Effects of methionine supplementation in the immune response of Atlantic salmon (*Salmo salar*) against salmon louse infection (*Lepeophtheirus salmonis*)

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Sworn Statement

I, Gonçalo Fernando de Oliveira Pinto, enrolled in the Master's Degree in Biological Aquatic Resources at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation reflects perspectives, research work and my own interpretations at the time of its submission.

By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

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Acknowledgements

GRINNAQUA was conceived in alignment with the “Farm to Fork” strategy and is focused on strengthening CIIMAR’s capacity to enhance the research profile of its staff and increase its innovation potential, positioning the institution among the leading players in the field of aquaculture. The project includes recognized leaders in R&D in European aquaculture with solid experience in relevant areas, working in partnership with CIIMAR. INIA-CSIC (Spain) has significant expertise in the study of the adaptive immune system and vaccination strategies; the team at the Roslin Institute – University of Edinburgh (UEDIN, United Kingdom) has essential knowledge in genetics and animal reproduction; and the University of Bergen (Norway) is proficient in the field of animal health and prophylactic strategies. The project has defined clear strategies to enhance the profile and research excellence of the staff through the implementation of training seminars and Summer Schools led by the partners. Several visits of CIIMAR staff to the partners facilities are planned, targeting senior researchers, technical and administrative training, and enabling the exchange of knowledge and best practices. The research project, integrating the scientific and technical strengths of the partners, will focus on the significant economic impact of viral and parasitic outbreaks in aquaculture, studying the prophylactic effects of functional diets against sea lice, a parasite that infects Atlantic salmon. This proposal outlines strategies to increase stakeholder engagement and mobilization towards scientific achievements, strengthening CIIMAR’s market-oriented research and innovation capacity in order to enhance its competitiveness at both national and international levels. GRINNAQUA will be key to improving CIIMAR’s innovation capacity, enabling it to become a leading institute in the blue revolution in aquaculture. A long-term collaboration is foreseen, contributing to the development of a more sustainable aquaculture sector in Europe.

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Resumo

A produção de salmão do Atlântico (*Salmo salar*) constitui uma parte importante para a diversificação da indústria da aquacultura, pelo facto de ser um dos peixes mais comercializados. Estes peixes poderão estar sujeitos a várias doenças infeciosas que irão colocar em causa a eficácia da produção. As infeções podem ter origem viral, bacteriana e/ou parasítica, como o caso da incidência de piolho-do-salmão (*Lepeophtheirus salmonis*). Os principais efeitos patológicos resultam das fases móveis do parasita, pois estes podem consumir grandes quantidades de tecido epitelial, criando lesões extensas através das quais podem desenvolver-se infeções secundárias. Assim é importante encontrar métodos e estratégias profiláticas para combater infeções por este parasita.

O presente trabalho teve como objetivo determinar se a dieta suplementada com metionina acima da necessidade nutricional estabelecida teve impacto positivo no sistema imunológico de salmão do Atlântico durante uma infeção com *Lepeophtheirus salmonis*. De modo a determinar o seu efeito foram analisados 60 salmões, dos quais foram recolhidos amostras de plasma para a análise dos parâmetros de resposta imune inata (peroxidase, protease, antiprotease, atividade bactericida e óxido nítrico), e amostras de rim cefálico para a expressão de diversos genes com função imunitária.

Os resultados mostram que a metionina teve um efeito positivo na expressão do gene CD4, sugerindo maior produção de glicoproteínas como coordenadoras das células T. Porém foi apenas em peixes não infetados e foi o único que demonstrou este tipo de relação entre o tipo de dieta e a expressão dos genes. Nos parâmetros de resposta imune, a infeção reduziu a atividade bactericida, possivelmente devido à migração de células imunes para o local de infeção. A atividade de proteases foi mais elevada em peixes infetados alimentados com dieta suplementada com metionina, indicando uma resposta inflamatória mais ativa.

Palavras chave: Salmão-do-Atlântico; Piolho-do-Salmão; Suplementação de metionina; Imunologia

Abstract

The production of Atlantic salmon (*Salmo salar*) represents an important component in diversifying the aquaculture industry, as it is one of the most commercially traded fish. These fish can be exposed to various infectious diseases that may compromise production efficiency. Infections can have viral, bacterial, and/or parasitic origins, such as the case of salmon lice (*Lepeophtheirus salmonis*) infestations.

The main pathological effects result from the mobile stages of the parasite, which can consume large amounts of epithelial tissue, causing extensive lesions through which secondary infections may develop. Therefore, it is important to find prophylactic methods and strategies to combat infections caused by this parasite.

The aim of the present study was to determine whether a diet supplemented with methionine above the established nutritional requirement had a positive impact on the immune system of Atlantic salmon during an infection with *Lepeophtheirus salmonis*. To evaluate this effect, 60 salmon were analyzed. Plasma samples were collected to assess parameters of the innate immune response (peroxidase, protease, antiprotease, bactericidal activity, and nitric oxide), and head kidney samples were taken to evaluate the expression of various immune-related genes.

The results show that methionine had a positive effect on the expression of the CD4 gene, suggesting greater production of glycoproteins involved in T cell coordination. However, this was observed only in non-infected fish, and it was the only gene that showed a relationship between diet type and gene expression. Regarding humoral parameters, infection reduced bactericidal activity, possibly due to the migration of immune cells to the infection site. Protease activity was higher in infected fish fed a methionine-supplemented diet, indicating a more active inflammatory response.

Keywords: Atlantic salmon; Salmon louse; Methionine supplementation; Immunology

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List of Abbreviations

FCUP - FACULTY OF SCIENCES OF THE UNIVERSITY OF PORTO

UP - UNIVERSITY OF PORTO

CIIMAR - INTERDISCIPLINARY CENTRE OF MARINE AND ENVIRONMENTAL RESEARCH

AA - AMINO ACIDS

CTRL - CONTROL DIET

MET - METHIONINE SUPLEMENTED DIET

RT – ROOM TEMPERATURE

HK - HEAD KIDNEY

RAS – RECIRCULATING AQUACULTURE SYSTEM

MALT - MUCOSA ASSOCIATED LYMPHOID TISSUE

SALT - SKIN-ASSOCIATED LYMPHOID TISSUE

GIALT - GILL-ASSOCIATED LYMPHOID TISSUE

GALT - GUT-ASSOCIATED LYMPHOID TISSUE

NALT - NASOPHARYNX-ASSOCIATED LYMPHOID TISSUE

ASC - ANTIBODY-SECRETING CELLS

IHNV - HEMATOPOIETIC NECROSIS VIRUS

ISAv - SALMON ANEMIA VIRUS

FLS – FLATSETSUND

EMB - EMAMECTIN BENZOATE

SAM - S-ADENOSYLMETHIONINE

GSH - GLUTATHIONE

Phdp - *Photobacterium damselae* subsp. *Piscicida*

UPLC - ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

MA – MARINE AGAR

MB - MARINE BROTH

CFU - COLONY-FORMING UNIT

HBSS - HANKS' BALANCED SALT SOLUTION

DMSO - DIMETHYL SULFOXIDE

qPCR - QUANTITATIVE PCR

Ct - THRESHOLD CYCLE

ef1α - ELONGATION FACTOR 1 α

β-act - β-ACTIN

il1β - INTERLEUKIN 1β

il10 - INTERLEUKIN 10

il12 - INTERLEUKIN 12

il4/13 - INTERLEUKIN 4/13A

il6 - INTERLEUKIN 6

il8 - INTERLEUKIN 8

ifnγ - INTERFERON γ

tnfα - TUMOUR NECROSIS FACTOR α

mmp9 - MATRIX METALLOPROTEINASE 9

saa5 - SERUM AMYLOID A

sms - SPERMINE SYNTHASE

sat2b - SPERMIDINE/SPERMINE N1-ACETYLTRANSFERASE FAMILY MEMBER 2b

cd4 - CLUSTER OF DIFFERENTIATION 4

mcsfr - MACROPHAGE COLONY-STIMULATING FACTOR 1 RECEPTOR 1

1. Introduction

1.1 – Aquaculture and Atlantic salmon

Aquaculture is one of the fastest-growing industries worldwide in food production, which constitutes an important source of protein for human consumption (Ahmad et al., 2021) and is defined by the cultivation of aquatic species in selected and controlled environments (Bouelet Ntsama et al., 2018). Fish production by aquaculture is increasing due to the high demand for animal protein. In 2018 it was estimated that 46% of the fish supply came from aquaculture, with 52% of that amount intended for human consumption («From the Millennium Development Goals to the Sustainable Development Goals», 2015). This demand is driven by global population growth and the decline in wild fish catches (Rahman et al., 2019). Moreover, aquaculture has been recognized as a rapidly advancing industry due to innovations in production systems (Guillen et al., 2019; Kumar & Engle, 2016) and has accounted for 8,8 % of the total food industry annually since 1980 (Belton & Thilsted, 2014). As a relatively young industry (compared to other sectors), there are many areas yet to be explored, allowing for its further development and growth in various aspects, including biological, economic, environmental and social (Føre et al., 2018). In 2018, the aquaculture industry produced approximately 82 million tons globally, a figure expected to reach 109 million tons by 2030. On average, the production growth rate was around 5.3 million tons per year between 2001 and 2018 (Ahmad et al., 2021), with fish being one of the main products, accounting for 54.3 million tons (*The State of World Fisheries and Aquaculture 2020*, 2020). Among the leading producers are India, Egypt, Bangladesh, Vietnam, Chile and Norway, which have contributed to global aquaculture production at varying levels over the years. Nigeria has become the second-largest aquaculture producer in Africa, although African aquaculture production accounts for only 2.7% of global production. Countries such as China, India, and Indonesia focus on freshwater aquaculture, while Greece, Norway, Japan, Canada, and Chile are the main producers of marine aquaculture, primarily cultivating cold-water salmonids (*The State of World Fisheries and Aquaculture 2020*, 2020). Marine aquaculture is practiced in the sea or along the coast, depending on water salinity levels, using manufactured structures such as cages and tanks, which can be operated traditionally, semi-intensively, or intensively. Currently, one-third of aquaculture production takes place in marine waters, but as the industry grows, it has been moving further offshore (Froehlich et al., 2017). Mariculture reached 30.8 million tons in 2018, accounting for 37.5% of global aquaculture production, with Asia contributing 88.89% (72.8 million tons). The main species produced include high-value fish such as Atlantic salmon (*Salmo salar*), sea bass (*Dicentrarchus labrax*),

gilthead seabream (*Sparus aurata*), giant perch (*Lates calcarifer*), and rainbow trout (*Oncorhynchus mykiss*), along with mollusks, bivalves, and seaweed (Ahmed & Thompson, 2019).

In nature, the Atlantic salmon cycle starts with the spawning in rivers all over the northern Atlantic in late autumn. The eggs hatch in early spring and the salmon lives the first period of its life, up to 6 years in fresh water depending on water conditions. When it reaches a certain size, it goes through a process known as smoltification to adapt to living in salt water, and it migrates out into the ocean with the spring flood. Here it feeds for 1–4 years before it returns targeting the river where it was born to spawn. The common production process in Atlantic salmon aquaculture follows the same stages (Asche & Bjørndal, 2011). The eggs are hatched, the fingerlings are transferred into fresh-water tanks in land-based facilities, and after the smoltification process, they are transferred to sea pens where they are fed until harvest (Afewerki et al., 2023), although the time from hatching to sea transfer is significantly shorter.

Atlantic salmon is one of the most successful farmed species, with a higher production growth than other farmed species (Kobayashi et al., 2015). This is due, to a large extent, to the fact that salmon producers are in the forefront in number of productivity enhancing categories such as production technology and supply chain development (Asche et al., 2018; Kumar et al., 2018; Kumar & Engle, 2016; Smith et al., 2010). Atlantic salmon is also produced in relatively few countries, exposed to different economic shocks and with substantial geographical dispersion and considerable differences in biophysical conditions (Iversen et al., 2020). Norway is the largest producer and was responsible for over half the total production (51%) in 2020, where most of the production is Atlantic salmon (1,232,200 metric tons) (Landazuri-Tveteraas et al., 2021). The second largest producer is Chile supplying around 25% of the global salmon production where, of the total Chilean production, 787,131 metric tons was Atlantic salmon (72.9 %) (Pandey et al., 2023).

In Norway salmon farming started in the late 1960 as a government supported activity to strengthen the livelihood of rural fishing communities facing depressed economies due to declining wild fisheries (Liu et al., 2011; Sønvisen, 2003). A decade later, many breakthroughs with biological and technological bottlenecks, such as rearing and development of dry feed advance the aquaculture industry (Aarset, 1998). One of the first successful pen in Norway was deployed off the island of Hitra in 1970 (Afewerki et al., 2023), most likely inspired by similar constructions in other aquaculture industries. The wood, styrofoam, and recycled tires used to construct the frame of the pen was soon

replaced by plastic and steel, allowing for larger structures as the building of the pens was transferred to specialized producers. The salmon farms started with small local family businesses scattered along the sheltered inlets, and with products targeting local markets (Liu et al., 2011). Later, due to high profitability and prospects of further expansion, these small-scale farms were merged and evolved into big multinational companies. This evolution gradually carried on through further capital concentration and vertical integration, where the initial demand from the home market was overtaken by exports (Liu et al., 2011). The early small farms often operated at locations with poor water quality and oxygenation that was exacerbated by the farms and provided incentives to move farms to more exposed locations. More recently, moving farms to even more exposed locations offshore are partly motivated by lower salmon lice levels. Since then, salmon aquaculture has experienced remarkable growth because of new locations, improved productivity and the growing of global markets (Liu et al., 2011). The Norwegian salmon aquaculture has gradually undergone a number of structural and technical changes making it expanded, intensified and diversified through time.

In recent years, the salmon industry has constituted an important part of the Norwegian economy, and farmed fish has become the fourth largest export commodity, after oil, gas and metals (Liu et al., 2011).

Currently the most important environmental challenge for the salmon industry is salmon lice, which in some regions in Norway limits the industry due to environmental regulations (Osmundsen et al, 2022). To overcome challenges posed by this parasite, fish-farming technology is advancing in several areas, including offshore farming, semi-enclosed sea pens, and land-based recirculating aquaculture systems (RAS). (Moe Føre et al., 2022; Øvrebø et al., 2022) and functional feeds that provide physiological benefits beyond basic nutritional requirements in reducing post-encounter infestation (Tacchiet al. 2011). For example, in 2017 the company SalMar began operating in an innovative exposed or offshore salmon farm concept: Ocean Farm 1. This facility is 68 m high and 110 m wide and is constructed in steel. It has 20,000 sensors for monitoring and feeding up to 1.5 million Atlantic salmon (Moe Føre et al., 2022). Functional feeds that improves the resistance against the salmon lice are already commercially available (Shield, Skretting; Robust, EWOS/Cargill) (Barrett et al., 2020) and their ingredients modify the mucus layer or modulate skin immune responses in order to reduce initial attachment success or facilitate effective immune responses against attached lice (Martin & Król, 2017).

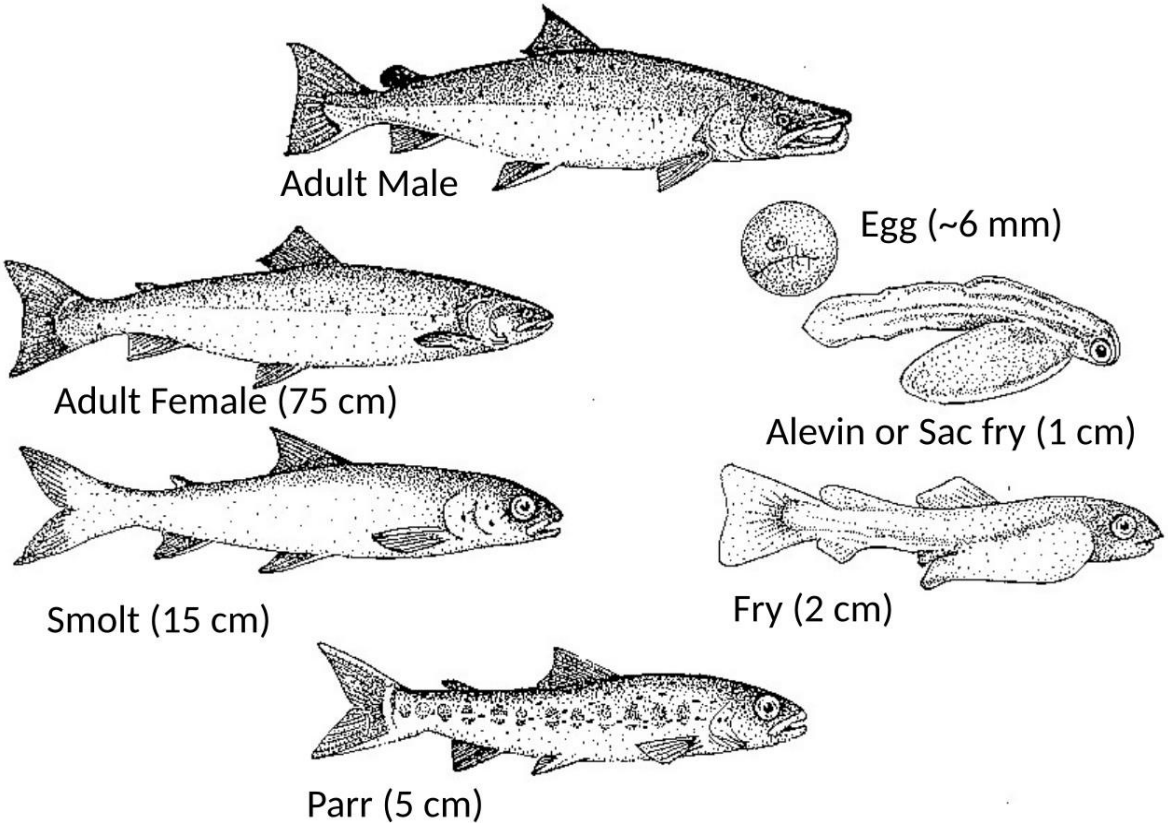


Figure 2. LIfe cycle of Atlantic Salmon (*Salmo salar*)

1.2 – Immune system

The immune system oversees the biological processes that detect and combat a wide range of agents, from viruses to parasites, protecting the organism from disease while distinguishing these threats from its own healthy tissues. The immune system present in all organisms is either a rudimentary immune system (the innate immune system) in the form of enzymes which protect against bacteriophage infections present in bacteria, in addition to the adaptive immune system (Rauta et al., 2012). Nonetheless, all vertebrate gnathostomes share a fundamental immune architecture, characterized by highly conserved innate immune mediators along with a limited yet functional adaptive system. This system provides layered, increasingly specific defense mechanisms against infection. For example, physical barriers such as the skin and gills prevent pathogens from entering the organism (Boyton & Openshaw, 2002; Rauta et al., 2012). If a pathogen breaches these barriers, the innate immune system provides an immediate, non-specific response (Rauta et al., 2012). Due to the inherent inefficiency of the adaptive immune response in fish—stemming from their evolutionary position and poikilothermic nature—the innate immune system plays a primary role in combating

infections. If a pathogen manages to evade the innate immune response, the adaptive immune system is activated, enhancing the organism's ability to recognize the invader and retain the response through immunological memory (Rauta et al., 2012).

1.2.1 - Innate immune system

In teleost fish species the skin, gills and gut act as the physical barriers that are considered the first defense against pathogens (Shephard, 1994). These mucosal barriers contain immune cells (lymphocytes) that form lymphoid tissue, an important component of which is the mucosa-associated lymphoid tissue (MALT). In fish, MALT is found across mucosal surfaces, which represent major sites of potential pathogen entry, and it hosts a range of both innate and adaptive defense mechanisms. (Gomez et al., 2013). MALT is subdivided into skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT), gut-associated lymphoid tissue (GALT), and nasopharynx-associated lymphoid tissue (NALT) (Salinas, 2015). SALT is a collection of various cell types, including secretory cells such as goblet cells and epidermal dendritic T cells, lymphocytes (T and B cells), granulocytes, macrophages, and Langerhans-like cells (Ángeles Esteban, 2012; Xu et al., 2013). Beyond its role as a physical barrier, fish skin is capable of secreting mucus, which plays a fundamental role in defense against pathogenic agents. The mucus contains several key immunological components, including lectins—sugar-binding proteins involved in molecular and cellular recognition processes such as opsonization and complement activation; antimicrobial peptides with broad activity against pathogens; lysozyme, a bactericidal enzyme; proteases that eliminate pathogens by cleaving their proteins or activating immune pathways; and antimicrobial IgM. (Ángeles Esteban, 2012; Dash et al., 2018).

The innate immune system is the response to initial infection and disease and does not retain memory of previous responses. This system is responsible for recognizing infections, initiating and resolving inflammation, and wound healing, through a complex network of various pro- and anti-inflammatory elements. These are generally characterized as either cellular—such as natural killer cells, phagocytes, and thrombocytes—or humoral components, including hormones, cytokines, protease inhibitors, lysins, precipitins, and agglutinins (Ellis, 2001; Robertsen, 1999; Scapigliati et al., 2001; Secombes et al., 2001). The inflammatory response starts when external barriers are trespassed, as a reaction to a wound or infection (Ellis, 2001). During an inflammatory response, various immune mediators are produced in reaction to circulating pathogens, such as cytokines, which mediate the immune response with pro-

or anti-inflammatory actions (Reyes-Cerpa et al., 2012). In addition, in response to an inflammatory state, leukocytes produce several immune components such as antiproteases, proteases, peroxidase, and reactive oxygen species (Secombes et al., 2001) .

The entire kidney contains immune cells: the anterior kidney has the highest concentration of developing B lymphoid cells, and also contains low levels of antibody-secreting cells (ASC) (Bromage et al., 2004; Zwollo et al., 2005). The head kidney/anterior kidney, (aglomerular) assumes hematopoietic functions (Zapata et al., 2006) and unlike higher vertebrates it is the principal immune organ responsible for phagocytosis (Rauta et al., 2012), antigen processing (Brattgjerd & Evensen, 1996), formation of IgM and immune memory through melanomacrophagic centers. In fish, the head kidney is a highly innervated organ that functions as an important endocrine center, homologous to the mammalian adrenal glands, and releases corticosteroids and other hormones. As such, it plays key regulatory roles and serves as a central hub for immune–neuro–endocrine interactions. In addition, it is the primary site of antibody production (Whyte, 2007). If the innate immune system is insufficient to clear the infectious agents, adaptive immunity is activated by non-specific immunity and interferes with pathogens by reacting with specificity and long-lasting protection (Rauta et al., 2012). The adaptive immune system is composed of highly specialized cells, T and B-lymphocytes, and proteins that destroy and inhibit the growth of invaders (Spiering, 2015). Consequently, the adaptive immune system exhibits remarkable specificity toward a wide range of pathogens. Another defining feature of adaptive immunity is its capacity for memory: it generates memory cells that confer long-lasting, pathogen-specific protection and enable rapid, efficient responses upon re-exposure to the same infectious agent. (Tangye & Tarlinton, 2009).

1.3 - Salmon Louse

The salmon louse, *Lepeophtheirus salmonis*, is one of the most significant pathogens affecting *Salmo salar* in the aquaculture industry. *Lepeophtheirus salmonis* causes major economic losses to the industry, with estimated losses ranging from approximately $500 million to around $1billion worldwide (Costello, 2006; Lafferty et al., 2015; Torrissen et al., 2013). The parasite follows a complex life cycle with three planktonic stages: nauplius I, nauplius II, and the infectious copepodid. Once the copepodid attaches to the host, it develops into the sessile chalimus I and II stages, during which it feeds on the host's epidermis. It then molts into the mobile pre-adult I and II stages before finally reaching

the sexually mature adult form. The life cycle is temperature-dependent and can take as little as 28 days at 14°C or several months in lower temperatures. The planktonic stages of the parasite do not harm the host, whereas the chalimus I and II stages cause only minor damage, restricted to the epidermis at the attachment site. This damage results from the extrusion and attachment of the frontal filament, as well as localized tissue erosion during feeding on the epithelium. Importantly, evidence suggests that the chalimus stages can suppress the host's immune response, potentially increasing susceptibility to other pathogens and secondary infections, particularly at the attachment site. (Barker et al., 2009; Jakob et al., 2011). Moreover, adult and pre-adult sea lice redistribute themselves on fish depending on the availability of potential mates, with males being much more mobile than females (Mordue (Luntz) & Birkett, 2009; Pino-Marambio et al., 2007).

The mobile stages of the parasite, which consume large amounts of epithelial tissue and cause extensive lesions in the skin, are primarily responsible for secondary infections. Moreover, it is believed that if these mobile stages feed on an already infected fish, they could act as vectors by transmitting pathogens to uninfected hosts. Indeed, studies have identified sea lice as vectors for diseases, including infectious hematopoietic necrosis virus (IHNV). (Jakob et al., 2011), infectious salmon anemia virus (ISAv) (Oelckers et al., 2015) and furunculosis (Nese & Enger, 1993). When the salmon lice reaches his adult stages, it starts feeding on the blood of the host, it secrets a saliva that inhibits blood clotting as well as inflammation, dendritic cell maturation, antigen presentation, T-cell proliferation and activation, neutrophil recruitment, antibody function and complement activation (Nuttall, 2019).

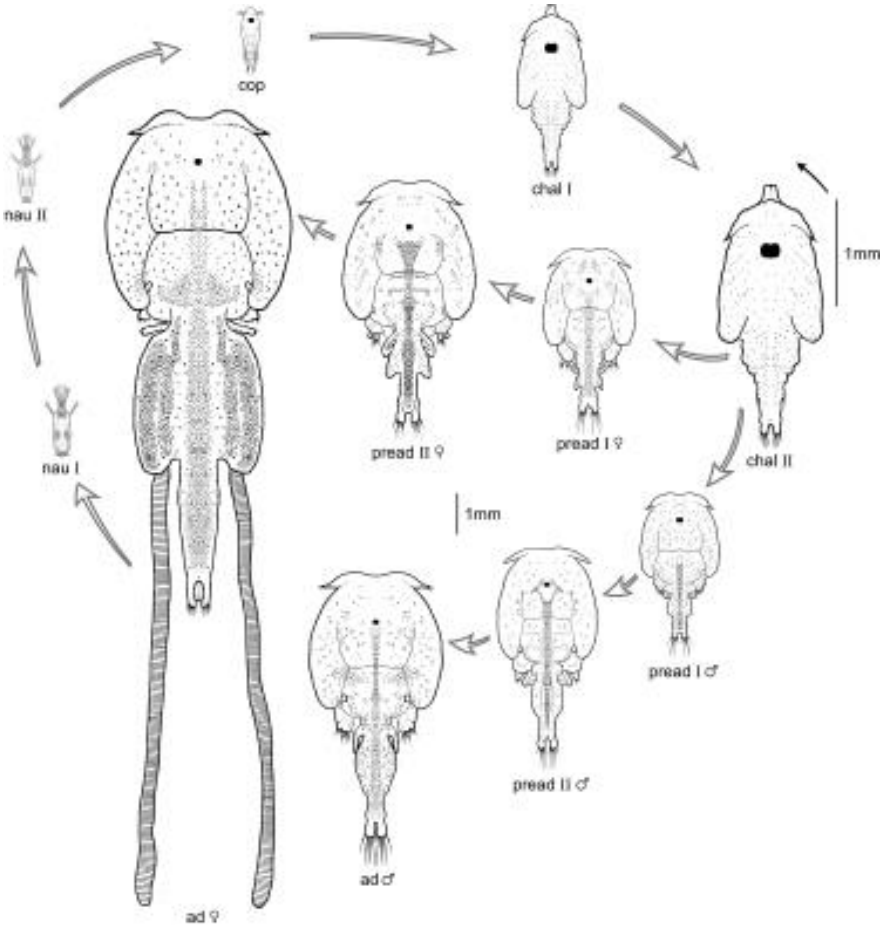


Figure 3. The stages in the life cycle of the sea louse Lepeophtheirus salmonis. (Sea Lice Research, 2020)

1.3.1 - Parasite control methods

In order to control sea lice infection in the production of Atlantic salmon, treatments like chemotherapeutants and non-medical treatments are used. Anti-parasitic chemotherapeutants are used to treat lice infestations in most countries where salmon aquaculture is practiced (Burridge et al., 2010). Because salmon lice reproduce year-round, effective control strategies aim both to prevent the establishment of internal infestation cycles and to eliminate gravid females (Burridge et al., 2010). Chemotherapeutants are used in two main ways: bath treatments and in-feed additives (Burridge et al., 2010). Organophosphates, pyrethroids, and hydrogen peroxide are applied via bath treatments, whereas avermectins—such as emamectin benzoate and diflubenzuron—are delivered as additives in medicated feeds (Burridge et al., 2010).

Because of the widespread resistance towards all available chemotherapeutants (Aaen et al., 2015), the industry has developed non-medicinal alternatives to control salmon lice. Five main species of cleaner fish (*Labrus bergylta*, *Ctenolabrus rupestris*, *Centrolabrus exoletus*, *Symphodus melops*; *Cyclopterus lumpus*) have been used as an alternative delousing method (Overton et al., 2019a). However, the sustainability of their use and their welfare have been of concern (Overton et al., 2019a; Skiftesvik et al., 2014). Mechanical and thermal delousing systems are recently developed technologies that serve as alternatives to chemotherapeutants. The limited reports available were primarily produced during their developmental phase, before these methods saw widespread industry adoption (Overton et al., 2019a).

Three separate companies provide the three mechanical delousing technologies currently in use: Flatsetsund (FLS) Engineering AS, SkaMik AS, and Hydrolicer. All three systems require the fish to be crowded and pumped into a treatment unit, where the lice are mechanically removed (Overton et al., 2019). In the FLS system, fish are first pumped into the system via a funnel and passed through two low pressure washers (0.2-0.8 bar), then the spray nozzles flush the lice off the fish as it passes through the pipe (Gismervik et al., 2017). SkaMik is similar to the FLS system but includes a brush system for removing lice (Helgesen et al., 2018). For last, the Hydrolicer pumps fish into a closed pipe filled with water, where inverse water turbulence ‘vacuums’ the lice off the fish (Overton et al., 2019).

Thermal treatment is based on inactivation of lice, as they detach from fish after short exposures to warm water (Brunsvik, 1997; Grøntvedt et al., 2015). Salmonids can survive in water temperatures of 20–34°C for short periods of time (Elliott & Elliott, 1995),

and while the upper thermal limit for lice is similar, due to the size difference between the host and parasite, lice survive for a shorter time (Grøntvedt et al., 2015; Roth, 2016). There are two competing thermal treatment systems: the Thermolicer and the Optilicer. In both, fish are first crowded in the sea cage and pumped past a dewatering strainer before entering a treatment chamber filled with warm seawater, with temperatures reaching up to 34°C. The key difference between the systems is that, in the Thermolicer, fish are pumped through the treatment chamber, whereas in the Optilicer, paddle wheels move the fish at a preset speed through a warm-water tank. (Overton et al., 2019). Although this treatment has showed elevated mortality of the fish at the end (Oliveira et al., 2021; Sviland Walde et al., 2021; Overton et al., 2019a).

There are in feed treatments against this parasite with emamectin benzoate (EMB), diflubenzuron and teflubenzuron, which have been used for several years (Aaen et al., 2015). However, no vaccine against salmon lice is commercially available, but there are studies aiming to develop a vaccine capable of combating the parasite (Contreras et al., 2020).

1.4 – Functionality of methionine as an additive

The dependence of the immune system on amino acid availability is associated with their role as essential signaling molecules for cellular function (Grimble & Grimble, 1998; Le Floc'h et al., 2011; Machado et al., 2018; Métayer et al., 2008), as methyl group donors, and as precursors of physiologically important molecules such as hormones, bioactive amines, enzymes, neurotransmitters and nitric oxide (Machado et al., 2018). Their essential role in the immune system is evidenced by the impaired immune responses observed in deficiency conditions (P. Li, 2007; Wu, 2009) and by their increased utilization due to metabolic shifts triggered by inflammation and infection (Le Floc'h et al., 2004).

Methionine is an example of an indispensable amino acid with a recognized role in the immune system, where its supplementation in diets has been shown to improve host immunity in fish (Machado et al., 2018, 2020). The methionine cycle participates in three pathways: methylation, transsulfuration, and aminopropylation. Methionine generates S-adenosylmethionine (SAM), serving as a methyl group donor in DNA methylation and thereby influencing gene expression. (Waterland, 2006). By inducing SAM-mediated methylation, methionine has been shown to inhibit autophagy, promoting growth in yeast (Sutter et al., 2013). Additionally, methionine contributes to the biosynthesis of polyamines (spermidine and spermine) through the aminopropylation pathway. In this process, S-adenosylmethionine (SAM) is decarboxylated and donates aminopropyl groups sequentially to the growing polyamines, a mechanism essential for cell

proliferation. (Igarashi & Kashiwagi, 2000). During the transsulfuration pathway, methionine serves as a precursor to cysteine, a component of glutathione (GSH). GSH helps eliminate free radicals, thereby protecting cells from oxidative stress, particularly during inflammation. (Grimble & Grimble, 1998). Methionine has also been shown to play an important role in processes responsible for controlling inflammation and apoptosis, such as protein ubiquitination and autophagy (Zinngrebe et al., 2014). Given that, the ideal inflammatory response is rapid yet specific (Ellis, 1999) and methionine shows significant potential as an immunomodulator during infections.

Methionine was shown to promote the European seabass (*Dicentrarchus labrax*) immune status by improving peripheral leucocytes response followed by a higher complement activity and bactericidal capacity (Machado et al., 2015). Also in the European seabass dietary methionine surplus enhance the cellular immune status without triggering pro-inflammatory indicators and this improved immune status allowed a better response against *Photobacterium damselae* subsp. *piscicida* (*Phdp*) (Machado et al., 2018). The modulatory effects of methionine on the immune system have also been investigated in other species, such as rainbow trout (*Oncorhynchus mykiss)*. A study demonstrated that doubling methionine levels above the species' nutritional requirements, in the absence of an inflammatory stimulus, reduced the expression of pro-inflammatory genes and increased peripheral neutrophil counts. (Machado et al., 2021). The authors suggest that this methionine supplementation may improve the status of neutrophils, potentially through the aminopropylation pathway (Machado et al., 2021; Wallace & Pegg, 2009). Furthermore, the work of Martín et al, 2023, provided new insights into methionine's effects on trout B cells and highlighted its positive influence on the adaptive immune response. Using in vitro splenic leukocyte cultures, the authors observed that supplementation with 1.5 mM L-methionine significantly increased the percentage of IgM^+ B cells.

1.5 – Objectives

The present study aims to determine whether diets supplemented with methionine above the established requirement will have a positive impact on the immune system of Atlantic salmon during infection by salmon lice.

2. Materials and Methods

2.1- Experimental diets

Two practical isonitrogenous (44% crude protein) and isolipidic (24% crude fat) diets were formulated to include fish oil as the main lipid source, fish meal and a blend of plant feedstuffs as protein sources. A CTRL diet was formulated to include the indispensable amino acids (AA) requirement levels estimated for Atlantic salmon. Following results from previous works, (Kaushik et al., 1993; Le Floc'h et al., 2011; Machado et al., 2015, 2018, 2020, 2021) a second diet was formulated similar to the CTRL but supplemented with DL-Methionine at 1 % of feed weight, at the expenses of wheat meal and wheat gluten (MET). All ingredients of the diet were finely ground, well mixed and dry pelleted in a laboratory pellet mill. Sparos Lda. (Olhão, Portugal) formulated and manufactured the diets. Proximate analysis and ingredient composition of the experimental diets are presented in Table 1.

Diets were analyzed for total AA content. Diet samples were hydrolysed in 6 M HCl at 116°C for 2 h in nitrogen-flushed glass vials. Samples were then pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analyses were done by ultra-high performance liquid chromatography (UPLC) in a Waters reversed-phase AA analysis system, using norvaline as an internal standard. During acid hydrolysis asparagine is converted to aspartate and glutamine to glutamate, so the reported values for these AA represent the sum of the respective amine and acid. Since it is partially destroyed by acid hydrolysis, tryptophan was not determined. The resultant peaks were analyzed with EMPOWER software (Waters, USA). The AA profile of the experimental diets and the relative percentage of methionine supplementation is presented in Table 2.

Table 1. Proximate analysis of the experimental diets and ingredient composition.

Ingredients	**%**	
	CTRL	**MET**
Fishmeal LT70	15.00	15.00
Krill meal	4.00	4.00
Soy protein concentrate	19.00	19.00
Wheat gluten	11.80	11.10
Corn gluten meal	5.00	5.00
Guar meal	5.00	5.00
Wheat meal	11.10	10.70
Faba beans (low tannins)	4.00	4.00
Vitamin and mineral premix	1.00	1.00
Choline chloride 50%	0.20	0.20
Antioxidant	0.20	0.20
Monoammonium phosphate	1.15	1.15
Astaxanthin 10%	0.05	0.05
L-Histidine	0.10	0.10
L-Lysine HCl 99%	0.50	0.50
DL-Methionine	0.00	1.10
Soy lecithin	1.00	1.00
Fish oil	7.20	7.20
Rapeseed oil	13.70	13.70
Total	100	100
Proximate Analysis (% feed)		
Ash	6.00	6.00
Protein	44.00	44.00
Fat	24.00	24.00
Phosphor	1.00	1.00
Energy (Mj/kg)	23.10	23.10
Fiber	12.00	11.70
Starch	6.00	6.00
EPA + DHA	2.50	2.50

Table 2. The AA profile of the experimental diets and the relative percentage of methionine supplementation.

Amino acids (%)	Experimental diets	
	CTRL	**MET**
Arginine	2.60	2.60
Histidine	1.10	1.10
Isoleucine	1.70	1.70
Leucine	3.40	3.40
Lysine	2.70	2.70
Threonine	1.50	1.50
Tryptophan	0.50	0.40
Valine	2.00	2.00
Methionine	**0.80**	**1.80**
Cystine	0.60	0.60
Methionine + Cystine	1.40	2.40
Phenylalanine	2.20	2.10
Tyrosine	1.50	1.50
Phenylalanine + Tyrosine	3.60	3.60
Taurine	0.20	0.20
Aspartic acid + Asparagine	3.60	3.60
Glutamic acid + Glutamine	9.70	9.40
Alanine	2.20	2.10
Glycine	2.10	2.10
Proline	3.00	3.00
Serine	2.10	2.10

2.2 – Experimental Design

The trial was conducted at the Sea Lice Research Centre - Department of Biological Sciences, University of Bergen, Norway. Experiments were performed in full compliance with national rules, by trained scientists, and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

Sixty juvenile Atlantic salmon (*Salmo salar*), averaging approximately 120 g, were randomly assigned to individual tanks. The experimental system consisted of 60 tanks arranged in a cascading, open-water configuration, organized into 20 groups of three interconnected tanks. Water quality parameters such salinity (34.08 ± 0.07 ppt) and temperature (12.01 ± 0.11°C) were controlled daily and the photoperiod was kept at 12 h dark: 12 h light and water flow rate of 120 L/h. During acclimatization (2 weeks), fish were fed the CTRL diet. At the onset of the feeding trial, half of the salmons were continuously fed the CTRL diet and the other half was fed MET at a daily ratio of 2 % biomass. The feeding trial lasted four weeks and after this feeding period, blood and head kidney samples were collected from 24 of the 60 fish (n= 12 per diet) to analyze plasma humoral response and gene expression, respectively (T4w samples). Of the remaining fish (n=36; n=18 per diet), half were infected by cohabitation with sea lice, and the other half were not (n= 9 per diet/ infection status), while maintaining the original dietary treatments. After two weeks of infection, blood and head kidney samples were collected from both infected and non-infected fish to assess plasma humoral response and gene expression (IT2w samples). Plasma was obtained by centrifugation (10,000 x *g*, 10 min) of blood collected from the caudal vein, and transferred to -80°C until analyzed, while HK samples were placed in RNAlater for 24h at 4ºC, and then stored at -80°C until gene expression analysis. Also, salmon louse were counted to determine which stages they were in following the infection trial.

2.3- Infection protocol

After the four-week feeding period and sampling, fish were experimentally infected with the salmon louse *Lepeophtheirus salmonis* via cohabitation, using a total of 100 copepods per tank / fish and the infection parameters where equal across all fishes. To simulate consistent handling stress across all groups, including non-infected controls, the water level in all tanks was temporarily lowered, although the system remained open and continuously flowing. Fish were maintained under these low-water conditions for approximately five hours, after which normal water flow was restored. Throughout the infection period (two weeks), fish continued receiving their respective diets.

2.4 – Plasma humoral response

Peroxidase Activity

The total peroxidase activity in plasma was measured following the procedure described by (Quade & Roth, 1997). In summary, 5 µl of plasma (in triplicate) was diluted with 145 µl of HBSS without Ca^{2+} and Mg^{2+} in flat-bottom 96-well plates. This was followed by the addition of 50 µl of 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma) at 20 mM and 50 µl of H_2O_2 at 5 mM. The reaction was stopped after 2 minutes by adding 50 µl of sulfuric acid (H_2SO_4) at 2M, and the optical density was read at 450 nm using a Synergy HT microplate reader. Wells without plasma were used as controls. Peroxidase activity (units ml/plasma) was determined based on the assumption that one unit of peroxidase produces a change in absorbance of 1 optical density unit.

Anti-protease Activity

Anti-protease activity was determined as described by Ellis, 1990, with some modifications (Machado et al., 2015). Briefly, 10 µl of plasma was incubated with an equal volume of a trypsin solution (trypsin diluted in $NaHCO_3$ 5 mg/ml; Sigma T-0646, 5G) for 10 minutes at 22°C in microtubes. To the incubation mixture, 100 µl of phosphate-buffered saline (PBS; NaH_2PO_4, 13.9 mg/ml, pH 7.0) and 125 µl of azocasein (20 mg/ml in $NaHCO_3$, Sigma A-2765, 5G, 2%) were added and incubated for 1 hour at 22°C in the dark. To stop the reaction, 250 µl of trichloroacetic acid (100 mg/ml, Sigma T-08657, 5G) was added to each microtube and incubated for 30 minutes at 22°C in the dark. The mixture was then centrifuged at 10 000g for 5 minutes at room temperature (RT). Subsequently, 100 µl of the supernatant was transferred to a flat-bottom 96-well plate, and 100 µl of NaOH (40 mg/ml, 1N) was added per well. The optical density was read at 450 nm using a Synergy HT microplate reader. Phosphate buffer in place of plasma and trypsin served as the blank, while the reference sample consisted of phosphate buffer with trypsin. The percentage of trypsin inhibition was calculated based on the percentage of uninhibited trypsin. All analyses were performed in triplicate.

Protease Activity

Protease activity was determined as described by Ellis, 1990. Briefly, 10 µl of plasma were incubated in microtubes with 100 µl of phosphate buffer (PBS; NaH_2PO_4, 13.9 mg/ml, pH 7.0) and 125 µl of azocasein (20 mg/ml in $NaHCO_3$, Sigma A-2765, 5G) for 24 hours at 22°C. Then, 250 µl of trichloroacetic acid (100 mg/ml, Sigma T-08657, 5G) was added to each tube and incubated for 30 minutes at 22°C. Next, the mixture was centrifuged at 10 000 g for 5 minutes at RT. Finally, 100 µl of the supernatant was

transferred to a flat-bottom 96-well plate, and 100 µl of NaOH (40 mg/ml, 1N) was added. The reading was carried out at 450 nm, which using a Synergy HT microplate reader. Phosphate buffer in place of plasma and trypsin served as the blank, while the reference sample consisted of phosphate buffer with trypsin. The percentage of protease activity was calculated by comparison with the reference sample. All analyses were performed in triplicate.

Bactericidal Activity

The bactericidal activity assay was performed using *Tenacibaculum maritimum* (strain 13.1). Inoculation was carried out on petri dishes with Marine Agar (MA) medium 25°C for 24 hours, followed by another 24-hour incubation in liquid Marine Broth (MB) medium. After this, a 1:2 dilution was made in MB, obtaining an absorbance of 0.584 (600 nm) and adjusting the colony-forming unit (CFU) value to 10^8. Plasma bactericidal activity was determined following the method described by Graham & Secombes, with modifications (Machado et al., 2015). Briefly, 20 µl of plasma was added to a round-bottom 96-well plate. HBSS (Hanks' Balanced Salt Solution with Ca^{2+} and Mg^{2+}, without phenol) and MB were used as positive controls. Then, 20 µl of *Tenacibaculum maritimum* was added to each well, and the plate was incubated for 2.5 hours at 25°C with agitation. Next, 25 µl of MTT (Thiazolyl Blue Tetrazolium Bromide) was added to each well and incubated for 10 minutes at 25°C with agitation to allow formazan formation. The plates were then centrifuged at 2000 g for 10 minutes, and the precipitate was dissolved in 200 µl of DMSO (Dimethyl Sulfoxide, Sigma). The absorbance of the dissolved formazan was measured at 560 nm, in direct proportion to the number of viable bacteria present. Viable bacteria were expressed as a percentage, calculated from the difference between the formazan dissolved in the samples and that formed in the positive controls (100%). Bactericidal activity was calculated as the percentage of non-viable bacteria.

Nitric Oxide

Nitric oxide in plasma was analyzed in duplicate using a colorimetric nitrite/nitrate assay kit (Ref.: 11746081001, Roche Diagnostic GmbH, Mannheim, Germany), adapted for 96-well microplates. Briefly, 100 µl of diluted plasma samples (90 µl of water and 10 µl of plasma), in duplicate, were added to a microplate. Then, 50 µl of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 4 µl of nitrate reductase enzyme were added to each well to reduce nitrate to nitrite. The plates were incubated for 30 minutes at 25°C. Subsequently, 50 µl of sulfanilamide and 50 µl of N-(1-naphthyl)-

ethylenediamine dihydrochloride were added and incubated for 10 minutes at 25°C. Absorbance was then read at 540 nm using a Synergy HT microplate reader after both incubation periods. Distilled water, instead of plasma, was used as a blank. Total nitrite was determined by comparison with a sodium nitrite standard curve prepared through serial dilutions.

2.5 – Head-kidney gene expression

Total RNA isolation from the head kidney was performed using the Maxwell RCS simplyRNA Tissue kit (Promega), following the manufacturer's specifications. The RNA samples were quantified by spectrophotometry using DeNovix DS-11 FX (Wilmington, DE, USA), and integrity of the samples was verified by electrophoresis gel (2%). The cDNA synthesis was carried out using the NZY First-Strand cDNA Synthesis kit (ref.: MB17002, NZYTech). Quantitative PCR (qPCR) assays were performed in CFX384™ Real-Time System (Bio-Rad, USA), using 1 µl of diluted cDNA (1:5 dilution) mixed with 5 µl of NZYSupreme qPCR Master Mix (ref.: MB419, NZYTech, Portugal) and 0.3 µl of each specific primer (10 µM), with a final volume of 10 µl.

The qPCR was carried out with specific primers (Table 3) for genes selected based on their involvement in immune responses. Some primers were designed using the NCBI Primer Blast Tool, considering known qPCR restrictions (amplicon size, melting temperature difference between primers, GC content, and formation of self or cross dimers).

Serial dilutions of cDNA were used to analyze the efficiency of the primer pairs by calculating the slope of the regression line of the threshold cycle (Ct) values against the relative cDNA concentration. The melting curve analysis was also performed to avoid amplification of primer dimers. The standard cycling conditions were: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, primer annealing temperature for 30 seconds, and extension at 72°C for 30 seconds. All reactions were performed in duplicate. Gene expression was normalized with the expression of the reference genes elongation factor 1 alpha (*ef1α*) and β-actin (*b-act*).

2.7 - Statistical Analysis

The data obtained were analyzed using: i) a one-way ANOVA to assess differences between dietary treatments at the end of a four-weeks feeding period and ii) a two-way ANOVA to assess differences between groups fed for four weeks, then submitted to a parasite challenge and sampled 2 weeks post-challenge, where diet and infection were considered the two main factors. The statistical analysis was performed using the SigmaPlot software (version 12.0) and a Tukey post-hoc test was conducted to identify differences between treatments. Outliers were previously identified and removed and normality of data and homogeneity of variances were previously ensured. The significance level used was $P \leq 0.05$ for all statistical analyses.

Table 3. Oligonucleotides used for real-time PCR (AT, annealing temperature (°C); AL, amplicon length (nt)).

Gene	Acronym	Accession nº	AT (°C)	AL (nt)	Efficiency (%)	Forward	Reversed	Reference
Elongation factor 1 α	*ef1α*	AF321836	62	88	108	GCTGTGCGTGACATGAGG	ACTTTGTGACCTTGCCGC	
β-actin	*β-act*	AF012125	60	163	86	GATGAAATCGCCGCACTGGTTGT	CGTCTCCAACGTAGCTGTCCTT	(Du et al., 2015)
Interleukin 1β	*il1β*	XM_014170479	62	73	95	GCTGGAGAGTGCTGTGGAAGA	TGCTTCCCTCCTGCTCGTAG	(Øvergård et al., 2023)
Interleukin 10	*il10*	EF165029	62	118	110	GGGTGTCACGCTATGGACAG	TGTTTCCGATGGAGTCGATG	
Interleukin 12	*il12*	AJ548830	62	62	111	TCTACCTACACGACATTGTCCAGCC	ATCCATCACCTGGCACTTCATCC	(Carvalho et al., 2020)
Interleukin 4/13A	*il4/13*	NM_001204895.1	62	72	89	CTCTCTGTTGCGATGGTGCTA	TTTATGCTGCCGGTACGCTG	
Interleukin 6	*il6*	NM001140710	60	170	110	CGTGGTGTTCGTTAAGGGGA	GCAAGGTGGTTACCTCGTGA	
Interleukin 8	*il8*	NM001140710	60	134	87	GAGGATTTCTAGTAGGATCATCT	ATGAGTCTACCAATTCGTCTGC	(Moore et al., 2017)
Interferon γ	*ifnγ*	AJ841811	60	241	98	CCGGAGCCACAGTGGAGATA	ACTTACAGATCTTGCTTCAACTGC	
Tumour necrosis factor α	*tnfα*	DQ787157.1	62	136	115	CGTCATGGCTTTTGCTTGTG	TGCAAAAGGCTGTAGGTCACT	
Matrix Metalloproteinase 9	*mmp9*	CA342769	62	72	107	ACTCTACGGTAGCAGCAATGAAGGC	CGTCAAAGGTCTGGTAGGAGCGTAT	(Carvalho et al., 2020)
Serum amyloid A	*saa5*	NM_001146565.1	59	82	107	CGGGAGATGGTTCAGGGTTC	CATTACGTCCCCAGCGGTTA	
Spermine synthase	*sms*	NM_001140162.1	62	97	108	TTCAACCTCTCAGCGCCAGAC	CCTCAGTGTCATGGACAGTCTC	
spermidine/spermine N1-acetyltransferase family member 2b	*sat2b*	NM_001173905.1	62	171	97	GAGTGCCTGATCGCTGAAGT	CAGTCCCTTTCCGATGCCTT	

Cluster of differentiation 4	*cd4*	AY973028	60	121	90	GAGTACACCTGCGCTGTGGAAT	GGTTGACCTCCTGACCTACAAAGG	(Mikalsen et al., 2012)
Macrophage colony-stimulating factor 1 receptor 1	*mcsfr*	XM_014195954.2	62	99	109	GGGTGGACACACACCAGTTTA	TTCCCGATGTTGCTTACTGGC	

3 – Results

3.1 – Salmon louce count

At the end of the experiment, the parasites were counted, and despite no statistically significant differences were observed between the experimental diets, a 100% prevalence was observed. It was observed that the majority of the parasites from the infection trial (≈ 70%) were at the Chalimus II stage, while the remaining 30% were at pre-adult stages (Table 4). The first clinical signs of infection (black spots) were found in the fins and altered skin pigmentation appeared two days post exposure, indicating successful parasite colonization of the host's skin and fins.

Table 4. Mean total number (n) of salmon louse on the skin and fin after two week of infection.

	CTRL	**MET**
Sum skin (n)	14.33 ± 5.73	16.10 ± 4.64
Sum fin (n)	9.00 ± 2.67	9.40 ± 3.83
Total sum (n)	23.38 ± 7.44	25.50 ± 4.18
Pre-adult (%)	32.80 ± 7.99	31.89 ± 7.68
Chamilus II (%)	67.20 ± 7.99	70.94 ± 13.05

3.2 - Innate immune response

Figure 2 shows parameters of humoral innate immune response in Atlantic salmon after the four week feeding period and two weeks after infection salmon lice. Within the parameters of the immune response, it was observed that peroxidase, anti-protease and nitric oxide activity (fig. 2B, 2C and 2D) did not show statistically significant differences ($P > 0.05$). Bactericidal activity (fig. 2A) was not significantly modulated by dietary treatment but it was observed to be significantly lower in infected fish compared to non-infected fish. Lastly, protease activity (fig. 2E) was observed to be significantly higher in infected fish fed with the MET diet compared to those fed with the CTRL diet.

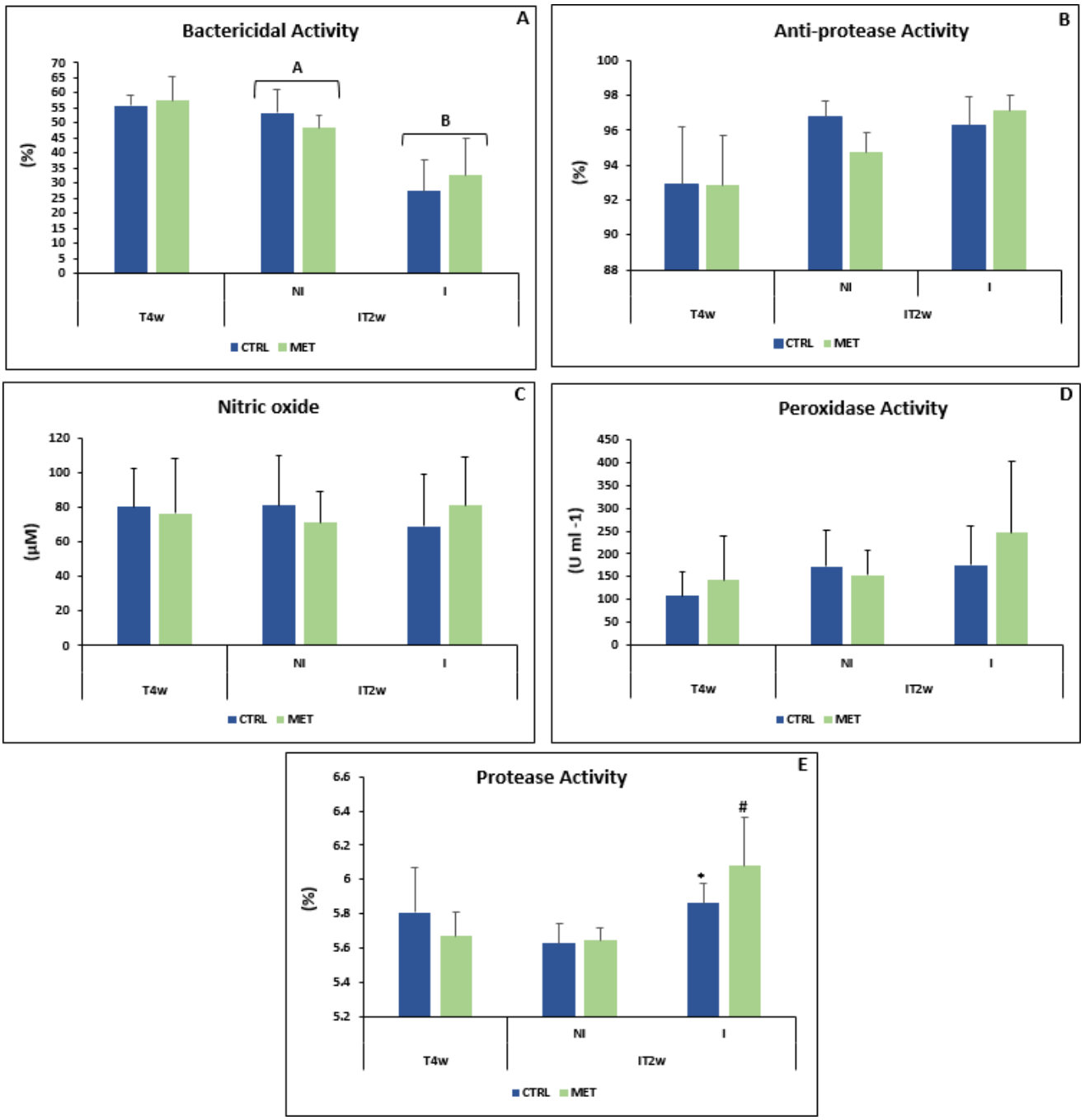
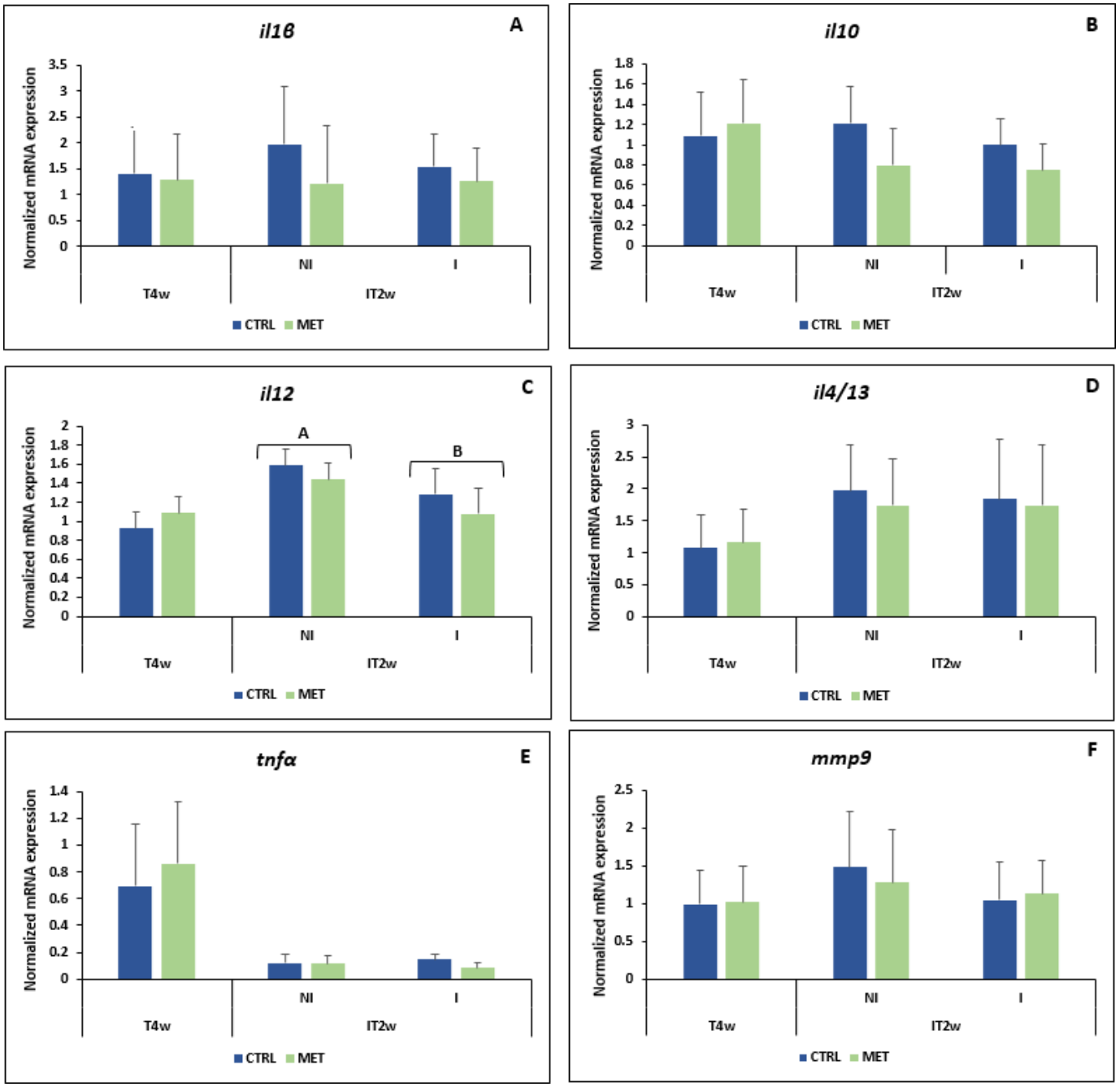
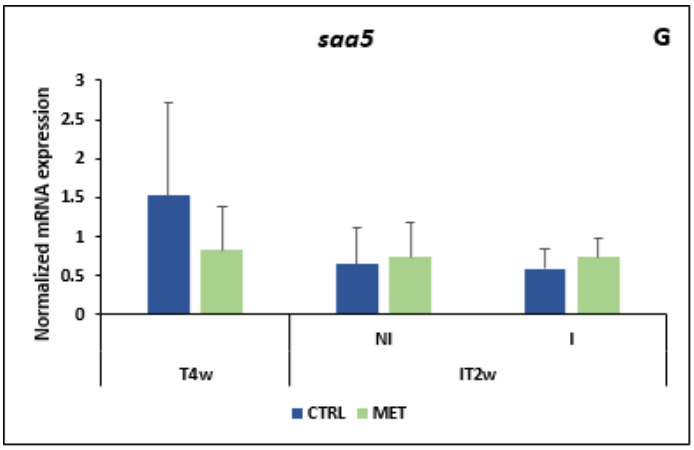


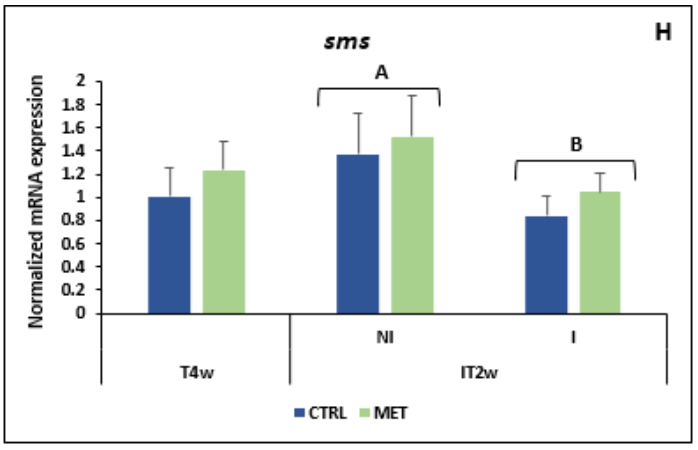
Figure 2. Humoral immune response of the Atlantic salmon fed dietary treatments for 4 weeks (T4w) and subsequently challenged with salmon louse via cohabitation (IT2w). Bactericidal Activity (A), Anti-protease Activity (B), Nitric oxide (C), Peroxidase Activity (D) and protease Activity (E). Uppercase letters indicate statistically differences between non-infected (NI) and infected fish (I) and symbols indicates differences between dietary treatments CTRL (control diet) and MET (methionine diet)). Multifactorial ANOVA; $p \leq 0.05$.

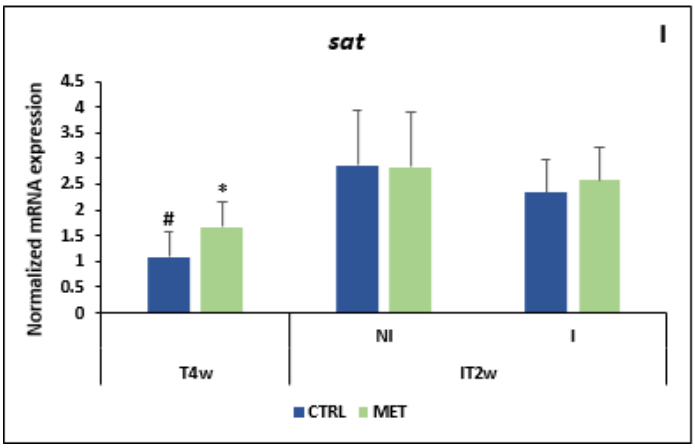
3.3 - Gene expression

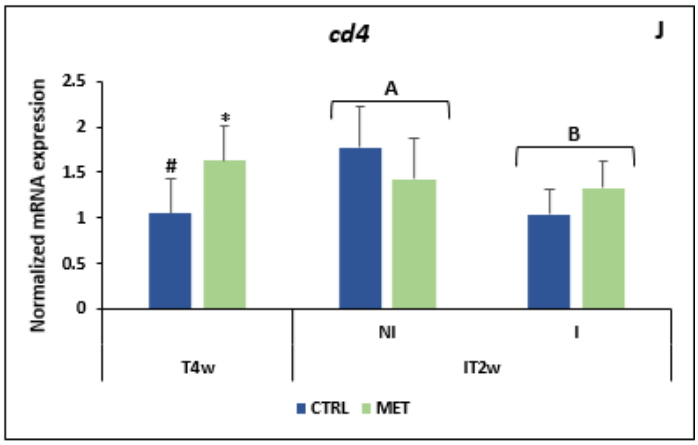
The results of gene expression in head kidney of Atlantic salmon after the feeding period and two weeks following infection are shown in Figure 3. The expression of the genes *il1β*, *il10*, *il4/13*, *tnfα*, *mmp9*, *saa5*, *il6*, *il8* and *ifnγ* did not show significant changes (fig. 3A, 3B, 3D, 3E, 3F, 3G, 3L, 3M and 3N, respectively). The expression of *il12*, *sms* and *cd4* was down-regulated after infection with salmon lice (fig. 3C, 3H and 3J, respectively) but the differences are small. Regarding dietary treatment, methionine dietary supplementation resulted in the increased expression of both *sat* and *cd4* at the end of the 4 weeks feeding period (fig. 3I and 3J, respectively). Moreover, it decreased the expression of *mcsfr* in non-infected fish sampled 2 weeks after the challenge (fig. 3K). *mcsfr* expression was also decreased by infection itself, but only in CTRL-fed fish.


il1β
A
Normalized mRNA expression
3.5
3
2.5
2
1.5
1
0.5
0
NI
I
T4w
IT2w
CTRL
MET
il10
B
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
il12
C
A
B
il4/13
D
tnfα
E
mmp9
F


saa5
G
Normalized mRNA expression
3
2.5
2
1.5
1
0.5
0
NI
I
T4w
IT2w
CTRL
MET


sms
H
Normalized mRNA expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A
B
NI
I
T4w
IT2w
CTRL
MET


sat
I
Normalized mRNA expression
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
#
*
NI
I
T4w
IT2w
CTRL
MET


cd4
J
Normalized mRNA expression
2.5
2
1.5
1
0.5
0
#
*
A
B
NI
I
T4w
IT2w
CTRL
MET

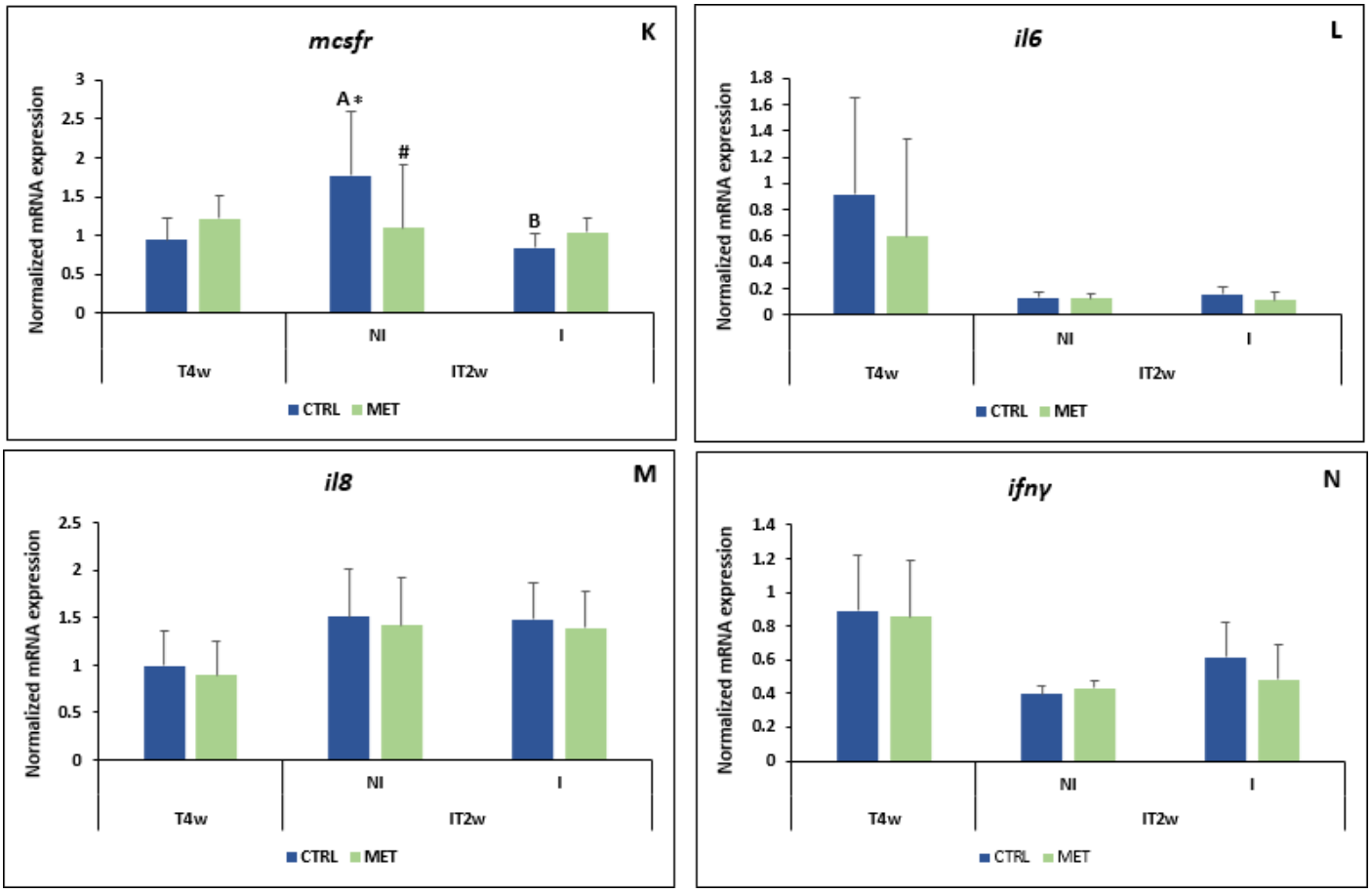


Figure 3. Gene expression in the head kidney of Atlantic salmon fed dietary treatments for 4 weeks (T4w) and subsequently challenged with salmon louse via cohabitation (IT2w). Interleukin 1β (*il1β,* A); Interleukin 10 (*il10*, B); Interleukin 12 (*il12*, C); Interleukin 4/13 (*il4/13*, D); Tumour necrosis factor α (*tnfα,* E); Matrix Metalloproteinase 9 (*mmp9*, F); Serum amyloid A (*saa5*, G); Spermine synthase (*sms*, H); spermidine/spermine N1-acetyltransferase family member 2b (*sat2b*, I); Cluster of differentiation 4 (*cd4*, J); Macrophage colony-stimulating factor receptor 1 (*mcsfr*, K); Interleukin 6 (*il6*, L); Interleukin 8 (*il8*, M); Interferon γ (*ifnγ*, N). Uppercase letters differences between non-infected (NI) and infected fish (I) and symbols indicates differences between dietary treatments CTRL (control diet) and MET (methionine diet)). Multifactorial ANOVA; $p \leq 0.05$.

4 - Discussion

The developmental cycle of the salmon louse parasite begins once it attaches to the host, initially consuming mucus and skin tissue (Fast, 2014). As the parasite mature, they progress to deeper tissue penetration and eventually incorporate host blood into their diet (Fast, 2014). In this study, parasitic infection was confirmed two days post-challenge by visible alterations in skin pigmentation and the formation of dark patches on the body and fins, consistent with earlier observations by Øverli et al. (2014). Since *L. salmonis* relies on host mucus, skin, and blood, the involvement of blood cells, plasma components, and enzymes is critical in mediating systemic immune responses during host-parasite interactions (Ross et al., 2000). Therefore, several changes are expected to occur amongst host immune defenses, set in motion to coordinately respond to the threat.

In the present study, plasma bactericidal activity, that measures the sum of humoral defenses activity against a pathogen, was significantly lower in the fish that were infected (IT2W), regardless of the diet they were fed. The decrease in activity in the infected fish could indicate an impaired immune response with a potentially reduced ability to synthesize key components involved in pathogen defense. Head kidney gene expression analysis, confirm such scenario. This important organ presents key regulatory functions and is the central organ for immune-endocrine interactions and neuro-immune connections (Whyte, 2007). Together with the reduced plasma bactericidal activity, gene expression of *il12*, *cd4* and *sms* demonstrated how some immune parameters are down-regulated during infection *per se*, i.e. regardless of what fish were being fed with. Interleukin 12 and *cd4* are genes related with Th1 response, and so their downregulation might mirror a natural, maybe transient suppression of this response, that only a time-course experiment would allow to clarify. Lower *sms* expression might indicate a lower polyamine synthesis in infected fish, a key process during protein synthesis and DNA structure maintenance (Clark et al., 2019).

In fact, there are strong evidences of host immune suppression since it has been described that ectoparasite is able to modulate host immune response, inducing an overall suppression of its inflammatory mechanisms, as the respiratory burst, decreased phagocytic activity of head kidney macrophages, and increased susceptibility to viral infections in Atlantic salmon (Øvergård et al., 2022).

In the present study, few changes resulted from dietary treatment, solely or in combination with infection. In the absence of immune stimulation, a methionine surplus seemed to favor Th1 response, given the higher expression of its molecular marker *cd4*.

Th1-mediated response has an important role in the adaptive immune response in the fish by assisting other cells as B cells and macrophages (Nakanishi et al., 2015; Tang et al., 2021). Methionine dietary supplementation also induced the expression of *sat*, a gene that codes for an enzyme involved in polyamine turnover. In fact, methionine has an indirect role in polyamine biosynthesis, as its decarboxylated form is a doner of an aminopropyl group during polyamine biosynthesis (Shahi et al., 2023). Methionine higher availability might therefore be a facilitator for the extension of polyamine synthesis.

In fish challenged with the parasite, gene expression was mostly unaltered by dietary methionine. However, 2 weeks after the infection procedure, salmon fed MET, submitted to the same confinement conditions but kept in a parasite-free environment showed a stable expression of the macrophage marker *mcsfr,* compared to those fed CTRL, in which *mcsfr* expression was higher. It seems to suggest that the dietary treatment granted a buffering effect to the stress itself. This gene is largely restricted to monocytes and macrophages, and codes for a receptor that, when triggered, leads to cell differentiation.

Beyond the role of circulating immune cells in defending against infection, fish plasma also contains a variety of antipathogenic factors, such as antimicrobial peptides and immune-related proteins, which contribute to protection against invasion (Ross et al., 2000; Mizaeva et al., 2023). Although no substantial changes were detected in blood cell counts—likely because the parasites had not yet reached a stage where they could access the bloodstream—protease activity was significantly elevated in fish fed the MET diet compared to those maintained on the basal diet. This suggests that the MET diet may enhance the production of plasma enzymes. As highlighted by Wagner et al. (2008), the release of proteases during sea lice infection is closely linked to the host's immune defense, given the direct involvement of these enzymes in counteracting parasite invasion (Ross et al., 2000). This changing pattern points to a greater capacity of the fish fed with MET to produce this plasma enzyme, but only under an infection scenario. Considered a key immune mechanisms of fish against sea lice infestation (Wagner et al., 2008), such increase could be an advantage during the host response against this particular pathogen.

The lack of further induced changes under an infection scenario may be associated with the fact that, at the end of the challenge, most parasites were at the Chalimus II stage, a stage that primarily feeds on mucus and skin (Fast, 2014). As a result, the immune response is likely concentrated in the skin, leading to a reduced response in the plasma and head kidney. In fact, only once the parasite reaches the adult stage, it begins feeding

on the host's blood, secreting factors with immunomodulatory and anticoagulant functions (Overgard et al., 2022). This induces an increased influx of immune cells to the site of infestation (Overgard et al., 2022) and can also lead to severe anaemia (Wagner et al., 2008). Since such scenario is absent in the present study, analyzing the salmon skin could provide more insights into the presence of a localized immune response. Additionally, a way to obtain a more pronounced response in the plasma would be to extend the infection trial period, allowing a greater number of parasites to reach more advanced stages that feed on the fish's blood.

5 – Conclusion

In conclusion, methionine dietary surplus was shown to promote the immune response of Atlantic salmon under infection conditions, but only in terms of protease activity. Regarding molecular markers, methionine also appeared to enhance immune-related gene expression such as *cd4* and sat though only in non-infection conditions. Finally, since most parasites found were at the Chalimus II stage, a stage that primarily feeds on mucus and skin this could partially explain the shy systemic response obtained.

6 – References

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