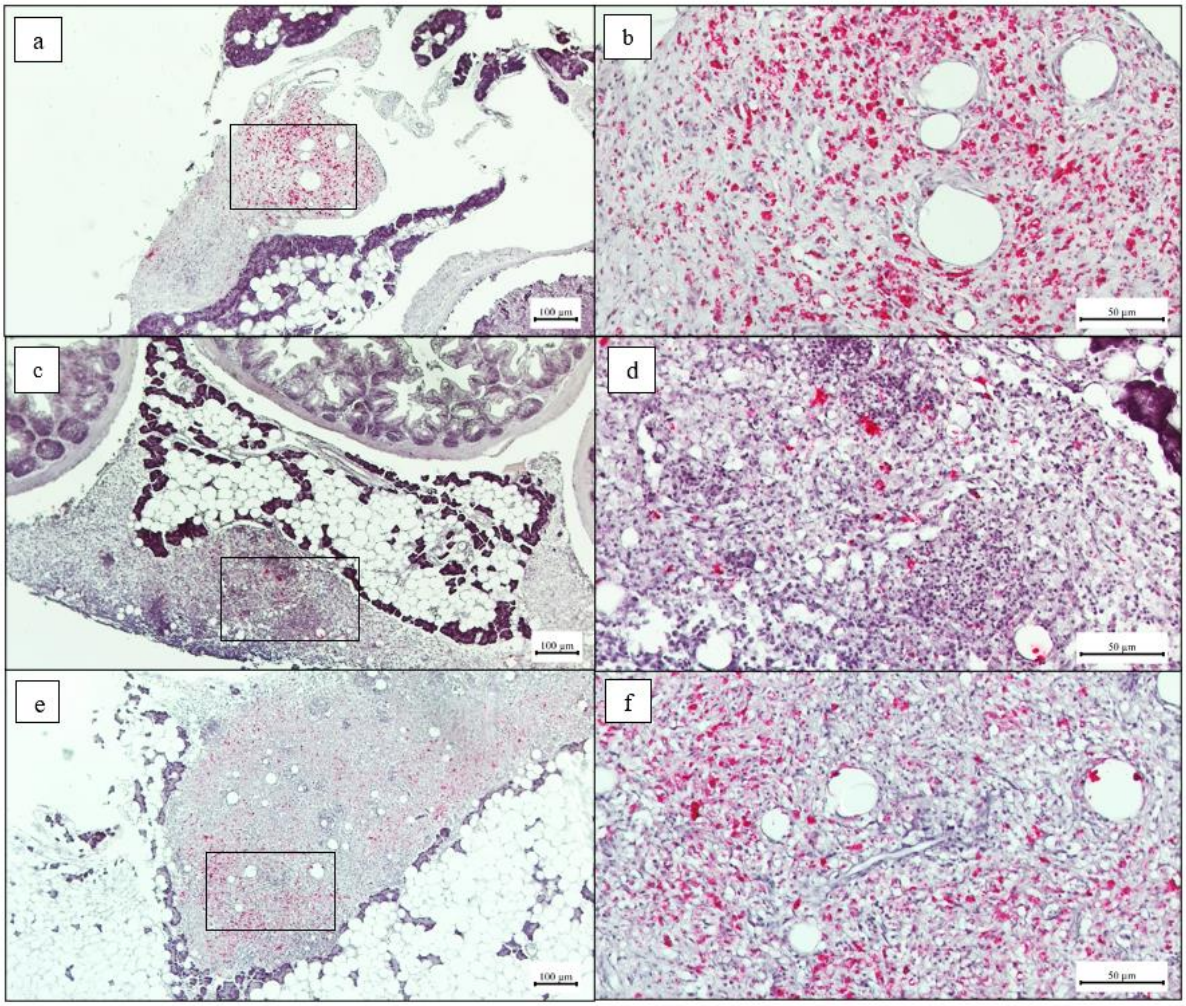

Figure 13: Immunohistochemical staining of A. salmonicida LPS in inflammatory lesion sections from vaccinated lumpfish in temperature groups 5 °C (a and b), 10 °C (c and d), and 15 °C (e and f) at 630 ddpi. Red colouration indicates presence of vaccine antigen. The inflammatory lesion sections are displayed at magnification 5x to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 20x to the right in the figure (b, d, f). Scale bar is 100 µm for a, c, e (5x) and 50 µm for b, d, f (20x).

The results displayed in Figure 13 show presence of *A. salmonicida* vaccine antigen in inflammatory lesions of vaccinated fish in temperature groups 5 °C, 10 °C, and 15 °C at 630 ddpi., which is indicated by the red stain. The staining pattern of *A. salmonicida* was similar to that observed in lesions of vaccinated fish at 18 wpi. including intense staining on the periphery of oils droplets (Figure 13: b, d and f).

Proliferating cells

Sections of the kidney, heart, spleen, liver, and inflammatory tissue were used for immunohistochemical detection of PCNA to identify proliferating cells within the different tissues.

Kidney

Figure 14 displays photomicrographs of kidney sections from vaccinated and control fish in the 5 °C, 10 °C, and 15 °C temperature groups at 18 wpi. following immunohistochemical staining with anti-PCNA. Positive staining is indicated by red colouration. For each temperature group, one photomicrograph (20x) is included for vaccinated and control fish.

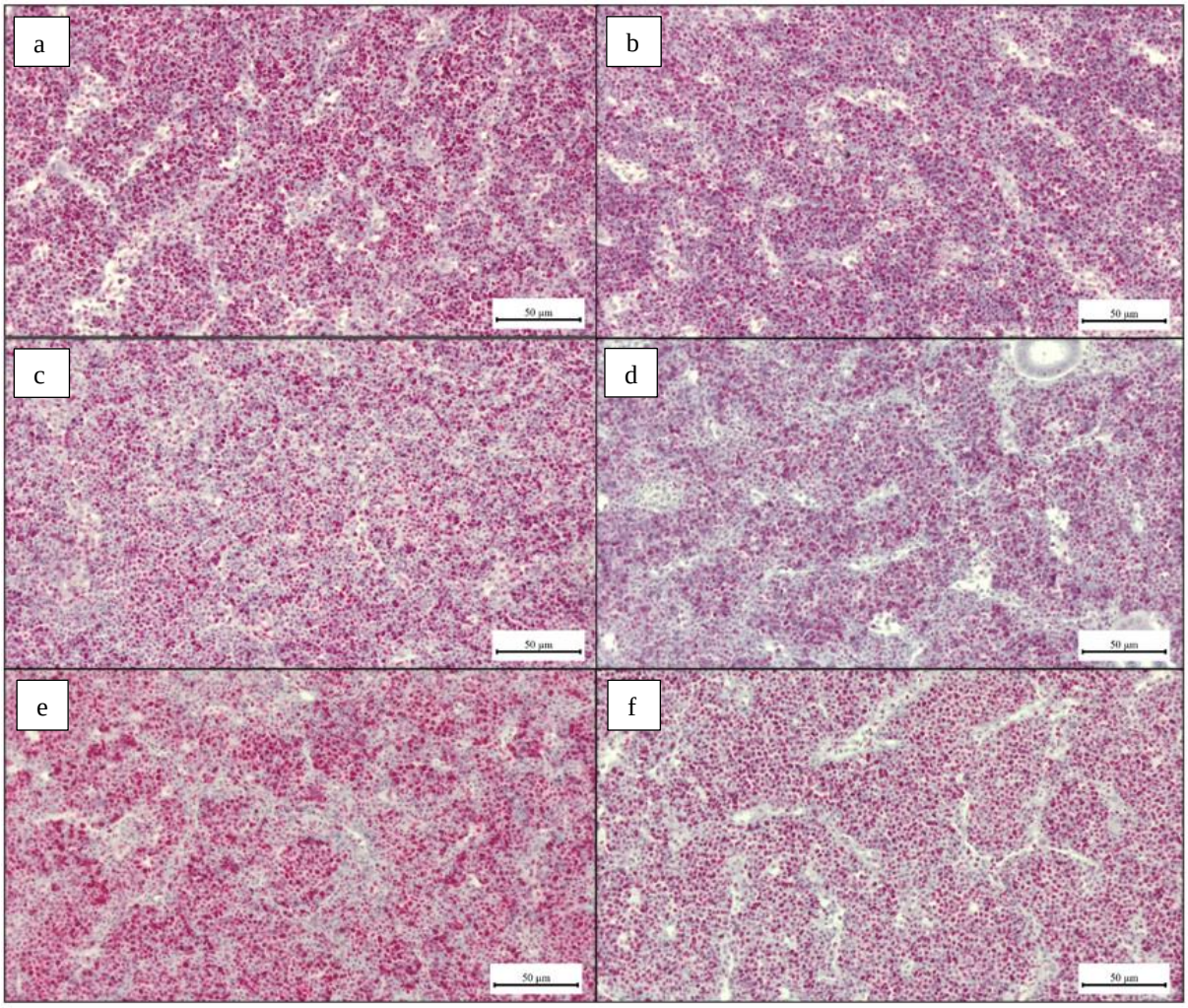

Figure 14: Immunohistochemical staining of PCNA in kidney sections of vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. Red colouration indicates presence of PCNA. Kidney sections of VAX and PBS fish are displayed to the left and right in the figure, respectively (a and b: 5 °C, c and d: 10 °C, e and f: 15 °C). Magnification is 20x and scale bar is 50 µm.

The results displayed in Figure 14 show presence of proliferating cells in the kidney of vaccinated and control fish at 18 wpi., which is indicated by the red stain. There were no apparent differences in PCNA staining intensity in kidney sections from fish in the 5 °C, 10 °C, and 15 °C temperature groups, including between vaccinated and control fish. Similar staining pattern of PCNA was observed in kidney sections of vaccinated and control fish in the 5 °C, 10 °C, and 15 °C temperature groups at 630 ddpi. (Appendix B).

Spleen

Figure 15 displays photomicrographs of spleen sections from vaccinated and control fish in the 5 °C, 10 °C, and 15 °C temperature groups at 18 wpi. following immunohistochemical staining with anti-PCNA. Positive staining is indicated by red colouration. For each temperature group, one photomicrograph (20x) is included for vaccinated and control fish.

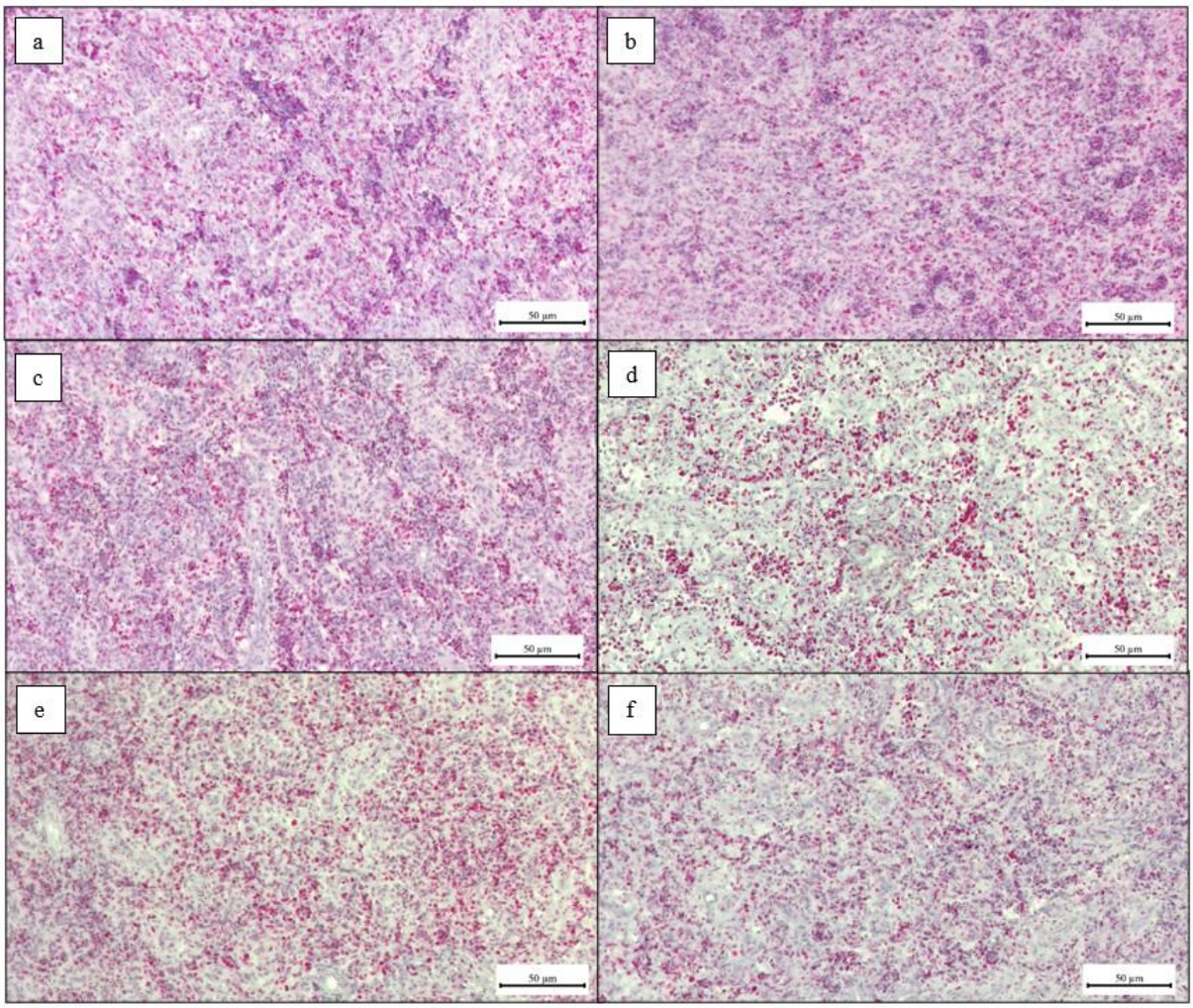

Figure 15: Immunohistochemical staining of PCNA in spleen sections of vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. Red colouration indicates presence of PCNA. Spleen sections of VAX and PBS fish are displayed to the left and right in the figure,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

The results displayed in Figure 15 show presence of proliferating cells in the haematopoietic tissue of the spleen derived from vaccinated and control lumpfish at 18 wpi., which is indicated by the red stain. There were no apparent differences in PCNA staining intensity in spleen sections from fish in the 5 °C, 10 °C, and 15 °C temperature groups, including between vaccinated and control fish.

Intra-abdominal inflammatory tissue

Immunohistochemical staining with anti-PCNA was performed on sections in which inflammatory lesions had been detected (HE-stain) to assess the presence and staining pattern of proliferating cells. Overall, there were many $PCNA^+$ cells scattered within the inflammatory lesions of vaccinated fish subjected to water temperatures of 5 °C, 10 °C, and 15 °C at both

sampling time points (18 wpi. and 630 ddpi.). The following results are presented according to sampling time points.

18 weeks post injection

Figure 16 displays photomicrographs of inflammatory lesions of vaccinated fish in the 5 °C, 10 °C, and 15 °C temperature groups at 18 wpi. following immunohistochemical staining with anti-PCNA. Positive staining is indicated by red colouration. For each temperature group, one area of lesion is displayed in photomicrographs taken at two different magnifications (20x and 40x).

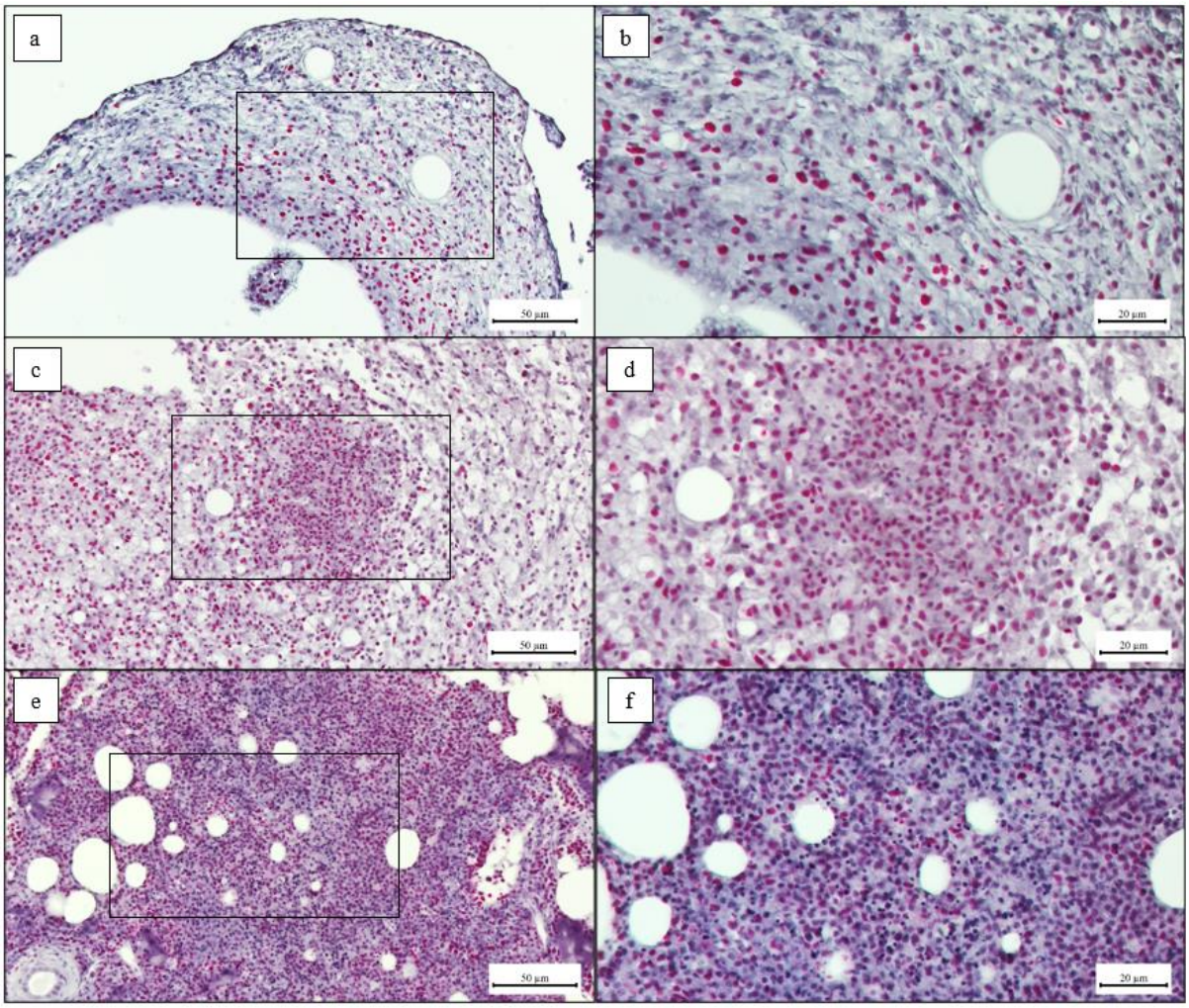

Figure 16: Immunohistochemical staining of PCNA in inflammatory lesion sections of vaccinated (VAX) lumpfish in temperature groups 5 °C (a and b), 10 °C (c and d), and 15 °C (e and f) at 18 wpi. Red colouration indicates presence of PCNA. The inflammatory lesion sections are displayed at magnification 20x to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 40x to the right in the figure (b, d, f). Note presence of proliferating cells around oil droplets. Scale bar is 50 µm for a, c, e (20x) and 20 µm for b, d, f (40x).

The results displayed in Figure 16 show presence of proliferating cells in inflammatory lesions of vaccinated fish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi., which is indicated by the red stain. Proliferating cells were detected in high numbers in the majority of lesions examined from vaccinated fish in all three temperature groups (Figure 16: a-f). The $PCNA^+$ cells were scattered in the loosely organized inflammatory tissue and tended to surround oil droplets. The most pronounced staining was observed among cells forming dense aggregations in the lesions (Figure 16: c-f) notably often surrounding oil droplets (Figure 16: e and f). The tissue in samples from control fish resembling inflammatory lesions of vaccinated fish did not show positive staining of PCNA.

630 day degrees post injection

Figure 17 displays photomicrographs of inflammatory lesions of vaccinated fish in the 5 °C, 10 °C, and 15 °C temperature groups at 630 ddpi. following immunohistochemical staining with anti-PCNA. Positive staining is indicated by red colouration. For each temperature group, one area of lesion is displayed in photomicrographs taken at two different magnifications (20x and 40x).

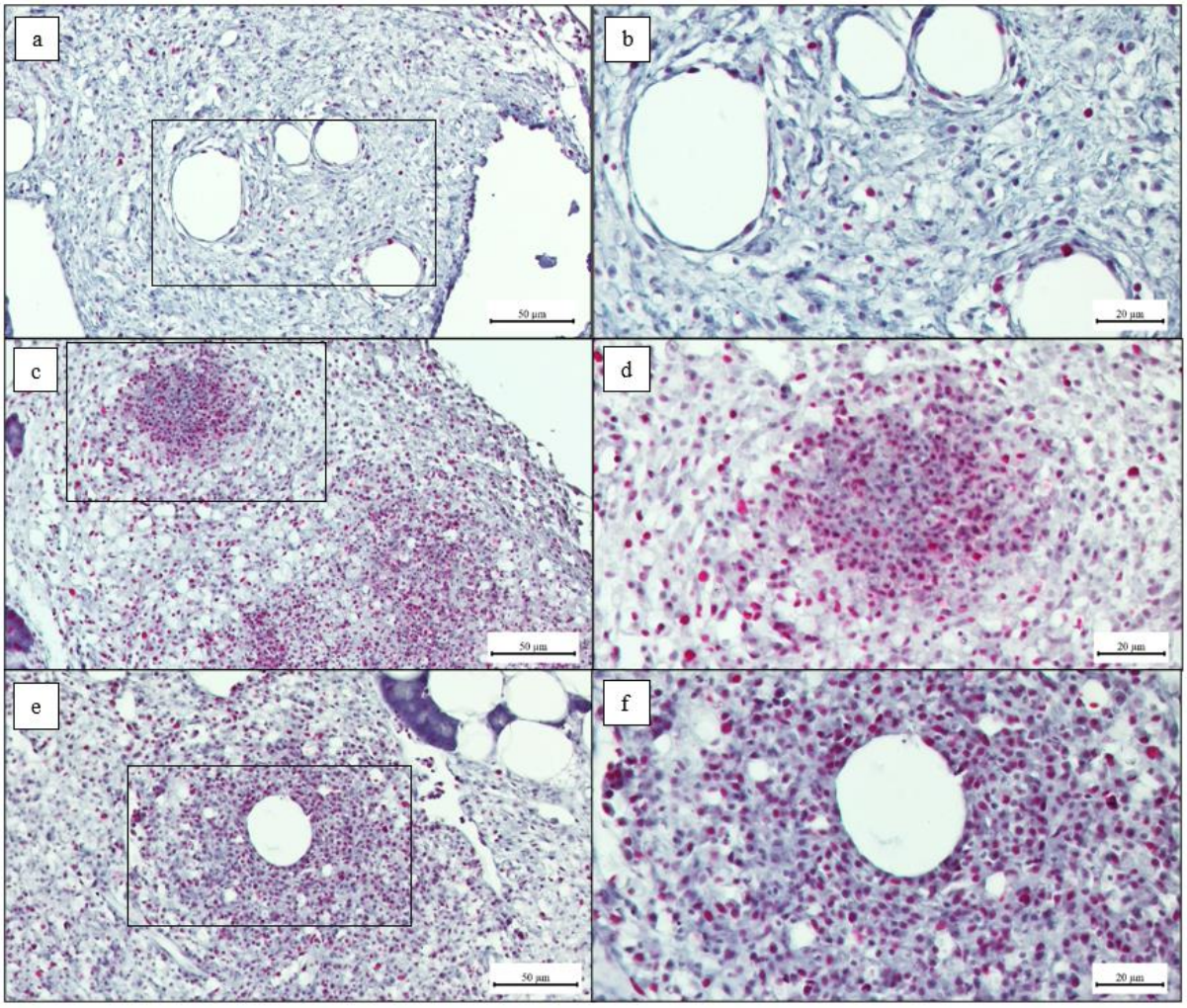

Figure 17: Immunohistochemical staining of PCNA in inflammatory lesion sections of vaccinated (VAX) lumpfish in temperature groups 5 °C (a and b), 10 °C (c and d), and 15 °C (e and f) at 630 ddpi. Red colouration indicates presence of PCNA. The inflammatory lesion sections are displayed at magnification 20x to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 40x to the right in the figure (b, d, f). Note aggregations of proliferating cells. Scale bar is 50 µm for a, c, e (20x) and 20 µm for b, d, f (40x).

The results displayed in Figure 17 show presence of proliferating cells in inflammatory lesions of vaccinated fish in temperature groups 5 °C, 10 °C, and 15 °C at 630 ddpi., which is indicated by the red stain. The observations of 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. Several proliferating cells were scattered in the tissue and staining tended to increase in intensity towards the areas centered around oil droplets, where cells were densely aggregated (Figure 17: e and f). The tissue in samples from control fish resembling inflammatory lesions of vaccinated fish did not show positive staining of PCNA.

Difference in PCNA staining pattern was observed in inflammatory lesions derived from the same fish in temperature group 15 °C sampled at 630 ddpi. Figure 18 displays two area of lesions (20x) following immunohistochemical staining with anti-PCNA. Positive staining is indicated by red colouration.

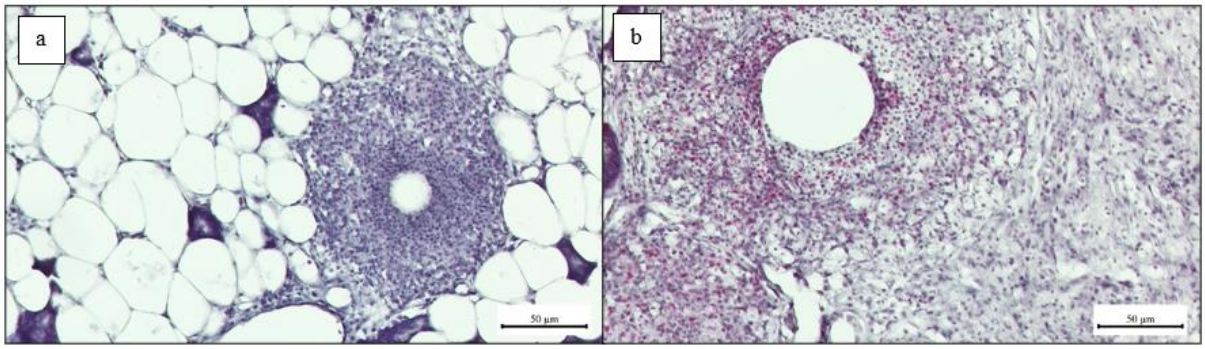

Figure 18: Immunohistochemical staining of PCNA in two inflammatory lesions from the same vaccinated lumpfish in temperature group 15 °C at 630 ddpi. Red colouration indicates presence of PCNA. Note the different staining pattern of PCNA in the two lesions. Magnification is 20x and scale bar is 50 µm.

The results displayed in Figure 18 show different staining pattern of PCNA in lesions from the same vaccinated individual in temperature group 15 °C at 630 ddpi., which is indicated by absence (a) and presence (b) of red stain. Cells were densely aggregated around a lipid droplet in both lesions, but proliferation was only occurring in one of the lesions (Figure 18: b).

Secretory and membrane-bound IgM

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

Kidney

The number of IgM^+ cells in the kidneys of vaccinated and control fish in the 5 °C, 10 °C, and 15 °C temperature groups at 18 wpi. and 630 ddpi. was determined by counting stained cells on four photographs taken from each of the kidney sections. Four photographs were chosen due to an insignificant p-value following a paired T-test of average values resulting from counting of cells on eight versus four photographs taken from kidney sections of ten fish. Table 11 lists the number of IgM^+ cells in kidney sections of individual fish in each temperature group at 18 wpi. (see Appendix B for 630 ddpi.).

Table 11: Number of IgM$^+$ cells in four photographs of kidney sections from vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. Values in bold are averages of groups. n=4 for VAX and PBS fish in the 5 °C group, n=6 for VAX and PBS in the 10 °C group, n=7 for VAX and PBS fish in the 15 °C group. Dash (-) indicates information not available.

Fish	**5 °C**		**10 °C**		**15 °C**	
	VAX	PBS	VAX	PBS	VAX	PBS
1	174	333	38	59	146	72
2	267	188	220	162	115	123
3	149	237	191	269	149	114
4	127	236	63	279	123	112
5	-	-	331	152	107	93
6	-	-	84	236	95	85
7	-	-	-	-	127	150
Average	**179.3**	**248.5**	**154.5**	**192.8**	**123.1**	**107**

The average number of IgM$^+$ cells in kidney sections of vaccinated and control fish was similar across the different temperature groups of 5 °C, 10 °C, and 15 °C, and at the sampling time points 18 wpi. (Table 11) and 630 ddpi. (Appendix B). No apparent trend in the number of IgM$^+$ cells was observed among vaccinated and control fish in the different temperature groups even though the numbers varied to some extent between individual fish.

Figure 19 displays photomicrographs of kidney sections from vaccinated and control fish in the 5 °C, 10 °C, and 15 °C temperature groups at 18 wpi. following immunohistochemical staining with anti-IgM. Positive staining is indicated by red colouration. For each temperature group, one photomicrograph (20x) is included for vaccinated and control fish.

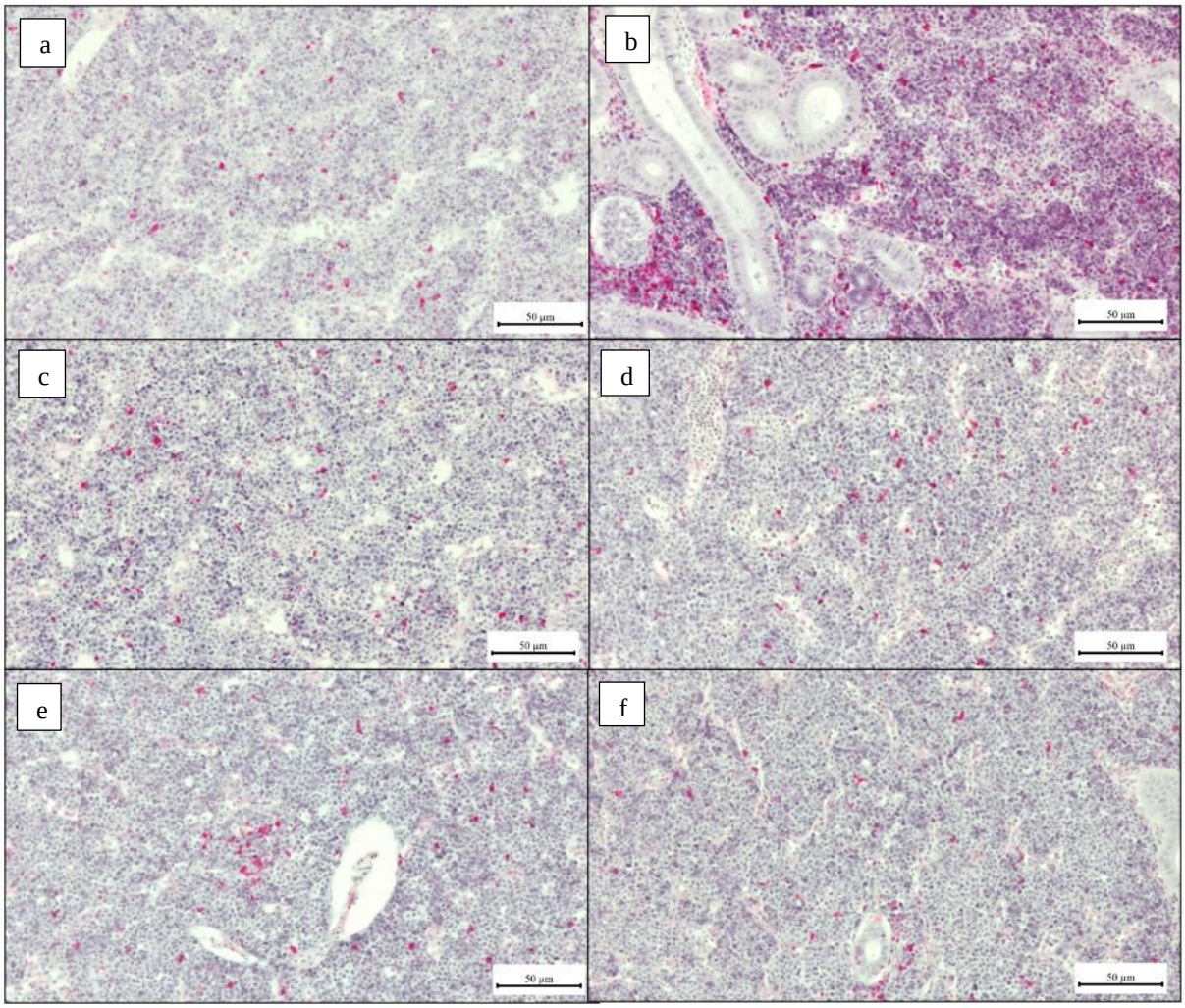

Figure 19: Immunohistochemical staining of IgM in kidney sections of vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. Red colouration indicates presence of IgM. Kidney sections of VAX and PBS fish are displayed to the left and right in the figure, respectively (a and b: 5 °C, c and d: 10 °C, e and f: 15 °C). Magnification is 20x and scale bar is 50 µm.

The results displayed in Figure 19 show presence of IgM^+ cells in the kidneys of vaccinated and control fish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi., which is indicated by the red stain. The IgM^+ cells were scattered in the tissue (Figure 19: a-f) and, in some instances, clustered around tubules or glomeruli in kidney sections from both vaccinated and control fish (Figure 19: b and f) in the different temperature groups. Diffuse staining was also observed 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 °C, 10 °C, and 15 °C temperature groups at 630 ddpi. (Appendix B).

Spleen

The number of IgM^+ cells in the spleen of vaccinated and control fish in the 5 °C, 10 °C, and 15 °C temperature groups at 18 wpi. was determined by counting stained cells on four photographs taken from each of the spleen sections. The spleen was not sampled from all fish in which inflammation was detected. Table 12 lists the number of IgM^+ cells in spleen sections of individual fish in each temperature group at 18 wpi.

Table 12: Number of IgM^+ cells in four photographs of spleen sections from vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. Values in bold are averages of groups. n=2 for VAX fish in the 5 °C group, n=3 for PBS fish in the 5 °C group, n=4 for VAX and PBS fish in the 10 °C group, n= 5 for VAX fish in the 15 °C group, n=6 for PBS fish in the 15 °C group. Dash (-) indicates information not available.

Fish	**5 °C**		**10 °C**		**15 °C**	
	VAX	PBS	VAX	PBS	VAX	PBS
1	270	3	89	10	20	172
2	387	481	250	33	39	11
3	-	654	9	94	115	111
4	-	-	72	0	56	60
5	-	-	-	-	45	87
6	-	-	-	-	-	17
Average	**328.5**	**379.3**	**105**	**34.6**	**55**	**76.3**

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 (Table 12). 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 as indicated by the average values of temperature groups.

Figure 20 displays photomicrographs of spleen sections from vaccinated and control fish in the 5 °C, 10 °C, and 15 °C temperature groups at 18 wpi. following immunohistochemical staining with anti-IgM. Positive staining is indicated by red colouration. For each temperature group, one photomicrograph (20x) is included for vaccinated and control fish.

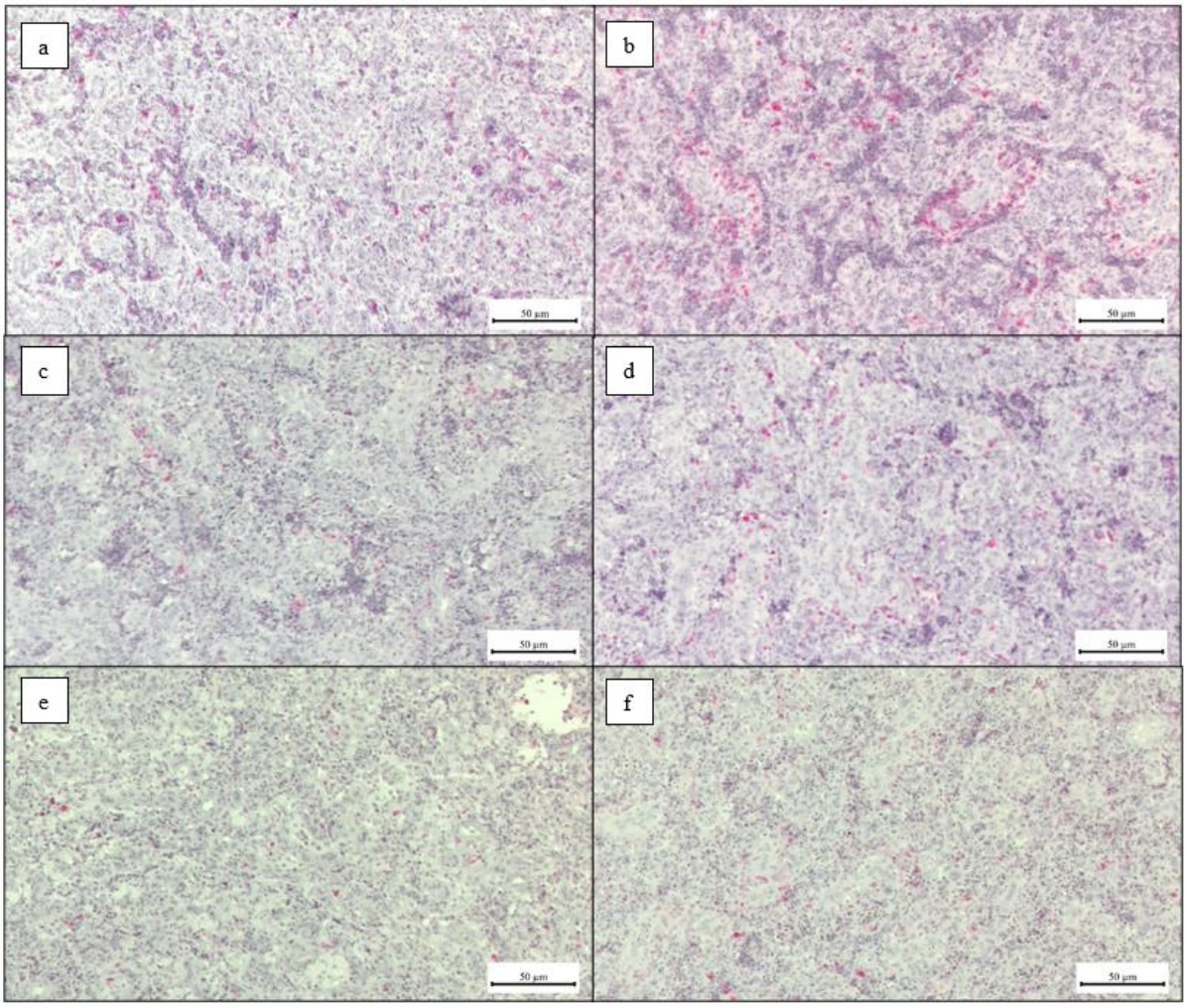

Figure 20: Immunohistochemical staining of IgM in spleen sections of vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. Red colouration indicates presence of IgM. Spleen sections of VAX and PBS fish are displayed to the left and right in the figure, respectively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

The results displayed in Figure 20 show presence of IgM in spleen sections of vaccinated and control lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. The IgM^+ cells were scattered in the tissue of vaccinated and control fish in all three temperature groups (Figure 20: a-f). The staining intensity of cells differed among the sections examined and no obvious staining pattern was detected. In some instances, however, clustering of IgM^+ cells around blood vessels was observed (Figure 20: b).

Intra-abdominal inflammatory tissue

Immunohistochemical staining with anti-IgM was performed on sections in which inflammatory lesions had been observed (HE-stain) to determine the presence of IgM^+ cells and to assess the distribution of such cells in the lesions. Staining revealed presence of IgM^+ cells

in lesions of vaccinated fish in the 5 °C, 10 °C, and 15 °C temperature groups at both sampling time points (630 ddpi. and 18 wpi.). The number of IgM^+ cells was determined by counting stained cells in each lesion and varied irrespective of temperature group ranging from 0-145 in the 5 °C group, 0-227 in the 10 °C group, and 1-120 in the 15 °C group. Table 14 lists the number of IgM^+ cells detected in lesions of vaccinated fish at 18 wpi. (see Appendix B for 630 ddpi.). For some fish, more than one lesion was detected on histological sections and IgM^+ cells were counted for each.

Table 14: Number of IgM^+ cells in inflammatory lesions of vaccinated lumpfish in temperature groups 5 °C, 10 °C, 15 °C at 18 wpi. More than one lesion was derived from some fish and the number of IgM^+ cells in each lesion is listed accordingly. Dash (-) indicates information not available.

Fish	5 °C	10 °C	15 °C
1	0	0	1
2	136	0, 33	2
3	26, 78	87, 72	4
4	145, 47	1, 6, 42	4,1
5	-	14, 12, 12	5, 3, 18
6	-	29, 9, 13, 12, 1, 144	24, 17, 17, 18
7	-	75, 106, 73, 227, 165	120, 56, 27, 12

The number of IgM^+ cells in lesions varied among fish within the 5 °C, 10 °C, and 15 °C groups at both 18 wpi. (Table 14) and 630 ddpi. (Appendix B) and differences in numbers were also observed between lesions from the same fish. There was no general trend detected in the abundance of IgM^+ cells in lesions of vaccinated fish in all groups. IgM^+ cells were detected in lesions from 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 in total. 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. The highest number of IgM^+ cells detected in lesions from fish vaccinated at 10 °C and 15 °C was 40 and 69, respectively. Less than ten IgM^+ cells were detected in lesions from four fish in the 10 °C group and five in the 15 °C group (Appendix B).

18 weeks post injection

Figure 21 displays photomicrographs of inflammatory lesions of vaccinated fish in the 5 °C, 10 °C, and 15 °C temperature groups at 18 wpi. following immunohistochemical staining with anti-IgM. Positive staining is indicated by red coloration. For each temperature group, one area of lesion is displayed in photomicrographs taken at two different magnifications (20x and 40x).

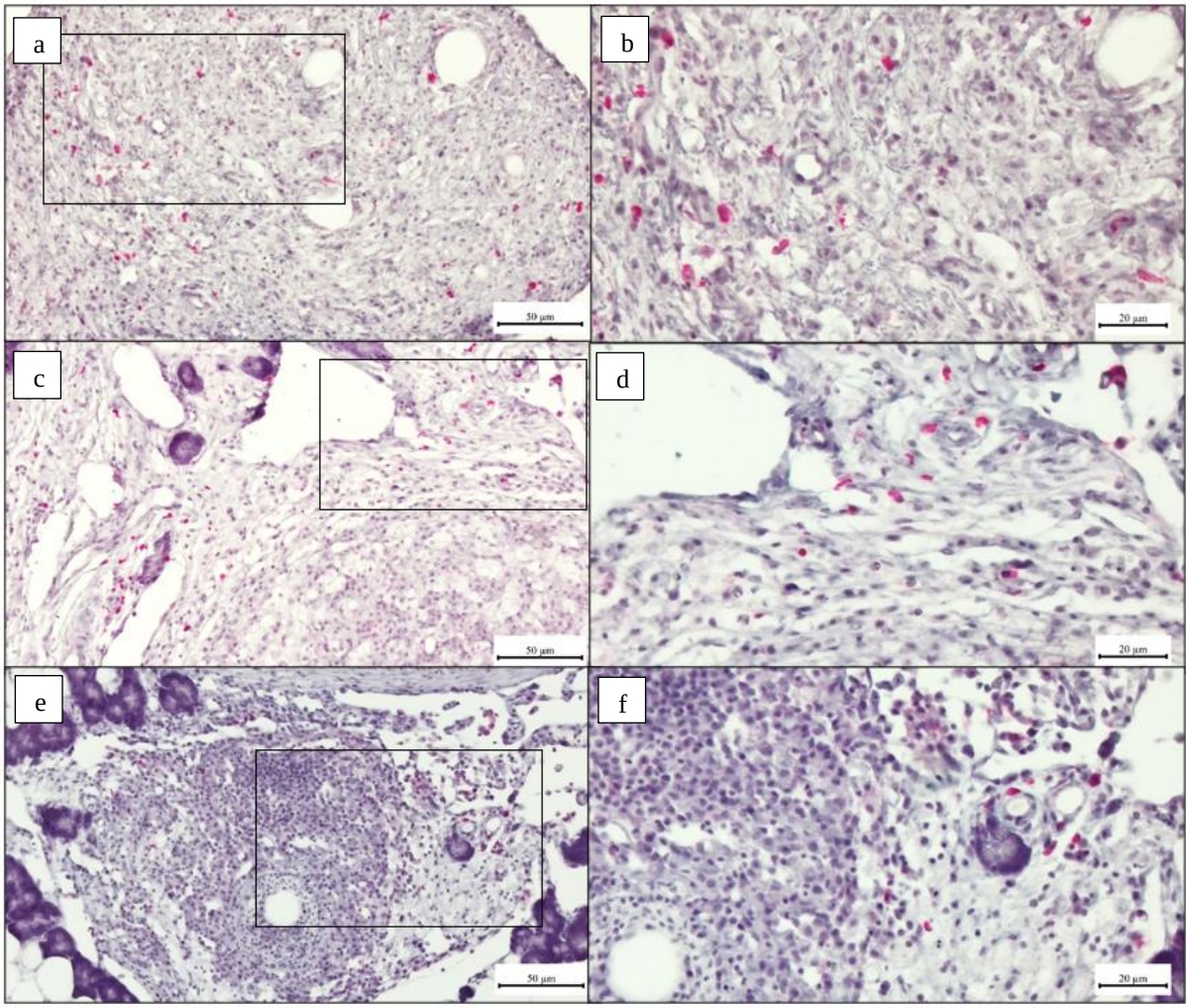

Figure 21: Immunohistochemical staining of IgM in inflammatory lesion sections of vaccinated (VAX) lumpfish in temperature groups 5 °C (a and b), 10 °C (c and d), and 15 °C (e and f) at 18 wpi. Red colouration indicates presence of IgM. The inflammatory lesion sections are displayed at magnification 20x to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 40x to the right in the figure (b, d, f). Note presence of cell associated IgM in the loose inflammatory tissue. Scale bar is 50 µm for a, c, e (20x) and 20 µm for b, d, f (40x).

The results displayed in Figure 21 show presence of IgM^+ cells in inflammatory lesions of vaccinated fish in temperature groups 5 °C, 10 °C, and 15 °C at 18 wpi. which is indicated by

the red stain. IgM^+ cells tended to be distributed in loosely arranged inflammatory tissue of vaccinated fish in all three temperature groups (Figure 21: a-f). This was especially noticeable in lesions derived from vaccinated fish in the 10 °C and 15 °C temperature groups which included areas of more densely arranged cells (Figure 21: c and 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 °C and 15 °C (Figure 21: d and f). Positive staining of IgM was detected in some of the sections from control fish; two in the 5 °C and 10 °C groups and three in the 15 °C group. Diffuse staining was observed in some arteries, while a couple of positive cells were found around arteries or in the pyloric caeca in the sections from control fish.

More than one lesion was present on sections derived from some of the vaccinated fish in the 10 °C and 15 °C temperature groups at 18 wpi. In three instances, there was an apparent difference in the number of IgM^+ cells in the lesions from the same fish. Figure 22 displays photomicrographs (20x) of two area of lesions from the same fish with different staining pattern of IgM. Positive staining is indicated by red colouration.

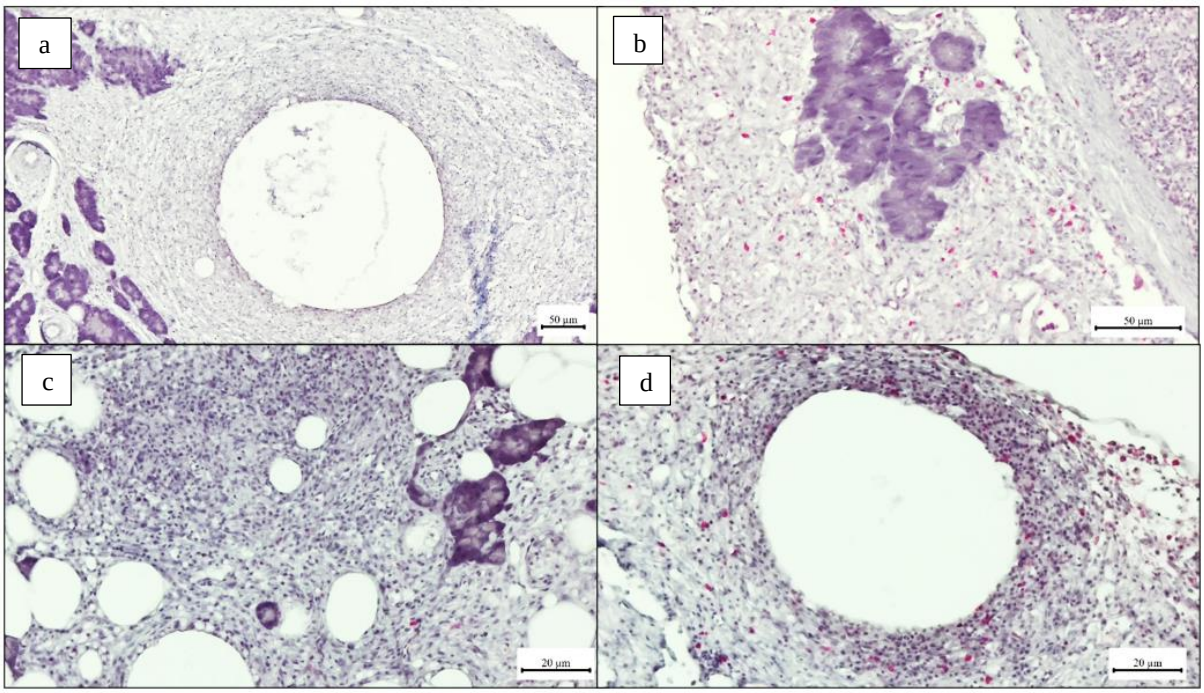

Figure 22: Immunohistochemical staining of IgM in two inflammatory lesions derived from the same vaccinated lumpfish at 18 wpi. The two lesions are from one fish in temperature group 10 °C (a and b) and another fish in temperature group 15 °C (c and d). Red colouration indicates presence of IgM. Note the different staining pattern of IgM in the two lesions derived from the same fish. Magnification is 10x with scale bar 50 µm for a, 20x with scale bar 50 µm for b, and 20x with scale bar 20 µm for c and d.

The results displayed in Figure 22 show different staining pattern of IgM in lesions from the same fish, which is indicated by red stain. The lesions are derived from one fish in the 10 °C group (Figure 22: a and b) and another fish in the 15 °C group (Figure 22: c and d). Both lesions from the fish vaccinated at 10 °C were characterized by loose inflammatory tissue. No IgM^+ cells were detected in the lesion with the prominent oil droplet located in between pancreatic tissue among the pyloric caeca (Figure 22: a), whereas several cells stained positive in the lesion adjacent to the spleen (Figure 22: b). Dense arrangements of cells associated with oil droplets were present in both lesions from the vaccinated fish in the 15 °C group. A small number of IgM^+ cells was detected in the periphery of the dense cell aggregate in one of the lesions (Figure 22: c), which was the general trend in observed staining pattern of IgM. Several IgM^+ cells were detected in the other lesion from the same fish. Some of these cells were distributed in loose inflammatory tissue while others were present in the dense cell aggregation surrounding the oil droplet (Figure 22: d).

630 day degrees post injection

Figure 23 displays photomicrographs of inflammatory lesions of vaccinated fish in the 5 °C, 10 °C, and 15 °C temperature groups at 630 ddpi. following immunohistochemical staining with anti-IgM. Positive staining is indicated by red colouration. For each temperature group, one area of lesion is displayed in photomicrographs taken at two different magnifications (20x and 40x).

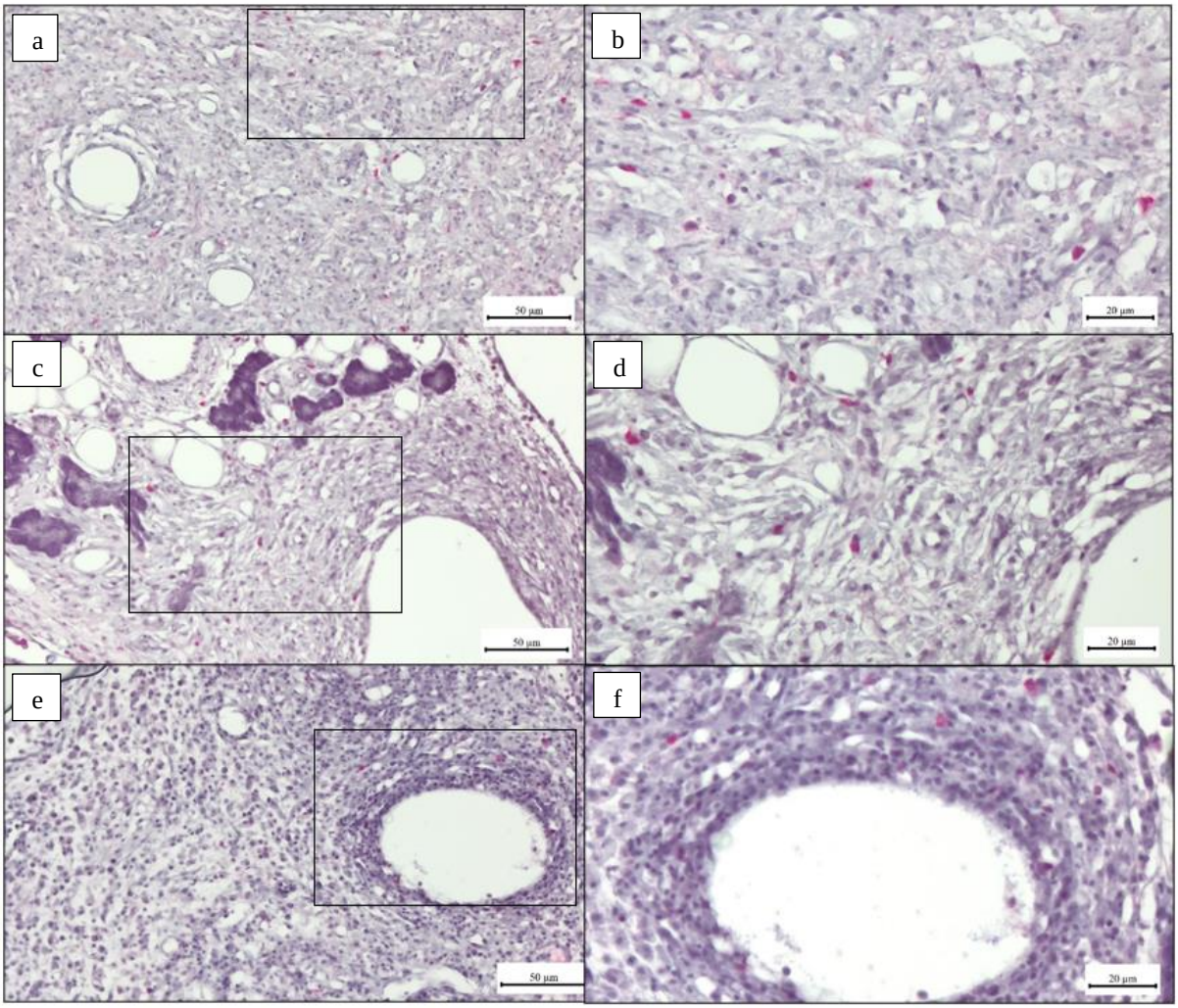

Figure 23: Immunohistochemical staining of IgM in inflammatory lesion sections of vaccinated (VAX) lumpfish in temperature groups 5 °C (a and b), 10 °C (c and d), and 15 °C (e and f) at 630 ddpi. Red colouration indicates presence of IgM. The inflammatory lesion sections are displayed at magnification 20x to the left in the figure (a, c, e). The black squares indicate the areas displayed at magnification 40x to the right in the figure (b, d, f). Note presence of cell associated IgM in the loose inflammatory tissue. Scale bar is 50 µm for a, c, e (20x) and 20 µm for b, d, f (40x).

The results displayed in Figure 23 show presence of IgM^+ cells in inflammatory lesions of vaccinated fish in temperature groups 5 °C, 10 °C and 15 °C at 630 ddpi. which is indicated by the red stain. 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 23: a-d) and were only detected among dense aggregations of cells in two lesions derived from different fish in the 15 °C group (Figure 23: f).

Comparison of stains

One area of inflammatory lesion from a vaccinated lumpfish in temperature group 15 °C sampled at 18 wpi. is displayed in Figure 24 for comparison of staining patterns produced following IHC with anti-*A. salmonicida* LPS, anti-PCNA and anti-IgM, including the routine histological HE-stain.

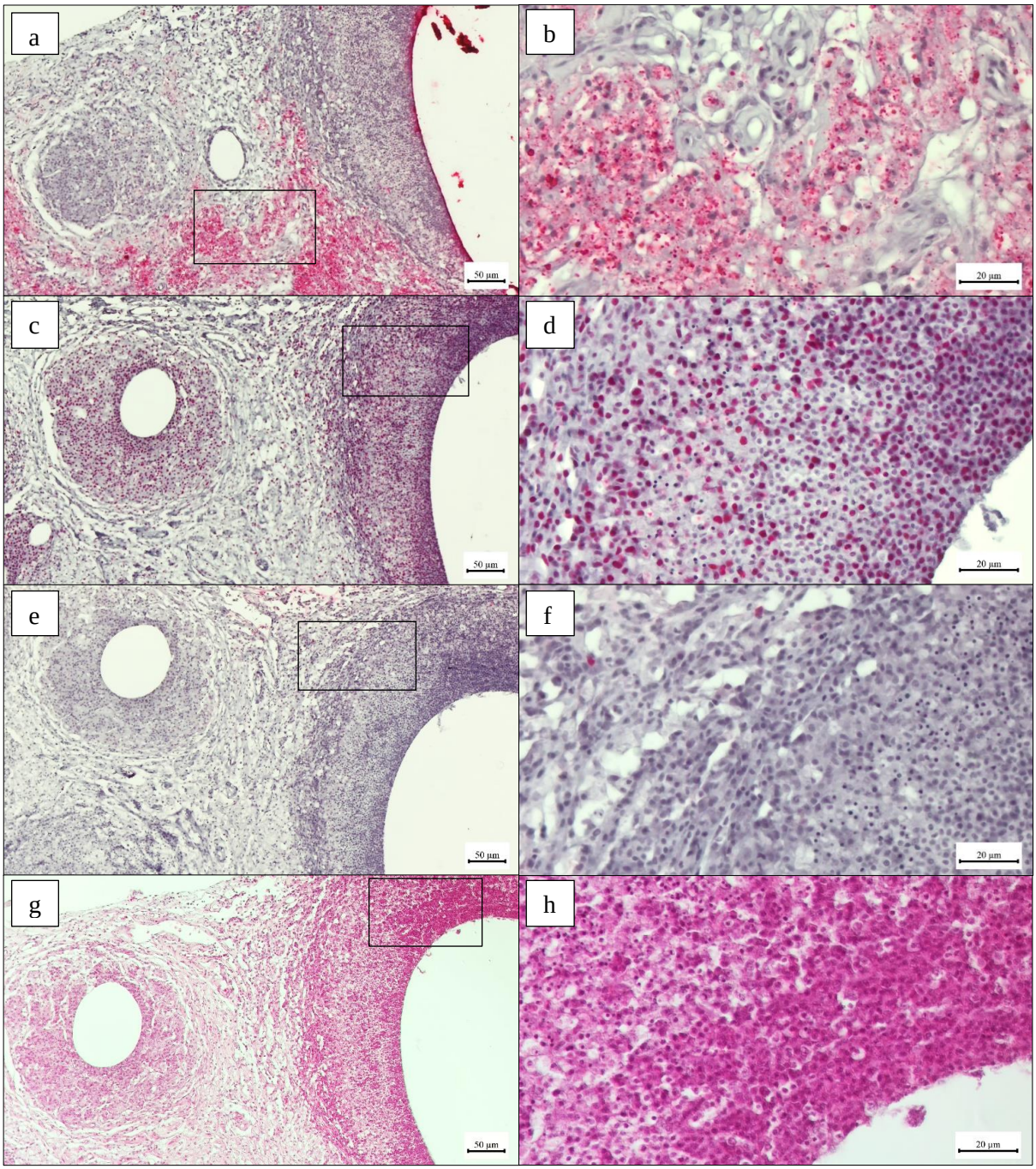

Figure 24: Immunohistochemical staining of A. salmonicida LPS (a and b), PCNA (c and d), and IgM (e and f) in an inflammatory lesion from a vaccinated lumpfish in temperature group 15 °C at 18 wpi. Red colouration indicates presence of antigens. HE-stain is also included for comparison (g and h). The inflammatory lesion is displayed at magnification 10x to the left in the figure (a, c, e, g). The black squares indicate the areas displayed at magnification 40x to the right in the figure (b, d, f, h). Scale bar is 50 µm for a, c, e, g (10x) and 20 µm for b, d, f, h (40x).

Figure 24 displays equivalent areas in one inflammatory lesion derived from a vaccinated individual in temperature group 15 °C sampled at 18 wpi. following staining with anti-*A. salmonicida* LPS (a and b), anti-PCNA (c and d), anti-IgM (e and f) and HE (g and h). Distinct

staining patterns of the different antigens detected by IHC are illustrated. *A. salmonicida* vaccine antigen was distributed on the periphery of the big oil droplet and in the loose inflammatory tissue (Figure 24: a and b) in which IgM^+ cells could be observed in small numbers (Figure 24: f). Proliferating cells formed aggregations in areas around oil droplets in the lesions which included necrotic tissue (Figure 24: c and d). In the areas of pronounced proliferation, many of the inflammatory cells showed bright pink cytoplasmic staining on the HE-stained section (Figure 24: g and h).

3.3 qPCR

The mRNA level of secretory and membrane-bound IgM in head kidney samples of vaccinated and control lumpfish subjected to different temperature regimes (5 °C, 10 °C, and 15 °C) was determined by using relative quantification. The expression of target genes in the samples is relative to that of the control average (control sample) in the respective temperature groups. Results displayed in figures are presented as log(2)-transformed gene expression ratios (or fold-changes) calculated from equation III, in which target genes are normalized to the reference gene EF1-α. On the log(2)-scale, values above and below zero correspond to increased and decreased gene expression relative to the control sample, respectively. An increase in one unit is equivalent to a doubling in gene expression and a decrease in one unit indicates a reduction of the same scope. See Appendix C for normal scale values. Two-way ANOVA and Tukey's post hoc test were used for statistical analyses of sIgM and mIgM expression at 18 wpi., including mIgM expression at 630 ddpi. A multiple T-test with correction for unequal variances was used to assess statistically significant differences in sIgM expression between vaccinated and control fish at 630 ddpi.

The relative expression ratios or fold changes of mIgM in head kidney samples of lumpfish at 18 wpi. are presented in Figure 25.

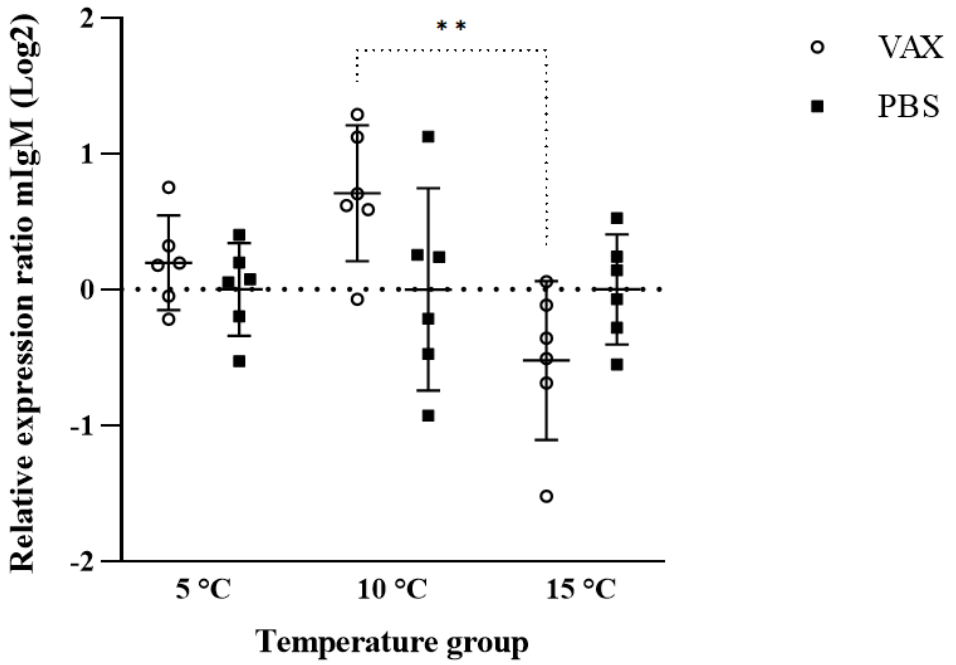

*Figure 25: Relative gene expression ratio of mIgM in head kidney of vaccinated (VAX) and control (PBS) lumpfish (n=6) at 18 wpi. Changes in gene expression are relative to average Cq values of control lumpfish in respective temperature groups. Error bars indicate ± 95% confidence intervals of the average expression ratio in each group. Statistically significant differences are indicated with asterisks (**: $p \leq 0.005$).*

The results illustrated in Figure 25 show 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 °C and 15 °C ($p = 0.0016$). Differences in relative mIgM expression between other groups of vaccinated and control fish were insignificant. There was a significant interaction effect of temperature and injection group on the relative expression ratio of mIgM in the head kidney of lumpfish at 18 wpi. (two-way ANOVA, $p = 0.0144$).

The relative expression ratios or fold changes of sIgM in head kidney samples of lumpfish at 18 wpi. are presented in Figure 26.

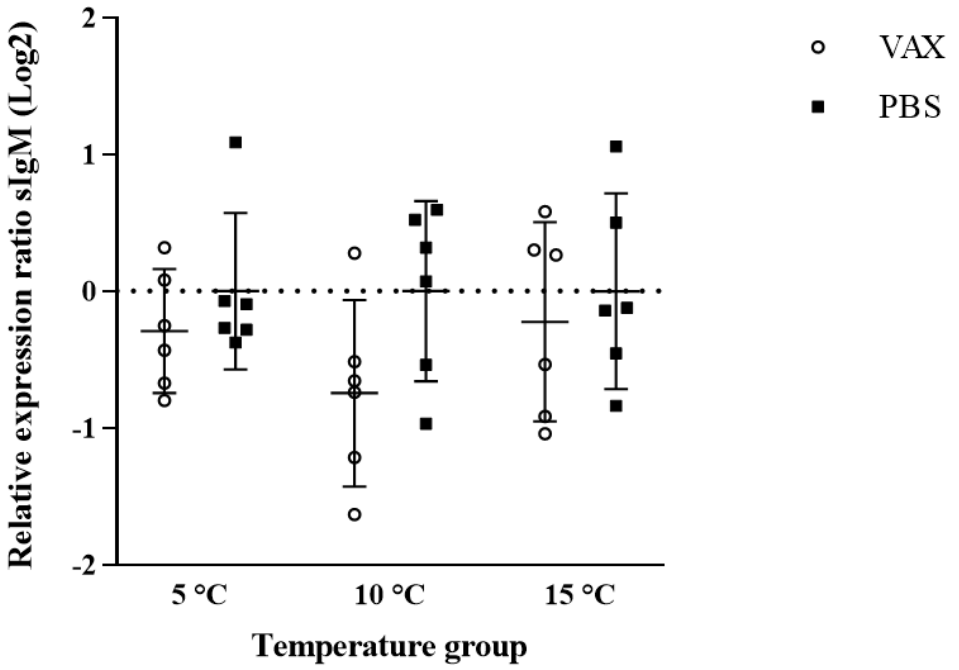

Figure 26: Relative gene expression ratio of sIgM in head kidney of vaccinated (VAX) and control (PBS) lumpfish (n=6) at 18 wpi. Changes in gene expression are relative to average Cq values of control lumpfish in respective temperature groups. Error bars indicate ± 95 % confidence intervals of the average expression ratio in each group.

The results illustrated in Figure 26 show a mean decrease in the relative expression of sIgM for vaccinated fish in all three temperature groups, 5 °C, 10 °C, and 15 °C, when compared to control fish. The differences in gene expression between vaccinated and control fish were insignificant, both within and between each temperature group. Vaccinated fish in the 10 °C group showed the biggest decrease in mean relative gene expression of -0.75-fold [-1.43, -0.064], in which five out of six individuals had values below zero. In general, the individual changes in the relative expression of sIgM showed high variability within every group, with some individuals demonstrating up- and others downregulation. Fish vaccinated at 15 °C, for instance, demonstrated spread in relative sIgM gene expression 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 27.

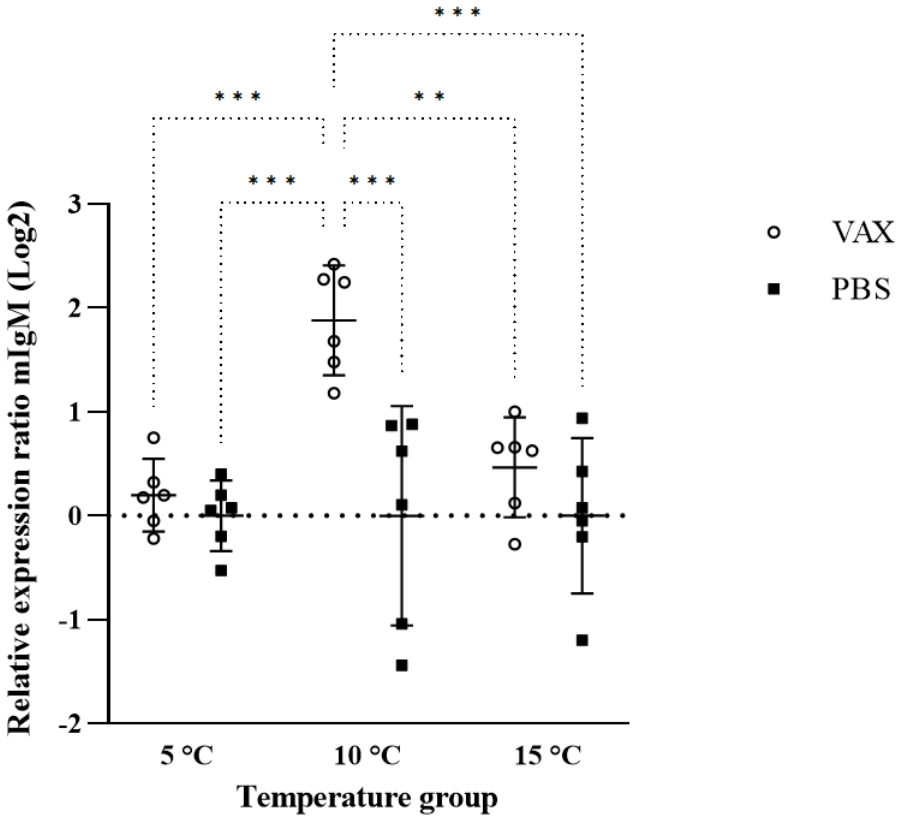

*Figure 27: Relative gene expression ratios of mIgM in head kidney of vaccinated (VAX) and control (PBS) lumpfish (n=6) at 630 ddpi. Changes in gene expression are relative to average Cq values of control lumpfish in respective temperature groups. Error bars indicate ± 95% confidence intervals of the average expression ratio in each group. Statistically significant differences are indicated with asterisks (**: $p \leq 0.005$, ***: $p \leq 0.0005$).*
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The results illustrated in Figure 27 show a general trend of increased mean mIgM expression in the head kidney of vaccinated fish in all three temperature groups, 5 °C, 10 °C, and 15 °C, relative to that of 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 their respective control group ($p = 0.0001$). Vaccinated and control fish subjected to water temperatures of 5 °C and 15 °C showed no significant differences in mean relative mIgM expression. There was a significant interaction effect of temperature and injection group on the relative expression ratio of mIgM in the head kidney of lumpfish at 630 ddpi (two-way ANOVA, $p = 0.004$).

The relative expression ratios or fold changes of sIgM in head kidney samples of lumpfish at 630 ddpi. are presented in Figure 28.

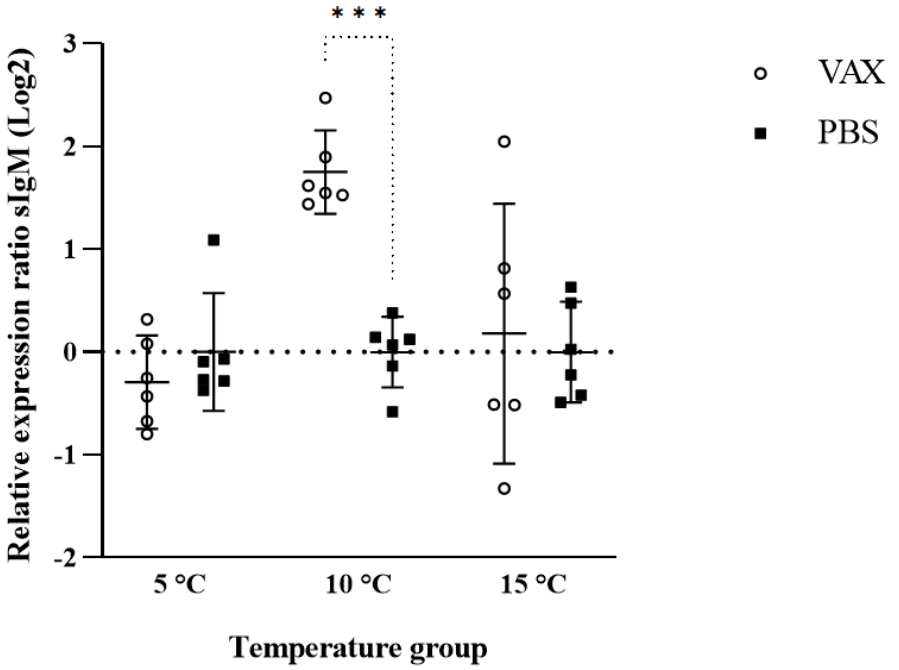

*Figure 28: Relative gene expression ratios of sIgM in head kidney of vaccinated (VAX) and control (PBS) lumpfish (n=6) at 630 ddpi. Changes in gene expression are relative to average Cq values of control lumpfish in respective temperature groups. Error bars indicate ± 95 % confidence intervals of the average expression ratio in each group. Statistically significant differences are indicated with asterisks (***: $p \leq 0.0005$).*

The results illustrated in Figure 28 show 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] relative to their respective control group ($p < 0.0001$). The individual fold-changes in sIgM expression of fish vaccinated at 15 °C were highly variable in contrast to fish vaccinated at 10 °C and 5 °C. One vaccinated individual in the 15 °C group showed an increase in the relative expression of sIgM at a level similar to 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 °C and 15 °C groups.

4 Discussion

In this study, inflammatory responses in lumpfish vaccinated at different water temperatures of 5 °C, 10 °C, and 15 °C were assessed. Samples included for analysis originated from a previous experiment in which antibody responses and possible vaccine side effects following intraperitoneal vaccination had been studied (Erkinharju et al. 2018). Intra-abdominal lesions were detected on haematoxylin and eosin (HE)-stained sections of vaccinated fish, 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 derived from fish vaccinated at different water temperatures and sampled at 630 day degrees post injection (ddpi.) and 18 weeks post injection (wpi). Furthermore, kidney and spleen sections of vaccinated and control fish showed no difference in staining pattern of cellular-associated IgM. Low relative gene expression of mIgM and sIgM in the head kidney of vaccinated fish sampled at 18 wpi. was observed.

4.1 Welfare indicators

Vaccination may cause side-effects negatively impacting on the welfare of fish by compromising growth and health (Poppe and Koppang 2014). As such, specific growth rate and Fulton's condition factor were calculated to give indications of the well-being of vaccinated fish and to assess possible differences between temperature treatment groups. The mean specific growth rates of lumpfish were in the range of 1.09-1.44, which is similar to earlier reports (Imsland et al. 2018a). Though no statistics were performed on these growth data, differences between vaccinated and control lumpfish appeared small. Likely, the growth rates of lumpfish throughout the experiment were not influenced by the vaccine. Higher values of fish reared at 10 °C (VAX: 1.25, PBS: 1.44) and 15 °C (VAX: 1.27, PBS: 1.42) are expected as specific growth rates tend to increase with increasing temperature, at least up to a certain point (Fry 1971). The results are consistent with a previous study using similar temperature treatments reporting higher specific growth rates for lumpfish reared at 10 °C and 16 °C compared to 4 °C (Nytrø et al. 2014). The mean condition factors of vaccinated and control lumpfish in all three temperature groups were high and not significantly different. Thus, vaccination did not seem to have influenced the condition of the fish either. In addition, the body form of fish affects condition factor (Treer et al. 2009). A condition factor above one implies general well-being of salmonid fish (Barnham and Baxter 1998), whereas it is normally much higher for lumpfish due to their rounded shape (Brooker et al. 2018). In this study, mean

calculated condition factors were 4.3, 3.8, and 4.9 for the 5 °C, 10 °C, and 15 °C temperature groups, respectively. These values are in the range reported in previous studies of lumpfish (Imsland et al. 2014, Imsland et al. 2018a). Possibly, the higher condition factors for both vaccinated and control lumpfish in temperature group 15 °C resulted from increased growth potential at a higher temperature. In summary, the results obtained from calculations of specific growth rate and condition factor did not indicate that the welfare of lumpfish was compromised by vaccination.

4.2 Histopathology

Development of intra-abdominal lesions of varying severity is a well-recognized side effect of oil-adjuvanted vaccines administered with intraperitoneal injection (Poppe and Koppang 2014). Histological examination of abdominal tissue sections derived from several vaccinated lumpfish revealed presence of perivisceral lesions that were positive for *A. salmonicida* and included negative imprints of oil droplets, which indicate that these were vaccine-induced. Moreover, most control fish did not show similar tissue reactions. In the literature, histological changes are normally not reported for control fish receiving injection with phosphate buffered saline. Mislabeling was probably not the reason for this observation, as low antibody responses against *A. salmonicida* were demonstrated for these control fish by ELISA. Furthermore, abdominal adhesions were absent among control fish in the study (Erkinharju et al. 2018). It is possible that the vaccination needle hit an abdominal organ inducing inflammatory responses in the control fish showing similar histological changes as those detected on sections from vaccinated fish.

The vaccine-induced lesions were located between the pancreas, pyloric caeca, and spleen, which is commonly reported for other species as well such as 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 loose inflammatory tissue and, in fish vaccinated at 10 °C and 15 °C, dense aggregations of cells were detected within the lesions notably surrounding the vaccine oil droplets. The cell aggregations resembled granulomas, which may form in response to chronic inflammation. Various agents can induce formation of granulomas, including pathogens and foreign material, which serve to isolate and eradicate infection (Petersen and Smith 2013). Accumulation of collagen, as detected by van Gieson staining, supports the presence of granulomatous inflammatory lesions. Collagen is a type of fibrous connective tissue synthesized by fibroblasts (Wynn and

Ramalingam 2012). Fibroblasts and connective tissue are commonly abundant in outer regions of older granulomas (Poppe 2002), which is consistent with what was observed. Some of the granulomas showed signs of necrosis as indicated by cells with pyknotic nuclei in the center. Development of granulomas in fibrous granulation tissue has been noted in several studies of the inflammatory response in different fish species, including lumpfish (Erkinharju et al. 2019), following vaccination with oil-adjuvant. Also, the severity of lesions typically increases with time, which has been shown for sea bass (Afonso et al. 2005) and Atlantic salmon (Midtlyng 1996). The examined samples were derived from lumpfish immunized with a vegetable-oil adjuvanted vaccine, however. Supposedly, non-mineral metabolizable oils lead to lower side effects and only induce weak inflammatory responses (Spickler and Roth 2003). This was not the case 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 observed following macroscopic examination (Erkinharju et al. 2018). The adherence scores (Speilberg scoring system) reported, however, were within the range of what is considered acceptable or "normal" for e.g. Atlantic salmon vaccinated with a multivalent oil-adjuvanted vaccine (PHARMAQ AS). A previous 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, contained four bacterial antigens (Erkinharju et al. 2017).

Innate immune components are essential for the arrangement of granulomas. The most prominent cell type in such aggregated collections of inflammatory cells is the macrophage (Petersen and Smith 2013). 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). Other cells reported to be present in vaccine granulomas of fish are neutrophils, lymphocytes, eosinophilic granular cells and multinucleated cells which have been identified morphologically on histological sections (Mutoloki et al. 2008, Haugarvoll et al. 2010, Mutoloki et al. 2004, Poppe and Breck 1997). Except for strong eosinophilic cytoplasmic staining, other cell characteristics of those forming aggregations in lesions on HE-stained sections were not identified. May-Grünwald-Giemsa (MGG) staining, however, revealed that many of these cells contained cytoplasmic eosinophilic granules. Inflammatory cells with similar staining characteristics were observed to increase in numbers over time following intramuscular vaccination in lumpfish (Erkinharju et al. 2019). These were termed eosinophilic-granulocyte-like cells, due to their resemblance to eosinophilic granule cells which have been

identified in various teleost fish (reviewed in Reite and Evensen, 2006). It is reasonable to assume that the same cell population was present in inflammatory lesions examined in this study. Regarding eosinophilic granule cells, these have been shown to actively participate in acute inflammatory responses releasing chemical mediators involved in recruitment of neutrophils. Also, an increase in the abundance of such cells seems to be a general feature of chronic inflammation in teleost fish (Reite and Evensen 2006).

Few studies have attempted characterization of lumpfish leukocytes within histological sections, but many types have been isolated including lymphocytes, monocytes, macrophages and granulocytes (Haugland et al. 2018) Some leukocytes in lumpfish have different morphology than those in Atlantic salmon, for instance, making identification somewhat challenging (Erkinharju et al. 2019, Haugland et al. 2018). Applying different specific cell markers or stains could perhaps 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 participating 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 likely due to incompatibility of the stains with formalin fixated histology samples, as they have been developed for use on fresh cytological preparations (T Erkinharju 2021, pers. comm., 13. May).

Inflammatory lesions were detected on sections derived from vaccinated fish in all three temperature groups, but the number of fish in the 10 °C and 15 °C groups exhibiting such lesions was higher than in the 5 °C group. Side effects of vaccination such as adhesions between internal organs and the abdominal wall, relate to peritonitis (Midtlyng et al. 1996). As such, lower occurrence of lesions among fish in the 5 °C group could have been be explained by lower adherence scores. There were no reports, however, on differences in scores between fish in the 5 °C, 10 °C, and 15 °C groups at all sampling time points (Erkinharju et al. 2018). Instead, the finding could be due to methodological challenges. Lesions must be present in the obtained tissue samples used for processing to be detected on histological sections. It is likely that lesions could have been missed as the samples trimmed from the fixated tissue were small (Mutoloki et al. 2008). Detection also relies on the correct positioning of tissue prior to sectioning and lesions can be missed if the paraffin blocks are either sectioned to deep or insufficiently. In addition, the number of individual replicates (fish) should be high enough and equal among

groups to obtain representative samples for comparative analysis. In this study, fewer replicates were included for the 5 °C group as the sampling time point 18 wpi. also corresponded to 630 ddpi.

4.3 Immunohistochemistry

Detection of vaccine antigen – *Aeromonas salmonicida*

Positive staining of *A. salmonicida* LPS was detected in some of the kidney and spleen sections from lumpfish. These organs are known to function in antigen trapping and clear exogenous substances from the circulation (Press and Evensen 1999, Espenes et al. 1996). 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 containing vaccine components have been detected in the head kidney and spleen of turbot following intraperitoneal injection (Noia et al. 2014). Detection of *A. salmonicida* antigen in kidney and spleen sections from lumpfish was not associated with any intracellular staining. Thus, there is no direct evidence for migration of inflammatory cells with phagocytosed antigen from the injection site to immune organs. The positive staining could have resulted from the vaccine needle penetrating a blood vessel leading the antigen through the blood to the kidney. However, immunohistochemistry also revealed positive staining in kidney sections from control lumpfish that received injection with phosphate buffered saline. Unspecific staining cannot be ruled out as a factor contributing to positive staining of *A. salmonicida* in the kidney of some lumpfish.

Oil-adjuvanted injection vaccines have been shown to induce long term protective immunity in farmed fish by stimulating (effective) local inflammatory responses and providing prolonged antigenic exposure to immunocompetent cells. The increased exposure time of antigen to the immune system is believed to be due to the oil adjuvant creating a depot at the injection site with the slow release of antigen (Poppe and Breck 1997). The abdominal tissue samples showing lesions on histological sections were sampled at 6, 9, and 18 wpi. This implies that the antigens persisted at the administration site stimulating the immune system for weeks (Noia et al. 2014). Moreover, immunohistochemical staining revealed presence of vaccine antigen (*A. salmonicid*a) in the inflammatory lesions of vaccinated fish at 6, 9, and 18 wpi. The intense staining of *A. salmonicida* antigen on the periphery of oil droplets is in accordance with observations noted in vaccine lesions of Atlantic cod (Mutoloki et al. 2008) and Atlantic salmon

(Mutoloki et al. 2004). This type of observed staining pattern has been attributed to the depot effect (Mutoloki et al. 2008).

Detection of PCNA

PCNA is an established marker for active phases of the cell cycle and DNA repair (Maga and 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 noted in diseased salmon hearts following viral infections and was linked to regeneration of tissue (Yousaf et al. 2013). Immunostaining of PCNA indicated proliferative cell activity in the inflammatory lesions of lumpfish at 630 ddpi. and 18 wpi. The $PCNA^+$ cells were scattered throughout the lesions, but staining was especially pronounced in cells forming aggregations and surrounding oil droplets. Many of the aggregated cells around oil droplets were found to contain pink/eosinophilic granules following MGG staining. Thus, it is possible that a population of the proliferating cells in lesions constituted granulocytes. The staining pattern observed with PCNA in the abdominal lesions is consistent with previous findings reported by Erkinharju et al. (2019). A high number of $PCNA^+$ cells was observed in intramuscular vaccine-induced lesions of lumpfish at 6 wpi., many of which were identified as eosinophilic-granulocyte-like cells.

Detection of IgM

In teleost fish, the kidney and spleen are considered as secondary lymphoid organs where immune responses are initiated (Soulliere and 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 (the spleen was not sampled at 630 ddpi.). Membrane-bound IgM can be expressed by B cells which mount humoral responses towards antigens. Upon activation by antigen, B cells undergo clonal proliferation and differentiate to plasma cells producing large amounts of specific antibodies (Castro et al. 2013). Clustering of B cells in lymphoid haematopoietic tissue around blood vessels has been suggested to represent areas of differentiation (Schrøder et al. 1998). In addition, numerical increases of B cells in the (head) kidney and spleen have been associated with the important role of these organs in initiating humoral responses (Bermúdez et al. 2006). These types of observations were not noted in kidney and spleen sections of vaccinated lumpfish in neither of the temperature groups (5 °C, 10 °C, and 15 °C) at both sampling time points (630 ddpi and 18 wpi.). IHC showed that the

IgM^+ cells in the kidney and spleen were mostly scattered in the tissue exhibiting no signs of distinct distribution patters such as grouping in clusters. IHC also revealed that the abundance of the B cells was not affected by immunization because no apparent differences in the number of positive cells were detected among sections derived from vaccinated and control fish. Corresponding results were reported previously for lumpfish immunized intramuscularly, in which the number of IgM^+ cells in the head kidney was no different from control fish (Erkinharju et al. 2019). This contrasts with a study in juvenile sea bass, where 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). In regard to temperature, percentage 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 and Kotterba 2002). The results in this study with respect to IgM staining in the kidney and spleen of vaccinated lumpfish are not in compliance with the findings just mentioned. It is important to mention that the semi-quantitative analysis performed on the spleen and kidney sections to determine the number of positive IgM cells is highly subjective. Thus, manual counting is not the most accurate method introducing some bias in the number of IgM^+ cells reported.

Size increase and proliferation of B cells are associated with differentiation to antigen secreting plasma cells (Bromage et al. 2004). No further characterization of the B cells was performed following IHC with anti-IgM such as determination of the cytoplasm:nucleus ratio, which is known to be high for differentiating B cells (Schrøder et al. 1998). IHC with anti-PCNA revealed intense staining in both the kidney and spleen of vaccinated fish. It was not possible, however, to identify the proliferating cells as IgM^+ B cells. In order to gain such insight, co-incubation with anti-PCNA and anti-IgM antibodies could have been a possible option. Still, the observed non-existing difference in staining intensity of both IgM^+ and proliferating cells among vaccinated and control fish in all three temperature groups at 18 wpi. and 630 ddpi. suggests that immunoreactivity in the kidney and spleen was not induced following vaccination. The possibility of an increase in number of IgM^+ B cells occurring at earlier timepoints cannot be dismissed, however. To verify the assumption of low immunological activity in the kidney, IHC should also be performed on samples collected earlier in the time course of the study from which they were derived from.

Low water temperatures suppress immune functions 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 in the 5 °C group, although no significant humoral responses were demonstrated for these fish (Erkinharju et al. 2018). Like fish immunized at 10 °C and 15 °C, IgM^+ B cells were detected in lesions from fish in the 5 °C group indicating local proliferation or recruitment to the peritoneum. No overall difference in number of IgM^+ cells in lesions of vaccinated fish in the three temperature groups was detected by the semi-quantitative analysis. As such, temperature does not seem to have influenced the local inflammatory response initiated following vaccination. Lower water temperature of 5 °C may have affected maturation of the IgM^+ B cells or their interactions with other cells culminating in decreased humoral responses.

Presence of IgM^+ B cells in the peritoneum following antigenic stimulation has been reported for other species as well, such as Atlantic salmon (Haugarvoll et al. 2010) and rainbow trout (Castro et al. 2017). IgM^+ B cells are considered central participants in inflammatory responses in the abdominal cavity of teleost fish (Korytář et al. 2013). In the abdominal tissue samples of lumpfish, the IgM^+ cells were mainly detected in loose inflammatory tissue surrounding granulomas. Similar distributional pattern of IgM^+ cells has been observed in granulomatous lesions of Atlantic salmon months after intraperitoneal vaccination (Haugarvoll et al. 2010). Administration of virus vaccine in rainbow trout led to mobilization of IgM^+ cells to the peritoneum (Martinez-Alonso et al. 2012). In another study concerning 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 to 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 to T cells (Rønneseth et al. 2017). The increase in IgM^+ cells in the peritoneum of vaccinated fish as opposed to control fish combined with no similar observations in the kidney and spleen could indicate local mechanisms in IgM responses to the vaccine. 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 systemic organs following challenge with salmonid alpha virus subtype 3 (SAV3).

However, intraperitoneal injection with inactivated virus did not produce local responses of the same extent (Tiruneh 2019). In rainbow trout, immune responses involving APCs ($CLEC4T1^+$) and T cells ($CD3^+$) have been demonstrated in visceral adipose tissue following intraperitoneal injection with inactivated *A. salmonicida*. In the same study, IgM^+ cells were observed to increase in numbers in the interstitial tissue between adipocytes (Veenstra et al. 2019). The differentiation of B cells into plasma cells may take place at different sites including that of inflammation as lymph nodes and germinal centeres are lacking in fish (Bakke et al. 2020). One may hypothesize that the significant increase in antigen specific antibodies in serum of fish vaccinated at 10 °C and 15 °C at 18 wpi. (Erkinharju et al. 2018) could have resulted from local 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.

Diffuse staining was not especially noticeable in the lesions of vaccinated fish following immunohistochemical staining with anti-IgM. In vaccine-induced intra-muscular lesions of lumpfish, diffuse staining indicative of sIgM has been observed. However, the staining intensity was shown to decrease over time (up to 6 wpi.) (Erkinharju et al. 2019). Late sampling time points, i.e. 6, 9, and 18 wpi., might be the reason for limited staining of sIgM in lesions of lumpfish examined in this study.

4.4 Gene expression analysis

The relative gene expression analysis revealed great spread of values of individuals belonging to the same group, such as those vaccinated at 15 °C. Normally, the response to vaccination is not uniform among fish unless domestication has greatly reduced individual variability. Often vaccine trials reveal responders and non-responders among groups (R Dalmo 2021, pers. comm., 16 April). Such an outcome, or at least different responses, may be reflected in variable relative gene expression values of individual fish (Thim et al. 2014). However, in this case, head kidney samples included for qPCR analysis were derived from (only) six vaccinated fish in each temperature group showing the highest concentration of specific IgM antibodies in response to the vaccine (*A. salmonicida* specific). Therefore, non-responders were likely not included, but the spread in gene expression ratios still reflect individual differences typically observed in experiments with 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 and Koppang 2021). In this study, insignificant differences in head kidney expression level of mIgM and

sIgM between vaccinated and control fish were mainly 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 immunoreactivity in the head kidney was induced by the vaccine for fish in 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 individual gene expression values of vaccinated fish in this group were centered around the mean producing quite narrow confidence intervals. Thus, the finding is not due to certain individuals adjusting the gene expression to higher levels and seems reliable. 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 uncertain. However, the expression of mIgM and sIgM shifted after some weeks. At 18 wpi., the mean mIgM expression was still higher but not significantly different from the control group. Regarding the expression of sIgM, this was no different from controls and fish in the other temperature groups at 18 wpi. For vaccinated fish in the 5 °C and 15 °C groups, differences in expression level of mIgM and sIgM from control fish were not detected at neither 630 ddpi. nor 18 wpi. Overall, the gene expression analysis of sIgM and mIgM of vaccinated fish in all three temperature groups gave no indication of immunoreactivity occurring in the head kidney. This finding is supported by immunohistochemical staining of IgM in the kidney showing no differences in positivity among vaccinated and control fish.

Previous studies have emphasized that terminally differentiated B cells, plasma cells, migrate to the head kidney and stay there for long time periods secreting antibodies (Zwollo et al. 2005). Our results show no such indications as the sIgM expression in the head kidney of vaccinated fish at 18 wpi. was low and no different from control fish in all three temperature groups. This is not a surprising finding for individuals in the 5 °C group as very low antibody responses were detected independent of time and day degrees (Erkinharju et al. 2018). For vaccinated fish in the 10 °C and 15 °C groups, however, high antibody titers in serum were detected at 18 wpi. As mentioned, the increase in concentration of specific antibodies could originate from B cells in non-systemic sites such as the peritoneal cavity where local immune responses were induced following vaccination. Yet, another possible explanation, is high antibody secretion capacity of few plasma cells in the head kidney of fish subjected to water temperatures of 10 °C and 15 °C (Tiruneh 2019). Further analyses are needed to substantiate the finding that local IgM responses were induced following vaccination.

4.5 Supplementary analysis and future perspectives

It would have been beneficial to analyse the expression of sIgM and genes involved in B cell differentiation in samples derived from the inflammatory site. Insight on whether terminal stage maturation of B cells occur locally could have been provided in this way. Furthermore, combining incubation with anti-IgM and anti-PCNA could have revealed B cell proliferation associated with activation and differentiation towards plasma cells. Other valuable analyses for the evaluation of B cell responses include ELISPOT and flow cytometry. ELISPOT can be used to determine the frequency of plasma cells (Castro et al. 2017), and flow cytometry can provide information on cell sizes which can be related to differentiation stages of B cells (i.e. increased size for plasma cells) (Martín-Martín et al. 2020). Performing such assays, however, require isolation of cells. T cells are central players in adaptive immune responses along with B cells. Extending the qPCR analysis to include quantification of additional genes such as TCR, CD3 and MHC class II may have provided information on whether the vaccine supported cell-mediated adaptive responses. For other fish species, antibodies directed against surface antigens have been used for identification of T cells in tissue (Nakanishi et al. 2015).

Future studies should look further into local inflammatory responses in lumpfish induced by vaccination. Development of cell markers for lumpfish leukocytes present in inflammatory tissue would contribute to a more detailed characterization of vaccine responses. The potential contribution of B cells present at the local injection site to the systemic antibody response represents an interesting area of research. Additionally, whether the IgM^+ B cells identified in inflammatory lesions resulted from local proliferation or trafficking from lymphoid organs remain to be answered. Studies on how B cells are differentiated upon encounter with antigen are also highly relevant. The possibility of *A. salmonicida* directly stimulating lumpfish B cells to undergo proliferation and differentiation should be addressed, which was recently shown for rainbow trout B cells (Soleto et al. 2020).

5 Conclusion

Local inflammatory responses were induced by intraperitoneal vaccination in lumpfish subjected to different water temperatures of 5 °C, 10 °C, and 15 °C. Some of the intra-abdominal lesions identified on 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 prolonged inflammatory responses. MGG-staining revealed participation of eosinophilic-granulocyte-like cells in the locally induced inflammatory responses. These cells tended to aggregate around vaccine oil droplets and showed apparent 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, staining intensity of cellular associated IgM in the kidney and spleen of lumpfish subjected to different water temperatures was negligible. This also applied to vaccinated and control fish in all three temperature groups. The relative expression of mIgM and sIgM in the head kidney of vaccinated fish was low at 630 ddpi. (except for fish vaccinated at 10 °C) and 18 wpi. These findings suggest that immunoreactivity in the kidney was not induced following vaccination. Instead, local mechanisms in regulation of B cell responses to the vaccine may have contributed to the increase in concentration of specific antibodies detected in serum of vaccinated fish in temperature groups 10 °C and 15 °C at 18 wpi. (Erkinharju et al. 2018). To verify these results, however, further analyses are needed, including gene expression studies on samples derived from the inflammatory site. Still, this study indicates similar vaccine responses as previously proposed for lumpfish (Erkinharju et al. 2019) and that temperature treatment did not exert any great influence on the locally induced inflammatory responses at the injection site.

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Appendices

Appendix A – Reagents

Appendix B – Immunohistochemistry

Appendix C – qPCR

Appendix D – Additional findings

Appendix A – Reagents

Table A: List of reagents used.

Reagents	Producer	Concentration/content
Acetic acid	Sigma-Aldrich	0.5 % in H_2O
Anti-*A. salmonicida* LPS	Roy Dalmo and Jarl Bøgwald, UiT	1:5000 in 1.5 % BSA
Anti-lumpfish IgM	Ivar Hordvik, UiB	1:6000 in 2.5 % BSA
Anti-PCNA, Clone PC10	Dako	1:3200 in 2.5 % BSA
BSA	Sigma-Aldrich	1.5 % in TBS 2.5 % in TBS
Citric acid, anhydrous	Sigma-Aldrich	
Citrate buffer (pH: 6.0)		Citric acid: 1.92 g MilliQ water: 1000 mL 1N NaOH for pH adjustment
Eosin Y, alcoholic	Sigma-Aldrich	
Ethanol	VWR Chemicals	96 %, abs.
Eukitt® - Quick hardening mounting medium	Sigma-Aldrich	
Fast SYBR® Green Master Mix	Applied Biosystems	
Giemsa's azur eosin methylene blue solution	Merck	
Haematoxylin solution modified acc. to Gill III	Merck	
Histo-Clear™	National Diagnostics™	
ImmPRESS®-AP Horse Anti-Mouse IgG Polymer Reagent	Vector Laboratories Inc.	
ImmPRESS®-AP Horse Anti-Rabbit IgG Polymer Reagent	Vector Laboratories Inc.	

ImmPACT® Vector® Red	Vector Laboratories Inc.	
ImmunoPen™	Merck	
May-Grünwald's eosin-methylene blue solution	Sigma-Aldrich	
Methanol	Sigma-Aldrich	
MilliQ water		
Nuclear fast red-aluminum sulfate solution 0.1 %	Sigma-Aldrich	
Paraffin Histowax®	HistoLab®	
Quantitech Reverse Transcription kit	Qiagen	
RNeasy mini kit	Qiagen	
Silver plating kit acc. to von Kossa	Merck	
TBS	NFH	
TBST		TBS: 250 mL TWEEN® 20: 250 µL
Trypsin-EDTA solution	Sigma-Aldrich	0.05 %
TWEEN® 20	Sigma-Aldrich	
Ultrapure water	Qiagen	
van Gieson solution		1 % acid fuchin: 10 mL Picric acid: 100 mL
Weigert's haematoxylin		50:50 W1 and W2 W1: 1 g haematoxylin in 100 mL abs. ethanol. W2: 4 mL $FeCl_3$ in 95 mL dH_2O. Addition of 1 mL HCl.

Xylene	Sigma-Aldrich

Appendix B – Immunohistochemistry

Figure B1 displays photomicrographs (20x) of kidney sections from vaccinated and control fish in temperature groups 5 °C, 10 °C, and 15 °C at 630 ddpi. following immunohistochemical staining with anti-PCNA.

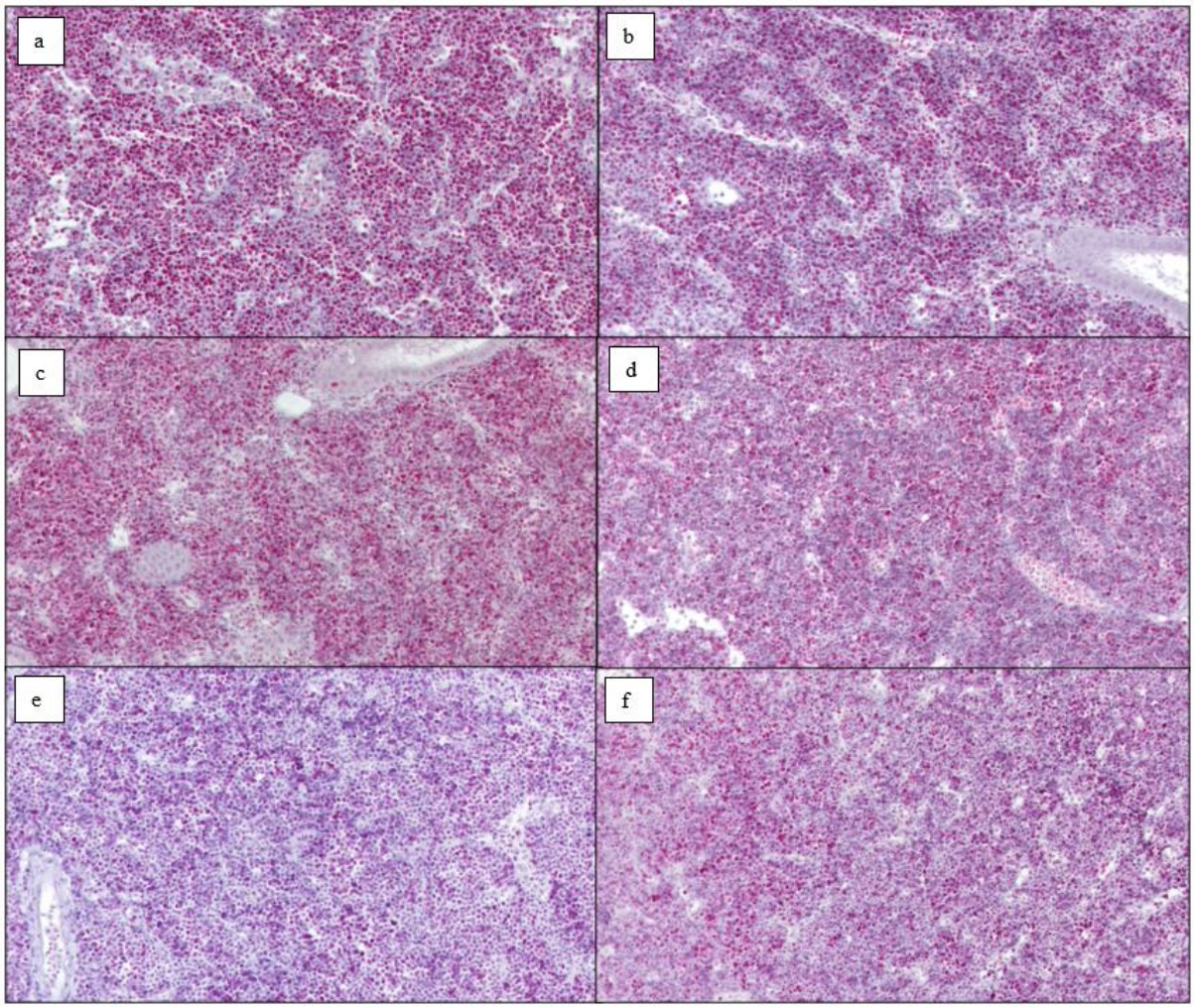

Figure B1: Immunohistochemical staining of PCNA in kidney sections of vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 630 ddpi. Red colouration indicates presence of PCNA. Kidney sections of VAX and PBS fish are displayed to the left and right in the figure, respectively (a and b: 5 °C, c and d: 10 °C, e and f: 15 °C).

Table B1 lists number of IgM^+ cells detected in kidney sections from vaccinated fish at 630 ddpi.

Table B1: Number of IgM^+ cells in four photographs of kidney sections from vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 630 ddpi. Values in bold are averages of groups. n=4 for VAX and PBS fish in the 5 °C group, n=7 for VAX fish in the 10 °C group, n=8 for PBS fish in the 10 °C group, n=9 for VAX and PBS fish in the 15 °C group. Dash (-) indicates information not available.

Fish	**5 °C**		**10 °C**		**15 °C**	
	VAX	PBS	VAX	PBS	VAX	PBS
1	174	333	325	84	165	131
2	267	188	86	71	191	60
3	149	237	23	83	129	33
4	127	236	20	38	141	181
5	-	-	126	96	189	71
6	-	-	31	86	118	285
7	-	-	148	35	81	23
8	-	-	-	98	171	62
9	-	-	-		101	102
Average	**179.3**	**248.5**	**108.4**	**73.9**	**142.9**	**105.3**

Table B2 lists the number of IgM^+ cells detected in lesions from vaccinated fish at 630 ddpi.

Table B2: Number of IgM^+ cells in inflammatory lesions of vaccinated lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 630 ddpi. More than one lesion was derived from some fish and the number of IgM^+ cells in each lesion is listed accordingly. Dash (-) indicates information not available.

Fish	**5 °C**	**10 °C**	**15 °C**
1	0	0	69
2	136	2	47
3	26, 78	9	0, 2
4	145, 47	25	3, 4
5	-	5, 1	1, 2
6	-	3, 2	10, 6
7	-	34, 40	5, 0, 3
8	-	-	5, 3, 8
9	-	-	12, 0, 0

Figure B2 displays photomicrographs (20x) of kidney sections from vaccinated and control fish in temperature groups 5 °C, 10 °C, and 15 °C at 630 ddpi. following immunohistochemical staining with anti-IgM.

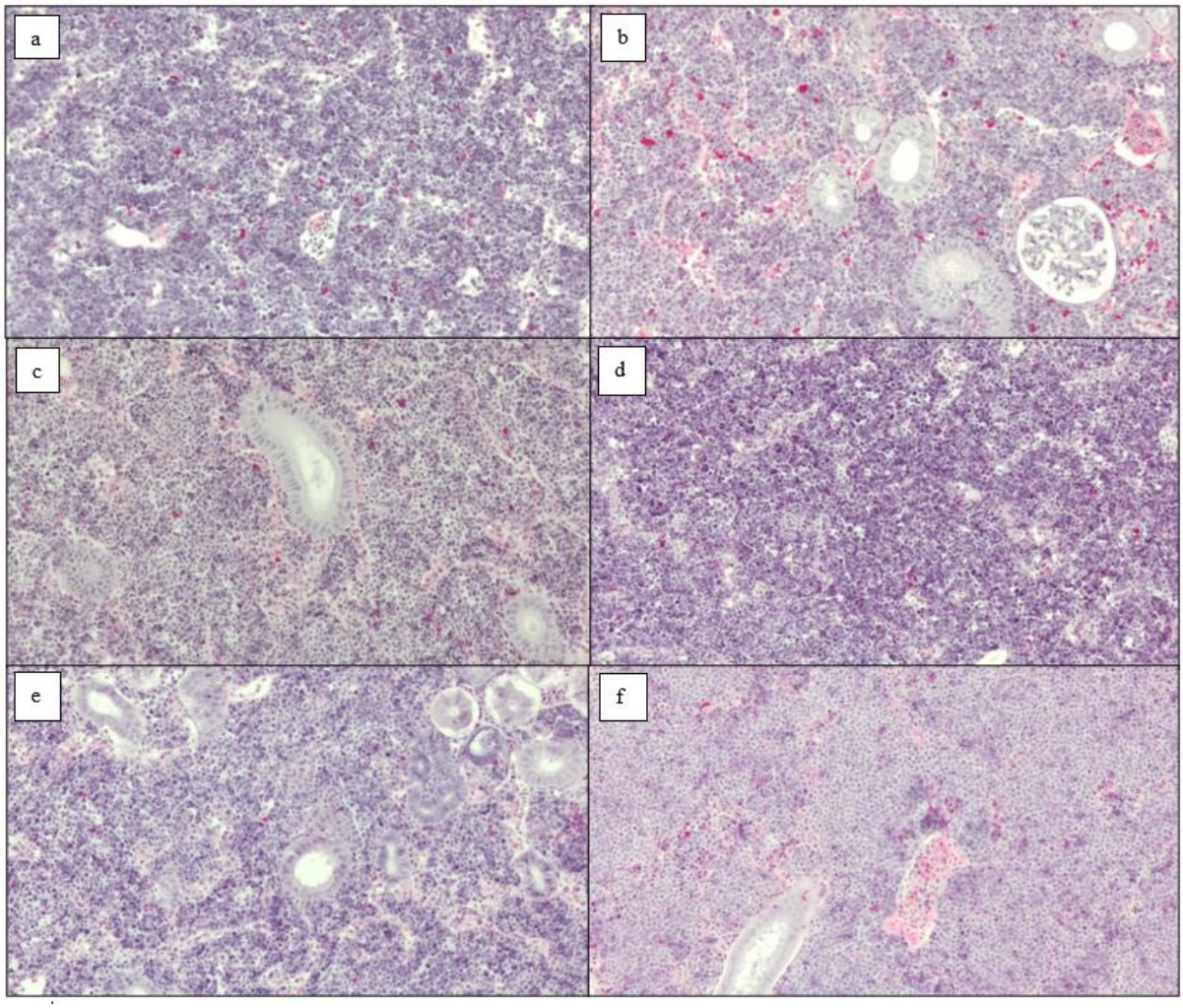

Figure B2: Immunohistochemical staining of IgM in kidney sections of vaccinated (VAX) and control (PBS) lumpfish in temperature groups 5 °C, 10 °C, and 15 °C at 630 ddpi. Red colouration indicates presence of IgM. Kidney sections of VAX and PBS fish are displayed to the left and right in the figure, respectively (a and b: 5 °C, c and d: 10 °C, e and f: 15 °C).

Appendix C – qPCR

Table C1: Calculated gene expression ratios (Pfaffel method) for mIgM and sIgM with log2-transformed values at 18 wpi. for temperature groups 5 °C, 10 °C, and 15 °C.

Sample number	Sampling time point (wpi)	Temperature °C	Group	Ratio mIgM	Log2(Ratio mIgM)	Ratio sIgM	Log2(Ratio sIgM)
T1r35	18	5	VAX	0,86	-0,22	0,74	-0,43
T1r36	18	5	VAX	1,68	0,75	1,06	0,08
T1r38	18	5	VAX	1,13	0,18	0,57	-0,80
T1r43	18	5	VAX	1,25	0,32	1,25	0,32
T1r48	18	5	VAX	1,15	0,20	0,84	-0,25
T1r49	18	5	VAX	0,97	-0,05	0,63	-0,67
T2r31	18	5	PBS	1,05	0,07	0,95	-0,07
T2r32	18	5	PBS	1,04	0,05	0,82	-0,28
T2r34	18	5	PBS	0,87	-0,20	2,13	1,09
T2r36	18	5	PBS	0,69	-0,53	0,83	-0,27
T2r38	18	5	PBS	1,15	0,20	0,94	-0,10
T2r40	18	5	PBS	1,32	0,40	0,77	-0,37
T3r33	18	10	VAX	0,95	-0,07	0,70	-0,52
T3r34	18	10	VAX	1,54	0,62	1,21	0,28
T3r37	18	10	VAX	1,63	0,71	0,64	-0,65
T3r40	18	10	VAX	1,51	0,59	0,32	-1,63
T3r49	18	10	VAX	2,44	1,29	0,60	-0,74
T3r50	18	10	VAX	2,18	1,12	0,43	-1,21
T4r33	18	10	PBS	0,53	-0,93	0,69	-0,54
T4r34	18	10	PBS	1,18	0,24	1,43	0,52
T4r35	18	10	PBS	0,86	-0,22	1,05	0,07
T4r36	18	10	PBS	1,19	0,26	1,25	0,32
T4r37	18	10	PBS	2,18	1,12	0,51	-0,97
T4r40	18	10	PBS	0,72	-0,47	1,51	0,60
T5r33	18	15	VAX	0,78	-0,36	0,49	-1,04
T5r37	18	15	VAX	0,35	-1,52	0,53	-0,92
T5r38	18	15	VAX	1,04	0,06	1,20	0,26
T5r40	18	15	VAX	0,62	-0,69	1,50	0,58
T5r47	18	15	VAX	0,70	-0,51	1,23	0,30
T5r48	18	15	VAX	0,92	-0,12	0,69	-0,53
T6r31	18	15	PBS	0,95	-0,07	0,92	-0,12
T6r34	18	15	PBS	1,10	0,14	0,73	-0,46
T6r35	18	15	PBS	1,44	0,52	1,42	0,50
T6r37	18	15	PBS	0,68	-0,55	0,56	-0,84
T6r38	18	15	PBS	1,18	0,24	2,08	1,06
T6r40	18	15	PBS	0,82	-0,28	0,91	-0,14

Table C2: Calculated gene expression ratios (Pfaffel method) of mIgM and sIgM with log2-transformed values at 630 ddpi. for temperature groups 5 °C, 10 °C, and 15 °C.

Sample number	Sampling time point (wpi)	Temperature °C	Group	Ratio mIgM	Log2(Ratio mIgM)	Ratio sIgM	Log2(Ratio sIgM)
T1r35	18	5	VAX	0,86	-0,22	0,74	-0,43
T1r36	18	5	VAX	1,68	0,75	1,06	0,08
T1r38	18	5	VAX	1,13	0,18	0,57	-0,80
T1r43	18	5	VAX	1,25	0,32	1,25	0,32
T1r48	18	5	VAX	1,15	0,20	0,84	-0,25
T1r49	18	5	VAX	0,97	-0,05	0,63	-0,67
T2r31	18	5	PBS	1,05	0,07	0,95	-0,07
T2r32	18	5	PBS	1,04	0,05	0,82	-0,28
T2r34	18	5	PBS	0,87	-0,20	2,13	1,09
T2r36	18	5	PBS	0,69	-0,53	0,83	-0,27
T2r38	18	5	PBS	1,15	0,20	0,94	-0,10
T2r40	18	5	PBS	1,32	0,40	0,77	-0,37
T3r14	9	10	VAX	5,36	2,42	3,07	1,62
T3r15	9	10	VAX	2,79	1,48	2,92	1,55
T3r17	9	10	VAX	4,75	2,25	5,55	2,47
T3r19	9	10	VAX	4,85	2,28	3,72	1,90
T3r20	9	10	VAX	3,20	1,68	2,71	1,44
T3r27	9	10	VAX	2,27	1,18	2,88	1,53
T4r11	9	10	PBS	0,37	-1,44	1,09	0,12
T4r13	9	10	PBS	0,49	-1,04	0,91	-0,13
T4r15	9	10	PBS	1,08	0,11	0,67	-0,58
T4r16	9	10	PBS	1,54	0,62	1,10	0,14
T4r17	9	10	PBS	1,82	0,87	1,30	0,38
T4r20	9	10	PBS	1,84	0,88	1,05	0,07
T5r16	6	15	VAX	1,58	0,66	4,13	2,05
T5r19	6	15	VAX	0,83	-0,27	1,48	0,57
T5r22	6	15	VAX	2,00	1,00	1,76	0,82
T5r23	6	15	VAX	1,09	0,12	0,40	-1,33
T5r26	6	15	VAX	1,55	0,63	0,70	-0,51
T5r28	6	15	VAX	1,57	0,66	0,70	-0,51
T6r11	6	15	PBS	1,06	0,08	1,02	0,03
T6r13	6	15	PBS	0,87	-0,20	0,86	-0,22
T6r14	6	15	PBS	1,35	0,43	1,39	0,48
T6r17	6	15	PBS	0,44	-1,20	0,75	-0,42
T6r18	6	15	PBS	0,97	-0,05	1,55	0,63
T6r19	6	15	PBS	1,92	0,94	0,71	-0,49

Table C3: qPCR raw data (average Cq-values for duplicate samples) for temperature group 5 °C at 630 ddpi./18wpi.

Sample number	Sample time point (wpi)	Temperature °C	Group	Cq EF1-α	Cq mIgM	Cq sIgM
T1r35	18	5	VAX	19,14	20,87	20,90
T1r36	18	5	VAX	19,79	20,56	21,06
T1r38	18	5	VAX	18,94	20,23	21,05
T1r43	18	5	VAX	18,57	19,67	19,55
T1r48	18	5	VAX	18,54	19,78	20,09
T1r49	18	5	VAX	18,74	20,25	20,72
T2r31	18	5	PBS	19,03	20,44	20,42
T2r32	18	5	PBS	18,72	20,12	20,30
T2r34	18	5	PBS	19,16	20,87	19,40
T2r36	18	5	PBS	19,38	21,45	20,98
T2r38	18	5	PBS	18,79	20,04	20,19
T2r40	18	5	PBS	19,61	20,72	21,32

Table C4: qPCR raw data (average Cq-values for duplicate samples) for temperature group 10 °C at 9 wpi./630 ddpi. and 18 wpi.

Sample number	Sample time point (wpi)	Temperature °C	Group	Cq EF1-α	Cq mIgM	Cq sIgM
T3r14	9	10	VAX	21,90	21,63	23,94
T3r15	9	10	VAX	19,43	19,92	21,44
T3r17	9	10	VAX	20,71	20,51	21,84
T3r19	9	10	VAX	20,78	20,55	22,49
T3r20	9	10	VAX	20,27	20,62	22,42
T3r27	9	10	VAX	18,92	19,69	20,93
T4r11	9	10	PBS	16,15	19,41	19,46
T4r13	9	10	PBS	17,26	20,20	20,87
T4r15	9	10	PBS	17,71	19,49	21,78
T4r16	9	10	PBS	18,77	20,10	22,16
T4r17	9	10	PBS	18,85	19,94	22,01
T4r20	9	10	PBS	19,40	20,52	22,89
T3r33	18	10	VAX	18,38	19,90	20,09
T3r34	18	10	VAX	18,79	19,61	19,71
T3r37	18	10	VAX	18,45	19,15	20,29
T3r40	18	10	VAX	18,03	18,82	20,84
T3r49	18	10	VAX	18,70	18,82	20,64
T3r50	18	10	VAX	19,48	19,84	21,92
T4r33	18	10	PBS	17,26	19,58	18,94
T4r34	18	10	PBS	18,37	19,56	19,04
T4r35	18	10	PBS	18,96	20,68	20,10
T4r36	18	10	PBS	19,26	20,51	20,17
T4r37	18	10	PBS	19,24	19,57	21,42
T4r40	18	10	PBS	20,29	22,40	20,96

Table C5: qPCR raw data (average Cq-values for duplicate samples) for temperature group 15 °C at 6 wpi./630 ddpi. and 18 wpi.

Sample number	Sample time point (wpi)	Temperature °C	Group	Cq EF1-α	Cq mIgM	Cq sIgM
T5r16	6	15	VAX	16,03	19,09	17,08
T5r19	6	15	VAX	15,96	19,99	18,48
T5r22	6	15	VAX	16,53	19,28	18,83
T5r23	6	15	VAX	16,14	19,77	20,56
T5r26	6	15	VAX	17,29	20,49	20,94
T5r28	6	15	VAX	16,62	19,74	20,25
T6r11	6	15	PBS	15,93	19,59	19,00
T6r13	6	15	PBS	16,09	20,06	19,41
T6r14	6	15	PBS	16,12	19,43	18,74
T6r17	6	15	PBS	16,81	21,88	20,35
T6r18	6	15	PBS	16,82	20,70	19,32
T6r19	6	15	PBS	17,22	20,09	20,85
T5r33	18	15	VAX	18,60	21,99	23,58
T5r37	18	15	VAX	16,37	20,78	21,14
T5r38	18	15	VAX	17,01	19,82	20,62
T5r40	18	15	VAX	16,39	19,93	19,66
T5r47	18	15	VAX	16,24	19,58	19,78
T5r48	18	15	VAX	18,46	21,57	22,92
T6r31	18	15	PBS	18,38	21,44	22,43
T6r34	18	15	PBS	17,17	19,91	21,51
T6r35	18	15	PBS	17,30	19,64	20,68
T6r37	18	15	PBS	16,48	19,89	21,18
T6r38	18	15	PBS	18,12	20,83	20,98
T6r40	18	15	PBS	16,73	19,88	20,74

Appendix D – Additional findings

Figure 1D displays photomicrographs (20x) of heart (atrium) sections following HE-staining. Within the atrium of both vaccinated and control fish, rounded basophilic structures were detected.

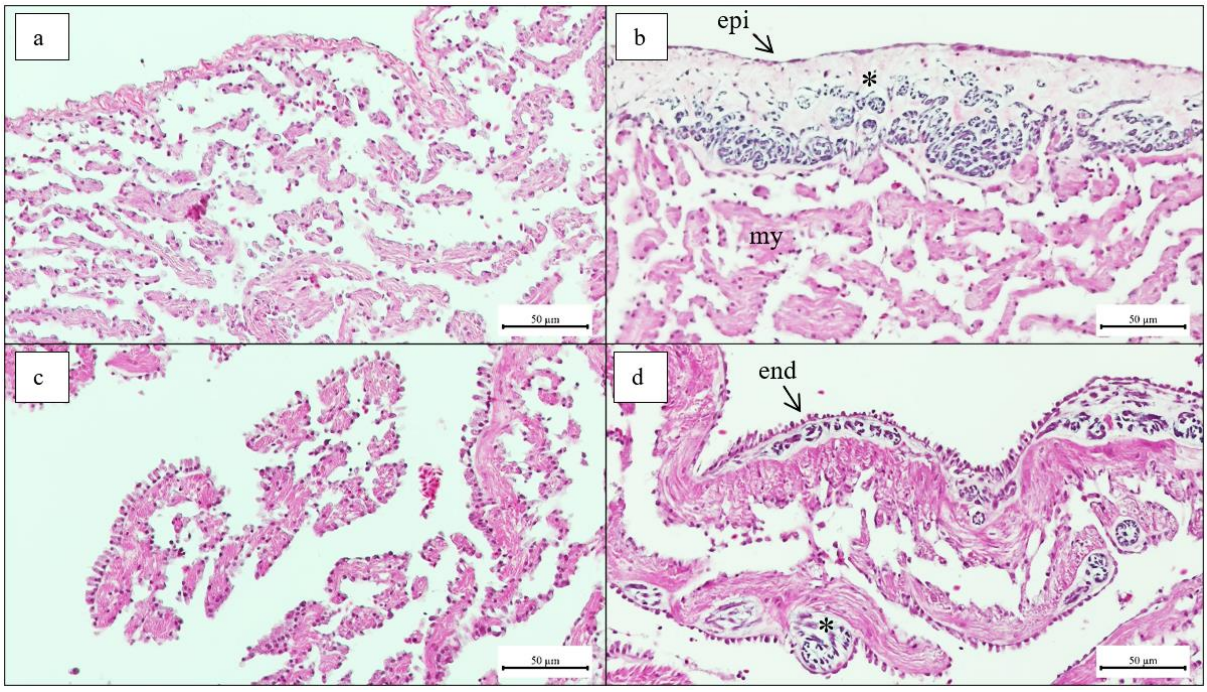

Figure D1: HE-staining of heart (atrium) sections from vaccinated (a, b) and control (c, d) lumpfish. b and d show basophilic structures indicated by asterisks (*) in the connective tissue between the epicardium (epi) and myocardium (my) and beneath the endocardium (end), respectively. a and c show no such structures and are included for reference. Magnification is 20x and scale bar is 50 µm for a, b, c, and d.

HE-staining revealed presence of rounded basophilic structures in the atrium of vaccinated and control lumpfish, as seen in Figure D1 (b and d). The structures were located in the subepicardial connective tissue layer covering the myocardium (Figure D1: b) and beneath the endothelium (Figure D1: d). For comparison, atria lacking such structures are also displayed in Figure D1 (a and c). Over half of the examined sections of the atrium from lumpfish in all three temperature groups (5 °C, 10 °C, and 15 °C) contained such structures.

The special staining technique von Kossa was performed on a heart section to test whether the observed rounded basophilic structures were calcium deposits. Figure D2 displays photomicrographs of a kidney section (20x) from salmon with nephrocalcinosis (positive control) and a section of an atrium (40x) with subepicardial basophilic structures from a vaccinated lumpfish. Calcium deposits are indicated by brown staining.

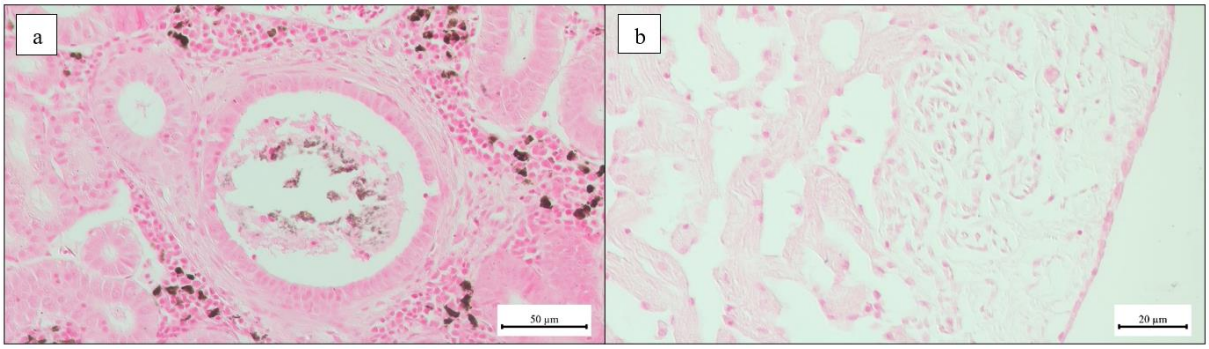

Figure D2: von Kossa staining of a kidney section (a) from Atlantic salmon and an atrium section (b) from vaccinated lumpfish. Note presence of calcium deposits (brown staining) within the kidney tubule, which was not evident in the subepicardial connective tissue of the atrium containing the detected structures on HE-sections. Scale bar is 50 for a (20x) and 20 for b (40x).

Staining with von Kossa revealed calcification within kidney tubules of the salmon diagnosed with nephrocalcinosis, which is indicated by the brown deposits (Figure D2: a). No positive staining was detected in the atrium of lumpfish suggesting that the observed basophilic structures were not calcium deposits (Figure D2: b).

Figure D3 displays a photomicrograph (40x) of erythrocytes in the heart (atrium) of a vaccinated fish following immunohistochemical staining with anti-PCNA. Positive staining is indicated by red colouration.

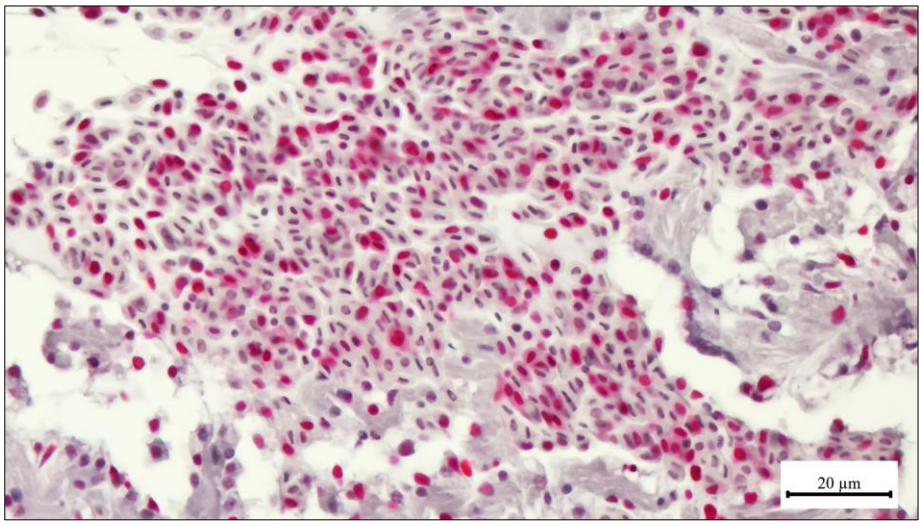

Figure D3: Immunohistochemical staining of PCNA in the atrium of a vaccinated lumpfish. Red colouration indicates presence of PCNA. Magnification is 40x and scale bar is 20 µm.

Figure D3 shows presence of proliferating erythrocytes in the heart of a vaccinated fish, which is indicated by the red stain. Positive staining of erythrocytes was apparent in the majority of heart sections examined from fish in all three temperature groups (5 °C, 10 °C, and 15 °C).

Figure D4 displays photomicrographs of HE-stained liver sections (20x) derived from one control and one vaccinated lumpfish showing normal liver histology and hepatitis, respectively.

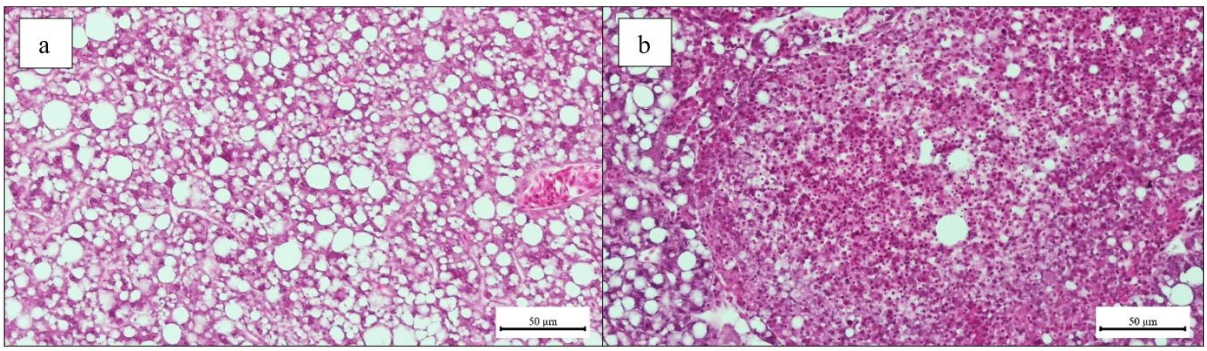

Figure D4: HE-staining of liver sections from control (a) and vaccinated (b) lumpfish. a shows normal liver histology with rounded hepatocytes and several lipid vacuoles. b shows a defined inflamed (necrotic) area, including pyknotic hepatocytes and cells with bright pink cytoplasmic staining, surrounded by normal liver tissue. Magnification is 20x and scale bar is 50 µm.

Figure D4 shows liver sections derived from one control (a) and one vaccinated (b) lumpfish following HE-staining. Normal lumpfish liver tissue has rounded hepatocytes and several vacuoles for fat storage, which is illustrated in the photographed liver section derived from a control fish (Figure D4: a). Multifocal hepatitis was detected in a liver section from a vaccinated fish in the 10 °C group indicated by several defined areas of degenerated liver tissue and clusters of cells with eosinophilic cytoplasmic staining. Such cell clusters were also noted in liver sections from two other vaccinated individuals.

Figure D5 display photomicrographs (20x and 40x) of a liver section with multifocal hepatitis following MGG-staining. The liver section is derived from the same sample pictured previously (Figure D4).

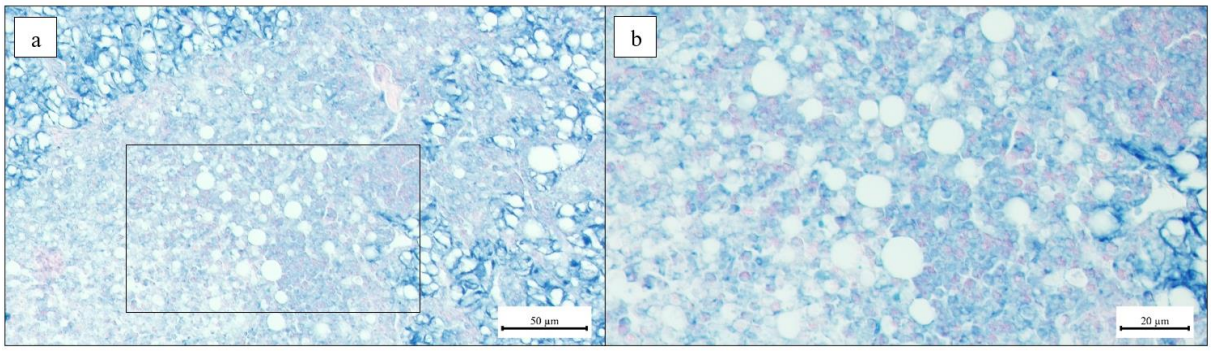

Figure D5: MGG-staining of a liver section with inflamed areas (multifocal hepatitis) from one vaccinated lumpfish in temperature group 10 °C at 18 wpi. Note presence of cells with eosinophilic cytoplasmic granules. The liver section is displayed at magnification 20x to the left in the figure (a). The black square indicates the area displayed at magnification 40x to the right in the figure (b). Scale bar is 50 µm for a (20x) and 20 µm for b (40x).

MGG-staining revealed that many of the cells in the inflamed areas of the liver sample derived from the vaccinated fish in the 10 °C group at 18 wpi. contained cytoplasmic eosinophilic granules (Figure D5).

Figure D6 displays photomicrographs (10x and 40x) of a liver section with multifocal hepatitis following immunohistochemical staining with anti-PCNA. The liver section is derived from the same sample pictured previously (Figure D4 and D5). Positive staining is indicated by red colouration.

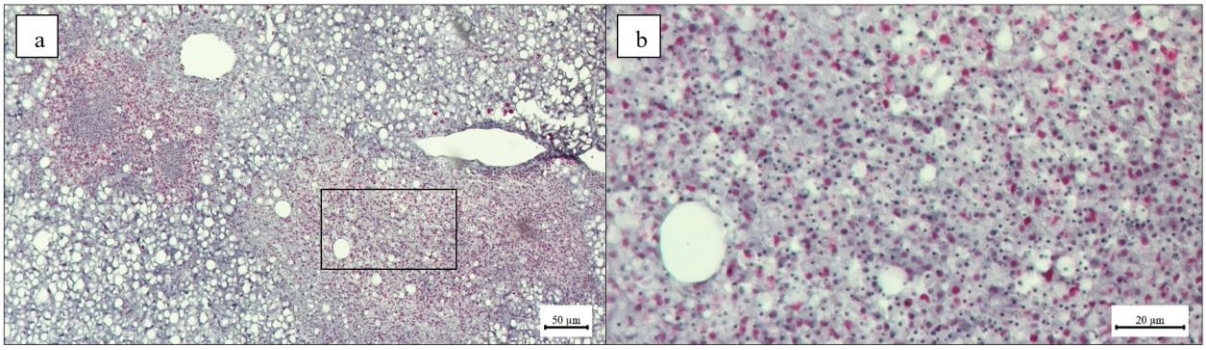

Figure D6: Immunohistochemical staining of PCNA in a liver section with inflamed areas (multifocal hepatitis) from one vaccinated lumpfish in temperature group 10 °C at 18 wpi. Red colouration indicates presence of PCNA. The liver section is displayed at magnification 10x to the left in the figure (a). The black square indicates the area displayed at magnification 40x to the right in the figure (b). Note presence of PCNA$^+$ cells among necrotic hepatocytes. Scale bar is 50 µm for a (10x) and 20 µm for b (40x).

Figure D6 shows presence of proliferating cells in inflamed areas of the liver sample derived from the vaccinated fish in the 10 °C group at 18 wpi. The PCNA$^+$ cells were located among the necrotic tissue. Some normal hepatocytes also stained positive following IHC with anti-PCNA.