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# **Welfare and stress in salmonids – blood based diagnostics in terms of feeding stress**

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*für*

*Knut Boysen, meinem Felsen in der Brandung, mit Nelson &*

*Gudrun und Wolfgang Seibel, in memoriam*



## List of publications

### Research Chapter I

**Seibel, H.,** Baßmann, B., & Rebl, A. (2021). Blood will tell: what hematological analyses can reveal about fish welfare. *Frontiers in Veterinary Science*, 8 (March), 1–21. <https://doi.org/10.3389/fvets.2021.616955>

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### Research Chapter IV

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## I. General introduction

### 1. Welfare in aquaculture

Welfare in aquaculture refers to the overall well-being of the aquatic animals being raised in captivity for various purposes, such as food production or ornamental trade (Broom, 2011)<sup>1</sup>. Ensuring good welfare practices in aquaculture is important due to ethical considerations, product quality, economic benefits, environmental sustainability, and public perception (Franks et al., 2021).

Farming of fish and other aquatic species has increased in recent decades (FAO, 2022) and never have there been more controversial debates on animal welfare in fish husbandry. As other vertebrates fish have the right of a healthy life with physical and mental integrity, under which they can perform adequately (Braithwaite et al., 2013). In 2020, a total of 87.5 million tonnes of aquatic animals were bred, at least 408 species, 59 to 129 billion vertebrates. This is 20 times as many species as farmed land animals. For only 30 % of the individuals, specific information on the welfare of the animals was available. For the remaining 70 %, there were either no publications on the welfare of the animals or the species was unknown (Franks et al., 2021).

The breeding of fish in aquacultures is an attempt to meet the challenge of feeding more than 9 billion people by 2050, considering climate change, economic and financial uncertainty and increasing competition for natural resources (FAO, 2022; Searchinger et al., 2018). Fish and its products supply protein and essential nutrient sources which support a balanced nutrition and good health. In 2013, fish accounted for about 17 percent of the global population's intake of animal protein and 6.7 percent of all protein consumed (FAO, 2016). Aquaculture is growing constantly. "In the period 1990–2020, total world aquaculture expanded by 609 percent in annual output with an average growth rate of 6.7 percent per year." (FAO, 2022). All these fish also want to be fed. Therefore, fish feed sources play an increasingly important role (Pauly et al., 2002). Especially for reasons of sustainability it is not possible to increase the amount of fishmeal any further, also due to the limited resources in the oceans, so that alternative feed ingredients must be explored in order to serve the rapidly growing aquaculture market (Bardey, 2019; FAO, 2022; Ramírez et al., 2022). Feeding fish with fish is not particularly sustainable.

Besides, maintaining good animal welfare practices can contribute to environmental sustainability in aquaculture. Healthy and well cared animals are less likely to suffer from disease, reducing the need for treatments that can have a negative impact on the environment. Furthermore, minimising stress in aquaculture facilities can lead to a more efficient use of resources. Good animal welfare practices result in good growth rates, good reproduction, and general health (Ashley, 2007; Cooke, 2016; Huntingford & Adams, 2006). This in turn can contribute to higher productivity and economic benefits for

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<sup>1</sup> The references for this chapter can be found at the end of the thesis.

aquaculture producers (Macaulay, Bui, et al., 2021). Healthy and well-cared animals are more likely to reach market size efficiently, reducing production costs and increasing profits (Searchinger et al., 2018). In addition, there is the Animal Welfare Act in Germany, which in § 2 determines that animals must be adequately nourished, cared for and behaved in a proper manner, and pain or avoidable suffering or damage must be avoided<sup>2</sup>.

The welfare of aquatic animals has a direct impact on the quality of the product. Stress and poor conditions can lead to inferior fish products (Ashley, 2007). In addition, consumers often prefer products that have been produced under humane suitable and ethical conditions. What defines good welfare in farmed fish is controversially discussed (Browman et al., 2018; Cooke, 2016; Dawkins, 2017; Goldberg, 2016; Toni et al., 2018). Consumers are becoming more conscious of how their food is produced and are increasingly interested in supporting industries that prioritize animal welfare (Carrassón et al., 2021). Press coverage influences the social construction of reality (Berger & Luckmann, 2007) and can influence purchasing decisions of consumers (Pieniak et al., 2007). Aquaculture operations that demonstrate a commitment to ethical practices may gain a competitive advantage in the marketplace. Consumers request more and more ecological and “animal friendly” produced products<sup>3</sup>, but the transparency concerning welfare of fish products is still very incomplete (Ahmed et al., 2020; Carrassón et al., 2021; Chandararathna et al., 2021; Dara et al., 2023; Franks et al., 2021).

For ethical reasons fish welfare needs to be critically determined and put into a measurable way for the consideration of husbandry effects and improvements. This requires methods to adequately determine health, welfare, and stress status and needs to include feeding.

## 2. Methods for analysing the welfare and stress status of salmonids

A combination of behavioural, physiological, and environmental assessments is described to analyse the welfare and stress levels of salmonids in aquaculture. Common methods used for evaluating the welfare and stress levels of salmonids are behavioural observations like feeding or swimming behaviour, physiological Parameters, classically cortisol or blood glucose levels, gill and fin scorings, mortality monitoring, condition indices, environmental parameters like water quality monitoring and crowding assessments as well as disease and pathogen monitoring (Ashley, 2007; Broom, 1986; Cooke, 2016; Håstein et al., 2005; Herrera et al., 2022; Huntingford & Adams, 2006; Noble et al., 2018; Segner et al., 2019; Stien et al., 2020). A combination of several methods and parameters is certainly the most comprehensive approach to assess the welfare and stress status of salmonids in aquaculture.

A differentiation must be made between the influence of stress and welfare in general, even if the two are usually related to each other. It should be noted that the outbreak of a disease does not always have to be associated with a poor welfare status.

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<sup>2</sup> <https://www.gesetze-im-internet.de/tierschg/BJNR012770972.html>

<sup>3</sup> <https://www.bund.net/themen/aktuelles/detail-aktuelles/news/deutsche-kaufen-immer-mehr-bio-produkte/>

However, fish that are not kept in the optimal conditions are more susceptible to disease (Ashley, 2007; Huntingford & Adams, 2006).

Physiologist Selye first defined "stress" as "the non-specific reaction of the body to a demand placed on it" (Selye, 1953, 1973). Stress is always the physiological reaction to some kind of demand, the "stressor" (Caspers, 1983). More specific concepts include an endocrine cascade as part of the non-specific response, while others favour a broader view of stress and see stress as a "... physiological cascade of events that occurs when the organism attempts to resist death or the homeostatic norms in the face of an insult" (Schreck, 2000; Schreck & Tort, 2016). Then again, there is the view that stress is in some way related to energetic efficiency (Emlen et al., 1998).

Stress in aquaculture can be caused by many different factors like water quality, handling, and transportation, crowding and stocking density, pathogens, environmental changes, noise, social interactions as well as diet and nutrition (Dara et al., 2023; Erikson et al., 1997; Martos-Sitcha et al., 2020; Pickering, 1992, 1993). Understanding and mitigating these stressors is critical to promoting fish health and welfare. The application of appropriate management practices, regular monitoring and the creation of optimal environmental conditions can help to reduce the impact of these stressors. It should always be part of professional aquaculture practice.

"Fish welfare" is a complex and multi-layered concept that can only be assessed with a holistic approach compared to the more concretised "stress".

The welfare of fish can be described very generally as "the quality of life perceived by the animal itself" (Stien et al., 2013). The requirements for fish welfare are very diverse and need to be species specific. So, fare, available information on welfare issues is not equally distributed across fish species and different strains, which may have a different adaptation to environmental conditions, and there is a discrepancy between scientific knowledge and practical experience.

Animal welfare has been divided into functional, emotional, and nature-based concepts that often complement each other (Fraser et al., 1997; Mendl, 2001). While fish farmers and livestock scientists tend to have a function-based understanding of animal welfare in their social role, animal welfare organizations and critical consumers may be more likely to express emotion- and nature-based animal welfare. Rational science-based assessments rely more on measurable welfare indicators. In science the aim must be to be as 100 % rational as possible and the chosen experimental approaches must not be based exclusively on the moral views of the researcher, so that the data analysis contains moral views. Animal welfare organizations and critical consumers usually come more from the ethical-moral side of indicators and human emotions such as compassion. This influences the normative assessment of artificial interventions in aquaculture by consumers (Vanhonacker et al., 2011).

The discussion about the conscious perceptual abilities of subjective states plays a central role in the debate about the welfare of fish (Yue Cottee, 2012). In animal husbandry, the "Welfare Quality®" principles are applied based on the five freedoms model (FAWC, 2009; McCulloch, 2013). These include "freedom from hunger, thirst and malnutrition", "freedom from discomfort", "freedom from pain, injury and disease", "freedom

from fear and suffering” and “freedom to express normal behaviour”. They describe various welfare indicators (Blokhuis, 2008; Keeling & Veissier, 2005).

In recent years, numerous studies have been conducted to assess the welfare of fish in aquaculture and a variety of laboratory-based and farm-based welfare indicators have been published. They include physical and behavioural needs (Gutierrez Rabadan et al., 2021; Müller-Belecke, 2019; Noble et al., 2020; Pedrazzani et al., 2022; Stien et al., 2013; Tschirren et al., 2021; Weirup et al., 2022a). The number of applied welfare indices within the studies differs from small (Gutierrez Rabadan et al., 2021) to large (Tschirren et al., 2021) and within the assessed methods, and complexity of calculation applied. However, the models still have their weaknesses, which makes further clarification necessary (Folkedal et al., 2016).

Many different methods are being tested: automatic water analyses, camera monitoring, AI-controlled evaluations of swimming behaviour, weight gain, size growth and others (Barreto et al., 2022; Jones et al., 2022; Macaulay, Warren-Myers, et al., 2021; Mandal & Ghosh, 2023; Martins et al., 2012). What they all have in common is that these parameters tend to reflect the consequences of a long-term stress impact or a sub-optimal husbandry. However, what about stress influences that occur much earlier or have a hidden influence without already having a clear impact on performance parameters or behaviour? Blood testing offers a good, minimally invasive option here.

Blood analyses provide substantial information about the physiological aspects of animal welfare assessment, including the neuroendocrine and immune system, acute and long-term impacts due to adverse husbandry conditions, potential diseases, and genetic predispositions (Burgos-Aceves et al., 2019; Fazio, 2019). However, fish blood is still not routinely analysed for the assessment of fish health and/or welfare.

Over the years, the investigative techniques have evolved from antibody-based or PCR-based single-parameter analyses to transcriptomic, metabolomic, and proteomic approaches and from haematological observations to fluorescence-activated blood cell sorting in high-throughput modes. The range of test methods that enable the monitoring of informative fish blood parameters is summarised in Research Chapter I, study 2 (page 9).

In most cases, other immunologically active organs such as the head kidney, liver or spleen are examined (Petitjean et al., 2019). Fish must be killed for this. One favourable option for rapid and non-lethal sampling of large numbers of fish individuals is blood analysis (Roche & Boge, 1996). Blood tests in fish are versatile tools for assessing various physiological and health parameters. They provide valuable information about the internal conditions of fish and offer many possibilities for analysing the health status.

Blood tests can help diagnose diseases in fish by detecting specific pathogens, antibodies, or other indicators of infection or can be examined for the presence of parasites or abnormal cellular changes associated with parasitic infections. Blood tests can provide information about the cellular components of blood, including red and white blood cell counts, haemoglobin concentration, and haematocrit levels. Changes in these parameters can indicate health status and potential stress. Analysis of blood chemistry can reveal information about metabolic processes, electrolyte balance, and organ function. Common parameters include glucose, cholesterol, enzymes, and ions (Ahmed et al., 2020; Esmaeili, 2021; Mueller et al., 2023). The measurement of the stress hormone cortisol in the blood has been used to obtain information about the stress response of fish to various

environmental or handling stressors for a long time but has its weaknesses (Aluru & Vijayan, 2009; Mommsen et al., 1999; Weyts et al., 1998). The same with increased lactate levels, which can indicate an anaerobic metabolism and can be associated with stress or lack of oxygen (Olsen et al., 1992). Nutrient levels could also be determined via the blood and provide information about the nutritional status of fish. Nutrients such as vitamins and minerals or protein and lipid profiles are particularly important here (Lall & Kaushik, 2021; Mueller et al., 2023; Roques et al., 2020). For the purpose of completeness, it should also be mentioned that blood tests can be used to assess the reproductive status of fish by measuring sex hormones, detect the presence of contaminants or pollutants in fish, providing information about environmental exposure and can be used to monitor the presence of drug residues in fish after therapeutic treatments (Azizi-Lalabadi & Pirsahab, 2021; Canton et al., 2021; Fernández et al., 2019; Shahjahan et al., 2022). In terms of stress detection and fish welfare, the use of blood for measuring parameters such as antibody levels, immune cell counts, or lysozyme activity becomes particularly meaningful. The combination of several blood parameters enables a comprehensive assessment of the general health and well-being of the fish.

### 3. Feeding stress and Soybean meal induced enteritis model

When searching for valid stress markers, it is particularly important to take a close look at the causative stress factor. A poor-quality or imbalanced diets can lead to nutritional stress and impact growth and overall health (Ciji & Akhtar, 2021; Schull et al., 2023).

This thesis primarily deals with the influence of feeding stress, which is often caused by incompatible substances in the feed and leads to inflammation in the digestive tract. Inflammation can be assessed as localised stress. Inflammation in the gut of fish, like in other living beings, can have a range of effects on the health and overall well-being of the fish. Inflammation is a natural response of the immune system to injury, infection, or irritation ('Immunology of fishes', 1998; Pauley, 2011; Rombout et al., 2011). While it is a protective mechanism, chronic or severe inflammation can lead to negative consequences. Chronic inflammation can weaken the immune system and make the fish more susceptible to diseases (Bakke-McKellep et al., 2007; Bøgwald & Dalmo, 2014). An altered digestive function, a compromised gut integrity, immunological responses, malabsorption and malnutrition with reduced growth rates and behavioural changes are classical effects of gut inflammation in fish. They can even result in production losses.

Different factors can cause gut inflammation, including water quality, diet, and overall husbandry practices. Nutrition obviously has a decisive influence on fish growth and performance, but it also affects fish health (Marjara et al., 2012; Tsai et al., 2023).

Feeding SBM-based diets to salmonids is a common practice in aquaculture, especially as a substitute for fishmeal, which is expensive and ecologically unsustainable as described above (Bjerkeng et al., 1997; Carter & Hauler, 2000; Olsen & Hasan, 2012; Wang et al., 2017). Plant-based aquafeeds are becoming more and more important for commercial aquaculture, due to overfishing and sustainability reasons (Gatlin et al., 2007).

Soybean meal (SBM) is used as a plant-based alternative to fishmeal. It has a relatively high protein content, and the amino acid profile is similar to fishmeal. At the same

time, it is much cheaper to produce (Barnes et al., 2014; Bruni et al., 2021; INRA 2018; Oliva-Teles et al., 1994; Voorhees et al., 2019).

Processed soybean meal and soya protein products are currently the most used alternative protein sources for rainbow trout and are not expected to affect fish growth (Bruni et al., 2021; Caballero et al., 2002; Choi et al., 2020; Olmos Soto et al., 2015; Voorhees et al., 2019). Similarly, the inclusion of proteins from other plant sources, such as wheat, barley, cottonseed, or canola, in rainbow trout diets can also lead to comparable or even better growth performance than that achieved with fishmeal (Apper-Bossard et al., 2013; Gatlin et al., 2007; Kissil et al., 2000; Y. Wang et al., 2017).

However, SBM contains a range of antinutritive ingredients. These substances may interfere with the digestion and utilization of nutrients and can potentially have negative effects on the health and performance of animals. As a side note, more and more humans are becoming allergic to soya products, as well (Jedrusek-Golinska et al., 2019). The antinutritional compounds include protease inhibitors, phytates, lectins, oligosaccharides, goitrogens, isoflavones and allergenic proteins that can trigger allergic reactions in some individuals (Francis et al., 2001; Vikram et al., 2020).

Protease inhibitors interfere with the activity of digestive enzymes, particularly trypsin. This inhibition can reduce the digestibility of dietary proteins and affect protein utilization (Krogdahl et al., 1994; Krogdahl, Sundby, et al., 2015). Phytates in soybeans can bind to minerals such as calcium, iron, zinc, and magnesium, forming insoluble complexes. This reduces the availability of these minerals for absorption in the digestive tract (Zheng et al., 2020). Lectins are proteins that can bind to carbohydrates. While lectins are generally resistant to digestion, their impact on animal health is not fully understood (Erdaw, 2023). Some studies suggest potential negative effects on gut health and nutrient absorption. Lectins may have immunomodulatory effects on fish. They can interact with immune cells and influence the immune response. It has also been described that lectins can trigger stress reactions in fish (Krogdahl et al., 2010). The goitrogens are compounds that can interfere with thyroid function. These compounds can potentially affect the metabolism of iodine, leading to thyroid dysfunction, which is also important for fish (Deal & Volkoff, 2020). Soybeans are rich in isoflavones, which are phytoestrogens. While these compounds have potential health benefits, their estrogenic properties can also have unintended effects on reproductive physiology in salmonids and immunomodulatory effects (Francis et al., 2001; Vikram et al., 2020).

It is important to note that many of these antinutritive compounds should be reduced or eliminated through processing methods such as heat treatment, fermentation, and solvent extraction (Vikram et al., 2020). This is the reason why processed soya products, as mentioned above, are usually referred to when used in commercial fish feed.

After enzymatic treatment, for example, rainbow trout (*Oncorhynchus mykiss*) showed significantly higher growth performance and protein retention compared to the control than when fed unprocessed soybean meal (Kumar et al., 2020). Fermented soybean meal was tolerated by juvenile coho salmon (*Oncorhynchus kisutch*) a better way than the non-treated variant (Xu et al., 2023; Zhang et al., 2023). However, recent studies show that negative effects on the health of salmon cannot be eliminated even with processing (Carter & Hauler, 2000; Kumar et al., 2020).

Decades of research have, however, revealed that soy diets not only reduce the growth of salmonids (Rumsey et al., 1994), but also provoke mild to severe enteritis and other health complications (van den Ingh et al., 1991) due to the high levels of antinutritional factors (Francis et al., 2001; Krogdahl et al., 1994, 2010; Krogdahl, Sundby, et al., 2015). For example, Atlantic salmon become highly susceptible to temperature stress when fed SBM-based diets (Uran et al., 2008), due to alterations in the intestinal microbiome (Green et al., 2013).

SBM has been demonstrated to cause morphological changes in the distal intestine of salmonids (Baeverfjord & Krogdahl, 1996; Rumsey et al., 1994; van den Ingh et al., 1991), which comprise increased numbers of inflammatory cells, shortening of folds and disruption of microvilli, widening of central stroma, increased formation of microvillar vesicles, elevated numbers of goblet and apoptotic cells as well as reduced supranuclear vacuolisation with apical nuclear displacement within enterocytes (Baeverfjord & Krogdahl, 1996; Chikwati et al., 2012; Knudsen et al., 2008; Merrifield, 2011).

The inflammatory reaction triggered by SBM is therefore of particular importance. The changes described are also summarised under the name soybean meal-induced enteritis (SBMIE).

The SBMIE is used to generate stress under study conditions and can be used as a kind of challenge model in fish. As fishmeal is increasingly being replaced by plant-based raw materials, this is also an application-related model. The SBMIE model provides a controlled environment for the study of inflammatory responses triggered by specific dietary components or, conversely, the study of anti-inflammatory effects of feed additives (Booman et al., 2018; Egerton et al., 2020). Therefore, the expression of pro-inflammatory cytokines, the infiltration of immune cells and other markers associated with inflammation in the fish intestine can be analysed (Djordjevic et al., 2021; Kiron et al., 2020).

#### 4. Aim of the study

Good, valid stress markers are needed that can be used for various examinations. In addition, the examination should be as minimally invasive as possible. Blood tests are a promising option, and the focus of this study is to investigate whether blood is suitable as diagnostic agent for detecting stress and welfare in fish. Salmonids (rainbow trout and Atlantic salmon) were used as model species and were challenged using the SBMIE model to generate feeding stress.

Rainbow trout were first exposed to a potentially deficient diet and their physiological response was investigated. Blood samples were analysed to determine and compare a wide range of conventional and novel health and welfare parameters. These were tested in different set-ups. The investigation of the effects of low, rather commercially used soybean meal quantities in connection with handling stress and the use of immunostimulant feed additives were part of this thesis.

The aim was to find suitable molecular stress markers detectable in blood of salmonids and to test whether blood is suitable for diagnostics in terms of feeding stress as tool for the assessment of stress and welfare.

Based on the presented background, four research chapters are provided.

**Research Chapter I** deals with a general introduction on blood as diagnostic agent. It provides an overview of the state of literature and addresses the following research questions:

- What are current simple plasma tests and multiparametric analyses of fish blood?
- How are the different techniques used to assess fish welfare and disease?

**Research Chapter II** presents a study in which blood is used as diagnostic agent for investigating stress and the immune status of salmonids. Thereby, the soybean meal-induced enteritis model is used as a challenge model for stress induction.

It should be clarified:

- How does the examination of molecular stress markers compare with classic methods such as the determination of cortisol in the blood or differential blood counts for the early assessment of feeding stress in trout?
- Which markers that can be determined in the blood of trout provide well-founded indications of feeding stress?

Various potentially suitable gene markers were found, which will be used in the following studies.

In **Research Chapter III** the usage of blood is considered from a more applied point of view with the addition of the SBMIE model.

The research question here was to investigate the influence of a predominantly plant-based diet on stress behaviour. It is analysed whether trout do have a different stress resilience depending on whether they are fed a classic fish meal diet or a predominantly plant-based diet.

Finally, in **research chapter IV**, the molecular markers of the stress model developed in the previous studies are tested in a specific application to improve feed with SBM content. Their transferability from trout to salmon is tested. In this further step, not only the influence of SBM is investigated, but also a feed additive is analysed for its stress-balancing, immune-stimulating effect.

Research questions that are answered in research chapter IV:

- Are stress and immune markers generated by the SBMIE model transferable from trout to Atlantic salmon?
- Can the stress and immune markers found demonstrate positive effects of immunostimulant feed additives using blood for diagnostics?



## II. Research Chapter I - General introduction on blood based diagnostics

### Blood Will Tell: What Hematological Analyses Can Reveal About Fish Welfare<sup>4</sup>

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Blood analyses provide substantial information about the physiological aspects of animal welfare assessment, including the activation status of the neuroendocrine and immune system, acute and long-term impacts due to adverse husbandry conditions, potential diseases, and genetic predispositions. However, fish blood is still not routinely analyzed in research or aquaculture for the assessment of health and/or welfare. Over the years, the investigative techniques have evolved from antibody-based or PCR-based single-parameter analyses to now include transcriptomic, metabolomic, and proteomic approaches and from hematological observations to fluorescence-activated blood cell sorting in high-throughput modes. The range of testing techniques established for blood is now broader than for any other biogenic test material. Evaluation of the particular characteristics of fish blood, such as its cell composition, the nucleation of distinct blood cells, or the multiple isoforms of certain immune factors, requires adapted protocols and careful attention to the experimental designs and interpretation of the data. Analyses of fish blood can provide an integrated picture of the endocrine, immunological, reproductive, and genetic functions under defined environmental conditions and treatments. Therefore, the scarcity of high-throughput approaches using fish blood as a test material for fish physiology studies is surprising. This review summarizes the wide range of techniques that allow monitoring of informative fish blood parameters that are modulated by different stressors, conditions, and/or treatments. We provide a compact overview of several simple plasma tests and of multiparametric analyses of fish blood, and we discuss their potential use in the assessment of fish welfare and pathologies.

**Keywords:** erythrocytes, hematology, leukocytes, teleost fishes, well-being, transcriptomics, proteomics, stress

#### INTRODUCTION

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A significant segment of the constantly growing aquaculture sector is represented by intensive farming practices (1) aimed at meeting the increasing demand of a growing world population. However, at the same time, this emphasis raises many concerns due to the competition for resources and the negative impact of intensive aquaculture on the environment and fish welfare (2). Consequently, the visionary concepts for future fish farming include welfare-friendly systems that can monitor animal-based parameters and quickly regulate disruptive environmental variables when necessary (3, 4). Certified farming procedures should be based on a comprehensive, science-based understanding of how teleost fish respond to anthropogenic environmental disruptions and challenging aquaculture-related conditions. This requires multidisciplinary investigations to define and evaluate optimal species-specific husbandry conditions, especially for newly introduced aquaculture species (5). Consequently, research groups and large scientific consortia are currently studying the influences of a variety of factors, ranging from global climate change, ocean acidification, and eutrophic habitats to introduced pathogens and pollution by environmental toxins or microplastics, on fish breeding and wild fish stocks. The use of more extensive research approaches to screen the effects of environmental conditions can increase the probability of detecting disturbances and even dangerous confounding factors, thereby allowing the discovery of relevant diagnostic biomarkers for fish health.

One favorable option for rapid and non-lethal sampling of large numbers of fish individuals is blood analysis. Blood is a complex mixture of heterogeneous cell populations (6–8) that include erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (analogs of mammalian platelets) (9). These fish blood cells are broadly similar in function to their mammalian counterparts and are found in all other tissues and organs throughout the body. Blood transports an immense variety of substances (gases, water, minerals, nutrients, hormones, immune effectors, toxins, microbial structures, or waste products), so its analysis can provide a wealth of information about fish physiology and health status. Alterations in informative blood-based indicators like metabolite concentrations, hormonal profiles, and transcript abundances can reflect systemic reactions to changes or disturbances in homeostasis that can alert scientists and veterinarians (as well as fish farmers), who monitor the physiological status of an individual fish or an entire population.

Blood tests on fish have been carried out for decades (10) in laboratory and field settings to assess endocrine, reproductive, and immune functions; maturation; nutrition and health status or to perform genetic studies (11). More analysis techniques have been established for blood as a test material (Figure II-1) than for any other tissue or fluid sample. Nevertheless, interpreting the obtained data to extract meaningful information on the individual condition remains difficult. On the one hand, a few excellent reviews have recently updated the history of selected hematological techniques (12, 13); however, remarkably, blood analyses using systems biological approaches or even PCR-based techniques are still underrepresented in fish physiology (14). On the other hand, the omics-based hypotheses put forward by fish geneticists and molecular biologists are correlated only on a small scale with non-transcript parameters extracted from fish blood.

A growing part of the research on farmed fish focuses on “animal welfare” involving optimization of husbandry conditions, stress avoidance, and improvement of fish quality of life (15, 16). The focus is largely on the ability of domesticated fish populations

to cope with environmental and/or anthropogenic challenges (17). In general, animal-welfare programs aim to ensure freedom from (i) thirst, hunger, and malnutrition; (ii) discomfort; (iii) pain, injury, and disease; (iv) restriction of normal behaviors; and (v) fear and suffering (18). These basic aspects also apply to some extent to the welfare of fish in aquaculture. However, fish require different treatment than terrestrial animals in many ways due to their aquatic nature and differences in their anatomy and physiology, as well as in their required husbandry conditions. According to Huntingford et al. (19), fish welfare can be expressed by (i) a feelings-based definition, focusing on a reduction in pain and negative emotions, and/or increased access to positive experiences; (ii) a nature-based definition assuming that every fish species must express its inherent biological nature; or (iii) a function-based definition targeting the ability of fish to adapt to environmental demands. The well-being of fish in aquaculture is impaired by acute environmental changes, coupled with husbandry practices, such as sorting, grading, and transport that can induce stress responses. Most research studies are based on the third concept (iii), which involves fish health and the adequate functioning of fish biological systems, especially those involved in managing a compromised homeostasis imposed by aquaculture conditions. The physiological state of a fish with disturbed homeostasis is usually captured by observing and recording indicative signs and measurable characteristics of the response to environmental challenges or stressors. Restoration of homeostasis usually requires the invocation of complex behavioral and physiological adaptive responses (20).

Primary stress responses are characterized by the release of catecholamines and corticosteroids (21). The subsequent secondary stress responses have a multitude of actions in many tissues, including blood, and can range from accelerated mobilization of energy via glucose, altered hydromineral balance, and increased lactate levels to decreased blood pH, hematocrit, and sodium levels and lower liver glycogen levels (20, 21). Increased cardiovascular activity and breath rate that enhance the uptake and transport capacity for oxygen are accompanied by a redistribution or suppression of immune functions (21, 22). Tertiary responses are reflected by behavioral adaptations, inhibited growth, decreased reproduction, and a compromised capacity to endure any additional stressors. The repeated exposure to a low-intensity stressor triggers the development of an adaptive response over time and attenuates acute stress reactions, whereas the exposure to harmful, persistent stressors is likely to intensify the physiological response (23). When stressors co-occur with a high pathogenic pressure, they provoke serious diseases (24) and strongly impair fish welfare (25).

Many previous and current studies on fish welfare have measured the main components of the primary physiological stress response, largely plasma cortisol and glucose (26– 28). Both components are informative stress markers, but they have limitations (29). The current scientific consensus is that the assessment of fish welfare is a complex task (30), in part because the absence of a physiological stress response does not necessarily imply adequate welfare (31). Each fish species has distinct “ecological and behavioral demands” (15); therefore, the responses to adverse conditions vary across taxa (32, 33). In recent years, the scientific evaluation of fish physiology has shifted from the conventional, limited biomarker approach to comprehensive and rather holistic approaches (34, 35). The spectrum of the parameters now recorded has expanded and is accompanied by an increasingly well-equipped fish-specific toolbox (Figure II-1). These advances now

facilitate the generation of weighted welfare scores (36, 37) that can integrate operational and laboratory- based parameters.

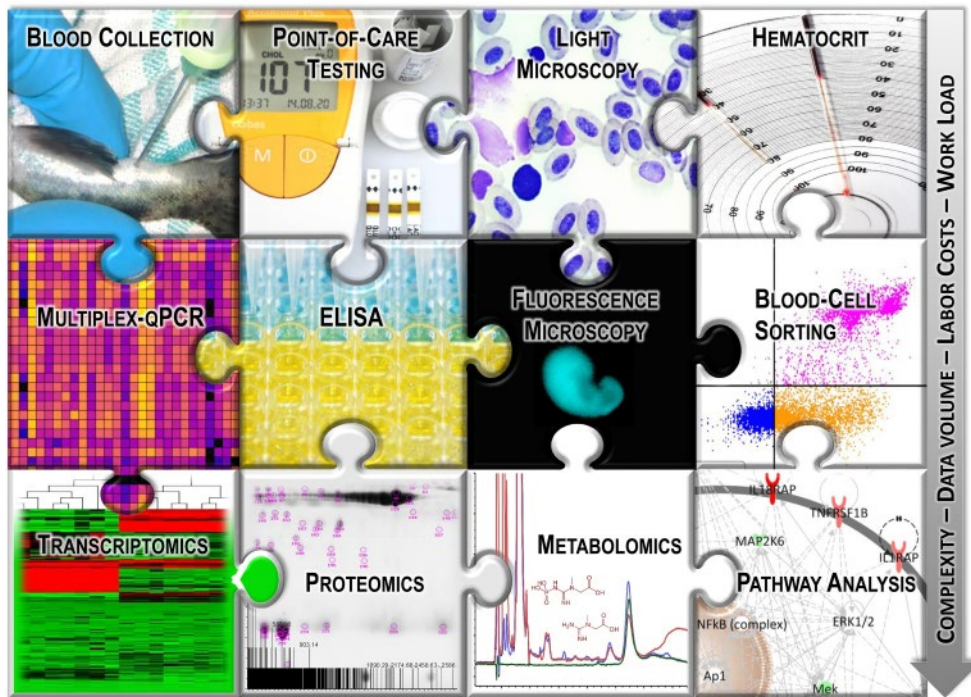


Figure II-1 Graphical overview of the selected analysis techniques used with fish blood.

Laboratory data, such as gene expression studies (30, 38, 39), are based on RNA specimens that are mostly obtained from the organs of previously killed fish. By contrast, fish blood collection can be conducted non-lethally (11); therefore, blood represents an alternative and preferable matrix for ethical reasons. Though relatively non-invasive, blood sampling is generally stressful for fish, but repeated blood sampling from the same individual provides the possibility of tracking the time course of various processes after the treatment or determining fish welfare during the developmental stages. Other operational parameters, such as the monitoring of exploratory and swimming behavior (40, 41) or the rate of gill ventilation (42), can readily be recorded on commercial farms, even those that lack the well-equipped wet laboratories.

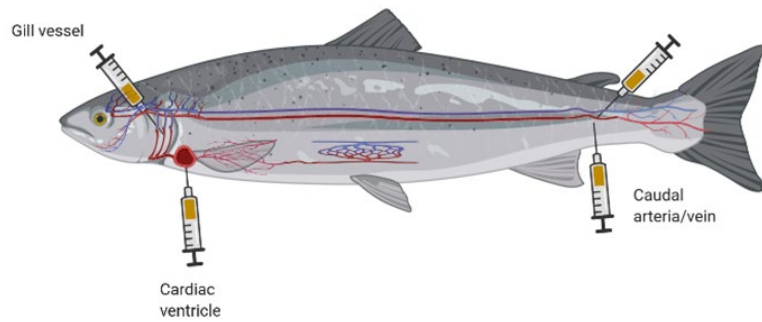
This review compiles the most commonly reported techniques for obtaining chemico-physical, cellular, metabolic, and transcriptional information from fish blood. It also provides interpretations of the various methods in terms of their importance in assessing the impact of changing environmental conditions or experimental treatments and the intensity and duration of the response of fish.

## ANESTHESIA AND BLOOD SAMPLING PROCEDURES

For gentle handling and for safety reasons, fish should be sedated prior to and during blood sampling to minimize pain or discomfort and to prevent defensive or flight reactions and subsequent injuries. An analgesic alone may mask the sensation of pain but will not prevent the perception of subsequent treatments. A loss of consciousness, therefore, ensures the welfare of the fish during blood collection. The most commonly used anesthetic is MS-222 (also known as Tricaine-S or 3-ethoxycarbonylanilinium methanesulfonate) (43), although clove oil (containing eugenol, 4-allyl-2-methoxyphenol), quinaldine (2-methylquinoline), 2-phenoxyethanol, and benzocaine (ethyl 4-aminobenzoate) are also commonly used to stun fish (44). The legal provisions of a given country determine the circumstances that justify the use of particular fish anesthetics. Anesthetics are usually administered as an immersion bath, but the capture of the fish and its transfer to the anesthetic bath, with the brief exposure to air, are likely to evoke a stress reaction (45, 46). Therefore, these steps should be carried out quickly. The anesthetic per se can also act as a stressor on the individual fish being treated.

MS-222 blocks the sodium channels and action potentials of neurons, but concentrations of up to 300 mg MS-222 per liter were shown to have no significant impact on the plasma cortisol levels of South American silver catfish (*Rhamdia quelen*) (47). By contrast, gilthead sea bream (*Sparus aurata*) showed significantly increased cortisol levels following the exposure to even 25 mg/L MS-222 or 0.075 mg/L 2-phenoxyethanol (48). In zebrafish, 15 mg/L of buffered MS-222 altered various hematological characters, including hematocrit, coagulation, and the amount of blood collected (49). The influence of a given anesthetic on the biochemical profile of a sample obviously depends on the dose and on the treated species. These considerations should therefore be taken into account during the evaluation of fish blood parameters. Nevertheless, the anesthesia certainly facilitates the blood collection and prevents a more pronounced stress response, so these advantages may outweigh the potential difficulties related to stress diagnostics.

Optimal blood collection depends on the size and anatomical characteristics of the investigated individual (11). The least traumatic and most widely used blood sampling procedure is to withdraw blood from the caudal vessels—laterally or ventrally—using a cannula and syringe filled with an anticoagulant (sodium citrate, heparin, or potassium ethylenediaminetetraacetic acid) (Figure II-2). Some analyses require quite large amounts of blood, which creates difficulties when the blood is collected from small teleosts with an estimated whole-blood volume of 3–7 ml per 100 g (50–52). Fish smaller than 8 cm in length are difficult to bleed, but one option is to sever the fin of an anesthetized small fish and then centrifuge the fish at low force (14). Larger teleosts may also be bled by puncture of the cardiac ventricle or other vessels, such as gill-blood vessels (Figure II-2) (53).



Created in BioRender.com 

Figure II-2 Schematic illustration of different cannula placements for blood collection from fish (created with BioRender.com).

These blood sampling techniques are reserved for studies aimed at answering distinct research questions (54), as they bear a high risk of late complications. In this context, a point worth noting is that the site of blood collection often influences the obtained biomarker profile (55). More detailed information on blood collection techniques has been provided in recent review articles (45, 56). After blood sampling, the fish should be transferred to an aerated recovery tank to allow it to regain perception, awareness, and ability to react. Immediate return of a fish to its usual environment poses a risk that it may be attacked by its tank mates if it remains in a state of non-reactivity.

The liquid part of the blood—the plasma fraction—transports diverse metabolites of interest, including hormones and catabolic products, such as proteins, amino acids, glucose, lipids, and organic acids. For this reason, many researchers separate the plasma via centrifugation with an anticoagulant or obtain the serum after blood clotting and subsequent centrifugation (57). Hence, plasma contains natural coagulation factors, whereas serum contains all plasma components except the coagulation factors. Thus, the choice of using plasma or serum depends largely on the research question and its related need for coagulation factors.

## IMMUNOASSAYS AND CLINICAL TEST KITS

Many studies that have evaluated fish welfare in aquaculture have revealed that aversive husbandry conditions alter the stress-hormone levels in the farm fish. Stressors and adverse conditions can trigger the chromaffin cells of the head kidney to rapidly release catecholamines, such as noradrenaline/norepinephrine and adrenaline/epinephrine. These reactions may also occur in direct response to the catching process, the subsequent anesthesia, and/or the blood sampling, so they complicate the evaluation of experimentally induced challenges. However, the hypothalamus generates a parallel production of corticotropin-releasing hormone (CRH), which then stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH) (19, 58, 59). This is followed by the synthesis and secretion of cortisol by the interrenal tissue in the head kidney. Many scientists prefer to measure cortisol or ACTH as blood-based indicators of acute stress responses due to the slow increase (within minutes) of these compounds.

Cortisol is probably the most commonly measured molecule for determining the effects of stress in fish (16, 35). Cortisol and ACTH are commonly measured using radioimmunoassays (RIAs). Typically, RIA incorporates a radioactive iodine isotope due to its decay properties. However, the higher technical effort required in an isotope laboratory has led to an increasing preference for ELISAs (60) as an alternative non-radioactive method for the cortisol and ACTH measurements. Both RIAs and ELISAs are antibody-based detection methods, but ELISAs use specific antibodies that bind to the substance (antigen) and rely on an enzymatic color reaction for antigen detection. Electrochemical immunoassays (61), gas or liquid chromatography (62), mass spectrometry (63), and fluorescence-based methods (64) are alternatives to RIA and ELISA for determining blood cortisol levels.

Increased cortisol levels stimulate a number of metabolic processes, such as gluconeogenesis and gluconeogenesis, to mobilize and allocate energy reserves (e. g. glucose release into the bloodstream) (21). Increased, as well as decreased, glucose levels are considered signs of stress (Table II-1) (88), as stress-induced alterations in muscle activity accelerate the conversion of glucose to lactate (or alternatively to pyruvate) by anaerobic glycolysis. The resulting plasma levels of lactate and glucose are often used in conjunction with cortisol levels to establish the physiological state and to assess the severity of a stress response (9, 87, 99–101). Some studies have demonstrated that even the removal of an individual *S. aurata* fish evoked an acute stress reaction in the remaining fish in the tank. The plasma cortisol levels in the tank fish returned to the initial level after 8 h, whereas the plasma glucose and lactate levels and other hematological parameters required 24 h for recovery (102). The plasma triglycerides or cholesterol levels can also reflect the metabolic changes and serve as physiological indices (86), as these lipids can be used as alternate sources of energy by the fish (103–105). Blood glucose, lactate, and triglyceride levels can be determined easily and quickly using appropriate cuvette tests or test strips in portable devices designed for clinical test kits (also known as point-of-care tests) (76). The advantages of point-of-care tests are their time-saving and simple operation, with their reliable and easily readable results.

Changes in cortisol levels indicate the magnitude of a primary stress response, but usually do not provide sufficient information to gauge the ability of an animal to cope with a challenging condition (106). Notably, cortisol levels can vary widely among individuals in response to diurnal or seasonal fluctuations and the ambient temperature (107–109) or according to gender and maturity (58, 106, 110, 111). The cortisol level depends on many factors that can have diverse interactions (29). In optimal cases, the cortisol level rises in correlation with the intensity of a stressor and returns to its baseline level if the stressor does not persist. Multiple stressors occurring simultaneously or persistent chronic stress conditions can complicate the interpretation of a measured cortisol level, as cortisol release might be suppressed by feedback interactions through the activated stress axis. In particular, differences in treatments can complicate comparisons between experiments (112); therefore, we recommend measuring other parameters of the secondary and tertiary stress response to establish the ability of the animal to cope with stressors and to assess its well-being.

## HEMATOLOGICAL PROFILING AND BLOOD CELL SORTING

The original technique for obtaining a differential blood count is simple and relatively inexpensive, but time-consuming and tedious (113). Hematological evaluations are usually based on blood smears and require a sound knowledge of blood cell morphology.

The vast majority of blood cells are erythrocytes, which ensure a sufficient supply of oxygen in the various body tissues. Metabolic alterations associated with physical work, excitement, and stress responses increase the tissue oxygen requirements, so large numbers of erythrocytes are additionally recruited and mobilized from depots in the spleen (114). For instance, rainbow trout (*Oncorhynchus mykiss*) responded to a 2-month food supplementation with roselle (*Hibiscus sabdariffa*) meal by significantly increasing the number of erythrocytes in the blood and simultaneously lowering the blood cortisol and glucose levels (92). By contrast, Indian major carp (*Labeo rohita*) exposed to water 6°C warmer than usual showed significantly increased glucose levels and a reduction in erythrocyte counts (115). A recent examination of the erythrocytes from grass carp (*Ctenopharyngodon idella*) did not reveal any significant alteration in numbers after 20 days of exposure to polystyrene nanoplastics, but numerous abnormalities were observed regarding the shape and size of the cells and their nuclei (95).

Adverse environmental conditions can affect the numbers and shapes of circulating erythrocytes, but they can also change the composition of leukocytes in circulation (58, 116, 117). Leukocytes are generally subdivided into monocytes, lymphocytes, and granulocytes (118). Granulocyte staining with Romanowsky/Wright or May–Grünwald–Giemsa stains can differentiate the eosinophilic, basophilic, and neutrophilic types (119, 120) that occur in tetrapods. Some fish species possess heterophilic granulocytes that are characterized by the presence of additional eosinophilic granules (121, 122).

As reported in other vertebrates (123), the ratios of certain leukocyte populations in fish blood can provide insights into the response to defined treatments or environmental variables. Stress hormones inhibit the proliferation of lymphocytes (58), the apoptosis of granulocytes (124), and the emigration of monocytes and neutrophilic granulocytes from the hematopoietic tissue of the head kidney into the peripheral blood (125). A high number of circulating leukocytes (leukocytosis) can therefore reflect an increased number of monocytes (monocytosis) and neutrophilic/heterophilic granulocytes (neutrophilia) and a concomitant decrease in the number of lymphocytes (lymphopenia) (126). The resulting dysregulation of the immune system can lead to a persistent inflammatory state in fish (127).

Although the neutrophil/heterophil-to-lymphocyte ratio is considered as an important indicator of distress across vertebrate species, it does not necessarily correlate with stress-hormone levels (128). The delayed mobilization of immune cells following the cortisol peak has been regarded as a mechanism that enables the immune system to respond once the primary threat has been overcome (129). The response of the neutrophil/heterophil-to-lymphocyte ratio to long-term environmental stressors is detectable over relatively long periods of time, in contrast to the temporary increase in hormone levels (130). Infections also have a decisive influence on the proliferation and trafficking of leukocytes (131), and the coincidence of stress and immune responses can have an antagonistic effect on the ultimate number of peripheral leukocytes (132).



Differential blood counts have been used to assess the effects of acute and chronic stress events, such as heavy metal exposure in common carp (*Cyprinus carpio*) (133), organochloride herbicide exposure in African catfish (*Clarias gairepinus*) (134) or crowding in Atlantic salmon (*Salmo salar*) (135). Nevertheless, conducting a differential blood count in fish is not very common in clinical laboratory diagnostics, often because reference values are missing.

Flow cytometry is an alternative method for studying the blood cell composition and has the advantage of high sample throughput. In the flow cytometer, individual blood cells successively pass by a laser beam, and the light they scatter is characteristic for a specific blood cell population, allowing their separation. The use of specific antibodies facilitates a more precise determination of the proportions of specific immune cell subpopulations in the blood. Fish cell sorting has depended more on cell characteristics, such as size and granularity, because of a general lack of fish-specific antibodies except for a few model fish species (7, 136).

Similar alterations in blood cell composition have been studied in different fish species exposed to various types of stress (137). For instance, in channel catfish (*Ictalurus punctatus*), the number of circulating neutrophil granulocytes increased after transportation stress (138), while the overall number of leukocytes (including lymphocytes) decreased. Another study on *I. punctatus* confirmed the occurrence of the previously observed neutrophilia simultaneously with decreasing numbers of peripheral B-lymphocytes in response to handling and transportation (Table II-1; 65). In Gulf killifish (*Fundulus grandis*) and sea trout (*Cynoscion nebulosus*), the response to crude-oil pollution was characterized by a significantly decreased number of circulating lymphocytes and an increased number of monocytes and eosinophilic granulocytes, respectively (139). Maraena whitefish (*Coregonus maraena*) exposed to high stocking densities (100 kg/m<sup>3</sup>) showed strong increases in the numbers of myeloid cells (granulocytes, monocytes, myeloid dendritic cells, and mast cells), whereas the number of thrombocytes was significantly reduced (Table II-1; 32). Overall, different types of stress apparently trigger an increased mobilization of myeloid cells and a reduction in the levels of lymphocytes in the circulation of the affected fish.

Table II-1 Overview of the selected physiological parameters that change in blood, plasma, or serum in response to stressful conditions in various fish species; Refer = Reference

Stressor/ condition	Species <sup>a</sup>	Duration of challenge	Putative indicator	Direction	Refer.
<b>Handling:</b>					
Transportation	<i>I. punctatus</i>	20 min	Circulating granulocytes and T cells	Up	(65)
Transportation	<i>I. punctatus</i>	20 min	Circulating B cells	Down	(65)
Catching, sorting, transportation	<i>R. rutilus</i> , <i>A. brama</i>	3 h	Sodium-ion contents	Down	(66)
Handling (15 s)	<i>S. salar</i>	2 weeks	O-phosphocholine, lactate, carbohydrates, alanine, valine, trimethylamine-N-oxide	Up	(67)
Handling (15 s)	<i>S. salar</i>	4 weeks	Di-O-acetylated sialic acids	Up	(68)
Transfer to another tank	<i>S. trutta</i>	1 min	Cortisol, glucose, lactate	Up	(69)
Aerial exposure	<i>H. sabinus</i>	30 min	Lactate, acidosis, glucose	Up	(9)
Aerial exposure	<i>S. senegalensis</i>	3 min	Cortisol, glucose, lactate, osmolality levels	Up	(70)
"Hook and line" stress <sup>b</sup>	<i>O. mykiss</i>	2 min	Cell counts, hematocrit, glucose, thrombocytes	Up	(71)
"Hook and line" stress <sup>b</sup>	<i>O. mykiss</i>	2 min	Clotting time	Down	(71)
Manual stripping procedure	<i>O. mykiss</i>	5 days	Cortisol, <i>TNF</i>	Up	(72)
<b>Temperature:</b>					
16°C	<i>G. morhua</i>	5 days	<i>B2M</i> , <i>MHC-I</i> , <i>IGL</i> mRNA	Up	(73)
20°C	<i>C. catla</i>	12 h	<i>TLR2</i> , <i>-4</i> , <i>-5</i> and <i>NOD1</i> , <i>-2</i> mRNA	Up	(74)
23°C	<i>D. labrax</i>	12 h	Cortisol, glucose, superoxide dismutase activity	Up	(75)
30°C	<i>P. mesopotamicus</i>	5 days	Glucose	Up	(76)
35°C	<i>C. catla</i>	12 h	<i>TLR2</i> , <i>-4</i> , and <i>NOD1</i> , <i>-2</i> mRNA	Up	(74)
36°C	<i>R. holubi</i>	7 days	Cortisol	Up	(28)
37°C	<i>E. coioides</i>	1 h	Immunoglobulin M	Up	(77)
<b>Oxygen saturation:</b>					
0.3 ppm	<i>O. niloticus</i>	3 days	Heat-shock protein 70	Up	(78)
1.0 ppm	<i>C. catla</i>	1 h	<i>HMBG1</i> , <i>TLR4</i> , <i>MYD88</i> , <i>NOD1</i> , <i>RICK</i> , <i>IL6</i> , <i>CXCL8</i> , <i>IL10</i> mRNA	Up	(79)
1.3 ppm	<i>S. aurata</i>	> 11 h	Hematocrit, hemoglobin, glucose, Lactate	Up	(80)
1.3 ppm	<i>S. aurata</i>	> 11 h	<i>UCP2</i> mRNA	Down	(80)

Table II-1 continued

Stressor/ condition	Species <sup>a</sup>	Duration of challenge	Putative indicator	Direction	Reference s
1.3 ppm	<i>S. aurata</i>	4 h	<i>GST3, UCP2, ATPAF2, SCO1, MIRO1a, TIM44, TIM10, ACAA2</i> mRNA	Down	(81)
1.3 ppm	<i>S. aurata</i>	4 h	Glucose, lactate, hematocrit, hemoglobin	Up	(81)
2.3 ppm coupled with stocking of 19 kg/m <sup>3</sup>	<i>S. aurata</i>	22 days	<i>NDUFAF2</i> mRNA	Down	(82)
2.3 ppm coupled with stocking of 9.5 kg/m <sup>3</sup>	<i>S. aurata</i>	22 days	Hematocrit, growth hormone, lactate, erythrocyte number	Up	(82)
<b>Stocking:</b>					
30 kg/m <sup>3</sup>	<i>S. salar</i>	10 weeks	Alkaline phosphatase	Up	(83)
30 kg/m <sup>3</sup>	<i>S. salar</i>	10 weeks	Immunoglobulin M	Down	(83)
30 kg/m <sup>3</sup>	<i>S. salar</i>	10 weeks	Cortisol	Up	(83)
30 kg/m <sup>3</sup>	<i>S. salar</i>	10 weeks	Maleic dialdehyde	Up	(83)
34 kg/m <sup>3</sup>	<i>S. aurata</i>	15 weeks	Cortisol, plasma proteins, hematocrit, hemoglobin, erythrocyte number	Up	(84)
40 kg/m <sup>3</sup> , 80 kg/m <sup>3</sup>	<i>O. mykiss</i>	9 months	Cortisol	Down	(85)
45 kg/m <sup>3</sup>	<i>O. mykiss</i>	1 month	Cholesterol	Down	(86)
45 kg/m <sup>3</sup>	<i>O. mykiss</i>	1 month	Glucose	Up	(86)
45 kg/m <sup>3</sup>	<i>O. mykiss</i>	1 month	Triglyceride	Down	(86)
70 kg/m <sup>3</sup>	<i>O. mykiss</i>	2 days	Lactate	Up	(87)
100 kg/m <sup>3</sup>	<i>C. maraena</i>	9 days	Circulating myeloid cells	Up	(32)
100 kg/m <sup>3</sup>	<i>C. maraena</i>	9 days	Circulating thrombocytes	Down	(32)
120 kg/m <sup>3</sup>	<i>S. fontinalis</i>	1 month	Glucose	Down	(88)
<b>Nutrition:</b>					
Food deprivation	<i>O. mykiss</i>	28 days	Very-low-density lipoproteins	Up	(89)
Food deprivation	<i>O. mykiss</i>	28 days	High-density lipoprotein, choline, β-glucose, lactate	Down	(89)
Plant-based diet with yeast fraction	<i>O. mykiss</i>	84 days	Histidine	Down	(90)
Food supplementation with menthol oil	<i>O. niloticus</i>	15 days	Hematocrit, erythrocytes, leukocytes, globulin and albumin content, protein concentration, lysozyme and phagocytic activity	Up	(91)
Food supplementation with roselle powder	<i>O. mykiss</i>	60 days	Erythrocytes, hematocrit, activities of superoxide dismutase and catalase	Up	(92)
<b>Pollution:</b>					
Metallic/organic compounds	<i>D. labrax</i>	15 days	Glucose, cortisol, superoxide dismutase activity	Up	(75)
Oxytetracycline	<i>O. mykiss</i>	14 days	Sodium dismutase, erythrocyte and leukocyte number	Down	(93)

Table II-1 continued

Stressor/ condition	Species <sup>a</sup>	Duration of challenge	Putative indicator	Direction	Reference s
Nitrite	<i>P. fulvidraco</i>	4 days	Sodium- and chloride-ion contents, erythrocyte number, hemoglobin, total antioxidant capacity, activities of superoxide dismutase and catalase and glutathione peroxidase	Down	(94)
Nitrite	<i>P. fulvidraco</i>	4 days	Sodium- and chloride-ion contents, leukocyte number, malondialdehyde content	Up	(94)
Polystyrene nanoplastics	<i>C. idella</i>	20 days	Erythrocyte nuclear abnormalities, altered erythrocyte morphometry	Up	(95)
<b>Other environmental conditions:</b>					
Low-dose ultraviolet B radiation	<i>C. carpio</i>	6 weeks	Total protein concentration, oxidative burst activity	Down	(96)
Low-dose ultraviolet B radiation	<i>O. mykiss</i>	6 weeks	Oxidative burst activity, cortisol, lymphocyte number	Up	(96)
High CO <sub>2</sub> levels	<i>H. hippoglossus</i>	14 weeks	Complement C3, fibrinogen	Up	(97)
Low salinity	<i>D. labrax</i>	12 h	Glucose, cortisol, hemoglobin, peroxidase and superoxide-dismutase activity	Up	(75)
Open field (absence of shelter)	<i>B. episcopi</i>	2 min	Cortisol	Up	(98)

## HEMATOCRIT MEASUREMENTS

As outlined in the previous section, the stress-related secretion of cortisol might provoke contractions of the fish spleen to induce the release of stored erythrocytes into the peripheral blood (114). This would then elevate the volume percentage of erythrocytes, also referred to as the hematocrit value. Along with the hemoglobin (Hb) content and the leukocyte count, the hematocrit is regarded as a key indicator of the secondary stress response. The hematocrit measurement is easy and relatively inexpensive, as the collected whole blood is simply centrifuged in heparinized micro-hematocrit capillaries that are then read off a measuring template (140). A substantially greater effort is required to establish leukocyte profiles; consequently, hematocrit measurements are about 50 times more common in fish studies, as evident from our recent literature search in the Web of Science.

One important aspect for fish physiologists is the association between the hematocrit and the blood viscosity (141, 142). Wells and Weber measured the blood viscosity in *O. mykiss* kept under stressful conditions and found that a 30% hematocrit indicates an optimal relative oxygen transport capacity in the presence of a blood viscosity with a variable hematocrit but a constant Hb concentration (142). The hematocrit in *S. salar* ranges between 44 and 49% (143); however, the levels depend on the temperature (115,

144), fish strain (145), diet (91), and body weight. Large, active fish generally have a high muscular oxygen demand, which can lead to a stimulation of erythropoiesis in the head kidney (146, 147). Accordingly, the physiological hematocrit differs between fast-swimming pelagic fish and fish living sedentarily or in benthic habits (148).

Anesthesia can increase the hematocrit (149), while malnutrition, infection, or environmental toxins can reduce hematocrit and Hb values in fish (150). Non-physiologically low hematocrit values are considered hallmarks of anemia, a specific pathophysiological stress response. Anemia in fish can be easily diagnosed by examining the gills, although more detailed blood analyses help to identify the cause of anemia (53). A significantly reduced hematocrit and an increased erythropoietin production, for instance, can be observed with experimental hemolysis induced by the hemolytic compound phenylhydrazine in *S. salar* (151).

The hematocrit and Hb values associated with erythrocyte count can also depend on the electrolyte–water balance of the fish blood (152). Stressed freshwater fish undergo a drop in their plasma sodium concentrations, and this drop activates counter-transporting ion channels on the erythrocyte membranes (66). The rising ion concentration then induces an inflow of water, causing the erythrocytes to swell and increase their binding capacity for oxygen. These responses are accompanied by the release of additional erythrocytes from splenic stores to compensate for the increased oxygen demands.

## MEASUREMENTS OF OSMOLALITY AND ION CONTENTS

The electrolyte–water balance in the body is termed its osmolality. The plasma osmolality and osmotic regulatory capacity are measured with an osmometer, whereas a spectrophotometer can identify the contributing ions; these are predominantly sodium, chloride, calcium, magnesium, potassium, and phosphate (46).

Freshwater fish species are hyperosmotic in relation to their habitat, whereas marine fishes are hypo-osmotic to the surrounding sea. The resulting differential osmotic pressures force teleost fishes to undergo continuous osmoregulation. Water and ions are exchanged primarily via the skin, gills, intestines, and kidneys (52, 153, 154), though freshwater and marine fishes have developed different strategies to maintain their internal blood osmolality within narrow limits. Osmoregulation is a persistently energy-intensive process, even in the absence of additional stress. However, since stress hormones control both the hydromineral balance and energy metabolism in fish, variations in the osmolality of the blood plasma, including changes in the ion composition, are part of the secondary stress response (155, 156). In general, aversive conditions decrease the osmolality in freshwater fish and increase osmolality in marine fish (Table II-1) (59, 69, 70, 145, 157, 158). The exposure to high nitrite concentrations, for example, caused a significant reduction in sodium and chloride ion contents in the blood of the freshwater species yellow catfish (*Pelteobagrus fulvidraco*) (94). Catching and a subsequent 3-h-long transportation of freshwater roach (*Rutilus rutilus*) and common bream (*Abramis brama*) reduced the level of plasma sodium by one-third (66). By contrast, the osmolality significantly increased in the plasma of the marine Japanese flounder (*Paralichthys olivaceus*) after the acute exposure to air (159).

Adverse environmental conditions (e. g. hypoxia, which is often associated with high ambient temperature) require an increased branchial activity to enhance the uptake

of oxygen. In (hypotonic) freshwater, this hyperventilation accelerates the loss of osmolytes. The conflicting demands of osmoregulation and respiratory gas exchange cause an increased oxygen uptake and loss of ions from the plasma in freshwater fishes. This concept of the “osmorepiratory compromise” has been well-researched in salmonids (160, 161) and hypoxia-tolerant and euryhaline fishes (162). The salinity level (162, 163), the species-specific cellular gill architecture (164), and various extreme environmental variables (165) influence the osmoregulatory ability of fishes under stressful conditions and can maintain physiological osmolality (166, 167) and ion levels (168) in response to diverse challenges in freshwater and saltwater species in their native osmotic environments. For example, ion concentrations were unaffected in Pacific hagfish (*Eptatretus stoutii*) exposed to hypoxia (169) or in Gulf toadfish (*Opsanus beta*) stimulated with spill oil (170).

Some fishes compensate relatively quickly for increased ion flux rates within a certain range; therefore, ion concentrations and osmolality can serve as suitable indicators of acute environmental stressors (140). On the other hand, both parameters change less rapidly compared to the dynamics of stress-hormone levels, making this difference advantageous for recording post-stress responses.

### **ASSESSMENT OF THE HUMORAL IMMUNE CAPACITY**

The response to distinct external signals involves the neuroendocrine system and the immune system (171). For decades, researchers have extracted various immune-relevant parameters from fish blood and evaluated their potential as indicators for compromised homeostasis (172). The central question addressed by these studies is the extent that stress and related adaptive responses influence immunocompetence in fish.

An initial test for assessing immunocompetence is to record the bacterial growth rate in blood plasma from treated vs. control fish (173–176). For example, the growth of the bacterium *Aeromonas salmonicida* after a 24-h incubation was significantly enhanced in plasma from *O. mykiss* with impaired immune capacity due to the exposure to high temperature coupled with crowding (177). By contrast, the non-stressed fish clearly showed potent bactericidal mechanisms that depended mainly on the concentration of a range of immunocompetent macromolecules known as humoral factors. These humoral factors consist of antimicrobial peptides, antibodies, and complement components (178) that circulate in the body fluids. Many fish physiologists have therefore examined the activity of these specific humoral factors rather than the general bactericidal activity of the plasma.

The bactericidal enzyme lysozyme and the microbe-clearing complement components are important humoral molecules of the teleostean innate immune defense (179, 180) and are frequently used as non-specific immune markers (181). In fish, lysozyme has a broader activity than its mammalian counterpart, and several complement components are present as multiple isoforms in bony fishes (182). Lysozyme and complement components are mainly synthesized by leukocytes and the liver, respectively (183–185), but they are secreted into the blood to eliminate invasive pathogens and harmful agents. However, the actual concentrations of the multiple immune factors that are synthesized and secreted into the blood of various fishes at different stages of an infection process or during a challenging situation are not known. Therefore, the measured values

for supposedly one factor could actually represent multiple variants and should be interpreted with caution.

The activity of lysozyme and the complement system are measured using turbidimetric assays (186), lysoplate assays (187), lysorocket electrophoresis (188), or microplate assays (189). These assays have demonstrated, for example, that acute handling increased the activity of lysozyme in *O. mykiss* (181) and goldfish (*Carassius auratus*) (190). By contrast, lysozyme activity decreased in Siberian sturgeon (*Acipenser baerii*) after heat stress (189), in sheatfish (*Silurus glanis*) after exposure to intense halogen light (191), in Nile tilapia (*Oreochromis niloticus*) after exposure to the pesticide chlorpyrifos (91), and in *O. mykiss* after transport and exposure to chemicals (192, 193) or low stocking densities (85). Subordinate *O. niloticus* individuals had lower lysozyme activity levels than their dominant conspecifics (194). Overall, stress seems to dampen the lysozyme activity in fish (193).

The influence of stress on the complement system is ambiguous. For example, heat-stressed *A. baerii* (189) and acutely stressed *S. aurata* (195) showed increased complement activity, whereas this activity decreased in European bass (*Dicentrarchus labrax*), red porgy (*Pagrus pagrus*), and *S. aurata* exposed to crowding (84, 196–198). The complement gene families are expanded in several fishes (180, 185), and this has often been associated with newly acquired or partitioned functions of the original complement factors. Consequently, complement-involving events are likely to be more complex than indicated by the snapshot provided by a complement test result.

Other parameters have also proven significant for the assessment of fish welfare. These include the coagulation capacity of the blood (71), the antibody titer (83, 96), the phagocytic activity (32, 91), or the oxidative burst (199, 200), including the activities of the myeloperoxidase (201), superoxide dismutase (75, 202), glutathione peroxidase (93, 94), or glutathione reductase (152). Together with the bactericidal activity, these parameters provide valuable downstream information about the effects of stress hormone release on the immune performance under challenging conditions. Hormones are also likely to induce rather subtle changes in the activity of these immune parameters, either daily or seasonally, and between the sexes (203–205). Most of these analyses are carried out *ex vivo* within a limited time after the previous collection. The oxidative burst assay or phagocytic tests can also be performed *in vitro* on cultured cells (206).

## IN VITRO TESTS ON PRIMARY BLOOD CELL CULTURES

The development of *in vitro* systems has allowed testing of the effect of certain stressors or challenging conditions delivered in defined doses, intensities, time frames, and/or time points. The dominant model systems for primary fish cell cultures are still derived from the head kidney and spleen (207–209); however, many protocols for blood cell cultures from different fish species have been established (210–213). These models are mainly used to investigate the influence of microbial structures/vaccines and viruses on particular immune cascades (212, 213). In addition, blood cells from fish are useful for investigating the influence of drugs or environmental toxins on the cellular homeostasis. For example, low concentrations of a halogenated hydrocarbon (once used as the insecticide lindane) were shown to increase intracellular calcium levels in peripheral blood leukocytes of *O. mykiss*, whereas high concentrations reduced the synthesis of vital cytokines and induced cell

death (214). The polycyclic aromatic hydrocarbon 3-methylcholantrene stimulated the proliferation of blood leukocytes from *C. carpio* but inhibited the lymphocyte proliferation in response to immunostimulants (215). Similarly, the toxin microcystin-LR (produced by cyanobacteria) and bisphenol A (the monomer component of polycarbonate plastics) modulated the proliferation of lymphocytes isolated from the blood of *O. mykiss* (216) and *C. auratus* (217), respectively. Leukocytes from *C. carpio* subjected to the “alkaline comet assay” have been used to assess the genotoxic potential of organic sediment by determining the DNA damage (218).

Apart from these toxicological studies, blood cell cultures are actually not suitable for modeling aquaculture-relevant problems, such as malnutrition or stocking density stress. For this reason, generalizing the data obtained from in vitro systems to an entire organism or a population is controversial, as the response to environmental stress is usually systemic and involves complex cellular networks and tissue systems that communicate with each other via stress-inducible mediators, such as steroids and amines. Only a few studies have investigated how stress hormones affect events like the in vitro proliferation of blood cells (219). The further development of three-dimensional cultures from fish cells (220) will bring in vitro data one step closer to their practical use as fish model systems.

## EXPRESSION PROFILING OF SELECTED GENES IN BLOOD CELLS

Quantitative PCRs (qPCRs) detect the smallest alterations in the expression of genes (221) that are subject to modulation by environmental challenges or stressors. Importantly, gene expression profiles rarely allow absolute statements about the functioning of biological systems, for a number of reasons. One is that many genes are not completely switched on or off in response to a specific treatment or under certain environmental conditions. Instead, most treatments induce a stronger (upregulated) or a reduced (downregulated) expression of a distinct set of genes, and these expression changes only become evident when treated cells are compared with an untreated matching control. One case in point is the expression of potential thermal indicator genes that correlate with the well-studied response of many fish to suboptimal water temperatures (189). Heat stress is well-known to induce the expression of certain heat-shock protein (HSP) genes, such as HSP70 (HSP1A1) and HSP90 (HSP90AA1) (189, 222, 223), whereas hypothermia also induces the copy number of HSP90 in the blood of *C. carpio* (224). The HSP-encoding transcripts have also been proposed as indicators of the potentially destructive effects of environmental toxins and pollutants. For example, the level of HSP90 copies dropped almost by half in the blood cells of *C. carpio* exposed to cadmium for 24 h (224). By contrast, the abundance of HSPA8 copies increased, together with the HSP70 level, in silver sea bream (*Sparus sarba*) exposed to sublethal concentrations of cadmium for only 2 h (225).

Several investigations have also demonstrated that thermal stress modulates the expression of immune genes in the blood cells of different fish species. In particular, cytokine-encoding transcripts appear to mirror the immune status during stress (226). The classic cytokines, such as interleukins (IL), tumor necrosis factor  $\alpha$  (TNF), interferon (IFN), and transforming growth factor (TGF), are relevant in this context (72, 226, 227). The head kidney, spleen, and liver are the usual tissue choices for quantifying immune-relevant



transcripts in stimulated or stressed fish, whereas the skin, gills, and blood are used to detect impaired homeostasis. For instance, a temperature study exposed Atlantic cod (*Gadus morhua*) to water temperatures rising from 10 °C to 16 °C or 19 °C and reported slightly increased plasma glucose and cortisol levels (73). In parallel, upregulated expression was observed for the genes encoding interleukin-1 $\beta$  (IL1B),  $\beta$ 2-microglobulin (B2M), major histocompatibility complex class I (MHC-I), and immunoglobulin M light chain (IGL) in leukocytes of the thermally challenged *G. morhua* (Table II-1). These findings were partly confirmed by a report of increased IgM-transcript levels in blood cells of orange-spotted grouper (*Epinephelus coioides*) after heat shock (77). The Indian major carp (*Catla catla*) exposed to temperatures above and below the optimum temperature of 25 °C showed a significant increase in blood expression levels of immune genes coding for toll-like receptors (TLR2, -4, -5) and nucleotide binding oligomerization domain containing proteins (NOD1, -2) (Table II-1; 74).

An expanded set of immune genes was profiled in *C. catla* exposed to an oxygen saturation below 3 ppm for 1 h (79). The increased expression of the genes coding for the transcriptional regulator high-mobility-group-box-1 protein HMBG1, the receptors TLR4 and NOD1, and their associated adapter proteins myeloid differentiation primary-response protein 88 (MYD88) and receptor interacting serine/threonine kinase 2 (RIPK2), as well as the cytokines IL6, CXCL8, and IL10 suggested an activation of early innate immune mechanisms by hypoxia (Table II-1). Other hypoxia studies reported that considerably fewer genes were regulated in fish blood cells, and most were downregulated. For example, a 1 h exposure of *S. aurata* to hypoxic conditions with an oxygen saturation of 1.3 ppm increased hematocrit, Hb content, glucose and lactate levels, but the level of uncoupling protein 2 (UCP2) transcripts in the blood cells was strongly decreased (Table II-1; 80). Another research group also investigated *S. aurata* under similarly acute hypoxic conditions but with an extended set of qPCR assays (81). They reported a significant downregulation of UCP2 along with reduced levels of transcripts coding for antioxidant enzymes (GST3), outer and inner membrane translocases (TIM44 and TIM10), respiratory enzyme subunits (SCO1 and NDUF2), and also markers of mitochondrial dynamics (MIRO1a) and fatty acid  $\beta$ -oxidation (ACAA2) (Table II-1). A subsequent experiment by the same group exposed *S. aurata* to a lowered oxygen saturation of 2.3 ppm combined with crowding (82). The slightly higher oxygen concentration (compared with the previous hypoxia experiment) or an antagonistic effect of hypoxia and density stress was proposed as reasons for the unexpected lack of modulation of either UCP2 or 42 other profiled genes (Table II-1). The exception was NDUF2 (Table II-1), which codes for an assembly factor of the Nicotinamide Adenine Dinucleotide - Hydrogen (NADH): ubiquinone oxidoreductase complex and had been included in the list of differentially regulated genes in their previous hypoxia experiment.

Gene profiling can significantly extend the list of stress response parameters beyond hematological, immunological, and metabolic types by identifying negative biomarkers whose values drop below the control levels (39, 80). A key advantage of gene profiling over the detection of stress hormones is that the stressful events that occur immediately prior to sampling (e. g., due to the capture, stunning, and killing of the animal) are usually not reflected in altered transcript levels, while the levels of ACTH or cortisol increase promptly (see section Immunoassays and Clinical Test Kits). Stress hormones

are stored as preformed molecules that can be released within seconds, whereas several minutes are required for activation of the appropriate signaling pathways that culminate in the activation and nuclear transfer of the respective transcription factors that initiate the gene transcription process (228). Nonetheless, stress is mechanistically defined by a hormonal response (229), and this response cannot be adequately demonstrated at the transcript level. Without accompanying data on stress hormone levels, alterations in gene expression might only reflect the adaptive changes in the pathways that reestablish homeostasis.

A shortcoming of transcript-specific assays is that the selection of supposedly suitable parameters is left to the skill and knowledge of the experimenters. Studying the complete set of transcripts facilitates the identification of novel indicators that may not previously have been recognized as relevant. However, in truth, exploratory omics approaches are less effective in elucidating mechanistic insights than they are in generating hypotheses on how subsequent experiments can validate a selection of meaningful biomarkers.

## BLOOD TRANSCRIPTOMICS

High-throughput transcriptomic approaches, such as microarray or RNA-sequencing (RNA-seq) analyses, allow monitoring of the transcriptional changes in a comprehensive panel of potential indicators for a defined research setting (230, 231). In this way, transcriptomic approaches help to arrange traditional and novel parameters into virtual pathways and/or gene networks. RNA-seq is certainly the transcriptomic method of choice over microarrays, which have only been available for a few fish species for decades (230, 232, 233). This shortage is unlikely to be overcome in the future since the availability of reference genomes from a constantly increasing number of fish species and the reduced costs of deep sequencing have now made RNA-seq analyses highly attractive. Another benefit is that RNA-seq analysis distinguishes between individual transcript variants and ohnologous genes, whereas standard PCRs and microarrays typically do not.

The blood cells of most fish species are nucleated (234), whereas the mature erythrocytes and thrombocytes of mammals lack nuclei. This fact alone makes teleostean erythrocytes and thrombocytes highly interesting for (comparative) transcriptomic analyses to record the constitutively expressed transcripts in either cell type (8, 231, 235). Moreover, erythrocytes have been proven to actively supplement allostatic reactions by the induced expression of certain genes. Most investigations have focused so far on the immune responses of erythrocytes after stimulation with pathogen-associated microbial patterns (236), bacteria (237), or viruses (238, 239).

Beyond this, the impact of only a few other stressors has been investigated on the transcriptional response of blood cells. Following an acute exposure of *O. mykiss* to a 25 °C water temperature, the erythrocytes showed altered (at least 2-fold) expression of 26 genes at 4 h and 33 genes at 24 h (240). The panel of upregulated genes comprised a cluster of molecular chaperones, including the genes coding for HSP70 (constitutive and inducible forms), HSP90, and the heat-shock factor-binding protein HSBP1. The genes HSP90, HSP70, and zinc-finger AN1-type-containing protein 2B (ZFAND2B) were later confirmed by literature-mining approaches to represent robust biomarkers for temperature stress in different tissues of salmonid fish (241, 242). Heat stress also increased the

transcription of additional stress-related genes, such as stress-induced-phosphoprotein (STIP1) and JUN, and also immune-relevant features, including NF- $\kappa$ B inhibitor  $\alpha$  (NFKBIA) and interferon regulatory factor 1 (IRF1) in erythrocytes from *O. mykiss*, whereas immunoglobulin-encoding genes were downregulated. This example once again points to the close interdigitation of immune and stress pathways in teleost fishes (171, 243, 244).

High water temperatures are often associated with oxygen depletion as a co-occurring stressor. The schizothoracine fish (*Gymnocypris eckloni*) is an established model for the study of adaptation (245) and hypoxia tolerance (246). A comparison of two *G. eckloni* cohorts exposed to water containing  $\sim 8$  mg oxygen per liter (normoxia) or  $\sim 3$  mg oxygen per liter (hypoxia) for 3 days revealed differential expression of about 70 genes in the blood (q-value  $< 0.05$ ) of the stressed fish (246). Insulin-like-growth-factor-binding protein 1 (IGFBP1) was among the upregulated genes and had previously been identified in the liver of hypoxia-stressed goby fish (*Gillichthys mirabilis*) (247). The differentially expressed genes were assigned to nine Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, including hypoxia-inducible factor 1- $\alpha$  (HIF1 $\alpha$ ) signaling and fructose/mannose metabolism. Both pathways are expected to be regulated in the context of oxygen depletion in particular and stress in general.

The transcriptome of blood samples from *C. auratus* was analyzed to identify a suitable anesthesia method for routine use in aquaculture (see section Anesthesia and Blood Sampling Procedures) (248). This study revealed that most genes were differentially regulated after percussive stunning (877 at least 2-fold regulated features, q-value  $< 0.05$ ), compared with two chemical anesthetics, MS-222 (487 genes) and eugenol (208 genes). Handling of *C. auratus* triggered the upregulation of a large cluster of genes involved in general stress responses (including heat and cold shock, oxidative stress, and endoplasmic reticulum stress), whereas the anesthetized groups showed comparably fewer differentially regulated stress genes. In addition, all three anesthetics effectively maintained the serum cortisol at low levels ( $< 100$  ng/ml).

Transcriptomic profiles are critical for understanding relevant functional pathways and networks, but they also have limitations. One serious problem regarding transcriptome analyses of blood samples (and in other samples from whole tissues and organs as well) is that blood is generally a very heterogeneous mixture of cells, so the transcripts represent an average over a broad range of populations. Single-cell RNA sequencing (scRNA-seq) is one step beyond whole-transcriptome analysis, as it identifies the entirety of the transcriptional changes at the level of an individual cell. The use of an scRNA-seq approach in zebrafish provided novel insights into the unique expression patterns of rare immune cell subsets in the teleostean spleen (249) and documented that scRNA-seq created multifold possibilities for recording the tailored response of distinct blood cells to a defined stressor.

## BLOOD PROTEOMICS

The debate regarding how well-transcript and protein levels correlate (250, 251) is fueled by continuously published confirmatory and contradictory results. Therefore, the safest policy is to consider transcriptome and proteome datasets as complementary. Before qPCR analyses became a standard method in research, antibodies were exploited to record specific biomarkers in blood. The parameters chosen for examination were mostly

the already established markers. An additional limitation was that the antibodies, which had generally been produced for mammalian antigens, needed to be cross-reactive (e. g. be able to recognize well-conserved epitope sequences). For instance, the elevated levels of HSP70 in the blood of *O. niloticus* (Table II-1) were reported to indicate acute hypoxia (78), whereas the elevated levels of ubiquitin in the erythrocytes of blue maomao (*Scorpius violaceus*) suggested confinement stress (252). The protein analysis conducted by 2D- or differential polyacrylamide-gel electrophoresis or by liquid chromatography coupled to tandem mass spectrometry and using matrix-assisted laser desorption/ionization and time-of-flight-mass analysis, now provides more comprehensive insights into the dynamic allostatic events occurring at the protein level (253).

The effects of handling stress in *S. salar* were examined by Liu et al. (68), who profiled the O-acetylation of sialic acids in the serum. They found that the levels of di-O-acetylated sialic acids increased (Table II-1), whereas the levels of mono-O-acetylated sialic acids decreased significantly in stressed fish (68). The exposure of Atlantic halibut (*Hippoglossus hippoglossus*) to an optimal temperature of 12 °C and a suboptimal temperature of 18 °C in combination with high-CO<sub>2</sub> water (1,000 matm) for 14 weeks resulted in increased levels of the complement component C3 and fibrinogen  $\gamma$  chain (FGB) in the plasma of both high CO<sub>2</sub>-exposed groups (Table II-1) (97). A synthesis of these two factors is triggered very early after injury and pathogen invasion. The plasma of salinity-stressed Mozambique tilapia (*Oreochromis mossambicus*) also showed high concentrations of C3, together with NADH dehydrogenase, Mg<sup>2+</sup>-dependent neutral sphingomyelinase, semaphorin, and caspase-3 (254). The phagocytic activity of leukocytes from the head kidney and spleen also decreased in parallel, suggesting that both aspects might be causally connected.

## BLOOD PLASMA METABOLOMICS

Metabolome research is increasingly finding its way into aquaculture research, but it still lags behind the metabolomic-based research in mammalian models (46). Most metabolite structures are identical across species, in contrast to gene and protein sequences; therefore, the analytical assays do not need to be customized for a particular investigated species (34). The metabolites found in the blood plasma include various intermediates from a wide range of biochemical pathways. For this reason, metabolomic analyses of blood serum can be used to understand nutritional (89, 90), developmental (255), or pathophysiological (256) aspects of fish physiology and are increasingly being used for disease diagnostics (257). The most commonly used analytical techniques for studying endogenous metabolite profiles are nuclear magnetic resonance spectroscopy in combination with mass spectrometry or vibrational spectroscopy (258). Most metabolomic studies that have been conducted on plasma samples from fish have dealt with toxicological questions (259–262). Application of a five-percent-by-weight concentration of heavy oil has been reported to increase the levels of several plasma metabolites, including amino acids, butyrate derivatives, creatinine, glycerol, and glucose, in *C. carpio* (259). These findings suggested a perturbed tricarboxylic acid cycle of energy metabolism. A similar conclusion was drawn following the analysis of plasma samples from zebrafish exposed to the herbicide acetamiprid (260). The insecticide chlorpyrifos was found to enhance gluconeogenesis (glucose and glycerol), fatty acid metabolism (3-D-

hydroxybutyrates and acetoacetate), energy metabolism (creatine), and glutamate generation (glutamine and proline) in *C. carpio* (261). *O. mykiss* exposed to the synthetic contraceptive estrogen ethinylestradiol revealed increased vitellogenin levels, concomitant with significant changes in the plasma lipid profiles that, in turn, were attributed to the high lipid content of vitellogenin (262).

Metabolomic approaches for blood plasma analysis have also been utilized to address aquaculture-related issues. Food deprivation in juvenile *O. mykiss* increased the level of very-low-density lipoproteins while reducing the concentrations of high-density lipoproteins, choline,  $\beta$ -glucose, and lactate, in fasted fish (Table II-1; 89). The daily netting of juvenile *S. salar* for 2 weeks disturbed the plasma metabolic balance, as reflected by altered levels of lipoproteins, lipids, lactate, carbohydrates, and specific amino acids (Table II-1; 67).

The concentration of a particular enzyme does not necessarily increase or decrease (coupled to an up- or downregulated gene expression) due to varying environmental conditions, though the efficiency in converting certain metabolites may vary. Therefore, metabolic profiling can provide an alternative list of highly sensitive potential biomarkers (34) that can complement the findings of PCR-based techniques and transcriptomics or antibody-based techniques and proteomics. This type of holistic approach can help to coordinate the differentially regulated features in blood and plasma/serum samples in cases where elevated concentrations of a certain metabolite co-occur with increased levels of the associated catalyzing enzyme and with upregulated expression of the enzyme-encoding gene. However, these holistic high-level approaches (cf. Figure II-1) remain to be performed in fish.

## CONCLUSIONS

Blood contains easily accessible information about the individual physiological state of a fish. Nonetheless, blood is not the appropriate matrix for every research question; for instance, not all aspects of “welfare” can be detected in the blood. Several studies have reported the isolation of steroids from matrices other than blood (i.e., mucus, scales, feces, or water) (263– 266); however, the data obtained directly from blood are still far more accurate, as the risk of rapid cortisol degradation and contamination from external cortisol sources are evidently lower (16). Most blood sampling techniques are considered minimally invasive for fish above a given size, though sampling activates primary stress responses within minutes. During the experimental manipulations, the researcher should remain aware that the sampling itself might conceal the hallmarks of a (stress) response to previous treatments, thereby biasing the interpretation of the extracted data. In general, the interpretation of blood-derived parameters requires caution, since particular physiological perturbations do not necessarily depend on a given experimental protocol. The metabolic changes, for instance, might also result from persistent chronic disturbances and/or causally independent events (e. g. circadian rhythms, seasonality, feeding times, conspecific aggressions, water quality, etc.) or substandard sampling and laboratory-specific procedures. The influence of sex and body weight/size of the individual fish should also not be underestimated. Multiple parameters should be recorded simultaneously, preferably from different analysis techniques, to disclose unsuitable husbandry conditions and to identify less obvious or previously unnoticed environmental stressors that exceed

the adaptive capacity of fish. This approach supports identification of the comprehensive signature of a distinct stressor, thereby allowing valid conclusions to be drawn regarding fish welfare aspects. Unfortunately, the question of which method(s) should be used to detect the signature of a distinct stressor cannot be answered given the current state of knowledge.

This manuscript reviews different methods for recording welfare-related physiological processes in fish blood. Over the past few decades, a broad repertoire of fish-specific tools and methods has been established that enables the quantification of the concentrations of numerous hormones, metabolites, immune factors, and relevant transcripts that now supplement the panel of traditional biomarkers in blood. In the future, high-throughput -omics technologies (particularly transcriptomics, proteomics, and metabolomics) are expected to provide holistic snapshots of the physiological state of an individual. Assembling the ever-growing number of -omics puzzle pieces will ultimately provide a comprehensive picture of the metabolic, transcriptional, and immunological activities in the blood (and other tissues) of fish. Recent technological innovations, such as scRNA-seq and spheroid cell cultures, will further boost the identification of transcriptional signatures in blood cells of farmed and model fish species.

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Anlage 3

<b>C   A   U</b>	Christian-Albrechts-Universität zu Kiel	Agrar- und Ernährungs- wissenschaftliche Fakultät
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**Declaration of co-authorship**

If a dissertation is based on co-authored articles, a declaration from each of the authors regarding the part of the work done by the doctoral candidate must be enclosed when submitting the dissertation.


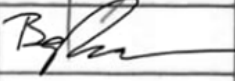
<b>1. Doctoral candidate</b>
Name: <b>Henrike Seibel</b>

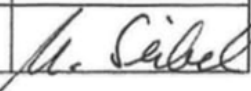
<b>2. This co-author declaration applies to the following article:</b>
<b>Blood Will Tell: What Hematological Analyses Can Reveal</b>

The extent of the doctoral candidate's contribution to the article is assessed on the following scale:

- A. Has contributed to the work (0-33%)
- B. Has made a substantial contribution (34-66%)
- C. Did the majority of the work independently (67-100%)

3. Declaration on the individual phases of the scientific work (A,B,C)	Extent
<b>Concept:</b> Formulation of the basic scientific problem based on theoretical questions which require clarification, including a summary of the general questions which, it is assumed, will be answerable via analyses or concrete experiments/investigations	B
<b>Planning:</b> Planning of experiments/analyses and formulation of investigative methodology, including choice of method and independent methodological development, in such a way that the scientific questions asked can be expected to be answered	B
<b>Execution:</b> Involvement in the analysis or the concrete experiments/investigation	C
<b>Manuscript preparation:</b> Presentation, interpretation and discussion of the results obtained in article form	C

4. Signature of all co-authors		
Date	Name	Signature
28.2.24	Alexander Rebl	
02.03.2024	Björn Balßmann	

5. Signature of doctoral candidate		
Date	Name	Signature
28.02.24	Henrike Seibel	



### III. Research Chapter II - Analysis of stress markers from blood using the SBMIE model

#### **Blood-based Parameters Indicating a Soybean Meal-Induced Enteritis in Rainbow Trout, *Oncorhynchus mykiss*<sup>6</sup>**

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This study evaluated a diverse range of markers for feeding stress to assess more precisely the welfare of rainbow trout in relation to inadequate husbandry conditions. A feeding stress model based on dietary soybean meal was employed to identify suitable minimally-invasive "classical" stress markers together with molecular signatures.

In a 56-day feeding experiment, rainbow trout were fed with different dietary inclusion levels of soybean meal. The impact of these soybean meal diets on rainbow trout was assessed by water quality analyses, clinical health observations, classic growth and performance parameters, gut histopathology, blood-parameter measurements and multigene-expression profiling in RNA from whole blood. The soybean meal-induced enteritis manifested phenotypically by an inflammatory reaction in the posterior section of the intestine and diarrhoea in some trout. These inflammatory changes were associated with decreased supranuclear vacuolation. The haematocrit values and the levels of plasma cortisol and circulating lymphocytes in the blood of trout were increased in trout having received high SBM dosages. Noteworthy, the increased haematocrit depended significantly on the body weight of individual trout. The transcript levels of certain genes (e. g. MAP3K1, NOD1, STAT1, HSP90AB) emerged as potentially useful indicators for unsuitable feed source inclusion levels, while the levels of other genes (e. g. SOD, IL1B, HSP47) did not alter significantly. The results are a first step towards well-founded, early assessments of feeding stress of trout, which are less invasive.

**Keywords:** Stress, inflammation, soybean meal, feeding stress, molecular marker

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## 1. INTRODUCTION

The increasing intensification of the global aquaculture industry is stimulating a growing public debate about the welfare of farmed fish (Farstad, 2018). Although product quality and profitability are of paramount importance to the aquaculture industry, fish welfare is becoming an increasingly prominent focus (Bergleiter et al., 2009; Hiller and Wichmann, 2010; Lasner et al., 2010). Meanwhile, aspects of fish welfare during the production process are included in various national, European and global research programmes. The aim of these research consortia is to define the organism's reduced adaptability to physical and psychological stress in general. Initially, the focus was placed on general environmental husbandry parameters including water temperature (Ings et al., 2012; Rebl et al., 2013) concentrations of dissolved oxygen (Bernier and Craig, 2005), stocking densities (LeBlanc et al., 2011; Sørensen et al., 2012) or tank characteristics such as background colour (Ebrahimi, 2011). In recent years, more and more working groups have established welfare-indicator indices (Gutierrez Rabadan et al., 2021; Müller-Belecke, 2019; Stien et al., 2013; Tschirren et al., 2021). However, reports on multifactorial signatures of feeding stress in fish are still rare.

Plant-based aquafeeds are becoming more and more important for commercial aquaculture, due to overfishing and sustainability reasons. Soybean meal (SBM) is a plant-based alternative to fish meal, as it provides a relatively high protein content and an amino-acid profile, which is nearly similar to fish meal at low costs (Barnes et al., 2014; Bruni et al., 2021; INRA, CIRAD, 2018; Oliva-Teles et al., 1994; Voorhees et al., 2019). Decades of research have, however, revealed that soy diets not only reduce the growth of salmonids (Rumsey et al., 1994), but also provoke mild to severe enteritis and other health complications (van den Ingh et al., 1991) due to high levels of antinutritional factors including saponins (Krogdahl et al., 2015), proteinase inhibitors (Krogdahl et al., 1994), lectins, oligosaccharides (Krogdahl et al., 2010), phytoestrogens and antivitamin (Francis et al., 2001). SBM has been demonstrated to cause morphological changes in the distal intestine of salmonids (Baeverfjord and Krogdahl, 1996; Rumsey et al., 1994; van den Ingh et al., 1991), which comprise increased numbers of inflammatory cells, shortening of folds and disruption of microvilli, widening of central stroma, increased formation of microvillar vesicles, elevated numbers of goblet and apoptotic cells as well as reduced supranuclear vacuolisation with apical nuclear displacement within enterocytes (Baeverfjord and Krogdahl, 1996; Chikwati et al., 2012; Knudsen et al., 2008; Merrifield, 2011). Of particular significance is the soy-induced inflammatory reaction. Unsuitable feed compositions have been reported to impair the immune system (Krogdahl et al., 2010) and chronic inflammations may have serious consequences for growth, health, and, of course, the well-being of fish.

The negative effects of SBM diets do not affect all fish species to the same extent (Booman et al., 2018). It is well established that carnivorous fishes like rainbow trout (*Oncorhynchus mykiss*) suffer from intestinal inflammation after feeding with high levels of terrestrial plant components that contain antinutritional factors, such as soyasaponin, b-conglycinin and glycinin in soybean (Bakke, 2011; Buttle et al., 2001; Zhang et al., 2013). The majority of the published findings on SBM-induced dietary intolerances in fish is essentially based on intestinal histology, enzyme-activity tests and microbiome analyses. Only a limited number of studies quantified the expression of distinct genes to characterize

the teleost response to nutritional deficiencies (Miao et al., 2018; Wang et al., 2017; Zhang et al., 2013).

The present study exposed rainbow trout to potentially deficient nutrition and recorded their physiological response. For the above reasons, we opted for three dietary soybean meal inclusion levels to induce different gradations of intestinal inflammation and varying degrees of compromised overall welfare. The focus was placed on non-lethal and less invasive blood sampling to detect and compare a broad panel of traditional and novel health and welfare parameters.

## 2. MATERIALS AND METHODS

### 2.1. SBM FEEDING TRIAL

A 56-day feeding trial was conducted at the research facility of the Gesellschaft für Marine Aquakultur mbH in Büsum<sup>7</sup>, Germany.

#### 2.1.1. FISH AND HUSBANDRY CONDITIONS

204 juvenile, female monosex rainbow trout (*O. mykiss*; Troutbreeder Troststadt GbR, Reurieth, Germany) were randomly stocked into twenty 150-L freshwater tanks in a recirculation system (Spranger Kunststoffe, Plauen, Germany) with a total volume of 5 m<sup>3</sup>. The transparent window of each tank was covered with a mirror spy foil (ESG Handels GmbH, Leutasch, Austria) to shield the fish from routine work. Fish were held under optimal conditions (18.5 ± 0.7 °C, 7.6 - 8.4 mg/L dissolved oxygen, pH 7.5 ± 0.1, NH<sub>4</sub> 0.3 ± 0.2 mg/L, NO<sub>2</sub> 1.3 ± 0.7 mg/L). The day-night rhythm was 14:10 hours. A 56-day feeding trial was conducted at the research facility of the Gesellschaft für Marine Aquakultur mbH in Büsum, Germany. All animal handling procedures were approved by the animal welfare officer of the “Gesellschaft für Marine Aquakultur mbH” and the local authority of Schleswig-Holstein according to the German animal welfare law.

#### 2.1.2. DIETS

The diets were designed and produced isonitrogenous and isoenergetic. The fishmeal portion (50 % in the control diet) was replaced with soybean meal at 33 %, 66 %, and 100 % to generate four feeds, SBM0 (no SBM included, control diet corresponding to the feed formulation of a commercially available trout feed), SBM33 (16.4 % SBM included in total ration), SBM66 (33 % SBM included in total ration) and SBM100 (50 % SBM included in total ration; Table III-1).

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<sup>7</sup> dato Department of Aquaculture and Aquatic Resources at Fraunhofer IMTE

Table III-1 Ingredients and composition of feeding stuffs used in the feeding experiment.

Diet composition	SBM0	SBM33	SBM66	SBM100
<b>Fish meal<sup>1</sup></b>	<b>50.00</b>	<b>33.50</b>	<b>17.00</b>	<b>0.00</b>
Blood meal <sup>3</sup>	5.00	5.00	5.00	5.00
Galantine <sup>4</sup>	3.00	3.00	3.00	3.00
<b>Soybean meal<sup>2</sup></b>	<b>0.00</b>	<b>16.40</b>	<b>33.00</b>	<b>50.00</b>
Pea protein isolate <sup>5</sup>	6.50	6.50	6.50	6.50
Wheat gluten <sup>6</sup>	7.00	10.59	14.05	17.25
Wheat starch <sup>7</sup>	19.50	15.00	10.55	5.50
Fish oil <sup>8</sup>	8.00	8.55	9.05	10.03
Vit / Minerals <sup>9</sup>	0.50	0.50	0.50	0.50
Kaolin <sup>10</sup>	0.00	0.47	0.85	1.22
Calcium hydrogen phosphate <sup>11</sup>	0.5	0.5	0.5	0.5
Biolys <sup>12</sup>	0.00	0.00	0.00	0.50

SBM = Soybean meal; 0, 16, 33 and 50, different amounts [%] of total SBM in diet

SBM0 = Control diet

<sup>1</sup> Sea lion, herring fishmeal, 68% rawprotein, 10% rawprotein (Bioceval GmbH & Co. KG, Cuxhaven, Germany)

<sup>2</sup> Soy bean meal 200 (P.C. Van Tuijl Kesteren b. v., Kesteren, Netherlands)

<sup>3</sup> Pig blood meal 90 (Sonac, Burgum, Netherlands)

<sup>4</sup> Edible gelatine gold (Gustav Ehlert GmbH & Co. KG, Verl, Germany)

<sup>5</sup> Pea protein isolate; heat treated (Empro E 86, Emsland Group, Emlichheim, Germany)

<sup>6</sup> Pregelatinised wheat starch: type 90 (Kröner starch, Ibbenbüren, Germany)

<sup>7</sup> Wheat gluten vital (Kröner starch, Ibbenbüren, Germany)

<sup>8</sup> United Fishmeal Works (Cuxhaven GmbH & Co. KG, Cuxhaven, Germany)

<sup>9</sup> Vitamin-mineral mixture: 517158 (Vitfoss, Grasten, Denmark)

<sup>10</sup> Kaolin: Sibelco (Ransbach-Baumbach, Germany)

<sup>11</sup> Calcium hydrogen phosphate (CaHPO<sub>4</sub>): JRS (Pharma GmbH & Co. KG, Rosenberg, Germany)

<sup>12</sup> "Biolys" Lysine (Evonik Industrier AG, Essen, Germany)

The Nutrient compositions of feed are shown in Table III-2.

Table III-2 Nutrient compositions of feed ingredients. Indicated are mean values and standard deviations of the percentage (n = 2)

	SBM0	SBM33	SBM66	SBM100
Dry matter (%)	89.83 ± 0.02	88.35 ± 0.04	90.00 ± 0.02	90.00 ± 0.02
Ash (% DM)	10.48 ± 0.05	09.16 ± 0.05	07.86 ± 0.03	06.38 ± 0.00
Crude protein (% DM)	54.63 ± 0.09	55.40 ± 0.23	54.44 ± 0.13	54.31 ± 0.18
Crude lipid (% DM)	15.72 ± 0.04	14.77 ± 0.05	13.94 ± 0.05	13.61 ± 0.05
Carbohydrates (% DM) <sup>1</sup>	19.17	20.67	23.76	25.70
Energy (MJkg <sup>-1</sup> )	22.10 ± 0.01	22.19 ± 0.03	22.19 ± 0.00	22.44 ± 0.02

<sup>1</sup> carbohydrates = 100 - (crude protein + crude ash + crude lipid); DM = dry matter

### 2.1.3. FEEDING TRIAL

After an acclimation period of 14 days, the feeding experiment was performed in triplicates with 17 fish ( $81.9 \pm 0.4$  g) per tank. The initial stocking density was 4-5 kg/m<sup>3</sup>. Trout were fed twice a day (9 am and 4 pm) to apparent satiation with one of the four experimental feeds (Table III-1). After each feeding, the uneaten pellets were collected and quantified to calculate the actual and precise feed intake.

### 2.1.4. SAMPLING

Samples of six fish each were taken at the start of the trial, after 4 weeks and after 8 weeks (at the end of the trial). Two fish per tank, i. e. six fish per feeding group, were stunned by a blow to the head. After collecting blood on EDTA for haematocrit, serum cortisol, differential blood cell count and molecular investigations, the length and weight of individual fish were measured. The fish were euthanized by severing the gill arches, then dissected and the liver and spleen weights measured for calculations of their organosomatic indices. Histology samples from the distal intestine were collected into 10% formalin for fixation.

The time required from catching the fish with subsequent direct anaesthesia by a blow on the head to the end of blood collection was documented and took not longer than five minutes, on average 2.5 minutes.

## 2.2. ASSESSMENT OF BODY CONDITION INDICES

The performance parameters specific growth rate (SGR), feed conversion ratio (FCR), daily feed intake (DFI), Fulton's condition factor (K), hepatosomatic index (HSI) and spleenosomatic index (SSI) were calculated based on group measures as follows:

$$\text{SGR} = [\ln(\text{FGW}) - \ln(\text{IGW})] / t * 100$$

$$\text{FCR} = (\text{Ft} / 1000) / (\text{FGW} - \text{IGW})$$

$$\text{DFI} = \text{SGR} * \text{FCR}$$

$$\text{K} = \text{FIW}/(\text{FIL})^3 * 100$$

$$\text{HSI} = \text{LiW}/\text{FIW} * 100$$

$$\text{SSI} = \text{SpW}/\text{FIW} * 1000$$

where FGW = final group weight [kg], IGW = initial group weight [kg], t = 59 days, Ft = total amount of feed intake [g], FIW = final individual weight [g], FIL = final individual length [cm], LiW = weight of liver [g]; SpW = weight of spleen [g].

For whole-body analysis, a pool of three randomly picked fish per feeding group were analysed. The nutrient content of diet and homogenised fish bodies were determined in duplicates using the Weender analysis method on the basis of EU Regulation (EC) No. 152/2009. To determine the quality of the dry substance, the homogenized samples were dried at 103 °C for 4 h (model ED 53, Binder GmbH, Tuttlingen, Germany) until their weight remained constant. The ash content was determined by the weight loss after 4 h of

incineration (P300, Nabertherm, Lilienthal, Germany) at 550 °C. The ash content was then determined by the ash concentration in the incinerator (P300, Nabertherm, Lilienthal, Germany). The protein content was determined using the Kjeldahl method (N = 6.25) by chemical digestion (InKjel 1225 M, WD, Behr Labor-Technik GmbH, Düsseldorf, Germany), distillation (Behrosog3, Behr Labor-Technik GmbH) and titration (Titrex 2000, Behr Labor-Technik GmbH) of the samples. Crude fat contents were determined by hydrolysis with hydrochloric acid (Hydrotherm HT 6 Gerhardt, Königswinter, Germany) and ether extraction in a Soxhlet unit (Soxtherm Multistat/SX PC, Gerhardt, Königswinter, Germany). The gross energy contents were determined by means of a bomb calorimeter (C 200, IKA-Werke GmbH & Co. KG, Staufen, Germany).

The carbohydrates were calculated based on the dry matter contents of crude ash, crude protein and crude fat subtracted from 100 % of the dry matter.

### 2.3. HAEMATOCRIT DETERMINATION

The collected EDTA-whole blood was immediately filled into three heparinized micro hematocrit capillaries (Art. No. 7493 11, Brand GmbH + Co KG, Wertheim, Germany) to approx. 75 % and centrifuged with a haematocrit centrifuge (Hämatokrit 210, Hettich GmbH & Co. KG, Tuttlingen, Germany) at 40.000 RZB/min at room temperature for 4 min. The hematocrit value was then read from a measuring template (Hettich GmbH & Co. KG, Tuttlingen, Germany).

### 2.4. DIFFERENTIAL BLOOD COUNT

For the haematological evaluation of the blood cells, a blood smear was taken from each fish sampled. A drop of EDTA-whole blood was applied to a previously cleaned slide and smeared with the narrow edge of a second slide. The blood smears were air-dried and then stained with a Giemsa stain (Dip Quick Stain, Jorgensen Laboratories, Loveland, Colorado). The stained blood cells were counted under light microscopy (CKX41, Olympus, Tokyo, Japan) at 1000 × magnification using oil immersion. The blood smears were counted meandering. For each smear, 100 cells (granulocytes, lymphocytes, thrombocytes and monocytes) were recorded at two different suitable locations on the slide. The percentage composition of the differential blood count was subsequently derived from the relative proportion of blood cells detected.

### 2.5. QUANTIFICATION OF SERUM CORTISOL LEVELS

For cortisol quantification, 500 µl whole blood was transferred immediately after blood collection into a 1.5 ml reaction vessel and centrifuged at 15,060 × g for 5 min (Eppendorf Centrifuge Model 5414, Eppendorf, Hamburg, Germany). The resulting supernatant (blood plasma) was decanted into a new 1.5 ml-reaction tube and immediately frozen at -80 °C until measurement.

Before measurement, blood plasma samples were purified with ethanol extraction (according to Schlachter, 2011). A commercial ELISA test kit for cortisol in human saliva—calibrated for rainbow trout—was used to determine the cortisol content in blood



plasma (Cortisol Salvia Elisa, RE52611, IBL International GmbH, Hamburg, Germany). The test was performed in duplicate. A microplate reader (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland) was used to measure the optical density, which was linearized using the logit-loglineare analysis (in IBM SPSS Statistics 20, Armonk, New York), as the decrease in optical density was not linear with the increase in cortisol content.

## 2.6. HISTOLOGICAL EVALUATION OF THE INTESTINE

The intestinal tissue sections were evaluated by light microscopy, with a focus on the characteristic morphological changes of SBM-induced enteritis (SBMIE) (Baeverfjord and Krogdahl, 1996; Bruce et al., 2018; Merrifield, 2011). Tissues were dehydrated and embedded in paraffin, cut in 4 µm thick slices and stained with haematoxylin and eosin according to routine methods of the histopathological surgery (Bleyer and Gruber-Dujardin, Göttingen, Germany). The results were obtained with a light microscope (Zeiss, Primo Star). Changes in mucosal fold length, width and cellularity of the submucosa and lamina propria, supranuclear enterocyte vacuolisation, frequency of intraepithelial lymphocytes, mitotic figures and apoptotic bodies within the epithelial layer were examined. The degree of morphological changes was analysed using a scoring system based on a scale from 0 to 4, where 0 was normal; 1 slight; 2 moderate; 3 severe; and 4 extremely severe changes. The allocation of the individual samples to the experimental groups was carried out after the histological evaluation had been completed.

## 2.7. REAL-TIME QPCR (RT-QPCR)

We designed a total of 34 oligonucleotide primer pairs (Table III-S1) for gene-expression profiling in blood samples using the multiplex-qPCR system Biomark HD (Fluidigm, California, USA). The panel of primer pairs covered genes coding for pattern-recognition receptors, pro- and anti-inflammatory cytokines, acute phase reactants, complement components, signalling molecules and heat-shock proteins. In addition, different marker genes for specific immune cell populations were included (Table III-3). ACTB (Rebl et al., 2009), EEF1A1 (Bowers et al., 2008) and RPS5 (Rebl et al., 2014) served as reference genes to normalize the data according to ref. (Kortner et al., 2011).

Total RNA was extracted from whole blood samples conserved in RNeasy (SIGMA-Aldrich/Merck, Hamburg, Germany) via a combination of QIAGEN RNeasy Plus Minikit (QIAGEN, Hilden, Germany) and RiboPure blood (Thermo Fisher Scientific) with additional treatment with DNAase (QIAGEN, Germany). Optical density of extracted RNA was measured photometrically (Tecan Nanoquant Infinite M200 Photometer, Tecan Group AG, Switzerland) and RNA concentration was set to 10 ng/ml. RNA samples were separately reverse-transcribed into cDNA using the Reverse Transcription Master Mix (Fluidigm). To increase the target concentration, a preamplification over 10 cycles was performed in 5 µl reactions using the PreAmp Master Mix (Fluidigm) followed by a treatment with exonuclease I (New England BioLabs, Frankfurt am Main, Germany). After injecting the control-line fluid (Fluidigm) into the two accumulators, the IFC (integrated-fluidic circuits) chip was primed in the MX-IFC Controller (Fluidigm). After priming, 5 µl of each primer assay and 5 µl of the preamplified cDNA samples were loaded into the respective inlets on

the IFC and analysed with the Biomark HD system using the recommended manufacturer's thermal protocol. EvaGreen fluorescence dyes (Bio-Rad) served as DNA-binding reporter molecules to allow the quantification of the amplified target fragments.

## 2.8. DATA ANALYSIS AND STATISTICS

The statistical evaluation was carried out with the statistics program SPSS 20.0 (IBM SPSS Statistics 20, Armonk, New York). The arithmetic mean and the standard deviation from the mean value were calculated as a measure of the dispersion. The examination for normal distribution was carried out with the Kolmogorov Smirnov test. The Levene test was used to check the variance homogeneity. With normally distributed, homogeneous data, a single-sector analysis of variance (ANOVA) was performed and the Tukey test was applied as a post-hoc test. If there was no normal distribution or variance heterogeneity, the data were evaluated by the Kruskal-Wallis test and the post-hoc Dunnett-T3. Pearson's correlation coefficient and linear regression analysis were used to assess a potential linear correlation between two variables. For all statistical tests,  $p \leq 0.05$  (two-sided) was considered significant.

Differences in histological values for the various evaluated morphological properties of the tissue were analysed for statistical significance using the Fisher test and a Chi-square post hoc test. Both statistical tests were performed in the R-Statistics package (version 3.4.2; 2017) within the RStudio interphase (version 1.1.383; 2017). The differences were considered significant with  $p \leq 0.05$ .

In order to minimize the variances between different qPCR runs, all data for genes of interest were grouped together by using multiple plate analysis (MX 1.0 Agilent Technologies, USA). gNorm and NormFinder (Andersen et al., 2004) were used to evaluate the stability of reference gene expression. The geometric mean of the copy numbers of the suitable reference genes was used to normalize the copy numbers of the target genes. Target gene expression was calculated and normalized to a correction factor calculated from the internal control genes according to Vandesompele and Bustin (Bustin et al., 2009; Vandesompele et al., 2009, 2002) using the formula  $2^{-\Delta\Delta Ct}$  (Livak and Schmittgen, 2001). Fluidigm data were analysed using the Fluidigm Real-Time PCR analysis software v. 4.0.1.

## 3. RESULTS

### 3.1. OVERALL HEALTH, GROWTH AND PERFORMANCE

Mortalities did not occur during the experimental period, but a strong growth divergence of fish was observed across all experimental groups. During the eight-week trial period, we recorded a minor but steady increase in weight of rainbow trout. The average weight gain ranged from 25 g (SBM100) to 64 g (SBM0, Table III-3). The exchange of fishmeal with SBM in the diet groups SBM33, SBM66 and SBM100 was not reflected by significant differences in weight gain but influenced the specific growth rates ( $p = 0.05$ ) and the FCR ( $p < 0.05$ ) between control (SBM0) and SBM100 groups. Increasing supplementation of soybean meal in trout feed correlated significantly negative with the SGR ( $r = -0.69$ ;  $p = 0.01$ ) and significantly positive with the FCR ( $r = 0.70$ ,  $p = 0.02$ ).

This indicates that rainbow trout fed at 100% fish meal exchange (SBM100) had a lower DFI compared with the SBM33 group ( $p = 0.045$ ) and also with the SBM66 group ( $p = 0.052$ ). There was a highly negative correlation ( $r = 0.939$ ,  $p = 0.000$ ) between SGR and DFI across the groups analysed, while various condition factors (including weight, K, HSI and SSI) were not significantly different (Figure III-1 and Table III-3).

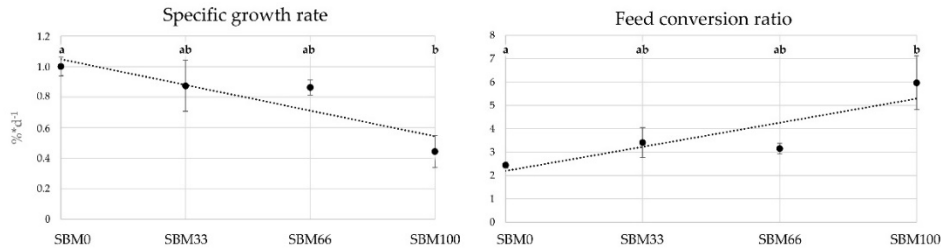


Figure III-1 Mean values of (a) the specific growth rate SGR (%·d<sup>-1</sup>,  $r = 0.69$ ;  $p = 0.013$ ) and (b) feed conversion ratio FCR ( $r = 0.669$ ,  $p = 0.017$ ) given for the feeding groups at the three different sampling times. <sup>a,b</sup> Letters indicate significant differences between groups ( $p \leq 0.05$ ). SBM0 = control, SBM33 – SBM100 = Soybean meal feeding groups with exchange of fish meal to 33%, 66% or 100%.

Table III-3 Growth and performance of rainbow trout investigated.

	SBM0	SBM33	SBM66	SBM100
Initial weight [g]	81.75 ± 0.16	82.09 ± 0.90	81.44 ± 0.85	82.15 ± 0.40
Final weight [g]	146.10 ± 10.49	138.26 ± 29.64	134.52 ± 8.66	106.74 ± 13.19
K	0.89 ± 0.24	0.95 ± 0.29	0.94 ± 0.22	0.88 ± 0.20
DFI [%·d <sup>-1</sup> ]	2.43 ± 0.13 <sup>ab</sup>	<b>2.70 ± 0.13<sup>a</sup></b>	<b>2.69 ± 0.07<sup>a</sup></b>	<b>2.32 ± 0.21<sup>b</sup></b>

<sup>a,b</sup> Letters indicate significant differences between groups ( $p \leq 0.05$ ).

Fish behaved in a normal manner, although some trout from the SBM100 group suffered from bloody diarrhoea ten days after the start of the experiment, which lasted until the end of the experimental period. From day 14 onwards, diarrhoea was also documented in trout from SBM66 group. No pathological anatomical findings were present during final sampling, with the exception of medium to highly soft intestinal contents and slight redness of the intestinal mucosa in the SBM66 and SBM100 groups. Altogether, the increasing substitution of fishmeal by SBM provokes a lower feed conversion, reduced growth and chronic diarrhoea.

### 3.2. QUANTITATIVE NUTRIENT COMPOSITIONS OF THE FISH BODY

The individuals of the SBM66 group had significantly lower protein contents ( $p = 0.04$ ) compared to the SBM0 group (Table III-4). The moisture, ash, fat and energy contents were not significantly different across groups. Although non-significant, the groups SBM33 and SBM66 differed from the SBM100 and SBM0 groups by a low-fat content, accompanied by increased levels of moisture and ash.

Table III-4 Nutrient composition of the total fish body per feeding groups compared to trout at the start of the experiment.

	Start	SBM0	SBM33	SBM66	SBM100
Moisture content [%]	71.75	70.14 ± 1.540	74.27 ± 5.120	78.15 ± 1.47	71.75 ± 1.34
Ash content [%]	02.79	02.79 ± 0.07	03.02 ± 0.330	03.23 ± 0.11	02.77 ± 0.12
Protein content [%]	16.26	<b>17.01 ± 0.62<sup>a</sup></b>	16.16 ± 0.65 <sup>a,b</sup>	<b>15.68 ± 0.15<sup>b</sup></b>	16.34 ± 0.21 <sup>a,b</sup>
Fat content [%]	09.20	10.05 ± 1.170	06.55 ± 4.800	06.55 ± 4.80	09.82 ± 1.04
Energy content [MJ/kg]	26.05	25.58 ± 0.440	23.58 ± 2.900	21.33 ± 1.19	25.73 ± 0.16

<sup>a,b</sup> Letters indicate significant differences between groups ( $p \leq 0.05$ ).

### 3.3. HAEMATOCRIT

The initial value of haematocrit at the start of the experiment ( $n = 3$ ) was  $22.13 \% \pm 3.36 \%$ . With increasing body weight, the haematocrit values increased (Pearson correlation; 4 weeks  $p = 0.003$ ; 8 weeks  $p = 0.015$ ) (Figure III-2).

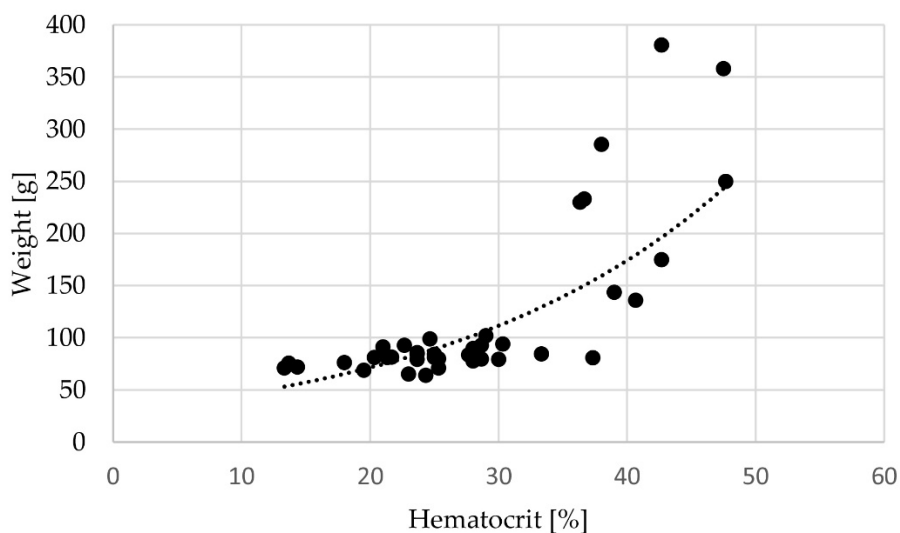


Figure III-2 Correlation between weight [g] and haematocrit [%] across all sampling times;  $r = 0.697$ ,  $p = 0.000$ .

In general, the haematocrit values strongly varied across individuals and experimental groups ranging on average from 22 % to 33 % (Table III-5).

Table III-5 Haematocrit values [%] of the rainbow trout per feeding group.

	<b>SBM0</b>	<b>SBM33</b>	<b>SBM66</b>	<b>SBM100</b>
4 weeks	30.33 ± 6.17	29.50 ± 1.53	33.22 ± 7.99	22.67 ± 9.50
8 weeks	23.39 ± 4.50	27.13 ± 11.40	30.40 ± 6.07	32.27 ± 11.25

### 3.4. DIFFERENTIAL BLOOD COUNT

The replacement of fishmeal with SBM in the feed did not significantly influence the cellular blood composition of rainbow trout (n = 52) as evaluated by means of differential blood count (Figure III-3).

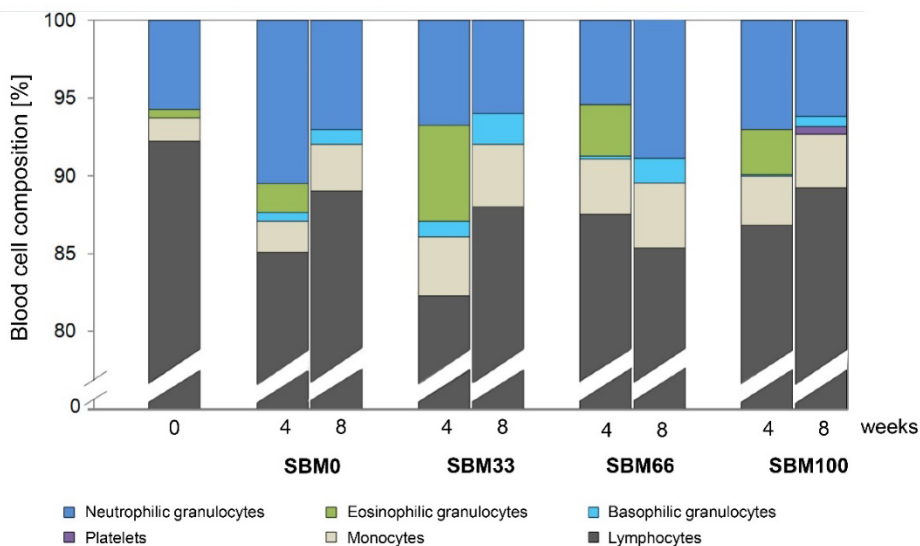


Figure III-3 Cellular blood composition of rainbow trout examined. Given is the percentage distribution of white blood cells and platelets of trout per feeding group (SBM0, SBM33, SBM66 and SBM100) at the start of the trial (start; n = 3), after 4 weeks (n = 9) and after 8 weeks (n = 9).

However, we observed a slightly lower percentage of lymphocytes and an associated increase in the percentage of granulocyte and monocyte across the groups investigated (SBM0, SBM33, SBM66 and SBM100) after 4 weeks in comparison to the start of the trial. With reference to week 4, the percentage of lymphocytes was slightly higher in SBM0, SBM33 and SBM100 groups at the end of the experiment after 8 weeks, except for trout from group SBM66, which then had the lowest mean percentage of lymphocytes. Here, the increase in lymphocytes correlated with a decrease in neutrophilic granulocytes, while the percentages of monocytes and basophilic granulocytes did not significantly change. Eosinophilic granulocytes were absent in the blood samples from week 8. The occurrence of platelets/thrombocytes (1 %) could only be documented in individuals of the SBM100 group at week 8.

### 3.5. CORTISOL

At the beginning of the experiment, rainbow trout ( $n = 4$ ) had the lowest cortisol values with an average concentration of  $2.6 \pm 0.53$  ng/ml (Figure III-4).

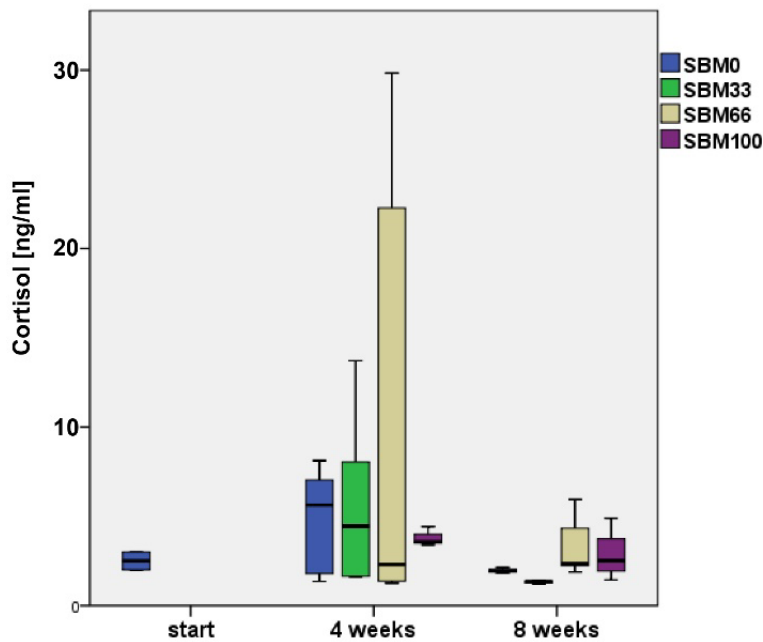


Figure III-4 Cortisol concentration [ng/mL] of rainbow trout across the time points analysed. Boxes represent the interquartile range (IQR), lower end = 25th percentile, upper end = 75th percentile, solid line = median, whiskers =  $1.5 \times \text{IQR}$ . SBM0 = control, SBM33 – SBM100 = Soybean meal feeding groups with exchange of fish meal to 33 %, 66 % or 100 %.

After a four-week period, sampled animals had double to five times higher cortisol levels, which decreased again after eight weeks. However, these levels did not reach the values of the initial sampling, except for SBM33 group with an average cortisol content of  $1.92 \pm 1.74$  ng/ml (Figure III-4).

Due to strong individual variations of the cortisol concentrations, we did not observe significant differences between the SBM and the control groups, but a tendency indicating a rising cortisol concentration that correlates with a rising SBM content in the feed (Figure III-4). Cortisol levels and weight did not significantly correlate.

### 3.6. HISTOPATHOLOGY

Distal intestinal sections of three rainbow trout per group showed slight to moderate signs of inflammation. All feeding groups contained at least one fish revealing a slight infiltration of the lamina propria and submucosa by diverse leukocyte subpopulations. The occurrence of inflammation correlated with the proportion of SBM in the diet. Inflammatory changes were most prevalent in the SBM100 group, followed by the SBM66 group (Figure III-5). The loss of supranuclear vacuolisation in the enterocytes was more pronounced compared to the infiltration of the submucosa and the lamina propria with inflammatory cells,

particularly in the SBM100 group, followed by the SBM66 group. These observations indicate an SBM-induced enteropathy.

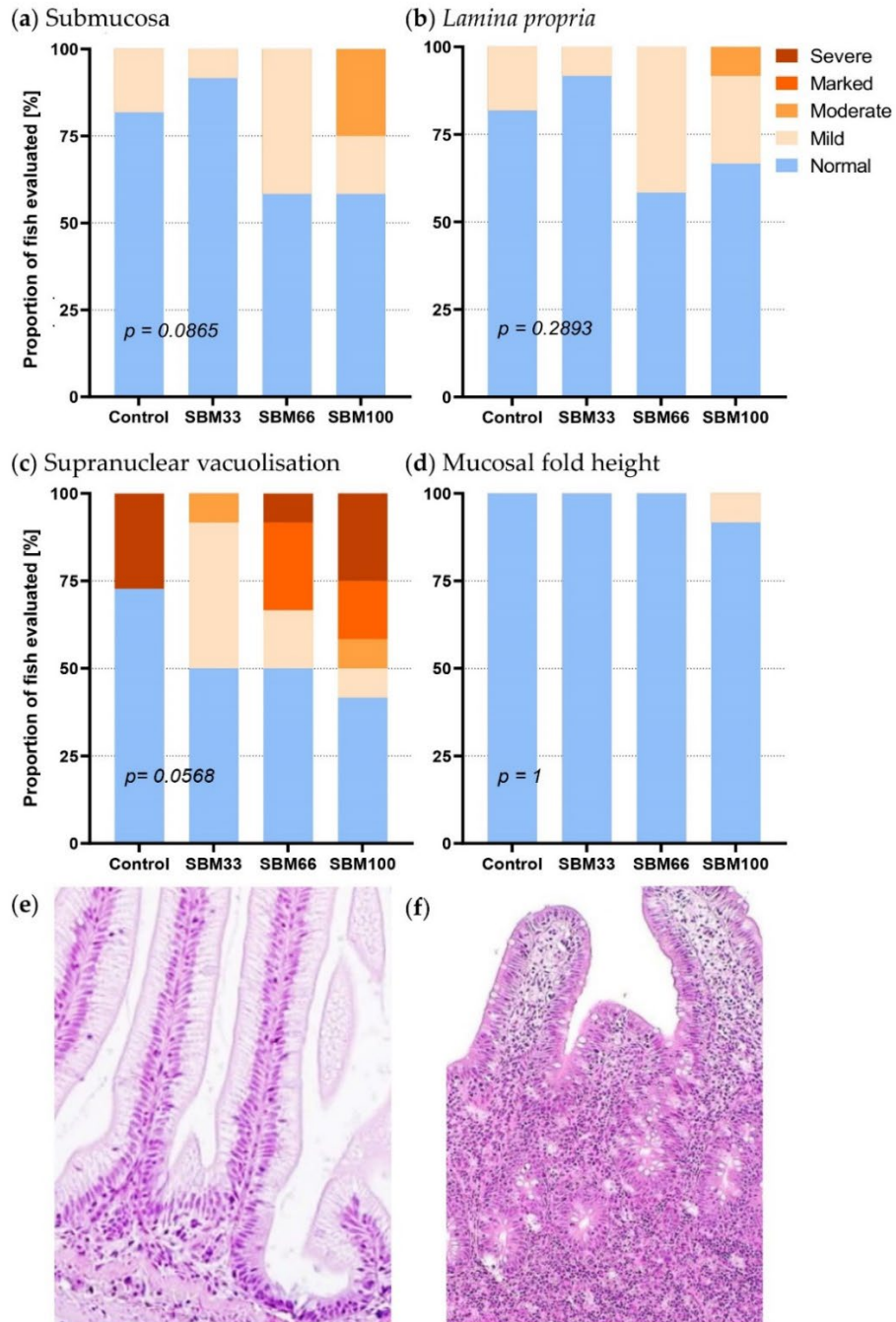


Figure III-5 Histological assessment of the intestinal sections from the feeding trial. The graphs show the number of intestinal sections ( $n = 6$ ) that were assessed as "normal", "mild", "moderate", "marked" and "severe". Various morphological characteristics were examined: (a) increase in width and inflammatory cell infiltration into the submucosa, (b) increase in width and inflammatory cell infiltration into the lamina propria, (c) reduction in supranuclear vacuolisation in enterocytes, and (d) height of mucosal villi. Histological images represent (e) a normal healthy gut (upper) and (f) a gut with severe inflammation characterised by widening and infiltration of the submucosa and lamina propria by inflammatory cells and loss of vacuolization on the enterocytes (lower). SBM33 – SBM100 = Soybean meal feeding groups with exchange of fish meal to 33 %, 66 % or 100 %.

The morphological changes in submucosa ( $p = 0.087$ ) and supranuclear vacuolation of enterocytes ( $p = 0.057$ ) were only slight across feeding groups (Figure III-5). The number of melanoma macrophages in liver and spleen was slightly increased in SBM100 and the macrophage clusters were proportionally larger than in the other feeding groups and tended to increase towards the end of the experiment.

### 3.7. GENE PROFILING

We established a panel of 34 immune- and stress-relevant qPCR assays to profile the transcriptomic response of rainbow trout to the three SBM supplemented diets. A comparison of the different diet groups revealed that distinct genes of the entire panel were significantly differentially regulated. Since our above described analyses already documented various more or less clear phenotypic and histological differences between the SBM100 diet and the other feeding treatments, the gene-expression data are presented in the Figure III-6 relative to the SBM100 group. MAP3K1 was the only out of 34 selected genes, which was significantly and consistently downregulated (approximately -2-fold;  $p \leq 0.04$ ) in the SBM100 group compared to the SBM33 and SBM66 groups after four and after eight weeks (Figure III-6).

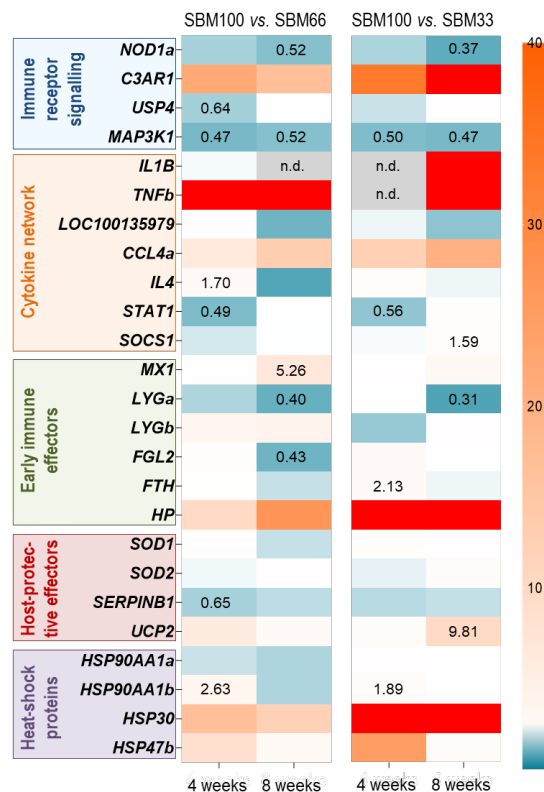


Figure III-6 The HeatMap illustrates the averaged fold-change values (according to the legend below) of the mRNA concentrations measured in the SBM100 relative to the SBM66 group (set as 1.0) and the SBM100 relative to the SBM33 group (set at 1.0) at the time points given below the scheme. The quantified transcripts are listed as gene symbols on the left and categorized according to their affiliation to immune- and stress-related processes. Transcripts that were undetectable in certain organs or cells were marked grey in the appropriate fields. Only significant copy-number changes ( $p \leq 0.05$ ) are given. All expression values were normalized against the geometric mean of three reference genes. SBM33 – SBM100 = Soybean meal feeding groups with exchange of fish meal to 33 %, 66 % or 100 %.



Other genes were found differentially expressed depending on the duration of the treatment. Four weeks of SBM100 diet reduced STAT1 levels ( $\leq -1.7$ -fold;  $p \leq 0.03$ ), but induced HSP90AB1a ( $\geq 1.7$ -fold;  $p = 0.01$ ) compared with the SBM33 and SBM66 groups. Additionally, FTH (2.1-fold;  $p = 0.02$ ) was upregulated in SBM100 group compared to SBM33, while IL4 (1.7-fold;  $p = 0.03$ ) and SERPINB1 (-1.5-fold;  $p = 0.03$ ) were modulated in the SBM100 group compared to the SBM66 groups.

Eight weeks of SBM100 diet downregulated NOD1a ( $< -1.9$ -fold;  $p = 0.05$ ) compared to the SBM33 and SBM66 groups. UCP2 (9.8-fold;  $p = 0.05$ ), TLR3 (2.4-fold;  $p = 0.006$ ), ITGA2B (1.9-fold;  $p = 0.002$ ) and SOCS1 (1.6-fold;  $p = 0.05$ ) were upregulated, whereas LYGa (-3.2-fold;  $p = 0.009$ ) and NCCRP1 (-1.6-fold;  $p = 0.05$ ) were downregulated in the SBM100 group compared to the SBM33 group. MX1 (3.2-fold;  $p = 0.05$ ) was upregulated and FGL2 (-1.9-fold;  $p = 0.007$ ) was downregulated in the SBM100 group compared to the SBM66 groups.

Furthermore, we found only few genes with higher (LYGb, 4.3-fold; ITGA2B, 1.74-fold) and lower transcript levels (CCL4, -1.64-fold) in the SBM33 compared with the SBM66 group after four weeks of feeding. After eight weeks, the levels of six genes analysed (FGL2, -2.1-fold; IL4/13, -2.1-fold; SOD1, -1.8-fold; SOD2, -1.49-fold; STAT1, -1.23-fold; TNF, -7.7-fold) were lower in the SBM33 compared with the SBM66 group (data not shown).

#### 4. DISCUSSION

The present study evaluated potential biomarkers that indicate the mid-term exposure of rainbow trout to three different concentrations of SBM. In essence, we observed that the increasing substitution of fishmeal by SBM decreased the growth and feed conversion. This is well in line with previous reports on SBM feeds for salmonid fish (Green et al., 2013; Knudsen et al., 2008; Zhang et al., 2013). These studies demonstrated consistently that feed intake and growth parameters are robust measures to assess the welfare status of trout in challenging long-term trials or under suboptimal husbandry conditions, provided that the disruptive stimulus is strong enough. Other parameters such as behaviour or colour changes of the skin can be standardised only to a limited extent and are primarily restricted to sick fish. Animal welfare goes beyond preventing the fish from suffering from serious diseases and weight loss. The German Animal Welfare Act (§ 1 TierSchG) prohibits pain, suffering and harm in fish and justifies implicitly the need for further, simply determinable markers to detect unsuitable husbandry conditions. Feed supply and food quality are crucial elements of animal husbandry and should be included in welfare monitoring strategies.

In this study, poor performance parameters were found across all feeding groups sampled. This is probably related to an increased frequency of necessary management measures (such as tank cleaning), which were necessary due to the less stable structure caused by a high SBM content of the feed pellets. The applied SBM diet models for a feed-induced enteritis manifested phenotypically by an inflammatory reaction in the posterior section of the intestine and diarrhoea in some trout as also described in other studies (Blaufuss et al., 2019; Krogdahl et al., 2000). These inflammatory changes were associated with supranuclear vacuolation, but this morphological feature is likely not limited to inflammation. The destruction of the intestinal epithelium deteriorates the absorption of

nutrients and massively disturbs the osmotic balance of the body. In this way, enteritis can finally even result in death (Blaufuss et al., 2019; Heikkinen et al., 2006).

The search for indicative parameters for SBM-induced enteritis at the molecular level turned out to be more challenging than expected. The histopathological analysis suggested that such markers are likely to be expected in the intestine, as supported by recent studies in various fish species (Blaufuss et al., 2019; Kemski et al., 2020; Valenzuela et al., 2021). As this requires the killing and dissection of the animals, we screened for potential markers that can be detected in small blood volumes taken non-lethally from farmed fish (Seibel et al., 2021). The haematocrit values and the levels of plasma cortisol and circulating lymphocytes in the blood of trout were elevated after the application of feed with high SBM dosages. Of note, these alterations were subject to strong individual variations, which presumably reflected social stress and the diverging growth of the fish. Strikingly, the increased haematocrit depended significantly on the body weight of individual trout. Thus, the above parameters do not seem suitable to indicate diet-related pathological effects.

The quantification of transcripts generally allows screening a large number of parameters involved in a variety of relevant functional pathways (Seibel et al., 2021). The gene-expression profiling suggested that the addition of a smaller SBM proportion in the feed ration has a slightly positive effect on the immune system, while a higher SBM proportion has an immunosuppressive effect in rainbow trout. Our data point at several genes (including SAA, MPO, NOS2, UCP2) that may be of interest for monitoring, but the observed modulations of their expression were rarely consistent across time points and/or diets. Only the levels of MAP3K1 were consistently reduced in trout having received feed with fully substituted fish meal. The MAP3K1 gene encodes the “mitogen-activated kinase kinase kinase 1” (alias MEKK1), which acts as an upstream activator of early transcription factors such as NF- $\kappa$ B (Hirano et al., 1996) or Jun (Yan et al., 1994). MAPK signalling pathways have previously been suggested to mediate the physiological effects of unbalanced dietary effects in fish (Holen et al., 2011; Wu et al., 2016; Zhang et al., 2017). STAT1 (coding for the transcription factor “signal transducer and activator of transcription 1”) was downregulated after four weeks of feeding with fully substituted fish meal. Only few studies reported the involvement of STAT1 in fish after feeding with particular diets (Booman et al., 2014; Zheng et al., 2010). At the same time point, HSP90AB1b (coding for a variant of the “heat-shock protein 90”) was upregulated in the same feeding group, which may reflect a cellular stress response to the challenging diet (Craig et al., 1993). After eight weeks of feeding with fully substituted fish meal, trout had reduced levels of NOD1a and LYGa (coding for variants of “nucleotide-binding oligomerization domain containing protein 1” and “lysozyme g”, respectively) suggesting a constrained bacteriolytic capacity (Dash et al., 2018; Kestra-Grunder and Tsolis, 2017). Apart from UCP2 (coding for “uncoupling protein 2”), none of the analysed transcript levels was strongly modulated in response to the SBM diets. A major bias was probably to expect pronounced systemic effects from a basically local (intestinal) response to certain feed additives. The systemic effects of trout fed with SBM-containing feed is obviously mild.

In conclusion, the present study underlines, once more, the necessity of tailoring the selection of diverse parameters to the type of challenge, which will be monitored. The results provide a basis for improved, minimally invasive monitoring of feeding regimes in

trout farming and may stimulate the development of practical detection devices for aquaculture 4.0 in the future - comparable to e. g. diabetes tests in human medicine.

**Supplementary Materials:** Table III-S1: primers used in this study.

**Author Contributions:** Conceptualisation, H.S., C.S.; methodology, H.S., A.R., and E.C.; investigation, H.S., A.R., and E.C.; writing, H.S. and A.R.; supervision, C.S.; project administration, H.S. and C.S.; funding acquisition, H.S. and C.S. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** All animal handling procedures were approved by the animal welfare officer of the "Gesellschaft für Marine Aquakultur mbH"<sup>8</sup> and the local authority of Schleswig-Holstein (MELUND<sup>9</sup>) according to relevant institutional and national guidelines for care and use of laboratory animals (German animal welfare law; TierSchG and Regulation for the Protection of Animals Used for Experimental and Other Scientific Purposes; TierSchVersV as the national implementation of the Directive 2010/63/EU).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data for this study were all collected at the Gesellschaft für Marine Aquakultur in Büsum<sup>8</sup>.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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Table III-S1 Primers used in this study.

	NCBI accession code	Gene symbol (Alias name)	Protein	Sense, antisense primer (5'→ 3')
Immune receptor signaling	XM_021626126	<i>NOD1a</i>	Nucleotide-binding oligomerization domain-containing protein 1	GACCGTGGTGAGATTGTGTG, AGTTCACCAGTATGGGTGGC
	NM_001124403	<i>C3AR1</i>	Complement 3a receptor 1	GGCTTATACTCGGCTTCCTCAT, TTCTTTGTGTGTTGGCTGCCTTA
	XM_021609985	<i>USP4</i>	Ubiquitin specific peptidase 4	GACGAGTGGTTTCTGATTGATAG, CTATCCCGGCCCTGTTGACAA
	XM_021601625	<i>MAP3K1</i>	Mitogen-activated protein kinase kinase 1	TCCTGGGAACCAATTGCCTTCAT, CACCTCGCTCATATTCAAGAT
Cytokine network	NM_001124347	<i>IL1B</i>	Interleukin-1 beta	GAGAGTGCTGTGGAAGAACATAT, ATGAATGAGGCTATGGAGCTGC
	NM_001124374	<i>TNF</i>	Tumour necrosis factor	AGCATGGAAGACTGGCAACGAT, CTTACCGCTGACACAGTGCAG
	XM_036943661	<i>LOC100135979</i>	CC chemokine with stalk CK2	TCCATGTTCCAGTCCAGGACC, CTGCAAGGATACCGCATCCAAA
	NM_001124489	<i>CCL4a</i>	C-C motif chemokine ligand 4 (variant a)	ATGGGTGAAGGACTACATCAACT, TATTTACAAGGACATGGCCAGT
	FN820501	<i>IL4/13</i>	Interleukin-4/-13	AACACATTTGACTGCGACCACC, AAACTTCAGAAGTGCACCGTTGT
	NM_001124707	<i>STAT1</i>	Signal transducer and activator of transcription 1	CCTGGCTGCTGCCAAAAGCA, AACAAGGTTCAAGGAAGCG-
	NM_001146166	<i>SOCS1</i>	Suppressor of cytokine signaling 1	GATCAGA ACGGATTCTGCGTCGGAAAATAT, ACGGATGCCAGGGAAGTGTGT
Early immune effectors	NM_001171901	<i>MX1</i>	MX dynamin like GTPase 1	AGGAGGGAATAGACTACTTTTGAA, ACATTACGAAGAGAAGGTGCCG
	XM_021560785	<i>LYGa</i>	Lysozyme g (variant a)	GCAGGTTGACAAGCGCTACCA, AAAGGGGGAATTTACGCTACAA
	XM_021557048	<i>LYGb</i>	Lysozyme g (variant b)	CAGGTTGATGTGGACCCAAATG, CTTAAAGGGGAATAGCTGCCT
	XM_021615386	<i>FGL2</i>	Fibrinogen-like protein 2	CAGCAACAAAATGGAGTCAGATAT, CATGGAACTAAAGGCCCAAG
	XM_021624437	<i>FTH</i>	Ferritin, heavy chain 1	AACCGGATGATCAACTTGGAGAT, GAGAGGTGGACGCATTTTACTC
	XM_021586024	<i>HP</i>	Haptoglobin	CGACCCAGGCTCCAGAACC, ATCATCACAGGCTGGGGCTTG
Host-protective effectors	XM_021590204	<i>SOD1</i>	Superoxide dismutase 1	TGCTTATGGAGACAACACCAAC, AATGTGGCTAAGATCAACATCCA
	XR_002474449	<i>SOD2</i>	Superoxide dismutase 2	TCCCTGACCTGACCTACGAC, GAGGTTTAATGGAGGAGGCC
	XM_021562188	<i>SERPINB1</i>	Serpin family B member 1 (leukocyte elastase inhibitor)	TACCAGTTCGTTGAGACGTTCC, ATCAAGAACCTATTGGCGGAGG
	XM_021622415	<i>UCP2</i>	Uncoupling protein 2	TTCGACCAGAGAGACGCCTTTA, GTAATGGACACTTTTTTGGGGG
Heat-shock	NM_001124231	<i>HSP90AB1a</i>	Heat-shock protein 90 alpha family class B member (variant a)	ATGATCAAGCTGGGCCTGGGA, CCTTATATAGCCTCACGTTTTCC

	XM_021598372	<i>HSP90AB1b</i>	Heat-shock protein 90 alpha family class B member (variant b)	GCTGGGTCTGGGAATCGATGA, GTCTCAAACCTACACACCTGTAT
	XM_021602849	<i>HSP30</i>	Heat-shock protein 30	AAGGGGTGAATCCTGAGACAGT, TGAAAACCCCAACAATCCTGTCA
	XM_021618128	<i>SERPINH1b (HSP47b)</i>	Heat-shock protein 47 (variant b)	ACCGCACAGGTCTCTATGGTTT, TTCAGCTTGCCCATCCAGTCAT
Leukocyte-specific markers	S63348	<i>IGHM</i>	Immunoglobulin, heavy constant chain mu (B-cell marker)	TACAAGAGGGAGACCGGAGGA, CTTGTTGACGATGAGCCGGTG
	EU072699	<i>TRB</i>	T cell receptor beta (T-cell marker)	GTCTTCTGCAAGTCAACAATGT, TCACCTGCATTGTCAGCTTTTAC
	XM_021617761	<i>NCCRP1</i>	non-specific cytotoxic cell receptor protein 1 (marker for non-specific cytotoxic cell)	TACGAAGCGAAGCCGTTCCGGT, GACTATACTGGCTGGACCACC
	XM_021600095	<i>ENPP3</i>	Ectonucleotide pyrophosphatase/phosphodiesterase 3 (marker for basophilic granulocytes)	CAGGCAACTCATCAAGGCTAATT, GATATTCTCATGCCGTTATGGGT
	XM_021582163	<i>MPO</i>	Myeloperoxidase (marker for neutrophilic granulocyte)	GTGAAGGACCAGATCATTGTATTA, TAGTATTGCCTCTTTAGGTGAGA
	XM_021581433	<i>CSF3R (GCSFR)</i>	Colony stimulating factor 3 receptor (granulocyte marker)	TACCACGGACCGGCTTTGTTC, AAGGTCCAAGCAGAGCCAACA
	XM_021559488	<i>CSF1R (MCSFR)</i>	colony stimulating factor 1 receptor (macrophage marker)	AACGAATGCTGGTGGTGTGAA, CTTAGTTTGATGCTGGACGTAATA
	NM_001124578	<i>TLR3</i>	Toll-like receptor 3 (marker for antigen-presenting cells)	AGTCCTCCACCTGTCGAATCTA, GAGCTTTCTGGGACAGCGATC
	XM_021603069	<i>ITGA2B (CD41)</i>	Integrin subunit alpha 2b (thrombocyte marker)	TATTATACAGTACGGGGTTGAGC, ATTGAATTTGCTCGACAGAACGC

**Anlage 3**

<b>C   A   U</b>	Christian-Albrechts-Universität zu Kiel	Agrar- und Ernährungs- wissenschaftliche Fakultät
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**Declaration of co-authorship**

if a dissertation is based on co-authored articles, a declaration from each of the authors regarding the part of the work done by the doctoral candidate must be enclosed when submitting the dissertation.

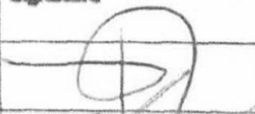
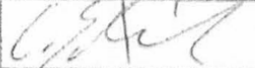
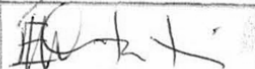
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<b>Name:</b>	Henrike Seibel

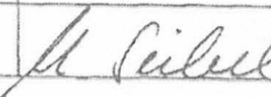
<b>2. This co-author declaration applies to the following article:</b>	
Blood-based Parameters Indicating a Soybean Meal-Induced	

The extent of the doctoral candidate's contribution to the article is assessed on the following scale:

- A. Has contributed to the work (0-33%)
- B. Has made a substantial contribution (34-66%)
- C. Did the majority of the work independently (67-100%)

3. Declaration on the individual phases of the scientific work (A,B,C)	Extent
<b>Concept:</b> Formulation of the basic scientific problem based on theoretical questions which require clarification, including a summary of the general questions which, it is assumed, will be answerable via analyses or concrete experiments/investigations	C
<b>Planning:</b> Planning of experiments/analyses and formulation of investigative methodology, including choice of method and independent methodological development, in such a way that the scientific questions asked can be expected to be answered	C
<b>Execution:</b> Involvement in the analysis or the concrete experiments/investigation	C
<b>Manuscript preparation:</b> Presentation, interpretation and discussion of the results obtained in article form	B

4. Signature of all co-authors		
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## IV. Research Chapter III - Soybean-meal and handling stress

### Interactions of plant-based feeding and handling stress on the expression of selected immune markers in rainbow trout (*Oncorhynchus mykiss*)<sup>10</sup>

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Substitutions for fishmeal in fish feed are important in the context of sustainability. However, nutrition has a decisive influence on well-being. An understanding of the impacts of botanicals on health or microbiomes is crucial when assessing the effect of potential immune modulators in the body of carnivorous fish. The present study examined the effect of a plant-protein based diet on the expression of selected immune markers in rainbow trout (*Oncorhynchus mykiss*) under handling stress. The amount of fishmeal was reduced to 7 % in the predominantly plant-protein based diet (P), in contrast to a diet based on 35% fishmeal (F). Triplicate groups of rainbow trout were fed for 59 days with experimental diets, and one experimental group of each feed type was stressed by shoing twice a day. The expression of the genes coding for tumour necrosis factor (TNF) $\alpha$ , interleukin (IL)1 $\beta$ , immunoglobulin (Ig) T and IgD was profiled in whole blood samples using real-time RT-qPCR. Significant expression differences between the F and P diets were observed for IL1 $\beta$  and IgD in nonstressed fish and TNF $\alpha$ , IgT and IgD in stressed fish. No differences were detected between nonstressed and stressed fish fed the same diet. After repetitive stress, gene expression patterns differed between trout fed the F or P diets, as IgT, IgD and TNF $\alpha$  were significantly downregulated in the stressed fish fed the P diet. In conclusion, growth and performance did not differ significantly with diet, although a pathohistological assessment of two intestinal segments revealed a histological response to the P dietfed ingredients. The molecular results indicate that fish performance should also be monitored under challenging conditions to fully assess the impact of dietary fishmeal substitutes.

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**Keywords:** immune marker, plant-based diet, rainbow trout, stress

## 1. INTRODUCTION

The well-being of aquaculture fish depends on several important factors, such as adequate feeding, tank environment and handling. Nutrition obviously has a decisive influence on fish growth and performance, but it also affects fish health. Current overfishing and sustainability concerns have led to the formulation of aquafeeds for carnivorous fish species, such as trout and salmon, with plant products as alternatives to fishmeal and fish oil (Hardy, 2010; INRA CIRAD, 2018). The nutritional value of plant-based proteins is less than that of fishmeal, but fishmeal-based diets can be substituted by a judicious selection of purified protein sources of plant origin (Hardy, 2003; Kissil et al., 2000; Mambrini et al., 1999; Stickney et al., 1996) without impairing growth performance.

Processed soybean meal and soy protein products are the most commonly used alternative protein sources for rainbow trout, for example, and do not compromise fish growth (Bruni et al., 2021; Caballero et al., 2002; Choi et al., 2020; Voorhees et al., 2019). Similarly, the inclusion of proteins from other plant sources, such as wheat, barley, cottonseed or canola, in rainbow trout diets can also lead to comparable or even better growth performance than that achieved with fishmeal (Apper-Bossard et al., 2013; Gatlin et al., 2007; Kissil et al., 2000; Wang et al., 2017). However, feeding soybean meal can cause morphological changes in the distal intestine of salmonids (Baeverfjord & Krogdahl, 1996; Booman et al., 2018; Krogdahl et al., 2015; Rumsey et al., 1994; Seibel et al., 2019; Uran et al., 2008; van den Ingh et al., 1991). These changes can include an increase in inflammatory cells, shortening of folds and disruption of microvilli, widening of the central stroma, increased formation of microvillar vesicles and elevated numbers of goblet cells and apoptotic cells, and reduced supranuclear vacuolization with apical nuclear displacement within enterocytes (Baeverfjord & Krogdahl, 1996; Chikwati et al., 2012; Knudsen et al., 2008; Merrifield, 2011).

Although plant-based proteins are broadly used in commercial fish diets, concerns have been raised about potential adverse reactions to these proteins in fish under stressful conditions. For example, Atlantic salmon becomes highly susceptible to temperature stress when fed soybean meal-based diets (Uran et al., 2008), due to alterations in the intestinal microbiome (Green et al., 2013). Stress in aquaculture can arise during the handling, sorting, grading and transportation of the fish and this increases the susceptibility to pathogenic diseases (Gabriel & Akinrotimi, 2011; Segner et al., 2019). Ethical concerns are now fueling increasing research on fish welfare (Seibel et al., 2020) with regard to both technical and nutritional aspects for the improved well-being of farmed fish. The aim of the present study was investigated whether feeding a plant-protein based diet would negatively impact the immune system of rainbow trout when the fish are exposed to handling stress in aquaculture.

Previous studies have used impaired growth performance as a biomarker of prolonged stress (Pickering, 1992; Tort, 2011; Van Weerd & Komen, 1998). An acute stress-related cortisol release promotes an elevation of plasma glucose and replenishment of glycogen stores in the liver, with a resulting depression in fish growth rates (Mommsen et al., 1999; Wendelaar Bonga, 1997). However, growth performance is not always a reliable stress marker, as growth changes only become apparent following long-term interference. A

better alternative is to use molecular markers, as these can reveal variations much earlier and more sensitively, while also covering a larger physiological target area, including the immune system (Seibel et al., 2021).

Teleost fish show stress-responsive production of interleukin (IL) 1 $\beta$ , a proinflammatory cytokine (Kono et al., 2002). The proinflammatory action of IL1 $\beta$  includes the stimulation of T cells (Mathew et al., 2002), increased phagocytosis (with enhanced phagocytic and lysozyme activity of macrophages) (Hong et al., 2004) and enhanced antibody production (Yin & Kwang, 2000; Zou & Secombes, 2016). IL1 $\beta$  production is stimulated by tumour necrosis factor alpha (TNF $\alpha$ ; Zou & Secombes, 2016), a key regulator of inflammatory responses (Zou & Secombes, 2016) that is produced by macrophages, lymphocytes, mast cells, astrocytes and tumour cells (Qin et al., 2001). In fish, TNF $\alpha$  in fish activates macrophages, induces their respiratory burst activity (Qin et al., 2001; Tort et al., 2004) and enhances the phagocytic activity of leucocytes (Grayfer et al., 2008; Zou et al., 2003).

In salmonid fishes, immunoglobulins (Ig), particularly IgT and IgD also play an important role in various processes (Fillatreau et al., 2013), including defences against stress due to exogenous substances. IgT is associated with digestive immunity and is therefore found in the intestinal mucosa and in serum (Zhang et al., 2010). IgD-armed basophils (membrane-bound isoforms) produce opsonizing and immune-stimulating factors (Chen et al., 2009); therefore, secreted IgD might enhance immune protection by regulating B-cell homeostasis and activation (Nitschke et al., 1993). IgD also has a role in immunomodulation and can be upregulated via signalling by proinflammatory cytokines (Chen et al., 2009).

The present study investigated the potential influences of rainbow trout nutrition on fish responses to chronic handling stress. We evaluated standard performance and health parameters, including a histopathological assessment of the fish gut, and recorded the effect of combined dietary and handling stress effects on the gene expression levels of the classical markers IL1 $\beta$  and TNF $\alpha$ , and of IgT and IgD.

## 2 MATERIAL AND METHODS

### 2.1 EXPERIMENTAL DESIGN, ANIMALS AND ETHICS STATEMENT

The experiment was conducted at the 'Gesellschaft für Marine Aquakultur mbH'<sup>11</sup> (GMA). The rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) used in this trial were all female fish originating from Troutlodge, Bonney Lake USA. They were obtained locally from Forellenzucht Troststadt GbR, Tautenhahn, Germany. All animal handling procedures were approved by the animal welfare officer of the 'Gesellschaft für Marine Aquakultur mbH' and by the local authority of Schleswig-Holstein (MELUND, V 241–36,754/2018), according to relevant institutional and national guidelines for care and use of laboratory animals (German animal welfare law; TierSchG and Regulation for the Protection of Animals Used for Experimental and Other Scientific Purposes; TierSchVersV as the national implemen-

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<sup>11</sup> dato Department of Aquaculture and Aquatic Resources at Fraunhofer IMTE

tation of the Directive 2010/63/EU). The research also adhered to Aquaculture Research ethical guidelines.

In total 216 rainbow trout were individually weighed ( $124.7 \text{ g} \pm 0.3$ ) and distributed among twelve 150 L tanks of a recirculating aquaculture system (RAS). The multi-factorial experimental block design resulted in 18 fish per tank (stocking density =  $14.94 \text{ kg/m}^3$ ) with a mean group weight of  $2.27 \text{ kg} \pm 0.05$  per tank. Experimental groups were reared in triplicate, resulting in four treatment groups. Two different diets (fishmeal diet F and a predominantly plant-protein based diet P; see Table IV-1) and two different stress treatments (handling stress S and no handling stress NS; see Figure IV-1) were applied as follows: FNS group (fishmeal diet, no stress), PNS group (predominantly plant-protein based diet, no stress), FS group (fishmeal diet, stress) and PS group (predominantly plant-protein based diet, stress). The fully recirculating system had an average water temperature of  $15.17 \text{ }^\circ\text{C} \pm 0.44$ , an average water salinity of  $4.62 \text{ ppt} \pm 0.63$ , and an average oxygen concentration in the water of  $9.67 \text{ mg/L} \pm 0.69$  throughout the experiment. A light to dark rhythm of 15:9 h was applied. Fish were fed twice a day with a restrictive feeding regime of 1.5 % of body weight per day for 59 days.

Prior to the trial, all fish were adapted to the RAS for 6 weeks and fed a commercial trout diet at 0.5% of body weight per day.

*Table IV-1 Components of fish diets used. The data regarding analytical components of the ingredients used (% of original substance) have been provided by SPAROS Lda, Olhão, Portugal. The calculation of data regarding nutritional values and energy content below was performed at GMA Büsum, Hafentörn 3, 25,761 Büsum Germany. F = predominantly fishmeal diet; P = predominantly plant-protein based diet; NfE = nitrogen-free extractives*

<b>Ingredients</b>	<b>F % feed</b>	<b>P % feed</b>
Fishmeal LT70	35.00	7.00
Fish protein concentrate	2.50	2.50
Soy protein concentrate	6.00	20.50
Wheat gluten	6.00	15.20
Corn gluten	5.00	5.00
Soybean meal 48		5.00
Wheat meal	15.00	11.40
Faba beans (low tannins)	6.00	6.00
Fish oil	13.92	14.64
Rapeseed oil	9.28	9.76
Vit & Min Premix INVIVO 1%	1.00	1.00
Lutavit C35	0.05	0.05
Lutavit E50	0.05	0.05
Antioxidant	0.20	0.20
Monocalcium phosphate		1.00
L-Lysine		0.10
L-Tryptophan		0.10
DL-Methionine		0.50
<b>Total</b>	<b>100.00</b>	<b>100.00</b>



As fed basis	F	P
Crude protein, % feed	41.75	42.66
Crude fat, % feed	26.50	26.48
NfE, % feed	18.43	20.79
Ash, % feed	7.74	4.85
Gross Energy, MJ/kg feed	23.54	23.92

## 2.2 EXPERIMENTAL TREATMENTS

Two isonitrogenous and isoenergetic diets (F, P) were designed and produced in accordance with standard commercial rainbow trout diets, varying only in the amounts of fishmeal and plant-based proteins (see Table IV-1; manufacturing by SPAROS Lda).

To generate a procedural stressor of continuous handling stress in the treatment groups PS and FS, the fish were shoed forcefully with aquarium nets for 60 s twice per day, 2 h after feeding (see Figure IV-1). In order to reduce random effects resulting from the shoing activity in nonstressed fish groups, stressed and nonstressed fish were spatially separated from each other in the RAS to account for activity in front of the tanks.

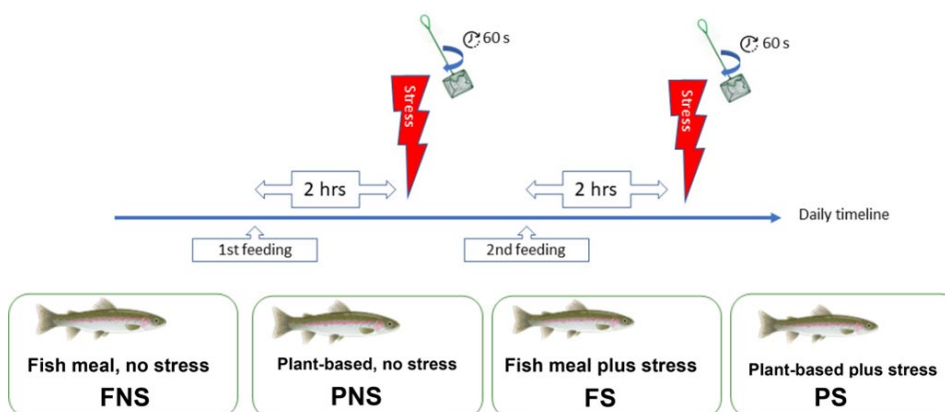


Figure IV-1 Feeding groups and schematic representation of daily trials. Water parameters were sampled every morning after the 1st feeding. After 2 h, a 1 min handling stress was induced. The treatment was repeated twice a day. FNS = fishmeal, no stress; FS = fishmeal, stressed; PNS = predominantly plant-protein based, no stress; PS = predominantly plant-protein based, stressed; Pictures of trout were created with biorender.

## 2.3 SAMPLING

Group weight was determined every 2 weeks and individual body weights of 10 fish per tank at the end of the trial. Classical performance parameters, including specific growth rate (SGR), feed conversion ratio (FCR), daily feed intake (DFI), protein efficiency ratio (PER) and protein retention efficiency (PRE), were calculated based on group measures, as follows:

$$\text{SGR} = [\ln(\text{FGW}) - \ln(\text{IGW})] / t * 100;$$

$$\text{FCR} = (\text{Ft} / 1000) / (\text{FGW} - \text{IGW});$$

$$\text{DFI} = \text{SGR} * \text{FCR};$$

$$\text{PER} = (\text{FGW} - \text{IGW}) * 1000 / \text{CPT};$$

$$\text{PRE} = \text{CPg} / \text{CPT} * 100;$$

where FGW = final group weight [kg], IGW = initial group weight [kg], t = 56 days, Ft = total amount of feed intake [g], CPT = total amount of crude protein intake [g] and CPg = total amount of body protein gained [g].

At the end of the trial, EDTA full blood samples were taken from 60 randomly selected and mechanically stunned trout (five fish per tank, 15 fish per treatment). Blood was taken from the caudal vein (vena caudalis) using sterile S-Monovette® (EDTA KE/1.2 ml; Sarstedt) and sterile safety-canula syringes (21G x 1 ½'; Sarstedt). Samples (250 µl) were suspended in 1.3 ml RNA-later (total volume 2 ml; Sigma Life Science) and stored at -80 °C until processing. Two intestinal sections - an anterior and posterior section - were excised from fish and subsequently fixed in 4 % buffered formalin solution for histopathological investigation.

#### 2.4 QPCR

RNA was extracted from blood using a modified RNeasy Midi kit (Qiagen), including two DNase treatments. The optical density of extracted RNA was measured with a Tecan Nanoquant Infinite M200 Photometer (Tecan Group AG) and the RNA concentration was set to 10 ng/ml. Genomic DNA was removed using the Qiagen gDNA Removal Mix (Qiagen) prior to reverse transcription (Quanti Nova Reverse Transcription Kit; Qiagen). Quantitative real-time PCR was performed using QuantiNova SYBR-PCR Kit (Qiagen) and the Aria Mx-Real Time PCR system (Agilent Technologies).

The mRNA concentrations were quantified for the potential reference genes actin beta (ACTB), elongationfactor1 (EEF1A) and ribosomalproteinS5 (RPS5). However, only EEF1A (Forward: GAAACTTGAGGATGCCCCCA; Reverse: AGCGAAACGACCAAGAGGAG; NM\_001124339) and RPS5 (Forward: ATGACATCTCACTGCAGGATTAC; Reverse: ATCAGCTTCTTGCCGTTGTTGC; NM\_001160519) were found suitable for normalizing qPCR data, since ACTB showed high standard deviations (tested with NormFinder). A normalization factor for each gene was determined by calculating the geometric mean of the quotient of these two reference genes. Raw copy numbers for each sample were corrected by this normalization factor.

Gene expression was investigated for membrane-bound IgT (Forward: GCCTCAGGGGTGGAATGCTT; Reverse: AGAAGGGGTCCACAGTTCATAA; GenBank accession no. AY870264), membrane-bound IgD (Forward: TGAACATATCCAAACCAGAGCTC; Reverse: GAGTGAAGCTGTAGAG; GenBank accession no. AY870260), and secreted IgD (Forward: CCCGCCTTCGTCAATCCATTTG; Reverse: ACTTCTGCCTCATCAACTGCTC; GenBank accession no. JQ034605), and IL1β (Forward: GAGAGTGCTGTGGAAGAACAT; Reverse: GCAGCTCCATAGCCTCATTCA; GenBank accession no. AJ298294) and TNFα (Forward:

AGCATGGAAGACTGGCAACGAT; Reverse: CTGCACTGTGTCAGCGGTAAG; GenBank accession no. AJ278085). The qPCR efficiency was evaluated by comparing the melting curves and  $T_m$ -values between samples. The variances between different qPCR runs were minimized by grouping all data for genes of interest together using multiple plate analysis (MX 1.0 Agilent Technologies).

## 2.5 HISTOPATHOLOGICAL INVESTIGATION

Tissues were dehydrated and embedded in paraffin, cut into 4  $\mu\text{m}$  thick slices, and stained with haematoxylin and eosin according to the routine methods used in histopathological surgery (Bleyer and Gruber-Dujardin). Samples were examined randomly with a light microscope (Primo Star, Carl Zeiss Microscopy GmbH). Corresponding sections were examined for various aspects associated with soybean meal- induced enteritis, including shortening of folds, increased widening of central stroma with increased cellularity, leucocytic cellular infiltration of the submucosa and lamina propria, elevated numbers of goblet cells and apical nuclear displacement within enterocytes, increased numbers of apoptotic cells among enterocytes, and disrupted folds (Baeverfjord & Kroghdal, 1996; Bruce et al., 2018; Merrifield, 2011).

## 2.6 STATISTICS

The R (version R-4.0.4) and RStudio (version 1.4.1106) statistical software were used to evaluate the data. An analysis of variance (ANOVA) was conducted to evaluate performance data based on group measurements, followed by multiple contrast tests (Bretz et al., 2011) using the R-package multcomp (Hothorn et al., 2008) to compare several levels of the two fixed factors diet and stress. A Kruskal–Wallis rank-sum test was applied to evaluate immune marker gene expression data, followed by nonparametric multiple comparisons for relative effects (Konietschke et al., 2012) using the R-package nparcomp (Konietschke et al., 2015). Test statistics and adjusted p-values were computed using a multivariate Satterthwaite t-Approximation. Data from the histopathological investigation were analysed using a Bayesian generalized linear model for binomial data (observation or no observation), followed by simultaneous tests for general linear hypotheses using the multcomp R-package (Hothorn et al., 2008). Based on the two fixed factors diet (F, P) and stress (NS, S), the following contrasts were considered in all statistical analysis: FNS vs. PNS, FS vs. PS, FNS vs. FS, and PNS vs. PS. A significance level of  $p < 0.05$  was assumed.

## 3 RESULTS AND DISCUSSION

The substitution of several plant-based proteins for fishmeal has been well documented to have little effect on fish growth (depending on inclusion levels), even among carnivorous species, such as salmonids (Collins et al., 2013; Kaiser et al., 2021; Michl et al., 2017). The present study used a 20.5 % soy protein concentrate and 5.0 % soybean meal in the predominantly plant-protein based diet P, whereas the primarily fishmeal-based diet F contained only 6.0 % soy protein concentrate and no soybean meal. Additional plant-based

ingredients were wheat and corn gluten, wheat meal, and faba bean meal. After 56 feeding days, no statistically significant differences were noted between treatments for the average final group weights or individual body weights ( $n = 3$ ; Table IV-2).

Table IV-2 Growth performance parameters of rainbow trout. Initial values and after 56 days of feeding. Mean  $\pm$  SD;  $n = 3$

	FNS	PNS	FS	PS
IBW	124.52 $\pm$ 0.55	124.91 $\pm$ 0.20	124.71 $\pm$ 0.23	124.62 $\pm$ 0.20
FBW	359.82 $\pm$ 2.09	347.80 $\pm$ 4.68	350.08 $\pm$ 0.52	343.01 $\pm$ 12.66
IGW	2.27 $\pm$ 0.04	2.23 $\pm$ 0.02	2.30 $\pm$ 0.00	2.27 $\pm$ 0.04
FGW	5.52 $\pm$ 0.10	5.38 $\pm$ 0.11	5.43 $\pm$ 0.08	5.38 $\pm$ 0.07
SGR	1.78 $\pm$ 0.02	1.76 $\pm$ 0.04	1.72 $\pm$ 0.03	1.73 $\pm$ 0.07
FCR	0.85 $\pm$ 0.02	0.88 $\pm$ 0.01	0.87 $\pm$ 0.02	0.89 $\pm$ 0.04
DFI	1.51 $\pm$ 0.01 <sup>A</sup>	1.54 $\pm$ 0.01 <sup>B</sup>	1.49 $\pm$ 0.01 <sup>A</sup>	1.54 $\pm$ 0.01 <sup>B</sup>
PER	2.77 $\pm$ 0.02	2.73 $\pm$ 0.05	2.70 $\pm$ 0.04	2.69 $\pm$ 0.12
PRE	45.79 $\pm$ 0.12	44.32 $\pm$ 1.12	41.49 $\pm$ 4.27	43.99 $\pm$ 7.32

Note: IBW (initial individual body weight, [g]); FBW (final individual body weight [g]); IGW (initial group weight, [kg]); FGW (final group weight, [kg]); FCR (feed conversion ratio); SGR (specific growth rate); DFI (daily feed intake); PER (protein efficiency ratio); PRE (protein retention efficiency). Values in the same row with different superscript letters are significantly different ( $p < 0.05$ ); FNS = fishmeal, no stress; PNS = predominantly plant-protein based, no stress; FS = fishmeal, stressed; PS = predominantly plant-protein based, stressed.

Neither the type of diet nor the stress application by shoing had a statistically significant effect on the growth performance of the fish. The dietary treatments also had little influence on most of the additional performance parameters investigated. However, we did observe a significant effect of diet on daily feed intake (DFI;  $p < 0.001$ ): the feed intake was lower in fish fed the fishmeal diet F than in fish fed the plant-protein based diet P. Despite reaching statistical significance, the differences in average DFI between feeding groups were 0.03 % and 0.05 % of the group weight and were therefore presumably not of biological significance.

Although soy products, such as soybean meal or soy protein concentrates, are of high nutritive value for fish (Caballero et al., 2002), they also may contain antinutritive factors that can impair the nutritional advantages. These antinutritive factors can only be eliminated to a certain extent through costly and energy-consuming processing (e. g. thermal treatment or separation). Moreover, during the processing of protein concentrates, some antinutritive factors (e. g. phytates) can be co-purified and accumulate, with subsequent adverse effects on the fed organisms (Kaiser et al., 2021). Soya saponins, for example, can have a negative influence on membrane permeability and can disturb the intestinal barrier (Francis et al., 2001). Other potential inhibitors in soy include lectins, anti-vitamins, allergens, and protease inhibitors (Apper-Bossard et al., 2013). Although both experimental diets were designed to be nutritionally balanced for rainbow trout, the predominantly plant-protein based diet might still include higher levels of antinutritive factors that could negatively affect gut health and general immune functions, despite the observation of identical growth performances in the present study.

We observed a slight histological alteration in fish fed the P diet (Figure IV-4). These included a tendency toward more abundant inflammatory cell infiltration within the submucosa and lamina propria and a significant increase in disrupted folds ( $p = 0.013$ ) and are probably attributable to soy-based saponins (Baeverfjord & Krogdahl, 1996; Bakke-McKellep et al., 2007; Krogdahl et al., 2000; Merrifield, 2011). All changes were more abundant in the posterior intestinal segments, which are the main nutrient retention areas. Slightly fewer destroyed folds were found in individuals from the stressed feeding groups (with  $p = 0.064$ ). We detected almost the same number of apoptotic cells in enterocytes, but these were again significantly more frequent in the posterior intestinal segments ( $p = 0.0001$ ) in both the FS and PNS groups, whereas the number in the PS group decreased considerably. The influence of soy was therefore to increase the number of cells undergoing cell repair and programmed cell death (Bakke-McKellep et al., 2007).

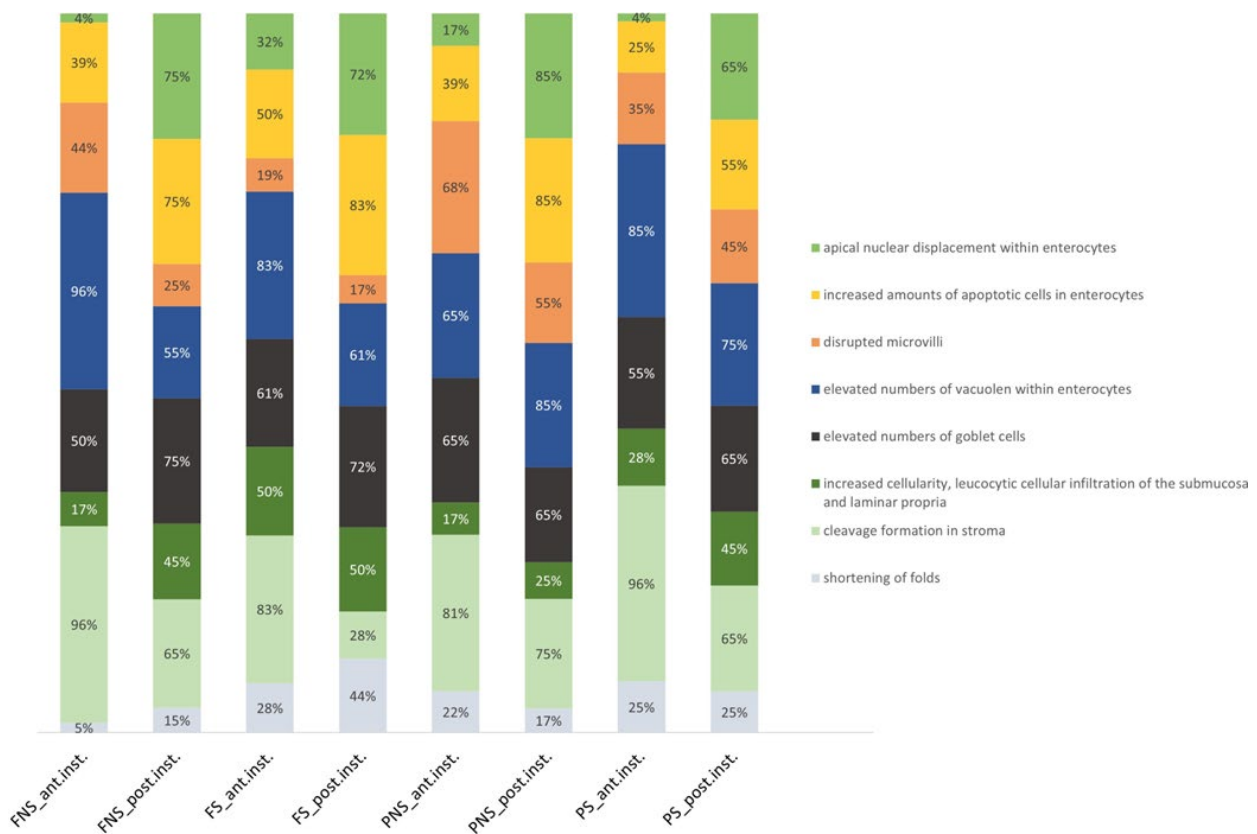


Figure IV-4 Probabilities of risk that the named change has occurred in investigated gut segments. ant. Inst., anterior intestine section; FNS, predominantly fishmeal, no stress; FS, predominantly fishmeal, stressed; PNS, predominantly plant-protein based, no stress; post. Inst., posterior intestine section; n = 6; PS, predominantly plant-protein based, stressed.

Previous work has indicated that the fish body weight or other classical performance parameters seem to be less suitable parameters for determining the stressor-dependent effects of inflammatory symptoms in the intestine, as these parameters remain apparently unaffected even in the presence of clear changes in the intestinal epithelium (Krogdahl et al., 2000; Yamamoto et al., 2010). Certainly, differences also arise due to the type of

stressor applied, the duration of the stress exposure, and the overall nutrient supply. Nine months of crowding stress (100 g/L) significantly decreased the weight of trout compared with their conspecifics kept at adequate density, and the weight decrease was correlated with significantly increased levels of plasma cortisol and pituitary growth hormone (Pickering et al., 1991).

Therefore, additional parameters should be quantified in future to determine the effects of plant-protein based diets on the well-being of fish exposed to even apparently minor stressors (i. e. aquaculture handling).

Rainbow trout prefer lipid and protein sources to fuel their energy demands (Hardy, 2003), since these carnivore salmonids lack adequate gut microbiota for carbohydrate digestion (Krogdahl et al., 2015). Fish meal contains large quantities of energy per unit and can quickly elevate glucose levels in the blood, as shown in experiments with cod, *Gadus morhua* (Hemre et al., 1989). Energy, and therefore an elevation of plasma glucose, is necessary to cope with stress (Wendelaar Bonga, 1997). Various proteins included in fish feeds can have distinct effects on the health of animals. For instance, antibody titers tested with sheep red blood cells were significantly lower in carp (*Cyprinus carpio*) fed a low-protein (15.2 % vs. 42.8 %) and low-fat (5.6 % vs. 8.2 %) diet than in the control group (Pilarczyk, 1995). In the current study, both feeding formulations had comparable levels of crude protein (F: 41.75 %; P: 42.66 %; Table IV-1) and crude fat (F: 26.50 %; P: 26.48 %; Table IV-1) and both were within the recommended range (NRC, 2011). Studies on gilthead seabream have shown an upregulation of the inflammatory cytokines IL1 $\beta$  and TNF $\alpha$  in the gut when fish oil was replaced 100 % by a mixture of vegetable oils, thereby confirming a link between the selected aqua-feed and a stress reaction (Montero et al., 2010). Both feed types in the present study had a comparable amount of energy in MJ/kg (F = 23.54; P = 23.92), crude protein, and fat (Table IV-1). However, the additional evaluation of digestibility was not part of this study; consequently, we cannot exclude the possibility that different energy retention of the two feeds may have caused different profiles of the immunological markers. In addition, local inflammation in the intestine certainly plays a role here. Nevertheless, we found similarly high numbers of goblet cells and vacuoles in enterocytes (Figure IV-4) in all groups, suggesting that nutrients had been appropriately taken up. The numbers of vacuoles were significantly different among the intestinal sections ( $p = 0.041$ ), with increased vacuole numbers observed in the posterior intestinal sections in all feeding groups. This is a known physiological phenomenon in trout and underlines the healthy development of the intestine.

### 3.1 TRANSCRIPT LEVELS OF TUMOUR NECROSIS FACTOR– ALPHA

Rainbow trout fed the primarily plant-protein based diet or the primarily fishmeal-based diet shared a similarly low TNF $\alpha$  transcript levels (FNS: 68 copies/ $\mu$ g cDNA, PNS: 27 copies/ $\mu$ g cDNA;  $p = 0.111$ ,  $n = 13$ ). However, exposure of fish to long-term handling stress significantly downregulated the average expression of TNF $\alpha$  in the fish fed the primarily plant-protein based diet (FS: 68 copies/ $\mu$ g cDNA, PS: 28 copies/ $\mu$ g cDNA;  $p = 0.042$ ,  $n = 15$ ; Figure IV-2).

TNF $\alpha$  is a proinflammatory cytokine that plays a crucial role in cell proliferation, differentiation, apoptosis, and necrosis. TNF $\alpha$  is upregulated in response to circulating stress hormones and in response to pathogenic activity (Castillo et al., 2009; Secombes et

al., 2001). Studies in seabream (Mulero & Meseguer, 1998) and catfish (Yin et al., 1997) have shown that TNF $\alpha$  activates macrophages and is therefore likely to increase phagocytosis processes needed to eliminate potentially harmful agents. LPS stimulation of leucocytes in rainbow trout also upregulates TNF $\alpha$  expression (Laing et al., 2001). An experiment with zebrafish infected with *Mycobacterium marinum* (an atypical water-borne Mycobacterium that affects soft tissues) showed that TNF $\alpha$  promoted macrophage survival, while restricting bacterial growth in already infected macrophages (Zou & Secombes, 2016). Overall, the TNF levels detected in this study were very low, suggesting that the fish feeding protocol did not lead to high-grade inflammatory symptoms. This was confirmed by the histological examination of the intestine, where the changes were rather mild.

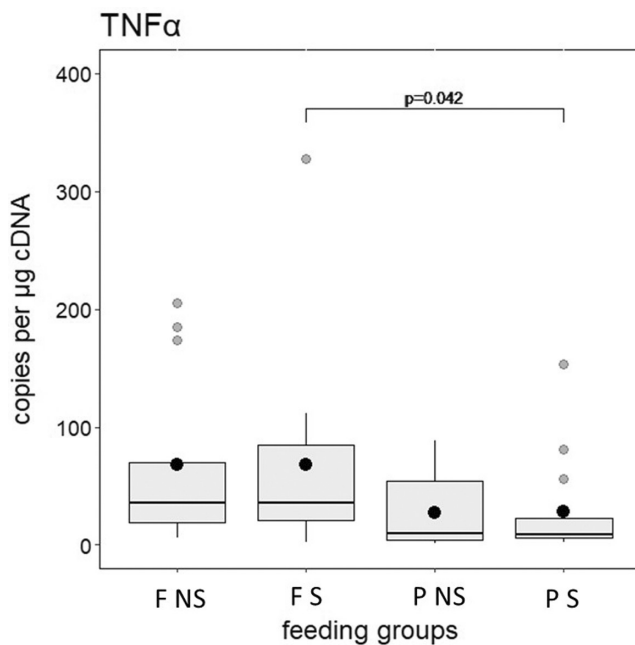


Figure IV-2 Box and whisker plot of the tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) copies per  $\mu$ g cDNA. FNS = fishmeal, no stress; FS = fishmeal, stressed; PNS = predominantly plant-protein based, no stress; PS = predominantly plant-protein based, stressed. Box represents the interquartile range (IQR): Lower end = 25th percentile, upper end = 75th percentile, solid line = median, whiskers = 1.5\*IQR, grey dots = potential outliers, black dots = mean of feeding group, significant differences between feeding groups are indicated with brackets and corresponding p-values.

### 3.2 INTERLEUKIN 1B GENE EXPRESSION

Overall, the mRNA levels of IL1 $\beta$  were higher in stressed than in non-stressed fish; however, the differences did not reach statistical significance. The IL1 $\beta$  mRNA levels also did not differ between the fish from the FS (292 copies/ $\mu$ g cDNA) and the PS (251 copies/ $\mu$ g cDNA) treatments. However, significant differences were found between fish from the nonstressed FNS and PNS groups (FNS: 107 copies/ $\mu$ g cDNA, PNS: 28 copies/ $\mu$ g cDNA;  $p = 0.048$ ; Figure IV-3 A,B).

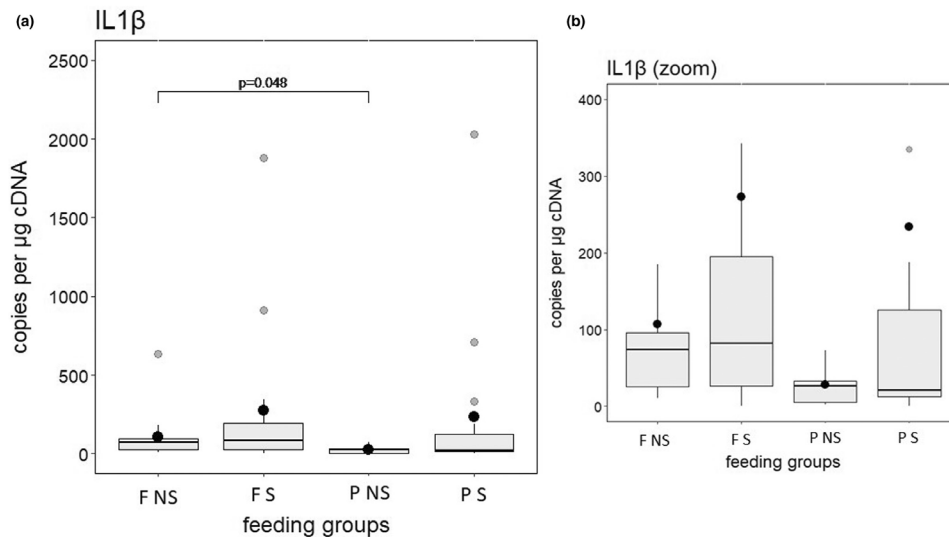


Figure IV-3 Box and whisker plot of the interleukin-1 $\beta$  (IL-1 $\beta$ ) copies per  $\mu$ g cDNA, normal presentation (a) and zoom (b). FNS = fishmeal, no stress; FS = fishmeal, stressed; PNS = predominantly plant-protein based, no stress; PS = predominantly plant-protein based, stressed. Box represents the interquartile range (IQR): Lower end = 25th percentile, upper end = 75th percentile, solid line = median, whiskers = 1.5\*IQR, grey dots = potential outliers, black dots = mean of feeding group, significant differences between feeding groups are indicated with brackets and corresponding p-values.

The cytokine IL1 $\beta$  is commonly upregulated in response to stress (Fast et al., 2008; Korytár et al., 2016) and is one of the first proinflammatory cytokines produced. It affects the expression of other proinflammatory cytokines (Uribe et al., 2011; Zou & Secombes, 2016), and this might explain the slightly higher IL1 $\beta$  copy numbers in the stressed trout groups.

In the present study, the highest IL1 $\beta$  expression was observed in the FS treatment group, and this may indicate a higher number of circulating leucocytes, because IL1 $\beta$  is robustly synthesized by leucocytes, such as monocytes/macrophages (Pelegrín et al., 2004). The histological examination confirmed this assumption, as the greatest numbers of inflammatory cells, and apoptotic cells, were found in the FS group (Figure IV-4). A similar tendency ( $p = 0.08$ ) was evident in the stressed versus nonstressed feeding groups, with fewer inflammatory cells in the submucosa and lamina propria of fish fed the predominantly plant-protein based diet than the fish-meal diet when the fish were not stressed.

Macrophages and other IL1 $\beta$ -producing cells are activated by PAMPs, such as LPS or other microbial features (Pressley et al., 2005; Zou et al., 2000; Zou & Secombes, 2016). Consequently, we cannot exclude an additional influence of pathogens on the expression of IL1 $\beta$  and TNF $\alpha$ , although this is very unlikely due to the low expression levels. Clinically, the trout examined here exhibited no outward symptoms that would indicate any acute signs of pathogen infection during the trials.

The expression of proinflammatory cytokines can change with diet. For instance, gilthead seabream reared on diets differing in fish oil content (replaced either partially [70 %] or totally by vegetable oil mixtures) showed significant increases in IL1 $\beta$  and TNF $\alpha$  expression in the intestine when fed exclusively vegetable oils, the fish were more susceptible when challenged with the pathogen *Photobacterium damsela* sp. *piscicida* (Montero et al., 2010). Increasing the amount of soybean meal in orange-spotted grouper diets also decreased growth performance and increased intestinal harm, while also



significantly increasing IL1 $\beta$  expression levels (Wang et al., 2017). In our experimental setup, the fish fed the primarily fishmeal-based diet showed proportionally higher expression levels of proinflammatory cytokines, although no high-grade histological changes were evident in the intestine.

### 3.3 IMMUNOGLOBULIN D AND T

Stress exposure and the type of feeding also influenced immune competence. We observed a significant downregulation of membrane-bound IgT and IgD transcripts in the stressed fish fed the plant-protein based diet. Overall, the expression was higher in trout fed a predominance of fishmeal in the diet.

The copy numbers of the genes coding for membrane-bound immunoglobulin D, IgD\_M (Figure IV-5 b) and the secreted immunoglobulin D, IgD\_S (Figure IV-5 a) were similar (they are henceforth termed IgD). Genes were significantly downregulated (IgD\_M:  $p = 0.007$ ; IgD\_S:  $p = 0.046$ ) in the fish fed the primarily plant-protein based diet and in stressed fish PS (IgD\_M:  $3.39E+05$  copies/ $\mu$ g cDNA; IgD\_S:  $3.33E+05$  copies/ $\mu$ g cDNA) compared with the treatment group FS (IgD\_M:  $7.71E+05$  copies/ $\mu$ g cDNA; IgD\_S:  $5.92E+05$  copies/ $\mu$ g cDNA).

The expression levels were significantly different (IgD\_M:  $p = 0.033$ ; IgD\_S:  $p = 0.037$ , Figure IV-5 a, b) between the PNS group (IgD\_M:  $4.42E+05$  copies/ $\mu$ g cDNA; IgD\_S:  $3.65E+05$  copies/ $\mu$ g cDNA) and the FNS group (IgD\_M:  $8.02E+05$  copies/ $\mu$ g cDNA; IgD\_S:  $7.56E+05$  copies/ $\mu$ g cDNA).

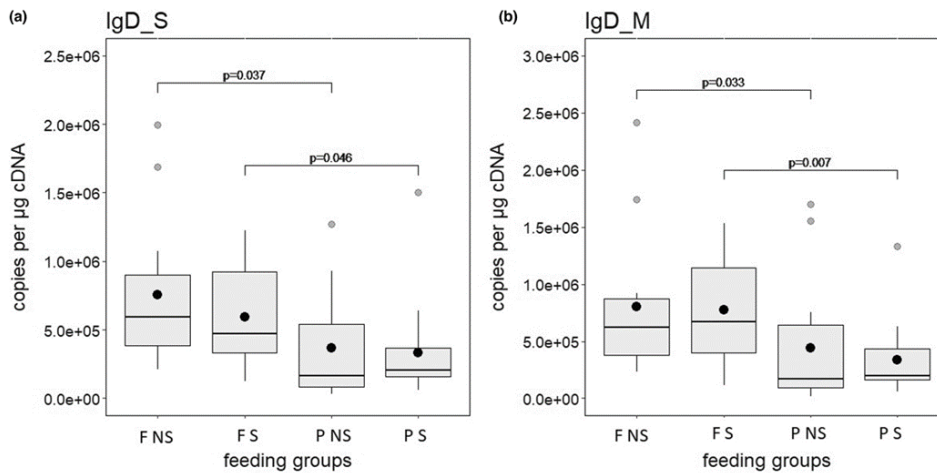


Figure IV-5 Box and whisker plot of the copies number of secreted immunoglobulin D (IgD\_S; a) and membrane-bound immunoglobulin D (IgD\_M; b) per  $\mu$ g cDNA. FNS, fishmeal, no stress; FS, fishmeal, stressed; PNS, predominantly plant-protein based, no stress; PS, predominantly plant-protein based, stressed. Box represents the interquartile range (IQR): Lower end = 25th percentile, upper end = 75th percentile, solid line = median, whiskers =  $1.5 \times$  IQR, grey dots = potential outliers, black dots = mean of feeding group, significant differences between feeding groups are indicated with brackets and corresponding p-values.

The copy numbers of the transcripts for IgT (membrane-bound immunoglobulin T; Figure IV-6) were significantly ( $p = 0.007$ ) downregulated in the PS feeding group (67

copies/ $\mu\text{g}$  cDNA) compared with the relatively high expression in the FS group (140 copies/ $\mu\text{g}$  cDNA). The average IgT\_M gene expressions in the PNS and FNS treatment groups were 87 copies/ $\mu\text{g}$  cDNA and 140 copies/ $\mu\text{g}$  cDNA, respectively, indicating a similar response in the primarily fishmeal-fed groups, regardless of the stress influence.

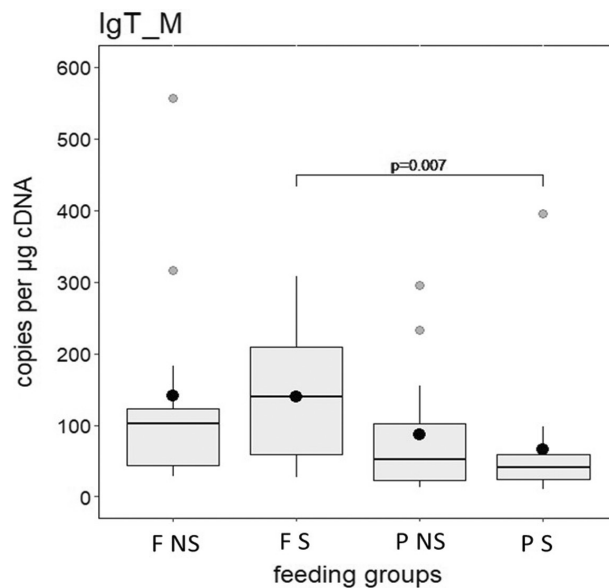


Figure IV-6 Box and whisker plot of the membrane-bound immunoglobulin T (IgT\_M) copies per  $\mu\text{g}$  cDNA. FNS, fishmeal, no stress; FS, fishmeal, stressed; PNS, predominantly plant-protein based, no stress; PS, predominantly plant-protein based, stressed. Box represents the interquartile range (IQR): Lower end = 25th percentile, upper end = 75th percentile, solid line = median, whiskers =  $1.5 \times \text{IQR}$ , grey dots = potential outliers, black dots = mean of feeding group, significant differences between feeding groups are indicated with brackets and corresponding p-values.

IgD is abundantly found in fish, and in various other vertebrates, since it plays an important role in immune activation and modulation (Wilson et al., 1997). Experiments in mice showed that secreted IgD (IgD\_S) might enhance immune protection by regulating B-cell homeostasis and activation (Nitschke et al., 1993). IgD was also shown to activate B-cell-stimulating programs in basophils (Chen et al., 2009). IgD-secreting cells are also found in many different tissues (Mashoof & Criscitiello, 2016), including blood. This prevalence of IgD in various tissues and its role in immune modulation could partially explain the higher values of IgD expression in our results.

Secreted IgD has been suggested to support mucosal homeostasis and regulate immune surveillance by arming myeloid effector cells, such as basophils and mast cells, with IgD antibodies against mucosal antigens (Chen et al., 2009). The affinity for low-density surface antigens is higher for IgD than for IgM, which is co-expressed with IgD (Løset et al., 2004). Immune surveillance of IgD also alerted immune-activating cytokines, such as TNF and IL1 $\beta$ .

Compared with IgD, the expression of IgT remained relatively low in the investigated treatment groups. IgT is expressed at relatively lower levels in the gut than in the blood serum (Zhang et al., 2010), so this could explain our low expression levels for membrane bound IgT. Since we have extracted the RNA from whole blood and not from the gut tissue, only a few IgT-positive B cells may be present in the blood of rainbow trout.

In conclusion, the fishmeal groups appeared to develop a higher inflammatory activity, which could be interpreted as better immune protection, reflected by a higher presence of immunoglobulins. The stressed plant-protein fed group showed a significant down-regulation of the expression of both TNF $\alpha$  and immunoglobulins, whereas these were increased in the fishmeal groups under stress. One explanation for this is an existing exhaustion syndrome. The gene expression of selected immune parameters and the histological appearance were significantly impacted by the feed formulation and the presence of stress. These results indicate that the performance of fish under stress should not be ignored when evaluating alternatives to fishmeal.

Further studies are needed on the microbiome composition, for example, to better understand these interactions.

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**Author contribution:** Mario Hasler contributed to data analysis.

**Conflict of interest:** The authors declare no conflict of interest.

**Data availability statement:** The data is available and was collected as part of the "Metabac" project. Original data were collected for the study to which the authors have full access. The integrity of the data is guaranteed and the authors confirm that the statistical data analysis was performed with the highest accuracy.

**Institutional review board statement:** All animal handling procedures were approved by the animal welfare officer of the 'Gesellschaft für Marine Aquakultur mbH'<sup>12</sup> and the local authority of Schleswig-Holstein<sup>13</sup> (MELUND) according to relevant institutional and national guidelines for care and use of laboratory animals (German animal welfare law; TierSchG and Regulation for the Protection of Animals Used for Experimental and Other Scientific Purposes; TierSchVersV as the national implementation of the Directive 2010/63/EU).

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## Anlage 3

C | A | U

Christian-Albrechts-Universität zu Kiel

Agrar- und Ernährungs-  
wissenschaftliche Fakultät

## Declaration of co-authorship

If a dissertation is based on co-authored articles, a declaration from each of the authors regarding the part of the work done by the doctoral candidate must be enclosed when submitting the dissertation.

## 1. Doctoral candidate

Name: **Henrika Seibel**

## 2. This co-author declaration applies to the following article:

Interactions of plant-based feeding and handling stress on the expression of selected immune markers in rainbow trout (*Oncorhynchus mykiss*)


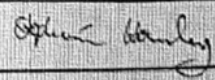
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Extent

<b>Concept:</b> Formulation of the basic scientific problem based on theoretical questions which require clarification, including a summary of the general questions which, it is assumed, will be answerable via analyses or concrete experiments/investigations	B
<b>Planning:</b> Planning of experiments/analyses and formulation of investigative methodology, including choice of method and independent methodological development, in such a way that the scientific questions asked can be expected to be answered	B
<b>Execution:</b> Involvement in the analysis or the concrete experiments/investigation	B
<b>Manuscript preparation:</b> Presentation, interpretation and discussion of the results obtained in article form	C

Date	Name	Signature
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Date	Name	Signature
28.02.24	Henrika Seibel	

Anlage 3

<b>C   A   U</b>	Christian-Albrechts-Universität zu Kiel	Agrar- und Ernährungs- wissenschaftliche Fakultät
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**Declaration of co-authorship**

If a dissertation is based on co-authored articles, a declaration from each of the authors regarding the part of the work done by the doctoral candidate must be enclosed when submitting the dissertation.

**1. Doctoral candidate**

Name: **Henrike Seibel**

**2. This co-author declaration applies to the following article:**

Interactions of plant-based feeding and handling stress on the expression of selected immune markers in rainbow trout (*Oncorhynchus mykiss*)

The extent of the doctoral candidate's contribution to the article is assessed on the following scale:

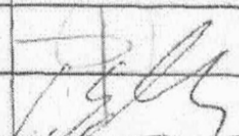
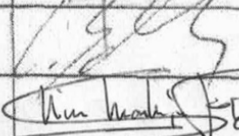
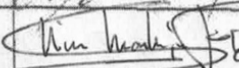
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- B. Has made a substantial contribution (34-66%)
- C. Did the majority of the work independently (67-100%)

**3. Declaration on the individual phases of the scientific work (A,B,C)**

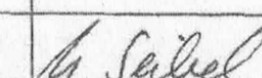
Extent

<b>Concept:</b> Formulation of the basic scientific problem based on theoretical questions which require clarification, including a summary of the general questions which, it is assumed, will be answerable via analyses or concrete experiments/investigations	<b>B</b>
<b>Planning:</b> Planning of experiments/analyses and formulation of investigative methodology, including choice of method and independent methodological development, in such a way that the scientific questions asked can be expected to be answered	<b>B</b>
<b>Execution:</b> Involvement in the analysis or the concrete experiments/investigation	<b>B</b>
<b>Manuscript preparation:</b> Presentation, interpretation and discussion of the results obtained in article form.	<b>C</b>

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## V. Research Chapter IV – Testing a feeding additive

### **Fungal fermentation product boosts the immune system of Atlantic salmon (*Salmo salar*) after soya challenge**

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#### **ABSTRACT**

The trial objective was to test the effect of two dietary inclusion rates of a fungal fermentation feed additive on the growth performance of juvenile Atlantic salmon (*Salmo salar*) and to quantify potential stimulatory effects on the immune system when fish were exposed to a dietary challenge. Salmon have been fed a control diet and two test diets containing 0.05 and 0.1 % of the feed additive for eight weeks and were then fed experimental feeds similar in composition but containing 20 % regular soybean meal in a 14-day challenge trial. The goal was to expose fish to a dietary challenge, as soybean meal is known to have inflammatory effects on the gut of Atlantic salmon.

While there were no significant treatment-dependent differences in growth performance parameters or feed efficiency detectable, a clear impact on different immune- and stress markers was observed. Gene expression patterns before and after exposition of salmon to the dietary challenge suggest a beneficial effect of the fungal fermentation feed additive on the gut health of treated salmon when included at a dietary level of 0.1 %. The genes analysed indicate that no inflammation occurred in the intestine and no acute phase reaction was activated during the soy challenge. Classical stress markers such as IL1 $\beta$  and TGF $\beta$  showed no upregulation. In comparison, these indications of an inflammatory immune response were found after feeding challenge with soya when only 0.05% of the product was used.

**Keywords:** Atlantic salmon, *Salmo salar*, growth trial, SBMIE, immune stimulation, *Aspergillus* spp.

## Introduction

Feeding soy-based diets to salmon is a common practice in aquaculture, especially as a replacement for fish meal, which can be expensive and environmentally unsustainable (Bjerkeng et al., 1997; Carter & Hauler, 2000; Olsen & Hasan, 2012; Wang et al., 2017). However, the use of soy-based diets in aquaculture, including salmon farming, can cause local inflammation in the gut, with its immunological effect and have growth depressing effects on the fish (Djordjevic et al., 2021; Hartviksen et al., 2014; Rumsey et al., 1994; Q. Zhang et al., 2023). Soy contains certain antinutritional factors, such as protease inhibitors and lectins, which can interfere with the digestion and absorption of nutrients (Francis et al., 2001; Krogdahl et al., 1994, 2010). Prolonged exposure to these substances affects the overall health and immune function of the fish. Main immunological effects associated with feeding soy to salmon are allergic reactions, which can manifest as inflammation and other immune responses and changes in gut health like alterations in the gut microbiota composition and accompanied influence on the overall immune response of the fish (Baeverfjord & Krogdahl, 1996; Booman et al., 2018; van den Ingh et al., 1991). Chronic inflammation can weaken the immune system and make the fish more susceptible to diseases (Bakke-McKellep et al., 2007; Bøgwald & Dalmo, 2014). From a different perspective, this soybean meal (SBM) induced effect (Knudsen et al., 2006; Kumar et al., 2020) can be exploited for research to expand our knowledge on the compatibility of diets for salmon. The soybean meal-induced enteritis (SBMIE), for instance, can be used as a challenge model in fish research (Seibel et al., 2022), particularly in studies aimed at understanding the effects of dietary components on fish health and immune responses. Therefore, the SBMIE provides a controlled environment for the study of inflammatory responses triggered by specific dietary components or, conversely, the study of anti-inflammatory effects of feed additives (Booman et al., 2018; Egerton et al., 2020; Seibel et al., 2022). The expression of e. g. pro-inflammatory cytokines, the infiltration of immune cells and other markers associated with inflammation in the fish gut can be used to investigate e. g. immune-balancing effects of feed additives (Djordjevic et al., 2021; Kiron et al., 2020).

This supports the development of functional feeds to promote gut health and improve the fish's ability to cope with nutritional challenges. This knowledge can be used to formulate feeds that support a healthy gut microbiota and immune system in aquaculture (Jalili et al., 2020).

Functional feeds are formulated aquafeeds designed to provide specific health and performance benefits to the aquatic animals (Olmos Soto et al., 2015). These feeds are tailored to contain various additives, supplements, or bioactive compounds that go beyond basic nutritional requirements. The goal of functional feeds is to enhance the overall health, disease resistance, growth, and quality of the aquaculture products. Functional feeds should be developed based on scientific evaluations and are continually evolving to address specific challenges in aquaculture, such as disease outbreaks, environmental stressors, and changing market demands (Dawood et al., 2018; Van Doan et al., 2023). These feeds play a crucial role in promoting sustainable and efficient aquaculture practices. However, the availability of products that have been specifically tested and authorised for different fish species is still limited as it depends on species,

developmental stage, and specific nutritional requirements. Examples include pro- or prebiotic-enriched or other immunostimulant feeds, feeds that are enriched with omega-3 fatty acid or pigments and others (Melo-Bolívar et al., 2020; Vallejos-Vidal et al., 2016). Prebiotics and probiotics refer to different things and play different roles in supporting gut health. Probiotics are living microorganisms, primarily bacteria and yeasts, which are consumed in sufficient quantities to help maintain a balanced and healthy gut microbiota while prebiotics are indigestible fibres and compounds that serve as a food source for beneficial bacteria in the gut (El-Saadony et al., 2021; Ringø et al., 2010). Probiotic-enriched feeds are made to promote gut health and improves digestion and contain for example *Bacillus* spp. or lactic acid bacteria as functionally effective ingredients (Chuphal et al., 2021; El-Saadony et al., 2021; Melo-Bolívar et al., 2020) while prebiotic feeds are designed to have an immunostimulant effect and thus strengthen the body's defence against disease include, for example, beta-glucans, nucleotides, or specific herbs with immunostimulant properties (Ahmadifar et al., 2021; Elumalai et al., 2021; Guzmán-Villanueva et al., 2014; Hossain et al., 2020; Vallejos-Vidal et al., 2016).

Fungi are also becoming increasingly interesting for use in aquaculture and can be used as probiotics in fish feed. Besides, some fungi produce enzymes that can help break down complex compounds in fish feed, improving nutrient utilization (Agboola et al., 2021; Amara and El-Baky, 2023; Strong et al., 2022). For example, certain fungal species produce phytase, which helps in the digestion of phytate-bound phosphorus. Fungi represent a new opportunity to feed fish sustainably with innovative and alternative protein sources (Amara and El-Baky, 2023). They have already found their way into the food industry. The number of patents in the field of microbial protein production has risen sharply in recent years. However, these generally relate to the production of meat alternatives based on filamentous fungi (Hüttner et al., 2020). The use as animal feed still has room for development. The fermentation fungal product, MAXFERM is produced through a solid-state fermentation process using selected strains of *Aspergillus* spp., here *Aspergillus tubingensis*, *Neurospora intermedia* and a specified range of substrates. MAXFERM is already approved for feeding to pigs and poultry to maximise feed conversion (Hein, 2022). It stabilizes the gastrointestinal system and increases the feed utilization and diet digestibility in pigs and chickens and therefore increases the pig production profits and improves the performance in broiler chickens (Hein, 2022; Provita Supplements, n.d.). It was designed to minimize the adverse effects of non-starch polysaccharides (NSPs) and to increase the bioavailability of nutrients for monogastrics (Provita Supplements, n.d.).

The aim of this study was to investigate whether these effects can also be transferred to Atlantic salmon. By carrying out a challenge trial with soybean meal, the aim was to investigate whether the addition of *Aspergillus tubingensis* and *Neurospora intermedia* also has other positive effects on gut health and whether the inflammation-inducing effects of SBM can be mitigated.

## Material and Methods

### Experimental setup

The study was conducted in accordance with islandic laws and regulations for the control of animal experiments. The trial was carried out at the Matis Aquaculture Research Station (MARS), Reykjavik, Iceland using juvenile Atlantic salmon (*Salmo sala*) reared in seawater ( $33.31 \pm 1.35$  ppm salinity). Fish were reared in 200 litre tanks (16 in total) that were part of a recirculating aquaculture system (RAS) with mechanical (sponge filter), biological (moving bed), physical (protein skimmer and UV) and chemical (ozone) water treatment at a water temperature of  $12.52 \pm 0.84$  °C, nitrite values of  $0.43 \pm 0.12$  mg/L, ammonia values of  $0.70 \pm 0.12$  mg/L and a pH of  $6.97 \pm 0.16$ . Water quality parameters were checked regularly (temperature and oxygen every 10 minutes via the automatic oxygen monitoring system OxyGuard® Pacific; salinity, pH, ammonia, and nitrite once a day, manually). Seawater was made using artificial sea salt (Aquamedic, Bissendorf, Germany) and replaced at a rate of 0 - 5 % per day. The water values remained stable over the entire test period and within the physiological range for salmon.

Atlantic salmon were supplied by a local salmon producer (Landeldi, Kópavogur, Iceland). The trial started after acclimating salmon over a period of two weeks. During the acclimation time, fish were fed with a commercial feed (Laxa ECO 2.5 mm). For the trial 12 salmon with an average weight of 235 g were assigned to 12 individual tanks. Feeds were tested in quadruplicates and randomly assigned to the 12 experimental tanks. The 70-day experiment was divided into an eight-week growth trial (Diets Control I, ISF AI and ISF BI), followed by a two-week dietary challenge where feeds containing regular soybean meal were fed (Control II, ISF AII and ISF BII). Throughout the trial, fish were fed six times per day to apparent satiation. The required amounts of feed were based on the expected growth rate at given temperature and salmon size. An excess of 10 – 15 % of feed was given to ensure an oversupply of feed for all groups. Feeding was performed using automatic feeders and uneaten feed collected once a day, frozen, and dried to assess the feed intake of fish. A 24 h light regime was maintained throughout the 10 weeks of the trial.

### Feed formulation

The diets were formulated based on the dietary requirements of Atlantic salmon post-smolts and produced at the Technologie-Transfer-Zentrum Bremerhaven (TTZ), Germany. The formulations used for the growth trial, the dietary challenge and their respective composition are shown in Table V-1 and Table V-2.



Table V-1 Composition of diets used during the 56-day growth trial (I) and the 14-day challenge trial (II)

Raw material (%)	Control I	ISF AI	ISF BI	Control II	ISF AII	ISF BII
<b>Soybean meal</b>	0.00	0.00	0.00	20.00	20.00	20.00
<b>Soy protein concentrate</b>	30.00	30.00	30.00	19.01	19.01	19.01
<b>Fish meal</b>	15.00	15.00	15.00	15.00	15.00	15.00
<b>Wheat gluten</b>	14.31	14.31	14.31	15.00	15.00	15.00
<b>Wheat</b>	11.99	11.99	11.99	8.00	8.00	8.00
<b>Fish oil</b>	10.70	10.70	10.70	9.43	9.43	9.43
<b>Rapeseed oil</b>	10.00	10.00	10.00	9.43	9.43	9.43
<b>Corn gluten meal</b>	6.00	6.00	6.00	4.00	4.00	4.00
<b>Rapeseed lecithin</b>	1.00	1.00	1.00	1.00	1.00	1.00
<b>Monoammonium phosphate</b>	0.78	0.78	0.78	1.15	1.15	1.15
<b>Vitamins</b>	0.50	0.50	0.50	0.50	0.50	0.50
<b>L-Lysine HCL</b>	0.43	0.43	0.43	0.27	0.27	0.27
<b>Choline chloride</b>	0.32	0.32	0.32	0.00	0.00	0.00
<b>Minerals</b>	0.20	0.20	0.20	0.20	0.20	0.20
<b>DL-Methionine</b>	0.16	0.16	0.16	0.14	0.14	0.14
<b>Astaxanthin 10%</b>	0.05	0.05	0.05	0.05	0.05	0.05
<b>Water</b>	-1.45	-1.45	-1.45	-3.19	-3.19	-3.19
<b>Test product ISF</b>	0.00	0.05	0.10	0.00	0.05	0.10

Table V-2 Proximate composition in % of experimental feeds used in the growth and challenge trial (dry matter basis).

	Control I	ISF AI	ISF BI	Control II	ISF AII	ISF BII
<b>Dry matter</b>	94.90	95.90	95.30	95.80	95.60	95.90
<b>Crude lipids</b>	19.92	18.14	17.84	20.67	24.48	21.38
<b>Crude protein</b>	54.06	55.37	54.46	54.07	52.93	53.08
<b>Crude Ash</b>	6.74	6.67	6.51	6.58	7.01	6.36

The ISF test product is a fungal biomass from inactivated fungi *Aspergillus tubingensis* and *Neurospora intermedia* (MAXFERM). It is a single feed product manufactured by Provita Supplements GmbH (Pinneberg, Germany; Reg. No. 27615 083 190 3668; European catalogue of feed materials: 12.2.9). It contains 30 % crude protein, 7.60 % crude ash, 3 % crude fat and 12 % crude fibre.

## Performance parameters and blood sampling

At the end of the eight-week growth period as well as after the subsequent dietary challenge that lasted for two weeks, blood samples were extracted from one fish per tank. A 1 ml syringe was inserted at the ventral side of the peduncle to penetrate the dorsal aorta. Blood samples were stored in EDTA fixated vacutainers (Saarstedt, Germany) and sent to Fraunhofer IMTE, Büsum, Germany for analysis.

The growth rate (SGR) was calculated as follows:  $SGR = (\ln(Wt) - \ln(W0) / days) * 100$  with  $\ln(Wt)$  = natural logarithm of the final individual weight and  $\ln(W0)$  = natural logarithm of the initial individual weight. The weight gain per tank per individual was calculated as:  $Weight\ gain = Wt - W0$ . The feed conversion ratio (FCR) was calculated as follows:  $FCR = feed\ intake\ (g) / weight\ gain\ (g)$ .

## Molecular investigations

RNA isolation was performed modified according to Qiagen® 2014 combining the QIAGEN RNeasy Plus Minikit (QIAGEN, Germany) and the RiboPure™-Blood Kit (Thermo Fisher Scientific Inc., USA). Additional DNase steps were added. The isolated RNA concentration was determined using a photometer (Tecan Nanoquant Infinite M200 Photometer, Tecan Group AG, Switzerland) and adjusted to 10 ng/µl. Integrity of extracted RNA was measured by NanoDrop One (Thermo Fisher Scientific). Using the Reverse Transcription Master Mix (Standard BioTools, San Francisco, CA, USA) cDNA was generated, preamplified by the PreAmp Master Mix (Standard BioTools) and at last treated with exonuclease I (New England BioLabs, Frankfurt/Main, Germany). All steps have been carried out according to the manufacturer's instructions.

In total, three reference genes (*eef1a1*, *rps20*, *actb*) and 45 target genes (see results and discussion) were chosen from studies regarding stress and immune competence in Atlantic salmon and rainbow trout (Krasnov et al., 2020; Lund et al., 2022; Seibel et al., 2019; Weirup et al., 2022 ) and analyzed using 48.48 gene expression biochips and the Biomark HD-System (Standard BioTools) according to the manufacturer's thermal protocol "GE Fast 48 × 48 PCR+Melt v2.pcl" (application type: gene expression; passive reference: ROX; assay: single probe). More information about the method as well all primer sequences of genes mentioned can be found in Weirup et al., 2022.

The raw qPCR Ct values were obtained using the Fluidigm real-time PCR analysis software v. 3.0.2 (Munich, Germany). Relative expression was calculated based on DD<sub>Ct</sub> with reference genes as internal normalisers and the mean Ct per gene for all samples as a calibrator during the calculation. Relative copy numbers were log<sub>2</sub> transformed prior to statistical analysis.

## Statistical analysis

R (version 4.1.2; R Core Team 2021) in R Studio was employed for all statistical analyses. FCR and final weights were compared by means of ANOVA and nested ANOVA respectively. Mixed-effect models were defined to explore the impact of diet and timepoint on the expression of the gene of interest. The model included diet, timepoint, and their interaction as fixed factors, with the tank included as a random factor. Inspection of model residuals through graphical analysis revealed approximately normal distribution and heteroscedasticity. Analysis of variance (ANOVA) was then performed to investigate potential effects of diet, timepoint, and their interaction, however due to heteroscedasticity ANOVA results were only consulted for support. Statistical significance  $p < 0.05$  among groups/timepoints was tested using multiple contrast tests for heteroscedastic data (Hasler and Hothorn, 2008).

## Results and Discussion

The trial objective was to test the effect of two dietary inclusion rates of fungal fermentation feed additive, MAXFERM (ISF GmbH, Germany) on the growth performance of juvenile Atlantic salmon (*Salmo salar*) and potential inflammatory effects and immunological reaction when exposed to a dietary soybean meal challenge. The SBMIE model was used to obtain a diet-induced stress effect as SBM is known to have inflammatory effects on the gut of Atlantic salmon (Bakke-McKellep et al., 2007; Bøggwald & Dalmo, 2014; Booman et al., 2014). It's important to note in this context that the specific immunological effects can vary based on factors such as the composition of the soy-based diet, the fish species, and the environment in which the fish are raised. In this study, the water parameters were kept stable, and the feed formulations are comparable except for the addition of the ISF supplement or the addition of soybean meal, also in terms of nutrient composition (Table V-2).

### Fish performance

Average initial and final weights of salmon, as well as their respective weight gain, SGR and FCR for the eight-week growth trial are shown in Table V-3. One fish died in one of the control tanks. Results did not show statistically significant differences in growth performance and feed conversion across trials. Results on fish performance for the dietary challenge are presented in Table V-4. The short trial duration does not allow a statistical assessment of treatment dependent differences. However, when looking at the results, it becomes clear that the values for the performance parameters of both the control and ISF groups are in the same range.

Table V-3 Initial weight (IW), final weight (FW), weight gain (WG), the specific growth rate (SGR) as well as the feed conversion ratio (FCR) of Atlantic salmon fed experimental diets containing two different concentrations of an ISF product relative to a control diet without test product; ISF A = 0.05 %; ISF B = 0,1 %.

	<b>Control I</b>	<b>ISF AI</b>	<b>ISF BI</b>	<b>ANOVA Diet</b>	
<b>IW (g)</b>	241.8 ± 6.85	231.0 ± 6.38	239.3 ± 6.13	F(3,12) = 1.79	p = 0.20
<b>FW (g)</b>	419.3 ± 15.17	412.8 ± 11.35	416.3 ± 36.21	F(3,12) = 0.96	p = 0.45
<b>WG</b>	177.5 ± 15.93	181.8 ± 6.40	177.0 ± 33.99	F(3,12) = 0.54	p = 0.67
<b>SGR</b>	0.98 ± 0.078	1.04 ± 0.023	0.98 ± 0.142	F(3,12) = 0.41	p = 0.75
<b>FCR</b>	1.05 ± 0.092	0.95 ± 0.025	1.00 ± 0.112	F(3,12) = 2.36	p = 0.12

Table V-4 Initial weight (IW), final weight (FW), weight gain (WG), the specific growth rate (SGR) as well as the feed conversion ratio (FCR) of Atlantic salmon exposed to a dietary challenge with feed containing regular soybean meal and two different concentrations of an ISF test-product relative to a control diet without test product; ISF A = 0.05 %; ISF B = 0,1 %.

	<b>Control II</b>	<b>ISF AII</b>	<b>ISF BII</b>
<b>IW (g)</b>	420 ± 14.76	413.8 ± 7.93	418.3 ± 48.41
<b>FW (g)</b>	476.3 ± 9.95	465.8 ± 8.66	460.8 ± 43.12
<b>WG</b>	56.3 ± 13.02	52 ± 2.83	42.5 ± 17.71
<b>SGR</b>	0.79 ± 0.193	0.74 ± 0.039	0.61 ± 0.263
<b>FCR</b>	1.1 ± 0.225	1.09 ± 0.045	1.52 ± 0.695

As the addition of only 20 % SBM was a relatively small amount, corresponding to the proportion of SBM in a commercial salmon feed, no effects on the performance parameters were to be expected (Seibel et al., 2022). Feeding 20 % SBM in such trials is not uncommon. It could be shown that after seven days the first significant pathological-anatomical changes of the intestine could be observed (Booman et al., 2018; Sahlmann et al., 2013). However, performance parameters were often not measured. In a 56-day feeding experiment with rainbow trout, fed with different levels of soybean meal, the animals showed histological changes in the intestine, a significant decrease in the SGR and a significant increase in the FCR only in the high inclusion quantities of 50 %. This involved replacing of 100 % fish meal in the diet with soybean meal (Seibel et al., 2022). Other studies show similar results (Green et al., 2013; Knudsen et al., 2008; Zhang et al., 2013). It is important to put into perspective that the severity of soybean meal enteritis and its immunological effects and impact on performance parameters can vary based on several factors, including the species of fish, the specific composition, degree of cultivation and processing of the soybean meal, and the duration of exposure (Venold et al., 2012; Wang et al., 2017; Zhou et al., 2018). Inflammation and damage to the intestinal lining can impair the absorption of nutrients, leading to malnutrition. Proper nutrition is essential for fish health, and impaired nutrient absorption can weaken the fish's immune responses. Changes in the performance parameters only occur at the very end of the stress cascade (Madaro et al., 2015; Tort et al., 2004), when the stress has already become chronic. In addition to the SBMIE model, a regular 56-day feeding trial was also carried out with the feed additive (I).

MAXFERM has been shown to improve feed conversion in pigs and chickens (Hein, 2022) and positive digestive effects of adding *Aspergillus* spp. to the diet of broilers and pigs are described (Saleh et al., 2014; Zentek & Goodarzi Boroojeni, 2020). This does not appear to be the case for Atlantic salmon.

Non-starch polysaccharides (NPS) are a category of complex carbohydrates that are not classified as starch. Unlike starch, which consists of glucose molecules linked together in

a linear or branched chain, non-starch polysaccharides have more complex structures and are not easily digestible. They often found in the cell walls of plants, like cellulose, hemicellulose or pectins (Căpriță et al., 2010; Marcotuli et al., 2020). Monogastric animals are organisms that only have one stomach or whose stomach is in one piece. As vertebrates do not have an enzyme that breaks down cellulose, they are dependent on the help of microorganisms to digest it to utilise the energy it contains. Salmonids also have difficulties digesting starch and these complex polysaccharides efficiently (Krogdahl et al., 2004). Starch is primarily used to facilitate binding during the pellet production process in salmon feed production (Kanmani et al., 2018; Romano & Kumar, 2019; Sørensen et al., 2010); however, NPSs are also increasingly found in fish food. They find their way into the feed by replacing the unsustainable fishmeal with plant-based raw materials (Sinha et al., 2011). In aquaculture, feed for salmon is often formulated to optimise the nutritional requirements of the fish. This may include the addition of easily digestible carbohydrates such as those from cereals to meet the energy requirements of the fish.

## Immunological reactions, molecular results

In this study, a short-term feeding stress was applied and no changes in performance parameters were expected. However, the observation of different genes over time, independent of the diets, shows that the expression of classical inflammation markers was induced as expected. Similar findings are also known from other studies (Booman et al., 2018; Sahlmann et al., 2013). Even if there are still no changes in the performance parameters, the stress and immune markers react clearly (Rubio Benito, 2023; Seibel et al., 2019).

The Biomark run was technically optimal, yet classic immune genes such as *c3-1*, *enpp3*, *tgfb\_2*, *tnfa*, *ucp2*, and *mpo* were not detectable, while the copy numbers of *il6*, *lect2*, *mcsfr*, *cxcl8/il8*, *hsp47a*, *il10* and *tnfb*, were detectable in small quantities at least in some individuals. The ten genes *nod1b*, *c3ar*, *socs1*, *cxcl8*, *ccl4a*, *crh*, *hp*, *usp4*, *fth*, *hsp47b* were only expressed in very small quantities. All genes from Table V-5 were expressed at medium to high amounts. They are grouped according to their functionality and significant differences, presented in Table V-5.

Table V-5 Analysed genes functionally grouped according to their function with given significancies ( $p < 0.05$ ). The following are indicated: (A) significant differences between the diets at one time point; (B) significant differences of a diet between initial trial (I) and after SBM challenge (II) and (C) significant differences between I and II respective of the diet. ISF A = includes 0.05 % MAXFERM; ISF B = includes 0.1 % MAXFERM.

functional category	Gen	(A) significant p values - diets		(B) Timepoint effect within diet	(C) Timepoint overall difference
		initial trial (I)	after challenge (II)		
<b>heat stress response</b>					
SERPINH1	Serpin family H member 1 (Heat shock protein 47 variant 1)				
HSP90AA1a	Heat shock protein 90 alpha family class A member 1 variant 1			ISF A ( $p < 0.01$ ), Control ( $p < 0.05$ )	
HSP90AA1b	Heat shock protein 90 alpha family class A member 1 variant 2				$p < 0.05$
<b>complement system (early defence)</b>					
C5AR	Complement 5			ISF A ( $p < 0.01$ )	
<b>coagulation (early defence)</b>					
FGL2	Fibrinogen-like protein 2				
<b>pathogen detection (early defence)</b>					
TLR3	Toll-like receptor 3	Control - ISF A ( $p < 0.001$ )		Control ( $p < 0.001$ )	
<b>early immune response (regulator)</b>					
MAP3K1	MAP3-kinase			ISF A & B ( $p < 0.05$ )	
STAT1	Signal transducer and activator of transcription 1			ISF A ( $p < 0.05$ )	
NOD1a	Nucleotide-binding oligomerization domain-containing protein 1 variant 1			ISF A ( $p < 0.01$ )	
NOD1b	Nucleotide-binding oligomerization domain-containing protein 1 variant 2				
USP4	Ubiquitin specific petidase 4				
<b>cytokine</b>					
IL1 $\beta$	Interleukin 1 beta				
TGF $\beta$ -1	Transforming growth factor beta variant 1			ISF A ( $p < 0.05$ )	

<b>chemokine</b>					
CCL4a	Chemokine (C-C Motif) ligand 4				
<b>cytokine (regulator)</b>					
SOCS1	Suppressor of cytokine signaling 1				
<b>acute phase</b>					
SAA	Serum amyloid A				
FTH	Ferritin heavy chain				
HP	Haptoglobin				
<b>immune cell marker</b>					
TCRB	T cell receptor	Control - ISF A (p < 0.05)	Control - ISF A (p < 0.05)		p < 0.01
IgD-secr	Immunglobulin D (secreted)			ISF A (p < 0.05)	
IgM-secr	Immunglobulin M (secreted)			ISF A (p < 0.05)	
IgM	Immunglobulin M (membran-bound)				p < 0.05
IgT-secr	Immunglobulin T (secreted)			ISF A (p < 0.05)	
GCSFR	Granulocyte colony stimulating factor receptor 3				
<b>Immune cell marker (non-specific cytotoxic cells)</b>					
NCCRP1	Non-specific cytotoxic cells receptor protein 1				
<b>antimicrobial protein</b>					
LYGa	Lysozyme G variant 1				p < 0.05
LYGb	Lysozyme G variant 2		ISF A & B (p < 0.01)		
LYGc	Lysozyme G variant 3		ISF A & B (p < 0.001)	ISF A (p < 0.01)	
<b>antiviral response</b>					
MX1	MX dynamin-like GTPase 1		ISF A & B (p < 0.05)		p < 0.01

Although, there were no significant differences within performance parameters, the genes analysed indicate that the challenge with soybean meal has achieved the desired effects. Differences in the expression of inflammatory markers were assessed pre- and post-exposing salmon to the dietary challenge as well in the control group. High levels of anti-nutritional factors in SBM and non-starch polysaccharides can trigger an inflammatory response in the fish's gastrointestinal tract (Carmona, 2008; Knudsen et al., 2008; Krogdahl et al., 2000; Urán et al., 2008). Inflammation is part of the immune response and can lead

to tissue damage and immunosuppression, which in turn can favour the onset of other diseases (Campos-Sánchez & Esteban, 2021; Dale et al., 2009).

The increased levels of transcripts coding for the complement 5a receptor or fibrinogen-like protein 2 (*fgl2*) suggest that healing processes have been set in motion in the ISF All group, significantly after SBM challenge (Figure V-1). The process of wound healing in fish involves the inflammatory response, as first stage of wound healing. Neutrophils and macrophages play a key role in phagocytosing debris and potential pathogens (“Handbook of Vertebrate Immunology,” 1998; Pastoret et al., 1998). In the next phase, cellular proliferation and migrating fibroblasts are essential for producing collagen, a structural protein that forms the basis of the extracellular matrix, providing structural support to the healing tissue. Looking at the gene expression, the ISF All group is currently in this phase. For the reason of completeness, subsequent phases of wound healing would be the extracellular matrix formation, angiogenesis, tissue remodelling and regeneration (Campos-Sánchez & Esteban, 2021; Cordero et al., 2017; Urán et al., 2008).

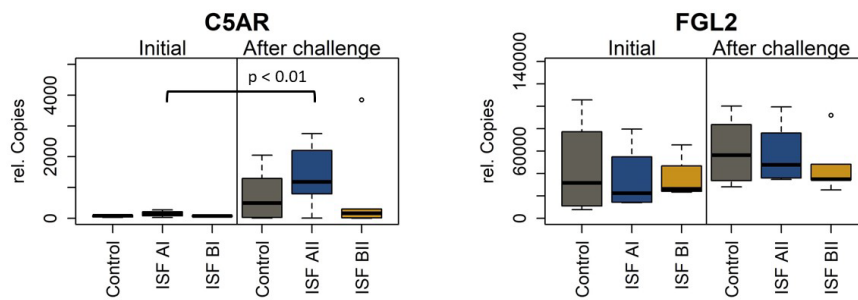


Figure V-1 Box and whisker plot of the complement 5 (*c5ar*) and fibrinogen-like protein 2 (*fgl2*) relative copies. Box represents the interquartile range (IQR): Lower end = 25th percentile, upper end = 75th percentile, solid line = median, whiskers = 1.5\*IQR, grey dots = potential outliers, significant differences between feeding groups are indicated with brackets and corresponding p-values; initial n = 4; after challenge n = 9. ISF A = includes 0.05 % MAXFERM; ISF B = includes 0.1 % MAXFERM.

The MAPK signalling transduction pathway is activated during stress and inflammation in mammals (Kyriakis & Avruch, 2012). After SBM challenging a significantly different expression between both ISF groups was detectable ( $p < 0.05$ ; Figure V-2). MAP3K1 is also significantly upregulated in ISF BII compared to ISF BI ( $p < 0.05$ ; Figure V-2). The MAP3K signalling pathway is involved in various physiological processes in teleosts including immune, and environmental stress responses. The activation of this pathway can be triggered by a variety of stimuli, such as growth factors, cytokines, and environmental stressors (Rombout et al., 2011; Wu et al., 2016). The MAPK signalling pathway has positive effects on cell survival as it induces an anti-apoptotic effect and represents an effective cellular stress coping mechanism (Larson et al., 2007). The fact that this study showed clear differences in the expression of various immune markers is all the more exciting because astaxanthin, which was added to the feed, prevented an LPS-induced increase in proinflammatory cytokines such as TNF- $\alpha$ , IL-6 and IL-1 $\beta$  and also the activation of the MAPK pathway (Li et al., 2018; Nakano et al., 1999; Rahman et al., 2016).



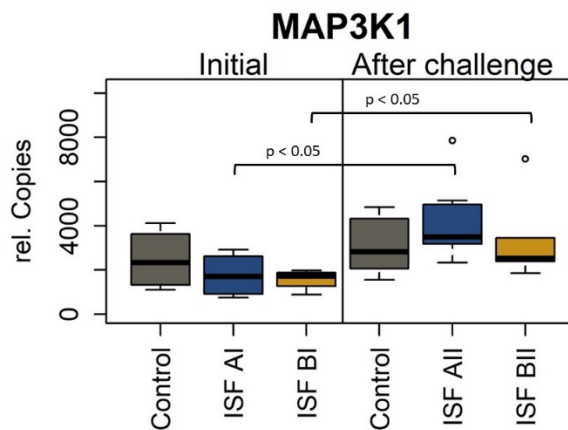


Figure V-2 Box and whisker plot of the MAP3-kinase signal transducer and activator of transcription 1 (MAP3K1); relative copies. Box represents the interquartile range (IQR): Lower end = 25th percentile, upper end = 75th percentile, solid line = median, whiskers = 1.5\*IQR, grey dots = potential outliers, significant differences between feeding groups are indicated with brackets and corresponding p-values; initial n = 4; after challenge n = 9. ISF A = includes 0.05 % MAXFERM; ISF B = includes 0.1 % MAXFERM.

Various cell groups involved in inflammation have been identified on a molecular level. Granulocytes are particularly active during inflammation and are also described for the SBMIE model (Li et al., 2022; Rubio et al., 2023). *G-csfr* the colony stimulating factor 3 receptor is the receptor for a cytokine that controls the production, differentiation, and function of granulocytes (Fingerhut et al., 2020; Mokhtar et al., 2023; Parra et al., 2015). This classic granulocyte marker was upregulated in the All group (Figure V-3).

An identical picture can be seen with *nccrp* which encodes the non-specific cytotoxic cells receptor protein, Figure V-3 (Jaso-Friedmann et al., 2001). It is produced by non-specific cytotoxic cells which belong to the natural killer cells (NK cells) as classic cell type of the innate immune system in teleost fish (Fischer et al., 2013; Meseguer et al., 1994). Therefore, *nccrp* is used as marker for NK cells. They have been shown to kill virus-infected, allogeneic, and xenogeneic target cells (Fischer et al., 2006).

T cells play an important role in the cell-mediated adaptive immune response and control the immunological response after the early non-specific defence reactions by identifying antigens. The T cell can then initiate the removal of the foreign material by phagocytosis or cytokine stimulation after antigen recognition by the T cell receptor complex (Castro et al., 2011). This can therefore be used as a marker for the detection of T cell involvement (*tcrb*, Figure V-3).

In summary, it can be concluded that granulocytes, NK cells and T lymphocytes are increased in the All, but not in the BII feeding group. Compared to the control group, ISF A appears to trigger *tcrb* overall, as there are significant differences compared to the control group both initially and after the SBM challenge.

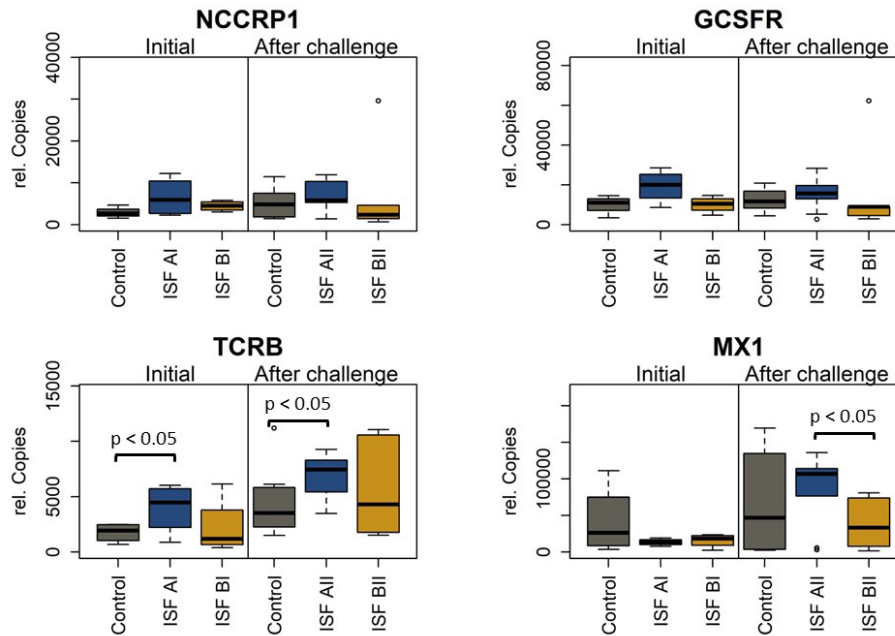


Figure V-3 Box and whisker plot of the non-specific cytotoxic cells receptor protein 1 (NCCRP), the granulocyte colony stimulating factor receptor 3 (GCSFR), the T cell receptor (TCRB) and the MX dynamin-like GTPase 1 (MX1); relative copies. Box represents the interquartile range (IQR): Lower end = 25th percentile, upper end = 75th percentile, solid line = median, whiskers =  $1.5 \times \text{IQR}$ , grey dots = potential outliers, significant differences between feeding groups are indicated with brackets and corresponding p-values; initial  $n = 4$ ; after challenge  $n = 9$ . ISF A = includes 0.05 % MAXFERM; ISF B = includes 0.1 % MAXFERM.

When evaluating the results, it must be considered that *mx1* was detectable after challenge and was even significantly ( $p < 0.01$ ) differentially expressed. *Mx1* was additionally tested as a kind of "safety marker". The so-called myxovirus 1 gene encodes an interferon-induced dynamin-like GTPase with antiviral activity against numerous RNA and some DNA viruses, which is used to detect existing viral infection (Larsen et al., 2004; Xiao et al., 2016). It is possible that a latent viral infection can be activated by stress events and the associated immunosuppression. The Piscine orthoreovirus (PRV) is, for example, a virus that infects various salmonid species, including Atlantic salmon and is widespread without appearing acutely in every case (Kannimuthu et al., 2023). PRV infection has been a subject of concern in the aquaculture industry. If this is the case here, ISF B may also have a certain antiviral effect as the *mx1* expression stays on the same level as the control, while it is significantly upregulated ( $p < 0.001$ ) after challenge in the ISF A group (Figure V-3). However, *mx1* can also be produced by numerous other events in the immune system. Since Atlantic salmon also react to PRV infection with a Th 1 immune response as a defence strategy, the beta subunit of the T-cell antigen receptor gene is also upregulated here (Vallejos-Vidal et al., 2022). In this experiment, *tcrb* is most likely controlled by the consequences of the immunosuppression triggered by the SBMIE model. There is a significant difference between the initial trial and the challenge trial across all groups,  $p < 0.01$ , and the ISF A group is significantly ( $p < 0.05$ ) upregulated compared to the control group.

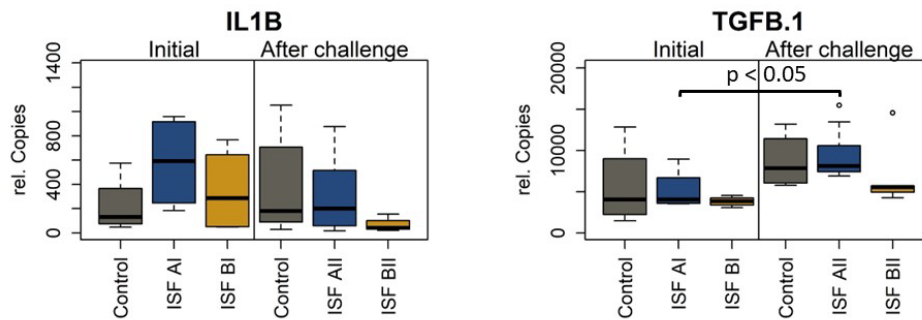


Figure V-4 Box and whisker plot of interleukin 1 beta (IL1B) and transforming growth factor beta variant 1 (TGFB.1); relative copies. Box represents the interquartile range (IQR): Lower end = 25th percentile, upper end = 75th percentile, solid line = median, whiskers = 1.5\*IQR, grey dots = potential outliers, significant differences between feeding groups are indicated with brackets and corresponding p-values; initial n = 4; after challenge n = 9. ISF A = includes 0.05 % MAXFERM; ISF B = includes 0.1 % MAXFERM.

To dive a little deeper into the effects of MAXFERM under a soybean meal challenge on Atlantic salmon, it is interesting to take a closer look at the expression of some other genes. The downregulation of the two genes *il1β* and *tgfbβ* likely reflect the positive immune equalising capacity of ISF B (Figure V-4). The transforming growth factor  $\beta$  (isoform.1) is a cytokine. All leukocyte lineages produce *tgfb1*, including lymphocytes, macrophages, and dendritic cells. Activated TGF $\beta$  forms a serine/threonine kinase complex that binds to type I and type II TGF $\beta$  cell surface receptors and activates a signalling cascade, thereby activating various downstream substrates and regulatory proteins, inducing the transcription of various target genes, and regulating differentiation, chemotaxis, proliferation, and activation of many immune cells. TGF $\beta$  inhibits the proliferation of T cells and cytotoxic T cells and the expression of immunoglobulin G and immunoglobulin T (Figure V-5), the major histocompatibility complex, Fc and IL2 receptor and deactivates macrophages. In addition to these anti-inflammatory, immune inhibitory effects, TGF $\beta$  also has pro-inflammatory effects by promoting macrophage and neutrophil chemotaxis and Th17 differentiation (Laing et al., 1999; Letterio & Roberts, 1998; Pauley, 2011; Rombout et al., 2011; Sanjabi et al., 2009). This upregulation of *tgfb* may indicate that the challenge with SBM has caused an inflammatory reaction. ISF B significantly alleviated this. Whether the substitute prevented the inflammation from developing or caused it to subside more quickly, cannot be determined.

It could almost be assumed that ISF A could not completely prevent the inflammatory reaction to the antinutritive ingredients of the SBM, but that the inflammation could be quickly contained by the body. This is supported by the fact that *il1β* has been detectable across all groups analysed initially, when soybean protein concentrate is already included into the feed. This classic inflammatory marker is one of the first to be released but is then very quickly degraded again (Pauley, 2011; Rombout et al., 2011). According to the SBMIE challenge, *il1β* was downregulated in the ISF B. If *il1β* is detectable at all, this indicates that the acute inflammatory phase is still in progress (Pauley, 2011). This is also reflected in the expression pattern of the immunoglobulins, which were only expressed in very small quantities (Figure V-5, IgD sec, IgT sec).

In fish, as in other vertebrates, immunoglobulins (*igs*) are produced as part of the adaptive immune response.

The timing of immunoglobulin production in fish varies depending on the species and the specific context of the immune challenge. Fish can produce immunoglobulins in response to infections, exposure to antigens, or environmental stressors. While IgM is the predominant immunoglobulin class in fish and is involved in systemic immune responses, IgT is another class specific to fish that plays a role in mucosal immunity. IgD, on the other hand, appears to be present in some fish species, but its exact functions are not as clear (Abós et al., 2016; Pauley, 2011; Rombout et al., 2011; Y. A. Zhang et al., 2010). The production of immunoglobulins in fish is part of their adaptive immune response and takes some time to develop. IgM was significantly upregulated in the mid and distal intestinal mucosa when Atlantic salmon were fed with soybean meal in a four-week challenge period (Krogdahl et al., 2000). All three immunoglobulins analysed were significantly upregulated after the challenge in this study in the ISF A group ( $p < 0.05$ ), while the ISF B group did not react. This again suggests that the addition of 0.1 % MAXFERM in the feed could prevent inflammation or the onset of disease despite challenge. Immunoglobulins are produced in fish as part of their adaptive immune response when they encounter pathogens or antigens. Considering the *mx1* expression, the theory of activation of a viral infection becomes more and more probable. Unfortunately, no PCR tests for PRV were carried out.

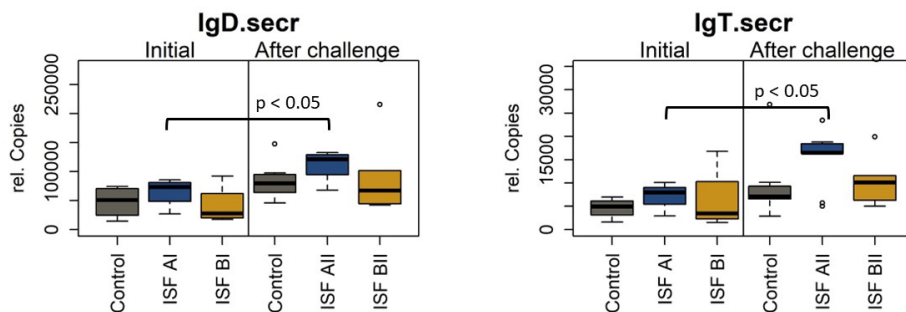


Figure V-5 Box and whisker plot of immunoglobulin D and T both secreted; relative copies. Box represents the interquartile range (IQR): Lower end = 25th percentile, upper end = 75th percentile, solid line = median, whiskers =  $1.5 \times \text{IQR}$ , grey dots = potential outliers, significant differences between feeding groups are indicated with brackets and corresponding  $p$ -values; initial  $n = 4$ ; after challenge  $n = 9$ . ISF A = includes 0.05 % MAXFERM; ISF B = includes 0.1 % MAXFERM.

## Conclusion

As there were no significant effects on the performance parameters, the effect of better digestibility of NSPs in salmon does not appear to play as clear a role as in chickens and pigs (Hein, 2022; Provita Supplements, n.d.), but a SBM challenge study showed that the addition of a functional fungal fermentation product at a dosage of 0.1 % MAXFERM (ISF B) has an anti-inflammatory and immune-stabilising effect on Atlantic salmon.

There were clear expression differences between the control group and the MAXFERM groups for 22 genes, but due to the outliers and the relatively small number of samples, only a few statistical significances were found. The expression pattern of the group with the high inclusion level (0.1 %; BII) was generally like the control group or even showed a

reduced gene expression, while often an upregulation was observed with the low inclusion level, 0.05 % (All). Since the upregulation of most genes indicates inflammation or an immunological response in general, the upregulations show that the additive did not have a sufficiently high balancing, immune stabilising effect at concentrations of 0.05 %, whereas 0.1 % did.

SBM contains antinutritive ingredients that can lead to reduced feed intake, impair growth performance, and interfere with digestion and nutrient absorption, leading to inflammation or other digestive issues (Cottrell et al., 2020; Francis et al., 2001). Phytic acid is one important antinutritive component in soybeans also known as inositol hexaphosphate (IP6). It acts as the primary storage form of phosphorus in plants (Chen & Xu, 2023; Reddy et al., 1982).

*Aspergillus* enzymes are involved in the degradation of plant cell wall polysaccharides (de Vries & Visser, 2001).

While *Aspergillus* spp. are often mentioned in fish nutrition in connection with intoxications, diseases and the production of aflatoxins (Dirican, 2018; Pinto et al., 2022; Shankar, 2013), there are certain aspergilli that have great potential (Strong et al., 2022). Different fungal species from the genera *Actinomucor*, *Amylomyces*, *Mucor*, *Rhizopus*, *Monascus*, *Neurospora*, *Aspergillus*, *Penicillium*, *Candida*, *Endomyces*, *Hansenula*, *Saccharomyces*, *Torulopsis*, *Trichosporon*, *Zygosaccharomyces* and others are reported to be involved in biotechnological food applications (Hyde et al., 2019). Filamentous fungi have a high secretion capacity to form enzymes. The utilisation of *Aspergillus niger*, for example, was the start of the commercial biochemical production of citric acid (Cairns et al., 2018) and *Aspergillus tubingensis* is even used biotechnologically to produce phytase, the enzyme that breaks down phytic acid. In the context of the SBM challenge in this study and the knowledge that the inflammatory problems in the gut of fish caused by SBM are caused by phytic acid (Francis et al., 2001; Zhong et al., 2019), among other things, it is likely that *Aspergillus tubingensis* has unfolded its effect precisely at this point. The positive effects of adding phytase are also known for other plant-based fish feed raw materials that contain phytic acid (Kaiser et al., 2021, 2022).

It remains unclear whether the positive immune effects are solely due to better availability of nutrients and the effect of phytase - according to the principle "the dose makes the poison" (Paracelsus). Further research is needed, and studies should investigate how high the phytase activity of the MAXFERM product actually is.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors.

## Author contribution

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HS: Data curation, Formal Analysis, Investigation, Visualization, Writing – original draft. DS: Data curation, Conceptualization, Writing – review & editing. SOM: Data curation, Methodology, Writing – review & editing. GL: Data curation, Methodology, Writing – review & editing. JM: Data curation, Formal Analysis, Visualization, Writing – review & editing. WK: Funding acquisition, Project administration, Supervision, Writing – review & editing. AR: Writing – review & editing, Data curation. MR: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing

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## Conflicts of interest

The authors declare no conflict of interest.

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**Anlage 3**

<b>C   A   U</b>	Christian-Albrechts-Universität zu Kiel	Agrar- und Ernährungs- wissenschaftliche Fakultät
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**Declaration of co-authorship**

If a dissertation is based on co-authored articles, a declaration from each of the authors regarding the part of the work done by the doctoral candidate must be enclosed when submitting the dissertation.

**1. Doctoral candidate**

Name: **Henrike Seibel**

**2. This co-author declaration applies to the following article:**

Fungal fermentation product boosts the immune system of Atlantic salmon (*Salmo salar*) after soya challenge




The extent of the doctoral candidate's contribution to the article is assessed on the following scale:

- A. Has contributed to the work (0-33%)
- B. Has made a substantial contribution (34-66%)
- C. Did the majority of the work independently (67-100%)

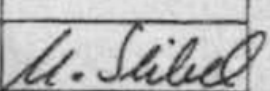
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Phase	Extent
<b>Concept:</b> Formulation of the basic scientific problem based on theoretical questions which require clarification, including a summary of the general questions which, it is assumed, will be answerable via analyses or concrete experiments/investigations	B
<b>Planning:</b> Planning of experiments/analyses and formulation of investigative methodology, including choice of method and independent methodological development, in such a way that the scientific questions asked can be expected to be answered	C
<b>Execution:</b> Involvement in the analysis of the concrete experiments/investigation	B
<b>Manuscript preparation:</b> Presentation, interpretation and discussion of the results obtained in article form	C

**4. Signature of all co-authors**

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Date	Name	Signature
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## Anlage 3

<b>C   A   U</b>	Christlan-Albrechts-Universität zu Kiel	Agrar- und Ernährungs- wissenschaftliche Fakultät
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**1. Doctoral candidate**

Name: **Henrike Seibel**

**2. This co-author declaration applies to the following article:**

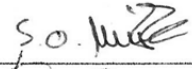
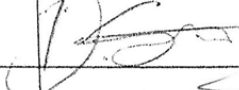
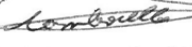
Fungal fermentation product boosts the immune system of Atlantic salmon (*Salmo salar*) after soya challenge

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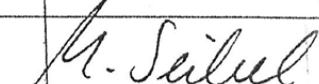
- A. Has contributed to the work (0-33%)
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3. Declaration on the individual phases of the scientific work (A,B,C)	Extent
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<b>Planning:</b> Planning of experiments/analyses and formulation of investigative methodology, including choice of method and independent methodological development, in such a way that the scientific questions asked can be expected to be answered	<b>C</b>
<b>Execution:</b> Involvement in the analysis or the concrete experiments/investigation	<b>B</b>
<b>Manuscript preparation:</b> Presentation, Interpretation and discussion of the results obtained in article form	<b>C</b>

**4. Signature of all co-authors**

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29.02.24	Georg Lamborelle	

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Date	Name	Signature
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**Anlage 3**

<b>C   A   U</b>	Christian-Albrechts-Universität zu Kiel	Agrar- und Ernährungs- wissenschaftliche Fakultät
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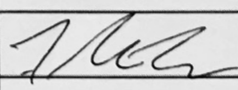
<b>1. Doctoral candidate</b>	
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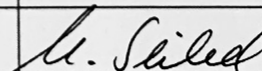
<b>2. This co-author declaration applies to the following article:</b>	
Fungal fermentation product boosts the immune system of Atlantic salmon ( <i>Salmo salar</i> ) after soya challenge	

The extent of the doctoral candidate's contribution to the article is assessed on the following scale:

- A. Has contributed to the work (0-33%)
- B. Has made a substantial contribution (34-66%)
- C. Did the majority of the work independently (67-100%)

3. Declaration on the individual phases of the scientific work (A,B,C)	Extent
<b>Concept:</b> Formulation of the basic scientific problem based on theoretical questions which require clarification, including a summary of the general questions which, it is assumed, will be answerable via analyses or concrete experiments/investigations	<b>B</b>
<b>Planning:</b> Planning of experiments/analyses and formulation of investigative methodology, including choice of method and independent methodological development, in such a way that the scientific questions asked can be expected to be answered	<b>C</b>
<b>Execution:</b> Involvement in the analysis or the concrete experiments/investigation	<b>B</b>
<b>Manuscript preparation:</b> Presentation, interpretation and discussion of the results obtained in article form	<b>C</b>

4. Signature of all co-authors		
Date	Name	Signature
28.02.24	Jonas Müller	

5. Signature of doctoral candidate		
Date	Name	Signature
28.02.24	Henrike Seibel	



## VI. Summarising discussion and prospects

Good, valid stress markers are needed that can be used for various examinations. In addition, the examination should be as minimally invasive as possible. Stress tests are used to evaluate the physiological and behavioural responses of fish to different environmental, handling, or experimental conditions. These tests help researchers, aquaculturists and scientists to understand the effects of stressors on fish and to develop strategies to minimise stress in aquaculture and research facilities. It is crucial to conduct stress tests ethically and minimise potential harm to fish during the testing process. Blood based tests are a promising option. Understanding the stress response of fish contributes to the development of better management practices in aquaculture and fisheries, leading to improved fish welfare and overall, more efficient production.

The aim of this work was to find suitable, valid molecular stress markers that are detectable in the blood of salmonids and to test whether blood based parameters are suitable for the diagnosis of stress and well-being in aquaculture.

### 1. Blood sampling

Blood sampling is considered minimally invasive in fish above a certain size (Cooke et al., 2019). For this purpose, mostly blood is collected from the caudal vessels (Duman et al., 2019). In very small fish, the technique is not minimally invasive, as vascular puncture is not possible. However, it must be noted that blood sampling without anaesthesia triggers a primary stress reaction within a few minutes, but that the anaesthesia itself can also trigger stress (Lawrence et al., 2020; Teixeira et al., 2021; Topic Popovic et al., 2012; Young et al., 2019). This must be considered when interpreting the test results. When comparing studies, it is important to pay close attention to how the blood sample was taken. The location of the blood sample can also influence the biomarker profile (Bando et al., 2010). To create a comparable significance of the test results, all fish in the studies listed here were stunned with a blunt blow to the head before the blood was taken by puncturing the caudal vein.

### 2. Phases of stress reaction and evaluation of released substances for stress and welfare diagnosis

Every organism must maintain complex and dynamic homeostatic processes (e.g. regulation of blood pressure), while these are regularly influenced by internal or external stressors. "Stress" thus describes the state in which the body's own homeostasis is impaired or is assumed to be out of normal physiological balance (Chrousos, 2009). To maintain homeostasis and thus protect itself, the body has a complex repertoire of behavioural and physiological adaptive responses that enable it to react to different stressors (Chrousos, 2009).

In fish, stressors are processed via the central nervous system and its sympathetic nerve fibres, which are connected to chromaffin cells. Chromaffin cells are glandular cells located mainly in the posterior cardinal vein and in the head kidney (Reid et al., 1998). The primary stress response is initialised by the cholinergic receptor in the chromaffin cells, which triggers the release of catecholamines (dopamine, noradrenaline,

and adrenaline) (Reid et al., 1996, 1998). In comparison to catecholamines, which are released immediately after the occurrence of a stressor, the release of corticosteroids is delayed. Cortisol levels, for example, rise within minutes and remain elevated for several hours (Strange et al., 1978). Therefore, Cortisol is probably the most measured molecule for determining the effects of stress in fish (Aluru & Vijayan, 2009; Sadoul & Geffroy, 2019a). In the hypothalamic-pituitary-adrenal axis, also known as the stress axis, the hypothalamus releases the corticotropin-releasing hormone (CRH). CRH stimulates the corticotropic cells in the pituitary gland, which leads to the release of adrenocorticotropin (ACTH) (Barton, 2002). Corticosteroids are synthesised in the interrenal cells located around the posterior cardinal vein in the head kidney. The released ACTH leads to a release of cortisol from the interrenal cells into the bloodstream (Barton, 2002; Wendelaar Bonga, 1997). Cortisol in turn influences the release of ACTH in a negative feedback mechanism (Wendelaar Bonga, 1997).

The released catecholamines play a role in increasing the respiration rate and oxygen uptake of the gills, in improving the body's blood flow and in increasing the oxygen transport capacity of the blood (Wendelaar Bonga, 1997). Elevated cortisol levels influence gluconeogenesis and glycogenolysis to mobilise and provide energy reserves (e. g. release of glucose into the bloodstream) (Wendelaar Bonga, 1997). Altered glucose levels are considered a sign of stress (Vijayan et al., 1990), as stress-induced changes in muscle activity accelerate the conversion of glucose to lactate (or alternatively to pyruvate) by anaerobic glycolysis. Lactate and glucose can be measured in blood plasma. These values are often used in conjunction with cortisol levels to determine the physiological state and assess the severity of a stress response (Acerete et al., 2004; Arends et al., 1999; Ashley, 2007; Conde-Sieira et al., 2010; Lambert et al., 2018). These mechanisms are also called the secondary stress response.

The stress hormones released influence the cells of the immune system (e. g. lymphocytes, leukocytes, macrophages) and lead to the release of messenger substances such as acute phase proteins, cytokines, and tumour necrosis factors (Tort et al., 2004). This is the starting point for the analyses in this study. Changes in cortisol levels indicate the extent of a primary stress response, but generally do not provide sufficient information to assess an animal's ability to cope with a difficult situation (Assem et al., 2014).

It has been shown that after a four-week period in which high doses of soybean meal (SBM) were fed, the animals sampled had cortisol levels twice to five times higher than the control group. After eight weeks, however, the cortisol levels decreased again. In addition, there were strong individual fluctuations in cortisol concentrations, so that statistically significant differences between the SBM and control groups could not be determined. Especially in the final stress phase, parameters such as the cortisol level, glucose or lactate values determined from the blood are no longer suitable.

Cortisol also has an inhibitory effect on growth, reproduction, and immune function (Wendelaar Bonga, 1997). If a stressor occurs over a longer period or becomes chronic, the adaptive stress response becomes maladaptive. The fish's ability to adapt and maintain homeostasis is exhausted, and the chronic stressors affect well-being, health, and mortality (Damsgård et al., 2006). The animal is now in the third stress phase, also known as the exhaustion phase. Ultimately, stress can therefore result in reduced growth and

reproduction, increased susceptibility to disease and behavioural changes (Barton, 2002; Damsgård et al., 2006; Ellis et al., 2002; Espmark & Baeverfjord, 2009).

Changes in performance parameters, such as poor growth, poor reproduction or even deaths, only occur at the end of a prolonged period of stress (Tort, 2011; Tort et al., 2004). In feeding studies, parameters such as initial weight (IW), final weight (FW), weight gain (WG), specific growth rate (SGR) as well as the feed conversion ratio (FCR) are still the main parameters investigated. However, with animal welfare in mind, additional blood analyses should be carried out to determine the possible influence of feed components on the immune system of fish. Both, the study on the influence of plant-based feed raw materials (Chapter IV, page 77) and the effects of handling stress and the investigation of fermented fungal feed additives (Chapter V, page 101) showed that there were clear differences in gene expression, while the performance parameters remained unchanged. The performance parameters only changed when a large amount of SBM was added, when histological changes in the intestinal epithelium and clinical diarrhoea were already detectable (Chapter III, page 51). Conversely, it can of course be said that if even the performance parameters change, special attention should be paid to this in feeding trials. Or it may also be that certain feed additives have a good anti-inflammatory or immunostimulant effect, but this is not yet reflected in the performance parameters and could therefore be underestimated in the feeding study.

## 2. Are molecular markers valid for stress detection

Since both the neuroendocrine system and the immune system are involved in the reaction to certain external signals (Tort, 2011), immune-relevant parameters have been extracted from fish blood for decades (Trust et al., 1981) to obtain information on how stress and the associated adaptive reactions affect the immune-competence of fish.

Various areas need to be examined more closely and can be mapped with specific molecular markers: influence on various immune cells, complement system, innate and adaptive immune response, effects of thermal stress, inflammatory parameters, superordinate regulatory systems and much more (Ferencz et al., 2012; Irwin & Cole, 2011; Simide et al., 2016). Blood is not the appropriate matrix for every research question; for example, not all aspects of "welfare" can be detected in blood. Isolation of steroids from matrices other than blood (i. e. mucus, scales, faeces, or water) have been reported in several studies (Cao et al., 2017; Carbajal Brossa, 2018; Félix et al., 2013; Guardiola et al., 2016); however, data obtained directly from blood is still far more accurate as the risk of rapid cortisol degradation and contamination from external cortisol sources is obviously lower (Sadoul & Geffroy, 2019). The data analysis and assessment are possible based on decades of basic research into the immune system of fish. It is known, that immunocompetence in fish is not dependent on age, but rather on size (Tort, 2011). Fish has – like other vertebrates – an innate and adaptive immune system (Mokhtar et al., 2023; Pauley, 2011) and lots is known about different immune markers and immunological reactions (Alvarez-Pellitero, 2008; Bayne & Gerwick, 2001; Magnadóttir, 2006; Mashoof & Criscitiello, 2016; Rebl & Goldammer, 2018; Rombout et al., 2011; Salinas, 2015; Salinas et al., 2011; Salinas & Magadán, 2017; Secombes et al., 2001; Stosik et al., 2021; Uribe et al., 2011; Wang & Secombes, 2013; Zhu et al., 2012; Zou & Secombes, 2011). Cytokine release in fish follows a recurring cascade, e. g. during inflammation (Campos-Sánchez &

Esteban, 2021) and quite a lot is known about different cell types in blood, already (Fingerhut et al., 2020; Zhu & Su, 2022).

Nevertheless, molecular markers from the blood of fish have rarely been analysed when it comes to questions of animal welfare and stress. Over 50 different genes were analysed in the present thesis. The model fish was the rainbow trout (*Onchorhynchus mykiss*) in two experiments and the Atlantic salmon (*Salmo salar*) in one experiment. The same markers were analysed for the salmon as for the trout. Table VI-1 shows the various markers and indicates whether they were detectable or not (marked in green or red).

Generally, the same genes were targeted, but there are also minor differences. Many genes were found that are also described by other groups in connection with stress (Li et al., 2017; Rebl et al., 2017; Schjolden & Winberg, 2007; Sun et al., 2017; Yarahmadi et al., 2016). The genes are usually isolated from organs such as the liver or head kidney. This thesis shows that they are also detectable in blood. Blood as a transport medium offers the possibility of analysing various substances without having to kill the fish. From an animal welfare perspective, this represents a major benefit compared to organ examinations.

A key advantage of analysing gene profiles instead of the detection of classical stress hormones such as cortisol or ACTH is that the stress events that occur immediately prior to sampling (e. g. due to capture, stunning and killing of the animal) are generally not reflected in altered transcript levels. A certain amount of time is required to activate the signalling pathways that ultimately lead to the release of transcription factors, which then initiate the gene transcription process (Vihervaara et al., 2018). Nonetheless, stress is mechanistically defined by a hormonal response (Lightman, 2008), and this response cannot be adequately detected at the transcriptional level. Accompanying data on stress hormone levels are needed if the changes in gene expression are to be assessed. Otherwise, they may only reflect the adaptive changes in the pathways to restore homeostasis.

Quantitative reverse transcription polymerase chain reactions (qRT-PCRs) can be used to detect minor changes in the expression of genes (Kralik & Ricchi, 2017) that are modulated by environmental influences or stressors. However, it must be noted that the system is also vulnerable. Genes may not be completely switched on or off in response to a particular treatment or under certain environmental conditions. Most treatments result in increased (upregulated) or decreased (downregulated) expression of a particular group of genes. These changes in expression only become apparent when the animals studied are compared with an untreated control group. This is the reason why such control / reference groups were analysed in all the studies presented in this thesis. Since animal welfare "diagnosis" also requires examination methods for use under practical field conditions, this fact represents a major weakness in the use of blood tests for stress and animal welfare diagnostics in field trials. Since a control group always seems to be necessary to obtain good information, the blood test is rather limited to scientific questions, since in practice the control group or the reference value of the analysed fish group is usually missing, and little is known about all the potential prior stress influences. However, blood samples are a very good and important tool for scientific purposes.

The challenge in interpreting the data also lies in the fact that a profound knowledge of the biochemical processes in the fish is necessary. As a rule, up-regulation indicates that the gene is currently active, but the interpretation of the significance of the up- or down-regulation of a gene must always be considered in the context of the function of this marker in the organism and the time frame of the reaction. The transcriptional stress response in fish involves the activation of specific genes and pathways to cope with stressors (Caspers, 1983; Mazeaud et al., 1977; Schreck & Tort, 2016; Wendelaar Bonga, 1997). The primary molecular mechanisms driving this response include the activation of stress-responsive transcription factors, modulation of gene expression, and the induction of stress-related proteins (Petitjean et al., 2019). In research chapter IV, page 77 is a table in which the genes studied were categorised according to their function given. The molecular mechanisms driving the transcriptional stress response in fish under feeding stress involve a complex interplay of signalling pathways and gene expression regulation. At this point it must be clearly stated that the SBMIE model is very specifically an inflammatory stress model. Enteritis in fish, particularly due to the inclusion of soybean meal in their diet, can trigger a stress response at the molecular level (Carmona, 2008; Krogdahl, Gajardo, et al., 2015; van den Ingh et al., 1991). The mechanisms driving the transcriptional stress response in fish experiencing enteritis due to soybean meal involve various pathways and gene expression changes (Agboola et al., 2022; Jalili et al., 2020; Sahlmann et al., 2013):

In response to the inflammatory conditions associated with enteritis, signalling pathways related to inflammation become activated. Pro-inflammatory cytokines, such as interleukins (ILs) and tumour necrosis factor-alpha (TNF $\alpha$ ) or the antiproliferative and proapoptotic transforming growth factor beta (TGF $\beta$ ), are released in response to the inflammatory signals (Campos-Sánchez & Esteban, 2021; Dale et al., 2009). These cytokines play a crucial role in coordinating the immune response and can influence the expression of genes involved in immune function and stress response. IL1 $\beta$ , for example, emerges as a classic stress and inflammatory marker. It is one of the first to be released but is then very quickly degraded. IL1 $\beta$  indicates the acute inflammatory phase when it is upregulated (Pauley, 2011; Rombout et al., 2014). was detectable in varying amounts in this thesis. When using the SBMIE model to investigate the potential immune-strengthening, anti-inflammatory competence of a feed additive (Research Chapter IV, page 76), the downregulation of this cytokine compared to the control group in the high-dose group (0.1 %; ISF IIB) is to be assessed as "positive", as it allows the statement that the inflammatory reaction is mitigated by the addition of the fermented fungal product in this concentration. Feeding SBM for 14 days did not lead to an inflammatory reaction in the Atlantic salmon, whereas this was the case for the treatment group with a lower amount of product added (0.05 %; ISF IIA). This statement is only valid if the other analysed markers are considered, as these clearly show that inflammatory reactions were detectable after the SBM challenge (e.g. upregulation of fibrinogen-like protein 2 (Fgl2) or complement factors). The increased levels of transcripts coding for the complement 5a receptor or Fgl2 suggest that healing processes have been set in motion (Boshra et al., 2006; Magnadóttir, 2006). The process of wound healing in fish involves the inflammatory response, as first stage and the presents of IL1 $\beta$  indicates that the acute inflammatory phase is still in progress (Kono et al., 2002; Zanuzzo et al., 2020). The fact that IL1 $\beta$  was still detectable in this study is probably because the SBM challenge trial was only relatively

short at 14 days. In the feeding trial with different amounts of SBM (Research Chapter III, page 50) fed over a period of 56 trout from the SBM100 group suffered from bloody diarrhoea ten days after the start of the experiment, which lasted until the end of the experimental period. From day 14 onwards, diarrhoea was also documented in trout from SBM66 group. Bloody diarrhoea is a clear symptom of inflammation in the intestines. The inflammation was therefore present until the end of the feeding trial, but IL1 $\beta$  was no longer detectable, which is most likely because the trout may already have been in the exhaustion phase, which is supported by the fact that cortisol was also no longer detectable after 8 weeks. It is therefore very important that only the observation of a well-chosen portfolio of different gene markers allows well-founded statements.

In response to the inflammatory conditions associated with enteritis, signaling pathways related to inflammation become activated. The nuclear factor-kappa B (NF- $\kappa$ B) pathway is a key regulator of inflammation and can be triggered by various stimuli, including components of soybean meal (Booman et al., 2018; Pasare, 2003; Sahlmann et al., 2013). The MAP3K1 gene encodes the "mitogen-activated kinase kinase kinase 1", which acts as an upstream activator of early transcription factors such as NF- $\kappa$ B (Hirano et al., 1996) or Jun (Yan et al., 1994). MAPK signalling pathways have previously been suggested to mediate the physiological effects of unbalanced dietary effects in fish (Holen et al., 2011; Wu et al., 2016; Zhang et al., 2017). The MAPK signalling transduction pathway is activated during stress and inflammation in mammals (Kyriakis & Avruch, 2012). An upregulation indicates that this signalling pathway has been activated. After SBM challenging a significantly different expression between both ISF groups was detectable (Research Chapter V, page 98) after 14 days. The levels of MAP3K1 in trout having received feed with fully substituted fish meal were consistently reduced after 8 weeks (Research Chapter III, page 51) which clearly illustrates the temporal dependence of the activation of the different cascades during stress response.

However, the selection of genes represents a choice made based on the investigators' prior knowledge and experience. Transcriptomic approaches with high throughput, such as microarray or RNA sequencing analyses (RNA-seq), could provide a remedy here. They enable the monitoring of transcriptional changes in a comprehensive panel of potential indicators in each research setting (Denslow et al., 2007; Götting & Nikinmaa, 2017). The challenge for blood is that it involves a very heterogeneous mixture of cells and a broad spectrum of cell populations. Erythrocytes in fish are also nucleated. Transcriptome analyses in fish are therefore primarily aimed at specific questions and the analysis of blood is underrepresented (Cao et al., 2019; Götting & Nikinmaa, 2017; Kemski et al., 2020; Kiron et al., 2020; Li et al., 2017). This also shows that the search for THE ONE or the few stress markers for fish is more of a search for a needle in a haystack and can only ever relate to a specific stress event, a specific fish species, breeding differences and others (Lund et al., 2022).

In this study, promising markers were found for feeding stress in connection with the feeding of SBM and salmonids. And, it has already been shown that analysing the genes mentioned here in different trout farms in combination with other parameters allowed



conclusions about the animal welfare status, even if there is no control group and the husbandry conditions are different (Weirup et al., 2022b).

Table VI-1 Overview of genes analysed in the various studies of this thesis. *green* = detectable, *red* = not detectable. For an explanation of the genes (names), see the paper. The three internal standard genes (housekeeping genes) *eef1a1*, *rps20*, *actb* were analysed in all studies.

Chapter III, page 511 "Blood based parameter indicating SBMIE ..."	Chapter IV, page 77 "Interactions of plant-based feeding ..."	Chapter V, page 101 "Fungal fermentation product boosts immune system ..."
Rainbow trout	Rainbow trout	Atlantic salmon
		C3-1
C3AR		C3AR
		C5AR
CCL4a		CCL4a
CD41		
		CXCL8/IL8
ENPP3		ENPP3
FGL2		FGL2
FTH		FTH
GCSFR		GCSFR
HP		HP
		HSP47a
HSP47b		
HSP30		
HSP90AB1a		HSP90AB1a
HSP90AB1b		HSP90AB1b
	IgD-secr	IgD-secr
	IgD	
		IgM
		IgM-secr
	IgT-secr	IgT-secr
	IgT	
IGHM		
		IL10
IL1 $\beta$	IL1 $\beta$	IL1 $\beta$
IL4/13		
		IL6
		LECT2
LOC100135979		
LYGa		LYGa
LYGb		LYGb
		LYGc
MAP3K1		MAP3K1
MCSFR		MCSFR
MPO		MPO
MX1		MX1
NCCRP1		NCCRP1
NOD1a		NOD1a
		NOD1b
SAA		SAA
SERPINB1		SERPINB1
SOCS1		SOCS1

SOD1		
SOD2		
STAT1		STAT1
		TCRB
		TGFb
		TGFB-1
TLR3		TLR3
TNF $\alpha$	TNF $\alpha$	TNF $\alpha$
TNF $\beta$		TNF $\beta$
TRB		
UCP2		UCP2
USP4		USP4

### 3. The SBMIE model, stress tests and “welfare” detection systems

The SBMIE model is an additional suitable model for investigating the effects of feeding stress and everything associated with it on fish. It is still used in many studies and is now regarded as a standard model (Li et al., 2022; Peng et al., 2020; Rubio Benito, 2023; Shan et al., 2023; Zhao et al., 2024). Antinutritive factors in soy, such as protease inhibitors and lectins interfere with the digestion and absorption of nutrients in fish and lead to inflammatory changes in the gut (Booman et al., 2018; Francis et al., 2001; Krogdahl et al., 1994, 2010). Compared to some other stress tests, such as evoke stress by temperature, air exposure or chemicals (Arends et al., 1999; Gingerich et al., 2007; Lim & Hur, 2018; Sharma, 2019; Vinagre et al., 2012), the SBMIE model is particularly suitable for testing feed additives. As the tests in this study show, even relatively small quantities of soybean meal / soy protein concentrate (~ 20 %) are sufficient to achieve a challenge effect. Feeding soy-based diets to salmonids is a common practice in aquaculture, especially as a replacement for fish meal, which can be expensive and environmentally unsustainable (Bjerkeng et al., 1997; Carter & Hauler, 2000; Olsen & Hasan, 2012). Therefore, the experiences from challenged fish using SBM has an important practical relevance. The evaluable markers are based on the inflammation caused by native SBM in the intestine in a controlled manner. The expression of proinflammatory cytokines, the infiltration of immune cells and other markers associated with inflammation are assessed (Djordjevic et al., 2021; Kiron et al., 2020).

Therefore, especially in studies aimed at understanding the effects of dietary components on fish health and immune responses, the investigation of blood is more sensitive compared to other welfare investigations like behavioural analyses by camera. Welfare examinations of fish using a camera involve using imaging technology to assess the health, behaviour, and overall well-being of fish in aquaculture or research settings (Barreto et al., 2022). Although the advantage of this method is that it is completely non-invasive, it cannot map the physiological state of the fish. This technique promises behavioural analysis (swimming patterns, feeding habits, social interactions, and behaviourally responses to environmental stimuli or stress) or health monitoring (detection of physical abnormalities, lesions, or other signs of disease, changes in coloration or skin condition) (Ellis et al., 2019; Wang et al., 2021). By using camera-based animal welfare assessments, researchers and aquaculture operators could make decisions to improve the overall health and conditions of the fish, ultimately increasing the efficiency and

sustainability of aquaculture practices. Work is underway to develop advanced computer vision and machine learning algorithms to analyse large amounts of image data relating to fish behaviour, identify abnormal patterns and detect health problems at an early stage (Li et al., 2022; Yang et al., 2021). However, it will probably be some time before detailed statements can be made about the state of health.

This is already possible by analysing blood parameters, but the expensive and time-consuming molecular examination need to be simplified in the future. The development of rapid tests is conceivable. For cortisol there are different rapid tests available. Rapid tests, in the context of cortisol or other biomarker assessments, are diagnostic or analytical methods designed to provide quick results. These tests prioritize speed and simplicity, making them suitable for on-site or point-of-care applications where immediate information is essential. They should be easy to use and portable. Non-experts should be enabled to conduct reliable results with it. Many rapid tests provide a visual readout, such as a colour change or other easily interpretable signals, allowing for straightforward result interpretation without the need for specialized equipment. In the case of cortisol, rapid tests for stress assessment in fish may involve the use of cortisol dipsticks, ELISA kits or portable analysers (Aiga et al., 2023; May et al., 1985; Tang et al., 2021).

It remains to be seen which technology can be used to develop rapid tests for the detection of stress or immune system weaknesses in fish. Conceivable techniques are ELISA, lateral flow immunoassays (LFIA), fluorescent immunoassays or chemiluminescent immunoassays. Biosensors are devices that integrate biological recognition elements (such as antibodies) with a transducer to convert the binding event into a measurable signal. Immunosensors, based on antibodies and colorimetric assays involve the use of colour changes. Microfluidic platforms allow for the manipulation of small volumes of fluids and are often employed in point-of-care and rapid testing applications and surface plasmon resonance (SPR) is a label-free technique that detects changes in refractive index on a sensor surface (Aiga et al., 2023; May et al., 1985; Qi et al., 2020; Ramamurthy et al., 2020; Whiting et al., 2005; Wongsrichanalai, 2001).

#### 4. Conclusion - Is blood suitable for diagnostics of stress in fish?

The assessment of the data remains a challenge, in which aspects such as fish species, husbandry system, type of stress marker (acute or chronic) and the mutual influence of the genes on each other must be considered. Inflammation or stress-induced immunosuppression can lead to secondary bacterial or viral infections. It has been shown that it is always a good idea to analyse a classic virus marker such as MX1 as well. So far, little is known about how stress affects the microbiome and, conversely, how microbiome changes influence classical bacterial markers (López Nadal et al., 2020; Suhr et al., 2023b, 2023a). Nevertheless, for many applications in research, the investigation of stress and immune parameters using blood as a minimal invasive method is very interesting, especially to better understand stress coping mechanisms in fish or impacts of feed supplements.

The examination of blood provides information about the individual physiological state of a fish. However, when interpreting the parameters obtained from the blood, it must be considered that metabolic changes may also be due to persistent chronic disturbances and / or causally independent events (e. g. circadian rhythms, seasonality, feeding times, aggression from conspecifics, water quality, etc.) or inadequate sampling and laboratory

procedures. The influence of sex and body weight / size of individual fish should also not be underestimated.

It needs to keep in mind, that welfare and stress are related concepts in the context of animal well-being, including fish, but they refer to different aspects. While welfare encompasses the overall state of an animal, considering its physical health, mental well-being, and the quality of its environment, stress is a physiological and behavioural response to challenging or threatening stimuli (stressors). It is a natural and adaptive reaction that prepares an organism to cope with a perceived threat or change in its environment. This is important to emphasise, as not all stress is the same and a sick stock does not always indicate a poor welfare status at the same time. The distinction between positive and negative stress is context-dependent, and what may be a positive stressor in one situation could become negative if the intensity or duration exceeds the fish's adaptive capacity. In contrast to negative stress, which can have a detrimental effect on the health and well-being of fish, positive stress can improve their ability to cope with environmental stress and increase their overall fitness. However, the same messenger substances are detected at the molecular level, as the body does not differentiate between eu- and distress in the first line, when the stress axis is activated.

Blood tests should be given more attention in basic research and feeding tests. As a diagnostic tool for practical use, there are still some hurdles, such as the lack of reference values, the strong dependence on the stress event and the fish species, as well as the costs. Rapid tests are currently only available for a few substances such as glucose, lactate, or cortisol so far.

## VII. Summary

Animal welfare aspects in aquaculture relate to the general welfare of aquatic animals reared for various purposes in a controlled environment. Ensuring good animal welfare practices in aquaculture is important for several reasons. Challenge trials can be conducted to assess physiological and ethological responses of fish to different environmental, handling or experimental conditions and to improve stress management where necessary. Soya bean meal induced enteritis (SBMIE) can be considered as such a stress test and was used in the studies of this thesis to provoke feeding stress.

Good, valid stress markers are needed to assess feeding stress and animal welfare and the test should be as non-invasive as possible. Blood tests are a promising option. Thus, the aim of this study was to investigate whether a blood-based diagnosis is suitable for the detection of stress and welfare in fish. Salmonids (rainbow trout and Atlantic salmon) were used as research species and tested for feeding stress using the SBMIE model.

In a general introduction (review) on the topic of blood as a diagnostic tool, an overview of the state of the literature was given and answers were given as to what simple plasma tests and multiparametric analyses of fish blood are. Various techniques for assessing the well-being and diseases of fish were presented. It was explained how the analysis of molecular stress markers compares to classical methods such as the determination of cortisol in the blood or differential blood counts and which markers from the blood can provide well-founded indications of feeding stress.

The following three empirical studies illustrate three different aspects of the use of blood for diagnostics and analysing stress. (1) Firstly, the influence of a soybean meal (SBM)-based diet on water quality, physiology, classical growth and performance parameters and the blood of rainbow trout was analysed in a 56-day feeding trial. In addition to investigating the haematocrit value, plasma cortisol and differential blood count, the focus of this study was on identifying suitable molecular stress markers that can be measured in blood. The study answered the question of how molecular stress markers behave in comparison to classical methods - such as the determination of cortisol in the blood or the differential blood count - and which markers that can be determined in the blood of trout provide well-founded indications of feeding stress. (2) In the second study, the influence of a predominantly plant-based diet on carnivorous rainbow trout was investigated. Individual markers of the innate and adaptive immune response under the influence of stress and SBM challenge were detected in blood. In the commercial diet of salmonids, soy is often a dietary component, this was modelled in this feed formulation and the effects were analysed under stress challenge. Handling stress was simulated by means of daily netting in the tanks. It turned out that blood tests should be given more attention in basic research and feeding trials, as they can provide important information for feeding even before changes in the performance parameters are found. However, as many factors influence the expression of the analysed genes, a control group treated in the same way is always required in the studies for valid evaluation. This has so far limited blood tests to scientific questions. (3) In the last study, the molecular markers investigated were used in the stress model to analyse the stress-balancing, immunostimulatory potential of a feed additive in a specific application to improve feed containing soya. The molecular markers from blood found in studies 1 and 2 were also successfully tested for their usability for

direct diagnostic applications for Atlantic salmon and the potential uses of blood-based diagnostics in the context of feeding stress tests were analysed in more detail.

In conclusion, in this study, promising markers for feeding stress associated with the feeding of SBM and salmonids were found. They can be used to detect feeding stress, to investigate the influence of feed additives and to analyse various functional additives for their anti-inflammatory and immunostimulatory properties. Since there are many factors that influence the expression of the analysed genes, a control group is required. This restricts blood tests to scientific questions, as in practice there is usually no control group or reference values for the group of fish being analysed.

## VIII. Zusammenfassung

Tierschutzaspekte in der Aquakultur beziehen sich auf das allgemeine Wohlbefinden von Wassertieren, die zu verschiedenen Zwecken in kontrollierter Umgebung aufgezogen werden. Die Gewährleistung guter Tierschutzpraktiken in der Aquakultur ist aus verschiedenen Gründen wichtig. Um physiologische und ethologische Reaktionen von Fischen auf verschiedene Umwelt-, Handhabungs- oder Versuchsbedingungen zu bewerten und das Stress-Management ggf. zu verbessern können Challengeversuche durchgeführt werden. Die Sojabohnenmehl induzierte Enteritis (SBMIE) kann als solcher Stresstest angesehen werden und wurde in den Studien dieser Arbeit eingesetzt, um Fütterungsstress zu provozieren.

Zur Beurteilung von Fütterungsstress und Tierschutz werden gute, valide Stressmarker benötigt und die Untersuchung sollte so wenig invasiv wie möglich sein. Bluttests sind eine vielversprechende Option. So war es das Ziel dieser Arbeit zu untersuchen, ob eine auf Blut basierende Diagnose zum Nachweis von Stress und Wohlbefinden bei Fischen geeignet ist. Salmoniden (Regenbogenforelle und Atlantischer Lachs) wurden als Forschungsspezies verwendet und mit Hilfe des SBMIE-Modells auf Fütterungsstress getestet.

In einer allgemeinen Einführung (Review) zum Thema Blut als Diagnostikum wurde ein Überblick über den Stand der Literatur gegeben und beantwortet, was einfache Plasmatests und multiparametrische Analysen von Fischblut sind. Verschiedenen Techniken zur Beurteilung des Wohlbefindens und von Krankheiten bei Fischen wurden dargestellt. Es wurde erläutert, wie sich die Untersuchung von molekularen Stressmarkern im Vergleich zu klassischen Methoden wie der Bestimmung von Cortisol im Blut oder dem Differentialblutbild darstellt und welche Marker aus dem Blut fundierte Hinweise auf Fütterungsstress liefern können.

Die sich anschließenden drei empirischen Studien veranschaulichen drei verschiedene Aspekte der Verwendung von Blut als Diagnostikum zur Analyse von Stress. (1) Zunächst wurde in einem 56-tägigen Fütterungsversuch der Einfluss einer auf Sojamehl basierenden Ernährung auf die Wasserqualität, die Physiologie, klassische Wachstums- und Leistungsparameter und das Blut von Regenbogenforellen untersucht. Neben der Untersuchung des Hämatokritwertes, des Plasmacortisols und des Differentialblutbildes lag der Schwerpunkt dieser Studie auf der Identifizierung geeigneter molekularer Stressmarker, die im Blut gemessen werden können. Die Studie beantwortet die Frage, wie sich molekulare Stressmarker im Vergleich zu klassischen Methoden - wie der Bestimmung von Cortisol im Blut oder dem Differentialblutbild - verhalten und welche Marker, die im Blut von Forellen bestimmt werden können, fundierte Hinweise auf Fütterungsstress liefern. (2) In der zweiten Studie wurde der Einfluss einer überwiegend pflanzlichen Ernährung auf karnivore Regenbogenforellen untersucht. Es wurden einzelne Marker der angeborenen und adaptiven Immunantwort unter Stresseinfluss und Soja Challenge im Blut nachgewiesen. In der kommerziellen Ernährung von Salmoniden ist Soja oft ein Fütterungsbestandteil, dies wurde in dieser Futterformulierung nachempfunden und die Effekte wurden unter Stressprovokation analysiert. Mittels täglichem Keschern in den Becken wurde Handling-stress simuliert. Dabei hat sich herausgestellt, dass Bluttests in der Grundlagenforschung und bei Fütterungsversuchen mehr Beachtung finden sollten, da sie wichtige Informationen für die Fütterung liefern können, noch bevor sich

Veränderungen in den Performanceparametern finden. Da jedoch viele Faktoren die Expression der untersuchten Gene beeinflussen, braucht es zur fundierten Auswertung immer eine gleichartig behandelte Kontrollgruppe in den Untersuchungen. Dies schränkt bisher Bluttests eher auf wissenschaftliche Fragestellungen ein. (3) In der letzten Studie wurden die untersuchten molekularen Marker im Stressmodell eingesetzt, um in einer konkreten Anwendung zur Verbesserung von Futtermitteln mit Sojaanteil das Stressausgleichende, immunstimulierende Potential eines Futtermittelzusatzstoffes zu analysieren. Dabei wurden die in den Studien 1 und 2 gefundenen molekularen Marker aus dem Blut auf ihre Verwendbarkeit für direkte diagnostische Anwendungen auch für den Atlantischen Lachs erfolgreich getestet und die Nutzungsmöglichkeiten von Blut basierter Diagnostik im Rahmen von Fütterungsstressuntersuchungen näher beleuchtet.

Zusammenfassend, konnten in dieser Studie wurden vielversprechende Marker für Fütterungsstress im Zusammenhang mit der Fütterung von Soja und Salmoniden gefunden werden. Sie können zum Nachweis von Fütterungsstress, zur Untersuchung des Einflusses von Futtermittelzusatzstoffen und zur Analyse verschiedener funktioneller Zusatzstoffe auf ihre entzündungshemmenden und immunstimulierenden Eigenschaften verwendet werden. Da es viele Faktoren gibt, die die Expression der untersuchten Gene beeinflussen, ist eine Kontrollgruppe erforderlich. Dies schränkt Bluttests auf wissenschaftliche Fragestellungen ein, da in der Praxis meist keine Kontrollgruppe oder Referenzwerte für die untersuchte Fischgruppe vorliegen.



## IX. Bibliography introduction and discussion

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