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# Effects of dietary histidine and plant lipids on the Atlantic salmon (*Salmo salar*) health status following transfer to seawater

Maria Inês Ribeiro Carvalho

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# EFFECTS OF DIETARY HISTIDINE AND PLANT LIPIDS ON THE ATLANTIC SALMON (*Salmo salar*) HEALTH STATUS FOLLOWING TRANSFER TO SEAWATER

Dissertação de Candidatura ao grau de Mestre em Ciências do Mar – Recursos Marinhos, submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Doutor Benjamín Costas
Categoria – Investigador Auxiliar
Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental

Co-orientador – Doutora Sofie Remø
Categoria – Research scientist
Afiliação – Institute of Marine Research

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Maria Inês Ribeiro Carvalho

## Abstract

With the growth of the aquaculture industry, issues regarding animal welfare and production sustainability have become determining. Among the challenges faced by Atlantic salmon (*Salmo salar* L.) farming, it is essential to reduce losses due to disease emergence, mainly after seawater (SW) transfer, and to minimize the industry dependency on finite marine-derived ingredients by replacing them with more sustainable plant-based ones. Thus, the present study was carried out to assess the effects of supplementing fish oil (FO) or vegetable oil (VO) diets with histidine (His) on the immunity and oxidative stress of Atlantic salmon following SW transfer. It also aimed to compare the effect of feeding on diets supplemented with His during both freshwater (FW) and SW phases with feeding on His surplus only before or after SW transfer on post-smolt salmon health, and regarding the dietary lipid source.

From the time Atlantic salmon were around 30 g and until SW transfer, triplicated groups were fed four experimental diets differing in His content (low or high His content, His− or His+, respectively) and lipid source (100 % FO or a blend of VO). At the SW transfer moment, the dietary His treatments were crossed, resulting in 8 experimental groups: fish fed His− or His+ throughout the entire feeding trial (FO His−−, FO His++, VO His−−, VO His++), those fed His+ diets during the FW stage (FO His+−, VO His+−) and those fed His+ diets during the SW stage (FO His−+, VO His−+). There were three sampling moments: one at the end of the FW stage and two after fish being transferred to SW, 2 and 4 weeks after it (SWT2 and SWT4, respectively).

The incorporation of high His levels in feeds affected plasma IgM levels, which decreased between SWT2 to SWT4 in fish fed His− diets, but not in those fed His+ diets. The plasma bactericidal activity was enhanced in fish fed His+ diets at FW, but this effect was no longer significant after SW transfer. Even the highest level of His supplementation did not seem to compensate for the oxidative stress observed after smolts exposure to the marine environment, nor the effects of replacing FO by VO on the post-smolts immune system. Although in the present experimental conditions Atlantic salmon seemed to beneficiate from feeding FO, it was in fish fed this same diet that the greatest oxidative damage in the liver was observed. The switch from FO His− diet to FO His+ after SW transfer seemed to weaken plasma lysozyme activity.

In general, the dietary His supplementation did not seem to significantly influence the post-smolt Atlantic salmon health status, whereas VO diets appeared to excerpt a negative effect on it.

**Keywords:** Atlantic salmon; histidine; vegetable oils; immunomodulation; oxidative stress.

## Resumo

Com o crescimento da indústria da aquacultura, questões relacionadas com o bem-estar animal e a sustentabilidade da produção ganharam maior importância e visibilidade. Entre os desafios enfrentados pela produção de salmão do Atlântico (*Salmo salar* L.), apresenta-se como fundamental reduzir as perdas causadas pelo aparecimento de doenças, as quais ocorrem principalmente após a transferência para a água do mar (SW), e minimizar a dependência da indústria de ingredientes marinhos finitos, substituindo-os por ingredientes de origem vegetal mais sustentáveis. Assim, o presente estudo teve como objetivo avaliar os efeitos da suplementação de dietas à base de óleos de peixe (FO) ou óleos vegetais (VO) com histidina (His) na imunidade e stress oxidativo dos salmões do Atlântico após a sua transferência para SW. Pretendeu também comparar o efeito da suplementação de His durante ambas as fases de água doce (FW) e salgada com a suplementação apenas antes ou após a transferência para SW, na saúde dos salmões e tendo em consideração a fonte lipídica da dieta.

A partir do momento em que os salmões do Atlântico atingiram cerca de 30 g e até à transferência para SW, grupos triplicados foram alimentados com quatro dietas experimentais que diferiam no conteúdo de histidina (His) (baixo ou alto teor, His− ou His+, respetivamente) e na fonte lipídica (100 % FO ou mistura de VO). No momento da transferência para SW, os tratamentos dietéticos no que diz respeito ao conteúdo em His foram cruzados, resultando em 8 grupos experimentais: peixes alimentados com o mesmo nível de His durante todo o ensaio de alimentação (FO His−−, FO His++, VO His−−, VO His++), os alimentados com dietas com elevada concentração de His apenas durante a fase de água doce (FO His+−, VO His+−) e aqueles alimentados com dietas com elevado nível de His durante a fase de água salgada (FO His−+, VO His−+). Foram realizadas três amostragens: uma no final da fase FW e duas após a transferência dos peixes para SW, 2 e 4 semanas após (SWT2 e SWT4, respetivamente).

A incorporação de elevados níveis de His nas dietas afetou os níveis plasmáticos de IgM, os quais diminuíram entre SWT2 e SWT4 nos peixes alimentados com dietas His−, mas não nos alimentados com dietas His+. A atividade bactericida do plasma estava aumentada nos peixes alimentados com deitas His+ na FW, mas este efeito deixou de ser significativo após a transferência para SW. Mesmo o nível mais alto de suplementação de His não pareceu compensar o stress oxidativo observado após a exposição dos smolts ao ambiente marinho, nem os efeitos da substituição dos FO pelos VO no sistema imune dos pós-smolts. Embora nas presentes condições experimentais os salmões pareçam ter beneficiado da alimentação com FO, foi nos peixes alimentados com esta mesma dieta

que se observaram os níveis mais altos de danos oxidativos no fígado. A mudança da dieta FO His− para a FO His+ após a transferência para SW, pareceu enfraquecer a atividade da lisozima.

De forma geral, a suplementação da dieta com His não pareceu influenciar significativamente a saúde dos salmões do Atlântico após a transferência para SW, enquanto as dietas VO pareceram ter um efeito negativo na mesma.

**Palavras-chave:** salmão do Atlântico; histidina; óleos vegetais; imunomodulação; stress oxidativo.

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**List of Abbreviations and Acronyms**

| | |
|---|---|
| **AAs** | Amino acids |
| **ACH50** | Alternative complement pathway activity |
| **ACP** | Alternative complement pathway |
| **ACTH** | Adrenocorticotropin hormone |
| **ALA** | α-linolenic acid |
| **ARA** | Arachidonic acid |
| **ATP** | Adenosine triphosphate |
| **BHT** | 2,6-Di-tert-butyl-4-methylphenol |
| **BSA** | Bovine serum albumin |
| **CA** | Catecholamines |
| **CAT** | Catalase |
| **CC** | Chromaffin cells |
| **CDNB** | 1-chloro-2,4-dinitrobenzene |
| **cfu** | Colony-forming unit |
| **CHR** | Corticotropin-releasing hormone |
| **CI** | Confidence interval |
| **CS** | Corticosteroids |
| **DHA** | Docosahexaenoic acid |
| **DMSO** | Dimethyl sulfoxide |
| **DTNB** | 2-nitrobenzoic acid |
| **DTPA** | Diethylenetriaminepentaacetic acid |
| **EDTA** | Ethylenediamine tetraacetic acid |
| **EFAs** | Essential fatty acids |
| **EGTA** | Ethylene glycol tetraacetic acid |
| **ELISA** | Enzyme-linked immunosorbent assay |

| | |
|---|---|
| **EPA** | Eicosapentaenoic acid |
| **FAO** | Food and Agriculture Organization |
| **FAs** | Fatty acids |
| **FO** | Fish oil |
| **FW** | Freshwater |
| **GH** | Growth hormone |
| **GPx** | Glutathione peroxidase |
| **GSH** | Total glutathione |
| **GST** | Glutathione S-transferase |
| **GVB** | Isotonic veronal buffered saline |
| **His** | Histidine |
| **His−** | Low histidine diet |
| **His+** | High histidine diet |
| **HPI** | Hypothalamus-pituitary-interrenal |
| **HSC** | Hypothalamic-sympathetic-chromaffin |
| **HUFA** | Highly unsaturated fatty acids |
| **IC** | Interrenal cells |
| **Ig** | Immunoglobulin |
| **IgD** | Immunoglobulin D |
| **IGF-I** | Insulin-like growth factor I |
| **IgM** | Immunoglobulin M |
| **IgT/IgZ** | Immunglobulin T/Z |
| **LA** | Linoleic acid |
| **LPO** | Lipid peroxidation |
| **MDA** | Malondialdehyde |
| **MHC** | Major histocompatibility complex |

| | |
|---|---|
| **MT** | Methyltransferase |
| **MTT** | 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide |
| **NADPH** | Nicotinamide adenine dinucleotide phosphate |
| **NAH** | N-acetyl-histidine |
| **OD** | Optical density |
| **PBS** | Phosphate-buffered saline |
| **PIT** | Passive integrated transponder |
| **PUFAs** | Polyunsaturated fatty acids |
| **RAFOA** | Researching Alternatives to Fish Oil for Aquaculture |
| **RaRBC** | Rabbit red blood cells |
| **RNS** | Reactive nitrogen species |
| **ROS** | Reactive oxygen species |
| **RT** | Room temperature |
| **SD** | Standard deviation |
| **SOD** | Superoxide dismutase |
| **SW** | Seawater |
| **SWT2** | Two weeks after seawater transfer |
| **SWT4** | Four weeks after seawater transfer |
| **TBA** | Thiobarbituric acid |
| **TCA** | Trichloroacetic acid |
| **TMB** | 3,3',5,5'-tetramethylbenzidine |
| **Tris-HCl** | Trizma hydrochloride |
| **VO** | Vegetable oil |
| **WBC** | White blood cells |

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## Introduction

During the last 20 years, the world population has increased by approximately one billion inhabitants and it is estimated to grow by two billion throughout the next 30 years, resulting in a global population of 9.7 billion by 2050 (Nations, 2019). Besides, people have never consumed as much fish as they consume nowadays. In 2018, the average fish consumption was 20.5 kg per capita, which means an increase of 11.5 kg since 1961 (FAO, 2018).

Fish is recognized as essential to a nutritious diet, as it constitutes an excellent source of valuable nutrients, essential fatty acids (EFAs) and amino acids (AAs), vitamins and minerals. In many parts of the world, it is the leading animal protein source. For instance, in countries like Portugal and Norway, its consumption surpasses the average of 50 kg/year per capita (FAO, 2018).

However, as fisheries resources are currently overexploited or depleted, capture fisheries for direct human consumption cannot sustain the population growth (Miranda *et al.*, 2018), neither the increased demand for fish. Therefore, and once global wild fish capture stagnated since the mid-1980s, aquaculture appears as an absolute and realistic need to ensure access to seafood (FAO, 2018).

Aquaculture, defined as “the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants”, where farming implies intervention during the rearing process and ownership of the stock (FAO, 1997), has been the fastest-growing food production sector in the world, with a six-fold increase between 1990 and 2016. In 2030, it is projected to provide 62 % of the world’s seafood for human consumption (FAO, 2018). According to the latest reports, Asia dominates the world aquaculture production, with 89.4 % of global fish farmed, followed by 4.2 % in the Americas, 3.7 % in Europe, 2.5 % in Africa and 0.3 % in Oceania. Regarding the largest producing countries, China is by far the world’s top aquaculture producer and Norway stands out as Europe’s leading fish farmer (FAO, 2018).

### 1. Atlantic salmon

Among the most economically important fish species, Atlantic salmon (*Salmo salar* Linnaeus, 1758) (Figure 1) is the most cultured fish in the sea (FAO, 2018). In addition to its ecological importance and high conservation value, it is also a nutritious fish produced for human consumption. The salmon farming industry contributes to the economy and employment in many countries (Houston & Macqueen, 2019).

Initially, *S. salar* farming was aimed at repopulating natural ecosystems due to the decline of wild stocks and the desire to introduce salmonids to new locals. It is believed that the first salmon farm with commercial purpose arose in 1866 (Laird, 1996). Since then, the production of this species has been growing and, in 2016, accounted for 4 % of the total fish farmed globally (FAO, 2018). Nowadays, Atlantic salmon aquaculture stands as one of the most profitable and technologically advanced fish production industry worldwide (FAO, 2020). It is led by Norway, which, in addition to being the world's largest salmon producer, is also the major exporting country (FAO, 2020). According to the FAO (2016) report, the salmon farming industry represents 93 % of the total Norwegian aquaculture production, having a positive impact on the country's economy and society. The success of this industry may be explained in part by the favourable natural conditions of the country for the specie growth, such as the long coastline with many protected fjords, inlets and bays, as well as the suitable ocean temperature; and by the support of the Norwegian government along with the appropriate policies and strict regulations concerning the sustainable development of the activity imposed by it (Tilseth *et al.*, 1991).

### *1.1. Biology and life cycle*

*S. salar* is a bony fish of the family Salmonidae, which lives in association with the bottom but has mechanisms to maintain buoyancy in the water column, called benthopelagic fish (Koslow, 1996). It is native to both east and west coasts of the North Atlantic Ocean. Although most populations are anadromous, which means have the ability to move between freshwater (FW) and seawater (SW), some only undertake brackish water migrations and others complete the entire life cycle in FW (Thorstad *et al.*, 2010).

In the wild, anadromous Atlantic salmon spawn in rivers, generally between late autumn and winter. Salmon females lay their eggs in nests called redds (i.e., gravel depressions) and males fertilize them by releasing milt into the water column. Upon eggs hatch, still within the redd, alevins feed on the yolk sac. Once it is entirely absorbed, fry emerge from the gravel and start feeding on small insect larvae. After these first life stages, and once fry grows to approximately 5 g, it reaches a size-related developmental stage called parr stage. Parr undergoes dramatic behavioural, morphological, physiological, as well as biochemical changes, to be able to swim downstream to ocean feeding areas and adapt to the marine environment – a process called parr-smolt transformation or smoltification. These highly adaptive specialized alterations are triggered by environmental changes, such as photoperiod and water temperature variations (McCormick *et al.*, 1998).

Among the behavioural changes, the enhanced negative rheotaxis (i.e., movement in favour of the stream) and schooling (i.e. shoal swimming synchronously) are evident. Regarding morphological transformations, fish acquires a slimmer body form, resulting in reduced condition factor (i.e. weight relative to length), darkened fins margins and external body silvery colour. The increase in the level of salinity tolerance appears as the most important physiological shift. It may result from the increase in gill $Na^+$, $K^+$ ATPase activity, number and size of gill chloride cells and intestinal water permeability (McCormick *et al.*, 1998). These alterations on the osmoregulatory mechanisms are stimulated by hormone-dependent changes in pituitary, thyroid and interrenal tissues (Johansson *et al.*, 2016). The whole process has high-energy costs, leading to a decrease in liver glycogen and whole-body lipid content (Stubhaug *et al*., 2006), which also contributes to the reduction of the condition factor (McCormick *et al.*, 1998).

Once in the sea, salmon are named post-smolt. They remain there for 1 to 5 years, until reach maturity, during which they experience rapid growth. Adult salmon exhibit remarkable homing instincts, being capable of locating and returning to the river where they had born, to complete sexual maturation and spawn. Atlantic salmon is iteroparous, which means it may spawn repeatedly. However, as it requires high stored energy levels, only a low percentage of salmon spawn a second time. The ones that survive to spawn are called kelt (Thorstad *et al.*, 2010).

### *1.2. Farming production*

Based on the life cycle of wild salmon, salmon intensive aquaculture has two distinct phases: the first in FW and next in SW. The process starts with the extraction of eggs and sperm from breeding salmon, a process called stripping. After fertilized, eggs are incubated in oxygenated FW until hatch. At this time, alevins are moved to hatchery trays, which mimic the natural redds, and kept under flowing water. When alevins had absorbed the entire yolk sack, farmers start feeding them. The artificial diets provided take into account the carnivorous feeding requirements of the species. Once fish begin to develop adult salmon characteristics, parr are transferred to FW tanks, where they grow until the parr-smolt transformation (Lucas *et al.*, 2019).

In aquaculture, the control of light and water temperature is the primary technique to optimize the smoltification process and reduce hatchery rearing time. The artificial photoperiod signals seasonal cycles, and by periodically changing water temperature, fish metabolism is regulated. These two variables, well manipulated, allow the production of

yearling smolts (Lucas *et al.*, 2019). After smoltification, fish are transported to open sea cages, where they grow until reaching the market size.

A considerable difference between the life cycle of wild and farmed Atlantic salmon is found at the SW transfer, a critical period in both salmon wild lifecycle and aquaculture production (Aunsmo *et al.*, 2008). While in the wild, migrating smolts are exposed to gradually increased water salinities, in aquaculture, smolts are directly transferred to SW (Figure 1). Although some authors suggest that it does not affect smolts' health once they can directly enter SW with minimal ionic disturbance (McCormick *et al.*, 1998), others believe that this abrupt introduction into a hyperosmotic environment can act as an effective stressor (i.e., physical, biological or chemical insult to the biological condition) (Franklin *et al.*, 1992).

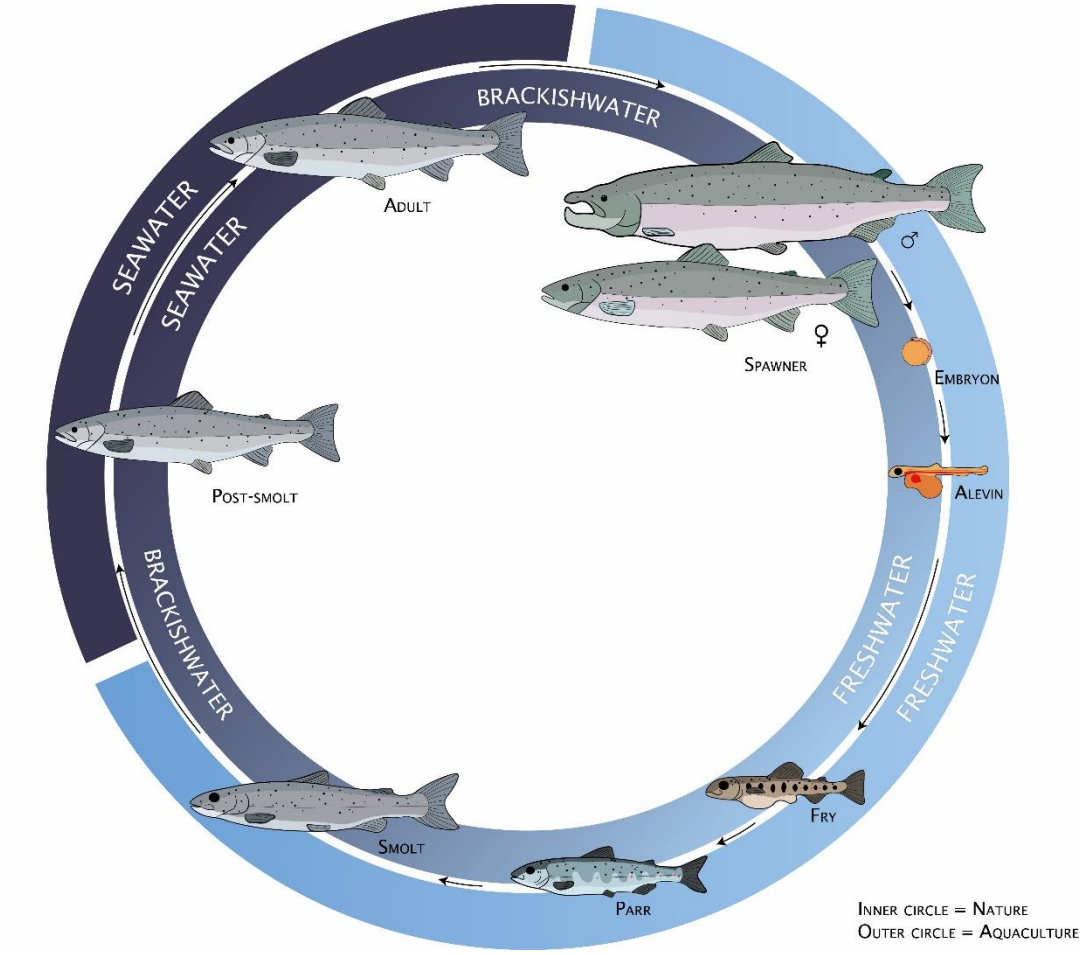


**Figure 1.** Representation of Atlantic salmon life stages highlighting the difference between nature and aquaculture production (inner and outer circle, respectively) concerning the transition of salmon from FW to SW. Adapted from: Kryvi *et al.* (2017).

Moreover, apart from the stress imposed by the abrupt introduction of smolts into SW itself, to move them from their land tanks to the respective sea sites, they must be gathered together, counted, captured and loaded in a well-boat or a helicopter, transported and

unloaded (Santurtun *et al.*, 2018). All these stressful procedures can affect fish immunocompetence, SW tolerance, growth and survival (Iversen *et al.*, 2005). Indeed, the emergence of diseases resulting from viral, bacterial and parasitic infections usually happens when the animal has previously been subjected to a stressful situation, and the most significant losses in Atlantic salmon farms take place chiefly during the first months after SW transfer (Johansson *et al.*, 2016).

## 2. Welfare of farmed fish

With the expansion of aquaculture practice in terms of intensification and technology, attention has been increasing with regard to fish welfare issues (Ashley, 2007). Broom (1999) defined animal welfare as the individual animal state concerning its environment. Good welfare combines the absence of hunger, thirst, discomfort, pain, injury, disease, fear and distress, and that the animal is allowed to express its natural behaviour (Ashley, 2007). If those conditions are not ensured, are liable to act as stressors, weakening fish defences and turning them more susceptible to diseases. Indeed, diseases rarely result from a simple association between a pathogen and a host; stressors often contribute to disease outbreak (Segner *et al.*, 2012).

Besides being an affront to animal welfare, the appearance of diseases in farm fish is a substantial source of monetary loss, jeopardising the industry's economic sustainability (Segner *et al.*, 2012). Furthermore, fish welfare is increasingly an essential issue regarding public perception, marketing and product acceptance (Broom, 1999). Therefore, it is the aquaculture industry's self-interest to guarantee proper husbandry conditions and avoid stressful situations to maximise fish production and sale.

### *2.1. Stress response*

To further understand how stress impacts fish health and welfare, it is important first to define "stress", which is a state of threatened homeostasis that is re-established by adjustments or adaptive responses (Chrousos, 1998). The stress response includes physiological, biochemical and behavioural changes to compensate for deviations resulting from stressors (Barton, 2002).

The physiological stress response involves two hormonal axes: the hypothalamic-sympathetic-chromaffin (HSC) and the hypothalamus-pituitary-interrenal (HPI). The HSC axis activates a fast stress response. It starts with the perception of the threat by the central nervous system, followed by the release of catecholamines (e.g., adrenaline, noradrenaline and dopamine) (Barton, 2002). Once in circulation, catecholamines, and mainly adrenaline, lead to the increase of ventilatory and heart rates, the amount of blood ejected and the blood perfusion in gills and muscle (Balasch & Tort, 2019). Then, the HPI axis is activated, leading to the release of corticosteroids (e.g., cortisol) and more catecholamines into circulation by the head-kidney (Barton, 2002) (Figure 2).

Once in circulation, the stress hormones, i.e., corticosteroids and catecholamines, elicit secondary stress responses, which include changes in plasma and tissue ion composition,

metabolite levels (e.g., increase in glucose and lactate blood levels, decrease in tissue glycogen), haematological features (e.g., hematocrit, haemoglobin), immune system (e.g., lysozyme activity, antibody production) and osmoregulation (e.g., chloride and sodium). When the stress becomes chronic, these alterations may lead to the tertiary stress response, affecting fish growth, swimming capacity, disease resistance and feeding activity (Segner *et al.*, 2012).

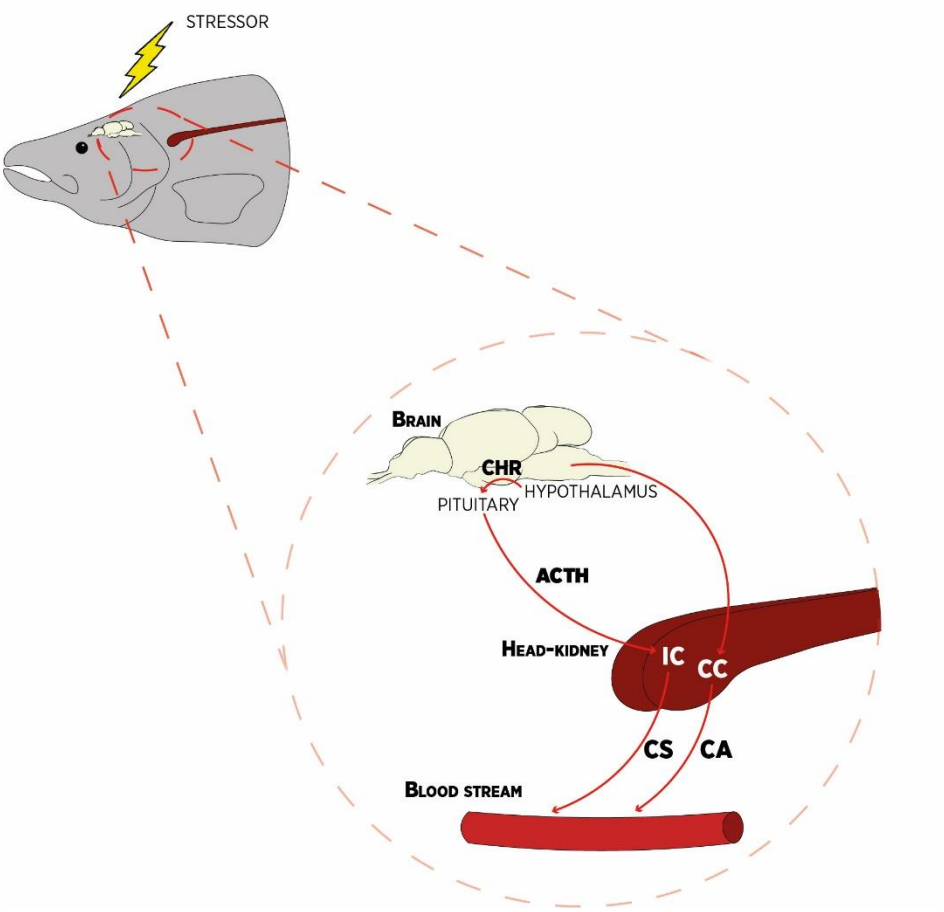


**Figure 2.** Principal hormones released during stressful events. CHR, corticotropin-releasing hormone; ACTH, adrenocorticotropin hormone; IC, interrenal cells; CC, chromaffin cells; CS, corticosteroids; CA, catecholamines. Based on: Tort (2011).

### *2.2. Teleost immune system*

In fish, the stress response cannot be disassociated from the immune response, as the first affects and becomes regulated by the second (Tort, 2011). The immune response comprises a network of biological structures (organs, tissues, cells, proteins and genes) and processes that interact to maintain the organism's integrity and protect it against non-self. It consists in two lines of defence with increasing specificity: the first is a rapid non-specific response, independent of prior exposure to a particular organism, known as innate

response; and the second, a specific response that takes longer time to mount and depends on the previous exposure to a pathogen, called adaptive or acquired immune response (Rauta *et al.*, 2012).

Even though fish possess both responses, the adaptive immunity parameters are more effective at higher temperatures (Alcorn *et al.*, 2002), being conditioned by the fish's poikilothermic nature (i.e., fish body temperature varies with the environment temperature). On the other hand, innate components are relatively independent of temperature. Thus, unlike mammals, fish strongly depend on efficient innate immune mechanisms (Magnadóttir, 2006).

### *2.2.1. Innate immune response*

The innate immune system includes anatomical barriers, cellular and humoral (circulating) components (Magnadóttir, 2006). Anatomical barriers, such as skin, gills and gut, are continuously exposed to the external environment, having a key role in preventing tissue colonisation by pathogens (Kiron, 2012). In addition to providing physical protection, they also provide chemical protection since they are in association with mucus, which contains several chemical mediators (e.g., lysozyme, lectins, complement proteins and antimicrobial peptides) (Magnadóttir, 2006). Indeed, mucosal surfaces, along with the stomach pH, form the chemical barrier against non-self. Biological barriers, like gut microbiota, are also part of the first line of defence (Trichet, 2010).

When the anatomical barriers are breached, the inflammatory response is activated to destroy the invading microorganism (Trichet, 2010). It starts with the recognition of infection by circulating leucocytes (i.e., neutrophils, monocytes/macrophages and lymphocytes) and the consequent activation of specific signalling pathways in immune-competent cells. Once the inflammatory response is initiated, the circulating leucocytes produce and release several mediators, such as eicosanoids and cytokines, as well as bactericidal components, such as lysozyme, complement factors, antiproteases, reactive oxygen species (ROS) and reactive nitrogen species (RNS), in order to fight the pathogen.

Lysozyme is a hydrolytic enzyme effective in the lysis of both Gram-positive and Gram-negative bacteria (Uribe *et al.*, 2011). It is also involved in other critical defence mechanisms, such as opsonisation and activation of the complement system (Magnadóttir, 2006). The complement system is a complex system of several glycoproteins little affected by temperature and that acts against a wide range of pathogens. Its functions include opsonisation and stimulation of phagocytosis, recruitment of phagocytes and cell lysis

(Kiron, 2012). It can be activated through three distinct pathways – the classical, lectin or alternative −, which differ on the molecules responsible for their initiation. The classical pathway is initiated by the binding of antibodies to the foreign microorganism's cell surface. The lectin pathway begins with the binding of a serum protein known as mannose-binding lectin to mannose residues on the surface of pathogens. The alternative complement pathway is activated by the direct binding to a microbial surface (Boshra *et al.*, 2006). Other enzymes efficient in preventing tissue colonisation by microorganisms are antiproteases or protease inhibitors, which impede the conversion of proteins to AAs by pathogen proteases, thereby inhibiting their adherence and establishment (Magnadóttir, 2006). ROS and RNS are covered in detail in the next section.

Stimulated by the chemical agents (chemotaxis) generated at the site of inflammation, monocytes/macrophages and neutrophils present along the endothelium of vessels migrate to the extravascular spaces (Magnadóttir, 2006). Neutrophils rapidly arrive at the local, followed by the macrophages. Both phagocytes are accountable for the foreign particles' engulfment by extending portions of the plasma membrane, forming an endocytose vesicle. When inside the phagocyte, lysosomes containing antimicrobial agents bind to the endocytosis vesicles and release their products to kill and digest pathogens. Phagocytosis is one of the most important defence mechanisms in poikilothermic animals because it is slightly influenced by temperature (Uribe *et al.*, 2011).

Finally, to avoid self-injury and promote the resolution of inflammation, anti-inflammatory compounds are produced and released to inhibit the recruitment of more neutrophils and stimulate the uptake of the neutrophils present in the inflammation site by macrophages (Barton, 2008).

#### *2.2.1.1. Respiratory burst and oxidative stress*

ROS and RNS production by phagocytic leucocytes, known as respiratory burst, plays a central role in innate immunity as it enables neutrophils and macrophages to eliminate internalised pathogens following phagocytosis (Fridovich, 1998). They are also produced during the mitochondrial respiration and in response to environmental stressors, such as changes in temperature, oxygen and salinity, and exposure to pollutants (Lushchak, 2011).

Inside the phagocytes, there are two different pathways that lead to the formation of either ROS or RNS, the superoxide and the nitric oxide pathways, respectively (Bogdan *et al.*, 2000). The superoxide pathway begins with the oxidation of the enzyme NADPH oxidase and results in the generation of three distinct ROS: the superoxide radical ($O_2^{\bullet -}$), hydrogen

peroxide ($H_2O_2$) and hydroxyl radical ($HO^{\bullet}$) (Fridovich, 1998). Even though hydrogen peroxide is not a radical specie, it can easily pass through cell membranes and be converted to hydroxyl radical (Birnie-Gauvin *et al.*, 2017). The nitric oxide pathway consists of the generation of nitric oxide by the enzyme nitric oxide synthase, which reacts with the superoxide radical, forming a highly reactive molecule, the peroxynitrite ($ONOO^-$) (Bogdan *et al.*, 2000). All superoxide radical, hydrogen peroxide, hydroxyl radical and nitric oxide are potent bactericides compounds, essential to destruct pathogens. When in contact with macromolecules, such as nucleic acids, proteins, lipids and carbohydrates, they can oxidize them, producing unpaired electrons and radical species, ensuing oxidative damage (Valko *et al.*, 2007). For example, polyunsaturated fatty acids (PUFAs), which are crucial components of cell membranes, are particularly prone to ROS attack, undergoing oxidative deterioration, also called lipid peroxidation (LPO). However, since ROS cannot distinguish between self and non-self molecules, they can attack the membrane structure and fluidity of the host own cells (Sargent *et al.*, 2003).

To protect themselves against the adverse effects of ROS, organisms developed an antioxidant protection mechanism (Figure 3). Antioxidants can be divided into enzymatic, which comprises superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST), and non-enzymatic, such as ascorbic acid (vitamin C), glutathione (GSH), α-tocopherol (vitamin E) and carotenoids (Valko *et al.*, 2007). Several studies also suggested the AA histidine (His) as a powerful antioxidant (Vera-Aviles *et al.*, 2018; Wade & Tucker, 1998).

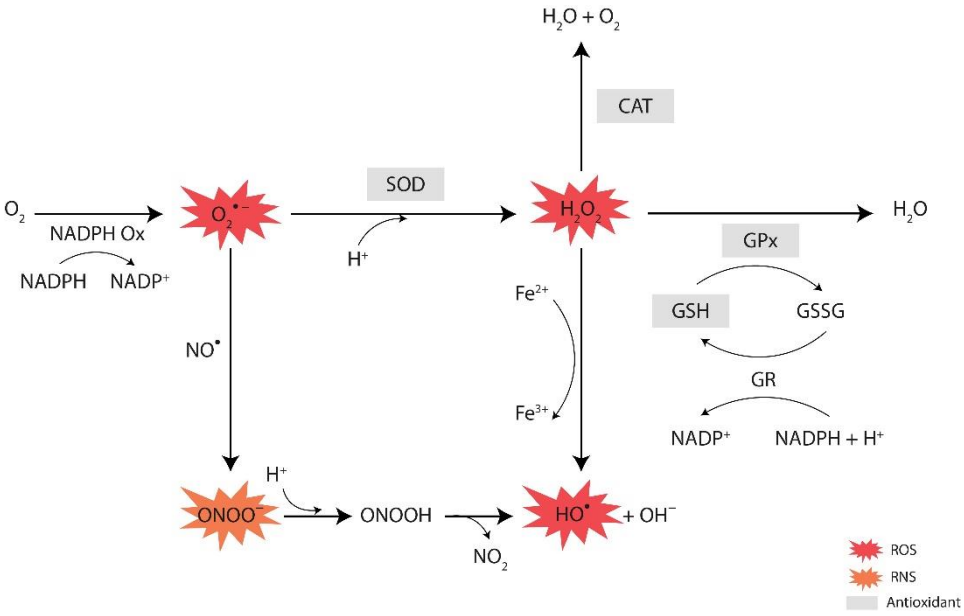


**Figure 3.** Schematic representation of the pathways producing reactive oxygen and nitrogen species (ROS and RNS, respectively) and key cellular antioxidant mechanisms. NADPH Ox, NADPH

oxidase; NADPH and oxidized $NADP^+$; $O_2^{\bullet-}$, superoxide radical; $NO^{\bullet}$, nitric oxide; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; $HO^{\bullet}$, hydroxyl radical; $OH^-$, hydroxide; SOD, superoxide dismutase; $H_2O_2$, hydrogen peroxide; CAT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase. Based on: Valko *et al.* (2007).

When ROS production exceeds the organism's capability to quench these reactive species through protective antioxidant mechanisms, it is called oxidative stress (Birnie-Gauvin *et al.*, 2017). It occurs when the antioxidant mechanisms are placed under stress (Niki, 2016), for example, due to dietary deficiencies of antioxidants or intake of oxidized foodstuffs (Sargent *et al.*, 2003). Oxidative stress is often associated with pathological and toxicological consequences.

### *2.2.2. Adaptive immune response*

Contrary to the innate immune response, the adaptive response takes longer to mount and depends on temperature (Alcorn *et al.*, 2002). It can be divided into two components: cellular, constituted by lymphocytes (B and T cells), and humoral, which includes inflammatory cytokines and immunoglobulins (Igs). Even though jawed fish are the earliest vertebrates to possess elements of the adaptive immune system (Rauta *et al.*, 2012), compared with mammals, they have a limited Igs repertoire, weak immunological memory and slower lymphocyte proliferation (Trichet, 2010).

Igs are glycosylated proteins produced by B lymphocytes in response to antigens with specific binding capacity to them. Igs can exist in the cell membrane and soluble form, also called antibody. The membrane form, or B-cell receptor, plays as an antigen sensor. After the antigen binds to the B-cell receptor, a cascade of reactions is triggered, leading to cell proliferation and differentiation. It generates a population of antibody-producing B cells (or plasma cells), as well as memory B cells. Memory cells remain inactive until new exposure to the same antigen, at which point they divide into plasma cells. Plasma cells secrete antibodies into the bloodstream and other body fluids, where they bind to the compatible foreign antigen, leading to the pathogen's inactivation by impeding them to bind to host cells' receptors (Secombes & Wang, 2012). Three major types of Igs have been recognised in teleost fish: IgM, IgD, and IgT/IgZ (Hordvik, 2015). The first to be expressed during the development of B cells is the IgM, which is also the most abundant class of Ig. Fish's IgM occurs in plasma and epithelial mucus (Randelli *et al.*, 2008).

Similarly, T cells have antigen-specific receptors on their surface, which allow the recognition of infected cells. Unlike B cells, they only recognise antigens that have been

processed and displayed on the surface of cells associated with a specific group of proteins encoded by a set of genes called the major histocompatibility complex (MHC). Indeed, this is the major pathway of T cells activation. There are two major classes of MHC molecules: MHC I, which are glycoproteins expressed on the surface of nucleated cells (except neurons); and MHC II, expressed primarily in antigen-presenting cells, such as macrophages, dendritic cells and B cells, but can be induced in other cells, such as naive T cells (i.e., inactivated mature T cells) and epithelium cells (Randelli *et al.*, 2008). Once activated, T cells divide into Cytotoxic T cells and Helper T cells. Cytotoxic T cells eliminate infected or abnormal cells by releasing cytotoxins, which cause the apoptosis of target cells. Helper T cells regulate the activity of other immune cells by releasing cytokines. They can activate B cells and cytotoxic T cells and increase phagocytosis (Secombes & Wang, 2012).

The adaptive immune response is particularly important for recurrent infections. After the first exposure to a specific pathogen, the immunological memory cells formed can remain in the organism for years, allowing the body to respond quickly and efficiently to a second infection by the same agent. This is the principle for vaccination (Secombes & Wang, 2012).

### *2.3. Effects of stress on immune functions*

As above-mentioned, stress and immune systems are strictly connected. They share common regulatory mechanisms and effector organs. Indeed, in fish, the head-kidney is a central organ for both systems as it combines endocrine, nervous and immune tissues. Its peculiar organization suggests that the stress response's end-products have direct paracrine access to the immune cells and vice versa (Tort, 2011). Leucocytes are known to have glucocorticoid, adrenergic, cholinergic and opioid receptors, being sensitive to a wide repertoire of neuroendocrine responses (Verburg-van Kemenade *et al.*, 2009). Although stress is usually associated with harmful effects on the immune system, depending on the time course and persistence of the stressor, it can both inhibit or activate immune factors (Yada & Tort, 2016).

Acute stressors, i.e., short and intense stimuli, are the most common in nature. They trigger a fight/flight/fright reaction, which is mounted in the presence of the threat and ceases in the absence of it (Dhabhar, 2002). They do not seriously affect the immune system. On the contrary, acute stress has been associated with advantages as it leads to immunoactivation or immunoenhancing processes (Tort, 2011). When stressful situations have beneficial effects on the organism, are called eustress (Schreck & Tort, 2016). Their stimulatory effects have been attributed to the activation of the HSC axis and the consequent release of catecholamines. Catecholamines stimulate the production and mobilization of blood cells,

including leucocytes. The number of leucocytes in blood increases and their subtypes' frequency varies: the number and percentage of lymphocytes and monocytes decrease, while neutrophils increase. Furthermore, the number of activated macrophages in circulation and the recruitment of activated T cells increase (Yada & Tort, 2016). Regarding the humoral immune components, some of them, such as lysozyme and complement C3 protein, may also increase (Tort, 2011). Moreover, eustress is usually associated with elevated levels of antioxidants and low levels of oxidative stress markers (Niki, 2016).

On the other hand, chronic stressors, i.e., persistent and low-lasting stimuli, trigger a physiological response that persists after the stressor has ceased or that is repeatedly activated, increasing the organism's exposure to the stress hormones released. Chronic stress may lead to the suppression of some defence mechanisms, undermining the organism's immune system. It is called distress (Tort, 2011). This type of stress is usually associated with aquaculture practices. The negative impacts of chronic stressors can be explained by the resource lack (allostatic load) for immune mechanisms that imply continuous energy availability (e.g., white cell production, synthesis of antibodies) and by the suppressive effect of cortisol for some immune mechanisms (Yada & Tort, 2016). For instance, cortisol is known to inhibit phagocytosis, respiratory burst, antibody production and bactericidal activity (Verburg-van Kemenade *et al.*, 2009). Regarding cellular changes during a persistent stressful situation, there is a decrease in the number of circulating leucocyte, since these cells will be mainly concentrated on the affected organs (Tort, 2011).

### *2.4. Effects of stress on osmoregulation*

Stress also affects osmoregulation in fish. Effective osmoregulation is particularly crucial at SW transfer and early adaptation of smolts to the new environment. As already stated, for anadromous Atlantic salmon to move from a river (hypoosmotic environment) to the sea (hyperosmotic environment), it has to pass through several physiological adaptions, such as the alteration of ion transport properties of the epithelium in the gills and gut (Hvas *et al.*, 2018) (Figure 4). Not surprisingly, those physiological responses are triggered by the neuroendocrine system, the primary system to alert the organism about environmental shifts (McCormick, 2001).

Despite cortisol's role during the stress response, it has been recognized as "the SW-adapting hormone" for a long time. Cortisol stimulates the gills sodium pump activity, affects the morphology and development of chloride cells, which are the responsible cells for the secretion of excess ions (McCormick, 1995). Recently, it has become clear that its function in promoting salt secretion is controlled by the growth hormone (GH). Indeed, there is

evidence that cortisol and growth hormone/insulin-like growth factor I (GH/IGF-I) axes work in synergy to increase the fish's hypoosmoregulatory ability (McCormick, 2001). GH/IGF-I and cortisol axes may interact through the regulation of cortisol receptors since GH treatment was shown to increase the gill cortisol receptors' numbers (McCormick, 2001).

However, it has been proved that stressed fish are prone to osmotic imbalances since stress turns osmoregulatory mechanisms less efficient. The elevation of plasma cortisol during stress is known to increase epithelial membrane permeability, jeopardizing the osmotic integrity by passive gain or loss of $Na^+$ and $Cl^-$, depending on the environment salinity (Fjelldal *et al.*, 2020). Ultimately, it can lead to the animal's death. For instance, the root cause of mortality for Atlantic salmon infected by salmon lice at SW is the elevated plasma ion concentrations resulting from the elevation of serum cortisol due to infection (Fjelldal *et al.*, 2020).

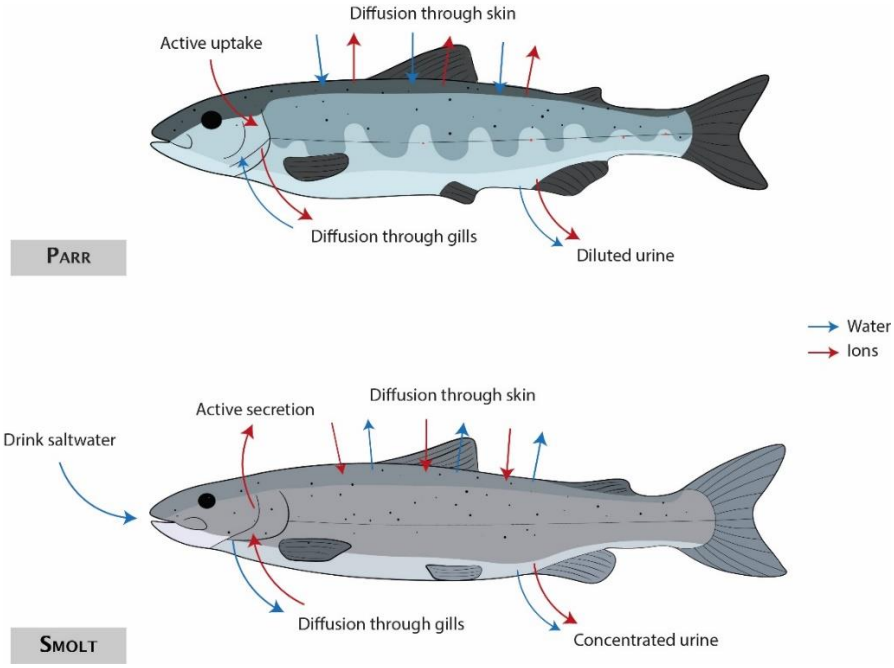


**Figure 4.** Osmoregulation in parr and smolts (FW and SW life stages of Atlantic salmon, respectively). Adapted from the work of Duane Raver, NOAA.

## 3. The role of nutrition on fish health

Proper nutrition, among other factors, has been recognized to have a critical role in fish health and welfare. The lack of a nutrient or the supply of an unbalanced diet may alter metabolic functions and interfere with processes with specific roles as modulators of the immune system, such as the stress response and the mechanisms of defence against infection, increasing fish's susceptibility to opportunistic disease-causing agents (Trichet, 2010). Artificial diets containing adequate quantities of all nutrients are the primary source of n utrition in intensive aquaculture (Gatlin, 2003).

However, under stressful situations, requirements for specific nutrients may be increased to cope with the needs of the defence mechanisms (Kiron, 2012). As stressful fish farming procedures are often unavoidable, nutritional strategies to mitigate their adverse effects are becoming usual in modern fish farming. Through the enrichment of feeds with specific additives, such as vitamins or AAs, that directly or indirectly participate in a given biological mechanism (Li *et al*., 2009), fish's immune system can be strengthened. These dietary formulations that promote health and well-being besides providing only the minimum nutritional requirements are called functional feeds (Herrera *et al*., 2019).

Moreover, to meet the world's demand for sustainable produced fish, there has been an increasing replacement of finite marine-derived ingredients by more sustainable plant-based ones in aquafeeds. However, those formulations might markedly deviate from fish natural feeding habits and affect the immune mechanisms (Trichet, 2010). By supplementing them with certain nutrients, their possible nutritional deficiencies may be counterbalanced and their detrimental effects mitigated.

### *3.1. The replacement of fish oil sources in aquafeeds*

Lipids are indispensable for all living organisms' nutrition. Apart from being the principal source of energy in fish feds, lipids and fatty acids (FAs) are also the primary sources of EFAs (Kiron, 2012). Although EFAs requirements vary between marine and FW fish species and, within a species, among development and physiological stages (Sargent *et al.*, 2003), all fish need three polyunsaturated FAs (PUFAs) for normal growth, reproduction and health: arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3).

Usually, EFAs requirements of FW species can be met by the supply of linoleic acid (LA, 18:2n-6) or α-linolenic acid (ALA, 18:3n-3), since they have enzymes that can elongate and desaturate them (elongases and Δ6- and Δ5-desaturases, respectively), leading to the

generation of ARA, EPA and DHA. Contrariwise, marine fish and salmonids (particularly post-smolts) lack or have very low activity of Δ5-desaturase and, consequently, cannot perform this conversion and efficiently synthesize n-3 highly unsaturated FA (HUFA) (Bell *et al.*, 1997; Sargent *et al.*, 2003). Therefore, they must be acquired through the diet.

Traditionally, fish oil (FO), obtained by pressing, centrifugation and separation of cooked fish, has constituted the preferred source of lipids in aquafeeds (Beheshti Foroutani *et al.*, 2018) because of its ready availability, high digestibility, HUFA content and EFA profile (Montero *et al.*, 2003). However, in recent years, there has been an increasing effort to reduce aquaculture dependency on finite fisheries-derived ingredients by replacing FO with more sustainable plant ingredients (FAO, 2020). Along with sustainability, the need for increasing the inclusion of vegetable oil (VO) sources has been exacerbated due to the exponential rise of FO prices as a consequence of its greater consumption by humans and the higher integration of long-chain n-3 FAs in pharmaceutical products over the last decades (Klinger & Naylor, 2012).

Notwithstanding the greater sustainability, high availability and lower price, the replacement of FO by VO sources is associated with variations on FAs profiles (Trichet, 2010). Unlike FO, VO has a lower level of n-3 FAs and do not contain EPA and DHA (Turchini *et al.*, 2009). Indeed, VO is rich in n-6 and n-9 FAs, such as LA. Thus, the replacement of FO by VO increases the intake of n-6 FAs (Oliva-Teles, 2012), altering the energy source for the immune cells, modifying lipoprotein profiles, and interfering with the cell membrane fluidity and permeability. It may also affect membrane receptors, receptor binding affinity, signalling pathways, and the regulation of the activation of transcription factors, modifying gene expression and altering the immune cell functions (Gatlin, 2003; Secombes & Wang, 2012; Trichet, 2010).

Moreover, a key element between FAs composition of the membrane of immune cells and the inflammatory response are eicosanoids (e.g., prostaglandins and leukotrienes). Eicosanoids are highly active biological hormone-like compounds in a wide range of processes, frequently synthesized under stressful and pathological conditions (Sargent *et al.*, 2003). They modulate the intensity and duration of the inflammatory response (Rowley *et al.*, 1995). Their amount is positively linked with the quantity of their precursors ARA and EPA, whose tissue content is influenced by the dietary FAs. Although fish cell membranes are rich in EPA, ARA is the preferred substrate for the synthesis of eicosanoids (Bell *et al.*, 1994). Moreover, eicosanoids synthesized from ARA are highly active, whereas the ones produced from EPA are a much less biologically active form (Sargent *et al.*, 2003) (Figure 5).

Thus, eicosanoids production and activity are determined by the ratio ARA/EPA in cell membranes, which in turn is imposed by the intake of n-6 and n-3 PUFAs (Sargent *et al.*, 2003). Since VO sources are rich in ALA, the precursor of ARA, it is expected that the greater inclusion of plant oils in fish feeds leads to the production of highly biologically active eicosanoids, increasing inflammation. Excessive inflammation contributes to a range of diseases (Calder, 2017) and so, when fish are fed with VO diets, more attention must be given to fish health.

In the Atlantic salmon aquaculture, the partial replacement of FO by VO has become a common practice (Stubhaug *et al.*, 2006), with the joint inclusion rate of FO and fish meal in salmon feeds being now often less than 10 % (FAO, 2020). Different VO, such as linseed, olive, palm, rapeseed, soybean and sunflower oil, have been investigated as alternative lipid sources for salmonids (Turchini *et al.*, 2009). Published studies on the complete or partial replacement of FO with VO reveal no adverse effects on Atlantic salmon growth. Importantly, in those studies, EPA and DHA sources were always provided either by the inclusion of FO (in partial FO replacement experiments) or fish meal (in complete FO replacement experiments) (Turchini *et al.*, 2009). However, according to the results from the project RAFOA (2005), salmon grown on VO diets may be under somewhat increased stress as suggested by some blood parameters (e.g., hematocrit, leucocyte numbers, macrophage respiratory burst).

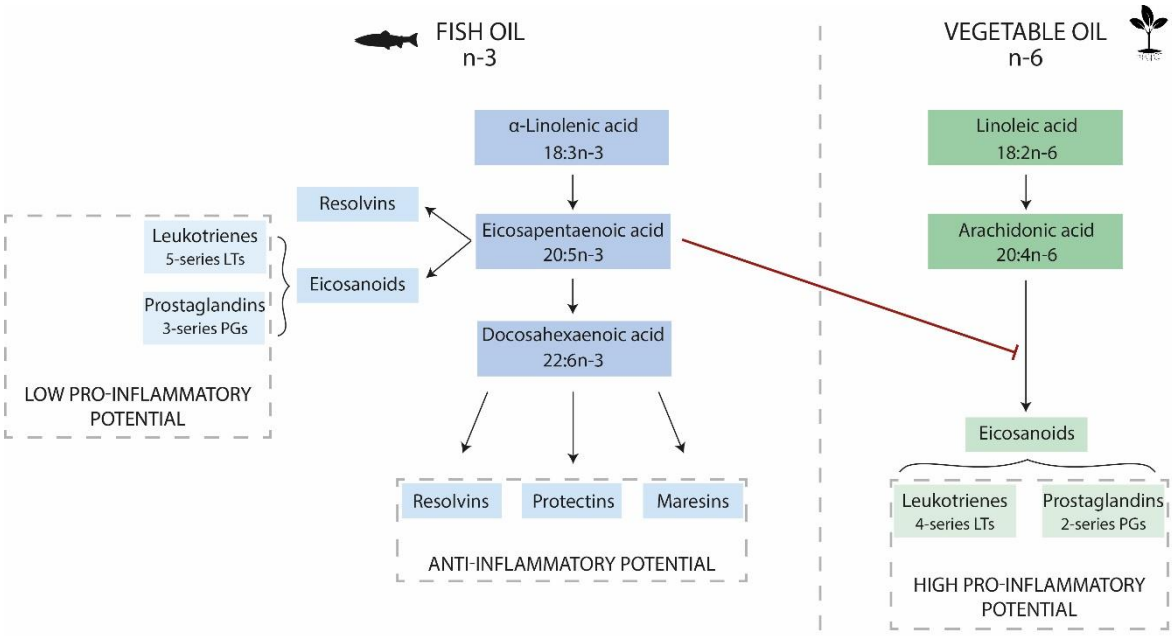


**Figure 5.** Synthesis of eicosanoids from n-3 and n-6 lipid sources (FO and VO, respectively) and their inflammatory potential. Based on: Calder (2017).

*3.2. Functional amino acids – histidine*

According to Herrera *et al.* (2019) review on diet supplementation to mitigate stress, AAs have been the most studied in this field, probably due to their versatility and direct involvement in the neuroendocrine response. Indeed, beyond being constituents of proteins and an energy source, AAs regulate key metabolic pathways and play important roles in antioxidative defence, stress response and cellular osmoregulation, among others (Li *et al.*, 2009). Under stressful conditions, the concentration of certain AAs in the plasma decreases as they are converted into requisite compounds to face the challenge (Herrera *et al.*, 2019). By supplementing feed with these AAs, it is possible to restore the plasma pool, avoiding the lack of resources and allowing fish to meet the augmented requirements.

Besides being an indispensable AA (i.e., cannot be synthesized by the animal, so must be acquired through diet), His and its derivatives are recognized to cover several functional roles that may improve animal health and welfare (Waagbø *et al.*, 2010). In addition to being used as building blocks in the protein synthesis, it has been shown that they behave as buffer (Abe *et al.*, 1985), antioxidant and have anti-inflammatory properties (Wade & Tucker, 1998) in the tissues in which they are present.

Migratory fish are known to contain large amounts of His and its dipeptides, such as anserine and carnosine, in the white muscle (Suzuki *et al.*, 1987). The greater deposition of histidine-related substances in the muscular tissue of active swimming fish species has been attributed to their buffering capacity, an important feature to protect muscle from acidosis (pH reduction) due to proton accumulation as a result of ATP consumption during burst swimming activity (Ogata & Murai, 1994; Ogata, 2002). Moreover, studies with different salmonid species (Cowey & Parry, 1963; Ogata & Murai, 1994) reveal their preference for accumulating anserine over other His derivatives (Abe, 1983). Beyond being much more metabolically stable, anserine has a higher buffering capacity within the physiological range than other His-related compounds (Abe *et al.*, 1985). For Atlantic salmon in farming conditions, the accumulation of a substance capable of maintaining intracellular pH homeostasis in white muscle might be especially relevant at SW transfer moment, as fish experience a rapid salinity fluctuation (Breck *et al.*, 2003). According to Ogata (2002), His buffering capacity also has benefits for human consume, since it leads to an enhancement of imidazole compounds in the fish muscle, improving fish's taste and texture by conferring sweetness, heaviness and thickness.

In the same way, increasing the level of His and its dipeptides in the white muscle can improve the tissue's buffering capacity, it can also increase its antioxidant activity (Ogata, 2002). Indeed, His is regarded as an efficient antioxidant as it acts as a metal-ion chelator

and a scavenger of singlet oxygen and hydroxyl radical. Moreover, this AA is recognized as the most active at scavenging singlet oxygen. Dipeptides containing His also appear to have antioxidative activity. Thus, it has been suggested that the antioxidant properties are conferred by the imidazole ring of L-His (Wade & Tucker, 1998). In addition to being antioxidant themselves, His and its related-compounds induce the expression and activity of some enzymatic antioxidants (e.g., CAT and GPx) (Remø *et al.*, 2014; Vera-Aviles *et al.*, 2018). Wade and Tucker (1998) also mentioned the anti-inflammatory potential of His in a range of diseases, in part due to the capacity to scavenge ROS generated by cells during the inflammatory response and also because they directly inhibit the secretion of some pro-inflammatory cytokines, particularly in chronic diseases (Vera-Aviles *et al.*, 2018).

Another known function of His is to maintain osmotic balance in the fish's eyes. This ability has been attributed to the presence of histidine imidazole N-acetyl-histidine (NAH) in the lens, whose content is dependent on the dietary His level (Breck *et al.*, 2003; Waagbø *et al.*, 2010). This compound is central for mitigating cataracts, which is a common eye disorder in Atlantic salmon intensive farming that, among other causes, can result from disturbances in osmotic homeostasis (Breck *et al.*, 2005). In a study conducted by Breck *et al.* (2003), it was found that the current recommended His content in the Atlantic salmon diet (8 g His/ kg dry matter) (NRC, 2011) did not meet the requirements concerning fish ocular health. This lack of His has been attributed to deficiencies in the diet, either due to the ban on the use of one of the major sources of His in aquafeeds (i.e., mammalian blood meal) (Breck *et al.*, 2003) or due to the increasing replacement of fish meal and FO by plant protein and oil sources, which are known to interfere with the osmoregulatory abilities of the lens (Remø *et al.*, 2014). By enriching feed with His, researchers were able to prevent cataracts development (Remø *et al.*, 2014; Waagbø *et al.*, 2010).

## Objectives

As the transfer of smolts from FW to SW is unavoidable and leads to stress, mitigate their effects is required to improve Atlantic salmon welfare, guaranteeing optimal immune status and increase salmon farming efficiency. After reviewing the literature on this field, His supplementation appears as a good candidate due to its potential buffer, antioxidant and anti-inflammatory properties. It is also important to assess how dietary VO sources affect salmon health status since VO are increasingly preferred over dietary FO and due to their knowing pro-inflammatory effects.

Therefore, this study aims to evaluate the effects of dietary His supplementation on the Atlantic salmon health status following transfer to SW and its interactive effects with dietary lipid sources. It also intends to compare the effect of feeding diets supplemented with His throughout smoltification and the first period in SW, with feeding His surplus only before or after SW transfer on post-smolt salmon health, and possible interactions with the dietary lipid source.

In particular, it is aimed to:

i. Assess if dietary His supplementation can modulate immunity and oxidative stress after SW transfer;

ii. Evaluate how salmon health status fed a diet rich in vegetable lipids might be affected by His surplus;

iii. Compare the immune and oxidative status of fish fed diets supplemented with His during both FW and SW with those fed His surplus either only during FW or SW phase;

iv. Investigate if a possible effect of switching dietary His treatment after SW transfer on post-smolts health status depends on the type of dietary lipid source.

The present study is in line with three targets of the Sustainable Development Goals predicted to 2030 Agenda: 2 – Zero Hunger; 12 – Responsible Consumption and Production; 14 – Life Below Water (Nations, 2015).

## Material and Methods

### 1. Experimental design

In this study, Atlantic salmon smolts that were part of a feeding trial carried out to investigate the long-term effects of different diet formulations on the development of cataracts were used. The feeding trial was conducted at the Institute of Marine Research (Matre Research Station, Norway), where fish from the AquaGen strain were reared by standard production procedures. From first feeding, 2 groups of Atlantic salmon fry were given diets containing either 100% FO (fish oil) or VO (plant oil mix, RAFOA). When fish were around 30 g (FO: 32 ± 7 g, VO: 30 ± 6 g), they were split into four groups (each reared in triplicate tanks, 12 (1x1 m) tanks in total), where fish continued on the diets with the same lipid source and with or without His supplementation, giving four experimental groups (FO His+, FO His−, VO His+, VO His−) during smoltification until SW transfer. The experimental diets were produced by BioMar AS, and the feed analysis was performed by the staff at IMR. The ingredients and chemical composition of the experimental diets are shown in Table 1, and their lipid composition is displayed in Table 2. Both diets had a His content above the optimum for growth. The fish were smoltified by giving them a winter period (photoperiod 12:12) followed by a period with continuous light (photoperiod 24:0). When the fish were around 50 g, around 600 DD prior to SW transfer, they were vaccinated (Clynav PD7 vet, MSD Animal health) to allow later transfer to net pens. The fish were fed daily to satiation by continuous feeders when the light was on. At the end of the FW period, smolts were 113 ± 25 g and there were no differences between dietary groups. The fish were PIT (passive integrated transponder)-tagged to allow mixing them in the tanks in SW (cross-over within each lipid group to investigate the effect of His supplementation before and after SW transfer).

At the time of SW transfer, 420 fish from each FW dietary group were distributed in 6 tanks per dietary lipid group, where fish previously given FO His+ and FO His- were mixed and VO His+ and VO His- were mixed, with 140 fish in each tank. The water was then switched from FW to SW. The fish were given the same diets (FO His+, FO His−, VO His+, VO His−), and due to the cross-over this gave eight experimental groups with fish given His+ supplementation both in FW and SW (FO His++, VO His++), His+ supplementation only in FW (FO His+−, VO His+−), His+ supplementation only in SW (FO His−+, VO His−+) and no His+ supplementation (FO His−−, VO His−−), as shown in Figure 6. In SW the photoperiod was 18:6 and the fish were given two meals, one in the morning and one in the afternoon. Throughout the trial, the temperature was kept at 13°C, water flow was adjusted according to the biomass as the fish grew and oxygen saturation was monitored according to standard production procedures.

The experiment respected the guidelines of the Norwegian Regulation and Animal Experimentation and EC Directive 2010/63/EU, and the National Animal Research Authority approved the protocol.

**Table 1.** Ingredients and chemical composition of the four experimental diets.

| | **Experimental diets** | | | |
|---|---|---|---|---|
| | **FO His−** | **FO His+** | **VO His−** | **VO His+** |
| **Ingredients (g / 100 g)** | | | | |
| Fish oil | 16 | 16 | − | − |
| RAFOA oil | − | − | 16 | 16 |
| Soya SPC | 13 | 18 | 13 | 18 |
| Pea Protein | 5 | 5 | 5 | 5 |
| Wheat Milling quality | 12 | 10 | 12 | 10 |
| Wheat Gluten | 15 | 11 | 15 | 11 |
| Fish meal | 39 | 40 | 39 | 40 |
| Lecithin Soy, Liquid | 0.50 | 0.50 | 0.50 | 0.50 |
| Additives and crystaline amino acids | 0.48 | 0.57 | 0.48 | 0.57 |
| Vitamins and minerals | 0.48 | 0.48 | 0.48 | 0.48 |
| Lucantin Pink | 0.03 | 0.03 | 0.03 | 0.03 |
| Mono-sodium Phosphate (MSP) | 1.49 | 1.59 | 1.49 | 1.59 |
| **Proximate Feed Composition** | | | | |
| Protein | 51 | 53.5 | 51 | 53 |
| Lipid | 20 | 18 | 19 | 18 |
| Dry matter | 95 | | 95 | |
| Ash | | | | |
| Histidine (mg/g) | 10.9 | 13.7 | 10.4 | 13.7 |

**Table 2.** Dietary lipid composition (mean ± SD) of the fish oil (FO) and vegetable oil (VO) experimental diets.

| | FO | | VO | |
|---|---|---|---|---|
| | **Mean** | **SD** | **Mean** | **SD** |
| **Lipid composition (mg / g ww)** | | | | |
| 06:0 | <0.01 | - | <0.01 | - |
| 08:0 | <0.01 | - | <0.01 | - |
| 10:0 | <0.01 | - | <0.01 | - |
| 12:0 | 0.21 | 0.02 | 0.19 | 0.02 |
| 14:0 | 11.3 | 1.2 | 2.2 | 0.1 |
| 14:1n-9 | 0.2 | 0.0 | <0.01 | - |
| 15:0 | 0.72 | 0.04 | 0.18 | 0.01 |
| 16:0 | 26 | 1 | 27 | 2 |
| 16:1n-9 | 0.5 | 0.1 | <0.01 | - |
| 16:1n-7 | 9.3 | 0.9 | 1.6 | 0.3 |
| 17:0 | 0.64 | 0.03 | 0.22 | 0.01 |
| 16:2n-4 | 1.30 | 0.24 | 0.35 | 0.01 |
| 18:0 | 4.0 | 0.5 | 4.8 | 0.3 |
| 16:3n-3 | 0.7 | 0.4 | <0.01 | - |
| 18:1n-11 | 0.5 | 0.2 | <0.01 | - |
| 18:1n-9 | 17 | 2 | 65 | 4 |
| 18:1n-7 | 3.8 | 0.7 | 3.7 | 0.5 |
| 16:4n-3 | 1.5 | 0.4 | <0.01 | - |
| 18:2n-6 | 5.9 | 0.4 | 25.3 | 1.8 |
| 18:3n-6 | 0.21 | 0.01 | <0.01 | - |
| 20:0 | 0.39 | 0.05 | 0.73 | 0.05 |
| 18:3n-3 | 2.1 | 0.1 | 19.3 | 1.7 |
| 20:1n-11 | 0.99 | 0.42 | 0.22 | 0.04 |
| 20:1n-9 | 9.4 | 3.7 | 2.6 | 0.1 |
| 20:1n-7 | 0.40 | 0.01 | <0.01 | - |
| 18:4n-3 | 4.17 | 0.59 | 0.61 | 0.03 |
| 20:2n-6 | 0.27 | 0.05 | <0.01 | - |
| 20:3n-9 | <0.01 | - | <0.01 | - |
| 20:3n-6 | <0.01 | - | <0.01 | - |
| 22:0 | 0.20 | 0.01 | 0.30 | 0.02 |

| | | | | |
|---|---|---|---|---|
| 20:3n-3 | <0.01 | - | <0.01 | - |
| 20:4n-6 (ARA) | 1.18 | 0.13 | 0.26 | 0.02 |
| 22:1n-11 | 16 | 8 | 2 | 1 |
| 22:1n-9 | 1.0 | 0.1 | 0.4 | 0.1 |
| 20:4n-3 | 1.0 | 0.1 | 0.2 | 0.0 |
| 20:5n-3 (EPA) | 17 | 2 | 3 | 1 |
| 24:0 | <0.01 | - | <0.01 | - |
| 22:4n-6 | <0.01 | - | <0.01 | - |
| 21:5n-3 | 0.91 | 0.03 | <0.01 | - |
| 24:1n-9 | 0.95 | 0.17 | 0.31 | 0.04 |
| 22:5n-6 | 0.36 | 0.02 | <0.01 | - |
| 22:5n-3 (DPA) | 2.1 | 0.2 | 0.3 | 0.1 |
| 22:6n-3 (DHA) | 16.1 | 0.5 | 3.7 | 0.3 |
| 24:5n-3 | 0.4 | 0.2 | <0.01 | - |
| 24:6n-3 | 0.2 | 0.0 | <0.01 | - |
| ∑unidentified | 8 | 2 | 1 | 0 |
| ∑identified | 159 | 10 | 166 | 9 |
| ∑fatty acids | 167 | 7 | 167 | 9 |
| ∑saturated | 43 | 1 | 36 | 2 |
| ∑16:1 | 9.8 | 0.9 | 1.6 | 0.3 |
| ∑18:1 | 22 | 2 | 69 | 4 |
| ∑20:1 | 10.8 | 4.1 | 2.7 | 0.2 |
| ∑22:1 | 17.5 | 7.6 | 2.8 | 0.5 |
| ∑mono-unsaturated | 61 | 9 | 76 | 4 |
| ∑EPA + DHA | 33 | 2 | 6 | 1 |
| ∑n-3 | 46 | 1 | 27 | 1 |
| ∑n-6 | 8 | 0 | 26 | 2 |
| ∑polyunsaturated | 55 | 1 | 52 | 3 |
| n-3/n-6 | 5.8 | 0.3 | 1.0 | 0.1 |
| n-6/n-3 | 0.2 | 0.0 | 1.0 | 0.1 |

## 2. Sampling

There were three sampling points: one at the end of the FW period and two after SW transfer, particularly after two and four weeks (SWT2 and SWT4, respectively) (Figure 6). In all samplings, six fish per tank were sampled, which means 18 fish per dietary treatment (n=18) at FW, and 9 fish per dietary treatment (n=9) at SW, since after the SW transfer there were two fish dietary groups mixed in each tank.

Fish were anaesthetized (100 mg Finquel $L^{-1}$), their weight and length recorded, and blood samples were taken using heparinized syringes. Right after blood collection, blood smears were prepared and air-dried. The remaining blood was centrifuged (13200 rpm, 2 minutes) and the plasma immediately collected, frozen in dry ice and stored at −80 ºC for posterior analyses. Single organ samples of liver were taken, flash-frozen in liquid nitrogen and stored at −80 ºC. All the samples were transferred to dry ice and sent to the Interdisciplinary Centre of Marine and Environmental Research (Porto, Portugal) for further analyses.

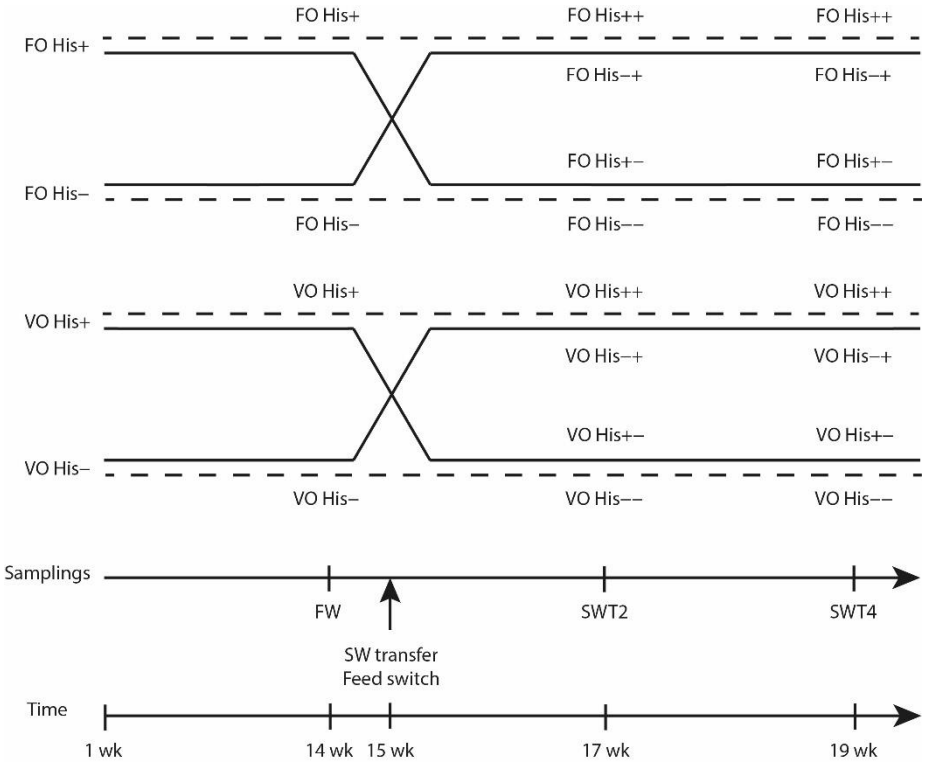


**Figure 6.** Design of the dietary His treatment cross-over. Fish were sampled at the end of the FW phase (FW) and two (SWT2) and four (SWT4) weeks after SW transfer.

## 3. Analytical procedures

### *3.1. Peripheral leucocyte count*

In order to count and categorize leucocytes as lymphocytes, monocytes, neutrophils and thrombocytes, air-dried blood smears were fixed with formol-ethanol (10 % of 37 % formaldehyde in absolute ethanol) for 1 minute and stained with Wright's stain (Haemacolor, Merck). The identification of neutrophils was achieved through the detection of neutrophils' peroxidase activity, according to the technique described by Afonso *et al.* (1998). Two hundred leucocytes per slide were counted under oil immersion (1000×). The relative percentage was calculated for each leucocyte type.

### *3.2. Analysis of plasma immune parameters*

#### *3.2.1. Alternative complement pathway (ACP) activity*

The ACP activity was evaluated according to the method described by Sunyer and Tort (1995), after validation and adjustment to Atlantic salmon. Three buffers were used: Isotonic veronal buffered saline (GVB), pH = 7.3, containing 0.1 % gelatine; EDTA-GVB, similar to GVB but with the addition of 20 mM EDTA; and Mg-EGTA-GVB, also as GVB but containing 10 mM $Mg^{+2}$ and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda, Portugal) were washed in GVB and resuspended in GVB to a concentration of $2.5 \times 10^8$ cells $\mu L^{-1}$. Then, 10 µL of this suspension were added to 40 µL of successively diluted plasma in Mg-EGTA-GVB buffer. Samples were incubated at room temperature (RT) for 100 minutes, continuously being shaken. The reaction was stopped by adding 150 µL of cold EDTA-GVB. After centrifuged (2.5 min at 120 × g), the supernatant's optical density was measured at 414 nm in a Synergy HT microplate reader (Biotek) to evaluate the extent of hemolysis. The positive control consisted of distilled water and the negative control of Mg-EGTA-GVB buffer. The ACH50 units were defined as the concentration of plasma leading to 50 % hemolysis of RaRBC. All procedures were carried out in triplicates.

#### *3.2.2. Lysozyme activity*

Lysozyme activity was determined following the procedure described by Costas *et al.* (2011). Plasma samples were pipetted to a flat-bottomed 96-well plate and mixed with 250 µL of a solution of *Micrococcus lysodeikticus* (0.5 mg $mL^{-1}$ 0.05 M sodium phosphate buffer, pH = 6.2) previously prepared, making a final volume of 265 µL. The reaction was performed at RT and the absorbance read at 450 nm over 20 min at 5 min intervals using

a Synergy HT microplate reader, Biotek. In order to quantify the amount of lysozyme in the samples, a standard curve was prepared through serial dilutions of lyophilized hen egg white lysozyme (Sigma) in the above buffer. The analysis was performed in duplicates. The results were expressed as μg $mg^{-1}$ protein.

### *3.2.3. Antiprotease activity*

Antiprotease activity was determined by plasma's ability to inhibit trypsin activity, as described by Machado *et al.* (2015). In Eppendorf tubes, 10 μL of plasma were mixed with an equal volume of trypsin solution (5 mg $mL^{-1}$ in $NaHCO_3$, 5 mg $mL^{-1}$, pH = 8.3) and incubated for 10 min at RT. Then, 100 μL of phosphate buffer ($NaH_2PO_4$, 13.9 mg $mL^{-1}$, pH = 7.0) and 125 μL of azocasein (20 mg $mL^{-1}$ in $NaHCO_3$, 5 mg $ml^{-1}$, pH = 8.3) were added and tubes incubated for 1 h at RT. After the second incubation period, 250 μL of trichloroacetic acid (TCA, 100 mg $mL^{-1}$) were pipetted and tubes incubated for another 30 min at RT. The solution was then centrifuged (10,000 × g, 5 min, RT) and the resulting supernatant transferred to flat-bottomed 96-well plates containing 100 μL of NaOH (40 mg $mL^{-1}$) per well. The OD was read at 450 nm in a Synergy HT microplate reader, Biotek. The negative control consisted of PBS and trypsin instead of plasma and the positive control of PBS. The analysis was carried out in triplicates. The plasma's antiprotease activity was expressed as % trypsin inhibition.

$$\%\ \boldsymbol{non\ inhibited\ trypsin} = \frac{\boldsymbol{sample\ Abs.}\ \times \mathbf{100}}{\boldsymbol{Abs.\ of\ the\ reference\ sample}}$$

$$\%\ \boldsymbol{inhibited\ trypsin} = \mathbf{100} - \%\ \boldsymbol{non\ inhibited\ trypsin}$$

### *3.2.4. Bactericidal activity*

Plasma's capacity to fight bacteria was measured according to Graham *et al.* (1988) with modifications (Machado *et al.*, 2015). Thus, *Tenacibaculum maritimum* were cultured for 48 h at 25 ºC in marine agar (Laboratorios CONDA, Spain). Then, exponentially growing bacteria were resuspended in marine broth (Laboratorios CONDA, Spain) and adjusted to $1\times10^8$ colony forming units (cfu) $mL^{-1}$.

In duplicates, 20 μL of plasma were incubated with the same volume of *T. maritimum* ($1\times10^8$ cfu $mL^{-1}$) for 2.5 h at 25 ºC with continuous shaking (100 rpm). After that, 25 μL of MTT (3-(4,5 dimethyl-2-yl)-2, 5-diphenyl tetrazolium bromide) were added to each well and the round-bottom 96-well plate incubated for another 10 min at the same conditions. After

the second incubation period, plates were centrifuged at 2,000 × *g* for 10 min. The supernatant was discarded and the precipitate dissolved in 200 µL of dimethyl sulfoxide (DMSO), of which 100 µL were further transferred to a flat-bottom 96-well plate. The absorbance was read at 560 nm. The negative control consisted of marine broth instead of plasma and bacteria and the positive control consisted of marine broth instead of plasma. The bactericidal activity was expressed as a percentage (%).

$$\%\ \boldsymbol{viable\ bacteria} = \frac{\boldsymbol{sample\ Abs.} \times \mathbf{100}}{\boldsymbol{Abs.of\ the\ reference\ sample}}$$

$$\%\ \boldsymbol{no\ viable\ bacteria}\ (\boldsymbol{bactericidal\ activity}) = \mathbf{100} - \%\ \boldsymbol{viable\ bacteria}$$

### *3.2.5. Total immunoglobulin M (IgM)*

To determine total plasma IgM levels, an enzyme-linked immunosorbent assay (ELISA) was performed. Briefly, 100 µL of diluted plasma (1:200 in 50 mM $Na_2CO_3$, pH = 9.6) were placed in flat-bottomed 96-well plates and incubated for 1 h at RT. Plate wells coated with plasma proteins were filled with 300 µL of blocking buffer consisting of 5 % low-fat milk in T-TBS (Tris Buffered Saline and 0.1 % Tween 20, pH = 7.6). After 1 h at RT, the plates were rinsed three times with T-TBS and incubated with 100 µL of a commercial mouse anti-Atlantic salmon IgM monoclonal antibody (1:500 in blocking buffer; Aquatic Diagnostics Ltd.). Plates were washed three more times with T-TBS and 100 µL of secondary antibody anti-mouse IgG-HRP (1:1000 in blocking buffer; Sigma) were added to each well. Following the incubation time (1 h at RT) and washing, 100 µL TMB (3,3',5,5'-tetramethylbenzidine) substrate solution for ELISA were added to each well. The reaction was stopped after 5 minutes by the addition of 100 µL $H_2SO_4$ (2M). The OD were read at 450 nm in a Synergy HT microplate reader, Biotek. The negative control consisted of $Na_2CO_3$ instead of plasma. The analyses were carried out in triplicates.

## *3.3. Liver antioxidant capacity*

### *3.3.1. Liver homogenization*

For assessing the liver's antioxidant capacity, samples were homogenized in K-phosphate buffer (0.1 M, pH = 7.4) at a ratio of 1:10 w/v. After sonicating, an aliquot for LPO analysis was taken, mixed with 4 % BHT (2,6-Di-tert-butyl-4-methylphenol) in methanol and immediately frozen at −80 ºC. The remaining sample volume was centrifuged (10,000 × *g*,

at 4 ºC, for 20 min) and the resultant supernatants were divided into aliquots and stored at −80 ºC.

### *3.3.2. Protein quantification*

To determine the maximal activity for each enzyme of interest, samples' protein concentration was measured with a colourimetric kit for detection and quantification of total protein (Thermo Scientific™ Peirce™ BCA Protein Assay Kit). Briefly, a standard curve was obtained through a series of dilutions of bovine serum albumin (BSA) according to the manufacture instructions. Both standards and diluted samples (1:50 in K-phosphate buffer, 0.1 M, pH = 7.4) were pipetted into 96-well microplates. Two hundred µL of the working reagent were then added to each well, making a final volume of 225 µL. After an incubation of 30 min at 37 ºC, the absorbance was measure at 562 nm on a Synergy HT microplate reader, Biotek. The analyses were carried out in triplicates.

### *3.3.3. Superoxide dismutase activity*

SOD (EC 1.15.1.1) activity was assessed using a spectrophotometric assay that measures the capacity of SOD to compete with ferricytochrome C for $O_2^{\bullet -}$ radicals generated by the xanthine/xanthine oxidase system (Pérez-Jiménez *et al.*, 2009). Two solutions were prepared: the reaction mixture, containing 50 mM-potassium phosphate buffer (pH = 7.8), 0.1 mM-NaEDTA, 0.7 mM-xanthine and 0.03 mM-cytochrome c; and the xanthine oxidase solution (0.03 U $mL^{-1}$). After 50 µL of each diluted sample (0.3 mg $mL^{-1}$) being plated in the 96-well microplates, 200 µL of the reaction mixture, followed by 50 µL xanthine oxidase solution were quickly pipetted. The absorbance was read over 3 minutes (one read every 20 seconds), at 550 nm (McCord & Fridovich, 1969), using a Synergy HT microplate reader (Biotek). In order to quantify the activity of SOD, a standard curve was created through serial dilutions of a stock solution of SOD (6000 U mL $protein^{-1}$) in 50 mM-potassium phosphate buffer (pH = 7.8). The negative control consisted of the above buffer. The analysis was carried out in triplicates. Results are presented in units of SOD $mg^{-1}$ of protein, where one unit of activity is defined as the amount of enzyme necessary to produce 50 % inhibition of the ferricytochrome C reduction rate.

### *3.3.4. Catalase activity*

The liver CAT (EC 1.11.1.6) activity was assessed by measuring the decrease of hydrogen peroxidase concentration (Aebi, 1984). Based on the results of protein quantification analysis, samples were diluted in K-phosphate buffer (0.1 M, pH = 7.4) to reach the required value of 0.7 mg $mL^{-1}$. In triplicates, 10 µL of the diluted sample and 140 µL of K-phosphate buffer (0.05 M, pH = 7.0) were added to a 96-well UV microplate. Quickly, 150 µL of a reaction buffer consisting of a hydrogen peroxide solution (30 % $H_2O_2$ in K-phosphate buffer, 0.05 M, pH = 7.0) were pipetted to each well and the absorbance immediately read at 240 nm for 1 min (one read every 15 sec. interval) in a Synergy HT microplate reader (Biotek). The negative control consisted of K-phosphate buffer (0.1 M, pH = 7.4) instead of sample. The activity of CAT was expressed as units of enzymatic activity per mg of protein. One activity unit was defined as the amount of enzyme needed to transform 1 µmol of substrate per min under the assay conditions.

### *3.3.5. Glutathione S-transferase (GST) activity*

Hepatic GST (EC 2.5.1.18) activity was determined through a colourimetric assay based on the GST-catalyzed reaction between reduced glutathione and the substrate for GST, CDNB (1-chloro-2,4-dinitrobenzene) (Habig *et al.*, 1974). For that, two solutions were prepared just before the assay: one consisting of L-glutathione reduced in K-phosphate buffer (0.2 M, pH = 6.5) and the other consisting of CDNB in ethanol. In triplicates, 50 µL of the diluted samples (0.7 mg $mL^{-1}$) were placed in 96-well microplates and 250 µL of the reaction solution (glutathione reduced solution 10 mM, CDNB solution 60Mm and phosphate buffer 0.2 M, pH = 6.5) were quickly pipetted to each well. The absorbance was immediately read at 340 nm, every 20 seconds for 5 minutes, in a Synergy HT microplate reader, Biotek. The negative control consisted of homogenisation buffer (0.1 M K-phosphate buffer, pH = 7.4) instead of sample. Results are presented as milli-units of enzymatic activity per mg of protein, where one unit of activity was defined as the amount of GST needed to transform 1 nmol of CDNB to reduced glutathione per min, under the assay conditions.

### *3.3.6. Total glutathione*

GSH was determined following a spectrophotometric assay method that measures the formation of a yellow derivate 5'-thiol-2-nitrobenzoic acid at 412 nm as a consequence of the oxidation of glutathione by the 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) (Rahman *et*

*al.*, 2006). After 50 µL of the diluted samples (0.7 mg $mL^{-1}$) being pipetted to 96-well microplates, 250 µL of a reaction mixture consisting of 0.2 M Na-K-phosphate buffer (pH = 8), NADPH solution, DTNB solution and glutathione reductase solution (214 U mg $protein^{-1}$) were added to each well. The reaction was read over 3 minutes (one read every 20 seconds), using a Synergy HT microplate reader (Biotek). To quantify the level of GSH in samples, a standard curve prepared through serial dilutions of a stock solution of L-glutathione reduced was used. The negative control consisted of 0.1 M K-phosphate buffer (pH = 7.4). The results are expressed as nmol $mg^{-1}$ of tissue.

### *3.3.7. Lipid peroxidation*

The concentration of malondialdehyde (MDA), a compound produced during LPO and which reacts with thiobarbituric acid (TBA) forming a pink compound, was used as a marker of LPO level in the liver (Pérez-Jiménez *et al.*, 2009). The aliquot previously made and destined for this assay was mixed with 100 µL of cold trichloroacetic acid (TCA) solution. Then, 1 mL of a solution containing 2-thiobarbituric acid (TBA), trizma hydrochloride (Tris-HCl) and diethylenetriaminepentaacetic acid (DTPA) was added. After an incubation of 1 hour at 100 ºC, the mixture was centrifuged (11,500 rpm, at RT, for 5 min) and 200 µL of the resultant supernatant transferred to a microplate. The absorbance was read on a Synergy HT microplate reader (Biotek), at 535 nm. Ultrapure water instead of homogenate was used as negative control. The analyses were performed in triplicates. Results were expressed as nanomole MDA per gram of wet tissue (nmol $g^{-1}$), calculated from a calibration curve.

## 4. Statistics

Statistical analyses were performed using the IBM SPSS Statistics version 26.0 for Windows. Data were tested for homogeneity of variances through Levene's test and the normality evaluated through the residual plots. Whenever necessary, outliers were removed and data logarithmic or arcsine transformed. Two different sets of data were statistically analysed. With data from fish that maintained the dietary His treatment from the start to the end of the trial, two different analyses were performed: a multifactorial ANOVA to investigate the effects of time, dietary histidine and lipid source; and a two-way ANOVA for each time, with dietary histidine and lipid source as variables. With the data from fish that maintained and those that switched dietary His treatment after being transferred to SW, a multifactorial ANOVA was performed with the lipid source, dietary His treatment at FW and dietary His

treatment at SW, as explanatory variables. When significant interactions between variables were present, a one-way ANOVA was performed to investigate the individual effects of the involved factors. Whenever the independent variable “time” was significant, a Tukey’s Honestly Significant Difference post hoc test was performed to identify significantly different groups. For all the tests, 95 % CI was used, giving a probability level of 0.05. Data are presented as means with their standard deviation (mean ± SD).

## Results

### 1. Fish growth

Data on fish weight during the SW phase are presented in Table 3. Although no significant differences were found among dietary treatments, fish fed the FO diet containing His+ throughout the experiment tended to weigh more at the final of the feeding trial, whereas those fed the FO diet supplied with His+ only during the SW phase or with no His+ supplementation throughout the entire trial tended to weigh less.

**Table 3.** Weight of Atlantic salmon fed FO or VO diets, supplemented with His+ throughout the entire trial (His++), during the FW phase (His+−), during the SW phase (His−+) or with no His+ supplementation (His−−), at SWT2 and SWT4.

| | | SWT2 | SWT4 |
|---|---|---|---|
| FO | His++ | 146,67 ± 28,18 | 186,44 ± 42,50 |
| | His+− | 142,67 ± 37,08 | 182,56 ± 43,42 |
| | His−+ | 134,89 ± 19,03 | 162,78 ± 28,59 |
| | His−− | 150,44 ± 35,90 | 162,78 ± 30,17 |
| VO | His++ | 158,67 ± 35,95 | 178,89 ± 25,48 |
| | His+− | 148,00 ± 54,10 | 185,89 ± 39,95 |
| | His−+ | 137,78 ± 38,36 | 176,44 ± 60,63 |
| | His−− | 144,22 ± 36,67 | 166,11 ± 27,65 |

Values are presented as means ± SD.

### 2. Peripheral leucocyte populations

The relative proportion of circulating leucocytes of fish fed dietary treatments over the feeding trial is presented in Table 4. The percentage of thrombocytes, lymphocytes and monocytes significantly changed among sampling times. Peripheral thrombocytes percentage decreased at two weeks after smolts were transferred to SW (SWT2), whereas the relative proportion of lymphocytes and monocytes increased at that time compared to the values found at FW specimens. Although non-significant, the percentage of neutrophils tended to increase at SWT2, regardless of dietary treatments. Four weeks after the transfer to SW (SWT4), the percentage of thrombocytes increased compared to the values found at SWT2, while lymphocytes numbers dropped. At that time, monocytes percentage returned to values similar to those observed at FW.

The relative proportion of circulating lymphocytes remained unchanged among Atlantic salmon fed dietary treatments, while the percentage of thrombocytes was increased in fish fed the VO His+ diet compared to fish fed the FO His+ diet (Table 4). However, within each time, the differences in thrombocytes among dietary treatments were non-significant (Figure 7). At SWT4, monocytes were increased in fish fed the FO His+ compared to those fed the FO His− diet (Figure 7).

An interactive effect of time, lipid source and dietary His concentration was observed in the percentage of peripheral neutrophils (Table 4). At FW, fish fed the VO His+ diet presented an increased proportion of neutrophils relative to fish fed the VO His− diet. At SWT4, the ones fed the FO His+ feed had a higher percentage of circulating neutrophils compared to those fed the FO His− feed, which, in turn, presented a lower percentage of this type of phagocyte compared to fish fed the VO His− diet (Figure 7). Moreover, in fish fed the VO His− diet the percentage of circulating neutrophils varied significantly along time, being lower at FW than at SWT2 and SWT4 (Table 4).

The differential WBC counts performed in both fish that received the same dietary His treatment after SW transfer and those that switched dietary His treatment after it is presented in Figure 8. At SWT2, the percentages of circulating thrombocytes, lymphocytes and neutrophils were affected by the dietary lipid source. Both thrombocytes and neutrophils percentages were higher in VO diet-fed fish than in FO diet-fed fish, whereas lymphocytes proportion was increased in the latter compared to the former. At SWT4, fish fed the FO His+ diet during the FW phase presented decreased thrombocytes percentage compared to those fed the FO His− diets during the FW phase and to the ones fed VO His+ diets during the FW stage. At the same time, monocytes percentage was higher in those fed the FO His+ diet during the SW than in the ones fed the FO His− diet during the same phase.

### 3. Immune parameters

Except for ACP activity, all the other immune parameters analyzed in fish fed His− or His+ throughout the entire trial were significantly different between FW and SWT2 (Table 5). Compared to the values observed at FW, at SWT2, both lysozyme activity and IgM levels increased, while antiprotease and bactericidal activities decreased.

At SWT4, both lysozyme and antiprotease activities returned to values similar to those found at FW, whereas the bactericidal activity remained to decrease over time. Variations in IgM levels, at that time, depended on the dietary His concentration. While fish fed on the His+ diet showed IgM values similar to those found at SWT2, fish fed the His− feed

presented decreased IgM levels at that time compared to the previous one (Table 5). Moreover, at SWT4, ACH50 values more than double compared to the values observed at the two previous times (Table 5) and, on that time, an effect of the dietary lipid source on plasma ACP activity was also detected, with FO diets-fed fish presenting increased ACH50 values than those fed VO diets (Figure 9).

Furthermore, regarding the effects of dietary treatments on humoral immune parameters, lysozyme activity was higher in fish fed the FO His− diet than in those fed the VO His− diet (Table 5). However, within each time, this effect was not observed (Figure 9). At SWT2, fish fed the VO His+ diet presented increased lysozyme activity compared to fish fed the VO His− diet (Figure 9). The analysis of plasma bactericidal activity over time revealed that fish fed FO diets had higher activity than fish fed VO diets, and that fish fed His+ feeds had greater activity relative to those fed His− diets (Table 5). However, within different sampling time, the bactericidal activity was affected either by the dietary His content or the lipid source (Figure 9). At FW, fish fed His+ diets displayed greater bactericidal capacity than fish fed His− diets, whereas at SWT2 fish fed FO diets presented higher bactericidal activities than those fed VO diets.

The immune parameters analysed in fish that maintained and those that changed dietary His treatment after being transferred to SW are displayed in Figure 10. At SWT2, fish fed the FO His− diet during the SW phase had increased ACP activity compared to the ones fed the same lipid source and His+ during the SW stage. At the same time, fish fed the FO His+ diet throughout the entire feeding trial presented enhanced bactericidal activity compared to the FO His−+ dietary group and the VO His++ group. At SWT4, the ACP activity was lower in the FO His−+ group compared to FO His++, to FO His−− and VO His−+ groups. The same dietary group (FO His−+) had decreased lysozyme activity than those fed the FO His+ diet throughout the trial and the ones from the VO His−+ group. Moreover, regardless of the lipid source, fish fed His− during FW and His+ after SW transfer (His−+ group) had decreased antiprotease activity compared to fish fed His+ or His− throughout the entire feeding trial.

### 4. Antioxidant enzymes, GSH and LPO

SOD and GST activities, as well as GSH levels, of fish that maintained the dietary treatment throughout the feeding trial significantly varied between FW and SWT2 (Table 6). At SWT2, fish presented lower SOD and GST activities, and remarkably decreased levels of GSH. At SWT4, while SOD activity remained to decrease, the liver GST activity increased compared to the values observed at the two previous times and GSH values slightly, but significantly,

increased relative to values observed at SWT2. LPO levels decreased between FW and SWT4.

An interactive effect of time, lipid source and His content of diets was found on CAT activity. In fish fed the FO His+ diet, CAT activity was increased at SWT4 compared to the values observed at both FW and SWT2. In those fed the FO His− diet, liver CAT activity was greater at FW than at SWT2, and in the ones fed the VO His+ diet this enzyme activity was higher at FW than at the two sampling points after SW transfer (Table 6). Moreover, at FW, the CAT activity was increased in fish fed the VO His+ diet compared to fish fed the FO His+ feed and to those fed the VO His− diet. At that time, fish fed the FO His+ diet presented lower CAT activity compared to the ones fed the FO His− diet (Figure 11). At SWT2, fish fed the VO His− diet had increased CAT activity compared to those fed the VO His+ diet and those fed the FO His− diet higher activity than fish fed the FO His+ diet (Table 6). However, the outcomes of the two-way ANOVA analysis at SWT2 showed no significant effects of the dietary treatments on CAT activity at that time (Figure 11). At SWT4, the liver CAT activity was higher in fish fed the FO His+ diet than in those fed the FO His− diet.

While through the outcomes of multifactorial ANOVA, SOD activity was affected by the dietary lipid source, with FO diets-fed fish presenting increased SOD activity compared to VO diets-fed fish (Table 6), within each time, this effect of the lipid source on the enzyme activity was not detected (Figure 11). Similarly, when considering the three sampling times, an effect of the dietary His content on the LPO levels can be observed, with fish fed His+ diets presenting increased levels of LPO than fish fed His− diets (Table 6). However, within each time, significant differences were only observed at SWT4, with higher LPO levels in fish fed His+ diets than in those fed His- diets (Figure 11).

The outcomes concerning the liver antioxidant capacity of fish that maintained and fish that switched dietary His treatment after SW transfer are presented in Figure 12. At SWT2, the CAT activity was higher in fish fed the VO His− diet during the SW phase compared to those fed the VO His+ diet during the SW stage and to the ones fed the FO His− diet during that same phase. At SWT4, fish fed FO diets had higher LPO levels than those fed VO diets.

**Table 4.** Percentage of peripheral thrombocytes, lymphocytes, monocytes and neutrophils of Atlantic salmon sampled at FW, SWT2 and SWT4, fed four different dietary treatments (FO His+, FO His−, VO His+, VO His−).

| WBC % | Experimental treatments | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FW | | | | SWT2 | | | | SWT4 | | | |
| | FO | | VO | | FO | | VO | | FO | | VO | |
| | His+ | His− | His+ | His− | His+ | His− | His+ | His− | His+ | His− | His+ | His− |
| Thrombocytes | 53.97 ± 10.39 | 57.21 ± 8.86 | 58.09 ± 6.43 | 56.39 ± 10.71 | 31.63 ± 7.11 | 33.99 ± 7.43 | 37.59 ± 6.43 | 32.25 ± 5.12 | 36.50 ± 7.96 | 47.69 ± 11.06 | 44.97 ± 6.09 | 46.03 ± 13.94 |
| Lymphocytes | 33.04 ± 9.41 | 31.71 ± 8.53 | 28.15 ± 6.56 | 32.79 ± 8.72 | 50.75 ± 7.76 | 52.05 ± 6.49 | 47.09 ± 7.58 | 52.67 ± 7.18 | 49.83 ± 7.15 | 46.94 ± 12.20 | 43.97 ± 6.46 | 41.14 ± 12.27 |
| Monocytes | 8.31 ± 3.77 | 7.36 ± 3.16 | 7.75 ± 1.65 | 7.03 ± 3.45 | 11.58 ± 4.26 | 10.65 ± 3.16 | 10.17 ± 3.69 | 9.96 ± 3.89 | 8.56 ± 2.62 | 5.56 ± 2.34 | 6.64 ± 2.37 | 7.83 ± 3.70 |
| Neutrophils | 3.68 ± 1.92 | 3.73 ± 2.44 | 4.96 ± 1.86$^{x}$ | 2.74 ± 1.69$^{by}$ | 4.24 ± 1.68 | 4.60 ± 2.12 | 5.15 ± 2.06 | 5.12 ± 1.86$^{a}$ | 5.11 ± 2.62$^{x}$ | 2.44 ± 0.98$^{By}$ | 4.42 ± 2.02 | 5.00 ± 2.08$^{aA}$ |

| WBC % | Multifactorial ANOVA | | | | | | | Time | | | Lipid × Histidine | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | FO | | VO | |
| | Time | Lipid | Histidine | Lipid × Histidine | Time × Lipid | Time × Histidine | Time × Lipid × Histidine | FW | SWT2 | SWT4 | His+ | His− | His+ | His− |
| Thrombocytes | < 0.001 | ns | ns | 0.024 | ns | ns | ns | a | c | b | B | - | A | - |
| Lymphocytes | < 0.001 | ns | ns | ns | ns | ns | ns | c | a | b | - | - | - | - |
| Monocytes | < 0.001 | ns | ns | ns | ns | ns | ns | b | a | b | - | - | - | - |
| Neutrophils | 0.043 | ns | 0.026 | ns | ns | ns | 0.007 | - | - | - | - | - | - | - |

Values are presented as means ± SD. Multifactorial ANOVA: ns: non-significant differences ($p > 0.05$); if interaction was significant, one-way ANOVA was performed; Tukey post hoc test was used to identify differences among sampling times. Different lowercase letters denote significant differences among sampling times. Significant differences between lipid sources are indicated by capital letters, while “x” and “y” stand for significant differences between dietary histidine treatments.

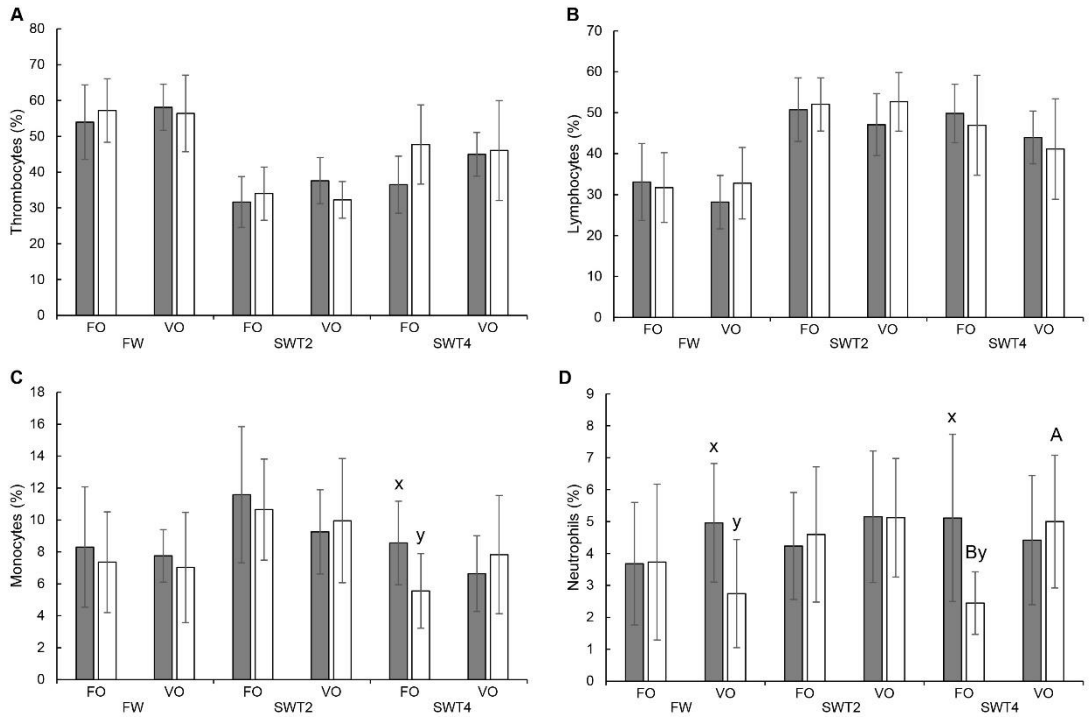


**Figure 7.** Percentage of peripheral thrombocytes (**A**), lymphocytes (**B**), monocytes (**C**) and neutrophils (**D**) of Atlantic salmon fed four dietary treatments differing on lipid source, FO or VO, and His content, high (■) or low (□), at FW, SWT2 and SWT4. Values are presented as means ± SD. P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, one-way ANOVA was performed. Within a time, significant differences between lipid sources are indicated by capital letters, while "x" and "y" stand for significant differences between dietary histidine treatments.

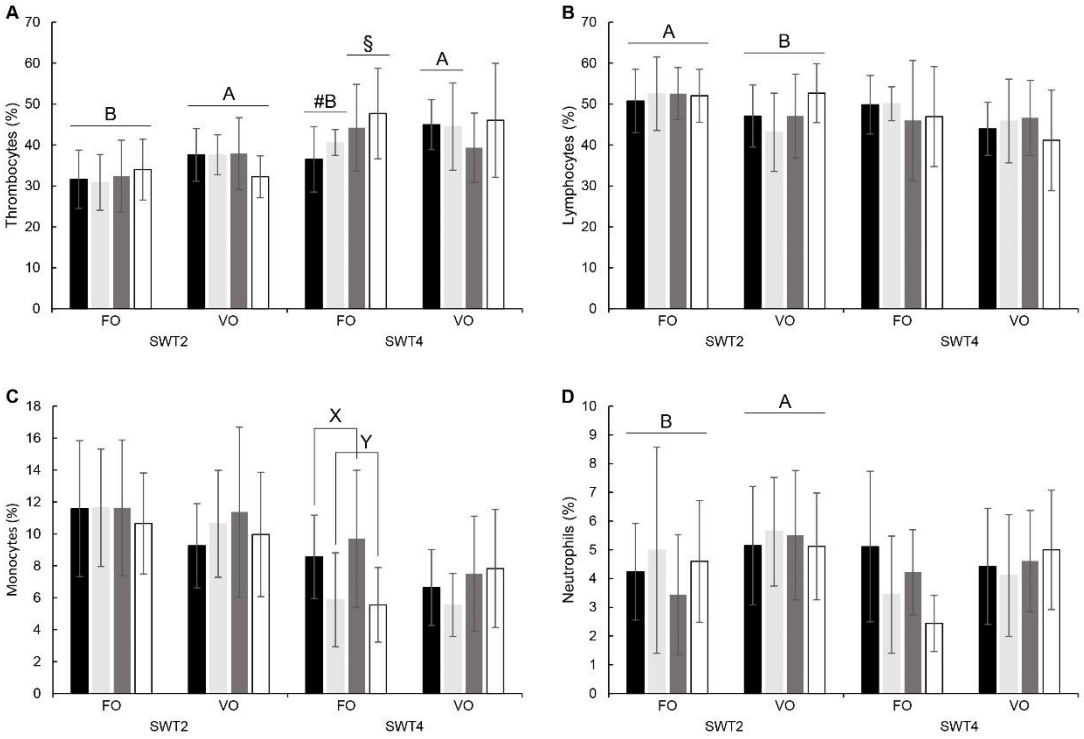


**Figure 8.** Percentage of peripheral thrombocytes (**A**), lymphocytes (**B**), monocytes (**C**) and neutrophils (**D**) of Atlantic salmon fed FO or VO-based diets, supplemented with His+ throughout the entire trial (His++, ■), during the FW phase (His+−, ■), during the SW phase (His−+, ■) or with no His+ supplementation (His−−, □), at SWT2 and SWT4. Values are presented as means ± SD. P-values from multifactorial ANOVA ($p \leq 0.05$). If interaction was significant, one-way ANOVA was performed. Within each time, significant differences between lipid sources are indicated by capital letters, while different symbols denote significant differences between dietary His treatments at FW, and "X" and "Y" stand for significant differences between dietary His treatments at SW.

**Table 5.** Plasma alternative complement pathway (ACH50), lysozyme, antiprotease activity, bactericidal activity and IgM levels of Atlantic salmon sampled at FW, SWT2 and SWT4, fed four different dietary treatments (FO His+, FO His−, VO His+, VO His−).

| Parameters | | Dietary treatments | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | FW | | | | SWT2 | | | | SWT4 | | | |
| | | FO | | VO | | FO | | VO | | FO | | VO | |
| | | His+ | His− | His+ | His− | His+ | His− | His+ | His− | His+ | His− | His+ | His− |
| ACH50 | (units $mL^{-1}$) | 9.84 ± 3.46 | 11.45 ± 3.26 | 12.19 ± 5.53 | 8.56 ± 3.67 | 9.97 ± 4.06 | 15.78 ± 10.01 | 16.32 ± 6.82 | 18.24 ± 12.51 | 52.37 ± 18.98 | 48.47 ± 19.30 | 36.79 ± 27.84 | 33.33 ± 15.81 |
| Lysozyme | (µg $mL^{-1}$) | 0.94 ± 1.21 | 1.50 ± 1.10 | 1.65 ± 1.24 | 0.82 ± 1.31 | 1.75 ± 1.28 | 2.15 ± 1.06 | 3.20 ± 1.16 | 1.49 ± 1.66 | 1.01 ±1.28 | 1.35 ± 1.77 | 0.40 ± 0.61 | 0.88 ± 0.96 |
| Antiprotease activity | (%) | 92.80 ± 2.68 | 93.40 ± 2.60 | 91.94 ± 3.70 | 93.54 ± 1.13 | 58.55 ± 5.59 | 65.34 ± 10.45 | 65.66 ± 13.59 | 63.03 ± 7.14 | 94.32 ± 5.79 | 94.90 ± 4.08 | 90.03 ± 7.15 | 94.58 ± 3.02 |
| Bactericidal activity | (%) | 70.57 ± 3.67 | 68.59 ± 3.91 | 69.25 ± 3.35 | 65.59 ± 3.98 | 63.64 ± 5.60 | 59.16 ± 6.71 | 55.90 ± 6.88 | 53.29 ± 6.73 | 52.43 ± 6.58 | 48.40 ± 5.77 | 46.62 ± 8.04 | 48.05 ± 6.11 |
| IgM | (OD 450 nm) | 0.33 ± 0.08 | 0.39 ± 0.11 | 0.38 ± 0.10 | 0.40 ± 0.08 | 0.93 ± 0.33 | 0.96 ± 0.26 | 0.76 ± 0.18 | 0.98 ± 0.21 | 0.77 ± 0.24 | 0.65 ± 0.30 | 0.87 ± 0.32 | 0.68 ± 0.27 |

| Parameters | Multifactorial ANOVA | | | | | | |
|---|---|---|---|---|---|---|---|
| | Time | Lipid | Histidine | Lipid × Histidine | Time × Lipid | Time × Histidine | Time × Lipid × Histidine |
| ACH50 | < 0.001 | ns | ns | ns | 0.019 | ns | ns |
| Lysozyme | < 0.001 | ns | ns | 0.011 | ns | ns | ns |
| Antiprotease activity | < 0.001 | ns | ns | ns | ns | ns | ns |
| Bactericidal activity | < 0.001 | 0.001 | 0.009 | ns | ns | ns | ns |
| IgM | < 0.001 | ns | ns | ns | ns | 0.014 | ns |

| Parameters | Time | | | Lipid × Histidine | | | | Time × Lipid | | | | | | Time × Histidine | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | FO | | VO | | FW | | SWT2 | | SWT4 | | FW | | SWT2 | | SWT4 | |
| | FW | SWT2 | SWT4 | His+ | His− | His+ | His− | FO | VO | FO | VO | FO | VO | His+ | His− | His+ | His− | His+ | His− |
| ACH50 | b | b | a | - | - | - | - | - | - | - | - | A | B | - | - | - | - | - | - |
| Lysozyme | b | a | b | - | A | - | B | - | - | - | - | - | - | - | - | - | - | - | - |
| Antiprotease activity | a | b | a | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Bactericidal activity | a | b | c | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| IgM | - | - | - | - | - | - | - | - | - | - | - | - | - | c | c | a | a | a | b |

Values are presented as means ± SD. Multifactorial ANOVA: ns: non-significant differences ($p > 0.05$); if interaction was significant, one-way ANOVA was performed; Tukey post hoc test was used to identify differences among sampling times. Different lowercase letters denote significant differences among sampling times. Significant differences between lipid sources are indicated by capital letters, while “x” and “y” stand for significant differences between dietary histidine treatments.

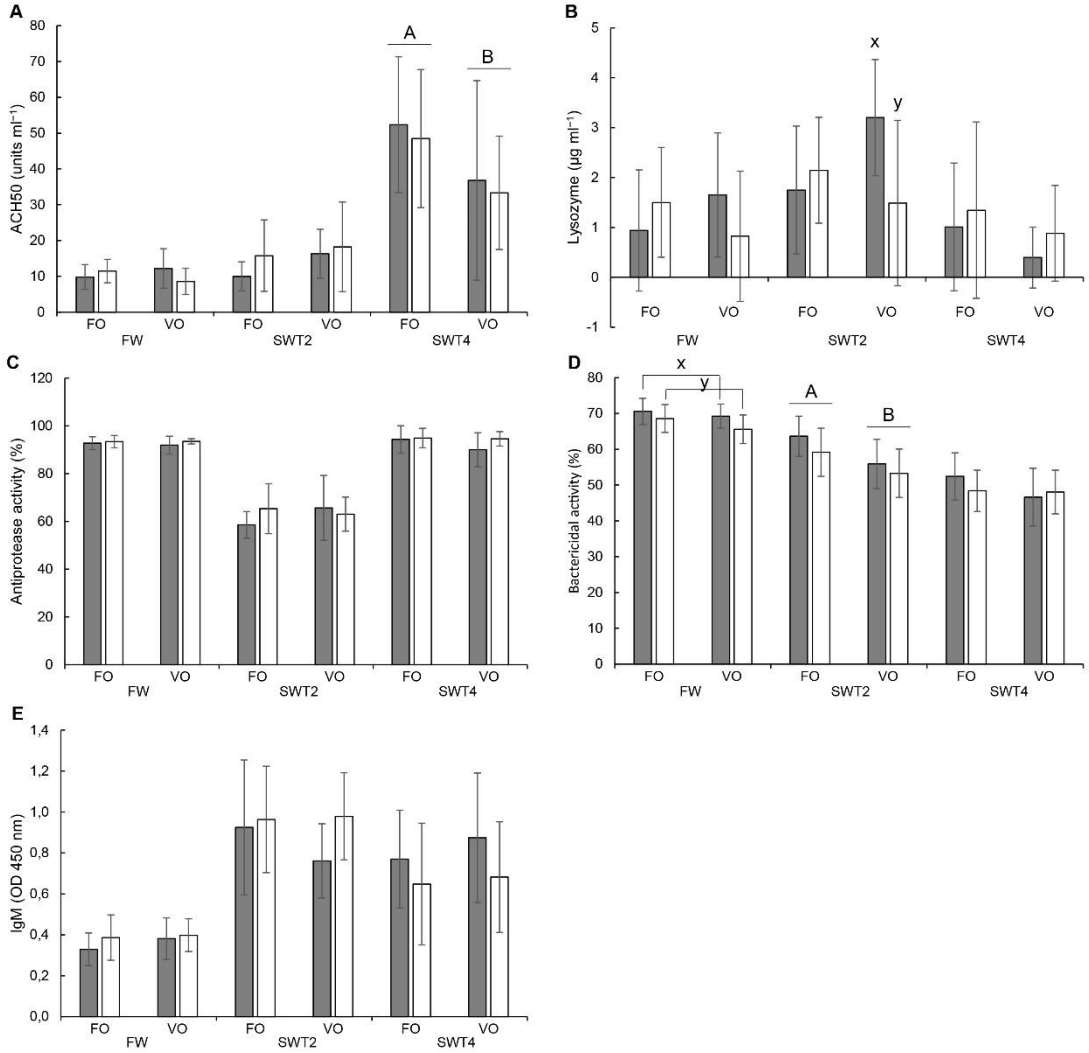


**Figure 9.** Plasma alternative complement pathway (**A**), lysozyme activity (**B**), antiprotease activity (**C**), bactericidal activity (**D**) and IgM levels (**E**) in Atlantic salmon fed four dietary treatments differing on lipid source, FO or VO, and His content, high (■) or low (□), at FW, SWT2 and SWT4. Values are presented as means ± SD. P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, one-way ANOVA was performed. Within each time, significant differences between lipid sources are indicated by capital letters, while "x" and "y" stand for significant differences between dietary histidine treatments.

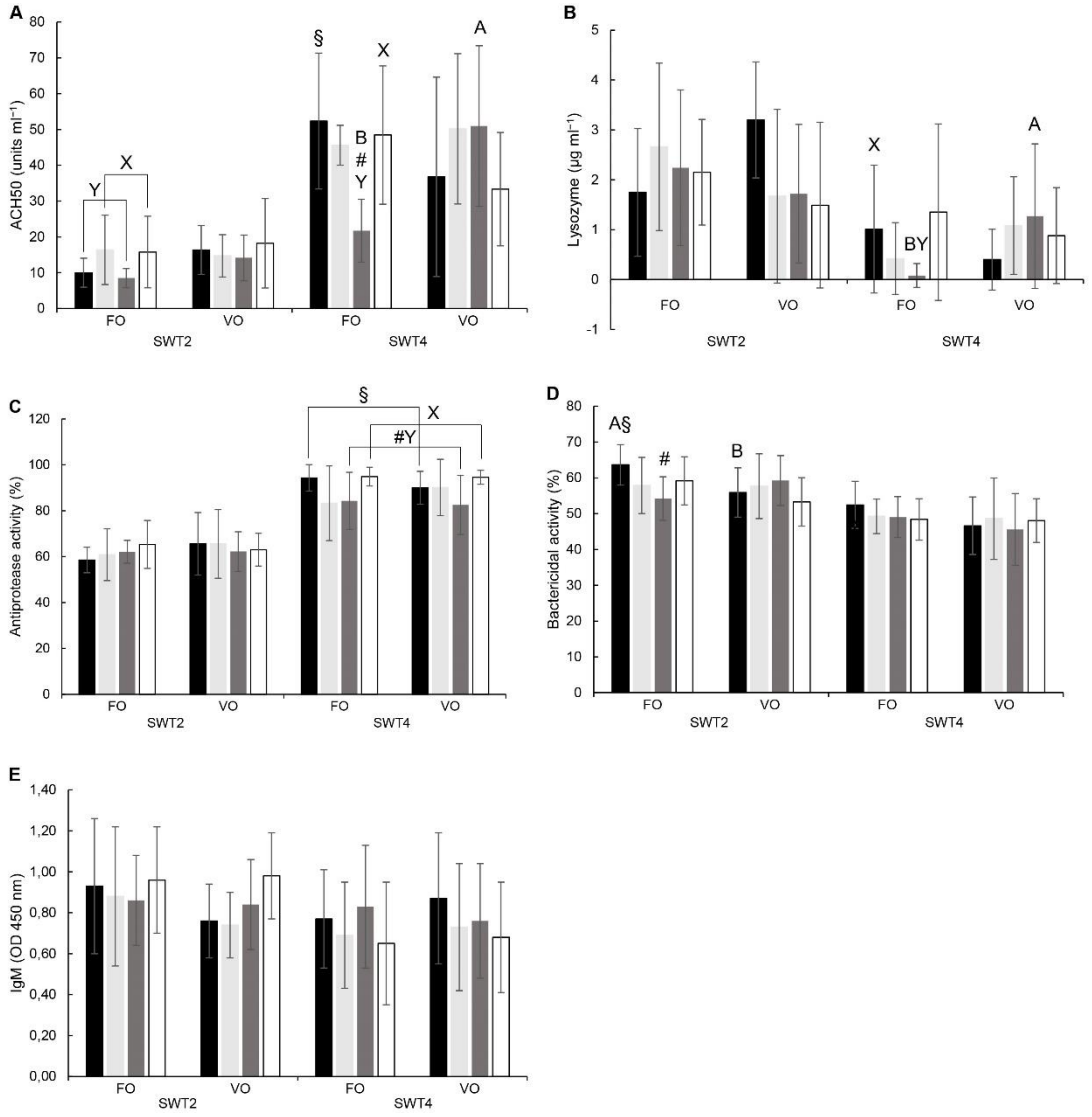


**Figure 10.** Plasma alternative complement pathway (**A**), lysozyme activity (**B**), antiprotease activity (**C**), bactericidal activity (**D**) and IgM levels (**E**) in Atlantic salmon fed FO or VO-based diets, supplemented with His+ throughout the entire trial (His++, ■), during the FW phase (His+−, ■), during the SW phase (His−+, ■) or with no His+ supplementation (His−−, □), at SWT2 and SWT4. Values are presented as means ± SD. P-values from multifactorial ANOVA ($p \leq 0.05$). If interaction was significant, one-way ANOVA was performed. Within each time, significant differences between lipid sources are indicated by capital letters, while different symbols denote significant differences between dietary His treatments at FW, and "X" and "Y" stand for significant differences between dietary His treatments at SW.

**Table 6.** Superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) activities, glutathione (GSH) and lipid peroxidation levels (LPO) in Atlantic salmon sampled at FW, SWT2 and SWT4, fed four different dietary treatments (FO His+, FO His−, VO His+, VO His−).

| Parameters | | Dietary treatments | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | FW | | | | SWT2 | | | | SWT4 | | | |
| | | FO | | VO | | FO | | VO | | FO | | VO | |
| | | His+ | His− | His+ | His− | His+ | His− | His+ | His− | His+ | His− | His+ | His− |
| SOD | (U mg protein$^{-1}$) | 45.3 ± 8.8 | 38.1 ± 12.9 | 38.0 ± 11.6 | 39.8 ± 12.1 | 34.6 ± 15.4 | 31.6 ± 7.7 | 29.6 ± 12.5 | 25.5 ± 9.4 | 21.3 ± 10.4 | 27.4 ± 10.0 | 20.0 ± 8.8 | 20.9 ± 10.5 |
| CAT | (U mg protein$^{-1}$) | 164.8 ± 22.6$^{bBy}$ | 186.0 ± 23.8$^{ax}$ | 198.7 ± 21.8$^{aAx}$ | 171.77 ± 22.81$^{y}$ | 162.1 ± 13.3$^{by}$ | 164.02 ± 21.2$^{bx}$ | 153.5 ± 17.0$^{by}$ | 172.7 ± 14.6$^{x}$ | 194.4 ± 29.2$^{ax}$ | 164.7 ± 9.3$^{aby}$ | 171.6 ± 20.5$^{b}$ | 186.2 ± 28.1 |
| GST | (mU mg protein$^{-1}$) | 100.8 ± 12.9 | 102.5 ± 13.6 | 105.3 ± 15.6 | 104.2 ± 11.07 | 96.9 ± 12.1 | 90.0 ± 15.0 | 94.8 ± 21.5 | 97.0 ± 21.0 | 111.7 ± 13.9 | 116.8 ± 13.9 | 109.2 ± 16.6 | 106.1 ± 14.9 |
| GSH | (nmol mg tissue$^{-1}$) | 14.0 ± 4.1 | 12.58 ± 2.80 | 12.5 ± 3.8 | 12.5 ± 4.8 | 2.8 ± 0.7 | 3.3 ± 1.3 | 3.4 ± 1.6 | 2.7 ± 1.1 | 3.6 ± 0.8 | 3.9 ± 1.5 | 3.2 ± 1.1 | 4.6 ± 2.2 |
| LPO | (nmol g tissue$^{-1}$) | 25.4 ± 6.6 | 25.3 ± 5.8 | 26.1 ± 4.3 | 22.0 ± 5.6 | 23.6 ± 2.1 | 23.5 ± 5.3 | 22.9 ± 5.3 | 22.7 ± 7.5 | 23.5 ± 4.1 | 19.2 ± 3.6 | 21.0 ± 3.1 | 19.6 ± 3.2 |

| | Multifactorial ANOVA | | | | | | | Time | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Time | Lipid | Histidine | Lipid × Histidine | Time × Lipid | Time × Histidine | Time × Lipid × Histidine | FW | SWT2 | SWT4 |
| SOD | < 0.001 | 0.029 | ns | ns | ns | ns | ns | a | b | c |
| CAT | 0.001 | ns | ns | ns | ns | ns | < 0.001 | - | - | - |
| GST | < 0.001 | ns | ns | ns | ns | ns | ns | b | c | a |
| GSH | < 0.001 | ns | ns | ns | ns | ns | ns | a | c | b |
| LPO | 0.002 | ns | 0.024 | ns | ns | ns | ns | a | ab | b |

Values are presented as means ± SD. Multifactorial ANOVA: ns: non-significant differences ($p > 0.05$); if interaction was significant, one-way ANOVA was performed; Tukey post hoc test was used to identify differences among sampling times. Different lowercase letters denote significant differences among sampling times. Significant differences between lipid sources are indicated by capital letters, while “x” and “y” stand for significant differences between dietary histidine treatments.

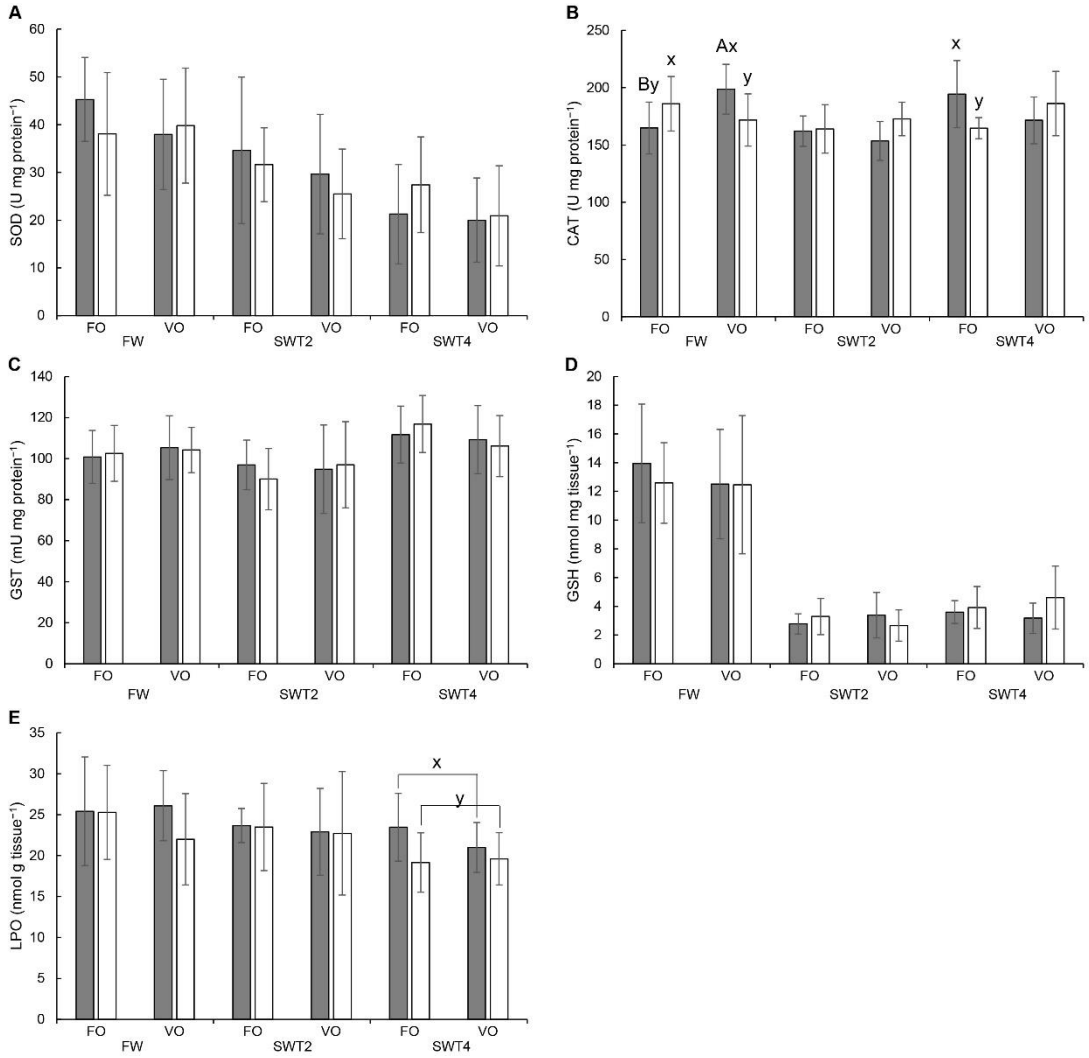


**Figure 11.** Superoxide dismutase (**A**), catalase (**B**) and glutathione S-transferase (**C**) activities, glutathione (**D**) and lipid peroxidation (**E**) levels in Atlantic salmon fed four dietary treatments differing on lipid source, FO or VO, and His content, high (■) or low (□), at FW, SWT2 and SWT4. Values are presented as means ± SD. P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, one-way ANOVA was performed. Within each time, significant differences between lipid sources are indicated by capital letters, while "x" and "y" stand for significant differences between dietary histidine treatments.

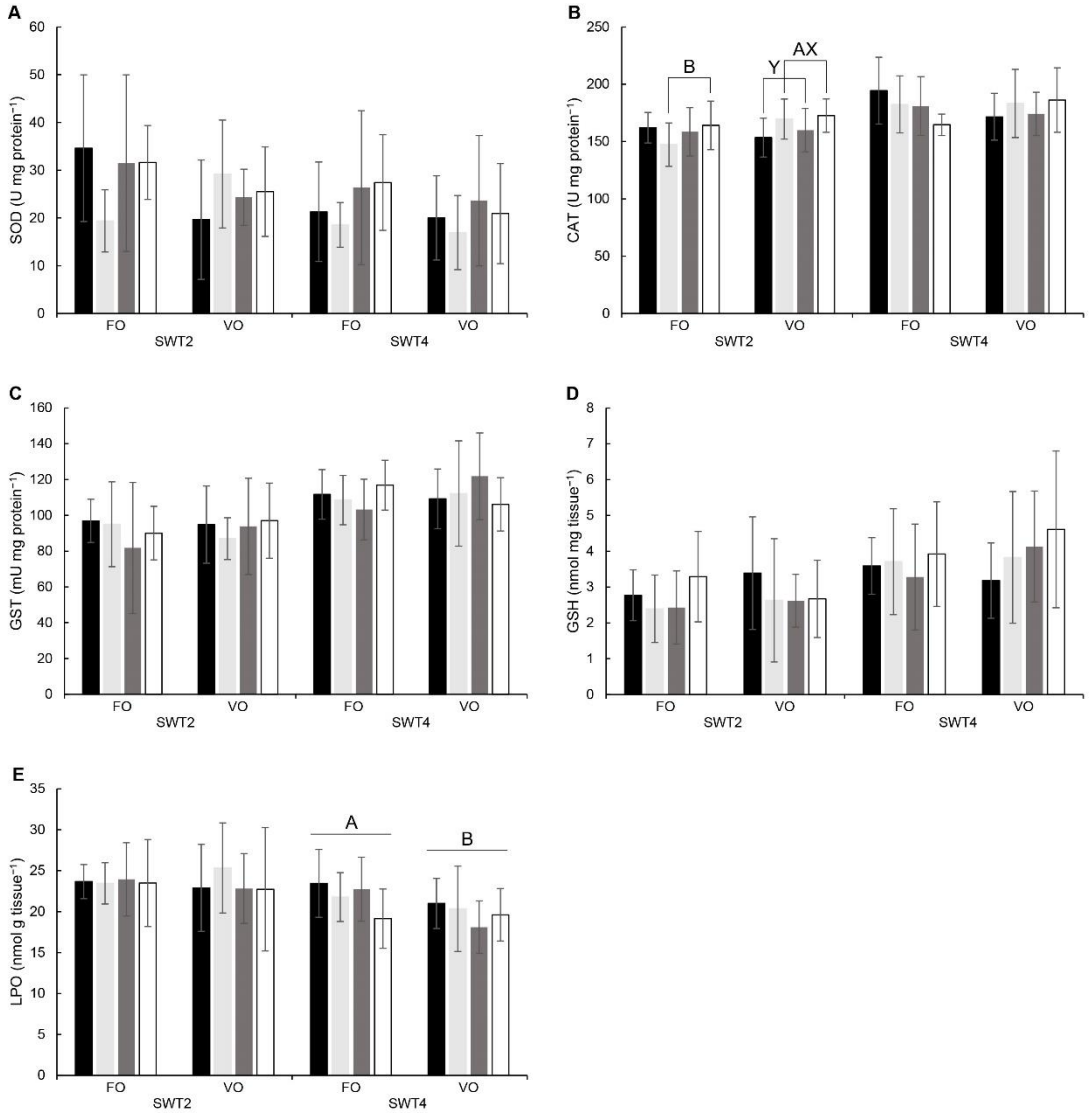


**Figure 12.** Superoxide dismutase (**A**), catalase (**B**) and glutathione S-transferase (**C**) activities, glutathione (**D**) and lipid peroxidation (**E**) levels in Atlantic salmon fed FO or VO-based diets, supplemented with His+ throughout the entire trial (His++, ■), during the FW phase (His+−, ▫), during the SW phase (His−+, ■) or with no His+ supplementation (His−−, □), at SWT2 and SWT4. Values are presented as means ± SD. P-values from multifactorial ANOVA ($p \leq 0.05$). If interaction was significant, one-way ANOVA was performed. Within each time, significant differences between lipid sources are indicated by capital letters, while "X" and "Y" stand for significant differences between dietary His treatments at SW.

## Discussion

### 1. Effects of transfer to seawater

Smoltification and subsequent transfer of smolts from FW to SW are crucial moments in the salmon's life cycle and are often associated with significant losses in Norwegian Atlantic salmon farms (Eggset *et al*., 1997). Still, few studies have focused on salmon immunocompetence variations during those stages (Johansson *et al*., 2016; Melingen *et al*., 1995; Muona & Soivio, 1992; Pettersen *et al*., 2003) and little attention has been given to the complex relationship between hormonal changes and the immune system (Johansson *et al*., 2016; Yada *et al*., 2012). Nevertheless, observations reveal massive immunosuppression during parr-smolt transformation and even more pronounced during the early SW adaptation (Johansson *et al*., 2016; Muona & Soivio, 1992; Pettersen *et al*., 2003).

In this study, the first sampling at SW stage was performed two weeks after the transfer from FW. Atlantic salmon sampled at SWT2 displayed increased relative proportion of circulating lymphocytes and monocytes, along with an enhancement in plasma lysozyme activity and IgM levels, compared to the values observed at the end of the FW stage. Apart from the stimulatory effect of an increased environment salinity on salmonid immune mechanisms (Yada *et al.*, 2001), the adaptation of smolts to SW is dependent on hormones that have immunomodulatory effects. For instance, it is known that fish GH levels increase after SW exposure (Bjornsson, 1997; Einarsdóttir *et al*., 2014). Besides its functions on regulating growth and metabolism, this hormone controls the development of hypoosmoregulatory mechanisms, being responsible for the good adaptation of smolts to the hyperosmotic environment (Bjornsson, 1997; Prunet *et al*., 1989). Moreover, GH can also act as a cytokine in the immune system under conditions of stress, counteracting immunosuppression by glucocorticoids in mammals (Jeay *et al.*, 2002). In fact, it stimulates lymphopoiesis (Pettersen *et al*., 2003) and phagocytosis, enhances lysozyme activity (Saurabh & Sahoo, 2008; Yada *et al*., 2001) and regulates IgM levels in fish (Yada *et al*., 1999). Nevertheless, the immune-modulatory role of GH in Atlantic salmon following transfer to SW still needs to be uncovered.

In the present study, the observed decrease in plasma bactericidal activity over time seems to be related to fish growth and normal development rather than the introduction of smolts into the marine environment. In contrast, plasma antiprotease activity appears to be compromised by the transfer of salmon to SW. Furthermore, it has been proposed that complement activity could be a good indicator of fish immunocompetence in stressed animals (Boshra *et al.*, 2006). In this study, ACP activity seems not to be affected by the

SW transfer since similar values were observed in individuals sampled at FW and SWT2, and further suggesting fish recovered well from SW transfer. Once these defence mechanisms are essential for restricting pathogens' ability to invade and grow in the organism, their enhanced activities at SWT4 might indicate an increased health status at that time.

Hereupon, some immunological mechanisms appeared to be stimulated by the introduction of smolts in the marine environment (lymphocyte and monocyte percentages, lysozyme activity and IgM levels), whereas others seem to be suppressed (antiprotease activity). Despite there is a two weeks gap between the FW sampling and the first sampling at SW, it is still reasonable to attribute changes in the humoral immune mechanisms between days as a consequence of the exposure to SW, presenting the fish with new challenges. However, the functional role of these variations is unclear and it is not possible to know how soon after the transfer they occurred. Regardless, although these results indicate a potential variation in Atlantic salmon immunocompetence, in opposition to the reported by Johansson *et al.* (2016), it seems that, in the present study conditions, smolts' immune defences were not impaired two weeks after SW transfer.

In the present study, data from hepatic antioxidant defence mechanisms revealed that the activities from both primary and secondary antioxidant enzymes, SOD and GST, respectively, were significantly reduced at SWT2 compared to the values observed at FW. Furthermore, the remarkable depletion of GSH levels found at that time is consistent with oxidative stress. Moreover, the drop in GST levels at SWT2 further suggests its importance to quench reactive molecules and catalyze the conjugation of GSH, thus protecting cells from the oxidative burst. Hamre *et al.* (2016) also observed that Atlantic salmon liver GSH levels decreased in the SW phase compared to those reared in FW. Although the induction of oxidative stress due to environmental salinity disturbance has been reported in other species (Lushchak, 2011), Hamre *et al.* (2016) suggested that, in Atlantic salmon, it may be rather related to surges of growth. Despite the decrease in antioxidant enzymes activity and liver GSH levels, LPO levels were not affected at that time. Thus, it seems that fish were able to protect themselves from oxidation through these antioxidants, preventing lipids oxidation. After four weeks of fish permanence in SW, although SOD activity remained to decrease over time, there was an improvement in GST activity and GSH levels, which was reflected in a decrease in LPO levels compared to the levels found at FW.

## 2. Effects of histidine

Free His and its derivates are known to cover several important roles in the tissues they are present, such as protection against oxidative damage (Remø *et al.*, 2011), regulation of intracellular pH (Ogata & Murai, 1994) and support of tissue osmoregulation (Rhodes *et al.*, 2010). In the present study, the increment in dietary His content was reflected in an increase in plasma bactericidal activity during the FW phase. Histidine is involved in the synthesis of antibacterial peptides, which are essential compounds of the plasma innate immune system (Magnadóttir, 2006). They effectively destroy bacteria by physically disrupting the structure of their cell membranes. Although the lack of studies in fish, an *in vitro* study with human cells conducted by Dong *et al.* (2019) found that His-rich antibacterial peptides exhibit high bactericidal activity against Gram-negative bacteria. This may explain the increased plasma bactericidal capacity of salmon fed His+ diets against *Tenacibaculum maritimum*.

However, after the introduction of smolts in SW, the effect of the dietary His content on the fish's bactericidal activity was no longer observed. One hypothesis is that the available His is redirected to other tissues with increased requirements at that first period in the sea (Li *et al.*, 2009), such as lens and, mainly, muscle. Indeed, during the transfer of fish from FW to SW, fish's lens are challenged by an increase in osmolality. The supply of dietary His has been shown to improve their osmoregulatory capacity through the accumulation of His in the form of NAH (Rhodes *et al.*, 2010). Moreover, several studies reported that anserine synthesis in muscle, which is limited by the free His in the tissue, is up-regulated by the transfer of smolts to SW (Breck *et al.*, 2005; Ogata *et al.*, 1998; Remø *et al.*, 2014). At that period, muscle anserine plays a key role in conferring buffering capacity to that tissue, an essential feature during the burst swimming activity of smolts after SW transfer (Ogata & Murai, 1994; Ogata *et al.*, 1998). Despite the importance of this AA in both tissues, comparing the amount of dietary His deposited in the lens and muscle, it is, in fact, in the muscle that most of His is accumulated in the form of anserine (Remø *et al.*, 2014).

In a study conducted by Melingen *et al.* (1995), the transient increase in plasma IgM levels of Atlantic salmon immediately after SW transfer was followed by a decrease to levels similar to those observed in FW phase four weeks after the transfer. In the present study, variations in plasma IgM levels of fish fed His− diets were similar to those described by Melingen *et al.* (1995), whereas in fish fed His+ diets, the IgM levels at SWT4 remained as high as in the previous time. This difference in IgM at the end of the feeding trial between fish fed different dietary His treatments indicates a possible modulatory effect of this essential AA. The prolonged administration of His+ surplus feeds may improve some adaptive immune mechanisms.

Surprisingly, although His is recognized as the most antioxidant AA (Wade & Tucker, 1998), no effects of the dietary His supplementation were observed on any of the liver's antioxidant enzymes activity, except on CAT activity, which was influenced by the interactive effects of dietary His content and lipid sources. At the last sampling time point of the present study, fish fed diets containing high His showed increased hepatic LPO levels compared to those fed His− diets. These results are not consistent with that reported by Remø *et al.* (2014), who also assessed the effect of dietary His concentration on the oxidative liver status of Atlantic salmon fed 10 to 18 g/kg His, 13 weeks after SW transfer. Thus, it is likely that the highest His concentration here tested was not above the threshold toxicity concentration for salmon, since other feeding studies with even higher His levels have not found His to be harmful (Remø *et al.*, 2014). It is also supported by the lack of differences in GST activity between dietary treatments observed in the present study. Although not statically significant, at SWT4, fish fed His+ diets weighed more than those fed His− diets. Therefore, the former could have higher hepatic lipid content and, consequently, be more susceptible to LPO.

### 3. Effects of lipid source

Contrary to the effects of dietary His supplementation on the fish systemic immune response, on which few studies have been published, the greater inclusion of more sustainable plant-based lipid sources in aquafeeds is well known to affect it (Radzikowska *et al.*, 2019). For instance, gilthead seabream (*Sparus aurata*) fed VO-based diets containing low levels of n-3 FAs were found to have decreased plasma bactericidal capacity (Montero *et al.*, 2010), while European seabass (*Dicentrarchus labrax*) fed a blend of VO displayed lower neutrophil numbers and plasma antiprotease activity (Machado *et al.*, 2019).

Concerning the effects of VO-based diets on the Atlantic salmon immune status, different results were observed depending on the type of oils supplied and their dietary inclusion levels, as well as the fish's developmental stage and the rearing temperature (Turchini *et al.*, 2009). For instance, Thompson *et al.* (1996) reported that parr fed a diet containing sunflower oil [(n-3/n-6) PUFA = 0.3] were less resistant to infection than those fed a diet containing fish oil [(n-3/n-6) PUFA = 5.2]. In a study conducted by Metochis *et al.* (2017), post-smolts fed six different dietary treatments were found to be able to satisfactorily utilize the VO diets without compromising their immune system. Moreover, several studies suggest vegetable-based diets to be beneficial for smoltification and successful SW adaptation (Bell *et al*., 1997; Bendiksen *et al*., 2003; Stubhaug *et al.*, 2006; Tocher *et al*.,

2000). In nature, wild pre-smolts feed mostly on FW aquatic insects, which are rich in n-6 FA. Only in the ocean, post-smolts change their diet as marine oil sources become available. Thus, in farming conditions, salmon might beneficiate from the supply of VO-based diets with higher levels of n-6 FA during the FW stage (Bell *et al.*, 1997).

In the present study, the dietary lipid source was not found to affect the fish immune response at the FW stage. However, after the transfer to SW, fish fed VO diets presented increased thrombocyte and neutrophil percentages and decreased lymphocyte percentages compared to fish fed FO diets. Similar changes in circulating leucocyte numbers have been described in other studies as a result of the exposure to stressful situations or upon an infection event (Ellsaesser & Clem, 1986; Engelsma *et al.*, 2003; Machado *et al.*, 2015). These data suggest that, in the present study, fish fed VO diets were in an increased inflammatory status at SWT2, probably due to the stress condition caused by the adaptation to SW and the consequent production of highly pro-inflammatory eicosanoids. Indeed, as described by Sargent *et al.* (2003), the intake of VO-based diets, rich in n-6 PUFAs, decreases the cellular ratio of n-3/n-6 PUFAs, promoting the synthesis of highly biologically active pro-inflammatory eicosanoids during stressful events. In contrast, the intake of higher EPA levels through FO-based diets increases the n-3/n-6 ratio and inhibits the production of pro-inflammatory eicosanoids from n-6 PUFAs (Sargent *et al.*, 2003). Therefore, the difference in the leucocyte dynamics between fish fed FO and those fed VO diets observed in this study may be imposed by the reduction of dietary n-3/n-6 ratio from 5.8 (± 0.3) to 1.0 (± 0.1) in the VO diets.

Furthermore, after SW transfer, fish fed VO diets rich in n-6 FA presented decreased plasma bactericidal capacity and ACP activity (Kiron *et al.*, 1995; Montero *et al.*, 2003; Montero *et al.*, 2010; Montero *et al.*, 1998). These results are consistent with those above mentioned, and support the knowledge that the supply of diets rich in n-6 FA may interfere with the fish immune response, leading to decreased protection against pathogens (Montero *et al.*, 2010). Indeed, considering the importance of these innate immune mechanisms in poikilothermic animals (Magnadóttir, 2006), it is likely that, in the present experimental conditions, individuals seem to beneficiate from FO-based diets.

Regarding the liver antioxidant defences, although fish fed VO diets throughout the trial presented significantly lower SOD activity compared to fish fed FO diets, within each time no differences on antioxidant mechanisms between dietary lipid sources were found. Moreover, in addition to the suggestion that VO diets do not seem to negatively impact the liver antioxidant protection, when analysing the set of data of both fish that maintained and that switched dietary His treatment after SW transfer, it was observed that at the end of the

feeding trial those fed FO diets had increased LPO levels compared to the ones fed VO diets. Remø *et al.* (2014) also found FO diets-fed fish to have higher liver oxidation product concentrations than fish fed a 70% inclusion of the oil blend used in the present study after fed the experimental diets for 13 weeks in SW. One possible explanation for the higher LPO levels in fish fed FO diets is that marine PUFA have higher susceptibility to oxidation and a lower content of natural antioxidant compounds than VO (Remø *et al.*, 2014).

### 4. Interactive effects of dietary histidine and lipid sources

At the end of the FW phase, the incorporation of high levels of His in VO diets led to increased percentages of circulating neutrophils, which are professional phagocytes and producers of large quantities of ROS. At FW, CAT activity in the liver was also enhanced in fish fed the VO His+ diet. CAT plays an essential role in controlling the formation and elimination of ROS, avoiding the excessive production of reactive species (Biller & Takahashi, 2018). Therefore, the higher CAT activity observed in fish fed VO His+ diets may be a strategy to control a possible increase in ROS as a result of the higher neutrophils percentages.

The same dynamic was observed at SWT2. At that time, the percentage of circulating neutrophils increased significantly in fish fed VO His− diet compared to the values observed at FW. In response to this increase, fish fed the VO diet containing His− either throughout the entire trial or only during the SW phase presented higher CAT activity than the ones fed the VO His+ diet during all trial or in the SW phase. However, the reason for the increase in circulating neutrophils proportions in fish fed the VO His+ diet at FW and in fish fed the VO His− diet at SWT2 remained to be uncovered.

Moreover, at SWT2, the plasma lysozyme activity was higher in fish fed the VO His+ diet than in those fed VO His−. Since at that time there were no differences in the proportion of circulating phagocytes between the two dietary treatments, the greater lysozyme activity was probably due to an improved activation of phagocytic cells in fish fed the VO His+ diet. Apart from this effect of incorporating high His levels in the VO diet on lysozyme activity at SWT2, no other effects of His+ diets on the lysozyme activity of fish that maintained the dietary treatment throughout the trial were observed. Han *et al.* (2013), who investigated the interactive effects of dietary arginine and histidine on the Japanese flounder (*Paralichthys olivaceus*) performance, also found that His did not influence lysozyme activity.

Regarding the effect of supplementing FO diets with His+ on post-smolt immune mechanisms, at SWT2, the plasma bactericidal activity was lower in salmon that were fed FO His− diet during the FW phase and changed to a FO His+ diet after SW transfer compared to those fed FO His+ diet throughout the entire trial. However, this effect was transient since no differences were found at SWT4. At SWT4, fish that switched from His− to His+ after SW transfer presented lower antiprotease activity than those that were fed the same His treatment during the entire feeding trial. However, despite the difference between these experimental groups, an increase in antiprotease activity from SWT2 to SWT4 was seen for all groups. Therefore, neither mechanisms appeared to be positively or negatively affected by the inclusion of His+ in the FO diet.

Independently on the dietary treatment, the lysozyme activity declined from SWT2 to SWT4. At SWT4, it was lower in fish from the FO His−+ experimental group than in the ones from FO His++ group. The differences between treatments observed in ACP activity at SWT4 were congruent with those in lysozyme at the same time, which supports the knowledge that lysozyme activates the complement system (Saurabh & Sahoo, 2008). However, the underlying reasons for these outcomes were not fully understood. Future studies should follow up the effects of switching dietary His treatments on lysozyme activity.

## Conclusion

Despite fairly intense research regarding the effects of dietary supplementation on the stress response and immune functions of fish, as far as the author knows, this was the first study to assess the impact of His surplus on the health status of Atlantic salmon during the early SW adaptation and as well as interactions with dietary lipid source. Results from the present study indicated that the incorporation of high levels of His in aquafeeds might be somewhat beneficial to the Atlantic salmon immune status, as suggested by the enhanced adaptive immune mechanisms (e.g., IgM levels) at the end of the trial, and the improved plasma bactericidal activity at FW. However, even the highest level of incorporation of this antioxidant AA in diet failed to compensate for the oxidative stress detected after exposure of smolts to SW, and its effects on improving plasma bactericidal activity were no longer observed after SW transfer.

In the present experimental conditions, regardless of dietary His treatment, fish fed VO diets after SW transfer seemed to be in an increased stress state, as suggested by the alterations in circulating leucocytes proportions and the general weaker plasma immune mechanisms compared to fish fed FO diets. Although the apparently less favourable effects of the lower ratio of n-3/n-6 PUFAs on post-smolts immune response, it was in the liver of fish fed FO diets that the highest LPO levels were observed.

Finally, there were no major differences between fish fed high dietary His levels throughout the entire feeding trial and those that switched dietary His treatment after SW transfer on post-smolt health status. However, at SWT4, the plasma lysozyme activity and, consequently, ACP activity seemed to be negatively affected by the switch from FO His− diet to FO His+ diet after SW transfer. It is suggested that future studies follow these effects of the switch of dietary His treatment on the lysozyme activity.

In summary, the supplementation of diets with His does not seem to have a significant impact on Atlantic salmon immunity and oxidative stress levels following SW transfer and it does not appear to compensate for the effects of replacing FO by VO diets on the fish's immune system.

## Future Approaches

In the present study, the first sampling point at SW occurred two weeks after the transfer. Future approaches could include two sampling moments, 4 and 24 hours after SW transfer, for evaluating how His surplus diets might modulate a possible acute stress response imposed by the abrupt introduction of smolts into the hyperosmotic environment. Moreover, to compare the impact of a gradual transfer of smolts from FW to SW with an abrupt transfer on immune responses and mortality rates over time would also be of interest.

Since the first months after SW transfer are often associated with significant losses in Atlantic salmon farms resulting from the emergence of diseases, to elucidate if the supply of His supplemented diets could improve fish resistance to disease, smolts could be challenged with a pathogen after the transfer and the immune parameters and mortality rates assessed.

Furthermore, since VO-based diets are known to increase intestinal inflammation, it would be important to evaluate if, in fish fed 100 % VO-based diets, His supplementation could modulate the local immune response at that tissue.

Finally, hormones mediate several immunomodulatory effects and are highly likely to be involved in the changes in immunological responses described in the present study. Future experiments should seek to clarify regulatory mechanisms leading to these changes.

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