Neuroendocrine and Gene-based Markers to Evaluate the Welfare Challenges of the Salmonid *Coregonus maraena*

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Summary

The teleost maraena whitefish *Coregonus maraena* is a salmonid fish native to the Baltic Sea region. With only a few natural populations remaining, they are maintained through large re-stocking programs and intensive aquaculture production. These high-yield aquaculture systems have to adapt the animal needs to the particular conditions of the farm and vice-versa. Within this framework, biological responses and demands that occur naturally in the wild face a number of unique challenges. These may be amplified conditions that are also present in the wild or new conditions related to human interventions. Animals will respond to culture-related stressors with a complex physiological reaction. This response, known as the stress response, is initiated in the brain and triggers the release of stress hormones in the head kidney (HK). Cortisol and catecholamines induce a series of compensatory physiological processes to re-direct the metabolic resources of the organism toward survival needs. Depending on the type of challenge, intensity and duration, the activation of the stress response can lead to decreased well-being. To understand and assess the impact and severity of these challenges, markers need to be developed that provide a reliable source of information, reflecting the interaction between the biological systems and the environmental factors of chemical, physical or biological origin. The main line of work of this project was divided between the impact of the stress response on the brain monoamine system, and the neuro-immune interactions in the head kidney at the cellular level. Therefore, we aimed at obtaining a range of indicators at different levels of the neuro-immune axis which can help understand the biological and welfare status of *C. maraena* when confronted with handling and immune challenges. In the search for promising markers, particular interest was placed on the siglec machinery for cell-cell interactions, as these are a key feature for the modulation of cellular immune responses in vertebrates. Additionally, gene-based markers have been used to characterize somatosensory networks in the adipose fin of salmonids, underpinning the functionality of this appendix and raising concerns about fin clipping as a means to mark fishes. The use of gene-based markers played a central role in this project as it allowed for the simultaneous evaluation of a large panel of promising markers in different tissues and under different challenges. The effects of handling were first observed in brain neurotransmitter dynamics and further assessed by changes in plasma cortisol concentration. This indicated the activation and recovery phases of the stress response at 3h and 24h post-challenge, respectively. The low stress response after 10d of repeated handling indicated the habituation of the animals to a recurrent stressor. In this sense, adrenaline receptor genes (*ADRA1D, ADRB3A*) and serotonin receptor genes (*HTR1A*) were found to be promising indicators of stress response activation and recovery (*HTR3C*) in the brain, while dopamine receptor genes (*DRD1* and *DRD4*) were potential indicators of habituation. The analysis of the HK cell-organization revealed the presence of B cells and granulocytes, and to a lesser extent T cells and monocytes. Interestingly, *ADRA2D* was the most expressed adrenergic receptor in the analyzed HK cells, suggesting this α2 receptor variant as a major target for catecholamine-immune interaction. The sensitivity of acute phase genes to immune challenges was confirmed by the *in vivo* experiments with *A. salmonicida* injection and the *in vitro* exposure of HK cells to TLR ligands. At the same time, the exposure of isolated cells to stress hormones alone resulted in the up-regulation of *IL1B*, *CXCL8* and *SAA*, indicating the sensitivity of these marker genes to stress stimuli of different origins. Combined exposure to stress hormones and pathogen-associated molecular patterns showed the suppressive effects of cortisol and adrenaline when the immune response is activated. In addition, noradrenaline was able to modulate the expression of hormone receptors but weak as an immune regulator. The study of siglecs in three teleost species showed a higher distribution of siglec genes in immune-related organs. At the same time, the exposure of *C. maraena* to a single episode of handling up-regulated *Siglec1* and *CD22* in the brain, and down-regulated MAG in the spleen. This suggests a

tissue-specific effect on siglec expression. Finally, the gene-based study of the salmonid adipose fin indicated the presence of nerves and neurons involved in nociception and mechanosensation. This supports the functionality of the adipose fin and raises concerns about fin clipping procedures. Overall, these studies collectively pursued a better understanding of *C. maraena* and how teleost biology responds to a challenging environment.

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Abbreviations

5-HIAA: 5-hydroxyindoleacetic acid
5-HT: serotonin
A: adrenaline
ADRA1-2: adrenoreceptor type α subtypes 1 and 2
ADRB1-3: adrenoreceptor type β subtypes 1 to 3
ASIC2: acid sensing ion channel subunit 2
BSC: Brain-sympathetic-chromaffin axis
cAMP: cyclic adenosine monophosphate
CD22: CD (cluster of differentiation) 22 molecule (Siglec 2)
CSF3R: colony stimulating factor 3R
CXCL8: Interleukin 8 (IL-8 or chemokine (C-X-C motif) ligand 8)
DA: dopamine
DAMPs: damage-associated molecular patterns
Dap12: DNAX-activating protein 12 kDa
DRD1-4: dopamine receptor subtypes 1 to 4
FB: forebrain section
GFAP: glial fibrillary acidic protein
NR3C1A-B: glucocorticoid receptor type 1 and 2
HB: hindbrain section
HK: head kidney
HK-cells: head kidney cells
HPI: hypothalamic-pituitary-interrenal axis
HSP90AA1: heat shock protein 90 Alpha family class A member 1
HSPA1A: heat shock protein family A (Hsp70) member 1A
HTR1-6: serotonin receptor subtypes 1 to 6
IGHM: immunoglobulin heavy chain Mu
IL12B: interleukin 12B
IL1B: interleukin 1β
IL6: interleukin 6
I.p.: intraperitoneal
ITAM: immunoreceptor tyrosine-based activation motif
ITIM: immunoreceptor tyrosine-based inhibitory motif
KCNK2: potassium two pore domain channel subfamily K member 2
MAG: myelin associated glycoprotein
MB: midbrain section
MPO: myeloperoxidase
NR3C2: mineralocorticoid receptor
NA: noradrenaline
NEFL: neurofilament light chain
NGFR: nerve growth factor receptor
PAMPs: pathogen-associated molecular patterns
PIEZO2: Piezo type mechanosensitive ion channel component 2
PRPH: peripherin
PRRs: pathogen recognition receptors
SAA: serum amyloid α
SIGLEC1: sialic acid binding Ig-like lectin 1 (Sialoadhesin)
SIGLEC15: sialic acid binding Ig-like lectin
TCR: T-cell receptor

TLRs: Toll-like receptors
TNFA: tumor necrosis factor α
TRPC1: transient receptor potential cation channel subfamily C member 1
ZAP70: zeta-chain associated protein kinase 70kDa

1. Introduction

1.1. Maraena whitefish and the welfare concerns in salmonid aquaculture

The teleost maraena whitefish *Coregonus maraena* (Bloch, 1779; Fig. 1A) is a salmonid fish present in the Baltic Sea area in anadromous and landlocked populations (Fig. 1B). In the northern Baltic, juveniles can undertake migrations of up to 700 km, from spawning rivers to foraging areas in the sea (Kottelat and Freyhof, 2007). With only a few natural populations remaining, its natural stocks are decreasing, only maintained by the large re-stocking programs carried out by the Baltic countries since the 90s (Jansen et al., 2008). It has been listed as vulnerable in the IUCN Red List of Threatened Species due to overfishing, water pollution and dam construction (Freyhof and Brooks, 2011). In Germany, maraena whitefish has been reared for intensive aquaculture production since 2005 (Jansen et al., 2008). The production in the Baltic area had an approximate average of 850 tons per year from 2009 to 2018 (Eurostat). Due to its high market value, it has been introduced in many lakes within its native range (Kottelat and Freyhof, 2007).

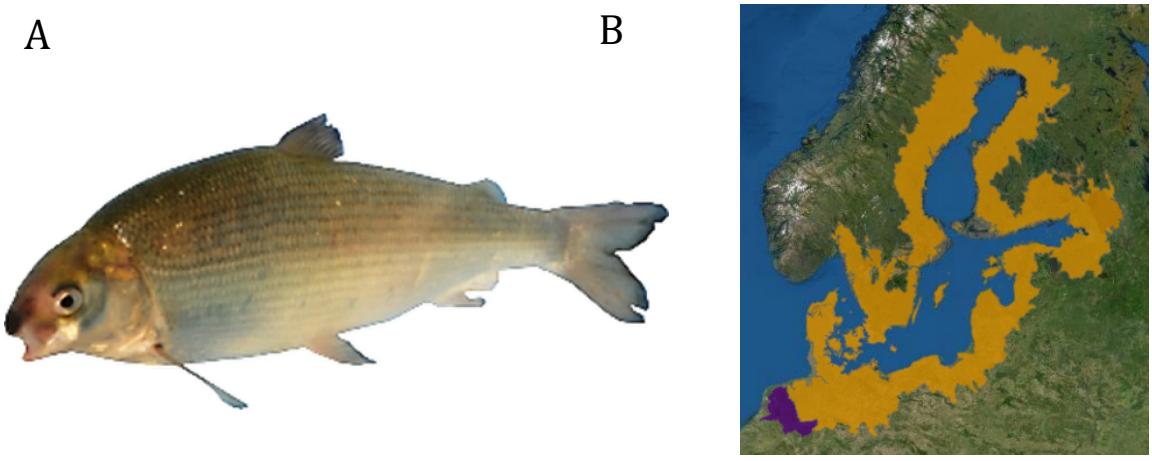

Figure 1. A) Adult individual of *C. maraena*. B) Distribution of *C. maraena* in the Baltic Sea region, orange area indicates native distribution and purple area indicates introduced populations. Map figure from IUCN Red List of Threatened species.

Nowadays, commercially profitable aquaculture implies high yield production based on intensive farms, as they have to supply for the increasing demand of finfish products while many wild stocks are being overexploited or depleted. In 2018, the world aquaculture production almost doubled capture fisheries yield (FAO, 2020). Such production systems have to adapt the animal needs to the particular conditions of the farm and vice-versa. In this framework, biological responses and demands that naturally occur in the wild are confronted with a series of exotic challenges of anthropogenic nature. These challenges can be amplified conditions also present in the wild (e.g. infections in closed systems) or new conditions related to human intervention (e.g. handling, finclipping). Over the years and due to their high economical interest, Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* have become the model animals to study salmonid physiology, and the main source of information about salmonid welfare in farming conditions.

Recently, two guidelines (Noble et al., 2018, 2020) summarized the welfare needs of these species in aquaculture conditions in four groups: health, feeding resources, behavior and quality of the environment. In summary, a proper health status is to the result of fighting against pathogen infections and diseases, including skin injuries related to handling or those caused by predators. Feeding resources have to be available to avoid starvation and cover the nutritional needs of the animals. Fish have to be able to display a natural behavior. This includes exploring their surroundings, rest, socialize with their peers and sexual behavior. The optimum quality of the environment is reached when water quality, temperature, salinity and oxygen concentration meet the demands for each species.

As most teleost species, salmonids are ectotherm which implies that the temperature of their bodies is dictated by the temperature of the surrounding water column. In particular to salmonids, different life stages have different preferable temperature ranges (Sauter et al., 2001). This makes thermal regulation a factor to be considered when setting the temperature conditions of the farm. Another characteristic of salmonids is that they are anadromous. They are born in freshwater and migrate to salt water towards adulthood, returning to freshwater for spawning. This ability to adapt their physiology to different osmolalities throughout their life is of major importance for the welfare needs at different life stages (McCormick, 2012). In some situations, anadromous fish can be physically blocked to reach the sea, becoming a landlocked population which will go through the entire life cycle in fresh water. This is the case of the different species of Coregonus inhabiting many lakes of northern Europe (Kottelat and Freyhof, 2007).

For the specific challenges studied in this work, handling procedures can cause injuries of the skin and especially the eyes, leading to secondary infections or loss of vision. Moreover, handling has been described as an activator of the stress response in salmonids (Barton et al., 1987). Optimized handling equipment and technics are essential to minimize the potential harm (e.g. vacuum pump system for life fish instead of group netting) (Noble et al., 2012). Removal of the adipose fin or fin clipping is a common practice in salmon aquaculture to differentiate farm escapees from wild individuals or to identify fish that are part of re-stocking projects (ICES WGBAST Report, 2007). This appendix was originally considered useless (Garstang, 1931). However, recent works point that it is a functional sensing organ (Buckland-Nicks, 2016) and that its removal has negative effects on fish performance (Reimchen and Temple, 2004). Finally, disease and infection are naturally occurring events that can also affect fish in otherwise good welfare condition. However, good hygiene standards and strict control of the water quality can avoid major pathogen outbreaks in aquaculture facilities.

1.2. The stress response in fish

The presence of multiple and overlapping stressors in aquaculture requires a complex physiological response. Depending on the type of challenge, intensity and duration, this condition can lead to a decreased welfare status (Broom and Corke, 2002; Huntingford et al., 2006). When confronted with a challenge, the stress response is activated, leading to the regulation of multiple systems in the organism in order to re-organize the energetic demands and to cope with the stressor. This implies the sudden overload of the organism's allostasis and the subsequent counter-reaction to return the system to an ideal allostatic range or homeostasis. The concept of homeostasis can be defined as the state of steady internal conditions that are most favorable to the biological processes of the organism and maintained by living systems, while allostasis is the energy-bound effort of the organism to reach this optimum range

(Wendelaar Bonga, 1997; Iwama et al., 1998; Ramsay and Woods, 2014). These physiological and molecular mechanisms involved in the stress response are highly conserved throughout the vertebrate sub-phylum and are key to the survival of the organism in a changing environment (Winberg and Nilsson, 1993).

The stress response is categorized in three levels (Fig. 2). The primary response is initiated in the brain and controlled by the hypothalamus and the surrounding regions of the forebrain (Winberg and Nilsson, 1993; Panula et al., 2010; Mueller, 2012). In this initial phase and during the stress response, brain monoamines play a major role as neurotransmitters and modulate the activity of the brain areas involved (Winberg and Nilsson, 1993; Kaslin and Panula, 2001). Upon initiation of the response, the hypothalamus activates the brain-sympathetic-chromaffin axis (BSC) (Wendelaar Bonga, 1997; Barton, 2002). Sympathetic fibers transfer the stress signals from the hypothalamus through the spinal cord and the sympathetic ganglia, these innervate the head kidney (HK) and form synapses with chromaffin cells. Synapse activity will trigger the release of the catecholamines adrenaline (A) and noradrenaline (NA) from the chromaffin cells into the blood stream (Reid et al., 1998). Following the activation of the BSC, the hypothalamus also initiates the activation of the hypothalamic-pituitary-interrenal (HPI) axis. CRH-releasing neurons project from the hypothalamus to innervate the pituitary gland and stimulate the release of ACTH in the circulation. This hormone will, in turn, induce the interrenal cells in the head kidney to release cortisol in the circulation (Wendelaar Bonga, 1997).

The secondary response (minutes to hours) starts once the stress hormones reach their target organs. This will induce changes in physiological parameters like blood pleasure and heart rate, as well as the increase of glucose and lactate concentration in plasma to prompt the availability of energy resources where needed. At this point and if the challenge is successfully overcome, this would be the end of the acute stress response also named “fight or flight” response. The tertiary response (days to weeks) is defined when the stress axes are continuously activated, due to constant or unavoidable stressors, and the system cannot return to homeostasis, the mechanisms conforming this response become dysregulated also impairing the functioning of the other systems under its control.

Energy resources and availability are limited. Therefore, the re-allocation of these resources to the systems needed for the stress response, will have trade-offs with other functions of the organism, which will subsequently affect growth, disease resistance, reproduction, behavior and swimming capacity in the long-term (Wendelaar Bonga, 1997; Barton, 2002; Segner et al., 2012).

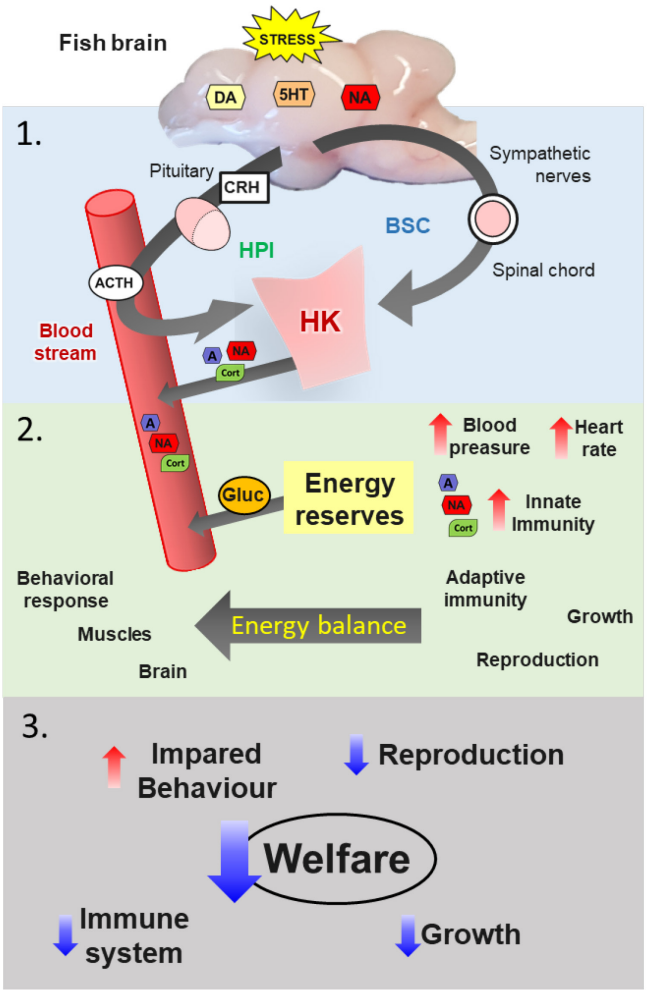

Figure 2. Summarizing view of the three levels of the stress response in fish. 1. Primary response: Stress axes activation resulting in stress hormone release from the HK. 2. Secondary response: The stress hormones induce the re-organization of energy resources. 3. Tertiary response: Systemic long-term effects of stressors.

1.3. Effects of the stress response on biological systems

It has been demonstrated that standard aquaculture practices can result in increased susceptibility of fish to disease (Mazur and Iwama, 1993; Conte, 2004; Dror et al., 2006). However, stressors can have a dual role in immune modulation. Acute stressors that do not affect homeostasis in the long term can positively stimulate the immune response in some cases. For instance, cells and molecules participating in the innate and adaptive immunity (T-cells) can be activated after acute stress (Dhabhar, 2002; Wojtaszek et al., 2002). This modulation of the immune response is initially driven by the different hormones of the HPI axis, mainly cortisol, as well as by catecholamines released in the HK (Weyts et al., 1999). Instead, chronic stressors generally have a suppressive role on the immune system. This is mainly driven by the continuous elevation of circulating cortisol which inhibits the release of pro-inflammatory cytokines while inducing the release of anti-inflammatory agents (Weyts et al., 1999). In addition, stress can reduce the amount of leukocytes in blood (Dhabhar, 2002; Tort, 2011) and

particularly affect B-cell populations, inducing apoptosis and reducing the production of antibodies (Weyts et al., 1998; Varsamos et al., 2006).

In homeostasis, the energy reserves of the fish will be allocated to growth and maturation. In this sense, the stress response and the regulatory actions of cortisol reduce the energy available for growth at different levels (Jentoft et al., 2005). First, stressors can impair feeding behaviors reducing feed-intake (Leal et al., 2011). Increased plasma cortisol regulates the endocrine control of appetite, inducing the release of leptin and inhibiting the release of ghrelin (Janzen et al., 2012; Madison et al., 2015). Second, monoamines can negatively affect the release of growth hormone (Gahete et al., 2009). Third, nutrient absorption in the gut will also be impaired due to activity of the HPI axis, reducing the available energetic resources (Barton et al., 1987).

The stress response can affect the pituitary-hypothalamic-gonadal (HPG) axis at different levels, acting directly or indirectly to the reproductive structures and decreasing the reproductive performance of the animals. Increased cortisol levels in plasma observed after acute handling or crowding were accompanied by a reduction of sexual hormones in plasma of salmonid fish (Campbell et al., 1994; Kubokawa et al., 1999). Stress can impair ovulation times, also affecting egg viability and size (Mileva et al., 2011). Moreover, exposure of adults to stressors can affect the survival of the progeny after hatching (Campbell et al., 1992; Mileva et al., 2011).

Fish behavior is defined by the constant activity of the animals in order to adapt to their external environment and internal cues, in a continuous pursuit of optimizing various parameters simultaneously: temperature, oxygen, pH, salinity, feeding, disease or danger. Accomplishing these demands will vary inter- and intra-specifically also taking into account the life stage of the animal, sexual maturity and the variability of its natural habitat (Barton, 2002; Pankhurst, 2011; Cockrem, 2013) The effect of stressors on behavior will change depending on which kind of challenge the animal is facing, how long it lasts, and how far it is from the optimal range. In addition, previous experiences with the same stressor can facilitate the habituation of the animal to that situation and change its behavioral response (Nilsson et al., 2012). The stress axes also participate in the setting of proactive and reactive behaviors in fish. Proactive fish show bold behavior and tend to aggression and dominance in the group, while reactive fish display shy behavior and subordination (Øverli et al., 2004; Huntingford et al., 2010). During the establishment of social hierarchies in rainbow trout, dominant fish had low cortisol levels in plasma, while subordinate individuals showed a continuous activation of the HPI and monoamine systems in the brain as described for chronic stress (Øverli et al., 1999). The activation of the HPI can also affect behavior in other circumstances. In Pacific salmon, elevated plasma cortisol has been linked to success in migration from sea to spawning rivers. Also, high cortisol levels are observed during migration periods (Carruth et al., 2002). Moreover, it has been demonstrated in zebrafish that teleost can adopt behavioral and emotional fever, choosing warmer water gradients when confronted with pathogens or stressors, respectively (Boltaña et al., 2013; Rey et al., 2015).

1.4. Evaluation of markers to monitor fish welfare under aquaculture-related challenges

The meaning of biomarkers from different scientific perspectives (e.g. clinical, toxicological, welfare) have all in common that they are substances or characteristics that indicate an up-coming condition (Mayeux, 2004). At the same time, biomarkers have to be understood as an objective and replicable measurement that reflects the interaction between a biological system and an environmental agent of chemical, physical or biological origin (World Health Orgnization, 1993). An excessive amount of stressors in farming conditions can impair the immune system of the animals, facilitating the appearance of diseases (Mazur and Iwama, 1993). In order to

understand and evaluate the impact and severity of these challenges, traditional and potential biomarkers are constantly validated and developed in the pursuit of a reliable source of information to the actual biological status and well-being of the fish. These markers will indicate the health status including the level of distress, factors that will ultimately have an impact on aquaculture production (Sadoul and Geffroy, 2019). In this sense, markers should be designed in a species-specific manner as the coping capacities and resilience greatly vary among teleosts (Castanheira et al., 2017; van Treeck et al., 2020). This is of special importance in species recently introduced to intensive aquaculture or with not yet developed breeding programs to obtain aquaculture-adapted animals (Mylonas and Robles, 2019). In order to obtain a reliable diagnostic and avoid false-negatives, a broad panel of markers should be appropriate, as relying on single indicators can overlook underlying challenges (Johansen et al., 2006). These should integrate different levels of the animal biological response, from gene expression to behavioral treats, including established physiological parameters like variations in plasma cortisol and glucose (Silbergeld, 1974; Sumpter et al., 1986; Broom, 2006). Physiological parameters can complemented with non-invasive measurements, such as growth rate, feeding intake or visual inspection for injury and infection (Johansen et al., 2006; Noble et al., 2020).

Gene-expression techniques have been used to search for candidate genes that are sensitive to environmental disturbances, and therefore useful as welfare markers for disease and distress (Gornati et al., 2005; Krasnov et al., 2005; Korytář et al., 2016). In addition, the distribution of gene transcripts that are related to particular biological functions can be used to indicate the functional attributes of cells, tissues and organs. The profile of gene expression in different organs and tissues can facilitate the selection of marker-genes with tissue-specific relevance (Son et al., 2005; Qin et al., 2016).
Different methodologies provide tools to investigate from small groups of genes (RT-qPCR), to whole transcriptomes (RNA-Seq), allowing simultaneous evaluation of potential markers in different tissues and under different challenges. However, gene expression markers should be supported by other physiological markers at the protein level (e.g. plasma hormones), as changes in RNA copies need not necessarily reflect protein availability (Seibel et al., 2021).

1.4.1. The brain and neuroendocrine response to anthropogenic stressors: Monitoring the activity of the brain monoamine system

As the awareness of fish sentience and farm-related stress-load are becoming a welfare concern (Bshary and Brown, 2014), efforts are being put towards breeds that can better cope with farm-related stressors (Øverli et al., 2001; Olesen et al., 2003). In this sense, neuroendocrine–related markers are key to understand and evaluate the level of distress at a given point. These markers can help to elucidate the coping range of the animal to unavoidable challenges and the return to homeostasis (Sadoul and Geffroy, 2019). Changes in plasma cortisol concentration are a standardized indicator of stressful events, as this hormone is the end-product of the HPI axis. Certain limitations appear with the use of cortisol due to its rapid fluctuations and a species-specific response. The range of cortisol response in each specie has to be addressed properly, as the cortisol response changes from one species to another and is also affected by life-stage and gender (Bermejo-Nogales et al., 2014; Castanheira et al., 2017; Martos-Sitcha et al., 2017). In addition, cortisol is involved in other processes independent from stress. For instance, cortisol usually increases after feeding and its concentration underlie a circadian rhythm being elevated at early hours (or start of daily activity) and slowly decreases during the day (Delaney and Klesius, 2004; Cui et al., 2010; Basu et al., 2016). Therefore, the complementation of cortisol

measurements with the analysis of other neuroendocrine markers, together with gene expression or behavior, can only improve the interpretation of the animal's stress status.
Measuring the changes in brain monoamine activity at the onset of the stress response allows to interpret the effects of a particular stressor (Gesto et al., 2013). This includes the observation of the patterns of release and synthesis of the different monoamines, which play different roles in stress coping and behavior (Øverli et al., 1999). In detail, monoamine release will be translated into changes in the neurotransmitter reserves of the pre-synaptic neuron. Neurotransmitters are rapidly released in the synaptic cleft and metabolized to secondary compounds. Both neurotransmitters and metabolites can be measured and quantified (Otten et al., 2010).

The study of the brain response implies a number of difficulties, ranging from the inherent anatomic complexity of the brain, which complicates sampling procedures, to the use of highly specialized equipment to measure neurotransmitter concentrations, and the necessity to euthanize the fish to obtain samples, making impossible to perform repeated measurements on the same individual at different time-points. Overall, the analysis of hormones and neurotransmitters involved in the stress axes helps to identify the stage of the stress response and the intensity of a challenge. This can be complemented with the gene regulation analysis during the stress response, which allows the selection of gene-based markers that facilitate the monitoring of potential stressors.

The brain as the center of systemic regulation is an interesting target to investigate the impact of stressors in cultured fish (Winberg and Nilsson, 1993). This organ and its many interrelated regions and neurotransmitters offer a wide range of potential welfare markers to be investigated and assessed. In particular to this thesis, the activity of monoamine neurotransmitters is closely related to the activation of the limbic system, this is composed of several interconnected brain regions including the hypothalamus and the amygdala (Kaslin and Panula, 2001). The limbic system is involved in the interpretation of external cues and the trigger of the neuroendocrine stress response, which can be followed by a systemic reaction and behavioral adaptation (Feldman et al., 1995; Viltart and Vanbesien-Mailliot, 2007; Lőrincz and Adamantidis, 2017).

Monoamine neurons and their respective neurotransmitters, serotonin (5-HT), dopamine (DA) and NA, modulate the activity of the different brain regions that process stressful cues and initiate the stress response (Winberg and Nilsson, 1993; Kaslin and Panula, 2001). Once released, brain monoamines interact with their correspondent receptors in the target neuron and regulate gene expression and the electrical signaling through the axon. Depending on the type of receptor, which can be activatory or inhibitory of monoamine activity, the neurotransmitter-receptor binding will decrease or enhance the signaling of the neuron (Flügge, 1999; Nichols and Nichols, 2008; Mishra et al., 2018). In mammals, seven types of serotonin receptors (5-Htr) with a total of 14 subtypes have been described. While 5-Htr1 and 5-Htr5 are inhibitory, 5-Htr2; 3; 4; 6; and 7 are activating (Nichols and Nichols, 2008; Fröhlich, 2016). There are five types of dopamine receptors (D1-5) described in mammals and fish (Maximino and Herculano, 2010), with D1 and D5 showing activating and D2; 3; and 4 inhibitory effects on dopamine actions (Mishra et al., 2018). Vertebrates have three types of adrenergic receptors (α1, α2 & β), which amount to 10 subtypes (Flügge, 1999). α1 and β have activating effects while α2 is inhibitory (Flügge, 1999).

The modulation of gene expression induced by monoamine neurotransmitters also affects monoamine receptor genes, involving a feed-back regulation of the monoamine systems. Hormones of the stress axis such as cortisol or ACTH also participate in this feed-back control

(Flügge, 1999; Bücheler et al., 2002). In this sense, monoaminergic neurons are self-regulatory (Bücheler et al., 2002), and control the number of synapses and receptors, including growth and apoptosis of other monoaminergic neurons (Flügge, 1999). This influences the neuronal plasticity mechanisms that will shape the formation of brain networks. Therefore, conforming the particular behavioral responses that individuals exert upon incoming challenges (Flügge, 1999; van den Heuvel, 2014).

The role of brain monoamine neurons and their respective neurotransmitters was firstly studied in mammals and has also been described in fish. In particular, 5-HT induced different activity patterns in proactive and reactive fish (Winberg et al., 1992; Øverli et al., 1999, 2001) and affected the expression of 5-HT receptors after a stressor was applied (Moltesen et al., 2016). Increases in DA activity in the forebrain have been linked to avoidance behavior (Höglund et al., 2005) and reward (Teles et al., 2013). In vertebrates, NA has a major role during wakefulness and arousal (Singh et al., 2015) and shows an increased turn-over in fish subjected to stressful stimuli (Øverli et al., 2001). Handling has been established as a common stressor in aquaculture as it has been shown to activate the stress response in several teleost species. After handling, increases in plasma cortisol levels have been reported (Barton et al., 1987; Barcellos et al., 2011), supported by altered gene expression in the brain (Krasnov et al., 2005) and changes in the brain monoaminergic activity (Gesto et al., 2013).

Due to this close relation between monoamines, stress hormones and monoamine receptors, this part of the thesis (Publication I) had a particular focus on the regulation of monoamine receptor genes during a stressful challenge, as the extended repertoire of monoamine receptors in the brain offered a wide range of promising stress markers. Most of the research on the brain monoaminergic response in fish has been performed on species that have been extensively reared and adapted to aquaculture (Øverli et al., 2001; Moltesen et al., 2016; Höglund et al., 2020). In this sense, a newly introduced aquaculture species like *C. maraena* proved its sensitivity to common aquaculture stressors like disease, crowding and temperature (Altmann et al., 2016; Korytář et al., 2016; Rebl et al., 2018), indicating that this species is a promising candidate to study the stress-related neuroendocrine response. However, a knowledge gap emerges regarding the monoamine system of *C. maraena* as this is, to our knowledge, the first study on the brain neurochemistry and monoamine related gene expression in *C. maraena.*

1.4.2. Effects of stress hormones on the early immune response: Markers of the neuro-immune modulation of HK leukocytes

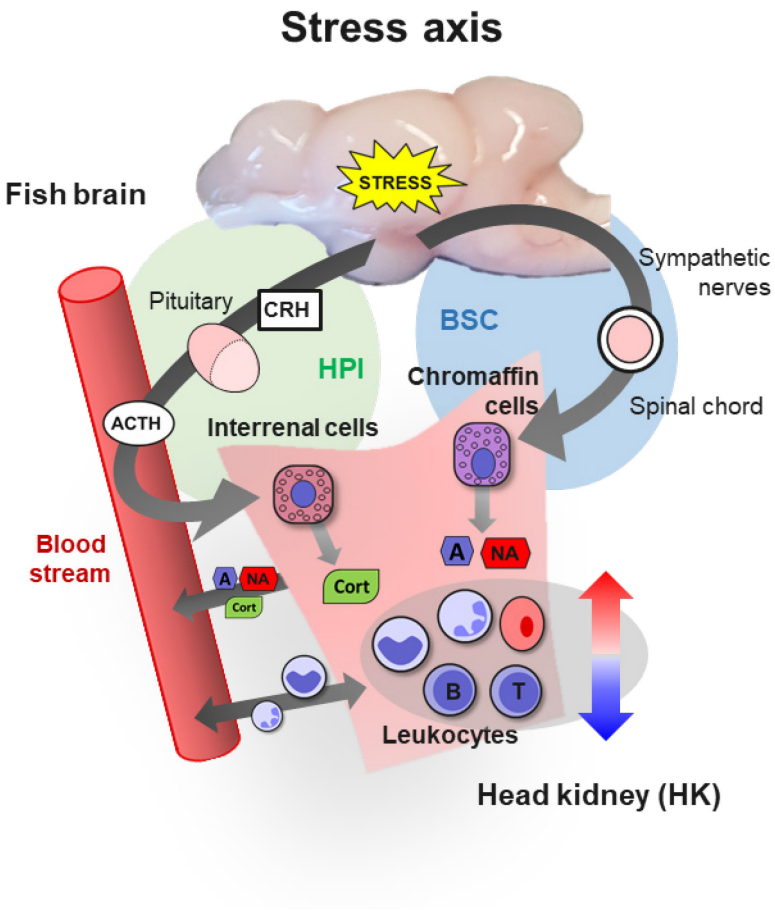

Figure 3. Summary of the stress-induced neuro-immune interaction in the HK. The initial activation of the stress axes in the brain lead to the release of stress hormones in the HK. HPI axis hormones and BSC nerves interact in the HK with interrenal and chromaffin cells, respectively. The release of cortisol and catecholamines will modulate the immune response, boosting innate immunity and reducing antibody production.

The use of *in vitro* methods in teleost research is highly valuable, as it reduces the welfare impact and ethical concerns of live experiments. Primary cell culture techniques are becoming a powerful tool, considering the large variety of species used in aquaculture and the lack of established cell lines. The combination of gene-expression analysis and primary-cell *in vitro* methods allow the researcher to target highly specific challenges in a short period of time. The aim is to obtain markers that are sensitive to a particular stimulus or cue, mimicking the cellular reaction naturally occurring in the organism. However, the obtained markers have to be assessed *in vivo* for life animal monitoring (Fierro-Castro et al., 2013; Seibel et al., 2021). The sensitivity of immune cells to pathogenic cues and stressors make them a promising choice for primary culture and allow to identify the cellular pathways that are most affected in each case.

The HK tissue is the primary source of leukocytes for primary culture in fish as it fosters important hematopoietic populations. In this organ, immune cells from the innate and adaptive response mature from early hematopoietic undefined stages to fully functional mature

leukocytes. In particular, the HK is the origin and maturing site for B cells together with several myeloid cell lineages, mainly monocytes, neutrophils and erythrocytes. In addition, the HK is the end-point of the primary stress response, here the above mentioned hematopoietic populations are mixed with interrenal and chromaffin cells, which are in charge of secreting cortisol and catecholamines, respectively (Gazola et al., 1995). In this sense, the head kidney becomes a hub for neuro-immune interactions in teleost fish (Fig. 3).

The interaction of stress hormones and immune cells has different effects in the innate and adaptive immune systems also varying under acute or chronic stress conditions (Dhabhar, 2002; Wojtaszek et al., 2002). In general, acute stressors modulate the immune response by enhancing the activity of innate immunity also increasing the traffic of neutrophils in the circulation and the migration of circulating macrophages into tissues. In contrast, antibody-producing lymphocytes migrate from the circulation to lymphoid tissues and their antibody production is reduced by stress (Weyts et al., 1999; Marino and Cosentino, 2013). Acute stressors can induce the activation of the acute phase response in the absence of pathogenic cues, stimulating the release of pro-inflammatory cytokines (Sunyer and Tort, 1995; Demers and Bayne, 1997). The constant release of stress hormones over time, as induced by chronic stress, has a general suppressive effect on the immune functions also related to desensitization processes due to a continuous exposure to stress hormones (Yada, 2007; Stolte et al., 2008a, 2008b; Teles et al., 2013).

In fish, three types of cortisol receptors have been described Gr1, Gr2 and MR (Greenwood et al., 2003). The receptor-ligand complex will actively enhance or inhibit the expression of particular genes and modulate the activity of immune cells (Mommsen et al., 1999; Aluru and Vijayan, 2009). In general, cortisol will have suppressive effects on the immune system, decreasing the synthesis and release of pro-inflammatory cytokines, leukocyte proliferation and antibody production (Wendelaar Bonga, 1997; Weyts et al., 1999). However, some parts of the innate immunity can be enhanced by the release of cortisol, facilitating the availability of acute phase proteins, macrophage activity and leukocyte traffic (White and Fletcher, 1985; Flory, 1989; Weyts et al., 1999).

Catecholamines interact with the immune system through the different adrenergic receptors present in immune cells (Fabbri and Moon, 2016). In particular, the β2 receptor type is the most commonly present among leukocytes and has shown inhibitory functions (Sanders and Kavelaars, 2007). In immune cells, the activation of adrenergic receptors by adrenaline or NA can negatively affect phagocytic activity and reduce the pro-inflammatory response. In B cells, the effects of catecholamines in antibody production depend on which adrenergic receptor is activated, therefore with inhibition or enhancing effects (Flory, 1990; Flory and Bayne, 1991). Furthermore, catecholamines can provoke increased pro-inflammatory activity of monocytes when β receptors are activated (Grisanti et al., 2010).

The initial immune response is mediated by the detection of pathogenic cues via specialized receptors in the cells known as pattern-recognition receptors or PRRs. These receptors recognize particular motifs in pathogen-borne structures or pathogen-associated molecular patterns (PAMPs). Among the different types of PRRs, toll-like receptors (TLR) are transmembrane receptors with a particular leucine-rich extracellular domain (Kawasaki and Kawai, 2014). TLRs can be found mainly in circulating macrophages and dendritic cells, which act as whistleblowers of early pathogen infection (Altmann et al., 2016). These initiate the acute phase response (Fig. 4) by enhancing the expression and release of acute phase cytokines (e.g. Il1β, Il6, Tnfα or Cxcl8). At the same time, anti-inflammatory cytokines, like Tgfβ, will be also

released to modulate the immune response (Geven and Klaren, 2017; Rebl and Goldammer, 2018). Subsequently, pro-inflammatory cytokines will induce the liver to release acute phase proteins (e.g. serum amyloid α, C-reactive protein) in the circulation (Bayne and Gerwick, 2001). These molecules participate in antimicrobial activities and tissue repair also enhancing the activity of the complement system (Mold et al., 1999). As mentioned in the previous chapter, the use of gene-expression techniques can cover a wide spectrum of the interrelated pathways involved in the acute phase response. This includes the processes of neuro-immune modulation carried out by hormones of the stress axes. The final result will be a selection of markers for the single and combined *in vitro* challenges.

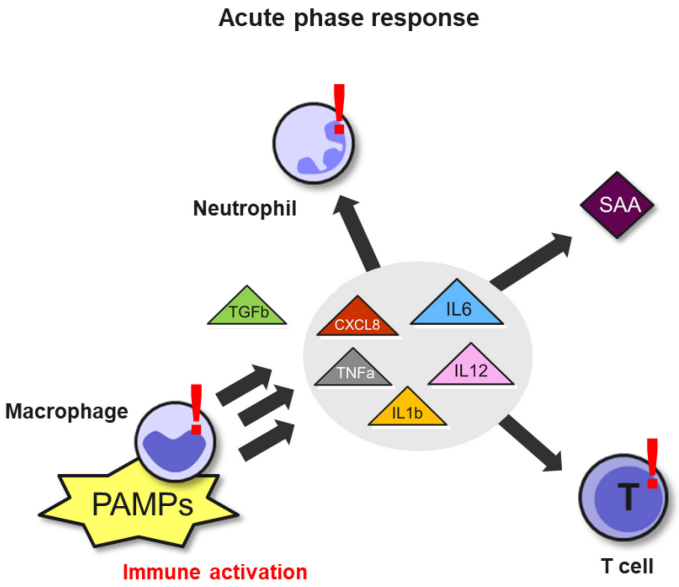

Figure 4. Schematic representation of the acute-phase response. Upon interaction with pathogenic cues (e.g. PAMPs; pathogen associated molecular patterns), macrophages release pro-inflammatory cytokines (CXCL8: Interleukin 8; IL6: Interleukin 6; IL12: Interleukin 12; IL1b: Interleukin 1β; TNFa: Tumor necrosis factor α) to facilitate the activation and recruitment of neutrophils and T cells, which subsequently mount the cellular immune response. The release of cytokines also induces the release of acute-phase proteins (SAA: Serum amyloid α) and anti-inflammatory factors (TGFb: Transforming growth factor β). Adapted from Bayne and Gerwick (2001); Magnadóttir (2006).

1.4.3. Siglecs as regulators of the immune cell response and the effects of stress on siglec signaling

Siglecs are lectin transmembrane proteins with an extracellular immunoglobulin-like domain that recognizes sialic acids present on the cell surface, participating on the cell-cell interaction machinery (Varki, 2011, 2017a, 2017b). The effects of stressors on the immune system and the immune response have been extensively studied (Weyts et al., 1999). However, there is a lack of research regarding stress-response and siglecs. Stressors may have an impact on siglec function as they are present in most types of immune cells and play an important role regulating the

innate and adaptive immune response. Previous transcriptomic studies showed that temperature and crowding stress affected the expression of *MAG* (Siglec 4) in different organs of *C. maraena* (Korytář et al., 2016; Rebl et al., 2018). In addition, the possible interaction between siglecs and stress-hormone receptors was pointed in recent works. It was described that bacterial sialic acids form interactions with adrenergic receptor β2 which is present in most immune cells, also hypothesizing that Sialoadhesin on macrophages might interact with the β2 from endothelial cells, initiating a signaling cascade (Virion et al., 2019).

The most conserved siglecs have been described in teleosts (Sialoadhesin, Mag, Cd22, Siglec 15) including a gene coding for Cd33 (Jeswin et al., 2018). However, there is no extensive research about teleost siglecs function, including tissue distribution or cell-specific expression. It can be assumed that the most evolutionary conserved siglecs also conserve their function in fish, as seen in the preserved function of various siglec orthologues throughout vertebrates. However, some functional variability has been described for teleost siglecs compared to mammalian siglecs (e.g. Mag) (Crocker and Varki, 2001; Bornhöfft et al., 2018).

Most siglecs play an inhibitory role on the immune activatory cascade via the ITIM motifs in their intracellular domain (Jandus et al., 2011). These ITIM motifs interact with neighbor activating receptors carrying ITAM domains and inhibit cellular activation (Ravetch and Lanier, 2000; Angata et al., 2007). In particular to the Siglecs studied in this thesis, Cd22 (Siglec 2) has been mainly described in B cells acting as a modulator of B-cell receptor activity and inhibiting antibody production. Down-regulation of B-cell receptor signaling is done through several ITIM motifs present in the intracellular domain of Cd22 (Crocker, 2005; Nitschke, 2005; Tedder et al., 2005; Varki and Angata, 2006). Sialoadhesin (Siglec 1) is specific for macrophages and regulates cellular adhesion processes, crucial for the endocytosis of pathogens and cell-cell interactions (Crocker et al., 2007; Chang and Nizet, 2014). As opposite to inhibitory siglecs, Siglec 15 interacts with Dap12 proteins to activate their ITAM domain. This facilitates the immune activatory pathways involving Zap70 (Angata et al., 2007). Siglec 15 is also involved in bone formation (Macauley et al., 2014). Mag (Siglec 4) is mostly characteristic of the nervous tissue of vertebrates and particularly of myelination-related cells, like oligodendrocytes and Schwann cells (Quarles, 2007). Mag has stabilizing functions in the interaction between axons and myelin (Sun et al., 2004). However, in fish it appears to have ITIM intracellular motifs, suggesting that fish Mag might have a role modulating signal transduction (Lehmann et al., 2004).

1.4.4. Functional markers to identify welfare concerns: The somatosensory function in the adipose fin

The process of adipose fin resection implies the potential loss of sensory functions and arises concerns on the welfare impact of this tagging method. The use of fin clipping has been demonstrated to reduce the swimming performance of rainbow trout juveniles when confronting water streams (Reimchen and Temple, 2004), as well as activating the stress response with elevated plasma cortisol (Gamperl et al., 1994). The presence of the adipose fin in particular fish lineages, has been related to the constant swimming conditions characteristic of turbulent waters (Temple and Reimchen, 2008). Mechanosensation in regular fins has been described throughout the vertebrate sub-phylum, becoming a key feature for the animal proprioception (Lowenstein, 1956; Bardach and Case, 1965; Williams IV et al., 2013). In detail, the action of water flow is translated to passive deformation of the adipose fin (Stewart and Hale, 2013). This structural change affects the collagen fiber network inside the fin and triggers a mechanosensory response by the adjacent nervous cells (Kimani, 1995). This mechanism has

been described in the adipose fin of the catfish *C. aeneus* (Reimchen and Temple, 2004; Buckland-Nicks, 2016). In addition, projections of nerves have been identified in the fin epidermis, these have been related to the sense of touch and water flow detection (Reimchen and Temple, 2004). In this sense, the profile of expression for cell-specific genes can be used in the adipose fin as markers to classify the cellular groups involved in sensory functions and clarify the mechanosensory mechanisms of this appendix.

2. Aims and Objectives

C. maraena is particularly sensitive to stressors which occur in aquaculture (Altmann et al., 2016; Korytář et al., 2016; Rebl et al., 2018). This species is a valued commercial fish, indicating the need of extended research to develop markers for the welfare evaluation of *C. maraena*. Here the line of work has been dedicated to the impact of the stress response on the brain monoamine system and the head kidney immune response. Therefore, we aimed at obtaining a range of indicators at different levels of the neuro-immune axes, which can help elucidate the welfare status of *C. maraena* when confronted with aquaculture-related stressors, in particular with handling and immune activation. In addition, the distribution of gene-based markers was used to characterize functional aspects of the monoamine and immune system in the brain and HK, respectively. In the search of potential markers, particular interest was put on the Siglec machinery for cell-cell interactions, as these are key features in the modulation of the cellular immune response. The gene-based characterization of the somatosensory networks in the adipose fin, allowed supporting the previous hypothesis on its functionality and point out the welfare concerns implied in fin clipping, which is performed in most cultured salmonid species.

Study I:
Considering the brain as the control center of the stress response, this study aimed at analyzing the effects of acute and repeated handling on the brain monoaminergic systems of *C. maraena*.

- The analysis of the basal distribution of transcripts and neurotransmitters intended to identify the brain regions with higher monoamine activity and the most relevant monoamine-related genes.
- After acute and repeated handling, this study endeavored to interpret the regulation of specific genes and monoamine activation patterns as indicators of stress response in the *C. maraena* brain. This was supported by measurements of plasma cortisol concentrations.

Study II:
The HK is the end-point of the primary stress response and hematopoietic center. Therefore, primary cultures were established to test the effects of the stress response on early immune activation in *C. maraena*.

- A preliminary *in vivo* approach with *C. maraena* aimed at identifying sensitive tissues and marker genes of early pathogen infection
- Combined methodologies were planned to reveal the most relevant immune populations in the HK of *C. maraena.* Additionally, a gene-marker panel was designed to profile the stress-related receptors in isolated HK-cells.
- Exposure of HK primary cultures to stress hormones and pathogenic stimuli was aimed at identifying gene-based markers of acute-phase response and neuro-immune modulation.

Study III:
As known immune regulators in mammals, siglecs were characterized in different teleost tissues to be assessed as markers for acute handling.

- In order to clarify the evolutionary conservation of siglec functions, the characterization of siglec structures was planned for three different teleost species, together with the expression profile of siglecs in different tissues.
- Analysis of tissue-specific expression of Siglecs after acute stress was expected to elucidate the potential of Siglecs and Siglec-related genes as stress markers.

Study IV:

Additionally, the use of gene-based markers was planned to profile the neuronal groups involved in the sensory functions of the salmonid adipose fin.

- A large panel of markers for cell-specific genes was developed to be analyzed in the adipose fin of *O. mykiss* and *C. maraena* with the aim to identify the different groups of neurons and neuron-related cells involved in mechanosensation and nervous innervation.

3. General Discussion

3.1. Characterization of functional aspects through marker distribution in unchallenged animals

The markers listed in the following table are intended to identify particular characteristics and functions in organs and tissues, based on the findings and referenced works of the four peer-reviewed publications included in this thesis. Monoamine-related gene expression was analyzed in different sections of *C. maraena* brain. Monoamine-related markers were selected when high expression was observed in comparison to other markers of the same group (e.g. 5-HT receptor genes) or comparing the expression of the three brain sections studied: FB, MB and HB (See Section 5.1 and publication I). The expression of cell-specific genes and stress-axis receptors was analyzed in isolated HK cells from *C. maraena*. Markers were selected when high expression was observed in comparison to other markers of the same group (e.g. ADR genes) or comparing the expression of the myeloid and lymphocytic fraction of the HK (See Section 5.2 and publication II). The expression of SIGLEC genes was analyzed in immune-relevant organs, brain sections and different cell fractions of the HK. Here are listed the organs with higher siglec-specific expression (See Section 5.3 and publication III). Mechanosensory-related gene expression was analyzed in different fins and organs of *O. mykiss* and *C. maraena*. The selected genes were highly expressed in the adipose fin compared to other organs (See Section 5.4 and publication IV).

Table 1. List of the most relevant gene-based markers measured in unchallenged animals to identify particular characteristics and functions in organs and tissues.

Selected gene	Highly expressed in	Marker for
Brain		
ADR genes	FB	Target region for NA
HTR genes	FB	Target region for 5-HT
ADRA2C	Whole brain	Noradrenaline signaling target/regulator
HTR1A	Whole brain	5-HT signaling target/regulator
DRD1	Whole brain	Dopamine signaling target/regulator
HTR4	FB	5-HT signaling target/regulator
HTR6	FB	5-HT signaling target/regulator
GR2	Whole brain	Cortisol signaling target/regulator
MR	Whole brain	Cortisol signaling target/regulator
HK cells		
ADRA2D	HK cells	Catecholamine signaling target
ADRA2A-2B; ADRB2-3	HK cells	Catecholamine signaling target

NR3C1A	HK cells; myeloid fraction	Cortisol signaling target
NR3C2	HK cells; lymphocytic fraction	Cortisol signaling target
MPO	HK cells; myeloid fraction	Microbicidal activity, myeloid cells
CSF3R	HK cells; myeloid fraction	Cell differentiation, myeloid cells
IGHM	HK cells; lymphocytic fraction	Antigen binding, B cells
TCR	HK cells; lymphocytic fraction	Cell-cell interaction, T cells
Siglec distribution		
SIGLEC1	Spleen	Macrophages, cell adhesion processes
CD22	Head kidney	B-cells/leukocytes, B-cell regulation.
MAG	HB, Head kidney cells	Schwann cells, myelinated nerve structures. Immune regulation in fish.
SIGLEC15	Gills, Head kidney cells	Immune-activatory capacities
Mechanosensation		
NEFL and PRPH	All fins	Small axons; nociception and mechanoreception
NGFR	Adipose fin, dorsal fin	Innervation, nerve structures
GFAP	Adipose fin, dorsal fin	Glia cells
TRPC1	Adipose fin, brain	mechanoreception
KCNK2	Adipose fin, brain	mechanoreception
ASIC2	Adipose fin, brain	mechanoreception
PIEZO2	All fins	mechanoreception

3.1.1. Basal neurochemistry and monoamine-related gene expression identify the telencephalon and hypothalamus as the predominant monoaminergic-activity areas in C. maraena

Unchallenged animals were analyzed to obtain the basal distribution of monoamine neurotransmitters and monoamine-related genes in the brain. This was aimed to identify the most relevant regions regarding monoamine activity and storage, including the basal levels of monoamines and metabolites in each brain section and reveal particular genes with a characteristic distribution or transcript abundance.

The analysis of neurotransmitter and metabolite concentration in the different brain sections indicated that the telencephalon and the hypothalamus including the optic tectum were the main sites of monoamine storage and synthesis. These findings correlated with the stronger expression of different monoamine receptor families in these areas. Therefore, the forebrain of maraena stands out as an important target for monoamine signaling of the adrenergic and serotonergic systems. Supporting these results in *C. maraena*, previous studies have described similar neurotransmitter distribution in the brain of other teleosts, indicating higher

concentration of monoamines in the forebrain and midbrain of *Labroides dimidiatus* and *Naso elegans* (Abreu et al., 2018), rainbow trout *O. mykiss* (Øverli et al., 2001) and Arctic charr *S. alpinus* (Backström and Winberg, 2017). Particular of the telencephalon was the high expression of *ADR* and *HTR* genes when compared with the other brain sections. In particular, *HTR4* and *HTR6* were specifically expressed in the telencephalon (Table 1). Together with the higher concentration of 5-HT and its metabolites in the forebrain areas, this underlines the importance of the serotonergic system in the telencephalon with more than 50% of the *HTR* genes analyzed being significantly higher expressed in the telencephalon than in the other sections of the brain. Similar distribution is reported in humans where most *HTR* genes are expressed in the amygdala and hippocampus which are equivalent regions of the fish telencephalon (Winberg and Nilsson, 1993; Panula et al., 2010; Mueller, 2012; Švob Štrac et al., 2016). Overall, this part of the study identified the forebrain and midbrain as the main areas of monoamine storage and synthesis in the brain of *C. maraena*, including higher expression of the adrenergic and serotonergic receptors.

As for the whole brain, *ADRA2C*, *HTR1A* and *DRD1* were the dominant transcripts of their respective receptor families. Interestingly, *ADRA2C* was almost absent in head kidney cells analyzed in publication II. This indicates that this receptor subtype has specificity for the brain. Preliminary unpublished data regarding *ADRA2C* expression in other tissues would support these findings as the highest expression of *ADRA2C* outside the brain tissue is found in the spleen but 80-fold lower compared to the brain and it is absent in other tissues such as head kidney (Figure 5).

In particular to the expression of cortisol receptor genes, we observed how *NR3C1B* (Gr2) and *NR3C2* (MR) were highly present in the brain compared to *NR3C1A* (Gr1). In contrast, *NR3C1A* expression was predominant in the isolated HK cells analyzed in publication II. This could indicate that Gr2 and MR are the main cortisol receptors involved in the feed-back regulation of the HPI axis in the brain, which directly affects the brain monoaminergic response (Medeiros and McDonald, 2013).

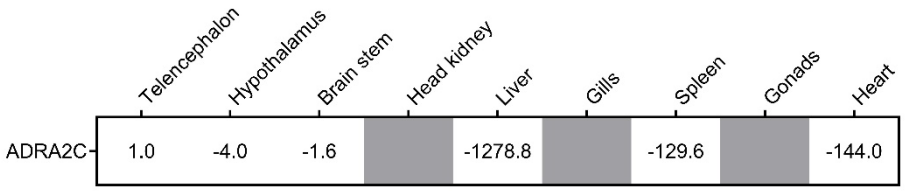

Figure 5. Unpublished data for the average expression of *ADRA2C* in different tissues from two individuals of *C. maraena*. The expression in the different tissues was normalized against the expression in the telencephalon and shown as fold-change. Blank cells in grey indicate non-detectable expression.

3.1.2. Characterization of HK cells as a tool to analyze the neuro-immune crosstalk in a non-model teleost

The characterization of the HK cells through microscopy, size/granularity sorting and cell-specific gene expression indicated that B cells and granulocytes were the most abundant immune sub-types in this organ, followed by monocytes and T cells. Microscopic inspection of HK cells prior to cell-sorting indicated the presence of cells with lymphocytic morphology. The selected cell-specific genes for T and B cells *TCR* and *IGHM*, respectively, were clearly higher expressed in the low-granularity/small-size cluster of the cell-sorting, as expected for lymphocytes, supporting the reliability of the selected markers. For the myeloid lineage, *MPO* and *CSF3R* were specifically highly expressed in the high-granularity/large-size cluster, confirming the specificity of these genes for granulocytic cells. Interestingly, genes with specific expression in monocytes (*LCP1*, *MPEG1*) were differentially expressed in both clusters of the cell sorting, possibly due to the large-size/low-granularity nature of these cells or the presence of different maturing stages of monocytes (Wulff et al., 2006).
The expression of stress-hormone receptor genes was analyzed in the two cell fractions obtained from HK cells after sorting. These results indicated the presence of six out of the eight adrenergic receptors targeted and three cortisol receptors. Overall, the receptor-expression panel was evenly distributed between the lymphocytic and the myeloid fractions with some exceptions. In detail, *GR1* was higher expressed in the myeloid fraction while *MR* was higher in the lymphocytic fraction (Table 1). This indicates the possible gates of cortisol signaling into innate and adaptive immunity, which it is supported by previous findings on the different distribution of cortisol receptors in different types of human leukocytes (Lu et al., 2017). The expression of four *ADRA2* and two *ADRB* adrenergic receptor genes was found in the analyzed HK cells, while *ADRA1* receptor genes were non-detectable. The adrenergic receptor β2 is the most commonly found in human immune cells and the only described for lymphocytes (Sanders and Kavelaars, 2007; Estrada et al., 2016). In our findings, the expression of *ADRB2* was even with most of the other adrenergic receptor genes and not specific for any of the two cell fractions. Interestingly, *ADRA2D* had higher expression than the sum of all the other receptor genes in both analyzed cell fractions indicating the potential role of this α2 variant in the interactions between catecholamines and immune cells. Unfortunately, there is not enough information about *ADRA2D*, as it was lost during evolution for mammals, and it is considered a paralog of *ADRA2A* (Céspedes et al., 2017). Altogether, these findings suggested that the innate and adaptive immune capacities of the head kidney can be observed in HK cells *in vitro*, and pointed the gates of neuro-immune communication in this organ.

3.1.3. Siglec gene-expression is biased towards immune organs and indicates a teleost-specific siglec distribution.

The expression analysis of *SIGLEC* genes in *O. mykiss*, *C. maraena* and *S. lucioperca* indicated that the pattern of distribution of *SIGLEC*s is higher in immune-related organs with the exception of MAG, which was also highly expressed in brain (Table 1). *SIGLEC1* expression was higher in immune-related organs of the studied fish such as spleen, head kidney and gills. Similar observations were made for *SIGLEC15*, which was also highly expressed in spleen of trout and *C. maraena*. This is in line with previous findings reporting *SIGLEC1* and *SIGLEC15* to be highly expressed in mammal macrophages (Crocker et al., 2007; O'Reilly and Paulson, 2009; Macauley et al., 2014). *CD22* was mainly expressed in the head kidney of all three fish studied. The known interaction of Cd22 with B-cell receptor in mammalian B cells (Nitschke, 2005; Tedder et al., 2005) supports our previous findings described in publication II, where *C. maraena* HK harbored

an important population of B cell-like lymphocytes as observed by the high numbers of *IGHM*, microscopic characteristics and light scatter properties. At the same time, *CD22* had high expression levels in erythrocytes and fraction I and II of the cell sorting, indicating that Cd22 in fish might not be restricted to lymphocytes, as is the case in mammals (Chappell et al., 2017; Clark and Giltiay, 2018). *SIGLEC15* and *CD22* were also the most expressed *SIGLEC*s in the different cell fractions of hematopoietic HK cells, which points to their immune-modulatory role in regulating the activation of intracellular ITAM domains of immune cells (Angata et al., 2007; Jandus et al., 2011).

As Mag is involved in the interaction between axons and myelin, it was expected to be highly expressed in brain and nervous tissue (Quarles, 2007). Our findings supported this hypothesis, with *MAG* being highly expressed in the hindbrain section. This part of the brain gathers bundles of myelinated axons from the brainstem joining the spinal cord (Duval et al., 2019). *MAG* was also highly expressed in the gills of the salmonid fish analyzed. The presence of *MAG* in this immune-related organ might be in accordance with the immune-regulatory capacities of piscine Mag. ITIM motifs in *MAG* have been described for zebrafish (Lehmann et al., 2004) and confirmed in the sequence analysis for *C. maraena* and trout in publication III. This ITIM motif has been lost in higher vertebrates (Lehmann et al., 2004).

When comparing the amino acid sequences of CD22 between different species, the results indicated that the most N-glycosylation sites in the human orthologue are conserved in the studied salmonids. At the same time, the presence of an ITIM motif was found in the *O. mykiss* sequence, indicating that the immune function of *CD22* is conserved throughout vertebrates. However, most of the amino acids involved in sialic acid binding in humans where absent in fish *CD22* and the 3D model comparison showed significant structural differences, altogether affecting the *CD22* binding capacities. The analysis of *SIGLEC15* amino acid sequence indicated that the immune-regulatory function of this siglec has been conserved throughout evolution, maybe due to the constant presence of particular pathogens with a defined sialic pattern. The cysteine residues forming the extracellular Ig domain were well-conserved between fish and mammals. At the same time, the key lysine residue for intracellular signal transduction with Dap12 proteins was also well conserved with high vertebrates. In summary, the characterization of salmonid *SIGLEC*s indicate the conserved nature of siglec functions, and the particular immune-modulatory role of teleost siglecs.

3.1.4. The gene expression analysis of the adipose fin indicates nervous innervation and mechanosensory functions

Previous studies based on histological analysis and animal trials have described the mechanosensory capacities of the adipose fin and identified an extensive network of neuronal innervations and the presence of astrocyte-like cells involved in sensory functions (Temple and Reimchen, 2008; Aiello et al., 2016; Buckland-Nicks, 2016). In the same direction, this project (Publication IV) aimed at the development of an extensive panel of genes that could be used as cell-specific functional markers in salmonid adipose fins, and further identify the possible cell types responsible for somatosensation and nervous innervation in this appendix. To this end, the expression patterns in the adipose fin were compared to the expression levels in other organs with known mechanosensory function or neuronal innervation, with the brain as the main reference tissue.

To elucidate the presence of neurons and neuronal innervation, genes whose mRNA is known to be present in the neuronal axons were used as indicators of innervation, together with marker genes for glial cells, astrocytes and Schwann cells based on the assumption that these cell-types are mostly present in tissues when associated to neurons and nerve fibers (Reinisch and Tschachler, 2012). The expression of *PRPH*, *NEFL*, and *NGFR* was particularly high in the adipose and other fins analyzed. In particular, *NGFR* was higher expressed in the adipose fin than in the brain (Table 1). *NGFR* is a known marker for neurons and innervated tissues (Hartmann et al., 2004; Li et al., 2018). In combination with *NEFL* and *PRPH*, the presence of these genes identify neurons with small unmyelinated axons (Lariviere and Julien, 2004; Zeisel et al., 2018). Small unmyelinated neurons are usually involved in nociception and mechanosensory functions, forming free nerve endings close to the epidermis as reception networks for incoming stimuli (Li et al., 2018). In this direction, *GFAP* was used as a marker for astrocytes and was mostly expressed in the adipose fin and the brain. Astrocyte-like cells expressing *GFAP* would interact between nerve endings and the structure of collagen fibers present in the adipose fin, acting as a motion receptor through deformation of the collagen structure (Buckland-Nicks, 2016). This is further supported by the particularly high expression of *TRPC1*, *PIEZO2*, and *KCNK*s in the adipose fin. These are defined markers of C-fiber low threshold mechanoreceptor neurons, which have afferent free-nerve endings and are involved in the sensation of touch (Li et al., 2018). Overall, the implementation of gene-based markers supported the nervous innervation of the adipose fin and the presence of various sensory networks related to pain, movement and touch. In this sense, the impact on fish welfare has to be considered, as the loss of the adipose fin due to marking purposes can diminish swimming performance, in addition to the stress load delivered by the painful procedure of fin resection and the chance of an opportunistic infection.

3.2. Response of the selected markers to aquaculture-related challenges

The following table shows the possible interpretation of the selected immune and stress response markers based on the findings of publications I, II and III and their correspondent discussions. Acute and chronic stress response was studied in *C. maraena* by the means of a single episode of handling or repeated handling for 10 days, respectively (See Section 5.1 and publication I). The concentrations of plasma cortisol and brain neurotransmitters were measured at different time-points for acute (3h, 24h) or repeated handling (10d). Neurotransmitter concentration was measured in three different sections of the brain (FB, MB and HB). Here the time-points and/or brain sections which showed significant changes in plasma cortisol or brain neurotransmitter concentrations are listed with respect to the control individuals. The expression of genes related to monoamines was analyzed using the same brain sections and time-points after handling. Genes significantly up- or down-regulated are summarized in the table. The expression of immune genes was analyzed at different time-points (1h, 3h and 24h) after HK-cells from *C. maraena* were stimulated *in vitro* with stress hormones and PAMPs. In addition, individuals from *C. maraena* were exposed to *A. salmonicida* (See Section 5.2 and publication II). In this case, the expression of immune-related genes was analyzed at different time-points (24h, 48h and 72h) in different organs and tissues. Here the most sensitive genes for the different immune stimulations applied are summarized. Siglec-related gene expression was analyzed in different organs and brain sections of *C. maraena* 3h after a single episode of handling (See Section 5.3 and publication III). The table shows in which tissue the siglec genes were most affected by handling.

Table 2. List of selected neuroendocrine and gene-based markers for *C. maraena* which were particularly sensitive to handling, *A. salmonicida* infection or *in vitro* to stress hormones or/and immune stimulation.

Neuroendocrine markers	**Tissue**	**Challenge**	**Response**	**Indication**
Brain and plasma				
Cortisol	Plasma	Acute handling	Increase concentration 3h post-challenge	Acute stress
5-HIAA	MB	Acute handling	Increase concentration 3h post-challenge	Acute stress
Noradrenaline	MB	Acute handling	Increase concentration 24h post-challenge	Stress recovery
Dopamine	MB	Acute handling	Increase concentration 24h post-challenge	Stress recovery
Serotonin	MB	Acute handling	Increase concentration 24h post-challenge	Stress recovery
Gene-based markers	**Tissue**	**Challenge**	**Response**	**Indication**
Brain				
HTR1A	HB	Acute handling	modulation 3h post-challenge	Acute stress
HTR6	MB	Acute handling	Up-regulation 3h post-challenge	Acute stress
ADRA1D and *ADRB3A*	HB	Acute handling	Down-regulation 3h post-challenge	Acute stress
HTR3C	FB	Acute handling	Down-regulation 24h post-challenge	Stress recovery
TPH1	FB	10d repeated handling	Down-regulation after repeated handling	Habituation
DRD1 and *DRD4*	FB and MB	10d repeated handling	Down-regulation after repeated handling	Habituation
Immune response *in vitro*				
IL1B, *SAA* and *CXCL8*	HK cells	PAMPs	Up-regulation at all time-points	Pro-inflammatory response
IL1B and *CXCL8*	HK cells	Adrenaline	Up-regulation at 1h and 3h	Pro-inflammatory response or/and stress response
SAA	HK cells	Cortisol	Up-regulation at all time-points	Pro-inflammatory response or/and stress response
IL1B and *CXCL8*	HK cells	PAMPs combined with adrenaline	Decreased up-regulation at all time-points	Stress response and dampened pro-inflammatory response
IL1B, *SAA* and *CXCL8*	HK cells	PAMPs combined with cortisol	Decreased up-regulation at all time-points	Stress response and dampened pro-inflammatory response

Immune response *in vivo*				
IL1B, *IL6*, *IL12B* and *TNFA*	HK tissue	*A. salmonicida* i.p. injection	Up-regulation after 48h, Down-regulation after 72h	Pro-inflammatory response
IL1B, *IL12B*	Peritoneal cells	*A. salmonicida* i.p. injection	Up-regulation after 72h	Pro-inflammatory response, cell-migration
CXCL8	Peritoneal cells	*A. salmonicida* i.p. injection	Up-regulation after 72h	Pro-inflammatory response
Siglecs				
CD22	FB and HB	Acute handling	Up-regulation after 3h	Anti-inflammatory response
MAG	Spleen	Acute handling	Down-regulation after 3h	Myelination/ immune regulation
SIGLEC1	MB	Acute handling	Up-regulation after 3h	Presence of macrophage-like cells, cell adhesion processes, endocytosis

3.2.1. A short episode of acute handling mobilizes the monoamine systems in the brain and the serotonergic response in particular.

The initial effects of a short handling episode (1 minute) were observed in the brain of maraena through the different analyzed levels of the serotonergic system, including the activation of the HPI as indicated by the increased cortisol levels 3h after the challenge (Wendelaar Bonga, 1997). Handling induced the release of 5-HT in the hypothalamic areas as indicated by the increased 5-HT metabolite 5-HIAA, this was corresponded with the modulation of adrenergic and serotonergic receptor gene-expression, mainly in the cerebellum and brainstem areas (Table 2). Particularly interesting was the down-regulation of *HTR1A* in the HB section 3h after handling, this gene codes for the 5-HT receptor 5-HT-1A which is actively involved on the modulation of HPI axis in fish and HPA in mammals by inhibiting 5-HT signaling (Fuller, 1992; Barnes and Sharp, 1999; Medeiros et al., 2010). *HTR1A* was the most expressed *HTR* gene in the whole brain of maraena, it can be hypothesized that changes in the number of *HTR1A* transcripts implied a stronger regulatory effort compared to other less expressed *HTR*s. This suggests *HTR1A* as a reliable marker for the initial response to acute handling on the brain serotonergic system.

The monoaminergic activity in the brain changed 24h after the acute handling. Monoamine metabolite concentration in the brain and cortisol in plasma returned to pre-stress levels, indicating the recovery to homeostasis after the initial activation of the stress response (Schreck et al., 2016). At the same time, elevated concentrations of all monoamines analyzed were observed in the midbrain areas. This can be understood as a compensatory action to the initial demand of monoamines during the stress-response to acute handling. *HTR3C* was the most affected gene 24h after handling with down-regulation in the telencephalon. This gene was also down-regulated 3h after the stressor in the hindbrain section but not with statistical significance. This repeatedly observed down-regulation at different time-points could indicate that acute stressors have a relatively long-lasting effect on the expression of *HTR3C*. As this receptor is involved in the activation of 5-HT signaling, this down-regulation could point to a

feed-back dampening of 5-HT activity after the initial 5-HT release triggered by acute handling (Sangkuhl et al., 2009).

Repeated handling for a period of 10 days had no effects on the activation of the HPI axis or monoaminergic activity. As observed by the unchanged plasma cortisol levels and neurotransmitter concentration in the brain. With no signs of a stress-recovery stage as seen 24h after acute handling, the lack of response to repeated handling might be explained as habituation to the stressor. In this sense, the modulation of *TPH1* and *TH* expression suggests the sensitivity of monoamine synthesis pathways to repeated handling and might be an indicator of habituation, although the expression of *TPH1* in FB section is inconsistent with previous findings (Lillesaar et al., 2009; Gaspar and Lillesaar, 2012). Modulation of DA receptor expression (*DRD1* and *DRD4*) in the frontal brain sections might indicate desensitization to DA signaling. Effects on gene expression for DA receptors and synthesis enzymes have been reported in fish after repeated handling in the past (Chabbi and Ganesh, 2015).

In summary, the FB and MB can be identified as promising areas of study regarding monoamine systems in the brain because they enclose a rich repertoire of monoamine receptors with high basal expression, and have a sustained high concentration of monoamine neurotransmitters in unchallenged animals (See section 3.1.1 and publication I). Considering the initial understanding of maraena as a stress-sensitive fish, here it presents as particularly resilient to stressful challenges when confronted with repeated short stressors over time. At the same time, the results of the acute stress experiment suggest that maraena has a stress-recovery period of at least 24h. These results indicate that maraena is sensitive to intense and unexpected challenges but also shows habituation capacities to repeated handling.

3.2.2. Acute-phase genes are promising markers to study neuro-immune interactions at the cellular level

The *in vivo* stimulation of *C. maraena* with *A. salmonicida* i.p. injections resulted in the strong up-regulation of pro-inflammatory genes *IL1B*, *IL6*, *CXCL8*, *TNFA*, *IL12B* in head kidney after 48 h and peritoneal cells after 72 h (Table 2). General down-regulation of the same group of genes was observed in the head kidney 72 h post-injection. These results could indicate the initial activation of immune cells 48h post-injection and the subsequent migration of these activated immune cells from the head kidney to the injection site in the peritoneum at 72 h. This has been reported in previous works in maraena and other salmonids and identifies the head kidney as a center for cell-mediated pro-inflammatory responses against bacterial infection (Jørgensen et al., 1993; Korytář et al., 2013; Brietzke et al., 2015; Altmann et al., 2016).

The response of acute-phase genes against pathogenic cues was further investigated *in vitro* with extracted HK cells from maraena, this time in combination with the major stress hormones of the HK and analyzing the expression of the stress-hormone receptors previously characterized in the immune populations of the HK (See section 3.1.2 and publication II). The exposure to PAMPs triggered the up-regulation of acute-phase genes similar to those previously observed in HK and peritoneal cells during the *in vivo* stimulation with *A. salmonicida*. In this case, *IL1B*, *CXCL8* and *SAA* had a strong initial up-regulation (Table 2). This confirmed the sensitivity of acute-phase response genes, which has been previously reported for various fish species (Jørgensen et al., 2000; Iliev et al., 2005; Seppola et al., 2008; Chettri et al., 2011), and indicates the importance of the head kidney in the initial response to pathogens and reliability of *IL1B*, *CXCL8* and *SAA* as early markers of pathogen-immune encounters.

Cultured cells exposed to cortisol responded with strong up-regulation of *SAA*, which increased from 1h to 24h. *SAA* has been well documented as a sensitive marker to various stressors, from inflammatory processes (Villarroel et al., 2008; Rebl et al., 2009; Kuçceukgul Guleç and Cengizler, 2012) to culture related challenges (Talbot et al., 2009; Korytář et al., 2016; Sveen et al., 2018). The exposure to cortisol also induced the early but weak up-regulation of *IL1B* and *IL6*, this was followed by down-regulation of most cytokines after 24h. When combined with PAMPs, cortisol greatly dampened the PAMP-induced pro-inflammatory response observed for cytokine genes and *SAA*. The results indicate an early pro-inflammatory influence of cortisol that would follow the HPI axis activation. This would be opposed by the increased anti-inflammatory capacities of cortisol when pathogenic cues are present. The exposure to adrenaline had similar effects, with stronger pro-inflammatory activity than cortisol when the hormone was exclusively added to primary cells, but it was less effective than cortisol at counteracting the pro-inflammatory activation when combined with PAMPs. This dual role of adrenaline as pro-inflammatory immune modulator has been reported for other teleosts with varying responses (Castillo et al., 2009; Khansari et al., 2017b, 2017a), indicating that catecholamine-immune interactions might be species-specific. The culture of HK-cells with stress hormones alone had different effects on the analyzed panel of adrenergic receptors. Catecholamines had in general an up-regulatory role of their own receptors starting from 3h of incubation and mainly through NA. The opposite was observed in the effects of Cortisol, which down-regulated most of the adrenergic receptors. Cortisol can act as a feed-back regulator of the monoamine activity including the expression of adrenergic and other monoamine receptors (Nakane et al., 1990; Flügge, 1999; Medeiros and McDonald, 2013). In summary, these results pointed the ambivalent role of stress hormones, when observing the modulatory effects exerted on immune cells with or without pathogenic cues.

Heat shock proteins are closely related to cortisol signaling and cellular stress (Bertorelli et al., 1998) and have also been reported to participate in immune-modulatory activities (Wachstein et al., 2012). Interestingly, the incubation with cortisol did not have major effects on HSP expression. However, the exposure to PAMPs and/or catecholamines had a similar effect on *HSP* genes by up-regulating them 24h after the start of the experiment. This indicates the sensitivity of *HSP* expression to stress and pathogenic cues. The ambivalent response to stress and pathogen cues of *IL1B*, *CXCL8* and *SAA* indicates the dual capacities of immune genes, as has been reported for cytokine transcripts (Irwin and Cole, 2011). This points the sensitivity of these genes as early markers for fish status and the necessity of a careful interpretation of the results.

3.2.3. Acute handling can influence the tissue-specific expression of siglecs and support their role as immune-regulators in fish

The effect of a short episode of acute handling (1 minute) on the expression of *SIGLEC*s was studied in different organs and brain sections of *C. maraena*. *SIGLEC1*, *CD22* and *MAG* were the most affected genes 3h after the handling challenge (Table 2). *SIGLEC1* was up to 3-fold up-regulated in the different brain sections of *C. maraena*. Interestingly, *SIGLEC1* is particularly expressed in macrophages and the expression levels of *SIGLEC1* in the telencephalon are similar to those observed in the HK (See publication III). This could indicate a similar set of macrophage-like cells expressing *SIGLEC1* in the brain. Having in mind that 3h after acute handling the brain stress response is still activated in *C. maraena* (See publication I), the up-regulation of *SIGLEC1* after handling might be related to the modulation of microglia or CNS-resident macrophages (Groh et al., 2016; Siew and Chern, 2018). These findings point *SIGLEC1* as a promising marker for the stress response of macrophage-like cells in the brain.

Handling up-regulated the brain expression of *CD22*. The function of Cd22 has been mostly described as a regulator of B-cell activity (Crocker et al., 2007). Interestingly, in the brain of mice it is produced by neurons to be released as an anti-inflammatory agent to modulate microglia activity (Mott et al., 2004). This could indicate that the increased levels of *CD22* are linked to microglia activation due to the brain stress response at the moment of sampling, making this gene an interesting marker for neurological reactivity to ongoing stress response.

The expression of *MAG* in *C. maraena* was previously reported to be affected by temperature in the microarray study of Rebl *et al* (2018). In detail, *MAG* was 2-fold up-regulated in the spleen after *C. maraena* was exposed to an increase from 18 to 24 °C in water temperature. This is opposed to the findings of this thesis (See publication III), where the exposure to handling down-regulated the expression of *MAG* in the spleen. In higher vertebrates, *MAG* is restricted to cells involved in the myelination of axons and has no immune-regulatory functions (Quarles, 2007). However, the *MAG* orthologue in teleost presents an ITIM motif and therefore the capacity to modulate the activity of the cell (Lehmann et al., 2004). These results support the modulatory effects of stressors on *MAG* gene expression, and identify the spleen as an interesting target organ to evaluate *MAG* immune-regulatory functions.

4. Conclusions and future perspectives

This project was directed towards the characterization and evaluation of a diverse group of markers for salmonid fish, and *C. maraena* in particular. The marker distribution analysis in unchallenged fish aimed to clarify the organization and function of the target tissues. At the same time, established markers and new candidates were assessed in *C. maraena* as indicators of the stress response when confronted to handling and immune stimulation. For the first time, the distribution and activity of the monoamine system was analyzed in the brain of *C. maraena*. Changes in brain monoamines proved to be a suitable complement to plasma cortisol concentrations, facilitating the interpretation of the stage and intensity of the stress response in fish after handling. Adrenergic and serotonergic genes were highlighted as sensitive markers of stress response activation and recovery, while dopaminergic genes were promising indicators of habituation. The use of isolated HK cells resulted in a powerful tool to simulate neuro-immune interactions at the cellular level, and revealed acute-phase genes as sensitive markers of such challenges. Moreover, the techniques and methodologies used in this part of the work for *C. maraena* can be easily adapted to other non-model species that are newcomers to aquaculture. The analysis of the structure and tissue expression of the studied *SIGLEC* genes indicated their conserved status across vertebrates with teleost-specific immune functions and distribution. In addition, *SIGLEC* gene-regulation proved to be sensitive to acute handling in a tissue-specific manner. Finally, the expression profile of cell-specific genes in the salmonid adipose fin, revealed the presence of neurons, neuron-related cells and somatosensory receptors, supporting the flow sensory capacities of this appendix at the gene level. Overall, the present work provides an extended panel of markers to better understand how *C. maraena* responds to a challenging environment.

The research on the effects of handling in the stress response of *C. maraena* is still an ongoing project with promising results. Here, further analyses have been done with a focus on the head kidney response to evaluate the effects of handling on the neuro-immune interaction *in vivo*. Cortisol and plasma parameters have been studied together with an extended transcriptomic analysis of the head kidney tissue at 3h and 24h post stress, and after 10d of repeated handling. The obtained transcriptomic data will be processed with IPA software to identify the regulatory pathways affected by handling. In addition, the phagocytic capacities of head kidney cells will be analyzed three hours after acute handling to discern the effects of and acute stressor to the immune performance of the myeloid population resident in the HK. The results of this study are in preparation for a peer-reviewed publication.

In publication II, the expression analysis of adrenergic receptor genes in isolated HK-cells revealed the unexpectedly high expression of *ADRA2D* compared to *ADRB2*, which is the most described adrenergic receptor in immune cells. There is little knowledge about *ADRA2D*, which is described as a paralog of *ADRA2A*. These results suggested the necessity to characterize this α2 receptor and further investigate its function in fish cells. To this end, the coding sequence of the gene will be sequenced and analyzed for specificities compared to *ADRA2A* and *ADRB2*. At the same time, *ADRA2D* will be cloned and transfected to an existing fish cell-line, which will be exposed to known α2 agonists and the catecholamines adrenaline and NA. The activity of cAMP and gene expression will be used to analyze the signal transduction capacities of the receptor and the response to its ligands.

Further research is planned in order to evaluate the effects of adipose fin rejection from a multiparametric approach. This is supported by the findings exposed in publication IV, together with the previous knowledge on the functionality of the adipose fin as a flow sensor, and the

reports on impaired swimming capacity after adipose fin rejection. High-throughput gene expression methods will be implemented to analyze the forebrain response of *O. mykiss* to fin rejection in a flow-current environment. This will be complemented with measurements of the brain monoamine activity and classic physiological stress markers. In addition, the swimming performance after fin rejection will be analyzed through video monitoring of the animals in a custom-built flow tank.

Different teleost species show a variety of stress responses and tolerance to up-coming challenges. In this respect, plasma cortisol concentration is an extensively used and accepted marker of the HPI activation. Therefore, it would be particularly beneficial to establish the ranges of plasma cortisol concentrations in *C. maraena* during the different stages of the stress response to obtain a maraena-specific range of plasma cortisol which would improve the stress-related studies using this particular specie. In this sense, the setting of a stress response experiment should be planned, involving a simple but effective challenge that clearly activates the stress response. Taking into account the previous experiences with handling stress, it could be estimated that the effects of an acute stressor to *C. maraena* last approximately 24h hours. With this in mind, plasma samples should be taken with 30 minutes intervals from the exposure to the stressor until 24h post-challenge. These samples would be later analyzed to determine the cortisol concentration in plasma, obtaining a detailed picture of the HPI activation after an acute challenge.

A promising field of study that could expand our understanding of *Coregonidae* is the stress and immune response of anadromous and landlocked populations in the Mecklenburg area. One characteristic of *Coregonus* species is the large number of isolated populations across Europe, which evolved in a great variety of species, subspecies and hybrids within the *Coregonus* genus. This indicates the speciation pressures on these fishes and their genetic adaptation to their actual environments. Research in this direction could open the chance to find well-adapted breeds to Mecklenburg culturing conditions from a stressor/resilience perspective. At the same time, it would give insights on how the stress response is affected by the different conditions of salinity, feeding or temperature of landlocked, anadromous and sea environments.

5. Publications

5.1. Publication I: Time-dependent effects of acute handling on the brain monoamine system of the salmonid *Coregonus maraena*

The stress response involves the activation of particular areas of the brain and the release of a variety of effector molecules including monoamine neurotransmitters. This study analyzed the stress response to handling in the brain of the salmonid *Coregonus maraena*, which was selected as a stress-sensitive species in adaptation to intensive aquaculture. Measurements of brain neurochemical activity were combined with plasma cortisol concentration and a panel of 37 genes related to the monoamine system as potential stress markers. In addition, brain neurochemistry and gene expression of undisturbed individuals aimed to identify the most relevant regions regarding monoamine activity and storage, as well as identifying particular genes with a characteristic distribution.

Highlights

- Brain monoamine activity responded to acute handling with increased serotonergic activity 3 h post-challenge, together with a significant increase of plasma cortisol. In addition, the expression of monoaminergic receptor genes was modulated in the hindbrain.

- Monoamine synthesis in the brain increased 24 h after acute handling and was accompanied by the modulation of serotonin-receptor expression. The concentrations of plasma cortisol returned to control levels.

- 10 days of repeated handling down-regulated dopamine receptor genes but had little effects on brain monoamine or plasma cortisol concentrations.

- Neurochemistry and gene expression of unchallenged animals identified the forebrain and midbrain as the main areas of monoamine storage and synthesis, including higher expression of the adrenergic and serotonergic receptors.

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Time-Dependent Effects of Acute Handling on the Brain Monoamine System of the Salmonid *Coregonus maraena*

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The immediate stress response involves the activation of the monoaminergic neurotransmitter systems including serotonin, dopamine and noradrenaline in particular areas of the fish brain. We chose maraena whitefish as a stress-sensitive salmonid species to investigate the influence of acute and chronic handling on the neurochemistry of monoamines in the brain. Plasma cortisol was quantified to assess the activation of the stress axis. In addition, we analyzed the expression of 37 genes related to the monoamine system to identify genes that could be used as markers of neurophysiological stress effects. Brain neurochemistry responded to a single handling (1 min netting and chasing) with increased serotonergic activity 3 h post-challenge. This was accompanied by a modulated expression of monoaminergic receptor genes in the hindbrain and a significant increase of plasma cortisol. The initial response was compensated by an increased monoamine synthesis at 24 h post-challenge, combined with the modulated expression of serotonin-receptor genes and plasma cortisol concentrations returning to control levels. After 10 days of repeated handling (1 min per day), we detected a slightly increased noradrenaline synthesis and a down-regulated expression of dopamine-receptor genes without effect on plasma cortisol levels. In conclusion, the changes in serotonergic neurochemistry and selected gene-expression profiles, together with the initial plasma cortisol variation, indicate an acute response and a subsequent recovery phase with signs of habituation after 10 days of daily exposure to handling. Based on the basal expression patterns of particular genes and their significant regulation upon handling conditions, we suggest a group of genes as potential biomarkers that indicate handling stress on the brain monoamine systems.

Keywords: catecholamines, marker genes, monoamine receptors, salmonids, serotonin, stress

Abbreviations: 5-HIAA, 5-hydroxyindolecacetic acid; 5-HT, 5-hydroxytryptamine; BSC, brain-sympathetic-chromaffin axis; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; FB, forebrain; MB, midbrain; HB, hindbrain; HPA, hypothalamic-pituitary-adrenal axis; HPI, hypothalamic-pituitary-interrenal axis; HPLC, high-pressure liquid chromatography; HVA, homovanillic acid; LC, *locus coeruleus*; NA, noradrenaline; NLV, *nucleus lateralis valvulae*; POA, pre-optic area; STR, *striatum*.

INTRODUCTION

The immediate stress response involves the activation of the brain monoaminergic systems, including serotonin (5-hydroxytryptamine; 5-HT), DA and NA as major neurotransmitters. Depending on the type of stressor and the duration and intensity of its occurrence (Barton, 2002), these monoamines modulate the neuronal responses in particular areas of the brain, principally the telencephalon, hypothalamus and brain stem (Winberg and Nilsson, 1993; Kaslin and Panula, 2001). In essence, monoamines affect behavior, the formation of memory and the activity of the brain regions that initiate the neuroendocrine stress axes (Feldman et al., 1995; Viltart and Vanbesien-Mailliot, 2007; Lõrincz and Adamantidis, 2017) to reprogram metabolism, immunity, growth and reproduction (Silbergeld, 1974; Hemre and Krogdahl, 1996). This complex response and the associated physiological changes aim to cope with environmental and/or anthropogenic challenges to reinstall homeostasis (Schreck et al., 2016). Handling procedures are a common anthropogenic disturbance in aquaculture. They activate the stress response in various fish species reflected by increased plasma cortisol concentrations (Barton et al., 1987; Barcellos et al., 2011), altered gene expression in the brain in a specific time-course (Krasnov et al., 2005) and elevated monoaminergic activity in particular brain regions (Gesto et al., 2013). The initial stress response of fish is translated into a systemic stress response via the hypothalamus. It directs the endocrine reaction by initiating the "fight-or-flight response" through the BSC axis (Feldman et al., 1995). This triggers the release of the catecholamines adrenaline and noradrenaline from the head kidney (Reid et al., 1998). Subsequently, the initiation of the BSC axis is followed by the activation of the HPI axis, which causes the release of cortisol into the circulation (Wendelaar Bonga, 1997; Schreck et al., 2016). Eventually, the HPI and the BSC induce a series of compensatory physiological processes to direct the body's metabolic resources to survival needs (Irwin and Cole, 2013; Aerts, 2018).

Monoaminergic neurons project into wide areas of the brain and in particular to those regions that form the limbic system, which evaluates sensory inputs such as visual or olfactory cues as possible threats (Vuilleumier, 2005) and initiates the central stress response with monoamines as active messengers (Morgane et al., 2005). Important monoaminergic neuron clusters are situated in the fish hypothalamus, a control center of the limbic system (Kaslin and Panula, 2001). In mammals, the limbic system is composed of the hypothalamus, hippocampus, amygdala and thalamus, while it is spread between the telencephalon and the midbrain of teleost fishes (Winberg and Nilsson, 1993; Panula et al., 2010; Mueller, 2012) (**Figure 1**).

Upon synaptic release, monoamines bind to their respective receptors on the target cell. Depending on the type of receptor, this binding can activate or inhibit neuronal functions (Flügge, 1999; Nichols and Nichols, 2008; Mishra et al., 2018). In mammals, several types of receptors for 5-HT (5-HT-1-7), dopamine (D1-5), adrenaline and NA (α1, α2 and β) have been identified (Flügge, 1999; Nichols and Nichols, 2008; Maximino and Herculano, 2010; Fröhlich, 2016; Mishra et al., 2018). However, there is little information on their orthologs in fish. For instance, the adrenergic receptor α2d is not present in mammals, but it is strongly expressed in salmonids (Martorell-Ribera et al., 2020).

In the fish brain, monoamines play an important role in the mechanisms of stress coping. In particular, 5-HT induces different activity patterns in proactive and reactive salmonids (Winberg et al., 1992; Øverli et al., 1999, 2001). In addition, the genes encoding 5-HT receptors (*HTR1Aα* and β) in the telencephalon of rainbow trout *Oncorhynchus mykiss* are down-regulated by stress (Moltesen et al., 2016). Such a downregulation of 5-HT receptors may reduce 5-HT uptake and lead to increased 5-HT-metabolism (Dwarkasing et al., 2016). In line with this, the chasing of rainbow trout increased the concentrations of 5-HIAA (the main 5-HT metabolite) in the telencephalon and hypothalamus (Gesto et al., 2013). DA has also an important role in modulating behavioral responses. For example, increased dopaminergic activity in the telencephalon of fish has been linked to avoidance behavior (Höglund et al., 2005) and reward (Teles et al., 2013). The expression of the dopamine receptor 2 (DRD2) gene was up-regulated in the brain of bold zebrafish *Danio rerio* compared to reactive individuals (Thörnqvist et al., 2019). Furthermore, handling and treatment with anesthetics increased the concentrations of DA metabolites in the telencephalon of Arctic charr *Salvelinus alpinus* (Backström et al., 2017) comparable with the aforementioned 5-HT levels in stressed rainbow trout. Also NA, which contributes to vigilance and arousal (Singh et al., 2015), has been subject to increased turnover in rainbow trout under stress conditions (Øverli et al., 2001). In Arctic charr, agonistic interactions increased NA concentrations in the telencephalon of dominant individuals (Backström et al., 2015). Taken together, the observed increase in monoamine metabolites is an indicator for the stress response in different salmonid species.

Maraena whitefish *Coregonus maraena* (Bloch) is a salmonid fish present in the Baltic region in anadromous and landlocked populations (Kottelat and Freyhof, 2007). In Germany, it has been reared for intensive aquaculture production since 2005 (Jansen et al., 2008). Our previous studies revealed that maraena whitefish is highly sensitive to stressors compared to salmonids that are better adapted to husbandry conditions, such as rainbow trout or Atlantic salmon *Salmo salar* (Altmann et al., 2016; Korytář et al., 2016; Rebl et al., 2018). This study investigates the influence of acute and repeated (chronic) handling stress on the monoaminergic systems in different brain regions of maraena whitefish. We focused our research on stress-related changes in plasma cortisol concentrations, monoamine neurotransmitters and metabolites and the expression profile of monoamine-related genes in the brain. Our specific aims were to elucidate (i) which brain regions were activated during handling; (ii) which monoaminergic systems showed increased sensitivity to this type of stressor; (iii) how handling can affect HPI activation; (iv) whether a single episode of handling was quickly overcome or whether it can have long-lasting effects on the monoamine neurochemistry of maraena whitefish and (v) how

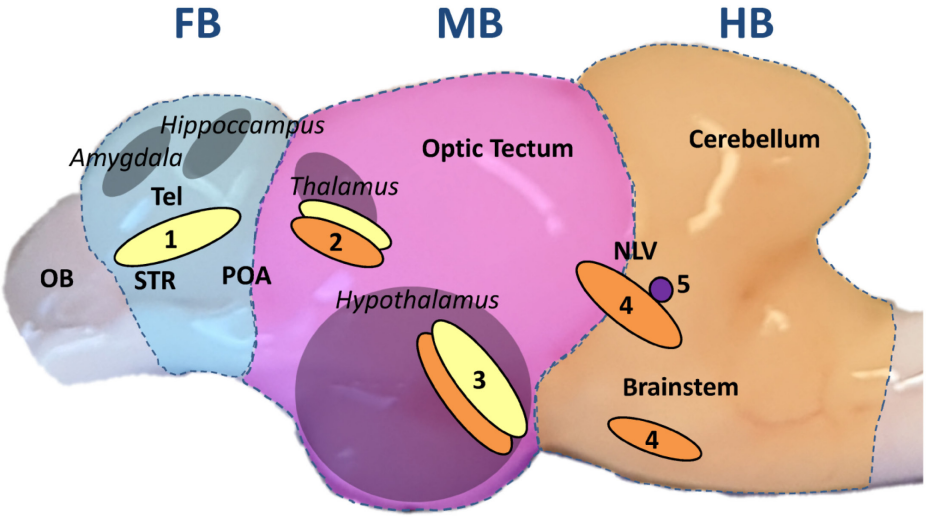


FIGURE 1 | Sagittal view of the whitefish brain. The brain was divided transversally, perpendicular to the rostral-caudal axis, in three parts throughout the experiment: forebrain section (FB, blue underlay); midbrain section (MB, pink); hindbrain section (HB, orange). The FB comprised the telencephalon (dorsal and ventral). The homologous regions to the mammalian amygdala and hippocampus are situated in the dorsal telencephalon while the ventral telencephalon comprises the equivalent to the *striatum* (STR) and dorsally the preoptic area (POA) as described by Mueller (2012); Vindas et al. (2017); Geng and Peterson (2019). The olfactory bulb (OB) was discarded. The MB included every region between optic tectum and diencephalon with the inferior lobes of the hypothalamus from a dorsal-ventral view. The pretectal complex, where the teleost thalamus has been described, is located in the dorsal part of the section (Kaslin and Panula, 2001; Mueller, 2012). The HB was composed of the cerebellum and the brainstem, including the *nucleus lateralis valvulae* (NLV), which was most probably partially divided between MB and HB during dissection (Gaspar and Lillesaar, 2012). From a transversal view, the anterior edge of the cerebellum marked the sectioning line on the brain stem. The different parts of the limbic system are marked in gray and the names in italics. Based on the aforementioned publications on teleost brain organization, monoaminergic neuron populations are marked in yellow for DA neurons; orange for 5-HT neurons and purple for NA neurons: (1) telencephalic complex; (2) pretectal complex; (3) hypothalamic paraventricular nucleus; (4) anterior *raphe nuclei*; (5) *locus coeruleus*.

the monoaminergic system responded over time to repeated episodes of handling. Furthermore, we sought to identify genes that might be suited as animal-based biomarkers for indicating stress in different regions of the brain.

MATERIALS AND METHODS

Husbandry of Maraena Whitefish

The Institute for Fisheries of the State Research Center for Agriculture and Fishery Mecklenburg-Western Pomerania (Born, Germany) and BiMES - Binnenfischerei GmbH (Friedrichsruhe, Germany) provided maraena whitefish for this study. Fish were reared in fresh-water recirculation systems with a stocking density of 10 kg/m^3 at 18°C and a 12:12-h day-night cycle. Water quality was maintained by automated purification and disinfection (bio-filter and UV light). The concentrations of NH_4^+, NO_2^-, NO_3^-, and NH_3 in the water, pH, temperature and oxygen saturation were constantly recorded. Commercial dry pellets (4.5 mm; Biomar, Inicio Plus, Aarhus, Denmark) were distributed by automatic feeders at a daily rate of 0.8–4.0% of the biomass of maraena whitefish in the tank.

Acute and Chronic Handling Experiments

All procedures have been approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany (LALLF M-V/TSD/7221.3-1-069/18). The fish (n = 48 in total) used in the experiments were juvenile with a starting size of about 20 cm. They were allowed to acclimatize to the recirculation system for at least 2 weeks in the reservoir tank (500 l) and were transferred to the experimental tanks 7 days before the experiments started. These tanks were identical dark polyethylene cylinders with a capacity of 150 L. Experiments were performed in the morning (between 8 and 11 a.m.) to minimize the influence of circadian rhythms on the measurements.

Each of the acute handling experiments started with eight rearing tanks with one pair of fish per tank, and four identical secondary tanks without fish (**Figure 2**). The fish were placed in the tanks in pairs to ensure that the sampling and treatment procedures disturbed the other animals that were part of the experiment as little as possible. At the start of the experiment and before the handling procedure, one fish per pair was euthanized. This fish was designated the control fish (0 h). After sampling of the control fish, the handling protocol was applied for 1 min

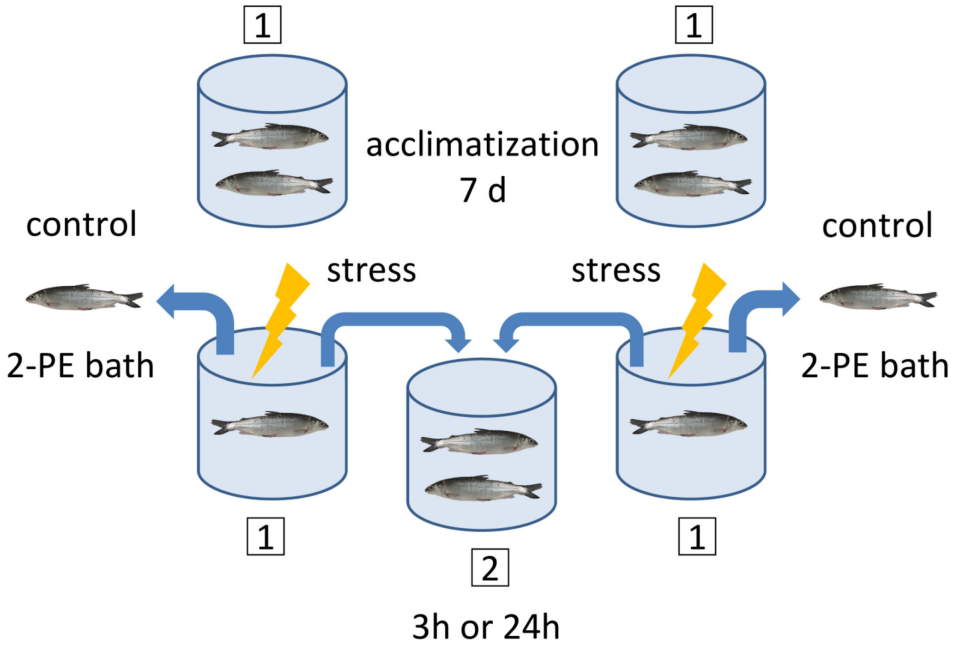


FIGURE 2 | Sampling and handling protocol for the acute experiments. A pair of maraena whitefish was placed in each experimental tank (1) and left undisturbed for acclimatization during 7 days prior to sampling. At day 7, the control fish were sampled for the 3 h or 24 h experiments before stress was applied to the test fish. The timing was as follows: The control fish were caught and placed in a 2-phenoxyethanol (2-PE) bath for anesthetic overdosage, which takes approximately 2–5 min. While the control fish were under anesthesia, the test fish remaining in the tank were subjected to the handling procedure treatment. The test fish were then transferred to the secondary tank (2), where they remained either 3 h or 24 h until sampling. This procedure was applied to two primary tanks simultaneously, where at the end two control fish were in the 2-PE bath and two test fish were transferred to the same secondary tank. The maraena whitefish were always in pairs of two, except for the handling procedure (1 min), during which they were alone in the tank.

to the remaining fish, which was designated as test fish. The handling protocol consisted of hunting and catching fish with nets, intermittently lifting the fish out of the water and finally transferring them to a secondary tank, where test fish pairs were left undisturbed for either 3 h or 24 h. After this period, test fish were euthanized and tissues samples were taken as described below (see section "Sampling of Tissues").

For the chronic handling experiment, control fish (n = 8) and test fish (n = 8) were kept in pairs in the same condition in eight separate tanks for the duration of the experiment. The experiment was divided into two rounds of eight fish each to swap the tanks for the test fish with the tanks for the controls. The test fish were hunted and netted once a day for 1 min for 10 days, while the controls were left undisturbed. The net lifting and tank-transferring steps performed in the acute-handling procedure were skipped in the chronic handling experiment to avoid skin injuries. To minimize habituation, handling was always performed at a random time within the 12 h 'daylight' period. The fish were euthanized 10 days after the onset of the chronic handling procedures, 24 h after the last handling procedure.

Sampling of Tissues

Before tissue sampling, fish were euthanized with an overdose of 2-phenoxyethanol (0.7 ml/l) followed by spine dissection at the skull level. Sampling and killing methods followed the standards described in the German Animal Welfare Act [§4(3) TierSchG]. Immediately after killing, blood was drawn from the caudal vein using a heparinized syringe and centrifuged at 270 × g for 15 min at 6°C to obtain plasma, which was stored at −80°C. Subsequently, the fish brain was quickly and carefully dissected in three parts transversally and perpendicular to the rostral-caudal axis: forebrain section (FB), midbrain section (MB), and hindbrain section (HB). **Figure 1** describes in detail the cutting lines of the dissection and the brain parts used in this study. The FB included the telencephalon (dorsal and ventral), while the olfactory bulb was discarded. From the dorsal-ventral view, the MB included each region between the optic tectum and the inferior lobes of the hypothalamus. The HB was composed of the cerebellum and the brainstem. In a transverse view, the anterior edge of the cerebellum marked the line of incision at the brainstem, thereby excluding the spinal cord. The three

different sections were separately weighed and homogenized in 500 µl ice-cold PBS. The resulting homogenate was divided into two aliquots and snap-frozen in liquid nitrogen for RNA isolation or monoamine analysis (see sections "Quantification of Monoamines and Metabolites" and "RNA Isolation and cDNA Synthesis").

Measurement of Plasma Cortisol

The cortisol concentration in plasma was quantified by a competitive enzyme-linked immunosorbent assay (ELISA; Cusabio Technology, Houston, TX, United States) according to the manufacturer's instructions. The level of absorbance was measured at 450 nm in a Beckman Coulter DTX 800/880 Series Multimode Detector (Beckman Coulter, Brea, CA, United States).

Quantification of Monoamines and Metabolites

Concentrations of NA, DA, 5-HT and their metabolites HVA, DOPAC and 5-HIAA were determined in the three brain regions using HPLC with electrochemical detection. This method has been described previously (Otten et al., 2010) and was used with a slightly adjusted extraction procedure. A 250-µl amount of brain samples homogenized in PBS was mixed on ice with 25 µl of 2 M and 25 µl of 0.2 M perchloric acid using a manual homogenizer. Following centrifugation (2,400 × *g*; 4°C; 10 min), the supernatant was preserved on ice while the pellet was resuspended in 300 µl of 0.2 M perchloric acid and centrifuged. The pooled supernatants were mixed and then centrifuged at 37,000 × *g* for 10 min at 4°C. The remaining pellet was weighed with a precision balance and the values obtained were used to calculate the concentration of neurotransmitters in each brain section. Aliquots of 40 µl were analyzed in duplicate. The HPLC system was equipped with a 125 mm × 4 mm reverse-phase column packed with Prontosil C18 AQ (Bischoff Analysentechnik, Leonberg, Germany). The mobile phase consisted of 58 mM sodium hydrogen phosphate buffer containing 1.2 mM octanesulfonic acid, 0.3 mM EDTA, 0.2 mM potassium chloride, and 9% methanol at pH 3.6, and was used at a flow rate of 1.2 ml/min. Electrochemical detection was achieved by a SenCell with a glassy carbon working electrode set at a potential of 600 mV (Axel Semrau GmbH, Sprockhövel, Germany). The HVA/DA, DOPAC/DA and 5-HIAA/5-HT ratios were calculated as an index of DA and 5-HT turnover.

RNA Isolation and cDNA Synthesis

Total RNA from previously homogenized brain samples was extracted using TRIzol Reagent (Life Technologies). RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to purify the extracted product. The quality of the RNA was analyzed by horizontal electrophoresis on 1%-agarose gels, which validated the presence of intact 18S and 28S rRNA bands for the individual RNA specimens. The RNA concentration was determined with a NanoDrop One^{C} spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). The RNA obtained was reverse-transcribed to single-strand cDNA using the SensiFAST cDNA Synthesis Kit (Bioline, London, United Kingdom). The reverse-transcriptase reaction was set at 42°C for 50 min with a subsequent inactivation step at 70°C for 15 min. Finally, the synthetized cDNA was diluted in 80 µl distilled water.

Real-Time Quantitative PCR (qPCR)

RNA samples from the brains of maraena whitefish exposed to the acute (sampled after 3 h or 24 h) and chronic handling experiments along with the respective controls were analyzed with multiplex qPCR technology (BioMark, Fluidigm, South San Francisco, CA, United States) to study the effects of handling on the transcript level. We designed a primer panel specific for the monoaminergic and neurological stress response. In this matter, orthologous gene sequences from Atlantic salmon *S. salar*, Coho salmon *Oncorhynchus kisutch*, and rainbow trout *O. mykiss* were aligned to the RNA-seq read collection of maraena whitefish *C. maraena* (Brietzke et al., 2016) using the program Bowtie2 (v 2.2.4). The resulting matching alignment with *C. maraena* was sorted and indexed with the Samtools package (v 1.6). Finally, the consensus sequences were visualized and redeemed using the software Ugene (v1.29). These sequences were used to design gene and species-specific primer pairs (see **Supplementary Table 1**) using the PSQ Assay Design Software 1.0.6 (Biotage AB, Uppsala, Sweden) for amplifying products with final lengths ranging from 140 to 180 bp. In addition, qPCR assays were carried out on 48.48-Dynamic Array IFC chips (Fluidigm) with EvaGreen fluorescence dyes (Bio-Rad, Hercules, CA, United States) using the BioMark HD-System (Fluidigm). One microliter of total RNA was reverse-transcribed using the Reverse Transcription Master Mix (Fluidigm). The resulting cDNA was adjusted to 10 ng/5 µl and underwent a subsequent pre-amplification of 11 cycles performed with the PreAmp Master Mix (Fluidigm) and the primers at a final concentration of 100 µM per primer pair. Afterward, preamplified cDNA was treated with exonuclease I (ExoI; New England BioLabs, Ipswich, MA, United States). Then, the cDNA samples were diluted in SsoFast EvaGreen Supermix with Low ROX (Bio-Rad) and the 20 × DNA Binding Dye Sample Loading Reagent. The cDNA samples and primer-pair mixes were transferred to the corresponding sample and assay inlets of the IFC chip using the IFC Controller RX (Fluidigm) and the 'Load Mix 48.48 GE' pre-set script. The IFC chip was transferred to the BioMark HD-System (Fluidigm) to perform the quantification reactions following the 'GE 48 × 48 Fast PCR + Melt v2.pcl' cycling program. The obtained qPCR data were analyzed using the Fluidigm RealTime PCR Analysis Software v.4.5.2. All qPCR products were run on a 2%-agarose gel to assess the integrity and specificity of the PCR products. An external standard was used to calculate the relative gene expression, which was normalized against the geometric mean of the copy numbers of *EEF1A1b*, *RPL9* and *RPL32* (Altmann et al., 2015). The 37 genes analyzed belonged to seven gene groups (**Table 1**). We studied a broad repertoire of genes involved in the synthesis (TH, TPH1, and TPH2) and degradation (MAO) of monoamines, and genes coding for monoamine receptors (ADR, DRD, and HTR) and markers for neuronal activity (FOSL) and neuronal plasticity (BDNF), downstream

TABLE 1 | Target genes.

	Official gene name; product	Function
Adrenergic receptors	ADRA1B; adrenoceptor α1B	Neuron signaling, regulation of transcription
	ADRA1D; adrenoceptor α1D	Neuron signaling, regulation of transcription
	ADRA2A; adrenoceptor α2A	Neuron signaling, regulation of transcription
	ADRA2B; adrenoceptor α2B	Neuron signaling, regulation of transcription
	ADRA2C; adrenoceptor α2C	Neuron signaling, regulation of transcription
	ADRA2D; adrenoceptor α2D	Neuron signaling, regulation of transcription
	ADRB2; adrenoceptor β2	Neuron signaling, regulation of transcription
	ADRB3A; adrenoceptor β3	Neuron signaling, regulation of transcription
Dopamine receptors	DRD1; dopamine receptor D1	Neuron signaling, regulation of transcription
	DRD2; dopamine receptor D2	Neuron signaling, regulation of transcription
	DRD3; dopamine receptor D3	Neuron signaling, regulation of transcription
	DRD4; dopamine receptor D4	Neuron signaling, regulation of transcription
	DRD5; dopamine receptor D5	Neuron signaling, regulation of transcription
5-HT receptors	HTR1A; 5-hydroxytryptamine receptor 1A	Neuron signaling, regulation of transcription
	HTR1B; 5-hydroxytryptamine receptor 1B	Neuron signaling, regulation of transcription
	HTR1D; 5-hydroxytryptamine receptor 1D	Neuron signaling, regulation of transcription
	HTR1E; 5-hydroxytryptamine receptor 1E	Neuron signaling, regulation of transcription
	HTR1F; 5-hydroxytryptamine receptor 1F	Neuron signaling, regulation of transcription
	HTR2A; 5-hydroxytryptamine receptor 2A	Neuron signaling, regulation of transcription
	HTR2B; 5-hydroxytryptamine receptor 2B	Neuron signaling, regulation of transcription
	HTR2C; 5-hydroxytryptamine receptor 2C	Neuron signaling, regulation of transcription
	HTR3A; 5-hydroxytryptamine receptor 3A	Neuron signaling
	HTR3C; 5-hydroxytryptamine receptor 3C	Neuron signaling
	HTR4; 5-hydroxytryptamine receptor 4	Neuron signaling, regulation of transcription
	HTR6; 5-hydroxytryptamine receptor 6	Neuron signaling, regulation of transcription
	HTR7; 5-hydroxytryptamine receptor 7	Neuron signaling, regulation of transcription
Monoamine synthesis and degradation	TPH1; tryptophan hydroxylase 1	Synthesis of 5-HT
	TPH2; tryptophan hydroxylase 2	Synthesis of 5-HT
	TH; tyrosine hydroxylase	Synthesis of catecholamines
	MAO; monoamine oxidase	Monoamine degradation
Neuronal activity	BDNF; brain derived neurotrophic factor	Growth factor, neurogenesis
	FOSL1; FOS Like 1, AP-1 transcription factor subunit	Transcription factor, neuronal activity
Microglia cell markers	CSF1R (MCSFR); Colony-stimulating-factor-1 receptor	Glia marker, cell differentiation
	MPEG1; Macrophage-expressed gene 1 protein	Glia marker, microbicidal activity
Cortisol receptors	NRC1a (GR1); glucocorticoid receptor 1	Regulation of transcription
	NRC1b (GR2); glucocorticoid receptor 2	Regulation of transcription
	NRC2 (MR); mineralocorticoid receptor	Regulation of transcription

Summary of the gene groups analyzed for differential expression in the brain of maraena whitefish. Official name, protein product and brief description of the function are indicated.

factors of the monoamine activation (Benito and Barco, 2015; Vindas et al., 2018). Cortisol receptor genes (GR1, GR2, and MR) were analyzed as feedback regulators of the monoamine system (Medeiros and McDonald, 2013). The expression of microglial cell markers (CSF1R and MPEG) was investigated as an indicator for stress effects on this immune cell population (Preston et al., 2018; Kuil et al., 2020).

Statistical Analysis

The effects of acute handling (fish sampled 3 and 24 h later) on the neurotransmitter concentration in the brain and cortisol in plasma were analyzed separately for statistical significance using one-way ANOVA and Dunnett's test for multiple comparisons. In this case, measurements of control groups from the 3 and 24 h experiments (n = 16 in total) were compared with the measurements of the fish at 3 h (n = 8) or 24 h (n = 8) after treatment. The control groups were also used to determine the basal level of neurotransmitters in the brain, which were analyzed using ANOVA followed by Tukey tests. For the 10-day chronic handling experiment, Student's t-test was used to compare the measurements of the control group (n = 8) with those of the treated group (n = 8) regarding neurotransmitter concentrations in the brain or cortisol levels in plasma. For the gene-expression analysis of the brain, t-test was used to compare the transcript level of each gene in the 3- and 24 h-acute-handling groups (n = 6 each) and the chronic-handling group (n = 6) with their corresponding control groups (n = 6 each). We reduced the sample size for gene expression to six

in each group to fit all groups onto the same Fluidigm chip for better comparability. The control groups from the acute handling experiment ($n = 12$) were used to determine the gene-expression profile of undisturbed fish between the different brain sections and within the gene groups. These were analyzed using the t-test. Tests were conducted using the software GraphPad Prism 8.0. We defined genes as being significantly expressed if they were up (>2-fold) or down-regulated (<−2-fold) with a p-value < 0.05.

RESULTS

Effects of Acute and Repeated Handling on Plasma Cortisol Concentration

The concentration of cortisol in plasma was analyzed for the acute (3 and 24 h post-challenge) and repeated handling experiments. In the acute handling experiment, plasma cortisol was significantly elevated (14.2 ± 3.1 ng/ml; $p < 0.05$) 3 h after handling compared to plasma concentrations in control fish (5.4 ± 0.7 ng/ml). At 24 h post-challenge, plasma cortisol (5.5 ± 0.8 ng/ml) did not differ significantly from control levels. After 10 days repeated handling, the cortisol concentration for the challenged fish (7.6 ± 2.3 ng/ml) showed no significant increase over the control fish (4.4 ± 0.5 ng/ml).

Basal Concentrations of Neurotransmitters and Acute-Handling Effects

We quantified the basal neurotransmitter concentrations in the different brain regions of undisturbed maraena whitefish (0 h; **Figures 3A–F**). The highest basal concentrations of neurotransmitters were found in the FB and MB while lowest were in the HB. The DA metabolites DOPAC and HVA were similarly distributed across the three brain regions at low concentrations (**Figures 3D,E**). The concentration of 5-HIAA showed a rostral-caudal gradient (**Figure 3F**).

The concentrations of monoamines and their metabolites in the different brain regions were measured at different times after a single episode of acute handling and compared to the baseline concentration of undisturbed fish (**Figure 3**). Brain neurochemistry was mainly affected by acute handling in the MB. The level of 5-HIAA significantly increased at 3 h post-handling in the MB compared to the undisturbed fish (**Figure 3F**). The three monoamine neurotransmitters NA, DA, and 5-HT significantly increased in the MB at 24 h after exposure to handling (**Figures 3A–C**). As the concentrations of metabolites DOPAC, HVA, 5-HIAA did not change (**Figures 3D–F**), the DOPAC/DA, HVA/DA, and 5-HIAA/5-HT ratios significantly decreased 24 h post-handling in the MB (**Figures 3G–I**) compared to the control group.

Expression Profiles of 37 Genes in Different Brain Regions, as Well as Acute-Handling Effects

We profiled the expression of 37 target and three reference genes (**Figure 4**) across the selected brain regions (**Figure 4A**) and compared the transcript levels according to their functional categories (**Figure 4B**). In the FB, the *ADR* and *HTR* genes were most strongly expressed (**Figure 4A**, gene groups 1 and 3). The 5-HT receptor gene *HTR6* was almost exclusively expressed in the FB and might thus be a suitable marker gene (**Figure 4A**, gene group 3). Besides this, *HTR4* and *HTR7* were strongly expressed in the FB compared to the MB (−2 to −12-fold) and the HB (−24 to −83-fold; **Figure 4A**, gene group 3). The *ADRA1d* and *DRD4* transcripts had a decreasing concentration gradient from rostral to caudal brain regions, similar to the 5-HT receptor genes *HTR1E*, *-1F*, *-2C*, *HTR4*, and *HTR7*. In contrast, the neurotrophin gene *BDNF* was highly expressed in the FB (**Figure 4A**, gene group 5), but less expressed in the MB and HB.

In the MB, *ADR*, and *HTR* expression was slightly lower than FB, while *DRD* genes shared similar expression levels in both FB and MB (**Figure 4A**, gene groups 1–3). *HTR3A* was specifically expressed in the MB and it may be a potential marker gene for this brain region. Similar to *HTR3A*, *HTR1D* was slightly stronger expressed in the MB than in the other brain regions. In contrast, there was less expression of the *ADRA1b* and *ADRA2c* genes in the MB (**Figure 3A**, gene group 1). The expression of *ADR* and *DRD* genes was markedly lower in the HB than in the other sections, especially for *ADRA2a* and *ADRA2d* (**Figure 4A**, gene group 1). In contrast, *ADRA1b* and *ADRA2c* were strongly expressed in the whole brain of maraena whitefish, while the *ADRB* genes were least strongly expressed (**Figure 4B**, gene group 1). Among dopaminergic receptor genes, *DRD1* was most strongly expressed in the brain compared to the other *DRD* genes, which shared similar (though lower) transcript levels (**Figure 4B**, gene group 2). Among the serotonergic receptor genes, *HTR1A*, *-2B* and *-3C* were the most widely expressed throughout the brain with no differences between brain sections (**Figure 4B**, gene group 3).

The microglial markers *CSF1R* and *MPEG* were evenly distributed across the three brain sections (**Figure 4A**, gene group 6), with *CSF1R* being expressed more than ten times as much as *MPEG* (**Figure 4B**, gene group 6). In the three brain sections, expression of *GR2* and *MR* was two to three times higher than *GR1* (**Figure 4B**, gene group 7). Among the genes coding for the tryptophan hydroxylase (Tph) enzymes, *TPH1* was slightly less expressed in HB than in FB, while *TPH2* was more strongly expressed in MB and HB than in FB. Regarding the expression of both *TPH* genes, *TPH1* was more strongly expressed in the FB and MB than *TPH2* (**Figures 4A,B**, gene group 4).

Acute handling affected the expression of genes coding for the adrenergic receptor subtypes *ADRA1* and *ADRB* in the HB region (**Figure 5**, gene group 1). In particular, *ADRA1d* (−2.2-fold) and *ADRB3* (−2.5-fold) were down-regulated 3 h after handling. Genes coding for the different subtypes of serotonergic receptors were also affected by acute handling throughout the brain (**Figure 5**, gene group 3). *HTR1D* transcript level increased 6.3-fold after 3 h in the HB and 2.8-fold after 24 h in the FB. *HTR6* was up-regulated (3.5-fold) in the MB 3 h post-handling. *HTR1A* and *HTR3C* were down-regulated (−2-fold) after acute-handling in the HB, while *HTR3C* was also down-regulated (−2.9-fold) in the FB after 24 h.

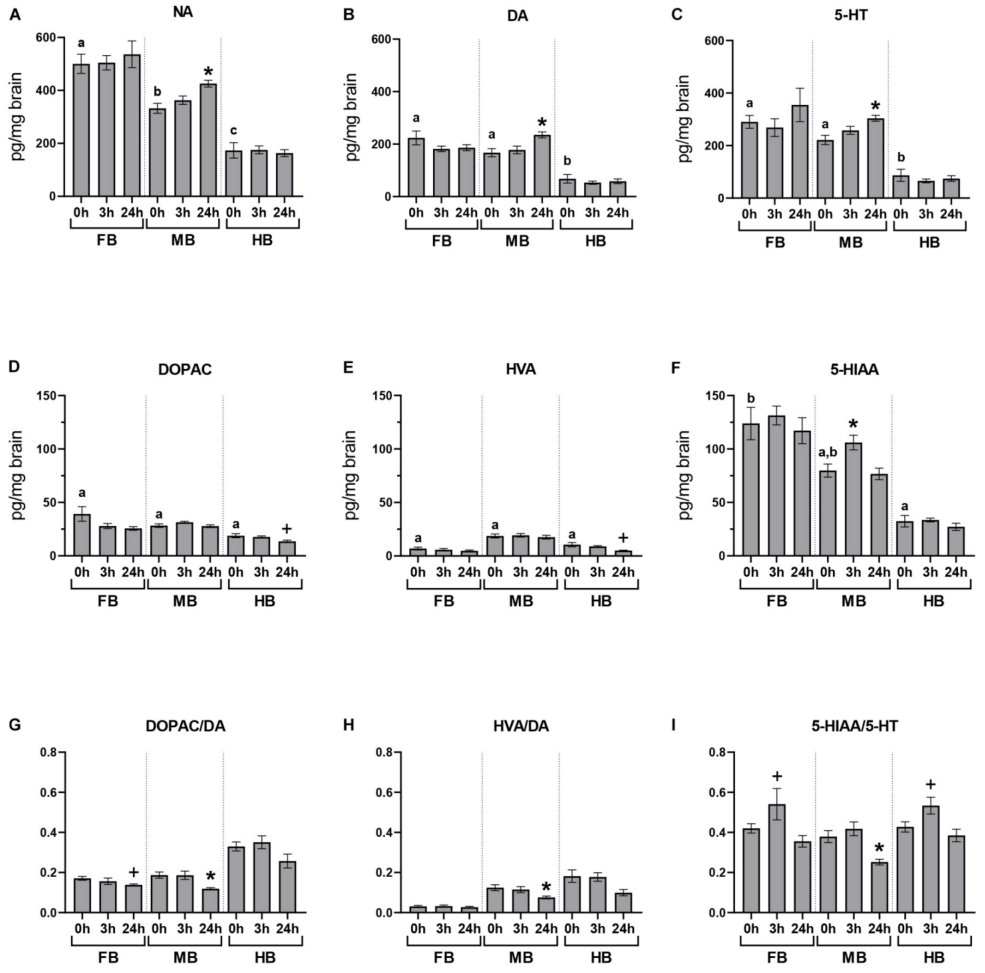


FIGURE 3 | Acute handling effects on neurotransmitter concentrations in the brain of maraena whitefish. The effects of acute stress were analyzed section-wise at 0 h (n = 16), 3 h (n = 8), and 24 h (n = 8) after exposure to acute handling for **(A)** NA; **(B)** DA; **(C)** 5-HT; **(D)** DOPAC; **(E)** HVA; **(F)** 5-HIAA concentrations (pg/mg tissue); and the ratios of **(G)** DOPAC/DA; **(H)** HVA/DA; and **(I)** HIAA/5-HT. Comparisons between handling and control groups were tested for statistical significance using one-way ANOVA and Dunnett's test for multiple comparisons and is indicated by (*) at $p < 0.05$ or non-significant tendency indicated as (+) at $0.05 < p < 0.10$. The controls of the acute stress experiments (0 h; n = 16) were used to determine the abundance and distribution of neurotransmitters and metabolites in the three brain sections of undisturbed maraena whitefish. Statistical analysis was performed using ANOVA with the Tukey test ($p < 0.05$). Error bars indicate SEM; different characters **(a–c)** above the bars indicate statistically significant concentration differences between brain regions and within time points.

Effects of Repeated Handling on Brain Neurotransmitter Concentrations and Gene Expression

After the 10-day period of daily handling, brain neurotransmitters were analyzed 24 h after the previous handling episode (**Figure 6**). NA concentration was slightly increased in FB and MB after 10 days of repeated handling ($p < 0.10$; **Figure 6A**), but the concentration of the other monoamines and metabolites was unaffected.

The expression of dopaminergic receptor genes was modulated in the FB and MB after 10 days of repeated handling (**Figure 5**, gene group 2). *DRD1* was −2.0-fold down-regulated

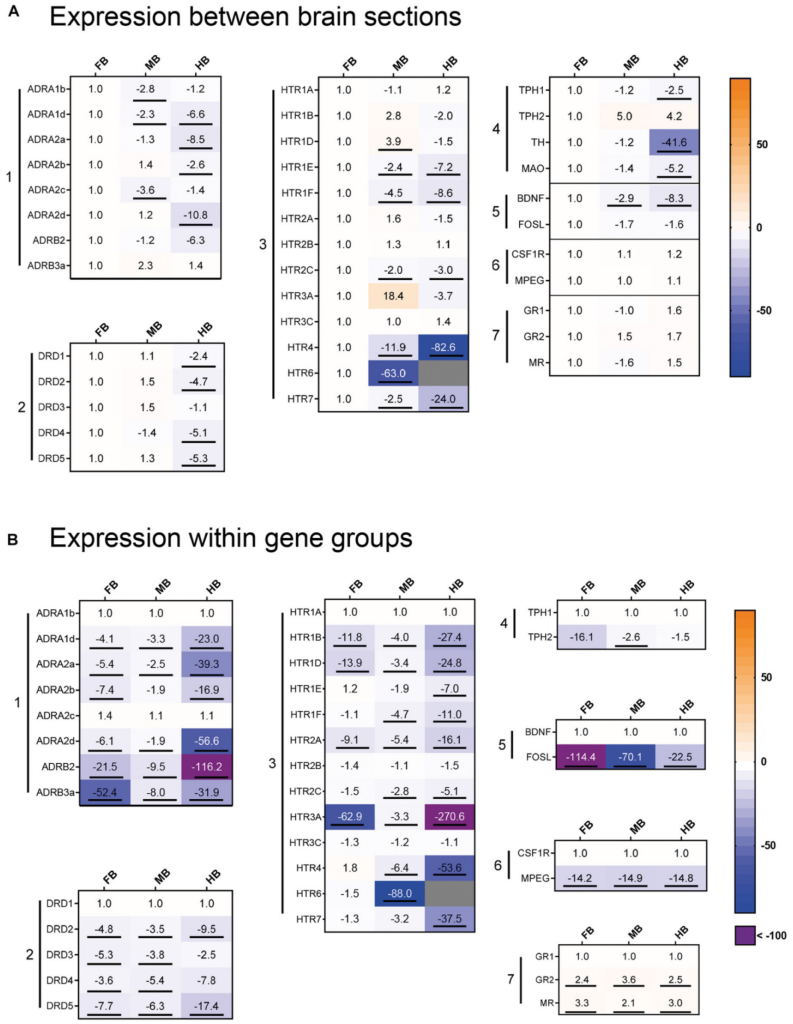


FIGURE 4 | Gene expression profile of maraena whitefish brain. The gene expression was profiled in the control animals of the acute stress experiment (3 and 24 h; $n = 12$) and analyzed with regard to **(A)** the different brain sections and **(B)** to the gene groups expressed. Results are expressed as fold-change values and colored according to the legend on the right. **(A)** To assess the gene expression in the different sections, the gene-specific transcript numbers in the MB and HB are shown relative to the respective transcript levels in the FB, which were set at 1.0. **(B)** Within the gene groups, the transcript numbers of the different genes are shown relative to the respective transcript levels of the gene in the first row, which were set at 1.0. The gene groups comprised (1) adrenergic receptor genes; (2) dopamine receptor genes; (3) 5-HT receptor genes; (4) monoamine synthesis genes; (5) neuronal activity markers; (6) microglia cell markers; (7) cortisol receptor genes. Genes were considered significantly regulated (marked by an underline) in the event of at least a twofold up or down-regulation of the transcript concentration and a p-value < 0.05 (Student's t-test).

in the FB and *DRD4* was −2.9-fold down-regulated in the MB. In addition, the transcript levels of the enzymes involved in monoamine synthesis were modulated in the FB and HB of maraena whitefish. *TPH1* was −3.6-fold down-regulated in the FB, while *TH* was 3.3-fold up-regulated in the HB (**Figure 5**, gene group 4).

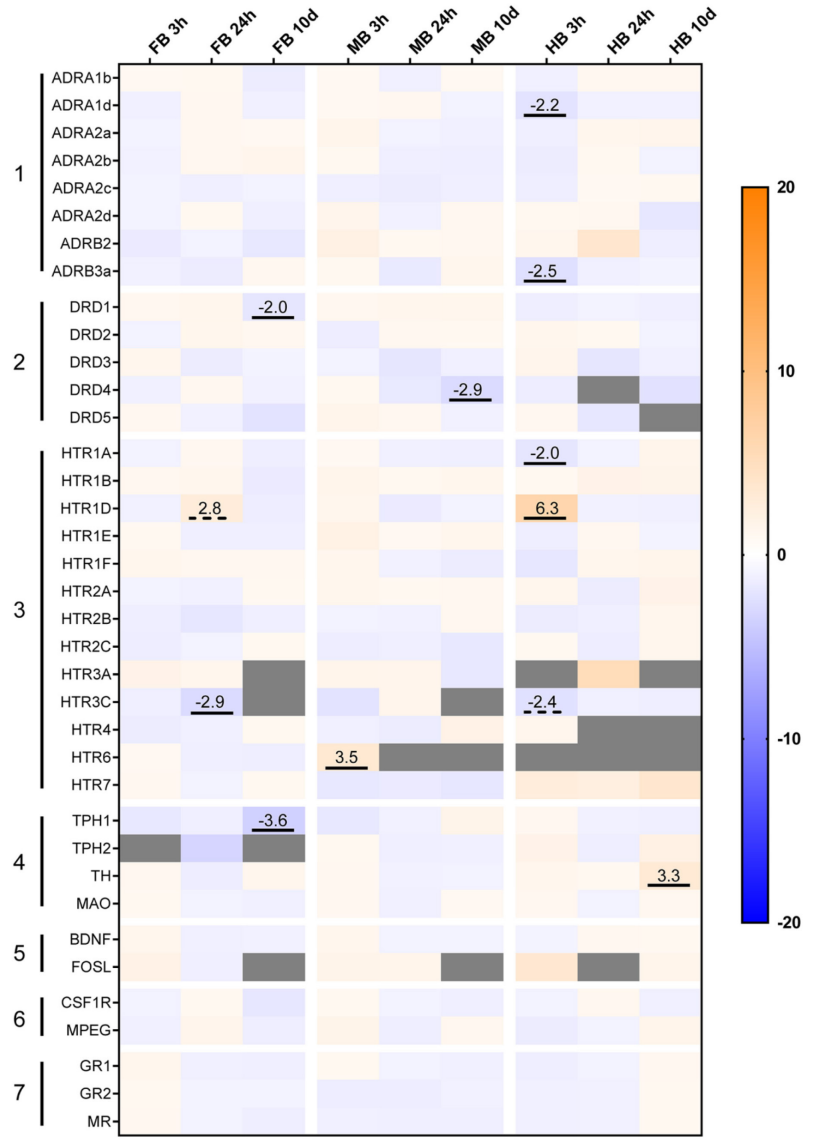


FIGURE 5 | Heatmap illustrating the effects of acute and chronic handling on gene expression in the brain of maraena whitefish. The heatmap shows the gene expression in fold-change values in the different brain sections after acute (3 and 24 h) and chronic (10 days) handling stress. The transcript level of each gene ($n = 6$) in the different brain sections and time-points was compared to its corresponding control group ($n = 6$ each). The scale bar indicates up-regulation (orange) or down-regulation (blue). Gray cells indicate non-detectable expression. The gene groups comprised (1) adrenergic receptor genes; (2) dopamine receptor genes; (3) 5-HT receptor genes; (4) monoamine synthesis genes; (5) neuronal activity markers; (6) microglial markers; (7) cortisol receptor genes. Genes were considered significantly regulated in the event of either an (at least) twofold up- or down-regulation of the transcript concentration and a p-value < 0.05 (marked by an underline) or non-significant tendencies at $0.05 < p < 0.10$ (are marked with dashed lines).

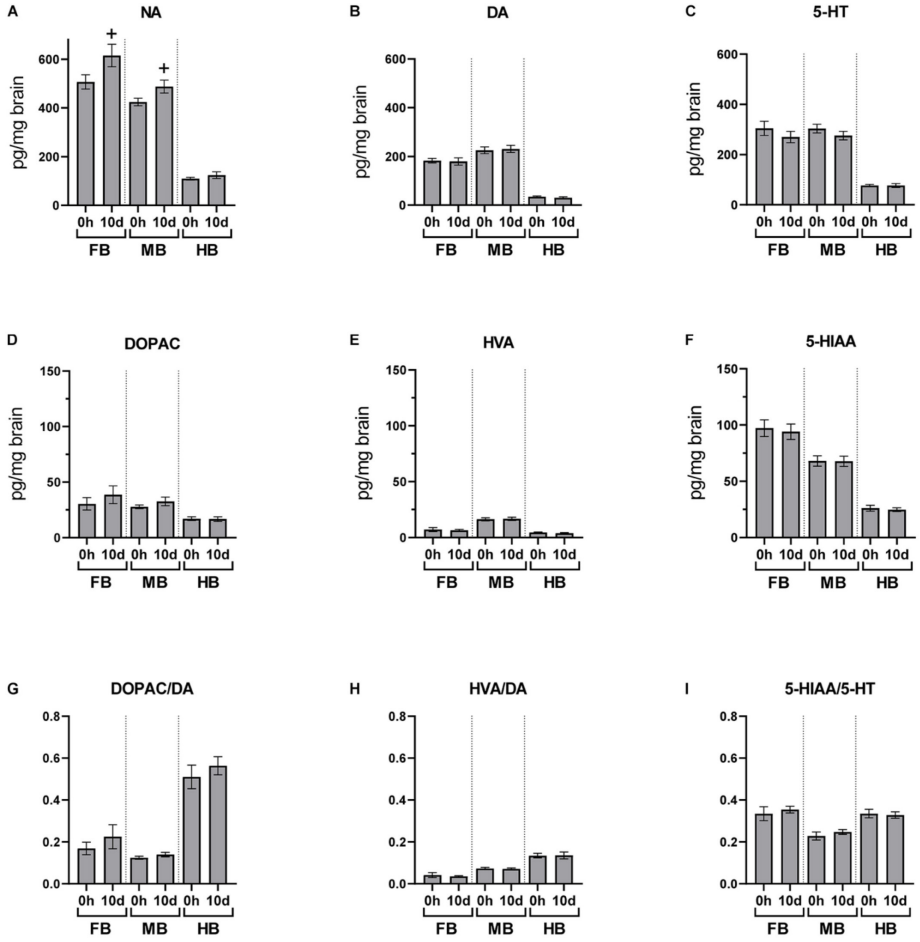


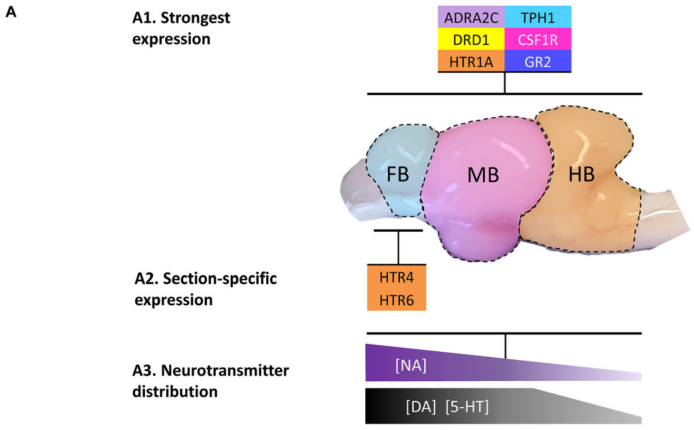
FIGURE 6 | Chronic handling effects on the neurotransmitter concentrations in the brain of maraena whitefish. The effects of repeated handling were analyzed section-wise at 0 h (n = 16) and 10 days (n = 8) after exposure to acute handling for **(A)** NA; **(B)** DA; **(C)** 5-HT; **(D)** DOPAC; **(E)** HVA; **(F)** HIAA concentrations (pg/mg tissue); and the ratios of **(G)** DOPAC/DA; **(H)** HVA/DA; and **(I)** HIAA/5-HT. Statistical significance was assessed with Student's t-test; non-significant tendency is indicated as (+) at $0.05 < p < 0.10$.

DISCUSSION

Low Monoamine Activity in the Hindbrain and Specific Gene Expression Patterns in Brain Sections of Maraena Whitefish

The first part of this study investigated the gene expression and neurochemistry of sixteen individual maraena whitefish, which served as reference individuals in the present study. Although this cohort was considered as "control fish," it was exposed to unavoidable challenges, i.e., husbandry in the anthropogenic environment as well as the sampling procedure.

Monoamine concentrations were analyzed in the three selected sections of the brain (**Figure 7**). We observed a decreasing concentration gradient of the investigated neurotransmitters from FB to HB (**Figure 7A**). This is consistent with our observation that the genes coding for monoamine receptors are expressed more strongly in FB and MB and less strongly in HB (**Figure 4A**; gene groups 1–3). This finding may indicate a lower influence of monoamines



B

			FB	MB	HB
ACUTE 3h	NTs		↑5-HIAA/5-HT⁺ ----	↑5-HIAA* ----	↑5-HIAA/5-HT⁺ ----
	Genes		---- ----	↑HTR6* ----	↑HTR1D* ↓ADRA1D* ↓ADRB3a* ↓HTR1A* ↓HTR3C⁺
ACUTE 24h	NTs		---- ↓DOPAC/DA⁺	↑NA* ↑DA* ↑5-HT* ↓HVA/DA* ↓5-HIAA/5-HT* ↓DOPAC/DA*	---- ↓DOPAC⁺ ↓HVA⁺
	Genes		↑HTR1D⁺ ↓HTR3C*	---- ----	---- ----
CHRONIC 10d	NTs		↑NA⁺ ----	↑NA⁺ ----	---- ----
	Genes		---- ↓DRD1* ↓TPH1*	---- ↓DRD4*	↑TH* ----

FIGURE 7 | Graphical summary about **(A1)** the most strongly expressed genes with a similar distribution across the three brain sections; **(A2)** those genes that were particularly high expressed in specific sections; and **(A3)** the spatial distribution of specific neurotransmitters. **(B)** Overview of the parameters that were affected by acute and chronic handling at the different time points in the analyzed brain sections. (↑) increased or (↓) decreased ratios/concentrations in case of neurotransmitters (NT) and >2-fold up- or <–2-fold down-regulation in the case of gene expression. Statistical significance is indicated with (*) at $p < 0.05$; non-significant tendency indicated as (+) at $0.05 < p < 0.10$. See **Figure 5** for heatmap with all target genes.

on HB. Higher concentrations of the monoamines investigated have already been described in the telencephalon and midbrain of the client reef fish *Labroides dimidiatus* and *Naso elegans* (Abreu et al., 2018), rainbow trout *O. mykiss* (Øverli et al., 2001) and Arctic charr *S. alpinus* (Backström et al., 2017). The high basal expression of *ADR* and *HTR* genes in the FB compared to other parts of the brain suggests that the telencephalon is the main target of 5-HT and NA. This assumption was supported by the high level of stored NA in this section, which may indicate the strong innervation of these areas by noradrenergic neurons. The high level of the metabolite 5-HIAA in the FB is most likely the result of 5-HT metabolization processes. The high concentration of monoamines and metabolites in the FB is coinciding with the high expression of *MAO*, which is essential for the degradation of monoamines (Winberg and Nilsson, 1993).

In our study, the expression of *DRD* genes in the HB was generally lower than in the other sections. *DRD1* was the most strongly expressed dopaminergic receptor gene in the three brain sections analyzed in maraena whitefish. In contrast to our findings, *DRD1* of zebrafish *D. rerio* was mainly found in the brain stem and hypothalamic region, but not in the forebrain (Maximino and Herculano, 2010). We found that the expression of *HTR* genes was particularly high in the FB of maraena whitefish compared to the other sections. In humans, prefrontal cortex and hippocampus express almost all *HTR*-encoding genes, which are a main target of serotonergic innervation (Švob Štrac et al., 2016). The corresponding regions of fish are presumably located

in the telencephalic area and include the equivalent to the amygdala, POA or the *striatum*, which are involved in the stress response and subjected to 5-HT signaling. Among the HTR genes analyzed, *HTR1A* was the most abundant transcript, which was present in all three brain sections. A similar observation has been made in Gulf toadfish *Opsanus beta* where 5-HT-1A modulates the HPI axis (Medeiros et al., 2010). In mammals, 5-HT-1A is also widely distributed in the brain, mainly present in the limbic areas and the raphe nuclei where it inhibits the 5-HT signaling and modulates the HPA axis (Fuller, 1992; Barnes and Sharp, 1999).

HTR4 and especially *HTR6* were highly expressed in the telencephalon compared to the rest of the brain (**Figure 7A**). In mammals, these 5-HT receptors act post-synaptically and induce cAMP signaling upon 5-HT binding (Branchek and Blackburn, 2000; Rosel et al., 2004). A high *HTR4* expression has been observed in progenitors of motor neurons in zebrafish *D. rerio* (Barreiro-Iglesias et al., 2015). In line with our results, mammalian *HTR4* is abundantly present in the mammalian nigrostriatal system that corresponds in teleosts to a region from the diencephalon to the telencephalon (**Figure 1**) (Barnes and Sharp, 1999; O'Connell et al., 2011). The expression pattern of *HTR6* in the telencephalon of maraena whitefish is similar to that in the brain of the cichlid *Astatotilapia burtoni* (Loveland et al., 2014). Previous studies on zebrafish (Lillesaar, 2011; Gaspar and Lillesaar, 2012) suggest that 5-HT populations express *TPH1* particularly in the hypothalamus (MB in this study), and *TPH2* in the pretectal complex and raphe (MB and HB in this study). Our finding of similar expression levels of *TPH1* in FB and MB of maraena whitefish was unexpected because 5-HT neurons, which normally express this gene, have not been described in telencephalon so far. We could speculate that *TPH1* is expressed by axonal projections of hypothalamic 5-HT populations into telencephalic regions similar to the expression of *TPH2* by neurons from the *raphe nuclei* (Lillesaar et al., 2009). Alternatively, we may have included in the dissection of the FB the pineal gland or part of the posterior *tuberculum*, which have been described to contain *TPH1*-expressing neurons (Gaspar and Lillesaar, 2012).

The LC is situated in the brainstem of teleosts and harbors an important noradrenergic cell population (Ekström et al., 1986). Therefore, the presence of *TH* and the synthesis of catecholamines would be expected here (Kaslin and Panula, 2001). However, *TH* transcripts were almost absent in HB. Concentrations of NA, as well as DA and its metabolites, were also low. The relatively small size of the LC in relation to the HB section might be an explanation for this finding.

Cortisol is the end product of the HPI axis and a strong regulator of the monoaminergic response in the brain (Medeiros and McDonald, 2013). The expression of the cortisol receptor genes *GR2* and *MR* was significantly higher throughout the brain than that of *GR1*, which suggests that these receptors might play a stronger role in cortisol signaling in the brain, compared to other organs where GR1 is predominant (Martorell-Ribera et al., 2020).

The expression of *BDNF*, which is an important factor for neurogenesis and cell proliferation, was particularly high in the FB. The brain of the killifish *Nothobranchius furzeri* displayed a similarly pronounced expression of *BDNF* in the dorsal telencephalic areas, suggesting that this region is essential for neuronal growth and plasticity (D'Angelo et al., 2014).

Acute Handling Increased 5-HT Turnover and Modulated Receptor Expression at 3 h Post-handling

Brain neurochemistry responded to acute handling with significantly increased 5-HIAA in the MB at 3 h post-handling, together with a slightly increased 5-HT turnover in FB and HB. We speculate that this is the result of an elevated release of 5-HT during and immediately after handling. A previous study demonstrated an increased 5-HT turnover in the telencephalon and hypothalamus of rainbow trout induced by chasing (Gesto et al., 2013). In addition, increased 5-HIAA concentrations have been linked to isolation and confinement stress of rainbow trout (Øverli et al., 2001). In Arctic charr, 5-HT and 5-HIAA concentrations in the brain stem of dominant fish were lower than in subordinate fish (Backström et al., 2015). In our study, serotonergic activation was accompanied by significantly elevated plasma cortisol levels 3 h after treatment, which indicates an activated HPI axis (Wendelaar Bonga, 1997). Although this cortisol concentration was significantly higher than that of control fish, it was still below the established stress levels of rainbow trout (Iwama et al., 1999; Barton, 2000; Schreck et al., 2016). This may be an indication that the cortisol concentration may have been higher before our first sampling, while it was already decreasing at the time of sampling. Acute handling up-regulated the *HTR6* expression in the MB of maraena whitefish. The basal expression of *HTR6* in the MB is extremely low and this condition might indicate its rather subordinate role in the response to handling, especially in view of the high *HTR6* expression in the FB of undisturbed fish.

The HB of maraena whitefish was expected to have important 5-HT neuron populations, which have been found in the NLV and *raphe nuclei* of several teleost species (Lillesaar, 2011). However, lower basal concentrations of 5-HT and 5-HIAA were observed in the HB compared to the other sections. This section displayed weakly increased 5-HT turnover 3 h after handling (**Figure 7B**). Contrary, acute stressors have been reported to increase of 5-HIAA/5-HT ratios in the brain stem of rainbow trout (Gesto et al., 2008).

Our results showed that the expression of the *HTR1* family genes was affected 3 h after handling. *HTR1D* was up-regulated, while *HTR1A* was downregulated. In mice, 5-HT-1D regulates the activity of 5-HT neurons in the *raphe nuclei* (Vogelgesang et al., 2017) by inhibiting 5-HT actions (Huang and Thathiah, 2015). 5-HT-1A controls the HPA axis in mammals and also inhibits 5-HT signaling and, at the same time, it is regulated by a negative feedback after cortisol release (Fuller, 1992; Zhong and Ciaranello, 1995; Barnes and Sharp, 1999). In rainbow trout, *HTR1A* was down-regulated in the telencephalon 1 to 4 h post-stress (Moltesen et al., 2016), while it was upregulated in subordinate Atlantic salmon parr (Thörnqvist et al., 2015).

5-HT-3C promotes 5-HT signaling in mammals (Sangkuhl et al., 2009). In this study, *HTR3C* was significantly down-regulated in the brain of maraena whitefish 24 h post-handling

(**Figure 7B**). The up-regulation of *HTR1D* and down-regulation of *HTR3C* are most likely compensatory responses to counteract this 5-HT release, which could affect the *raphe* 5-HT populations of the brain stem, as described in brook trout *Salvelinus fontinalis* and other teleosts (Bolliet and Ali, 1992; Lillesaar, 2011). The adrenergic receptor genes *ADRA1D* and *ADRB3a* were also down-regulated in the HB 3 h post-handling. Both adrenergic receptors act post-synaptically as activating modulators of NA actions in the target neuron (Graybiel and Penney, 1999; Huang and Thathiah, 2015; Maletic et al., 2017). *ADRA1D* has been shown to be up-regulated in the hippocampus of rats under restraint stress (Campeau et al., 2010). *ADRB3* genes code for β_3 receptors, which are generally involved in NA release and neuron plasticity and are down-regulated during chronic stress in mammals (Seki et al., 2018). Although no changes in NA brain levels were observed 3 h post-handling, the down-regulation of these two genes suggests that acute handling might result in desensitization to NA in the HB.

The Initial Response to Handling Was Compensated by Increased Monoamine Concentrations at 24 h Post-handling

The initial serotonergic response 3 h post-handling was followed after 24 h by increased concentrations of NA, DA and 5-HT in the MB compared to control fish (**Figure 7B**). We assume that the increased monoamine concentrations are the compensatory response to the stress-related demand of monoamines to cope with stress. The observed increase in neurotransmitters could be explained by a reduced monoamine metabolism. However, the metabolites were not reduced compared to the value at 0 h and the gene expression of *MAO* was unaffected by stress. Alternatively, the elevated monoamine concentrations could be the result of an increased synthesis as a consequence of an accelerated enzymatic activity of TPH and TH or the increased availability of their substrates tryptophan and tyrosine. Both enzymatic activity and substrate availability are likely to be affected by stress as previously shown (Dunn and Welch, 1991; Chen and Miller, 2012). However, we did not detect an increased *TPH* and *TH* gene expression at sampling times of 3 and 24 h. Unfortunately, our data do not permit a final conclusion because we have neither analyzed the protein content expression nor the activity of the relevant enzymes. We think that the unchanged metabolite concentrations and return of plasma cortisol levels to baseline 24 h after the challenge reflect recovery from stress and a return of HPI activity to baseline between 3 and 24 h after the challenge. In teleost fish, low reactivity to stress may indicate an increased allostatic load (Madaro et al., 2015; Moltesen et al., 2016), which also impairs the natural stress-response mechanisms (Schreck et al., 2016). This implies the stress-dependent activation of the DA and NA systems, although we did not detect any significantly altered levels of DA or NA in the brain of maraena whitefish 3 h post-handling. After 24 h post-handling, only the expression of 5-HT-related genes in the FB was affected (**Figure 7B**). The down-regulation of *HTR3C*, which activates 5-HT signaling, and the up-regulation of *HTR1D*, which antagonizes the 5-HT signaling, suggest a balanced 5-HT activity that migrated from the rear brain at 3 h post-handling to the telencephalon 24 h post-handling.

Repeated Handling Evoked a Weak Monoaminergic Response

The 10-day repeated handling had no significant effects on the brain neurochemistry (**Figure 7B**). Although the fish in the chronic experiment were sampled 24 h after the last treatment, the monoaminergic activity was not consistent with the observed recovery period 24 h after acute treatment. Explanations for these observations could be habituation processes, exhaustion of neural responses or the effects of chronically elevated cortisol levels on the monoaminergic systems. The possibility that the decreased neurochemical response could be due to continuously elevated cortisol levels in the brain could be excluded, since the cortisol levels of repeatedly handled fish did not differ from those of control fish at the end of the 10-day experiment and were not significantly elevated even 24 h after acute handling. Moreover, the observed plasma cortisol levels remained below the stress levels described for rainbow trout (Schreck et al., 2016). Increased DA metabolite concentrations without altered plasma cortisol levels have been previously described in Arctic charr in response to handling (Backström et al., 2017). Depletion or exhaustion of neuronal responses are not very likely as we did not observe a reduction of the neurotransmitter pool compared with controls, which might decrease the monoaminergic response. Instead, we found subtle modulations of the *TPH1* and *TH* expression. This suggests that the monoamine synthesis pathways were sensitive to repeated handling and may indicate habituation.

Isolated rainbow trout had increased NA levels in the telencephalon and the optic tectum after 1 week (Øverli et al., 1999, 2001). Our results are consistent with these reports, since we also observed slightly increased concentrations of noradrenaline together with an upregulation of *TH* in HB.

Previous studies on the POA in the brain of cichlid fish revealed increased levels of tyrosine hydroxylase after handling stress, and a modulated expression of dopamine receptors and genes related to 5-HT synthesis after repeated handling (Chabbi and Ganesh, 2015).

DRD1 and *DRD4* were down-regulated in the FB and MB of maraena whitefish, respectively. D1 activates the dopamine signaling, while D4 inhibits it (Svingos et al., 2000; Centonze et al., 2003; Mizuno et al., 2007). In studies with rodents, acute and chronic stress modulated the expression of *DRD1*, depending on the forebrain region analyzed (Rasheed et al., 2012). The DA receptor D4 participates in the dopaminergic stress response of primates (Arnsten et al., 2000). Taking into account the aforementioned studies, the down-regulation of *DRD1* and *DRD4* in our results points to the desensitization of the DA system in the FB and MB.

Our results suggest that the 5-HT system was desensitized by a repeated stressor compared to the 5-HT response to a single episode of handling (see sections "Acute Handling Increased 5-HT Turnover and Modulated Receptor Expression at 3 h Post-handling" and "The Initial Response to Handling was Compensated by Increased Monoamine Concentrations at 24 h

Post-handling"). However, markers derived from the synthesis pathway of 5-HT are apparently promising indicators of the serotonergic response to chronic stressors. TPH controls the rate-limiting step of synthetizing 5-HT from tryptophan. Recently, a study demonstrated a downregulation of *TPH1* and *TPH2* after heat stress in medaka fish *Oryzias latipes* (Shimomura et al., 2019). In our experiment, the repeated handling reduced *TPH1* expression in the FB section of maraena whitefish. However, as discussed in Section "Low Monoamine Activity in the Hindbrain and Specific Gene Expression Patterns in Brain," the expression of *TPH1* was detected in 5-HT neurons, which should not be present in the brain parts included in our FB sections. Therefore, further investigations based on precise histology are required to confirm our data.

CONCLUSION

The effects of stress on the brain neurochemistry vary remarkably throughout the numerous interrelated regions of the brain. In this study, we observed that acute handling activates the HPI axis and serotonergic activity of the brain shortly after the challenge. At 24 h after acute handling, the return of plasma cortisol to baseline together with an increase of brain monoamine concentrations indicated a recovery. After 10 days of repeated handling, the modest neurochemical response and the low cortisol levels might reflect a habituation to the persistent challenge, as the increase of the NA concentration is much weaker than at 24 h after the single handling event. Based on the basal expression and the significant regulation under handling conditions, we selected a group of genes as potential markers that indicated the effects of handling as a stressor on the brain monoamine systems, i.e., *DRD1*, *DRD4*, *ADRA1D*, *ADRB3a*, *HTR1A*, and *HTR3C*. Our results suggest that maraena whitefish should be left undisturbed for at least 24 h following routine aquaculture procedures that include intense handling, such as size sorting or transportation. This salmonid species apparently habituates to repeated handling in the long term. Our study shows that a stress-sensitive species such as maraena whitefish is able to adapt to the anthropogenic stressors related to aquaculture conditions if enough time is made available between challenges.

DATA AVAILABILITY STATEMENT

Datasets generated for this study are available from the corresponding authors on request.

ETHICS STATEMENT

The animal study was reviewed and approved by Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, Germany (LALLF M-V/TSD/7221.3-1-069/18).

AUTHOR CONTRIBUTIONS

UG, AR, JM-R, and TG designed the research. UG, AR, and TG supervised the experiments. RB organized the husbandry of the maraena whitefish. JM-R and MV performed stress experiments and sampled fish. JM-R and WO performed HPLC analyses. JM-R and AR conducted RT-qPCR analyses. JM-R performed statistical analyses. JM-R wrote the manuscript. AR and UG edited the manuscript. All the authors have read and agreed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.591738/full#supplementary-material

Supplementary Table 1 | Target genes and primers designed for RT-qPCR analysis.

REFERENCES

Abreu, M. S., Messias, J. P. M. M., Thörnqvist, P.-O. O., Winberg, S., and Soares, M. C. (2018). Monoaminergic levels at the forebrain and diencephalon signal for the occurrence of mutualistic and conspecific engagement in client reef fish. *Sci. Rep.* 8:7346. doi: 10.1038/s41598-018-25513-6

Aerts, J. (2018). Quantification of a glucocorticoid profile in non-pooled samples is pivotal in stress research across vertebrates. *Front. Endocrinol.* 9:635. doi: 10.3389/fendo.2018.00635

Altmann, S., Korytář, T., Kaczmarzyk, D., Nipkow, M., Kühn, C., Goldammer, T., et al. (2016). Toll-like receptors in maraena whitefish: evolutionary relationship among salmonid fishes and patterns of response to *Aeromonas salmonicida*. *Fish Shellfish Immunol.* 54, 391–401. doi: 10.1016/j.fsi.2016.04.125

Altmann, S., Rebl, A., Kühn, C., and Goldammer, T. (2015). Identification and de novo sequencing of housekeeping genes appropriate for gene expression analyses in farmed maraena whitefish (*Coregonus maraena*) during crowding stress. *Fish Physiol. Biochem.* 41, 397–412. doi: 10.1007/s10695-014-9991-y

Arnsten, A. F., Murphy, B., and Merchant, K. (2000). The selective dopamine D4 receptor antagonist, PNU-101387G, prevents stress-induced cognitive deficits in monkeys. *Neuropsychopharmacology* 23, 405–410. doi: 10.1016/S0893-133X(00)00133-0

Backström, T., Heynen, M., Brännäs, E., Nilsson, J., Winberg, S., and Magnhagen, C. (2015). Social stress effects on pigmentation and monoamines in Arctic charr. *Behav. Brain Res.* 291, 103–107. doi: 10.1016/j.bbr.2015.05.011

Backström, T., Heynen, M., Brännäs, E., Nilsson, J., Winberg, S., and Magnhagen, C. (2017). Anaesthesia and handling stress effects on pigmentation and monoamines in Arctic charr. *Environ. Biol. Fishes* 100, 471–480. doi: 10.1007/s10641-016-0563-0

Barcellos, L. J. G., Volpato, G. L., Barreto, R. E., Coldebella, I., and Ferreira, D. (2011). Chemical communication of handling stress in fish. *Physiol. Behav.* 103, 372–375. doi: 10.1016/j.physbeh.2011.03.009

Barnes, N. M., and Sharp, T. (1999). A review of central 5-HT receptors and their function. *Neuropharmacology* 38, 1083–1152. doi: 10.1016/S0028-3908(99)00010-6

Barreiro-Iglesias, A., Mysiak, K. S., Scott, A. L., Reimer, M. M., Yang, Y., Becker, C. G., et al. (2015). Serotonin promotes development and regeneration of spinal motor neurons in zebrafish. *Cell Rep.* 13, 924–932. doi: 10.1016/j.celrep.2015.09.050

Barton, B., Schreck, C., and Barton, L. (1987). Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. *Dis. Aquat. Organ.* 2, 173–185. doi: 10.3354/dao002173

Barton, B. A. (2000). Salmonid fishes differ in their cortisol and glucose responses to handling and transport stress. *N. Am. J. Aquac.* 62, 12–18. doi: 10.1577/1548-84542000062

Barton, B. A. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr. Comp. Biol.* 42, 517–525. doi: 10.1093/icb/42.3.517

Benito, E., and Barco, A. (2015). The neuronal activity-driven transcriptome. *Mol. Neurobiol.* 51, 1071–1088. doi: 10.1007/s12035-014-8772-z

Bolliet, V., and Ali, M. A. (1992). Immunohistochemical study of the development of serotoninergic neurons in the brain of the brook trout salvelinus fontinalis. *Brain Behav. Evol.* 40, 234–249. doi: 10.1159/000113915

Branchek, T. A., and Blackburn, T. P. (2000). 5-HT6 receptors as emerging targets for drug discovery. *Annu. Rev. Pharmacol. Toxicol.* 40, 319–334. doi: 10.1146/annurev.pharmtox.40.1.319

Brietzke, A., Borchel, A., Altmann, S., Nipkow, M., Rebl, A., Brunner, R. M., et al. (2016). Transcriptome sequencing of maraena whitefish (*Coregonus maraena*). *Mar. Genomics* 29, 27–29. doi: 10.1016/j.margen.2016.05.006

Campeau, S., Nyhuis, T. J., Kryskow, E. M., Masini, C. V., Babb, J. A., Sasse, S. K., et al. (2010). Stress rapidly increases alpha 1d adrenergic receptor mRNA in the rat dentate gyrus. *Brain Res.* 1323, 109–118. doi: 10.1016/j.brainres.2010.01.084

Centonze, D., Grande, C., Usiello, A., Gubellini, P., Erbs, E., Martín, A. B., et al. (2003). Receptor subtypes involved in the presynaptic and postsynaptic actions of dopamine on striatal interneurons. *J. Neurosci.* 23, 6245–6254. doi: 10.1523/JNEUROSCI.23-15-06245.2003

Chabbi, A., and Ganesh, C. B. (2015). Evidence for the involvement of dopamine in stress-induced suppression of reproduction in the cichlid fish oreochromis mossambicus. *J. Neuroendocrinol.* 27, 343–356. doi: 10.1111/jne.12269

Chen, G.-L., and Miller, G. M. (2012). Advances in tryptophan hydroxylase-2 gene expression regulation: new insights into serotonin-stress interaction and clinical implications. *Am. J. Med. Genet. Part B Neuropsychiatr. Genet.* 159B, 152–171. doi: 10.1002/ajmg.b.32023

D'Angelo, L., De Girolamo, P., Lucini, C., Terzibasi, E. T., Baumgart, M., Castaldo, L., et al. (2014). Brain-derived neurotrophic factor: mRNA expression and protein distribution in the brain of the teleost *Nothobranchius furzeri*. *J. Comp. Neurol.* 522, 1004–1030. doi: 10.1002/cne.23457

Dunn, A. J., and Welch, J. (1991). Stress- and endotoxin-induced increases in brain tryptophan and serotonin metabolism depend on sympathetic nervous system activity. *J. Neurochem.* 57, 1615–1622. doi: 10.1111/j.1471-4159.1991.tb06359.x

Dwarkasing, J. T., Witkamp, R. F., Boekschoten, M. V., Ter Laak, M. C., Heins, M. S., and van Norren, K. (2016). Increased hypothalamic serotonin turnover in inflammation-induced anorexia. *BMC Neurosci.* 17:26. doi: 10.1186/s12868-016-0260-0

Ekström, P., Reschke, M., Steinbusch, H., and Van Veen, T. (1986). Distribution of noradrenaline in the brain of the teleost *Gasterosteus aculeatus* L.: an immunohistochemical analysis. *J. Comp. Neurol.* 254, 297–313. doi: 10.1002/cne.902540304

Feldman, S., Conforti, N., and Weidenfeld, J. (1995). Limbic pathways and hypothalamic neurotransmitters mediating adrenocortical responses to neural stimuli. *Neurosci. Biobehav. Rev.* 19, 235–240. doi: 10.1016/0149-7634(94)00062-6

Flügge, G. (1999). Regulation of monoamine receptors in the brain: dynamic changes during stress. *Int. Rev. Cytol.* 195, 145–213. doi: 10.1016/S0074-7696(08)62705-9

Fröhlich, F. (ed.) (2016). "Neuromodulators," in *Network Neuroscience* (Amsterdam: Elsevier), 59–71.

Fuller, R. W. (1992). The involvement of serotonin in regulation of pituitary-adrenocortical function. *Front. Neuroendocrinol.* 13, 250–270.

Gaspar, P., and Lillesaar, C. (2012). Probing the diversity of serotonin neurons. *Philos. Trans. R. Soc. B Biol. Sci.* 367, 2382–2394. doi: 10.1098/rstb.2011.0378

Geng, Y., and Peterson, R. T. (2019). The zebrafish subcortical social brain as a model for studying social behavior disorders. *Dis. Model. Mech.* 12:dmm039446. doi: 10.1242/dmm.039446

Gesto, M., Lopez-Patino, M. A., Hernandez, J., Soengas, J. L., and Miguez, J. M. (2013). The response of brain serotonergic and dopaminergic systems to an acute stressor in rainbow trout: a time course study. *J. Exp. Biol.* 216, 4435–4442. doi: 10.1242/jeb.091751

Gesto, M., Soengas, J. L., and Míguez, J. M. (2008). Acute and prolonged stress responses of brain monoaminergic activity and plasma cortisol levels in rainbow trout are modified by PAHs (naphthalene, β-naphthoflavone and benzo(a)pyrene) treatment. *Aquat. Toxicol.* 86, 341–351. doi: 10.1016/j.aquatox.2007.11.014

Graybiel, A. M., and Penney, J. B. (1999). "Chemical architecture of the basal ganglia," in *Handbook of Chemical Neuroanatomy*, eds F. E. Bloom, A. Björklund, and T. Hökfelt (Amsterdam: Elsevier), 227–284.

Hemre, G.-I., and Krogdahl, Å (1996). Effect of handling and fish size on secondary changes in carbohydrate metabolism in Atlantic salmon, Salmo salar L. *Aquac. Nutr.* 2, 249–252. doi: 10.1111/j.1365-2095.1996.tb00067.x

Höglund, E., Weltzien, F.-A., Schjolden, J., Winberg, S., Ursin, H., and Døving, K. B. (2005). Avoidance behavior and brain monoamines in fish. *Brain Res.* 1032, 104–110. doi: 10.1016/j.brainres.2004.10.050

Huang, Y., and Thathiah, A. (2015). Regulation of neuronal communication by G protein-coupled receptors. *FEBS Lett.* 589, 1607–1619. doi: 10.1016/j.febslet.2015.05.007

Irwin, M. R., and Cole, S. W. (2013). Reciprocal regulation of the neural and innate immune systems. *Nat. Rev. Immunol.* 11, 625–632. doi: 10.1038/nri3042.Reciprocal

Iwama, G. K., Vijayan, M. M., Forsyth, R. B., and Ackerman, P. A. (1999). Heat shock proteins and physiological stress in fish. *Am. Zool.* 39, 901–909. doi: 10.1093/icb/39.6.901

Jansen, W., Jennerich, H.-J., Wenzel, H.-J., and Schulz, S. (2008). Zur Haltung von Ostseeschnäpeln in einem Kaltwasserkreislauf in Hohen Wangelin. *Fischerei Fischmarkt M V* 4, 36–39.

Kaslin, J., and Panula, P. (2001). Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (*Danio rerio*). *J. Comp. Neurol.* 440, 342–377. doi: 10.1002/cne.1390

Korytář, T., Nipkow, M., Altmann, S., Goldammer, T., Köllner, B., and Rebl, A. (2016). Adverse husbandry of maraena whitefish directs the immune system to increase mobilization of myeloid cells and proinflammatory responses. *Front. Immunol.* 7:631. doi: 10.3389/fimmu.2016.00631

Kottelat, M., and Freyhof, J. (2007). *Handbook of European Freshwater Fishes*. Delémont: Publications Kottelat.

Krasnov, A., Koskinen, H., Pehkonen, P., Rexroad, C. E., Afanasyev, S., and Mölsä, H. (2005). Gene expression in the brain and kidney of rainbow trout in response to handling stress. *BMC Genomics* 6:3. doi: 10.1186/1471-2164-6-3

Kuil, L. E., Oosterhof, N., Ferrero, G., Mikulášová, T., Hason, M., Dekker, J., et al. (2020). Zebrafish macrophage developmental arrest underlies depletion of microglia and reveals Csf1r-independent metaphocytes. *eLife* 9, 1–27. doi: 10.7554/eLife.53403

Lillesaar, C. (2011). The serotonergic system in fish. *J. Chem. Neuroanat.* 41, 294–308. doi: 10.1016/j.jchemneu.2011.05.009

Lillesaar, C., Stigloher, C., Tannhäuser, B., Wullimann, M. F., and Bally-Cuif, L. (2009). Axonal projections originating from raphe serotonergic neurons in the developing and adult zebrafish, Danio Rerio, using transgenics to visualize raphe-specific pet1 expression. *J. Comp. Neurol.* 512, 158–182. doi: 10.1002/cne.21887

Lőrincz, M. L., and Adamantidis, A. R. (2017). Monoaminergic control of brain states and sensory processing: existing knowledge and recent insights obtained with optogenetics. *Prog. Neurobiol.* 151, 237–253. doi: 10.1016/j.pneurobio.2016.09.003

Loveland, J. L., Uy, N., Maruska, K. P., Carpenter, R. E., and Fernald, R. D. (2014). Social status differences regulate the serotonergic system of a cichlid fish, *Astatotilapia burtoni*. *J. Exp. Biol.* 217, 2680–2690. doi: 10.1242/jeb.100685

Madaro, A., Olsen, R. E., Kristiansen, T. S., Ebbesson, L. O. E., Nilsen, T. O., Flik, G., et al. (2015). Stress in Atlantic salmon: response to unpredictable chronic stress. *J. Exp. Biol.* 218, 2538–2550. doi: 10.1242/jeb.120535

Maletic, V., Eramo, A., Gwin, K., Offord, S. J., and Duffy, R. A. (2017). The role of norepinephrine and its α-adrenergic receptors in the pathophysiology and treatment of major depressive disorder and schizophrenia: a systematic review. *Front. Psychiatry* 8:42. doi: 10.3389/fpsyt.2017.00042

Martorell-Ribera, J., Nipkow, M., Viergutz, T., Brunner, R. M., Bochert, R., Koll, R., et al. (2020). Early response of salmonid head-kidney cells to stress hormones and toll-like receptor ligands. *Fish Shellfish Immunol.* 98, 950–961. doi: 10.1016/j.fsi.2019.11.058

Maximino, C., and Herculano, A. M. (2010). A review of monoaminergic neuropsychopharmacology in zebrafish. *Zebrafish* 7, 359–378. doi: 10.1089/zeb.2010.0669

Medeiros, L. R., Mager, E. M., Grosell, M., and McDonald, M. D. (2010). The serotonin subtype 1A receptor regulates cortisol secretion in the Gulf toadfish, *Opsanus beta*. *Gen. Comp. Endocrinol.* 168, 377–387. doi: 10.1016/j.ygcen.2010.05.004

Medeiros, L. R., and McDonald, M. D. (2013). Cortisol-mediated downregulation of the serotonin 1A receptor subtype in the Gulf toadfish, Opsanus beta. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 164, 612–621. doi: 10.1016/j.cbpa.2013.01.014

Mishra, A., Singh, S., and Shukla, S. (2018). Physiological and functional basis of dopamine receptors and their role in neurogenesis: possible implication for parkinson's disease. *J. Exp. Neurosci.* 12:117906951877982. doi: 10.1177/1179069518779829

Mizuno, T., Schmauss, C., and Rayport, S. (2007). Distinct roles of presynaptic dopamine receptors in the differential modulation of the intrinsic synapses of medium-spiny neurons in the nucleus accumbens. *BMC Neurosci.* 8:8. doi: 10.1186/1471-2202-8-8

Moltesen, M., Laursen, D. C., Thörnqvist, P.-O., Andersson, M. Å, Winberg, S., and Höglund, E. (2016). Effects of acute and chronic stress on telencephalic neurochemistry and gene expression in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* 219, 3907–3914. doi: 10.1242/jeb.139857

Morgane, P., Galler, J., and Mokler, D. (2005). A review of systems and networks of the limbic forebrain/limbic midbrain. *Prog. Neurobiol.* 75, 143–160. doi: 10.1016/j.pneurobio.2005.01.001

Mueller, T. (2012). What is the thalamus in zebrafish? *Front. Neurosci.* 6:64. doi: 10.3389/fnins.2012.00064

Nichols, D. E., and Nichols, C. D. (2008). Serotonin receptors. *Chem. Rev.* 108, 1614–1641. doi: 10.1021/cr078224o

O'Connell, L. A., Fontenot, M. R., and Hofmann, H. A. (2011). Characterization of the dopaminergic system in the brain of an African cichlid fish, *Astatotilapia burtoni*. *J. Comp. Neurol.* 519, 75–92. doi: 10.1002/cne.22506

Otten, W., Kanitz, E., Couret, D., Veissier, I., Prunier, A., and Merlot, E. (2010). Maternal social stress during late pregnancy affects hypothalamic-pituitary-adrenal function and brain neurotransmitter systems in pig offspring. *Domest. Anim. Endocrinol.* 38, 146–156. doi: 10.1016/j.domaniend.2009.09.002

Øverli, Ø, Harris, C. A., and Winberg, S. (1999). Short-term effects of fights for social dominance and the establishment of dominant-subordinate relationships on brain monoamines and cortisol in rainbow trout. *Brain Behav. Evol.* 54, 263–275. doi: 10.1159/000006627

Øverli, Ø, Pottinger, T. G., Carrick, T. R., Øverli, E., and Winberg, S. (2001). Brain monoaminergic activity in rainbow trout selected for high and low stress responsiveness. *Brain Behav. Evol.* 57, 214–224. doi: 10.1159/000047238

Panula, P., Chen, Y.-C., Priyadarshini, M., Kudo, H., Semenova, S., Sundvik, M., et al. (2010). The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases. *Neurobiol. Dis.* 40, 46–57. doi: 10.1016/j.nbd.2010.05.010

Preston, A. N., Farr, J. D., O'Neill, B. K., Thompson, K. K., Tsirka, S. E., and Laughlin, S. T. (2018). Visualizing the brain's astrocytes with diverse chemical scaffolds. *ACS Chem. Biol.* 13, 1493–1498. doi: 10.1021/acschembio.8b00391

Rasheed, N., Ahmad, A., and Alghasham, A. (2012). Combined analysis of mRNA expression of dopamine receptors D1, D2 and cfos in different brain regions of stressed rats. *Pharmacologia* 3, 574–582. doi: 10.5567/pharmacologia.2012.574.582

Rebl, A., Verleih, M., Nipkow, M., Altmann, S., Bochert, R., and Goldammer, T. (2018). Gradual and acute temperature rise induces crossing endocrine, metabolic, and immunological pathways in maraena whitefish (*Coregonus maraena*). *Front. Genet.* 9:241. doi: 10.3389/fgene.2018.00241

Reid, S. G., Bernier, N. J., and Perry, S. F. (1998). The adrenergic stress response in fish: control of catecholamine storage and release. *Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol.* 120, 1–27. doi: 10.1016/S0742-8413(98)00037-1

Rosel, P., Arranz, B., Urretavizcaya, M., Oros, M., San, L., and Navarro, M. A. (2004). Altered 5-HT2A and 5-HT4 postsynaptic receptors and their intracellular signalling systems IP3 and cAMP in brains from depressed violent suicide victims. *Neuropsychobiology* 49, 189–195. doi: 10.1159/000077365

Sangkuhl, K., Klein, T. E., and Altman, R. B. (2009). Selective serotonin reuptake inhibitors pathway. *Pharmacogenet. Genomics* 19, 907–909. doi: 10.1097/FPC.0b013e32833132cb

Schreck, C. B., Tort, L., Farrell, A. P., and Brauner, C. J. (2016). *Biology of Stress in Fish*. Cambridge, MA: Academic Press.

Seki, K., Yoshida, S., and Jaiswal, M. (2018). Molecular mechanism of noradrenaline during the stress-induced major depressive disorder. *Neural Regen. Res.* 13:1159. doi: 10.4103/1673-5374.235019

Shimomura, Y., Inahata, M., Komori, M., and Kagawa, N. (2019). Reduction of tryptophan hydroxylase expression in the brain of medaka fish after repeated heat stress. *Zool. Sci.* 36:223. doi: 10.2108/zs180135

Silbergeld, E. K. (1974). Blood glucose: a sensitive indicator of environmental stress in fish. *Bull. Environ. Contam. Toxicol.* 11, 20–25. doi: 10.1007/BF01685023

Singh, C., Oikonomou, G., and Prober, D. A. (2015). Norepinephrine is required to promote wakefulness and for hypocretin-induced arousal in zebrafish. *eLife* 4, 1–22. doi: 10.7554/eLife.07000.001

Svingos, A. L., Periasamy, S., and Pickel, V. M. (2000). Presynaptic dopamine D4 receptor localization in the rat nucleus accumbens shell. *Synapse* 36, 222–232. doi: 10.1002/(sici)1098-2396(20000601)36:3<222::aid-syn6>3.0.co;2-h

Švob Štrac, D., Pivac, N., and Mück-Šeler, D. (2016). The serotonergic system and cognitive function. *Transl. Neurosci.* 7, 35–49. doi: 10.1515/tnsci-2016-0007

Teles, M. C., Dahlbom, S. J., Winberg, S., and Oliveira, R. F. (2013). Social modulation of brain monoamine levels in zebrafish. *Behav. Brain Res.* 253, 17–24. doi: 10.1016/j.bbr.2013.07.012

Thörnqvist, P.-O., Höglund, E., and Winberg, S. (2015). Natural selection constrains personality and brain gene expression differences in Atlantic salmon (*Salmo salar*). *J. Exp. Biol.* 218, 1077–1083. doi: 10.1242/jeb.114314

Thörnqvist, P.-O., McCarrick, S., Ericsson, M., Roman, E., and Winberg, S. (2019). Bold zebrafish (Danio rerio) express higher levels of delta opioid and dopamine D2 receptors in the brain compared to shy fish. *Behav. Brain Res.* 359, 927–934. doi: 10.1016/j.bbr.2018.06.017

Viltart, O., and Vanbesien-Mailliot, C. C. A. (2007). Impact of prenatal stress on neuroendocrine programming. *Sci. World J.* 7, 1493–1537. doi: 10.1100/tsw.2007.204

Vindas, M. A., Fokos, S., Pavlidis, M., Höglund, E., Dionysopoulou, S., Ebbesson, L. O. E., et al. (2018). Early life stress induces long-term changes in limbic areas of a teleost fish: the role of catecholamine systems in stress coping. *Sci. Rep.* 8:5638. doi: 10.1038/s41598-018-23950-x

Vindas, M. A., Gorissen, M., Höglund, E., Flik, G., Tronci, V., Damsgård, B., et al. (2017). How do individuals cope with stress? Behavioural, physiological and neuronal differences between proactive and reactive coping styles in fish. *J. Exp. Biol.* 220, 1524–1532. doi: 10.1242/jeb.153213

Vogelgesang, S., Niebert, S., Renner, U., Möbius, W., Hülsmann, S., Manzke, T., et al. (2017). Analysis of the serotonergic system in a mouse model of rett syndrome reveals unusual upregulation of serotonin receptor 5b. *Front. Mol. Neurosci.* 10:61. doi: 10.3389/fnmol.2017.00061

Vuilleumier, P. (2005). How brains beware: neural mechanisms of emotional attention. *Trends Cogn. Sci.* 9, 585–594. doi: 10.1016/j.tics.2005.10.011

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol. Rev.* 77, 591–625. doi: 10.1152/physrev.1997.77.3.591

Winberg, S., and Nilsson, G. E. (1993). Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. *Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol.* 106, 597–614. doi: 10.1016/0742-8413(93)90216-8

Winberg, S., Nilsson, G. E., and Olsén, K. H. (1992). Changes in brain serotonergic activity during hierarchic behavior in Arctic charr (*Salvelinus alpinus* L.) are socially induced. *J. Comp. Physiol. A* 170, 93–99. doi: 10.1007/BF00190404

Zhong, P., and Ciaranello, R. D. (1995). Transcriptional regulation of hippocampal 5-HT1a receptors by corticosteroid hormones. *Mol. Brain Res.* 29, 23–34. doi: 10.1016/0169-328X(94)00225-4

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.2. Publication II: Early response of salmonid head-kidney cells to stress hormones and toll-like receptor ligands

The head kidney (HK) is a suitable candidate to study stress-related neuro-immune interactions in teleosts, as it gathers hematopoietic populations and endocrine cells, which are in charge of secreting the stress hormones cortisol and catecholamines. The immediate immune response in the HK of *C. maraena* was tested *in vivo* after *A. salmonicida* i.p. injection. Subsequently, we characterized the primary culture of head-kidney cells from *C. maraena* using flow cytometry, microscopy and an extensive panel of qPCR assays. Finally, the HK primary culture was used to compare the influence of stress hormones cortisol, adrenaline and noradrenaline in parallel approaches on the establishment of an immune response and the expression of endocrine receptors.

Highlights

- The HK of *C. maraena* showed a pronounced response to *A. salmonicida*.
- Primary cell culture of the HK reflected well the composition of the entire organ. In addition, HK cells expressed a high density of endocrine receptors.
- Cortisol decreased the expression of cytokine genes stronger than catecholamines, while noradrenaline increased the expression of hormone receptors.
- The sensitivity of acute phase genes to immune challenges was confirmed by the *in vivo* experiments with *A. salmonicida* injection and the *in vitro* exposure of HK cells to TLR ligands.

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Early response of salmonid head-kidney cells to stress hormones and toll-like receptor ligands

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ABSTRACT

The functional spectrum of the teleostean head kidney covers haematopoietic, immune and endocrine signalling pathways with physiological effects that are likely to conflict if activated at the same time. An *in vivo* experiment on the salmonid fish maraena whitefish (*Coregonus maraena*) revealed that the head kidney shows a remarkably strong response after injection of *Aeromonas salmonicida* within 48 h. In order to investigate the potential influence of endocrine signalling on the initiation of immune responses, we established a primary culture of head-kidney cells of maraena whitefish. For the characterisation of this model system, we used flow cytometry complemented with an extensive panel of immunological/haematological and stress-physiological/neuroendocrinological qPCR assays. More than one third of the cells expressed the characteristic signature of myeloid cells, while more than half of the cells expressed those genes typical for lymphocytes and monocytes. In parallel, we quantified the expression of genes encoding endocrine receptors and identified *ADRA2D* as by far the most highly expressed adrenergic-receptor gene in head-kidney cells. The stimulation of the head-kidney cells with toll-like receptor ligands induced the expression of typical immune genes (*IL1B*, *CXCL8*, *TNF*, *SAA*) after only 1 h. The incubation with the stress hormones cortisol, adrenaline and noradrenaline also had an immune-activating effect, though less pronounced. However, cortisol had the strongest suppressive effect on the stimulation-induced immune response, while adrenaline exerted a comparably weaker effect and noradrenaline was almost ineffective. Moreover, we found that cortisol reduced the expression of genes coding for adrenergic and some glucocorticoid receptors, while noradrenaline increased it. In conclusion, the primary head-kidney cells of maraena whitefish reflect the immunological and neuroendocrinological diversity of the entire organ. This *in vitro* system allowed thus identifying the correlative changes between the activities of hormones and immune factors in salmonid fish in order to contribute to a better understanding of the regulation circuit between stress and immune defence.

1. Introduction

Strict hygiene standards, vaccination and/or resistant fish strains are among the most effective measures to encounter and prevent disease outbreaks and epidemics in finfish aquaculture. In addition, husbandry stress should generally be kept at low levels, as it influences many immunological processes and may therefore increase the susceptibility to infections [1]. The signalling cascades that are stimulated by stress and that activate the immune system eventually converge in the teleostean head kidney. Whenever antigenic structures are transported *via* the blood

Abbreviations: Aa, amino acid; ADR, adrenoreceptor; DAMP, damage/danger-associated molecular pattern; FACS, fluorescence-activated cell sorting; FSC, forward scatter; IL, interleukin; LPS, lipopolysaccharide; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B cells; NK cells, natural-killer cells; PAMP, pathogen-associated molecular pattern; p.i., post injection; PRR, pattern-recognition receptor; qRT-PCR, quantitative reverse-transcriptase polymerase-chain reaction; SAA, serum amyloid A; SSC, side scatter; TLR, toll-like receptor

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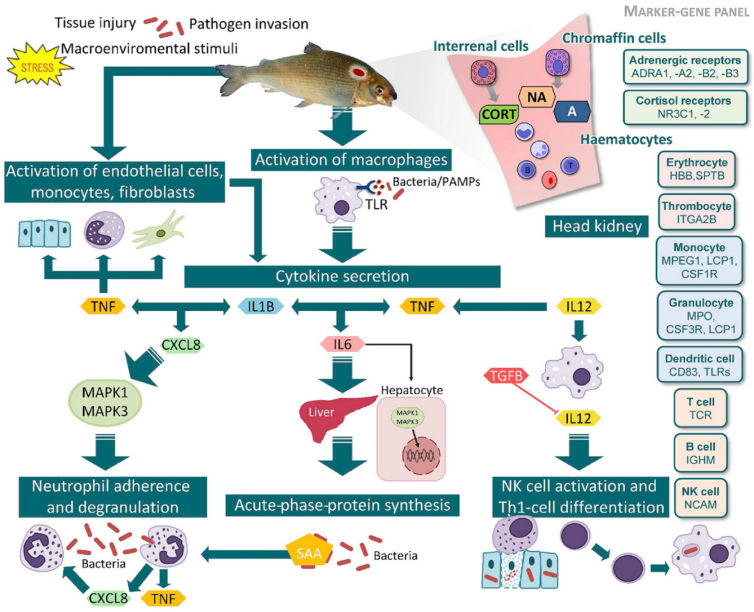

Fig. 1. Schematic representation of the teleost head kidney as important endocrine, haematopoietic, and immune-activating tissue. The major steps during the establishment of an immune response in the head kidney are illustrated; immune genes profiled in this study are highlighted by coloured hexagons. Gene names are abbreviated as listed in Table 1; the depicted stress hormones are CORT, cortisol; A, adrenaline; NA, noradrenaline. The panel of marker genes used for the characterisation of primary head-kidney cells is listed on the right side.

stream into the head kidney, they are recognised by pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs). These PRRs trigger the expression of pro-inflammatory cytokines (Fig. 1) including interleukin-1β (IL1B), IL6, IL12B, CXC-chemokine ligand-8 (CXCL8), tumour-necrosis factor α (TNF), as well as acute-phase proteins, such as serum-amyloid α (SAA) [2,3]. Simultaneously, anti-inflammatory mechanisms (e.g. by the cytokine TGFB) are induced to limit the extent of the immune response. In addition, the head kidney is the main site of haematopoiesis [4] and antibody production [5].

The haematopoietic and immunocompetent cells are in intimate association with interrenal steroidogenic and monoaminergic chromaffin cells [3,6,7] (Fig. 1). Stressful conditions stimulate the interrenal cells to release glucocorticoid hormones including the major stress hormone cortisol [8,9]. When cortisol binds to the glucocorticoid receptor (NR3C1) in the cytosol, this complex migrates to the nucleus and binds to its specific response elements to modulate the expression of a broad range of genes [10,11]. Thereby, cortisol influences the viability and proliferation of monocytic cells [12], the migration of lymphocytes, the mobilisation of myeloid cells and the production of pro-inflammatory mediators [13]. In parallel, the sympathetic fibres that innervate the head kidney trigger chromaffin cells to release the catecholamines adrenaline and noradrenaline. In fish, these catecholamines bind to α- and β-adrenoreceptors (ADRA, ADRB) in the membrane and modulate, together with other hormones, a multitude of physiological processes [14,15]. In particular, adrenaline and noradrenaline inhibit antibody production and suppress pro-inflammatory cytokines and phagocytic activity [16,17]. However, human monocytes showed enhanced pro-inflammatory responses when stimulated with an ADRB agonist [18]. In this manner, the neuro-immune circuit triggers a series of compensatory physiological processes to enable the sick individual to overcome its disease-related weakness and direct its metabolic resources to survival needs [19]. Conversely, this means that particular aspects of the immune system are constantly suppressed during periods of prolonged stress [20–23] augmenting the risk of pathogen invasion and infection. On the other hand, short-term stress is known to exert a stimulating effect on the immune system *via* the sympathetic nervous system [24–26].

Altogether, the effects of stress on the teleostean immune system are highly complex, since they depend on the type of stressor and its exposure time as well as the identity of the affected (immune-) cell populations and the species. So far, the influence of stress hormones in general, and catecholamines in particular, on the expression of immune genes in head-kidney cells has rarely been examined and provided in part contradictory results [16,17,27].

We chose maraena whitefish *Coregonus maraena* for our investigations, as this salmonid fish proved to be a suitable model organism for studying aspects of the salmonid stress physiology [22,28]. Maraena whitefish is remarkably more sensitive to particular stressors than its close relatives rainbow trout *Oncorhynchus mykiss* [22,29] and Atlantic salmon *Salmo salar*, which have long-term adaptations to intensive farming conditions [30,31]. Concomitant with the increasing commercial importance of maraena whitefish, efforts are intensifying to convert their production from extensive to intensive systems [32,33].

Given the increasing importance of maraena whitefish and taking into account the 3-R principles of animal experimentation, we established primary cultures of head-kidney cells to address the following questions: (i) Do gene-expression profiles of immune responses to bacterial challenges *in vivo* correspond to *in vitro* responses of head-kidney cells to PAMPs and are the expression dynamics comparable? (ii) To what extent do primary-cell preparations from head kidneys represent the immune-cell composition of the entire organ? (iii) How do stress hormones modulate the expression of genes involved in the immune-neuroendocrine crosstalk in head-kidney cells, and (iv) how do they influence the pro-inflammatory immune response to PAMPs?

2. Materials and methods

2.1. Husbandry and sampling of maraena whitefish

Maraena whitefish were held in fresh-water recirculation systems at

18 °C at a 12:12-h day-night cycle. Water quality was assured by continuous purification and disinfection (bio-filter and UV light). Water parameters including NH_4^+, NO_2^-, NO_3^- concentrations were measured twice a week, pH value, temperature and oxygen saturation were constantly recorded. The fish were fed commercial dry pellets (1.5–4.5 mm; Biomar) by automatic feeders at a daily rate of 0.8–4.0% of their total biomass.

The killing and sampling method followed the standards described in the German Animal Welfare Act [§ 4(3) TierSchG].

2.2. In vivo challenge of maraena whitefish with A. salmonicida

Details of the *in vivo* challenge have already been published [34]. In brief, we injected a 100-µl suspension containing 5×10^6 inactivated *A. salmonicida* ssp. *salmonicida* (wild-type strain JF 2267) into the peritoneal cavity of 18 maraena whitefish (n = 6 per time point). As a negative control, 15 maraena whitefish (n = 5 per time point) received an intraperitoneal injection of 100 µl sterile PBS solution. Fish were sampled 24 h, 48 h and 72 h post treatment for liver, spleen, head kidney, gills, and peritoneal cells.

2.3. Isolation and staining of head-kidney cells

Head-kidney tissues were isolated from freshly slaughtered fish and either homogenised for RNA isolation (*cf.* 2.6) or placed into a sterile container with Dulbecco's Modified Eagle's Medium (DMEM; Gibco) for FACS sorting or primary-cell isolation.

The isolation of head-kidney cells for cultivation followed in essence published protocols [13,35]. To obtain a single-cell suspension, head-kidney tissue was homogenised in DMEM solution using a steel sieve (500 µm, Roth). This head kidney-cell suspension was then successively sieved through two cell strainers of 200 µm (both PluriSelect) and thereafter 100 µm (Falcon). Subsequently, cells were centrifuged at 524 × g for 5 min and resuspended in 3.0 ml DMEM. The cell suspension was layered onto an isotonic Percoll gradient (3 ml; ρ = 1.084 g/ml) and centrifuged at 800 × g for 30 min (6 °C, minimum deceleration). The cell band in the DMEM/Percoll interface was collected and washed with DMEM. After an additional centrifugation step (524 × g, 5 min), the cell pellets were resuspended either in DMEM for primary-cell culture or in phosphate-buffered saline (PBS; Biochrom) for FACS sorting. For comparison, the Percoll-gradient centrifugation was omitted to assess its efficiency in reducing the number of erythrocytes. Prior to further steps, cell number and viability were measured with the Cellometer Auto 2000 (Nexcelom Bioscience). Finally, cells were subjected either to flow cytometry (*cf.* 2.4) or to cultivation in 6-well plates (*cf.* 2.5).

For a basic characterisation of the isolated immune-cell populations, head-kidney cells were placed on glass slides for May-Grünwald-Giemsa staining. May-Grünwald solution (Brand) was applied on the cells for 1 min, washed with distilled water and transferred to a 1:20-diluted Giemsa solution (Roth) for 30 min. After cleaning the slides with distilled water, microscopic observation was performed using the Nikon TMS-F microscope and a Nikon Coolpix E5000 camera with MDC Lens (Nikon).

2.4. Flow cytometry

Head-kidney cells suspended in 2 ml PBS solution (with a final concentration of ~1×10^7 cells/ml) were sorted using a MoFlo XDP high-speed cell sorter (Beckman Coulter) with an incorporated air-cooled Coherent Sapphire laser (488 nm, 100 mW). The cells were sorted through a 70-µm nozzle at 60 psi in purify mode. The forward scatter (cell size) was the trigger signal for all measurements. The side scatter (cell granularity) was employed to sort the cells in two fractions, low side-scattering intensity (fraction R1) and high side-scattering intensity (fraction R2). These fractions were then collected in PBS and re-analysed, with a final sorting efficiency of 94% ± 3%. This procedure was performed with head-kidney cells from five different individuals separately. To obtain a dry pellet of the fractions, cells were centrifuged at 500 × g for 5 min and used for RNA extraction (*cf.* 2.6).

2.5. Primary culture and in vitro stimulation experiments

Purified head-kidney cells were cultivated in DMEM (Gibco) with 10% foetal bovine serum (Gibco) and 0.1% antibiotic-antimycotic solution (Sigma) in 6-well plates (1.5×10^6 cells/ml). Cells were incubated at 18 °C in a 3%-CO_2 atmosphere to adjust the pH of the culture medium to that of the fish's body fluids.

Prior to conducting the stimulation experiments, we tested different incubation times and PAMP/hormone concentrations [36]. Eventually, primary head-kidney cells were cultured for 1 h, 3 h or 24 h with either cortisol (hydrocortisone, 600 ng/ml, Sigma-Aldrich/Merck), adrenaline ((−)-epinephrine (+)-bitartrate 10 µM, Sigma-Aldrich/Merck) or noradrenaline (L-(−)-norepinephrine (+)-bitartrate 10 µM, Sigma-Aldrich/Merck). Because cortisol is diluted in ethanol (Roth), we stimulated the primary cells also with this solvent to exclude that it affected the expression of the chosen target genes.

In addition, head-kidney cells were stimulated with highly pure natural or synthetic TLR ligands (all purchased from Invivogen), in the following referred to as pathogen-associated molecular patterns (PAMPs), either alone or in combination with the above hormones. We applied a mixture of different PAMPs mimicking the heterogeneous cocktail of microbial structures present during (co-)infections in the host's circulation. It included the TLR4 ligand lipopolysaccharide (LPS) from *Escherichia coli* (10 µg/ml), synthetic TLR2 ligands FSL-1 (100 ng/ml), Pam_2CSK_4 (100 ng/ml) and Pam_3CSK_4 (300 ng/ml), TLR5 ligand flagellin from *Salmonella typhimurium* (FLA-ST, 100 ng/ml) and TLR3 ligand poly(I:C) of low molecular weight (10 µg/ml). The individual stimulations were performed in triplicate in cells of three fish separately. Post-stimulation, the cells were harvested in 100 µl lysis buffer (Bioline) and 2 µl TCEP (tris(2-carboxyethyl)phosphine) reducing agent (Bioline).

2.6. RNA isolation and cDNA synthesis

Tissue samples were homogenised by 2.8-mm ceramic beads (Precellys, VWR/Avantor) at 6000 × rpm for 30 s using the Precellys24 Homogeniser (VWR/Avantor). Subsequently, total RNA was extracted using TRIzol Reagent (Life Technologies) and purified with the RNeasy Mini Kit (Qiagen). RNA from lysed cells was isolated and purified using the Isolate 2 RNA Micro Kit (Bioline) without previous TRIzol (Invitrogen) treatment. Finally, the RNA quality was checked by horizontal electrophoresis on 1%-agarose gels. The RNA concentration was determined using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

The SuperScript II Reverse Transcriptase Kit (Invitrogen, Life Technologies) was used to synthesise first-strand cDNA from purified total RNA. Reverse transcription was performed at 42 °C for 50 min followed by an inactivation step at 70 °C for 15 min. The resulting cDNA was purified using the High Pure PCR Product Purification-Kit (Roche) and diluted in 100 µl distilled water.

2.7. Real-time quantitative PCR (qPCR)

Since the sequences of most target genes were not available in the public databases, we searched our RNA-seq read collection from maraena whitefish [37]. The program Bowtie2 (v 2.2.4) was used to align orthologous sequences from *Oncorhynchus mykiss*, *Oncorhynchus kisutch* and *Salmo salar* with our reads. The Samtools package (v 1.6) was used to sort and index the obtained alignment. Then, the Ugene software (v 1.29) compiled the consensus sequences. Primers for qPCR assays were designed using the PSQ Assay Design Software 1.0.6 (Biotage AB). The

selected primer pairs amplified products with lengths between 121 bp and 239 bp with PCR efficiencies between 86.5% and 101.4%.

Small-scale qPCR assays were carried out with the LightCycler 96 System (Roche) using the SYBR Green fluorescence dye of the SensiFAST SYBR No-ROX Kit (Bioline) in clear LightCycler 480 multi-well plates (Roche). In detail, 1.5 μg of total RNA were reverse-transcribed into cDNA prior to the qPCR run. The qPCR cycling program included an initial pre-incubation step at 95 °C for 10 min, followed by 40 cycles with a 15-s denaturation at 95 °C, a 10-s annealing step at 60 °C, and a 20-s extension period at 72 °C. Relative quantification of expression was calculated using LightCycler 96 Software v1.1.

Multiplex-qPCR assays were carried out on 192.24 Dynamic Array IFC chips (Fluidigm) with EvaGreen fluorescence dyes (Bio-Rad) using the BioMark HD-System (Fluidigm). One microliter of total RNA was reverse-transcribed using the Reverse Transcription Master Mix (Fluidigm). Subsequently, a pre-amplification step was carried out using the PreAmp Master Mix (Fluidigm) and a "primer master mix" with a final concentration of 500 nM per primer pair. Following the cDNA pre-amplification, a treatment with exonuclease I (ExoI; New England BioLabs) was performed. Then, the cDNA samples were diluted in SsoFast EvaGreen Supermix with Low ROX (Bio-Rad) and 192.24 DELTAgene Sample Reagent (Fluidigm). The primer- and sample-master mixes were transferred to the assay and sample inlets on the IFC chip. After loading primers and samples onto the IFC chip using the IFC Controller RX (Fluidigm), this IFC chip was transferred to the BioMark HD-System (Fluidigm) to perform the quantification reactions following the GE 192 × 24 Fast PCR + Melt v2.pcl cycling program. The obtained qPCR data were analysed using the Fluidigm RealTime PCR Analysis Software v. 3.0.2.

The LightCycler-qPCR products were run on a 2%-agarose gel to assess the integrity and specificity of the PCR products. A standard curve based on serial dilutions (10^2-10^7 copies) of each specific PCR product was used to calculate the corresponding copy numbers. The geometric mean of the reference genes *EEF1A1b*, *RPL9,* and *RPL32* [38] was used to normalise qPCR data.

2.8. Statistical analysis

Multiple t-tests were conducted using the software GraphPad Prism 8 (Statcon) to calculate the statistical significance of the qPCR expression data. The traditional p-value of 0.05 was corrected according to the Bonferroni method to avoid type-I errors. Thus, the threshold for the gene-expression analysis of FACS-sorted cells was set at $p = 0.002$. For the expression data of the cell-stimulation experiments, a corrected $p < 0.0024$ indicated statistical significance.

3. Results

3.1. The head kidney of maraena whitefish shows a remarkably strong response to challenge with A. salmonicida

To compare *in vivo* and *in vitro* responses, we first studied gene expression following bacterial challenge *in vivo*. To this end, we established 24 immunological and/or haematological and 15 stress-physiological and/or neuroendocrinological qPCR assays (Table 1; Fig. 1) based on our 1.3 million RNA-sequencing reads from maraena whitefish [37]. These assays included the pro-inflammatory and acute-phase genes *IL1B*, *IL6*, *CXCL8*, *TNF, IL12B*, *SAA, MAPK1,* and *MAPK3*, which were used to profile the *in vivo* response in head kidney, spleen, liver, gills and in peritoneal cells of maraena whitefish at three different time points (24 h, 48 h, 72 h) after intraperitoneal injection of inactivated *A. salmonicida*.

All selected immune genes were significantly regulated after *A. salmonicida* injection, but their profiles reflected a particular tissue/cell type (Fig. 2). In the peritoneal cells from the site of injection, the transcript concentrations of *IL1B*, *CXCL8,* and *TNF* increased with progressive time post injection (p.i.) resulting in fold-change values between 21.1 (*CXCL8*) and 42.1 (*TNF*). In the head kidney, the concentration of particular transcripts followed a more pronounced dynamic. Here, *IL1B, IL6, IL12B, TNF,* and *MAPK1* showed nearly congruent expression patterns, starting with no or a slight up-regulation at 24 h, which then turned into a strong up-regulation at 48 h with fold-change values between 16.0 (*TNF, MAPK1*) and 122.0 (*IL1B*). Finally at 72 h, expression was even downregulated with fold-change values ranging from −2.7 (*MAPK1*) to −33.2 (*IL6*). In the spleen and gills, transcript levels of the selected immune genes were only moderately (less than 10-fold) regulated at the different time points after stimulation compared to non-stimulated controls. At 72 h p.i., *SAA* in spleen (9.5-fold) and *IL12B* in gills (4.9-fold) were the most highly up-regulated genes. In the liver, *IL1B*, *SAA,* and *MAPK1*-transcript levels were 2.1- to 8.0-fold increased across the three time points p.i., while *IL6* and *MAPK3* were downregulated (~-3-fold) at 72 h.

In summary, the head kidney of maraena whitefish exhibited strong dynamics in the expression of early immune genes within the first 48 h p.i. of *A. salmonicida*, whilst the spleen and gills responded only to a limited extent.

3.2. A primary-cell culture of head kidney from maraena whitefish sufficiently represents the immune-cell composition of the entire organ

Although widely used as a model system [39] the cellular composition of primary cultures of head-kidney cells has rarely been thoroughly analysed, especially in non-model fish species, for which detection antibodies are scarce. To characterise an unstimulated *in vitro* model of the head kidney from maraena whitefish with or without prior purification by Percoll-gradient centrifugation, we combined traditional staining and microscopy techniques with FACS sorting and real-time qPCR. The May-Grünwald-Giemsa staining of cells isolated from the head kidney confirmed the presence of erythrocytes (ellipsoid cells with orange-stained cytoplasm and blue-stained nuclei), lymphocytes (spherical cells with a thin rim of cytoplasm in dark blue and purple nuclei), monocytes (spherical cells with clear blue cytoplasm and kidney-shaped nuclei), and granulocytes (spherical cells with clear blue cytoplasm and lobulated purple nuclei) (Fig. 3A). All cells were apparently smaller than 10 μm. The flow-cytometric sorting of the unstimulated head-kidney cells allowed the separation of two cell suspensions, R1 enriched with less granular and smaller cells and R2 with more granular and larger cells (Fig. 3B). In repeated analyses with a total of five fish, more than half (55.5% ± 3.0%) of all cells isolated from the head kidney were sorted to fraction R1 and slightly more than a third (36.6% ± 2.7%) to fraction R2.

To determine the composition of these two cell suspensions R1 and R2, we subjected both fractions to cell-type specific gene-expression profiling (Fig. 3C1). To this end, we used 15 markers, which had previously been reported as characteristically expressed genes in distinct immune-cell populations of fish [22,34,40–42]. These qPCR analyses revealed that the R1 suspension contained significantly higher levels of *TCR* (specific for T cells), *NCAM1* (NK cells), *MPEG1* (monocytes/macrophages) and *SPTB* (erythrocytes) compared with the R2 suspension (with $p < 0.001$). Moreover, the R1 fraction contained pronounced copy numbers of *IGHM* (B cells) and *ITGA2B/CD41* (thrombocytes) compared to R2 ($p < 0.04$). *DNTT* (specific for lymphoblasts) was expressed at a very low level (1–10 copies/ng RNA) in both fractions, though significantly higher in R1 (with $p < 0.0001$). In the R2 fraction, we recorded significantly higher copy numbers of *MPO*, *CSF3R* (both specific for granulocytes), and *LCP1* (myeloid cells) compared with the R1 fraction (with $p < 0.0001$). *CSF1R* (monocytes) was weakly expressed in R2, but tended to be stronger in R1. *TLR22b*, *TLR9,* and *CD83* transcripts (indicating the presence of dendritic cells) as well as *HBB* (erythrocytes) were similarly expressed in both fractions.

The purification of the isolated cells *via* a Percoll gradient significantly reduced the number of erythrocytes but did not eliminate

Table 1
Gene-specific qPCR primers used in this study.

Gene symbol (alias name)	Sense, antisense primer (5'→ 3')	Gene product; function	Reported site of expression
ADRA1B	CCGGGAGAAGAAAGCAGCTAAA, AGTTGAAGTAGCCCAGCCAGAA	Adrenoceptor α1B; contraction of blood vessels	Spleen, kidney, blood vessel
ADRA1D	TCCGTGCGCTTGATGAAGTTCT, AGACCATGTCAGATGGCTTCAG	Adrenoceptor α1D; contraction of blood vessels	Blood vessel
ADRA2A	TCCGCCCTATAGCCTCCAGAT, GACGCCAACGAAACCAGGAAG	Adrenoceptor α2A; inhibition of NA release, hypotension	Brain, spleen, kidney
ADRA2B	AGCCACAGCGCTCTTTGCTAC, GGATGATGAGGGTCGCCACTA	Adrenoceptor α2B; regulation of vascular tone	Kidney
ADRA2C	TGTGGCTGATCTCGGCTGTTAT, AACCTGGTAGATCCTGATGAAGA	Adrenoceptor α2C; inhibition of adrenaline secretion	Brain, adrenal gland
ADRA2D	GTAGTGGCCGTTTTCACCAGC, CACACCAGGTGCTCCCAAAGT	Adrenoceptor α2D; unknown function	Brain, heart
ADRB2	TTCCAGCGGCTGCAGACGGT, AAGACGTCCGCCGCTGTCCA	Adrenoceptor β2; activation of glycogenolysis	Lung, lymphocyte
ADRB3a	GTTTGGATTGTGTCTGCTCTGAT, TGAGCAAAGGGATGTAGAATGAC	Adrenoceptor β3; lipolysis, ion transport	Adipose tissue
CD83	GTGGTACAAGCTCGGTGAGGA, TTACATTGGGCAGGAAGATATCG	CD83 antigen; antigen presentation	Dendritic cell
CSF1R (MCSFR)	TGCAACGTCCCGGTGAATACAA, ACAGCTACCTGGAGATGAGGC	Colony-stimulating-factor-1 receptor; cell differentiation	Macrophage
CSF3R (GCSFR)	ACAGCCACTCCTGGAGGACG, GCCAAAGCCCTAAGCAAGGGA	Colony-stimulating-factor-3 receptor; cell differentiation	Myeloid cell/granulocyte
CXCL8 (IL8)	TAGACTCATTAAGAAGGTGGAGAT, TGTTGGCCAGCATCTTCTCAAC	C-X-C-motif-chemokine ligand 8; chemoattraction	Leukocyte
DNTT	TGCAGAGAGCTGGTATCCTGTA, CTCCTTCCCCCTGCGAAAGC	DNA nucleotidylexotransferase; cell maturation	Lymphoblast
HBB	GGGAAGAGTTCTGATCGTCTAC, ACTTGTATGTGGCCAAGATGTTG	Haemoglobin subunit beta; oxygen transport	Erythrocyte
HSP90AA1 (HSP90)	AGATACGAGAGCTTGACAGACC, TGCCAGACTTGGCGATGGTTC	HSP90α-family-class A member 1; chaperone function	Ubiquitous
HSPA1A (HSP70)	CTCCTCTGGGTTGAAGGATTTG, GGCCATGAACCCCAACAACAC	Heat-shock-70kD protein 1A; chaperone function	Ubiquitous
IGHM	AATTCCAATTCATTGAGCCAACTC, TCCCTCACGTTCGTCATATTCTT	Immunoglobulin, heavy mu chain; antigen binding, cell/cell interaction	B cell
IL12B	GTTCGTCATGCTCCTCTGTGTT, CATTCTCGCCCTCGTACTTCC	Interleukin-12B; NK-cell activation	Leukocyte
IL1B	AAGGACAAGGACCTGCTCAACT, ACCCAGCTCTTGTTCTCAGAGT	Interleukin-1β; inflammation	Leukocyte
IL6	AGAAGCCTGTGGAAGAGATGTC, ATTGTGGGTATTCAGCCTTAACG	Interleukin-6; inflammation	Leukocyte
ITGA2B (CD41)	GACAAGCAAGCATAAACAACTATC, TGCAGTTATAGGGAGTAAAACAAG	Integrin subunit alpha 2b; cell-adhesion	Thrombocyte
LCP1	ATGTTGGAGCAGGCTGACCGT, CTCTGGTCTCACCCTCGATTG	Lymphocyte cytosolic protein 1; cell adhesion	Monocyte
MAPK1 (ERK2)	GTACATCCACTCAGCTAACGTC, GCTACATACTCCGTCAGGAAAC	Mitogen-activated-protein kinase 1; regulation of transcription	Ubiquitous
MAPK3 (ERK1)	CTATCCGTGCCAGCCCAAAGT, GTAACGACCACACCTGCTACTT	Mitogen-activated-protein kinase 3; regulation of transcription	Ubiquitous
MPEG1	CATCAACACTTGGCTGGAACAG, GTTCCGTACTTGAACACGAGCG	Macrophage-expressed gene 1 protein; microbicidal activity	Macrophage
MPO	TCTGCAACAACCTGAAGTACCC, TACGGTTAGAGACCTCCCTTAC	Myeloperoxidase; microbicidal activity	Neutrophil granulocyte
NCAM1	AAACCAGAGATCACCGTAACCC, AGGTGATCCTTTGGTAGAACTTG	Neural cell adhesion molecule 1; cell-to-cell interaction	NK cell
NR3C1a (GR1)	CACAGTACCAAAGGATGGATTGA, TGCATGGAGTCCAGTAGCTTAG	Glucocorticoid receptor 1; regulation of transcription	Ubiquitous
NR3C1b (GR2)	TGCCCCTCAGACTTTTACGTAG, TGGTGGTGTTGGAACCGCTAAA	Glucocorticoid receptor 1, variant b; regulation of transcription	Ubiquitous
NR3C2 (MR)	TGCTCAGCCCAGTTCCCAAAGA, TGCATGGCGTCCAGTAGCTTG	Mineralocorticoid receptor; regulation of transcription	Ubiquitous
SAA	TTCCCTGGTGAAGCTGCTCGA,TGACTCCTGCTGCCCACCTG	Serum-amyloid A; chemoattraction	Liver
SPTB	CACTAAGGGGCGCATGCGTAT, CAATGTCTTGGATCTGGAATCTG	Erythrocytic spectrin beta; membrane stability	Erythrocyte
STAR	TGTGCGCTGGCATCTCCTACA, ATCCGCAAGCTGAGCAGTGAG	Steroidogenic Acute Regulatory Protein; synthesis of steroid hormones	Interrenal cell
TGFB1b	CATTCCAAGGTGCTAGGTCTGT, TCTTGGGGGTCTTGCCGATGT	Transforming-growth factor b1, variant b; anti-inflammatory function	Leukocyte
TH	GTTCGAGACGTTTGAAGCTAAGA, TTTTGACGTCCTCTGCGATCCT	Tyrosine hydroxylase; synthesis of catecholamines	Chromaffin cell
TLR22b (TLR13)	CGGAGAGCAACAACCAAACA, GAGATAAGCCAGGAAGAGGTAGT	Toll-like receptor 22b; pattern recognition	Dendritic cell
TLR9	CCGCTCCTGCCAGGAAATCTAT, AGCTGGGTGTACCCCTTGTGTC	Toll-like receptor 9; pattern recognition	Dendritic cell

(*continued on next page*)

Table 1 (*continued*)

Gene symbol (alias name)	Sense, antisense primer (5'→ 3')	Gene product; function	Reported site of expression
TNF (TNFα)	TTTTCCCAGGGTGGGTTCGAG, GGAGTCTGAATAGCGCCAAATAA	Tumour-necrosis factor; inflammation	Leukocyte
TCR (TCRa)	GTAAAAGATGACATTGCAGGTGAA, CAACGATCACAACAGAACTGAAG	T-cell receptor α; cell-cell interaction	T cell

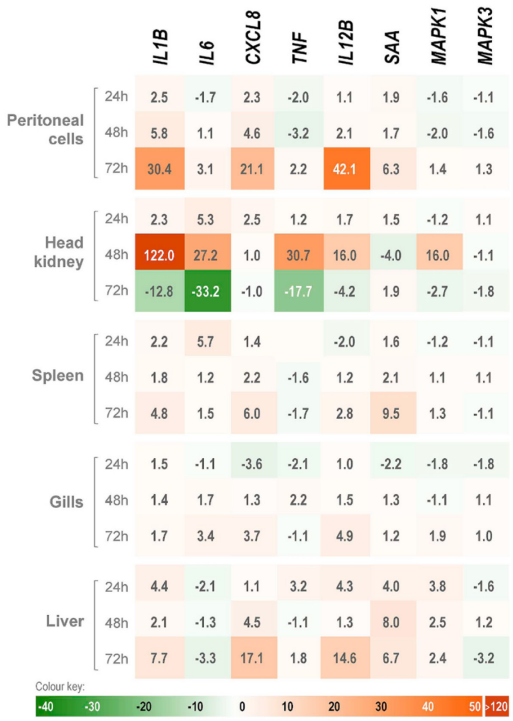

Fig. 2. Modulation of immune genes after injection of inactivated *A. salmonicida* into maraena whitefish. The heat map illustrates average expression ratios as fold-change values of the *A. salmonicida*-treated group relative to the control groups (n = 5 each) for the immune genes *IL1B*, *IL6*, *CXCL8*, *TNF*, *IL12B*, *SAA*, *MAPK1*, and *MAPK3* (listed in columns) as measured by multiplex qPCR. *EEF1A1b*, *RPL9*, and *RPL32* were used as reference genes to normalise the data. The analysed cells and tissues are listed on the left together with the time points p.i.

them completely. However, it hardly altered the outcome of the cell-sorting procedure. This was observed after microscopic examination and confirmed in a parallel qPCR analysis revealing that the copy numbers of *HBB* were significantly reduced. *Vice versa*, *HBB* was the dominant transcript (86.7%) among the 15 blood-cell markers in the discarded fraction after the Percoll purification (data not shown).

Additionally, we wanted to determine the "neuroendocrine capacity" of R1 and R2. These two fractions contained substantial copy numbers of *TH* (between ~ 1100 and 1200 copies/ng RNA) and *STAR* (between ~ 260 and 300 copies/ng RNA) indicating the presence of chromaffin and interrenal cells, respectively (data not shown). Almost all transcripts of the 11 selected receptors for cortisol (*NR3C1a, -b, 2*) and catecholamines (*ADRA1B, -A1D, -A2A, -A2C, -A2D, -B2, -B3A*) were similarly well represented in both fractions (Fig. 3C2). Only *ADRA1B* and *-A1D* were not detectable (although three different primer pairs were applied to each). *ARDA2D* had the highest transcript level in both fractions (> 2500 copies/ng RNA). Notably, we detected in fraction R1 slightly, but significantly higher levels of *NR3C2*, while fraction R2 contained higher levels of *NR3C1* transcripts.

Finally, we recorded the gene-expression patterns of the panel of immune-cell markers (*cf.* Fig. 3C1, w/o *HBB* and *SPTB*) in (i) the entire head kidney of untreated fish and (ii) isolated, Percoll-purified head-kidney cells in order to determine whether certain immune-cell types were enriched or depleted in the eventual primary-cell culture. We note in this context that the strength of the expression of a marker gene does generally not correspond directly with the percentage of an immune-cell population. The quantification of the immune-cell genes in a tissue sample of the entire head kidney revealed that the B-cell marker *IGHM* accounts for more than half (54.6%) of all selected immune cell-marker transcripts, followed by the markers *MPO* (33.9%) and *CSFR3* (5.4%), which are both characteristic of granulocytes (Fig. 3D1). The proportion of the T-cell marker *TCR* (2.5%) and the thrombocyte marker *ITGA2B* (1.3%) were detectable in the single-digit percentage range, while the proportion of the marker genes for monocytes, dendritic cells, lymphoblasts, and NK cells were below 1%. These expression data are essentially similar to the data collected for the primary head-kidney cells (Fig. 3D2). Nevertheless, the copy number of the dendritic cell marker *CD83* increased from 0.1% in the entire head kidney to 3.3%, while the copy number of the thrombocyte marker *ITGA2B* decreased from 1.3% to 0.7%.

Taken together, our expression profiling proved that a primary-cell culture of the head kidney from maraena whitefish represents the broad spectrum of chromaffin, interrenal, and immune cells of lymphoid and myeloid origin and thus well reflects the cell composition of the entire head kidney.

3.3. Cortisol is more effective than catecholamines in reducing the expression of cytokine genes in head-kidney cells

To study the immune-neuroendocrine crosstalk, we stimulated head-kidney cells with (i) stress hormones including cortisol, adrenaline or noradrenaline; (ii) PAMPs including LPS, FSL-1, Pam_2CSK_4, Pam_3CSK_4, flagellin, and poly(I:C); and (iii) PAMPs together with one of the stress hormones each.

The incubation of head-kidney cells with one of the three stress hormones revealed that adrenaline had the strongest inducing effect on the expression of the acute-phase genes *IL1B* (14.8-fold), *CXCL8* (10.0-fold), *TNF* (2.8-fold), *IL6* (2.8-fold), and *SAA* (7.4-fold) within 3 h of incubation (Fig. 4A). After 24 h, the *SAA* transcripts remained at an elevated level, while the transcript levels of the cytokine genes returned to the level found in unstimulated cells. Additionally, adrenaline slightly induced (up to 3.0-fold) the expression of heat-shock genes after 24 h of incubation (Fig. 4B). Cortisol had a comparatively weaker effect on the expression of *IL1B* (3.8-fold), *CXCL8* (2.8-fold), and *SAA* (14.7-fold) during a 3-h period of incubation. After 24 h, *SAA* continued to increase (up to 20.8-fold), while the transcript levels of most cytokine genes were partially reduced (−2.5- to −2.7-fold) compared to control cells. Concomitantly, cortisol decreased (−1.4- to −3.5-fold) the level of transcripts encoding adrenergic and mineralocorticoid

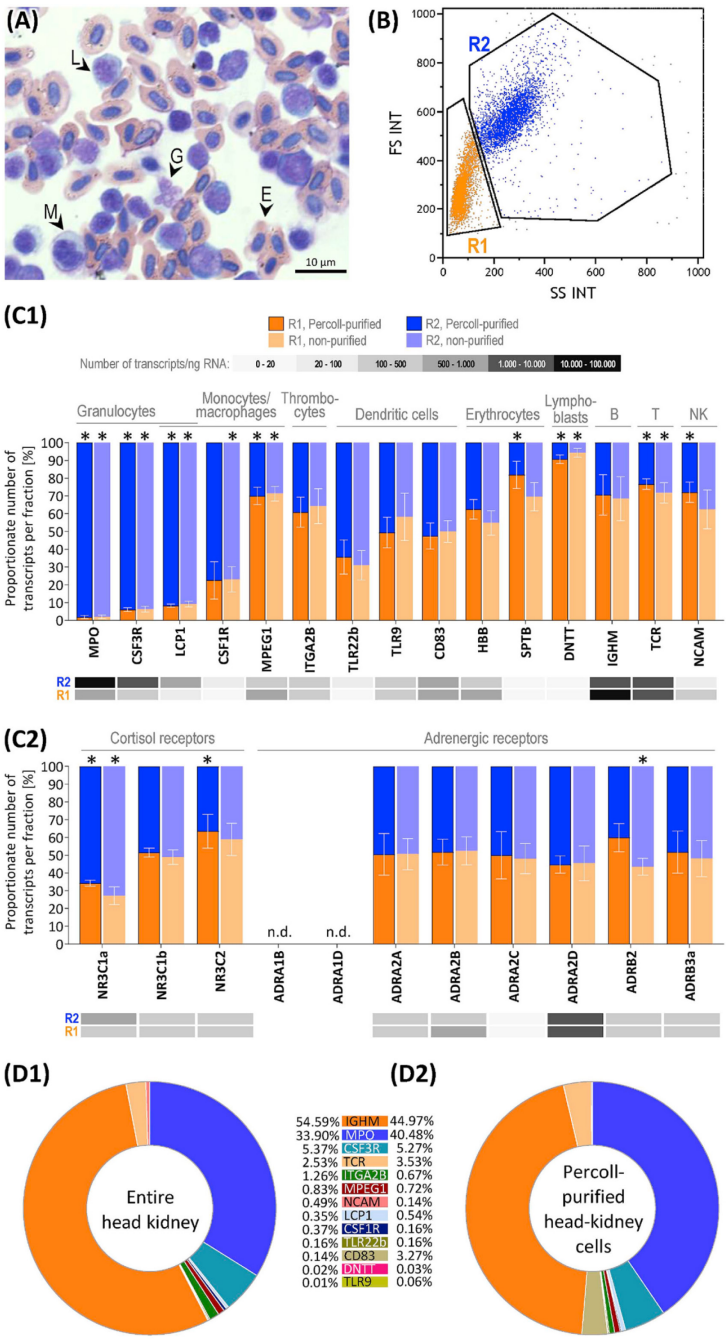

Fig. 3. Composition of unstimulated head-kidney cells isolated from maraena whitefish. (**A**) Microscopic observation of freshly isolated head-kidney cells from maraena whitefish, stained with May-Grünwald-Giemsa solution. Four morphologically distinct blood-cell types are labelled: E – erythrocyte; G – granulocyte; L – lymphocyte; M – monocyte. Scale bar represents 10 µm. (**B**) Representative dot plot of the flow-cytometric analyses (n = 5) of head-kidney cells isolated from five untreated maraena whitefish. The sorting of these cells was carried out according to the parameters cell volume (abscissa) and granularity (ordinate). The resulting two cell fractions are indicated as orange (R1) and blue (R2) clouds. qPCR profiling was performed in both fractions with a panel of (**C1**) blood cell-marker genes and (**C2**) genes encoding stress-hormone receptors. Two bars per gene are shown. The left bars (dark blue/orange) represent the results measured in head-kidney cells having been purified by Percoll density-gradient centrifugation; the right bars (light blue/orange) represent measurements in non-purified head-kidney cells. The individual bars represent the percentage of the transcript numbers recorded in fraction R1 (orange) and R2 (blue), respectively, relative to the summed transcript numbers of R1 + R2. Expression values were normalised using *EEF1A1b*, *RPL9*, and *RPL32* as reference genes; n.d. indicates not detectable expression. Error bars indicate SEM; asterisks mark significant ($p < 0.05$) transcript differences between fraction R1 and R2. The heat map below each graph indicates the average level of absolute copy numbers per fraction. Profiling of immune-cell markers in (**D1**) entire head kidney isolated from three untreated maraena whitefish and (**D2**) Percoll-purified head-kidney cells (n = 3) from the head kidneys of three fish. The average percentage of the three individual transcripts in the total transcript number is given. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

receptors (Fig. 4B). The 24-h incubation with noradrenaline elevated the transcript abundance of some immune genes by 2- to 3-fold (*IL1B*, *IL6*, *IL12B*), while others (*CXCL8*, *SAA*) were downregulated to a similar extent (Fig. 4A). Nonetheless, the transcript level of almost all investigated adrenergic receptors increased at least 2.3-fold after 24-h incubation with noradrenaline (Fig. 4B).

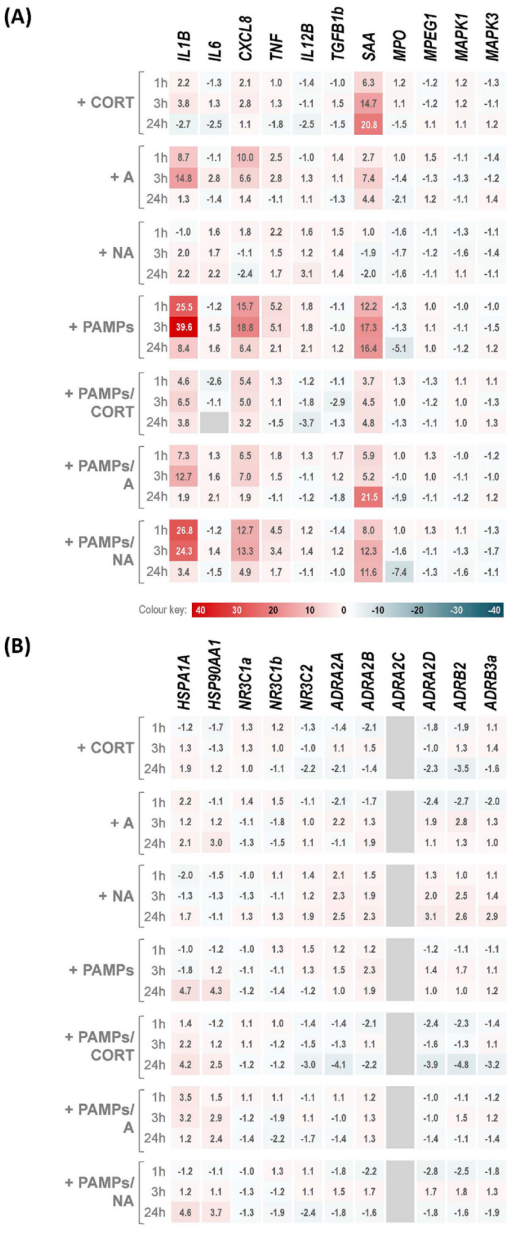

Fig. 4. Modulation of immune and stress-related genes after *in vitro* stimulation of Percoll-purified head-kidney cells with stress hormones and PAMP reagents. The heat map illustrates the average expression ratios as fold-change values of cells incubated with cortisol (CORT), adrenaline (A), noradrenaline (NA), PAMPs and combinations hereof relative to the untreated controls for (**A**) the panel of immune genes and (**B**) the panel of stress-related genes, as indicated above the drawings. *EEF1A1b*, *RPL9,* and *RPL32* served as reference genes. The different sampling time points together with the treatment categories are given on the left hand; grey fields in the heat map indicate non-detectable transcripts. The experiments were repeated with cells of three individual fish.

The stimulation of head-kidney cells with the PAMP cocktail alone had the greatest effect on the expression of the selected immune genes. The copy numbers of *IL1B*, *CXCL8* und *SAA* were strongly upregulated (~17- to 40-fold) as early as 3 h after PAMP stimulation (Fig. 4A). After 24 h, *HSPA1A* and *HSP90AA1* also exhibited a moderate increase (~4- to 5-fold) in copy number (Fig. 4B). The combination of PAMPs plus cortisol reduced the transcript levels of *IL1B*, *CXCL8*, *TNF,* and *SAA* by ~ 3- to 6-fold compared to the stimulation with PAMPs alone (Fig. 4A). The incubation with PAMPs plus adrenaline resulted in most cases in similar copy numbers as recorded after incubation with adrenaline alone. However, the transcript levels of *IL1B*, *CXCL8,* and *TNF* were significantly lower after PAMPs/adrenaline stimulation compared to stimulation with PAMPs alone. The transcript level of *SAA* was the only exception, as it was almost five times higher after stimulation with PAMPs/adrenaline compared with stimulation with adrenalin alone or with PAMPs alone. The incubation with PAMPs plus noradrenaline resulted in only minor changes in transcript levels compared to stimulation with PAMPs alone. After 24 h of stimulation with PAMPs and noradrenaline, we detected, moreover, a slight downregulation of adrenergic-receptor genes, although this was less pronounced than the effects induced by cortisol.

In summary, our experiments suggest that the three stress hormones regulate early immune functions in an ambivalent manner. Nevertheless, adrenaline mostly has a stimulatory impact on the immunity of head-kidney cells, while cortisol acts rather as an immune-suppressive. In addition, noradrenaline upregulated the expression of adrenergic-receptor genes, while cortisol reduced the expression of the respective genes with increasing time of incubation.

4. Discussion

The teleostean head kidney is not only a lympho-myeloid compartment, but also the key mediator between the brain and endocrine cells (reviewed in Refs. [2,3,8,43]). Consequently, different signals, which are likely to have conflicting physiological effects, may reach the head kidney at the same time. The major goal of the present study was thus to analyse the early regulation of immune and endocrine genes after stimulation of primary head-kidney cells from maraena whitefish.

4.1. Profiling of head kidney-cell cultures from maraena whitefish reveals the tissue-characteristic composition of immune-cell populations and a high density of hormone receptors

For the comparison of *in vivo* immune responses with head-kidney cells *in vitro,* we first studied the expression profile of a panel of immune-relevant genes within 72 h p.i. of *A. salmonicida* into maraena whitefish. In the head kidney, we found the highest levels of transcripts encoding pro-inflammatory cytokines at 48 h p.i., which generally characterise a strong inflammatory response, similarly as described for other salmonid species upon bacterial infection [44–46]. After 72 h p.i., a strong down-regulation of some of the cytokine genes in the head kidney occurred concomitantly with the significant up-regulation of the same genes in peritoneal cells. These dynamics are consistent with our

previous study on maraena whitefish, which revealed that the proportion of granulated cells increased in the peripheral blood accompanied by altering concentrations of immune-relevant transcripts in certain tissues in the course of the progressive inflammation [34]. The tissue-dependent variations in copy numbers suggested that immune cells migrate from the head kidney to the site of bacterial injection and to the spleen as a secondary lymphoid organ. A chemokine gradient most likely directs this trafficking, as previously described in other studies on salmonid fish species [44,47,48]. Hence, these data together document once more that the head kidney is of outstanding importance for establishing a local and/or systemic immune response by the enhanced proliferation of immune cells and the expression of pro-inflammatory mediators.

Since cell cultures allow analysing particular functions under precisely defined and adjustable conditions, we used a primary culture of head-kidney cells to investigate the crosstalk between stress hormones and immune activation. Primary cells are generally characterised by the high integrity of the original biological activity compared to cell lines [49]. For these reasons, they are considered the preferred tool for *in vitro* analyses [39]. However, a primary culture of head-kidney cells represents a complex composition of leucocytes, adipocytes and connective-tissue cells [50,51] that exert highly cell-type- and moreover species-specific stress and immune responses [11,52,53]. Our flow-cytometric analysis of the isolated head-kidney cells from maraena whitefish revealed the presence of two distinct cell populations, as previously reported for head-kidney cells from rainbow trout [48]. About 56% of the head-kidney cells from maraena whitefish were smaller and less granular and contained high levels of transcripts encoding the B- and T-cell receptors IGHM and TCR, respectively, and transcripts encoding the perforin MPEG1. Based on these results, this fraction appears to be a mixture of lymphocytes and monocytes/macrophages. About 37% of the head-kidney cells were larger cells with dominant intracellular granules and high levels of *MPO*, *CSF3R,* and *LCP1*, all of which are considered as markers for granulocytes. The markers for chromaffin (*TH*) and interrenal cells (*STAR*) were strongly expressed in both fractions.

The quantity of cells included in both FACS-sorted fractions correlated well with the results of the qPCR profiling of primary cells of the head kidney. These measurements showed that on average ~58% of the recorded immune-marker transcripts indicate lymphoid cells, while less than 40% are granulocyte-specific transcripts. In this context, we note again that the expression level of (a) particular marker gene(s) does not allow predicting the actual relative number of cells that are positive for this respective marker. Remarkably, the qPCR analyses of the isolated head-kidney cells compared with the entire head kidney suggested similar blood-cell compositions. This underscores the suitability of primary-cell cultures of teleostean head kidneys as a valuable model system for fish immunologists.

A further aspect of our study was the qualitative and quantitative detection of the repertoire of receptors for both cortisol and catecholamines in the head kidney of maraena whitefish. All selected receptors were abundantly expressed in both fractions of head-kidney cell except for *ADRA1B* and *–D*. Those are restricted to distinct mammalian neuronal cell populations [54] and our preliminary results confirmed that the same applies to fish (data published elsewhere). The copy number of *ADRA2D* was almost three times higher than the sum of all copy numbers for the other ten receptor genes together, which had been quantified in both cell fractions of the head kidney. This was a rather unexpected result, since *ADRA2D* is considered to be a “dispensable” paralog of *ADRA2A*, which has been “lost two independent times during the evolutionary history of vertebrates” [55]. The expression profiles obtained for head-kidney cells of whitefish suggest that the receptor density of the α-adrenoreceptors 2D is almost as high as that of the colony-stimulating factor-3 receptor on granulocytes in fraction R2, and more than half as high as the T cell-receptor density in cell fraction R1. This example illustrates that the immune cells of the teleostean head kidney are intimately involved in stress-hormone signalling, which in turn modulates their own immune activity [56,57]. This has been proven by our subsequent *in vitro* tests.

4.2. Immune and neuroendocrine responses of head-kidney cells in response to cortisol and catecholamines are species-specific

The classes of molecular patterns that communicate with the defence system are well established. The damage-/danger- and the pathogen-associated molecular patterns (DAMPs and PAMPs) alert the host either after injury or in the presence of microorganisms and viruses [58]. Beyond that, there are neural and endocrine signals that transmit information from macroenvironmental sensing to modulate immunological processes, as well [8,19].

The *in vitro* stimulation with PAMPs induced a rapid and strong up-regulation of the pro-inflammatory cytokine genes *IL1B* and *CXCL8* in maraena whitefish, which matches well with results from other studies on stimulated head-kidney cells of diverse fish species [59–61]. *SAA* was also immediately and significantly up-regulated at all three time points of PAMP stimulation. Although the liver is generally considered the main source of SAA in fish [62–64], our data prove, once again, that the head kidney has the potential to produce considerable amounts of SAA. Previous studies demonstrated that the level of *SAA* transcripts is remarkably increased in fish after the exposure to various stressors including *in vitro* stimulation with PAMPs [63] or *in vivo* infection with microorganisms [64–68] or parasites [69], rising temperatures [70], hypoxia [71], skin lesions [72], hormonal treatment [73], high stocking density [22,74], and confinement [75]. These correlations suggest that the concentrations of SAA increase in fish not only in the context of inflammation, but also during stress responses, as known for mammals [76–78]. In line with these reports, we observed that the incubation of the head kidney-cell cultures with cortisol continuously increased the copy numbers of *SAA* over time, similar to the stimulation with PAMPs. In contrast, within combination with PAMPS, cortisol antagonised PAMP effects and led to a downregulation not only of *SAA*, but of the cytokine-encoding genes as well.

Cortisol-immune interactions have been well explored in studies on primary [13,16,17,79,80] and secondary [81] cell cultures from various fish species, most of which agree that cortisol suppresses the stimulation-dependent synthesis of cytokines, SAA and other pro-inflammatory mediators. Although considered anti-inflammatory, cortisol on its own initially (after 3 h) upregulated the pro-inflammatory cytokine genes *IL1B*, *IL6,* and *TNF* and downregulated them after 24 h of incubation. This response pattern deserves further attention. The concentration of cortisol used here is in the same order of magnitude as observed in the plasma of cortisol-treated salmons [82] and of other salmonid species after stress [83] with some variations depending on species and stressor.

In contrast to cortisol, there are only a few, partially contradictory, studies that investigated the influence of catecholamines on the immune activity in fish cells. A previous study in head-kidney cells from gilthead seabream *Sparus aurata* showed that adrenaline weakly downregulated *IL1B* and *TGFB1* within 2 h of incubation, but strongly decreased the LPS-induced *IL1B* expression [17]. Another study on head-kidney cells from two different fish species demonstrated that the 2-h incubation with adrenaline reduced the expression of several cytokines in rainbow-trout cells, but enhanced the expression of those genes in the cells of gilthead seabream [27]. In combination with PAMPs, adrenaline also stimulated the expression of cytokines in head-kidney cells of seabream, but not of rainbow trout [16]. The authors reasonably argued that the different husbandry environments generally influence the individual amplitude of responses to stress hormones. In addition, the domestication history of a species should be taken into account as well. The intensive farming of rainbow trout and Atlantic salmon over decades accumulated phenotypes of low-cortisol responders [84] with high levels of stress tolerance. This could explain

why head-kidney cells from maraena whitefish, a relatively novel aquaculture species, showed a measurable sensitivity towards cortisol and adrenaline.

Noradrenaline only had a subtle impact on the expression of immune genes in unstimulated cells. Furthermore, we could not demonstrate a substantial influence of noradrenaline on the stimulation-dependent regulation of immune genes. Although this is (to our knowledge) the first study on noradrenaline in head-kidney cells of any fish species yet, it has been reported that splenic macrophages from spotted snakehead *Channa punctatus* showed a reduced phagocytic activity after incubation with noradrenaline [85]. Notwithstanding this observation, species-specific differences were again evident from another study on peripheral-blood leucocytes from tilapia *Oreochromis aureus*, which did not alter their phagocytic activity in response to different doses of noradrenaline [86]. Of note, the catecholamine concentration of 10 μM for both adrenaline and noradrenaline may not have the same biological relevance for the head-kidney cells. As adrenaline is secreted from chromaffin cells into the blood, the head-kidney cells are normally exposed to plasma levels, which are three orders of magnitude lower *in vivo* than in our study (9 nM; [87]). However, head kidney—as with most tissues with sympathetic innervation—is not only constantly exposed to plasma levels of noradrenaline (1.8 nM; [87]), but also to noradrenaline that "leaks out" from the synaptic cleft. It has been shown in mammals [88] and carps [89] that the noradrenaline levels are between 0.4 and 10 μg/g of various tissues. Assuming that 1 g tissue is approximately 1 ml and the molecular weight of noradrenaline bitartrate salt used here is ~337 g/mol, sympathetically innervated tissues are constantly exposed to concentrations of noradrenaline of about 10 μM. Therefore, the noradrenaline levels, which the head-kidney cells are adapted to, may have been in the same order of magnitude as the concentration we used in our study. This may explain the rather small effects of noradrenaline compared to those of adrenaline.

4.3. Stress hormones modulate the sensitivity to stress signalling in head-kidney cells of maraena whitefish

Our *in vitro* experiments showed that cortisol reduced the expression of adrenergic-receptor genes during 24 h of incubation, while noradrenaline increased the expression of genes coding for adrenergic and some glucocorticoid receptors. The simultaneous stimulation with PAMPs resulted rather in a common downregulation. Indeed, it is established in mammals that cortisol modulates the expression of adrenergic receptors [90] depending on the duration of the stress exposure [91]. Short-term treatment with cortisol reduced, for instance, the density of adrenoreceptors in several brain regions of male tree shrews *Tupaia belangeri* [91]. We may have recorded this specific time window in which cortisol down-regulates adrenoreceptors in head-kidney cells of maraena whitefish. The fact that glucocorticoids are known to increase β-adrenergic receptors in all cell types studied so far [92] contradicts our findings. It is unlikely that our cells have been exposed to high concentrations of bioactive adrenaline or noradrenaline for 24 h, but it is rather likely that cells became stimulated initially before adrenaline and noradrenaline were degraded. The observed gene-expression patterns would then be the result of this initial stimulation followed by 24 h of cellular responses.

Another remarkable observation is that adrenaline slightly increased the transcript level of the investigated heat-shock genes. This finding is supported by a previous study on adrenaline, which increased the transcript levels of *HSP1A1* in hepatocytes [93]. The immune stimulation with PAMPs triggered the expression of the investigated heat-shock genes even stronger than adrenaline and these elevated transcript levels were barely reduced by the presence of stress hormones. A similar observation has been reported for rainbow-trout hepatocytes, where lipopolysaccharide upregulated the transcript level of *HSP1A1,* while cortisol did not alter it [80]. In addition to being well established markers indicating cellular stress, HSPs act as vital interacting partners with glucocorticoids [94] and are hence intimately involved in the suppression of immune responses. It is well documented for fish that stress hormones regulate the synthesis of HSPs [95], but it remains to be investigated how HSPs interfere with immunosuppression. The observation that HSPs support the suppressive activities of regulatory T cells in mammals [96] is a starting point for further research on stress physiology in fish.

5. Conclusions

In conclusion, we show that a primary culture of head-kidney cells of maraena whitefish corresponds well with similar models from other fish species, which facilitate simulations of particular aspects of endocrine and immune functions of the head kidney. Our *in vitro* tests in this regard revealed that distinct immune parameters (*IL1B* > *SAA* > *CXCL8* > *TNF* > *IL6*) are well suited as a read-out system to assess the impact of the analysed hormones on the immunity (cortisol > adrenaline > noradrenaline) and the sensitivity to stress signalling (noradrenaline > adrenaline > cortisol). They reflect a section of the species-specific interdigitation of responses to microbes and stress in the head kidney of maraena whitefish. In future studies, we will use the here established qPCRs assays to investigate *in vivo* how adverse social environments manipulate the neuro–immune crosstalk in salmonid fish.

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References

[1] D.A. Padgett, R. Glaser, How stress influences the immune response, Trends Immunol. 24 (2003) 444–448 http://www.ncbi.nlm.nih.gov/pubmed/12909458.
[2] A. Rebl, T. Goldammer, Under control: the innate immunity of fish from the inhibitors' perspective, Fish Shellfish Immunol. 77 (2018) 328–349, https://doi.org/10.1016/j.fsi.2018.04.016.
[3] E.J.W. Geven, P.H.M. Klaren, The teleost head kidney: integrating thyroid and immune signalling, Dev. Comp. Immunol. 66 (2017) 73–83, https://doi.org/10.1016/J.DCI.2016.06.025.
[4] E.-S.H. Abdel-Aziz, S.B.S. Abdu, T. El-Sayed Ali, T.E.-S. Ali, H.F. Fouad, Haemopoiesis in the head kidney of tilapia, *Oreochromis niloticus* (Teleostei: cichlidae): a morphological (optical and ultrastructural) study, Fish Physiol. Biochem. 36 (2010) 323–336, https://doi.org/10.1007/s10695-008-9297-z.
[5] G.A. Ingram, J.B. Alexander, The primary immune response of brown trout (*Salmo trutta*) to cellular and soluble antigens: enumeration of antibody-secreting and antigen-binding cells, and the production of antibody, Acta Biol. Med. Ger. 40 (1981) 317–330 http://www.ncbi.nlm.nih.gov/pubmed/7029986.
[6] R. Gazola, M.I. Borella, M. V Val-Sella, F.F. Moraes, G. Bernardino, Histophysiological aspects of the interrenal gland of the pacu female, *Piaractus mesopotamicus* (Telostei, Cyprinidae), Bol. Tec. Do CEPTA Pirassununga [BOL. TEC. CEPTA]. 8 (1995) 1–12 http://bdpi.usp.br/single.php?_id=000976695.
[7] L. Mastrolia, V.P. Gallo, A. La Marca, The adrenal chromaffin cells of *Salmo gairdneri* Richardson (Teleostei, Salmonidae), J. Anat. 138 (Pt 3) (1984) 503–511 http://www.ncbi.nlm.nih.gov/pubmed/6735912.
[8] L. Tort, Stress and immune modulation in fish, Dev. Comp. Immunol. 35 (2011) 1366–1375, https://doi.org/10.1016/j.dci.2011.07.002.
[9] B.A. Barton, G.K. Iwama, Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids, Annu. Rev. Fish Dis. 1 (1991) 3–26, https://doi.org/10.1016/0959-8030(91)90019-G.

[10] T.P. Mommsen, M.M. Vijayan, T.W. Moon, Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation, Rev. Fish Biol. Fish. 9 (1999) 211–268, https://doi.org/10.1023/A:1008924418720.
[11] N. Aluru, M.M. Vijayan, Stress transcriptomics in fish: a role for genomic cortisol signaling, Gen. Comp. Endocrinol. 164 (2009) 142–150, https://doi.org/10.1016/j.ygcen.2009.03.020.
[12] K.B. Pagniello, N.C. Bols, L.E.J. Lee, Effect of corticosteroids on viability and proliferation of the rainbow trout monocyte/macrophage cell line, RTS11, Fish Shellfish Immunol. 13 (2002) 199–214 http://www.ncbi.nlm.nih.gov/pubmed/12365731.
[13] S. MacKenzie, D. Iliev, C. Liarte, H. Koskinen, J.V. V Planas, F.W.W. Goetz, H. Mölsä, A. Krasnov, L. Tort, Transcriptional analysis of LPS-stimulated activation of trout (*Oncorhynchus mykiss*) monocyte/macrophage cells in primary culture treated with cortisol, Mol. Immunol. 43 (2006) 1340–1348, https://doi.org/10.1016/j.molimm.2005.09.005.
[14] M.A. Sheridan, Effects of epinephrine and norepinephrine on lipid mobilization from coho salmon liver incubated in vitro, Endocrinology 120 (1987) 2234–2239, https://doi.org/10.1210/endo-120-6-2234.
[15] J.S. Cameron, S.E. Brown, Adrenergic and cholinergic responses of the isolated heart of the goldfish *Carassius auratus*, Comp. Biochem. Physiol., C 70 (1981) 109–115 http://www.ncbi.nlm.nih.gov/pubmed/6117401.
[16] A.R. Khansari, D. Parra, F.E. Reyes-López, L. Tort, Modulatory in vitro effect of stress hormones on the cytokine response of rainbow trout and gilthead sea bream head kidney stimulated with Vibrio anguillarum bacterin, Fish Shellfish Immunol. 70 (2017) 736–749, https://doi.org/10.1016/j.fsi.2017.09.009.
[17] J. Castillo, M. Teles, S. Mackenzie, L. Tort, Stress-related hormones modulate cytokine expression in the head kidney of gilthead seabream (*Sparus aurata*), Fish Shellfish Immunol. 27 (2009) 493–499, https://doi.org/10.1016/j.fsi.2009.06.021.
[18] L.A. Grisanti, J. Evanson, E. Marchus, H. Jorissen, A.P. Woster, W. DeKrey, E.R. Sauter, C.K. Combs, J.E. Porter, Pro-inflammatory responses in human monocytes are β1-adrenergic receptor subtype dependent, Mol. Immunol. 47 (2010) 1244–1254, https://doi.org/10.1016/j.molimm.2009.12.013.
[19] M.R. Irwin, S.W. Cole, Reciprocal regulation of the neural and innate immune systems, Nat. Rev. Immunol. 11 (2011) 625–632, https://doi.org/10.1038/nri3042.
[20] A.G. Maule, R.A. Tripp, S.L. Kaattari, C.B. Schreck, Stress alters immune function and disease resistance in chinook salmon (*Oncorhynchus tshawytscha*), J. Endocrinol. 120 (1989) 135–142 http://www.ncbi.nlm.nih.gov/pubmed/2918264.
[21] A.J. Ainsworth, C. Dexiang, P.R. Waterstrat, T. Greenway, Effect of temperature on the immune system of channel catfish (*Ictalurus punctatus*)–I. Leucocyte distribution and phagocyte function in the anterior kidney at 10 degrees C, Comp. Biochem. Physiol. A Comp. Physiol. 100 (1991) 907–912 http://www.ncbi.nlm.nih.gov/pubmed/1685384.
[22] T. Korytář, M. Nipkow, S. Altmann, T. Goldammer, B. Köllner, A. Rebl, Adverse husbandry of maraena whitefish directs the immune system to increase mobilization of myeloid cells and proinflammatory responses, Front. Immunol. 7 (2016), https://doi.org/10.3389/fimmu.2016.00631.
[23] C. Xu, E. Li, Y. Suo, Y. Su, M. Lu, Q. Zhao, J.G. Qin, L. Chen, Histological and transcriptomic responses of two immune organs, the spleen and head kidney, in Nile tilapia (*Oreochromis niloticus*) to long-term hypersaline stress, Fish Shellfish Immunol. 76 (2018) 48–57, https://doi.org/10.1016/j.fsi.2018.02.041.
[24] L.N. Franco Montoya, T.P. Martins, R.Y. Gimbo, F.S. Zanuzzo, E.C. Urbinati, β-Glucan-induced cortisol levels improve the early immune response in matrinxã (*Brycon amazonicus*), Fish Shellfish Immunol. 60 (2017) 197–204, https://doi.org/10.1016/j.fsi.2016.11.055.
[25] V. Aballai, J.E. Aedo, J. Maldonado, M. Bastias-Molina, H. Silva, C. Meneses, S. Boltaña, A. Reyes, A. Molina, J.A. Valdés, RNA-seq analysis of the head-kidney transcriptome response to handling-stress in the red cusk-eel (*Genypterus chilensis*), Comp. Biochem. Physiol. Genom. Proteonom. 24 (2017) 111–117, https://doi.org/10.1016/j.cbd.2017.09.002.
[26] C.M.A. Caipang, I. Berg, M.F. Brinchmann, V. Kiron, Short-term crowding stress in Atlantic cod, *Gadus morhua* L. modulates the humoral immune response, Aquaculture 295 (2009) 110–115, https://doi.org/10.1016/j.aquaculture.2009.06.036.
[27] A.R. Khansari, D. Parra, F.E. Reyes-López, L. Tort, Cytokine modulation by stress hormones and antagonist specific hormonal inhibition in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) head kidney primary cell culture, Gen. Comp. Endocrinol. 250 (2017) 122–135, https://doi.org/10.1016/j.ygcen.2017.06.005.
[28] A. Rebl, M. Verleih, M. Nipkow, S. Altmann, R. Bochert, T. Goldammer, Gradual and acute temperature rise induces crossing endocrine, metabolic and immunological pathways in maraena whitefish (*Coregonus maraena*), Front. Genet. 9 (2018) 241, https://doi.org/10.3389/FGENE.2018.00241.
[29] A. Rebl, M. Zebunke, A. Borchel, R. Bochert, M. Verleih, T. Goldammer, Microarray-predicted marker genes and molecular pathways indicating crowding stress in rainbow trout (*Oncorhynchus mykiss*), Aquaculture 473 (2017) 355–365, https://doi.org/10.1016/j.aquaculture.2017.03.003.
[30] W. Tymchuk, D. Sakhrani, R. Devlin, Domestication causes large-scale effects on gene expression in rainbow trout: analysis of muscle, liver and brain transcriptomes, Gen. Comp. Endocrinol. 164 (2009) 175–183, https://doi.org/10.1016/j.ygcen.2009.05.015.
[31] E. Petersson, T. Jaurvi, N.G. Steffner, B. Ragnarsson, The effect of domestication on some life history traits of sea trout and Atlantic salmon, J. Fish Biol. 48 (1996) 776–791, https://doi.org/10.1111/j.1095-8649.1996.tb01471.x.
[32] R. Bochert, T. Horn, P. Luft, Maraena whitefish (*Coregonus maraena*) larvae reveal enhanced growth during first feeding with live Artemia nauplii, Arch. Pol. Fish. 25 (2017) 3–10, https://doi.org/10.1515/aopf-2017-0001.
[33] R. Sebesta, D. Kucharczyk, J. Nowosad, M. Sikora, V. Stejskal, Effect of temperature on growth and survival of maraena whitefish *Coregonus maraena* (Bloch 1779) larvae in controlled conditions, Aquacult. Res. 49 (2018) 3151–3157, https://doi.org/10.1111/are.13778.
[34] S. Altmann, T. Korytář, D. Kaczmarzyk, M. Nipkow, C. Kühn, T. Goldammer, A. Rebl, Toll-like receptors in maraena whitefish: evolutionary relationship among salmonid fishes and patterns of response to aeromonas salmonicida, Fish Shellfish Immunol. 54 (2016) 391–401, https://doi.org/10.1016/j.fsi.2016.04.125.
[35] S. MacKenzie, J. V Planas, F.W. Goetz, LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and in vitro differentiated macrophages, Dev. Comp. Immunol. 27 (2003) 393–400 http://www.ncbi.nlm.nih.gov/pubmed/12631521.
[36] C. Liu, W. Liu, L. Fan, J. Liu, P. Li, W. Zhang, J. Gao, Z. Li, Q. Zhang, X. Wang, Sequences analyses and expression profiles in tissues and embryos of Japanese flounder (*Paralichthys olivaceus*) PRDM1, Fish Physiol. Biochem. 42 (2016) 467–482, https://doi.org/10.1007/s10695-015-0152-8.
[37] A. Brietzke, A. Borchel, S. Altmann, M. Nipkow, A. Rebl, R.M. Brunner, T. Goldammer, Transcriptome sequencing of maraena whitefish (*Coregonus maraena*), Mar. Genom. 29 (2016) 27–29, https://doi.org/10.1016/j.margen.2016.05.006.
[38] S. Altmann, A. Rebl, C. Kühn, T. Goldammer, Identification and de novo sequencing of housekeeping genes appropriate for gene expression analyses in farmed maraena whitefish (*Coregonus maraena*) during crowding stress, Fish Physiol. Biochem. 41 (2015) 397–412, https://doi.org/10.1007/s10695-014-9991-y.
[39] F.W. Goetz, J.V. Planas, M. Díaz, D.B. Iliev, S. MacKenzie, Culture of fish head kidney mononuclear phagocytes and muscle satellite cells: valuable models for aquaculture biotechnology research, Aquac. Biotechnol, Wiley-Blackwell, Oxford, UK, 2011, pp. 207–221, https://doi.org/10.1002/9780470963159.ch13.
[40] E. Bassity, T.G. Clark, Functional identification of dendritic cells in the teleost model, rainbow trout (*Oncorhynchus mykiss*), PLoS One 7 (2012), https://doi.org/10.1371/journal.pone.0033196.
[41] D. Le Guyader, M.J. Redd, E. Colucci-Guyon, E. Murayama, K. Kissa, V. Briolat, E. Mordelet, A. Zapata, H. Shinomiya, P. Herbomel, Origins and unconventional behavior of neutrophils in developing zebrafish, Blood 111 (2008) 132–141, https://doi.org/10.1182/blood-2007-06-095398.
[42] S. Partula, Surface markers of fish T-cells, Fish Shellfish Immunol. 9 (1999) 241–257, https://doi.org/10.1006/FSIM.1998.0190.
[43] C. Uribe, H. Folch, R. Enriquez, G. Moran, Innate and adaptive immunity in teleost fish: a review, Vet. Med. (Praha). 56 (2011) 486–503, https://doi.org/10.1016/j.pestbp.2010.09.001.
[44] A. Brietzke, T. Korytář, J. Jaros, B. Köllner, T. Goldammer, H.-M. Seyfert, A. Rebl, Aeromonas salmonicida infection only moderately regulates expression of factors contributing to toll-like receptor signaling but massively activates the cellular and humoral branches of innate immunity in rainbow trout (*Oncorhynchus mykiss*), J. Immunol. Res. 2015 (2015) 901015, https://doi.org/10.1155/2015/901015.
[45] M.M. Monte, K. Urquhart, C.J. Secombes, B. Collet, Individual monitoring of immune responses in rainbow trout after cohabitation and intraperitoneal injection challenge with *Yersinia ruckeri*, Fish Shellfish Immunol. 55 (2016) 469–478, https://doi.org/10.1016/j.fsi.2016.05.041.
[46] R. Castro, J. Coll, M. del M. Blanco, A. Rodríguez-Bertos, L. Jouneau, J.F. Fernández-Garayzábal, A. Gibello, Spleen and head kidney differential gene expression patterns in trout infected with *Lactococcus garvieae* correlate with spleen granulomas, Vet. Res. 50 (2019) 32, https://doi.org/10.1186/s13567-019-0649-8.
[47] J.B. Jørgensen, G.J.E. Sharp, C.J. Secombes, B. Robertsen, Effect of a Yeast-Cell-Wall Glucan on the Bactericidal Activity of Rainbow Trout Macrophages, Fish Shellfish Immunol. 4 (1993) 267–277, https://doi.org/10.1006/fsim.1993.1026.
[48] T. Korytář, J. Jaros, M. Verleih, A. Rebl, G. Kotterba, C. Kühn, T. Goldammer, B. Köllner, Novel insights into the peritoneal inflammation of rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 35 (2013) 3–10, https://doi.org/10.1016/j.fsi.2013.07.032.
[49] B.H. Dannevig, B.E. Brudeseth, T. Gjøen, M. Rode, H.I. Wergeland, Evensen, C.M.L. Press, Characterisation of a long-term cell line (SHK-1) developed from the head kidney of Atlantic salmon (*Salmo salar* L.), Fish Shellfish Immunol. 7 (1997) 213–226, https://doi.org/10.1006/fsim.1996.0076.
[50] C.J. Bayne, Pronephric leucocytes of *Cyprinus carpio*: isolation, separation and characterization, Vet. Immunol. Immunopathol. 12 (1986) 141–151, https://doi.org/10.1016/0165-2427(86)90118-2.
[51] E. Siegl, S. Albrecht, B. Lüdtke, Long-term liquid culture of haematopoietic precursor cells from the head kidney and spleen of the rainbow trout (*Oncorhynchus mykiss*), Comp. Haematol. Int. 3 (1993) 168–173, https://doi.org/10.1007/BF00186102.
[52] F.A.A. Weyts, N. Cohen, G. Flik, B.M.L. Verburg-van Kemenade, Interactions between the immune system and the hypothalamo-pituitary-interrenal axis in fish, Fish Shellfish Immunol. 9 (1999) 1–20, https://doi.org/10.1006/fsim.1998.0170.
[53] G. Nardocci, C. Navarro, P.P. Cortés, M. Imarai, M. Montoya, B. Valenzuela, P. Jara, C. Acuña-Castillo, R. Fernández, Neuroendocrine mechanisms for immune system regulation during stress in fish, Fish Shellfish Immunol. 40 (2014) 531–538 http://www.ncbi.nlm.nih.gov/pubmed/25123831.
[54] K.L. Hillman, V.A. Doze, J.E. Porter, 1A-adrenergic receptors are functionally expressed by a subpopulation of cornu ammonis 1 interneurons in rat hippocampus, J. Pharmacol. Exp. Ther. 321 (2007) 1062–1068, https://doi.org/10.1124/jpet.106.119297.
[55] H.A. Céspedes, K. Zavala, M.W. Vandewege, J.C. Opazo, Evolution of the α2-adrenoreceptors in vertebrates: ADRA2D is absent in mammals and crocodiles, Gen. Comp. Endocrinol. 250 (2017) 85–94, https://doi.org/10.1016/j.ygcen.2017.06.006.

[56] L.D. Estrada, D. Ağaç, J.D. Farrar, Sympathetic neural signaling via the β2-adrenergic receptor suppresses T-cell receptor-mediated human and mouse CD8(+) T-cell effector function, Eur. J. Immunol. 46 (2016), https://doi.org/10.1002/eji.201646395 1948–58.
[57] K.D. Lu, S. Radom-Aizik, F. Haddad, F. Zaldivar, M. Kraft, D.M. Cooper, Glucocorticoid receptor expression on circulating leukocytes differs between healthy male and female adults, J. Clin. Transl. Sci. 1 (2017) 108–114, https://doi.org/10.1017/cts.2016.20.
[58] M.E. Bianchi, DAMPs, PAMPs and alarmins: all we need to know about danger, J. Leukoc. Biol. 81 (2007) 1–5, https://doi.org/10.1189/jlb.0306164.
[59] M. Seppola, A.N. Larsen, K. Steiro, B. Robertsen, I. Jensen, Characterisation and expression analysis of the interleukin genes, IL-1beta, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.), Mol. Immunol. 45 (2008) 887–897, https://doi.org/10.1016/j.molimm.2007.08.003.
[60] J.K. Chettri, M.K. Raida, L. Holten-Andersen, P.W. Kania, K. Buchmann, PAMP induced expression of immune relevant genes in head kidney leukocytes of rainbow trout (*Oncorhynchus mykiss*), Dev. Comp. Immunol. 35 (2011) 476–482, https://doi.org/10.1016/j.dci.2010.12.001.
[61] D.B. Iliev, C.Q. Liarte, S. MacKenzie, F.W. Goetz, Activation of rainbow trout (*Oncorhynchus mykiss*) mononuclear phagocytes by different pathogen associated molecular pattern (PAMP) bearing agents, Mol. Immunol. 42 (2005) 1215–1223, https://doi.org/10.1016/j.molimm.2004.11.023.
[62] L.E. Jensen, S. Thiel, T.E. Petersen, J.C. Jensenius, A rainbow trout lectin with multimeric structure, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 116 (1997) 385–390 http://www.ncbi.nlm.nih.gov/pubmed/9149391.
[63] J.B. Jørgensen, H. Lunde, L. Jensen, A.S. Whitehead, B. Robertsen, Serum amyloid A transcription in Atlantic salmon (*Salmo salar* L.) hepatocytes is enhanced by stimulation with macrophage factors, recombinant human IL-1β, IL-6 and TNFα or bacterial lipopolysaccharide, Dev. Comp. Immunol. 24 (2000) 553–563, https://doi.org/10.1016/S0145-305X(00)00022-7.
[64] A. Rebl, T. Goldammer, U. Fischer, B. Köllner, H.-M. Seyfert, Characterization of two key molecules of teleost innate immunity from rainbow trout (*Oncorhynchus mykiss*): MyD88 and SAA, Vet. Immunol. Immunopathol. 131 (2009) 122–126, https://doi.org/10.1016/j.vetimm.2009.03.006.
[65] F. Villarroel, A. Casado, J. Vásquez, E. Matamala, B. Araneda, R. Amthauer, R. Enriquez, M.I. Concha, Serum amyloid A: a typical acute-phase reactant in rainbow trout? Dev. Comp. Immunol. 32 (2008) 1160–1169, https://doi.org/10.1016/J.DCI.2008.03.004.
[66] P.W. Kania, J.K. Chettri, K. Buchmann, Characterization of serum amyloid A (SAA) in rainbow trout using a new monoclonal antibody, Fish Shellfish Immunol. 40 (2014) 648–658, https://doi.org/10.1016/j.fsi.2014.08.018.
[67] A. Kuçceukgul Guleç, I. Cengizler, Determination of acute phase proteins after experimental *Streptococcus iniae* infection in tilapia (*Oreochromis niloticus* L.), Turk. J. Vet. Anim. Sci. 36 (2012) 380–387, https://doi.org/10.3906/vet-1011-2.
[68] L.E. Jensen, M.P. Hiney, D.C. Shields, C.M. Uhlar, A.J. Lindsay, A.S. Whitehead, Acute phase proteins in salmonids: evolutionary analyses and acute phase response, J. Immunol. 158 (1997) 384–392 http://www.ncbi.nlm.nih.gov/pubmed/8977214.
[69] H. Jodaa Holm, S. Wadsworth, A.-K. Bjelland, A. Krasnov, Ø. Evensen, S. Skugor, Dietary phytochemicals modulate skin gene expression profiles and result in reduced lice counts after experimental infection in Atlantic salmon, Parasites Vectors 9 (2016) 271, https://doi.org/10.1186/s13071-016-1537-y.
[70] F. Swirplies, S. Wuertz, B. Baßmann, A. Orban, N. Schäfer, R.M. Brunner, F. Hadlich, T. Goldammer, A. Rebl, Identification of molecular stress indicators in pikeperch *Sander lucioperca* correlating with rising water temperatures, Aquaculture 501 (2018) 260–271, https://doi.org/10.1016/j.aquaculture.2018.11.043.
[71] S.D. Mohapatra, V. Chakrapani, K.D. Rasal, P. Jayasankar, H.K. Barman, E. Subudhi, Hypoxia-induced Gene Expression Profiling in the Liver of Freshwater Fish, Channa striatus, Turk. J. Fish. Aqat. Sc. 17 (2017) 565–579 https://www.semanticscholar.org/paper/Hypoxia-Induced-Gene-Expression-Profiling-in-The-of-Mohapatra-Chakrapani/8adc64e6b89f55f6543b85ccfba95e0804829d73.
[72] L.R. Sveen, G. Timmerhaus, A. Krasnov, H. Takle, S. Handeland, E. Ytteborg, Wound healing in post-smolt Atlantic salmon (*Salmo salar* L.), Sci. Rep. 9 (2019) 3565, https://doi.org/10.1038/s41598-019-39080-x.
[73] A. Krasnov, M.S. Wesmajervi Breiland, B. Hatlen, S. Afanasyev, S. Skugor, Sexual maturation and administration of 17β-estradiol and testosterone induce complex gene expression changes in skin and increase resistance of Atlantic salmon to ectoparasite salmon louse, Gen. Comp. Endocrinol. 212 (2015) 34–43, https://doi.org/10.1016/J.YGCEN.2015.01.002.
[74] L.R. Sveen, G. Timmerhaus, A. Krasnov, H. Takle, S.O. Stefansson, S.O. Handeland, E. Ytteborg, High fish density delays wound healing in Atlantic salmon (*Salmo salar*), Sci. Rep. 8 (2018) 16907, https://doi.org/10.1038/S41598-018-35002-5.
[75] A.T. Talbot, T.G. Pottinger, T.J. Smith, M.T. Cairns, Acute phase gene expression in rainbow trout (*Oncorhynchus mykiss*) after exposure to a confinement stressor: a comparison of pooled and individual data, Fish Shellfish Immunol. 27 (2009) 309–317, https://doi.org/10.1016/j.fsi.2009.05.016.
[76] S.P.M. Alsemgeest, I.E. Lambooy, H.K. Wierenga, S.J. Dieleman, B. Meerkerk, A.M. van Ederen, T.A. Niewold, Influence of physical stress on the plasma concentration of serum amyloid-a (SAA) and haptoglobin (HP) in calves, Vet. Q. 17 (1995) 9–12, https://doi.org/10.1080/01652176.1995.9694521.
[77] C.F. Thorn, Z.-Y. Lu, A.S. Whitehead, Tissue-specific regulation of the human acute-phase serum amyloid A genes,SAA1 andSAA2, by glucocorticoids in hepatic and epithelial cells, Eur. J. Immunol. 33 (2003) 2630–2639, https://doi.org/10.1002/eji.200323985.
[78] C. Piñeiro, M. Piñeiro, J. Morales, R. Carpintero, F.M. Campbell, P.D. Eckersall, M.J.M. Toussaint, M.A. Alava, F. Lampreave, Pig acute-phase protein levels after stress induced by changes in the pattern of food administration, Animal 1 (2007) 133–139, https://doi.org/10.1017/s1751731107283909.
[79] J.P.J. Saeij, L.B.M. Verburg-van Kemenade, W.B. van Muiswinkel, G.F. Wiegertjes, Daily handling stress reduces resistance of carp to *Trypanoplasma borreli*: in vitro modulatory effects of cortisol on leukocyte function and apoptosis, Dev. Comp. Immunol. 27 (2003) 233–245 http://www.ncbi.nlm.nih.gov/pubmed/12590974.
[80] A.M. Philip, S. Daniel Kim, M.M. Vijayan, Cortisol modulates the expression of cytokines and suppressors of cytokine signaling (SOCS) in rainbow trout hepatocytes, Dev. Comp. Immunol. 38 (2012) 360–367, https://doi.org/10.1016/j.dci.2012.07.005.
[81] R. Castro, J. Zou, C.J. Secombes, S.A.M. Martin, Cortisol modulates the induction of inflammatory gene expression in a rainbow trout macrophage cell line, Fish Shellfish Immunol. 30 (2011) 215–223, https://doi.org/10.1016/j.fsi.2010.10.010.
[82] R. Wiik, K. Andersen, I. Uglenes, E. Egidius, Cortisol-induced increase in susceptibility of Atlantic salmon, *Salmo salar*, to *Vibrio salmonicida*, together with effects on the blood cell pattern, Aquaculture 83 (1989) 201–215, https://doi.org/10.1016/0044-8486(89)90033-1.
[83] B.A. Barton, Stress in Fishes: a diversity of responses with particular reference to changes in circulating corticosteroids, Integr. Comp. Biol. 42 (2006) 517–525, https://doi.org/10.1093/icb/42.3.517.
[84] S.E. Fevolden, T. Refstie, K.H. Røed, Selection for high and low cortisol stress response in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), Aquaculture 95 (1991) 53–65, https://doi.org/10.1016/0044-8486(91)90072-F.
[85] B. Roy, U. Rai, Role of adrenoceptor-coupled second messenger system in sympatho-adrenomedullary modulation of splenic macrophage functions in live fish Channa punctatus, Gen. Comp. Endocrinol. 155 (2008) 298–306, https://doi.org/10.1016/j.ygcen.2007.05.008.
[86] W.H. Chen, L.T. Sun, C.L. Tsai, Y.L. Song, C.F. Chang, Cold-stress induced the modulation of catecholamines, cortisol, immunoglobulin M, and leukocyte phagocytosis in tilapia, Gen. Comp. Endocrinol. 126 (2002) 90–100, https://doi.org/10.1006/gcen.2001.7772.
[87] J.J. Woodward, Plasma catecholamines in resting rainbow trout, *Salmo gairdneri* Richardson, by high pressure liquid chromatography, J. Fish Biol. 21 (1982) 429–432, https://doi.org/10.1111/j.1095-8649.1982.tb02848.x.
[88] C. Bell, J.S. Gillespie, Dopamine and noradrenaline levels in peripheral tissues of several mammalian species, J. Neurochem. 36 (1981) 703–706, https://doi.org/10.1111/j.1471-4159.1981.tb01645.x.
[89] Z. Jeney, J. Nemcsók, G. Jeney, J. Oláh, Acute effect of sublethal ammonia concentrations on common carp (*Cyprinus carpio* L.). I. Effect of ammonia on adrenaline and noradrenaline levels in different organs, Aquaculture 104 (1992) 139–148, https://doi.org/10.1016/0044-8486(92)90144-A.
[90] T. Nakane, T. Szentendrei, L. Stern, M. Virmani, J. Seely, G. Kunos, Effects of IL-1 and cortisol on beta-adrenergic receptors, cell proliferation, and differentiation in cultured human A549 lung tumor cells, J. Immunol. 145 (1990) 260–266 http://www.ncbi.nlm.nih.gov/pubmed/2162889.
[91] G. Flügge, Effects of cortisol on brain alpha2-adrenoceptors: potential role in stress, Neurosci. Biobehav. Rev. 23 (1999) 949–956 http://www.ncbi.nlm.nih.gov/pubmed/10580309.
[92] A.O. Davies, R.J. Lefkowitz, Regulation of β-adrenergic receptors by steroid hormones, Annu. Rev. Physiol. 46 (1984) 119–130, https://doi.org/10.1146/annurev.ph.46.030184.001003.
[93] P.A. Ackerman, R.B. Forsyth, C.F. Mazur, G.K. Iwama, Stress hormones and the cellular stress response in salmonids, Fish Physiol. Biochem. 23 (2000) 327–336, https://doi.org/10.1023/A:1011107610971.
[94] G. Bertorelli, V. Bocchino, D. Olivieri, Heat shock protein interactions with the glucocorticoid receptor, Pulm. Pharmacol. Ther. 11 (1998) 7–12, https://doi.org/10.1006/pupt.1998.0119.
[95] G.K. Iwama, P.T. Thomas, R.B. Forsyth, M.M. Vijayan, Heat shock protein expression in fish, Rev. Fish Biol. Fish. 8 (1998) 35–56, https://doi.org/10.1023/A:1008812500650.
[96] J. Wachstein, S. Tischer, C. Figueiredo, A. Limbourg, C. Falk, S. Immenschuh, R. Blaszcyk, B. Eiz-Vesper, HSP70 enhances immunosuppressive function of CD4 + CD25 + FoxP3 + T regulatory cells and cytotoxicity in CD4 + CD25 − T cells, PLoS One 7 (2012) e51747, , https://doi.org/10.1371/journal.pone.0051747.

5.3. Publication III: Characterization of sialic acid-binding immunoglobulin-type lectins in fish reveals teleost-specific structures and expression patterns

Siglecs are immunoglobulin-type lectins that modulate the response of vertebrate leukocytes upon recognition of extracellular sialic acid residues. While several siglecs are conserved among vertebrates, little is known about their function in fish. In this work, the expression profile of four known siglecs in teleost was analyzed in different tissues of *C. maraena*, *O. mykiss* and *S. lucioperca*. In addition, the aminoacid sequence of these piscine siglecs was analyzed for immune-regulatory motifs. To confirm previous reports on the sensitivity of siglec genes to aquaculture related stressors, the expression of siglecs was analyzed in the tissues of *C. maraena* after a short episode of handling.

Highlights

- The expression profile of siglec genes in three teleost species showed a higher distribution of siglecs in immune-related organs.

- Siglec sequence analysis revealed that some teleost siglecs have regulatory capacities not present in higher vertebrates

- The expression analyses of siglecs and associated enzymes indicated a gene- and tissue specific regulation after *C. maraena* was exposed to handling stress

cells

MDPI

Article

Characterization of Sialic Acid-Binding Immunoglobulin-Type Lectins in Fish Reveals Teleost-Specific Structures and Expression Patterns

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Abstract: The cellular glycocalyx of vertebrates is frequently decorated with sialic acid residues. These sialylated structures are recognized by sialic acid-binding immunoglobulin-type lectins (Siglecs) of immune cells, which modulate their responsiveness. Fifteen Siglecs are known to be expressed in humans, but only four Siglecs are regularly present in fish: Siglec1, CD22, myelin-associated glycoprotein (MAG), and Siglec15. While several studies have dealt with the physiological roles of these four Siglecs in mammals, little is known about Siglecs in fish. In the present manuscript, the expression landscapes of these Siglecs were determined in the two salmonid species *Oncorhynchus mykiss* and *Coregonus maraena* and in the percid fish *Sander lucioperca*. This gene-expression profiling revealed that the expression of *MAG* is not restricted to neuronal cells but is detectable in all analyzed blood cells, including erythrocytes. The teleostean MAG contains the inhibitory motif ITIM; therefore, an additional immunomodulatory function of MAG is likely to be present in fish. Besides *MAG*, *Siglec1*, *CD22*, and *Siglec15* were also expressed in all analyzed blood cell populations. Interestingly, the expression profiles of genes encoding Siglecs and particular associated enzymes changed in a gene- and tissue-specific manner when *Coregonus maraena* was exposed to handling stress. Thus, the obtained data indicate once more that stress directly affects immune-associated processes.

Keywords: acute stress; ITIM; Salmonidae; siglecs; sialic acids; vertebrate evolution

1. Introduction

Innate immunity is of even more paramount importance for fish health than adaptive immune mechanisms [1]. More than 50 regulators of innate immunity are known in fish today [2]; these regulators maintain the balance between pathogen defense and pathophysiological manifestations. Some of these innate-immune regulators recognize self-associated molecular patterns (SAMPs), a heterogeneous group of molecules that stimulate inhibitory receptors to dampen immune responses [3–5]. The family of sialic acids consists of more than 50 members [6] and belongs to the group of SAMPs [7]. Sialic acids are frequently located at the terminal ends of glycans on glycoproteins and the glycolipids that coat all mammalian cells in a glycocalyx. Immune cells abundantly express sialic acid-binding

immunoglobulin-type lectins (Siglecs), which interact with these sialylated glycoconjugates and thus prevent an excessive immunological response [8,9].

The Siglecs are divided into two subgroups: the CD33-related Siglecs and the highly conserved Siglecs, including Siglec1 (sialoadhesin), CD22 (Siglec2), MAG (Siglec4), and Siglec15. Both subgroups comprise Siglecs that suppress the immune response or activate the immune system [9,10]. The inhibitory function of Siglecs is mediated by an intracellular immune-receptor tyrosine-based inhibition motif (ITIM; signature: [I/V/L/S]-X-Y-X-X-[L/V] [9]), which is absent in immune-activating Siglecs (Siglec14, Siglec15, and Siglec16 in humans). These Siglec receptors interact with DNAX proteins (DAP10/DAP12), which carry immune-receptor tyrosine-based activation motifs (ITAMs) [9,11]. To date, fifteen and nine Siglecs have been identified in human and mouse, respectively, each expressed by defined cell types and responding to a particular set of ligands [12–14]. Remarkably, Siglec1, CD22, MAG, and Siglec15 were already present in the animal kingdom before the separation of tetrapods and teleost fishes more than 400 million years ago [15]. However, little is known about the physiological role of Siglecs in teleosts [16,17] including their tissue-specific basal expression profiles. For this reason, the present study aimed at the analysis of the expression landscapes of Siglec1, CD22, MAG, and Siglec15 in teleost fish. The expression profiling was complemented by in-depth analyses of the structures of the aforementioned Siglec receptors. These investigations focused on three aquaculture fish species of economic importance, (i) pikeperch (*Sander lucioperca*) as a representative of diploid percid fish, (ii) rainbow trout (*Oncorhynchus mykiss*) and (iii) maraena whitefish (*Coregonus maraena*) as representatives of the pseudotetraploid salmonid fishes that are currently undergoing species-specific reploidization processes [18]. Notably, the rainbow trout has been adapted to intensive farming for decades, whereas maraena whitefish is a novel aquaculture species and a useful model for dissecting the response of a pseudo-wild fish to anthropogenic environments. Our previous transcriptomic analyses on the stress physiology of the maraena whitefish revealed that the level of MAG transcripts is modulated by a factor of approximately two in different organs after exposure to temperature and stocking-density stress [19,20]. These findings suggested that Siglecs could potentially be biomarkers for immune and stress responses in teleost fish. To address whether stress affects the expression of Siglec-encoding genes, maraena whitefish were exposed to handling procedures [19,20]. The presently outlined study provides novel insights into the physiological roles of Siglecs in teleost fish.

2. Materials and Methods

2.1. Fish Husbandry and Experimental Treatment

The animals used for this study were provided by the Institute for Fisheries of the State Research Centre for Agriculture and Fishery Mecklenburg–Western Pomerania and BiMES Binnenfischerei GmbH (Friedrichsruhe, Germany). Fish were held in freshwater recirculation systems under a 12:12 day-and-night cycle at 18 °C. Water quality was maintained by automated purification and disinfection (bio-filter and UV light). In addition, the concentrations of NH_4^+, NO_2^-, NO_3^-, and NH_3 in the water, pH value, temperature, and oxygen saturation were constantly recorded. The feeding material consisted of commercial dry pellets (4.5 mm, INICIO Plus; BioMar, Brande, Denmark), which were distributed by automatic feeders at a daily rate of 0.8–4.0%, depending on the biomass of the fish.

Maraena whitefish were acclimatized in the experimental tanks connected to a recirculation system for a period of at least three weeks. Acute handling stress was induced by chasing, netting and the transfer of fish to another tank for a period of one minute. Eight stressed and eight control fish were euthanized with an overdose of 2-phenoxyethanol (0.7 mL/L; Sigma-Aldrich/Merck, Munich, Germany) and then underwent spine sectioning at the skull level. These procedures followed the standards described in the German Animal Welfare Act (§ 4(3) TierSchG) and were approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (Mecklenburg–Vorpommern, Germany; approval ID: LALLF M-V/TSD/7221.3-1-069/18; date of approval: January 16, 2019). The brain of each maraena whitefish was dissected into the hypothalamus, telencephalon, and hindbrain and sampled together

with the spleen, liver, white muscle, gills, head kidney (HK), and heart. Samples were snap-frozen in liquid nitrogen and stored at −80 °C. Husbandry and sampling of pikeperch and rainbow trout have been described elsewhere [21,22].

2.2. Isolation and Microscopic Characterization of Head Kidney Cells from Maraena Whitefish

For HK cell isolation and cell sorting, HKs from freshly slaughtered maraena whitefish were dissected and put into a Dulbecco's Modified Eagle Medium (DMEM; Gibco/Thermo Fisher Scientific, Bremen, Germany). The tissue was homogenized using a steel sieve (500 µm, Carl Roth, Karlsruhe, Germany) and then filtered through two cell strainers (200 µm, pluriSelect Life Science, Leipzig, Germany; 100 µm, Falcon/Fisher Scientific, Schwerte, Germany). After centrifugation (524× *g*, 5 min) and resuspension in 3 mL DMEM, the cell suspension was added onto an isotonic Percoll (Sigma-Aldrich/Merck) gradient (3 mL, ρ = 1.084 g/mL) and centrifuged at 800× *g* for 30 min at 6 °C with minimum deceleration. The erythrocyte pellet was stored at −80 °C for further RNA extraction, while the cell band at the interface was collected in the DMEM and the volume was adjusted for cell counting. Cell number and cell viability were determined using the Cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA, USA). In addition, a portion of the cells separated by the Percoll gradient were placed on glass slides, stained with a May-Grünwald-Giemsa solution (Brand, Wertheim, Germany; Carl Roth), and then microscopically observed using a Nikon TMS-F microscope and a Nikon Coolpix E5000 camera with an MDC Lens (Nikon, Tokyo, Japan).

2.3. Flow Cytometry

Flow cytometry was performed using a MoFlo XDP high-speed cell sorter (Beckman Coulter, Krefeld, Germany) with an incorporated, air-cooled sapphire laser (488 nm, 100 mW). A total of ~20 million HK cells were sorted through a 70-µm nozzle at 60 psi on purify mode into two fractions, low side-scattering intensity (fraction I) and high side-scattering intensity (fraction II). Fractions I and II were collected in phosphate-buffered saline (PBS), centrifuged at 500× *g* for 5 min, and used for RNA extraction. Subsequently, cell type-specific gene expressions were profiled, as described in detail in [23].

2.4. RNA Isolation

Approximately 50 µg of each of the individual tissue samples were placed in separate reaction tubes containing 1 mL of TRIzol Reagent (Life Technologies/Thermo Fisher Scientific) and homogenized using the Precellys24 Homogeniser (6000 rpm, 30 s). After the addition of chloroform and a centrifugation step (12,000× g, 15 min, 4 °C), the RNA contained in the resulting aqueous phase was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was isolated from cells and purified using the Isolate 2 RNA Micro Kit (Bioline, Luckenwalde, Germany) according to the manufacturer's instructions and without a previous treatment with TRIzol Reagent. The quality of the purified RNA was checked using horizontal agarose-gel electrophoresis. RNA concentration was determined using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

2.5. Primer Design and Quantitative PCR

Species-specific quantitative PCR (qPCR) primers were designed for the target genes using PSQ Assay Design 1.0.6 software (Biotage AB, Uppsala, Sweden). Amplicon length ranged from 140 to 180 bp (Table S1). Coding sequences for rainbow trout were retrieved from the NCBI public database. To identify Siglec sequences from pikeperch or maraena whitefish, we aligned the orthologous sequences from yellow perch (*Perca flavescens*) or rainbow trout, Atlantic salmon (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*), respectively, with the recently published pikeperch genome [24] or our RNA-seq read collection from maraena whitefish [25] using Bowtie 2 software (v. 2.2.4; http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). These alignments were then indexed and sorted with the Samtools software package (v. 16; http://www.htslib.org/), and final consensus

sequences were obtained with Ugene software (v. 1.29; http://ugene.net/). The amplicon sequences selected from pikeperch were highly identical to the counterpart sequences selected from yellow perch (*SIGLEC1*: 92%; *CD22*: 98%; *MAG*: 91%), but they shared only moderate levels of identity with the respective sequences of other fish species. Moreover, we did not find any *SIGLEC15/CD33L3* sequences from percid fish species in the public databases, so we used a *CD33* sequence from barred knifejaw (*Oplegnathus fasciatus*) instead. To take the uncertain assignment of the aforementioned sequences into account, we extended the gene names of the pikeperch sequences by '-like' (abbreviated as 'L').

The integrity and specificity of the PCR products were assessed via standard PCRs (HotStarTaq Plus DNA Polymerase, Qiagen) and single qPCR analyses (LightCycler 480 System, Roche, Mannheim, Germany; SensiFAST SYBR No-ROX Kit, Bioline). A multiplex qPCR analysis was performed with the 48.48 Dynamic Array IFC chip (Fluidigm, South San Francisco, CA, USA) and the BioMark HD-System (Fluidigm) using the nucleotide-binding EvaGreen fluorescence dye (Bio-Rad, Feldkirchen, Germany). In detail, 1 µL of the extracted RNA was reverse-transcribed with the Reverse Transcription Master Mix (Fluidigm). Then, the designed primer pairs and the PreAmp Master Mix (Fluidigm; 100 µM of mix per pair) were used to perform 11 pre-amplification cycles with the individual cDNA samples adjusted at 10 ng/5 µL. The pre-amplified products were treated with exonuclease I (ExoI; New England BioLabs, Frankfurt am Main, Germany) and subsequently diluted in a pre-mixed solution of SsoFast EvaGreen Supermix with Low ROX (Bio-Rad) and 20 × DNA binding dye sample loading reagent. The sample and primer mixes were transferred to the respective inlets of the 48.48 Dynamic Array IFC chip, which was thereafter primed in the BioMark IFC Controller MX (Fluidigm), running the Load Mix 48.48 GE script. The loaded array chip was then placed in the BioMark HD-System (Fluidigm) to proceed with the qPCR according to the GE 48 × 48 Fast PCR+Melt v2.pcl cycling program. Fluidigm RealTime PCR Analysis Software (v. 3.0.2, https://www.fluidigm.com/software) was used to analyze the qPCR results. To obtain the relative copy number of each transcript, a serial dilution-based standard curve (10^2–10^6 copies) was used and the copy number was normalized with the geometric mean of three suitable reference genes (*EEF1A1b*, *RPL9*, and *RLP32* for maraena whitefish; *EEF1A1*, *ACTB*, and *RPS5* for rainbow trout; and *EEF1A1*, *RPS5*, and *RLP32* for pikeperch) [21,26–28].

2.6. Cloning

Since we retrieved only gene fragments of *CD22* and *MAG* from our transcriptome of maraena whitefish, we derived primers from the 5′ and 3′ ends of the respective open reading frames. First, a SuperScript II Reverse Transcriptase Kit (Invitrogen/Thermo Fisher Scientific) was used to transcribe a total of 1 µg of RNA into cDNA. This reverse transcription was carried out at 42 °C for 50 min, followed by an inactivation step at 70 °C for 15 min. Purification of the cDNA was performed using a High Pure PCR Product Purification Kit (Roche), and the resulting cDNA was diluted in 100 µL of distilled water. Subsequently, we used the HotStarTaq Plus DNA Polymerase (Qiagen) to generate the PCR products of the full-length open reading frames. The purified (High Pure PCR Product Purification Kit; Roche) amplicons were inserted into a pGEM-T-Easy vector (Promega, Walldorf, Germany). The obtained plasmids were sequenced using the universal SP6/T7 primers and a MegaBACE capillary sequencer (GE Healthcare, Freiburg im Breisgau, Germany). Twelve clones were picked and analyzed per amplified sequence fragment.

2.7. In Silico Analyses

Sequence alignments were performed using the Clustal Omega tool of EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/clustalo/). The sequences of CD22, MAG and Siglec15 were derived by blasting the orthologous sequences of rainbow trout (*O. mykiss*; Om), coho salmon (*O. kisutch*; Ok), Atlantic salmon (*S. salar*; Ss), yellow perch (*P. flavescens*; Pf), and barred knifejaw (*O. fasciatus*; Of) against the sequence assemblies of maraena whitefish and pikeperch [24,29]. In addition, the CD22 and MAG sequences of maraena whitefish were completed by sequencing the results.

We retrieved the following sequences from the NCBI database: *Danio rerio* (zebrafish) MAG: XP_021337068; *Takifugu rubripes* (pufferfish) MAG: XP_011616490; *Mus musculus* (mouse) Siglec15: NP_001094508, MAG: XP_030098048; CD22: NP_033975; and *Homo sapiens* (human) Siglec15: NP_998767, MAG: AAB58805, CD22: NP_001762. The positions of the transmembrane domains of murine and human Siglec15 were retrieved from Uniprot; the transmembrane areas of Siglec15 from maraena whitefish and rainbow trout were predicted using SMART (http://smart.embl-heidelberg.de/). The V-set, Ig-like domains of murine and human Siglec15 were determined using the SMART program; the V-set, Ig-like domains of MAG and Siglec15 from fishes were estimated based on sequence alignments; and the V-set, Ig-like domains of human MAG and murine MAG (pdb sequence: 5LF5) were determined using the SMART program. In addition, the V-set, Ig-like domain of human and mouse CD22 was determined using the SMART program, while the V-set, Ig-like domains of CD22 from maraena whitefish and rainbow trout were defined by sequence alignment.

The 3D modelling of the sialic acid-binding domain (V-set, Ig-like domain) of CD22 from human and maraena whitefish was performed using YASARA 19.9.17. The structure of human CD22 was given by the pdb sequence: 5VKM, published by Ereño-Orbea et al. [30]. Based on Uniprot, the sequence was shortened to the first Ig-domain, responsible for sialic acid recognition. The sequence of the V-set domain of CD22 from maraena whitefish was obtained by sequence alignment with the sequence of the V-set, Ig-like domain of human CD22.The 3D-modelling of the sialic acid-binding domain (V-set, Ig-like domain) of MAG from mouse and maraena whitefish was also performed using YASARA 19.9.17. The structure of the murine MAG was determined by Pronker et al. [31]. Using the generated alignments, the sequence of murine MAG was shortened to the first Ig domain, which is responsible for sialic acid-binding. The sequence of the V-set, Ig-like domain of MAG from maraena whitefish was based on our cloned sequences.

The mechanistic interaction of Siglecs and associated factors was illustrated using the Ingenuity Pathways Analysis software (IPA; Qiagen Bioinformatics software solutions) based on the Ingenuity Knowledge Base. The constructed informal diagram was manually edited using the Path Designer tool (IPA).

3. Results and Discussion

3.1. The Expression of Siglec-Encoding Genes in Different Tissues of Salmonid and Percid Fishes

The expression of Siglec-encoding genes in different lymphoid and non-lymphoid tissues of the economically important farm-fish families *Salmonidae* and *Percidae* has, to our knowledge, not yet been described in detail. To pave the road for future immunological studies of the sialic acid-dependent regulation of immune processes in bony fish, we performed structural analyses and multiplex qPCR measurements of the piscine Siglecs *Siglec1*, *CD22*, *MAG*, and *Siglec15*.

Siglec1 plays an indispensable role in innate and humoral immunity, even though it contains no immunomodulatory motifs [32]. The expression analyses (Figure 1) demonstrated that *Siglec1* mRNA was most abundant in the spleens and HKs of pikeperch ($>2 \times 10^7$ copies/µg RNA) and in the spleens and gills of maraena whitefish ($>2 \times 10^3$ copies/µg RNA) and rainbow trout ($>3 \times 10^3$ copies/µg RNA). The teleostean HK is considered the functional counterpart of the mammalian bone marrow [1]. It contains considerable amounts of lymphocytes and macrophages [33], similar to the spleen and gills. Probably, *Siglec1* is mainly expressed on those immune cells, since it has been reported that mammalian *Siglec1* is highly expressed on splenic and lymph-node macrophages [9,13].

CD22 was assigned as an activation marker for mature B cells in mammals, and the interaction of CD22 with the B-cell receptor (BCR) has been well established in mammals. The highest levels of *CD22* were found in the HKs of the salmonids maraena whitefish and rainbow trout ($>2 \times 10^5$ copies/µg RNA) as well as in the HKs of pikeperch ($>3 \times 10^6$ copies/µg RNA). The high *CD22* copy number might indicate an analogous interaction between the CD22 and B cells of the teleostean HK.

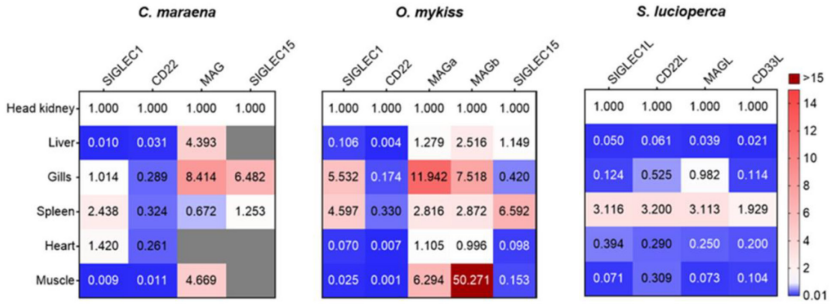


Figure 1. Tissue-specific expression of *Siglec1*, *CD22*, *MAG*, and *Siglec15*/*CD33L* in maraena whitefish (*C. maraena*), rainbow trout (*O. mykiss*), and pikeperch (*S. lucioperca*). The qPCR data were normalized by three reference genes. The resulting transcript numbers from the head kidney (HK) were set at 1.0, and the transcript numbers of the same gene in all other tissues were expressed as fractions. Lower and higher transcript values than those of the HK are highlighted in blue and red, respectively, according to the given color code. Non-detectable transcript numbers are indicated by gray fields.

Siglec15 is an immune-activating Siglec interacting with DNAX proteins [9,11]. The salmonid fish species shared high expression levels of *Siglec15* in the spleen (>15 × 10^3 copies/µg RNA). This observation was in line with the fact that mammalian macrophages are the dominant expression site of *Siglec15* [13,34].

MAG is mainly involved in the stabilization of axon-myelin interactions, the inhibition of neurite growth, and the inhibition of axon regeneration in mammals [35]. The copy numbers of *MAG* were present in a range of tissues across the three analyzed fishes, with the highest *MAG* levels in the gills (>8 × 10^3 copies/µg RNA). Notably, two *MAG* ohnologs were expressed in rainbow trout: *MAGa* and *MAGb*, which are located on chromosomes 2 and 3, respectively. *MAGa* showed at least twice as many transcript numbers as *MAGb* in the analyzed tissues, with muscle containing the highest number of *MAGb* transcripts (>2 × 10^3 copies/µg RNA).

Since in mammals the highest amounts of MAG can be found in the central nervous system [35], we also inspected the expression of *MAG* together with that of *SIGLEC1*, *CD22*, and *SIGLEC15* in different regions of the brains (hypothalami, telencephalons, and hindbrains) of maraena whitefish (Figure 2). While the transcripts of *Siglec1*, *CD22*, and *Siglec15* were detected at low or moderate levels (<2.5 × 10^3 copies/µg RNA), we detected extremely high levels (~2.6 × 10^7 copies/µg RNA) of *MAG* in the brains of maraena whitefish, especially in the hindbrains (>5 × 10^7 copies/µg RNA). The *MAG* levels in the hindbrains exceeded even the relatively high *MAG* copy numbers in the gills, muscles, and livers of maraena whitefish by 300- to 4000-fold (Figures 1 and 2).

The expression of *Siglec15* was absent in telencephalon, but comparably high in the hypothalamus of maraena whitefish (~2.5 × 10^3 copies/µg RNA). The murine SiglecH has previously been described as activating an immune response in microglia cells [36]. SiglecH is known to interact with DAP12 and enhance the phagocytotic activity of glioma cells in mice [36,37]. It is conceivable that Siglec15 may play a similar immune-regulatory function in fish brains. This also applies to CD22, which is expressed in mammalian microglia cells to decrease inflammatory effects [38].

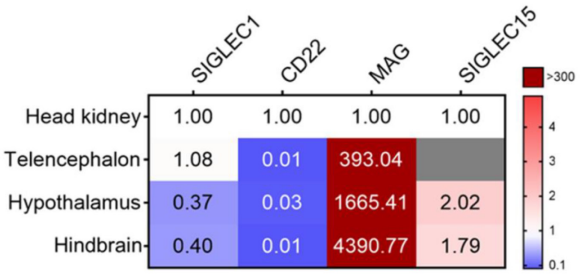


Figure 2. Expression of *Siglec1*, *CD22*, *MAG*, and *Siglec15* in the different brain regions of maraena whitefish. The qPCR data were normalized by the reference genes *RPL9*, *EEF1A1b*, and *RPL32*. The resulting transcript numbers of the same genes in different brain regions are shown relative to the respective transcript levels in the HKs, which were set at 1.0. Transcript values compared with those found in HKs are colored according to the code on the right. Non-detectable transcript numbers are indicated by a gray field.

3.2. *The Expression Patterns of Siglec-Encoding Genes in Cell Populations of Maraena Whitefish*

Since in mammals Siglecs are heterogeneously expressed in immune cells [39], we conducted a subsequent qPCR analysis to determine which immune-cell populations express Siglecs in maraena whitefish. To this end, we isolated the HKs (Figure 3A1) and extracted cells from these tissues (Figure 3A2). The resulting cell suspension was then separated into leukocyte (Figure 3A3) and erythrocyte (Figure 3A4) suspensions via Percoll treatment. Eventually, the leucocyte suspension was further separated into a fraction I, enriched with less granular and smaller cells (presumably lymphocytes and monocytes/macrophages), and a fraction II with more granular and larger cells (presumably granulocytes) (Figure 3B). Profiling the copy numbers of *Siglec1*, *CD22*, *MAG*, and *Siglec15* via multiplex qPCR revealed that the erythrocytes were the main cell population expressing all four Siglecs (Figure 3C1–6).

The main function of mammalian erythrocytes is the transportation of oxygen, whereas teleostean erythrocytes also exert immunological functions [40–43]. This may be due to the fact that teleostean erythrocytes contain nuclei and are capable of regulating their gene expression if necessary [44], in contrast to their mammalian counterparts [45,46]. In particular, fish erythrocytes are considered as antigen-presenting cells, recognize pathogen-associated molecular patterns (PAMPs), phagocytose, and influence the activity of other immune cells [40–43]. It is likely that Siglecs are also involved in these erythrocyte-pathogen interactions. A closer look at the Siglec expression in erythrocytes from maraena whitefish revealed that *Siglec15* and *CD22* were most highly expressed. This also applied to all other immune cell fractions indicating that a high expression of *CD22* in fish is not restricted to B cells [47]. The expression of mammalian *CD22* has been described as being predominately located in B cells [47]. As the genomes of fish obviously lack any inhibitory CD33-related Siglecs (containing the ITIM motif), it might be possible that CD22 may take over this role in the immune cells of fish. In addition, the expression of *MAG* and *Siglec1* was detectable in the analyzed immune cell populations of maraena whitefish.

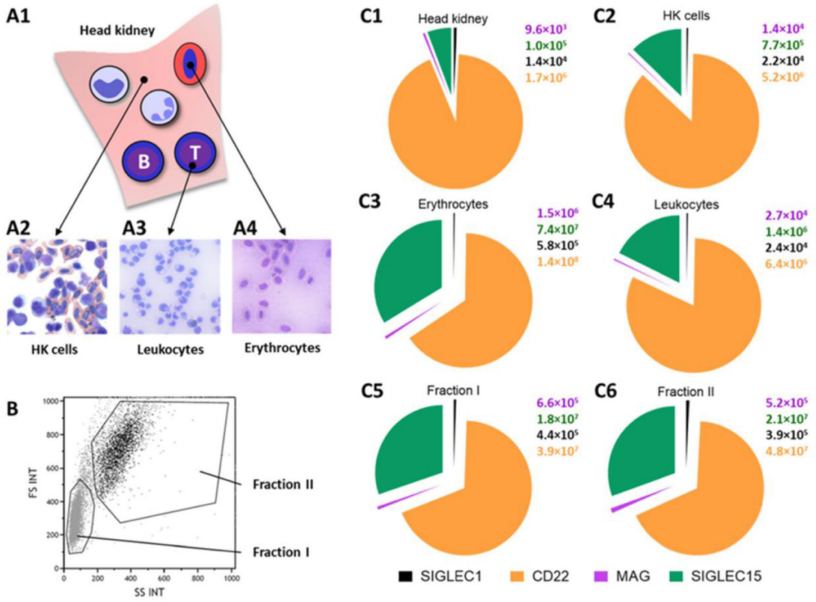


Figure 3. Copy numbers (per µg RNA) of *Siglec1*, *CD22*, *MAG*, and *Siglec15* in (**A1**) whole head kidneys (HKs), (**A2**) extracted HK cells, (**A3**) a heterogeneous leukocyte suspension, (**A4**) erythrocytes, and (**B**) one sorted cell fraction (I), enriched with lymphocytes and monocytes/macrophages, and one fraction (II) enriched with granulocytes (n = 5). (**C**) Siglec expression in the isolated cell fractions (**C1**) HK, (**C2**) HK cells, (**C3**) erythrocytes, (**C4**) leukocytes, (**C5**) fraction I, and (C**6**) fraction II. QPCR expression data for *Siglec1* (black), *CD22* (orange), *MAG* (purple), and *Siglec15* (green) was normalized against the GeoMean of the reference genes *RPL9*, *EEF1A1b*, and *RPL32*.

3.3. Sequence Comparison of Siglecs Expressed by Salmonid and Percid Fishes

We analyzed the nucleotide and amino-acid sequences of the evolutionarily conserved Siglecs in fish and mammals in more detail because their expression profiles exhibited remarkable differences. Siglec1 contains no regulatory domains; for this reason, only the sequences of CD22, MAG, and Siglec15 are presented here. Since the Siglec sequences of the pikeperch (SIGLEC1L, CD22L, MAGL, and CD33L) revealed poor homology with its piscine orthologs (see Section 2.5), we disregarded these sequences in the more detailed sequence analysis.

3.3.1. Sequence Comparison of Siglec2 (CD22)

In mammals, CD22 can counteract the BCR-triggered activation of B cells, when defined sialylated structures are detected simultaneously with an antigen [48,49]. The clustering of a BCR with CD22 molecules causes the recruitment of SHP1 and SHP2 (encoded by the genes *PTPN6* and *PTPN11*), which leads to the inhibition of the kinase-dependent signaling pathway, along with the reduced production of antibodies against the autoantigen [48,49]. This mechanism inhibits thus the synthesis of autoantibodies. Teleosts produce three main types of immunoglobulins (IgM, IgD, and IgT/IgZ) that act as BCRs [50,51]. The interplay between these immunoglobulins and CD22 might be regulated in a mammalian-analogous way on teleostean B cells.

The *N*-glycosylation status of mammalian CD22 seems to be important for its activity. Twelve *N*-glycosylation sites are known in mammalian CD22, six of which are close to the sialic acid-binding domain (N_{67}; N_{101}; N_{112}; N_{135}; N_{164}; N_{231}). According to Orbea et al., N_{67}, N_{112}, N_{135}, N_{164}, N_{231} can

be exchanged by an alanine without functional loss, while the mutation of N_{101} disrupted protein expression [30]. N-glycans at N_{101} are probably involved in the correct folding of the receptor. Regarding the sequence comparison of the V-set Ig-like domain, which is responsible for sialic acid-binding (Figure 4A), N_{101} seems to be conserved from mammals to lower vertebrates (N_{105} in fishes), while N_{67} and N_{135} seem to be absent. Recently, Wasim et al. determined that mutations of N_{67}, N_{112}, N_{135}, N_{164} and N_{231} resulted in a higher density of CD22 nanoclusters, along with a decreased CD22-phosphorylation rate and an increased B-cell signaling, culminating in a reduced functionality of CD22 [52]. Therefore, we took a closer look at the N-glycosylation sites in the CD22 orthologs from maraena whitefish and rainbow trout. The alignment of the CD22 sequences from maraena whitefish and rainbow trout suggested (Figure 4A and Figure S1) that the majority of N-glycosylation sites are also present in CD22 of fish, although they are at slightly different positions compared to their human orthologs. The N-glycosylation at N_{67}, N_{101}, N_{112}, N_{164} and N_{231} in human corresponds to N_{59}, N_{105}, N_{112}, N_{167} and N_{221} in fish. Moreover, we searched for ITIM domains in the CD22 orthologs of maraena whitefish and rainbow trout, since these domains characterize inhibitory receptors in mammals [53]. An ITIM is present in CD22 of rainbow trout but not in the orthologous sequence of maraena whitefish (Figure S1). However, our CD22 sequence of maraena whitefish was severely truncated, and we cannot exclude the possibility that an ITIM is present there.

Furthermore, we analyzed the sialic acid-binding domain in more detail. Human CD22 preferentially binds α2,6-linked sialic acid. The binding is mediated by the amino-acid residues R_{120}, R_{131}, E_{126} and W_{128} in addition to Y_{64}, which is responsible for the preference for α2,6-linked sialic acid [30]. The sequence comparison (Figure 4A) demonstrated that R_{120} is conserved from mammals to fish, while almost all other amino acids, necessary for sialic acid-binding in humans, are missing in the investigated fish. In mice, Y_{64} is replaced by F, indicating the conservation of the aromatic properties, and also in fish, W resides close to Y_{64} in human [30].

In addition, we simulated 3D models of the V-set Ig-like domain of CD22 from maraena whitefish based on the known 3D structure of the human counterpart [30]. This 3D model exhibited remarkable structural differences between the CD22 orthologs from human and maraena whitefish (Figure 4B–D). Hence, based on the modelling of CD22 from maraena whitefish (Figure 4B–D) combined with the sequence alignment (Figure 4A), we suggest that the binding properties of CD22 have changed during evolution. However, experimental data is needed to define the glycan-binding properties of CD22 in fishes.

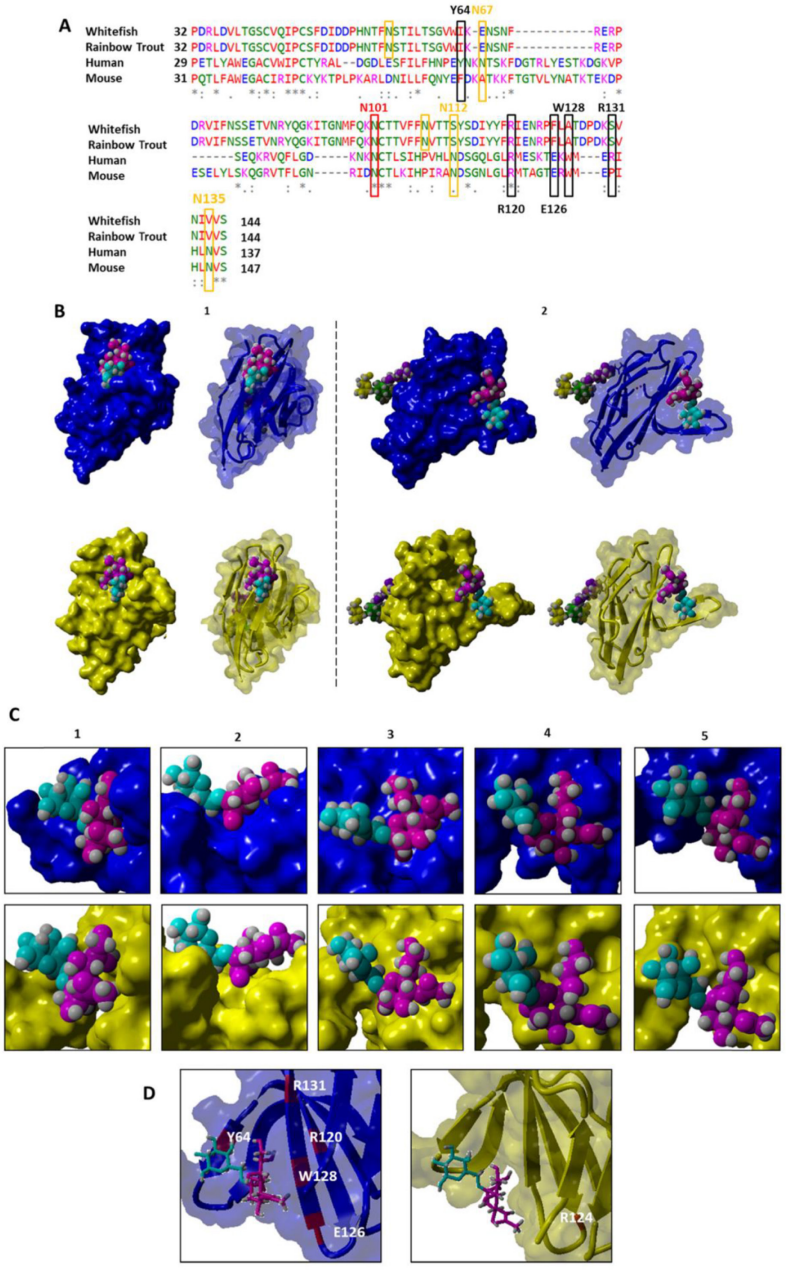


Figure 4. Sequence and structural comparison of CD22 from human and maraena whitefish. (**A**) The first Ig domain of maraena whitefish and rainbow trout was determined by aligning sequences. The different colors indicate the chemical properties of the amino acids as follows: red, small hydrophobic/aromatic

amino acids; blue, acidic amino acids; magenta, basic amino acids; green, hydrophilic, polar, and small amino acids. Black boxes show the amino acids that are essential for sialic acid-binding; red boxes show the conserved N-glycosylation site N_{101}. In addition, the orange boxes indicate further amino acids known to be a target for N-glycosylation. Numbering is based on the human CD22 sequence. (**B**) YASARA was used to model the 3D structure of the sialic-acid-binding domain (V-set, Ig-like domain) of CD22 from human (pdb: 5VKM, including 5 point mutations N_{67}A, N_{112}A, N_{135}A, N_{164}A, N_{231}A [30]) and maraena whitefish. The sequence of the human MAG was shortened to the first Ig domain. The sequence of the V-set, Ig-like domain of CD22 from maraena whitefish was based on the alignments (see (**A**)). CD22 models from human and whitefish are labelled in blue and yellow, respectively. Two different perspectives of the surface with a transparency of 30% are shown along with the corresponding secondary structures. (**C**) Enlargement of the sialic-acid-binding domain of CD22 from maraena whitefish in five different perspectives. (**D**) Amino acids responsible for sialic acid-binding by CD22 from human and maraena whitefish. Surface and secondary structures are shown. Bound glycans are specifically labeled as follows: galactose, cyan; *N*-acetyl-D-galactosamine, purple; sialic acid, pink; α-D-mannose, yellow; β-D-mannose, green.

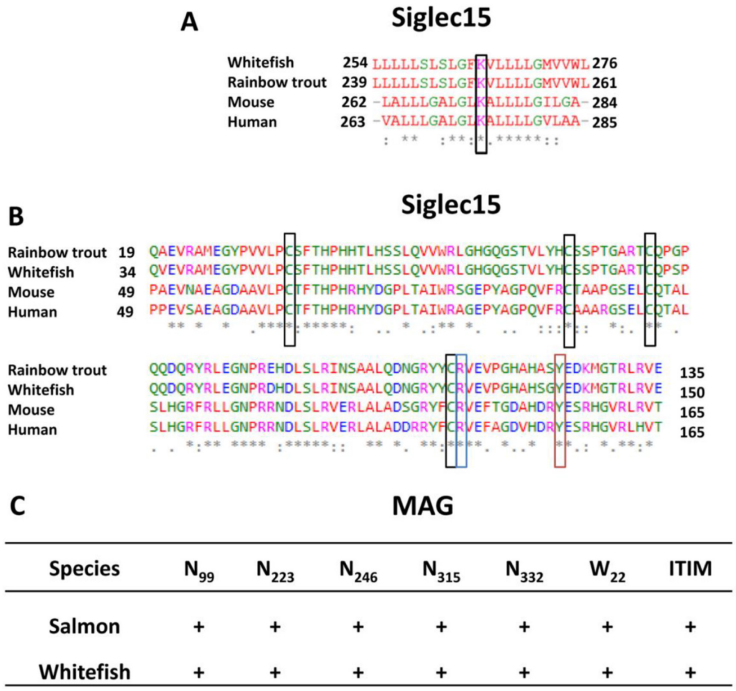
3.3.2. Sequence Comparison of Siglec15

Siglec15 belongs to the activating receptors, interacting with DAP10/12 via a lysine residue in the transmembrane domain [17]. This residue is well-conserved from fish to mammals (Figure 5A). The exchange of lysine by alanine has been demonstrated to abrogate the interaction of Siglec15 with DAP10 or DAP12; this confirms the functional importance of the lysine residue. However, this exchange might not impact the minimal FcRγ interaction with Siglec15 [17].

Furthermore, sequence alignments of the first Ig domain were performed. Angata and colleagues showed that an exchange of R_{143} to alanine results in the loss of sialic acid-binding and thus, in a loss of the immune regulatory function of Siglec15 [17]. This amino acid is conserved from mammals to lower vertebrates (Figure 5B). In addition, cysteine residues in the V-set, Ig-like domain are highly conserved across vertebrates. One likely reason for this remarkable conservation is pathogen-driven selection pressure. Our sequence alignments of the first Ig domain of the Siglec15 orthologs suggested that the well-conserved cysteine residues that contribute to the tertiary structure by forming disulfide bonds are present in the Siglec15 of the investigated salmonid species (Figure 5B). Nevertheless, the cloned ortholog from zebrafish did not show strong binding to the tested glycans ex vivo, irrespective of the presence or absence of cysteine residues [17].

3.3.3. Sequence Comparison of Siglec4 (MAG)

In mammals, MAG is involved in myelination processes through interactions with gangliosides. The expression of *MAG* is restricted to Schwann cells and oligodendrocytes. The dimerization of MAG is essential for specific axon-myelin spacing (9–12 nm) and strongly depends on the glycosylation pattern of MAG [15,35,54,55]. Therefore, we also inspected potential N-glycosylation sites of the MAG sequences from maraena whitefish and Atlantic salmon. Eight sites of N-glycosylation are known in human MAG (N_{99}, N_{223}, N_{246}, N_{315}, N_{332}, N_{406}, N_{450}, and N_{454}). The residue W_{22} is targeted by *C*-mannosylation and conserved from fish to humans (W_{21} in fish). Therefore, it is likely that this residue contributes to the functionality of MAG [31]. Our sequence alignment showed that the N-glycosylation sites of the three Ig-domains of the MAG were conserved together with residue W_{22} of the murine MAG sequences (Figure 5C and Figure S2).



C

MAG

Species	N_{99}	N_{223}	N_{246}	N_{315}	N_{332}	W_{22}	ITIM
Salmon	+	+	+	+	+	+	+
Whitefish	+	+	+	+	+	+	+

Figure 5. Analysis of specific properties of Siglec15 and MAG. (**A**) Sequence alignment of the transmembrane domain of Siglec15. The positions of the transmembrane domains of murine and human Siglec15 were retrieved from Uniprot, while the transmembrane area of Siglec15 from maraena whitefish and rainbow trout were predicted using SMART. The black box highlights the transmembrane lysine residue, which interacts with DAP10/12. (**B**) Sequence alignments of the first Ig domain of Siglec15. The alignment illustrates the conserved cysteine residues (black boxes) of Siglec15 as well as the conserved arginine residue (blue box) and a conserved hydrophobic amino acid (Y, red box), which are involved in sialic acid-binding [17]. Sequence alignments were performed using the Clustal Omega tool of EMBL-EBI. Different colors label the chemical properties of the amino acids: red, small hydrophobic/aromatic amino acids; blue, acidic amino acids; magenta, basic amino acids; green, hydrophilic, polar, and small amino acids. (**C**) The presence of specific N-glycosylation sites and IIM motifs of MAG from salmon and whitefish are shown. The position of the amino acid residue refers to the human sequence. For accession numbers, see Section 2.7.

In addition, these MAG sequences harbor immune-regulatory ITIMs in their intracellular sections, whereas their mammalian counterparts lack these motifs. In 2004, Lehmann et al. detected a proximal ITIM motif in the MAG orthologs of zebrafish and pufferfish [16]. Lehmann et al. suggested that these motifs are involved in signal transduction and contribute to biological functions different from those described for mammalian MAG [16]. Furthermore, our previous sequence analysis [15] confirmed the presence of ITIMs in coelacanth, while almost all investigated higher vertebrates were ITIM-negative. Consequently, the ITIM motif seems to be an ancestral feature that has persisted in most Siglecs but was lost during the evolution of higher vertebrates [15,16]. The presence of ITIM motifs in the intracellular part of MAG in salmonid fish might contribute to inhibiting immunological responses, while mammalian MAGs merely stabilize axon-myelin interactions by binding to GD1a and GT1b, two gangliosides present in the brain. Unlike the MAG in mammals, the MAG in fish seems to be involved

in immunomodulatory processes. To analyze its carbohydrate-binding pocket in more detail, the 3D structure of MAG from maraena whitefish was simulated and compared with its murine ortholog (Figure 6A–C).

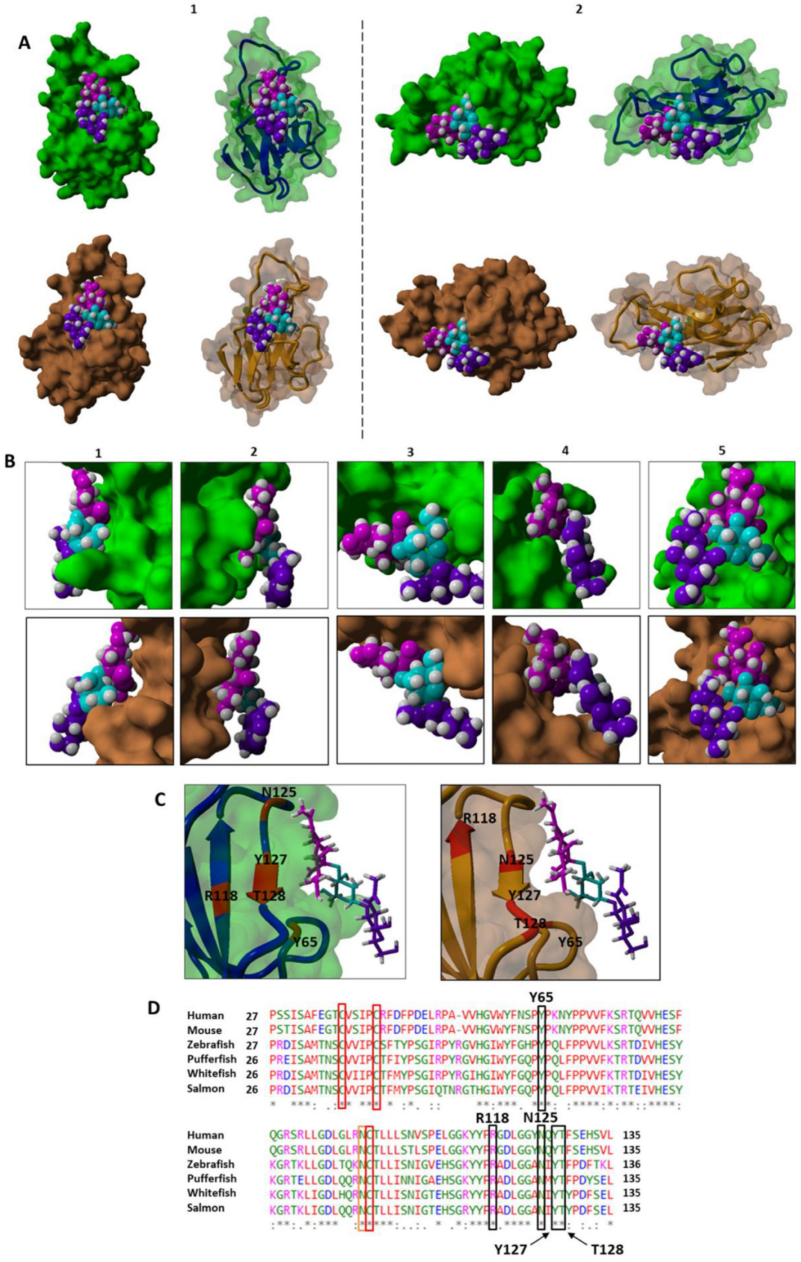


Figure 6. Sequence and structural comparison of MAG from mouse and maraena whitefish. (**A**) YASARA was used to model the 3D structure of the sialic-acid-binding domain (V-set, Ig-like domain) of MAG from mouse and maraena whitefish. Based on the sequence alignments performed, the sequence of the

murine MAG was shortened to the first Ig domain. The sequence of the V-set Ig-like domain of MAG from maraena whitefish was based on the performed alignments (see (**D**)). MAG models from mouse and maraena whitefish are labelled in green and brown, respectively. Two different perspectives of the surface with a transparency of 30% are shown along with the corresponding secondary structures. (**B**) Enlargement of the sialic-acid-binding domain of MAG from maraena whitefish in five different perspectives. (**C**) Amino acids responsible for sialic acid-binding by MAG from mouse and maraena whitefish. Surface and secondary structures are shown. Bound glycans are specifically labeled as follows: galactose, cyan; *N*-acetyl-D-galactosamine, purple; sialic acid, pink. (**D**) Sequences of MAG from zebrafish, pufferfish, human, and mouse were available in the NCBI database: zebrafish MAG: XP_021337068; pufferfish MAG: XP_011616490; murine MAG: XP_030098048; human MAG: AAB58805. The V-set, Ig-like domain of the mouse MAG was determined by analyzing the pdb sequence (pdb: 5LF5) with SMART. The V-set Ig-like domain of the human MAG was assessed using SMART. For zebrafish, pufferfish, maraena whitefish, and rainbow trout, V-set, Ig-like domains were detected using sequence alignment. The different colors indicate the chemical properties of the amino acids as follows: red, small hydrophobic/aromatic amino acids; blue, acidic amino acids; magenta, basic amino acids; green: hydrophilic, polar, and small amino acids. Black boxes show the amino acids that are essential for sialic acid-binding, red boxes show conserved cysteine residues [16] and the orange box indicates a glycosylation site.

Based on the crystal structure of murine MAG, the program determined that the secondary structure of MAG from maraena whitefish contains 37.6% beta sheets, 18.3% alpha-turn-helices, and 44.0% coiled coils, whereas that of murine MAG contains only 34.9% beta sheets, 22.0% alpha-turn-helices, and 43.1% coiled coils (Figure 6A). Although several amino acid residues differed between the MAG sequences from mouse and maraena whitefish, no significant changes were visible with regard to the sialic-acid-binding domain (Figure 6B,C). The binding pockets of MAG from mouse and maraena whitefish for Neu5Ac-α2,3-Gal-β1,3-GalNAc are highly comparable. The data suggest analogous functions of MAG in maraena whitefish and mammals. In addition, we aligned the sequences of the selected MAG orthologs to compare the presence of essential amino acids that mediate the protein-carbohydrate interaction. In human MAG, amino acids R_{118}, Y_{65}, N_{125}, T_{128}, and Y_{127} are responsible for sialic acid-binding [31]. These amino acids were conserved throughout evolution (Figure 6D), indicating once more [16] the preserved potential of MAG to bind to sialic acid across a range of vertebrate classes.

3.4. The Influence of Handling Stress on the Expression of Siglecs in Maraena Whitefish

Stress is known to affect immune processes [2]. To investigate the impact of stress on a panel of nine selected target genes related to the Siglec signaling, we exposed maraena whitefish to one-minute handling procedures (including chasing and exposure to air) and sampled the fish three hours after this treatment. Subsequently, we recorded the expression of the genes encoding the four Siglecs present in fish (*Siglec1*, *CD22*, *MAG*, and *Siglec15*), the associated non-receptor tyrosine kinases *LYN, SYK,* and *ZAP70* in addition to the non-receptor tyrosine phosphatase *PTPN6* (alias SHP1) and *PTPN11* (alias SHP2) (Figure 7A). Gene profiling revealed that the transcript levels of the four Siglecs were modulated in a tissue-specific fashion after exposure to stress (Figure 7B). *Siglec1* and *CD22* were 2.0- to 4.3-fold upregulated in telencephalon and hindbrain but substantially downregulated in the heart as well as the spleen (*Siglec1*) and muscle (*CD22*). All other tissues exhibited comparable values in untreated and stressed fish.

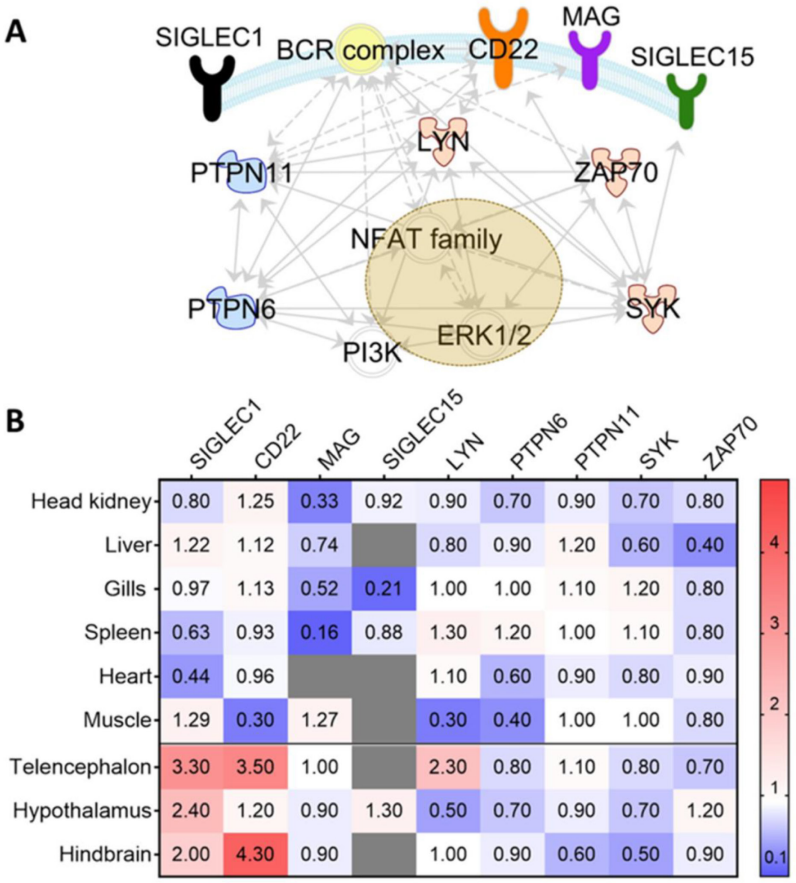


Figure 7. (**A**) Functional relationship between Siglec receptors and associated kinases (red symbols) and phosphatases (blue symbols) in a schematic B cell. Interactions are displayed by broken (indirect influence) or full (direct influence) lines. Cellular and nuclear membranes are colored in light blue and brown, respectively. Please note that these relationships are supported by at least one reference, which are based exclusively on investigations in mammalian species (accessible in the Ingenuity Knowledge Base). (**B**) Tissue-specific expression of genes encoding Siglecs (*Siglec1*, *CD22*, *MAG*, *Siglec15*) and downstream factors (*LYN*, *PTPN6*, *PTPN11*, *SYK*, *ZAP70*) in maraena whitefish exposed to three hours of handling stress. QPCR data were normalized by the reference genes *RPL9*, *EEF1A1b*, and *RPL32*. The heat map shows the averaged fold-change values in the respective tissue relative to the same tissue from unstressed fish, colored according to the code on the right. Non-detectable transcript numbers are indicated by gray fields.

Surprisingly, *MAG* expression was not influenced in the brain and was downregulated in nearly all other tissues. This effect was especially pronounced in both lymphoid organs spleen and HK. Since MAG from maraena whitefish contains an ITIM, this data might indicate the immunomodulatory capacity of MAG in salmonid fish. Furthermore, few *MAG* transcripts were detected in gills of stressed maraena whitefish, and these fish also exhibited reduced *Siglec15*-transcript level in their gills. As the

respiratory organs of fish, gills are directly exposed to significant environmental changes, including exposure to air, and are thus expected to induce fast responses.

The genes encoding the three Siglec-associated kinases (*LYN*, *SYK*, and *ZAP70*) and two phosphatases (*PTPN6* and *PTPN11*) were expressed at high levels (between ~2200 and ~71,500 copies/µg RNA) in HK, gills and spleen, but at low levels (<450 copies/µg RNA) in muscle, telencephalon, hypothalamus, and hindbrain. Handling stress did not affect the expression of the aforementioned enzyme genes, except for *LYN* in muscle (~4-fold downregulated), hypothalamus (~2-fold downregulated), and telencephalon (~2-fold upregulated), as well as *PTPN6* in muscle (~3-fold downregulated) (Figure 7B). Since we recorded these alterations in those tissues that had only relatively low basal concentrations of the respective transcripts, the observed expression data should not be overestimated. We rather assume that the stress-dependent regulation of the activity of Siglec-associated enzymes in fish does not occur at the transcript level. In contrast, the expression patterns of Siglec-encoding genes showed characteristic factor-specific alterations, both under homeostatic conditions and in response to handling stress. Although the biological significance of these changes remains unknown, the data obtained in the present study strongly suggests that the function of individual Siglecs has partially changed during the evolution of vertebrates.

4. Conclusions

The present study draws three main conclusions:

- Our qPCR analyses suggested that the basal gene-expression patterns of *Siglec1*, *CD22*, *MAG*, and *Siglec15* are largely conserved across salmonid and percid fishes. In contrast to mammals, *CD22* is highly expressed in several blood-cell populations. Similarly, the expression of *MAG* in fish is not restricted to the cells of the nervous system but is detectable in a range of blood cells.
- Stress modulates the expression of Siglecs (but not of the associated enzymes) in a tissue-dependent fashion and most likely influences the cellular reactivity against PAMPs and DAMPs.
- The genomes of fish lack CD33-related Siglecs, which exert inhibitory functions. Our structural analyses indicated that CD22 and MAG contain inhibitory motifs (ITIM) in salmonid fish. We speculate that these ITIM-containing Siglecs may compensate the deficiency of the canonical inhibitory Siglecs. This first assumption might be the starting point for subsequent studies to clarify whether CD22 and MAG have an immunosuppressive effect in fish.

Supplementary Materials: The following materials are available online at http://www.mdpi.com/2073-4409/9/4/836/s1: Figure S1: Sequence alignment of CD22 of rainbow trout (XM_021620093) and the obtained sequence of CD22 of maraena whitefish, Figure S2: Sequence alignment of MAG from salmon and the obtained sequence of MAG of maraena whitefish, Table S1: Primer sequences and accession codes.

Author Contributions: S.P.G., A.R., and U.G. designed the study. K.F.B. analyzed the sequences and generated the 3D models. J.M.R. and M.T.V. performed the qPCR analyses and calculated the data. J.M.R. and M.T.V. conducted stress experiments. T.V. performed flow cytometric cell sorting. K.F.B. wrote and S.P.G., A.R., and J.M.R. edited the manuscript. All authors have read and approved the final article. All authors have read and agreed to the published version of the manuscript.

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

References

1. Uribe, C.; Folch, H.; Enríquez, R.; Moran, G. Innate and adaptive immunity in teleost fish: A review. *Vet. Med.* **2011**, *56*, 486–503. [CrossRef]
2. Rebl, A.; Goldammer, T. Under control: The innate immunity of fish from the inhibitors' perspective. *Fish Shellfish Immunol.* **2018**, *77*, 328–349. [CrossRef] [PubMed]
3. Varki, A. Are humans prone to autoimmunity? Implications from evolutionary changes in hominin sialic acid biology. *J. Autoimmun.* **2017**, *83*, 134–142. [CrossRef]
4. Varki, A. Biological roles of glycans. *Glycobiology* **2017**, *27*, 3–49. [CrossRef] [PubMed]
5. Varki, A. Letter to the glyco-forum: Since there are pamps and damps, there must be samps? Glycan "self-associated molecular patterns" dampen innate immunity, but pathogens can mimic them. *Glycobiology* **2011**, *21*, 1121–1124. [CrossRef]
6. Bloemendal, V.R.L.J.; Moons, S.J.; Heming, J.J.A.; Chayoua, M.; Niesink, O.; van Hest, J.C.M.; Boltje, T.J.; Rutjes, F.P.J.T. Chemoenzymatic synthesis of sialic acid derivatives using immobilized n-acetylneuraminate lyase in a continuous flow reactor. *Adv. Synth. Catal.* **2019**, *361*, 2443–2447. [CrossRef]
7. Coady, A.; Nizet, V. Samp-ending down sepsis. *Ann. Transl. Med.* **2016**, *4*, 509. [CrossRef]
8. Varki, A.; Angata, T. Siglecs—The major subfamily of i-type lectins. *Glycobiology* **2006**, *16*, 1R–27R. [CrossRef]
9. Crocker, P.R.; Paulson, J.C.; Varki, A. Siglecs and their roles in the immune system. *Nat. Rev. Immunol.* **2007**, *7*, 255–266. [CrossRef]
10. Angata, T.; Margulies, E.H.; Green, E.D.; Varki, A. Large-scale sequencing of the cd33-related siglec gene cluster in five mammalian species reveals rapid evolution by multiple mechanisms. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 13251–13256. [CrossRef]
11. Ravetch, J.V.; Lanier, L.L. Immune inhibitory receptors. *Science* **2000**, *290*, 84–89. [CrossRef]
12. Pillai, S.; Netravali, I.A.; Cariappa, A.; Mattoo, H. Siglecs and immune regulation. *Annu. Rev. Immunol.* **2012**, *30*, 357–392. [CrossRef] [PubMed]
13. O'Reilly, M.K.; Paulson, J.C. Siglecs as targets for therapy in immune-cell-mediated disease. *Trends Pharmacol. Sci.* **2009**, *30*, 240–248. [CrossRef] [PubMed]
14. Crocker, P.R.; Varki, A. Siglecs in the immune system. *Immunology* **2001**, *103*, 137–145. [CrossRef] [PubMed]
15. Bornhöfft, K.F.; Goldammer, T.; Rebl, A.; Galuska, S.P. Siglecs: A journey through the evolution of sialic acid-binding immunoglobulin-type lectins. *Dev. Comp. Immunol.* **2018**, *86*, 219–231. [CrossRef]
16. Lehmann, F.; Gathje, H.; Kelm, S.; Dietz, F. Evolution of sialic acid-binding proteins: Molecular cloning and expression of fish siglec-4. *Glycobiology* **2004**, *14*, 959–968. [CrossRef]
17. Angata, T.; Tabuchi, Y.; Nakamura, K.; Nakamura, M. Siglec-15: An immune system siglec conserved throughout vertebrate evolution. *Glycobiology* **2007**, *17*, 838–846. [CrossRef]
18. Robertson, F.M.; Gundappa, M.K.; Grammes, F.; Hvidsten, T.R.; Redmond, A.K.; Lien, S.; Martin, S.A.M.; Holland, P.W.H.; Sandve, S.R.; Macqueen, D.J. Lineage-specific rediploidization is a mechanism to explain time-lags between genome duplication and evolutionary diversification. *Genome Biol.* **2017**, *18*, 111. [CrossRef]
19. Korytar, T.; Nipkow, M.; Altmann, S.; Goldammer, T.; Kollner, B.; Rebl, A. Adverse husbandry of maraena whitefish directs the immune system to increase mobilization of myeloid cells and proinflammatory responses. *Front. Immunol.* **2016**, *7*, 631. [CrossRef]
20. Rebl, A.; Verleih, M.; Nipkow, M.; Altmann, S.; Bochert, R.; Goldammer, T. Gradual and acute temperature rise induces crossing endocrine, metabolic, and immunological pathways in maraena whitefish (coregonus maraena). *Front. Genet.* **2018**, *9*, 241. [CrossRef]
21. Swirplies, F.; Wuertz, S.; Baßmann, B.; Orban, A.; Schäfer, N.; Brunner, R.M.; Hadlich, F.; Goldammer, T.; Rebl, A. Identification of molecular stress indicators in pikeperch sander lucioperca correlating with rising water temperatures. *Aquaculture* **2019**, *501*, 260–271. [CrossRef]
22. Rebl, A.; Rebl, H.; Verleih, M.; Haupt, S.; Kobis, J.M.; Goldammer, T.; Seyfert, H.M. At least two genes encode many variants of irak3 in rainbow trout, but neither the full-length factor nor its variants interfere directly with the tlr-mediated stimulation of inflammation. *Front. Immunol.* **2019**, *10*, 2246. [CrossRef] [PubMed]
23. Martorell Ribera, J.; Nipkow, M.; Viergutz, T.; Brunner, R.M.; Bochert, R.; Koll, R.; Goldammer, T.; Gimsa, U.; Rebl, A. Early response of salmonid head-kidney cells to stress hormones and toll-like receptor ligands. *Fish Shellfish Immunol.* **2020**, *98*, 950–961. [CrossRef] [PubMed]

24. Nguinkal, J.A.; Brunner, R.M.; Verleih, M.; Rebl, A.; de Los Rios-Perez, L.; Schafer, N.; Hadlich, F.; Stueken, M.; Wittenburg, D.; Goldammer, T. The first highly contiguous genome assembly of pikeperch (sander lucioperca), an emerging aquaculture species in europe. *Genes (Basel)* **2019**, *10*, 708. [CrossRef] [PubMed]
25. Langmead, B.; Salzberg, S.L. Fast gapped-read alignment with bowtie 2. *Nat. Methods* **2012**, *9*, 357–359. [CrossRef] [PubMed]
26. Altmann, S.; Rebl, A.; Kuhn, C.; Goldammer, T. Identification and de novo sequencing of housekeeping genes appropriate for gene expression analyses in farmed maraena whitefish (coregonus maraena) during crowding stress. *Fish Physiol. Biochem.* **2015**, *41*, 397–412. [CrossRef]
27. Rebl, A.; Rebl, H.; Korytar, T.; Goldammer, T.; Seyfert, H.M. The proximal promoter of a novel interleukin-8-encoding gene in rainbow trout (oncorhynchus mykiss) is strongly induced by cebpa, but not nf-kappab p65. *Dev. Comp. Immunol.* **2014**, *46*, 155–164. [CrossRef]
28. Rebl, A.; Anders, E.; Wimmers, K.; Goldammer, T. Characterization of dehydrodolichyl diphosphate synthase gene in rainbow trout (oncorhynchus mykiss). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2009**, *152*, 260–265. [CrossRef]
29. Brietzke, A.; Borchel, A.; Altmann, S.; Nipkow, M.; Rebl, A.; Brunner, R.M.; Goldammer, T. Transcriptome sequencing of maraena whitefish (coregonus maraena). *Mar. Genomics* **2016**, *29*, 27–29. [CrossRef]
30. Ereño-Orbea, J.; Sicard, T.; Cui, H.; Mazhab-Jafari, M.T.; Benlekbir, S.; Guarné, A.; Rubinstein, J.L.; Julien, J.P. Molecular basis of human cd22 function and therapeutic targeting. *Nat. Commun.* **2017**, *8*, 764. [CrossRef]
31. Pronker, M.F.; Lemstra, S.; Snijder, J.; Heck, A.J.R.; Thies-Weesie, D.M.E.; Pasterkamp, R.J.; Janssen, B.J.C. Structural basis of myelin-associated glycoprotein adhesion and signalling. *Nat. Commun.* **2016**, *7*, 13584. [CrossRef] [PubMed]
32. Chang, Y.C.; Olson, J.; Louie, A.; Crocker, P.R.; Varki, A.; Nizet, V. Role of macrophage sialoadhesin in host defense against the sialylated pathogen group b streptococcus. *J. Mol. Med. (Berl).* **2014**, *92*, 951–959. [CrossRef] [PubMed]
33. Zapata, A.; Diez, B.; Cejalvo, T.; Gutierrez-de Frias, C.; Cortes, A. Ontogeny of the immune system of fish. *Fish Shellfish Immunol.* **2006**, *20*, 126–136. [CrossRef] [PubMed]
34. Macauley, M.S.; Crocker, P.R.; Paulson, J.C. Siglec regulation of immune cell function in disease. *Nat. Rev. Immunol.* **2014**, *14*, 653–666. [CrossRef]
35. Quarles, R.H. Myelin-associated glycoprotein (mag): Past, present and beyond. *J. Neurochem.* **2007**, *100*, 1431–1448. [CrossRef]
36. Kopatz, J.; Beutner, C.; Welle, K.; Bodea, L.G.; Reinhardt, J.; Claude, J.; Linnartz-Gerlach, B.; Neumann, H. Siglec-h on activated microglia for recognition and engulfment of glioma cells. *Glia* **2013**, *61*, 1122–1133. [CrossRef]
37. Blasius, A.L.; Colonna, M. Sampling and signaling in plasmacytoid dendritic cells: The potential roles of siglec-h. *Trends Immunol.* **2006**, *27*, 255–260. [CrossRef]
38. Mott, R.T.; Ait-Ghezala, G.; Town, T.; Mori, T.; Vendrame, M.; Zeng, J.; Ehrhart, J.; Mullan, M.; Tan, J. Neuronal expression of cd22: Novel mechanism for inhibiting microglial proinflammatory cytokine production. *Glia* **2004**, *46*, 369–379. [CrossRef]
39. Duan, S.; Paulson, J.C. Siglecs as immune cell checkpoints in disease. *Annu. Rev. Immunol.* **2020**, *38*. [CrossRef]
40. Puente-Marin, S.; Nombela, I.; Ciordia, S.; Mena, M.C.; Chico, V.; Coll, J.; Ortega-Villaizan, M.D.M. In silico functional networks identified in fish nucleated red blood cells by means of transcriptomic and proteomic profiling. *Genes (Basel)* **2018**, *9*, 202. [CrossRef]
41. Passantino, L.; Massaro, M.A.; Jirillo, F.; Di Modugno, D.; Ribaud, M.R.; Modugno, G.D.; Passantino, G.F.; Jirillo, E. Antigenically activated avian erythrocytes release cytokine-like factors: A conserved phylogenetic function discovered in fish. *Immunopharmacol. Immunotoxicol.* **2007**, *29*, 141–152. [CrossRef] [PubMed]
42. Morera, D.; Roher, N.; Ribas, L.; Balasch, J.C.; Donate, C.; Callol, A.; Boltana, S.; Roberts, S.; Goetz, G.; Goetz, F.W.; et al. Rna-seq reveals an integrated immune response in nucleated erythrocytes. *PLoS ONE* **2011**, *6*, e26998. [CrossRef]
43. Workenhe, S.T.; Kibenge, M.J.; Wright, G.M.; Wadowska, D.W.; Groman, D.B.; Kibenge, F.S. Infectious salmon anaemia virus replication and induction of alpha interferon in atlantic salmon erythrocytes. *Virol. J.* **2008**, *5*, 36. [CrossRef] [PubMed]

44. Puente-Marin, S.; Thwaite, R.; Mercado, L.; Coll, J.; Roher, N.; Ortega-Villaizan, M.D.M. Fish red blood cells modulate immune genes in response to bacterial inclusion bodies made of tnfα and a g-vhsv fragment. *Front. Immunol.* **2019**, *10*, 1055. [CrossRef] [PubMed]
45. Raja-Sabudin, R.Z.; Othman, A.; Ahmed-Mohamed, K.A.; Ithnin, A.; Alauddin, H.; Alias, H.; Abdul-Latif, Z.; Das, S.; Abdul-Wahid, F.S.; Hussin, N.H. Immature reticulocyte fraction is an early predictor of bone marrow recovery post chemotherapy in patients with acute leukemia. *Saudi Med. J.* **2014**, *35*, 346–349.
46. Ney, P.A. Normal and disordered reticulocyte maturation. *Curr. Opin. Hematol.* **2011**, *18*, 152–157. [CrossRef]
47. Clark, E.A.; Giltiay, N.V. Cd22: A regulator of innate and adaptive b cell responses and autoimmunity. *Front. Immunol.* **2018**, *9*, 2235. [CrossRef]
48. Nitschke, L. The role of cd22 and other inhibitory co-receptors in b-cell activation. *Curr. Opin. Immunol.* **2005**, *17*, 290–297. [CrossRef]
49. Tedder, T.F.; Poe, J.C.; Haas, K.M. Cd22: A multifunctional receptor that regulates B lymphocyte survival and signal transduction. *Adv. Immunol.* **2005**, *88*, 1–50.
50. Parra, D.; Korytář, T.; Takizawa, F.; Sunyer, J.O. B cells and their role in the teleost gut. *Dev. Comp. Immunol.* **2016**, *64*, 150–166. [CrossRef]
51. Parra, D.; Takizawa, F.; Sunyer, J.O. Evolution of b cell immunity. *Annu. Rev. Anim. Biosci.* **2013**, *1*, 65–97. [CrossRef]
52. Wasim, L.; Buhari, F.H.M.; Yoganathan, M.; Sicard, T.; Ereño-Orbea, J.; Julien, J.-P.; Treanor, B. N-linked glycosylation regulates cd22 organization and function. *Front. Immunol.* **2019**, *10*, 699. [CrossRef]
53. Muller, J.; Obermeier, I.; Wohner, M.; Brandl, C.; Mrotzek, S.; Angermuller, S.; Maity, P.C.; Reth, M.; Nitschke, L. Cd22 ligand-binding and signaling domains reciprocally regulate B-cell Ca^{2+} signaling. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 12402–12407. [CrossRef]
54. Sun, J.; Shaper, N.L.; Itonori, S.; Heffer-Lauc, M.; Sheikh, K.A.; Schnaar, R.L. Myelin-associated glycoprotein (siglec-4) expression is progressively and selectively decreased in the brains of mice lacking complex gangliosides. *Glycobiology* **2004**, *14*, 851–857. [CrossRef]
55. Cagnoni, A.J.; Pérez Sáez, J.M.; Rabinovich, G.A.; Mariño, K.V. Turning-off signaling by siglecs, selectins, and galectins: Chemical inhibition of glycan-dependent interactions in cancer. *Front. Oncol.* **2016**, *6*, 109. [CrossRef]

5.4. Publication IV: Gene profiling in the adipose fin of salmonid fishes supports its function as a flow sensor

The adipose fin is present in several teleost species and has been described to act as a flow sensor in certain water conditions. In consequence, the tagging method consisting on adipose fin resection has proven to impair sensory functions and activate the stress response in fish. To further characterize the sensory functions of the salmonid adipose fin, this project established a large panel of marker genes for the adipose fin of *O. mykiss* and *C. maraena*. These markers were intended to classify the cellular groups involved in sensory functions and clarify the mechanosensory mechanisms of this appendix.

Highlights

- The expression of the selected markers indicated the nervous innervation of the adipose fin, and the presence of neurons with small unmyelinated axons.

- Marker genes specific for astrocytes were highly expressed in the adipose fin. Also highly expressed were genes related to mechanoreceptor neurons with afferent free-nerve endings.

genes

MDPI

Article

Gene Profiling in the Adipose Fin of Salmonid Fishes Supports its Function as a Flow Sensor

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Abstract: In stock enhancement and sea-ranching procedures, the adipose fin of hundreds of millions of salmonids is removed for marking purposes annually. However, recent studies proved the significance of the adipose fin as a flow sensor and attraction feature. In the present study, we profiled the specific expression of 20 neuron- and glial cell-marker genes in the adipose fin and seven other tissues (including dorsal and pectoral fin, brain, skin, muscle, head kidney, and liver) of the salmonid species rainbow trout *Oncorhynchus mykiss* and maraena whitefish *Coregonus maraena*. Moreover, we measured the transcript abundance of genes coding for 15 mechanoreceptive channel proteins from a variety of mechanoreceptors known in vertebrates. The overall expression patterns indicate the presence of the entire repertoire of neurons, glial cells and receptor proteins on the RNA level. This quantification suggests that the adipose fin contains considerable amounts of small nerve fibers with unmyelinated or slightly myelinated axons and most likely mechanoreceptive potential. The findings are consistent for both rainbow trout and maraena whitefish and support a previous hypothesis about the innervation and potential flow sensory function of the adipose fin. Moreover, our data suggest that the resection of the adipose fin has a stronger impact on the welfare of salmonid fish than previously assumed.

Keywords: adipose fin; fin-clipping; welfare; *Oncorhynchus mykiss*; *Coregonus maraena*; salmonids; mechanoreceptors; innervation

1. Introduction

Salmonid fishes, including rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792), Atlantic salmon *Salmo salar* L., and maraena whitefish *Coregonus maraena* (Bloch, 1779) are farmed in aquaculture facilities all over the world [1]. Their common characteristic is the adipose fin, which is situated on the dorsal midline between dorsal and caudal fin, although a total of 6000 species from eight orders of the Teleostei all possess an adipose fin [2]. Large numbers of artificially bred juvenile salmonids are released into sea-ranching procedures every year to produce 4.4 million tons of top-class food fish [3]. Furthermore, billions of salmonids are released in restocking or stock-enhancement projects [4]. Most of these animals are tagged to monitor the success of those research projects or indicate ownership relations [5] and to identify escapees from aquaculture farms. Those are considered as a serious problem since they reduce the natural gene pool [6].

In order to determine the most appropriate method suitable for routine large-scale screenings of all salmonids bred in Norwegian aquaculture systems, the Panel on Animal Health and Welfare of the Norwegian Scientific Committee for Food Safety evaluated all available marking techniques in 2016. These comprised (i) externally attached visible tags, (ii) visible internal tags, (iii) chemical marking, (iv) remotely detectable internal tags, (v) freeze branding, and (vi) fin clipping. The clipping of fins, especially of the adipose fin, was found to be the most applied and was evaluated as the only persistent and cost-efficient technique available. Unlike other fin structures [7], the adipose fin does not regrow when clipped completely [8–11]. In addition, fin clipping compromises the welfare of the fish [12]. Nonetheless, in European countries, such as Sweden, Estonia, and Latvia, all hatchery-reared salmon are mandatorily marked by adipose-fin clipping to facilitate the differentiation of farmed fish from natural stocks [13]. Adipose-fin clipping of Pacific salmon species is performed on a much larger scale. In the State of Washington (US) alone, more than 200 million juvenile salmonids are adipose fin-clipped every year [14]. Dozens of recapture studies reveal inconclusive influences on the growth and survival of fin-clipped animals [8–11,15–21]. Noteworthy in the context of fish welfare is that the resection of the adipose fin significantly reduces the swimming efficiency of *O. mykiss* juveniles in a flowing current [22]. Subsequent studies proved the innervation of the adipose fin in brown trout *Salmo trutta* [23] and a mechanoreceptive function of the adipose fin in catfish *Corydoras aeneus* [24]. These studies underscore that the adipose fin is not a useless body appendage, as originally claimed [25], but a mechanosensor contributing to optimal swimming performance [26].

Fin-clipping not only removes a supposedly useful organ. It can be assumed that the process itself causes pain. Nowadays, it is indisputable that fish are sentient beings [27–30], at the latest since damage- and pain-signaling nociceptors have been discovered in *O. mykiss* [27,29–31].

Somatosensory perception involves the activation of primary sensory neurons, whose somas reside within the dorsal root ganglia (DRG) or cranial sensory ganglia in the head region of the lateral line system [32–34] (Figure 1). The DRG neurons are pseudo-unipolar [33]. The axon has two branches, one penetrating the spinal cord to synapse with central nerve-system (CNS) neurons, and the other forms free peripheral endings or associates with peripheral targets. They respond to a wide range of stimuli comprising noxious mechanical or thermal stimuli as well as different kinds of touch [33,35]. Previous studies on higher vertebrates based on single-cell RNA-seq [35–47] and immunohistology [48–54] have identified particular sets of genes that indicate either specific sections and/or specific functions of the neuron and glial cells. The discovery of local mRNA translation within the axon outside the neuronal soma (reviewed in [55]) allows further analysis of the quality and functions of the nerves. All relevant genes selected in this study were shown to be present within the axon of sensory neurons (supplementary materials of [41]).

In order to evaluate the influence of the adipose-fin resection based on measurable and thus objective criteria, we profiled the expression of a panel of 35 genes in the adipose fins (AF) of *O. mykiss* and *C. maraena*. The obtained qPCR data were compared against the expression in a range of further tissues, including dorsal and pectoral fin, brain, skin, muscle, head kidney (HK), and liver.

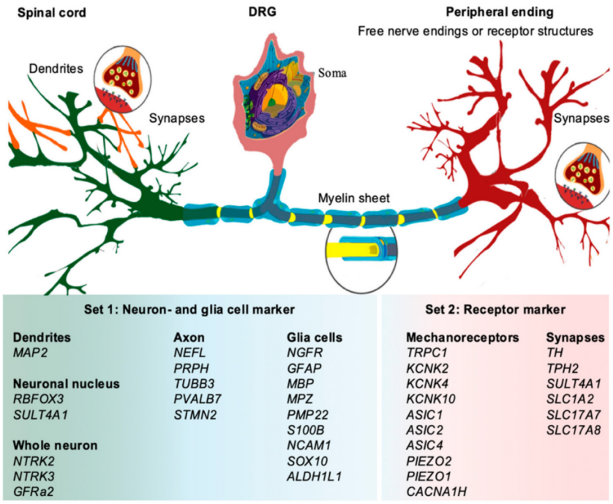

Figure 1. The pseudo-unipolar dorsal root ganglion (DRG) of a vertebrate neuron cell. Dendrites and synaptic connections within the spinal cord (**green**) are shown on the left side. The soma (**pink**) lies within the DRG beside the spinal colon. The axon (**yellow**) connects dendrites and soma to the peripheral ending (**red**) where the axon ramifies into free nerve endings or builds up receptor structures like Merkel, Ruffini, Meissner, or Pacinian corpuscles. The signal transmission between the peripheral ending and receptor structures is similar to synapses, which use equal neurotransmitters. The axon can be enveloped by myelinating Schwann cells (**turquoise**). The panel of genes selected for the present study and the expected site of translation are listed below the illustration. The graph is based on a free illustration (Wikipedia) by Mariana Ruiz Villarreal.

2. Materials and Methods

2.1. Fish and Sampling

Juvenile *O. mykiss* of the German selection strain BORN at the age of 15 months (n = 7, 26.24 ± 0.78 cm, 302.71 ± 42.78 g) were selected for this analysis, as these salmonids are naturally adapted to flow regime. Fish were kept in a flow-through aquaculture system of the State Research Centre for Agriculture and Fisheries (LFA-MV). Additionally, we chose *C. maraena* (n = 3, 17.5 ± 1.08 cm, 64.37 ± 10.88 g) as a second salmonid species for our investigations, also kept in recirculating aquaculture systems from the LFA-MV. After stunning and killing fish by electrical flow, brain, muscle, skin, HK, and liver were sampled. In addition, we resected AF, dorsal fin, (DF), and pectoral fin (PF) as entire target tissues without removing the skin. Samples were immediately transferred to liquid nitrogen and stored at −80 °C until further processing.

The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Mecklenburg-Western Pomerania (Landesamt für Gesundheit und Soziales LAGuS; approval ID: 7221.3-1-012/19).

2.2. Gene Selection and Primer Design

We selected 35 genes, which have been described as selectively expressed by either nerve cells, glial cells, or receptor corpuscles. Unlike mammals, the common ancestor of the extant salmonid species underwent an additional teleost- and an additional salmonid-specific round of whole-genome duplication (WGD) [1,56]. These two events multiplied the number of particular genes in, for instance, *O. mykiss* and *C. maraena*. The WGD-derived paralogous genes are known as ohnologs

[56], but their individual functions are largely unknown yet and it is to be expected that they are expressed to varying degrees. Our designed primer pairs detect either multiple paralogs/ohnologs or specifically a particular paralog/ohnolog. These details are listed together with the accession numbers and putative functions of the 35 target genes in Appendix A, Table A1. BLAST searches were performed using the NCBI server to identify possible gene duplicates and transcript variants in salmonids. All identified sequences were aligned using the Clustal Omega Multiple Alignment tool. Gene-specific oligonucleotides were designed applying Pyrosequencing Assay Design software v.1.0.6 (Biotage). The same primer pairs were used for the quantification of cDNA samples from *O. mykiss* and *C. maraena*.

2.3. RNA Preparation and cDNA Synthesis

The tissues were homogenized and RNA was isolated using Trizol (Life Technologies - Thermo Fisher Scientific, Karlsruhe, Germany), followed by a purification with the RNeasy Mini Kit (Qiagen, Hilden, Germany) with 15 min in-column DNase treatment. Spectrophotometry (NanoDrop One, Thermo Fisher Scientific, Karlsruhe, Germany) and gel electrophoresis were used to evaluate the quality and quantity of the isolated RNA. SuperScript II Reverse Transcriptase Kit (Thermo Fisher Scientific, Karlsruhe, Germany) was used to generate cDNA according to the manufacturer's instructions.

2.4. Gene-Expression Profiling via qPCR

Quantitative real-time PCR (qPCR) was carried out on the LightCycler 96 system (Roche, Basel, Switzerland) to detect and quantify specific transcript amounts. The LightCycler protocol used was optimized for a 12-µl reaction volume. A ready-to-use SensiFAST SYBR No-ROX Mix (Bioline, Luckenwalde, Germany) was mixed with the cDNA aliquots and applied to Light Cycler 480 Multiwell 96 plates (Roche). The qPCR program included an initial denaturation (95 °C, 5 min.), followed by 40 cycles of denaturation (95 °C, 5 min.), annealing (60 °C, 15 s) and elongation (72 °C, 15 s) steps and the fluorescence measurement (72 °C, 10 s). All melting curves were inspected to validate the absence of unspecific amplicons. In addition, PCR products were visualized on agarose gels to assess product size and quality. Individual copy numbers were calculated based on external gene-specific standard curves (10^7–10^3 copies per 5 µL). To control for variations in isolation, reverse-transcription yield, and amplification efficiency [57], the obtained copy numbers were then normalized with a factor based on the geometric mean of the three reference genes *EEF1A1*, *RPS5* and *18S* (*O. mykiss*) and *RPL9*, *RPL32*, and *EEF1A1b* (*C. maraena*), respectively [58–60].

Due to the lack of sequence information regarding transcript variants of *C. maraena*, the amplicons of the *C. maraena* genes were sequenced. Sequencing was performed with qPCR primers using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit and ABIPrism DNA sequencer (Applied Biosystems, Waltham, MA, USA), following the modified Sanger method [61].

2.5. Data Analysis

All data were evaluated for statistical significance using IBM SPSS Statistics 25. Global analysis of variance or Kruskal–Wallis H-Test was used with subsequent post-hoc tests. In all tests, a p-value of ≤ 0.05 indicated significance. Standard error of the mean (SEM) was calculated as described by [62].

3. Results

Sequences from 37 genes (including orthologue variants) were identified for *O. mykiss* and *C. maraena*, and expression profiling was performed in eight selected (strongly and weakly innervated) tissues. The gene panel was divided into two sets. Set 1 contained genes that indicate the presence of nervous cells, particularly those that are expressed exclusively in the neuronal axon, dendrites, or nucleus. Set 2 contained genes that indicate the presence of specific receptor structures. In general, expression of all analyzed marker genes was detectable in the adipose fin, often exceeding the expression levels of other nerve-traversed tissues. The presentation in this section is limited to those

genes that have been identified as informative markers in previous studies and that showed significant differences in expression between tissues in the present study. (Data on other genes are given in Figure 4a and Appendix Figure A1).

3.1. The Adipose Fin Showed High Levels of Neuron Marker Expression

The neuron-marker genes *NEFL*, *PRPH*, *PVALB7*, *NGFR*, *GFAP*, *MPB*, *MPZ* (Figure 2), and *PMP22*, *S100B*, *NCAM1*, *SOX10* (Figure A1) were detectable at high levels (between 1×10^3 and 4×10^6 transcripts per 1 μg RNA) in the three fin types (AF, DF, and PF) investigated in the rainbow trout and maraena whitefish (Figure A2). In most cases, the expression represented only a fraction of that detected in brain samples. On the other hand, the expression levels of all above-mentioned neuron marker genes (except for *PVALB7* in the fins) significantly surpassed the expression in the liver by >4.5-fold (*PRPH*) to >210.3-fold (*NCAM1*) and in the HK by >1.2-fold (*NCAM1*) to >37.2-fold (*MBP*). Particularly, the genes coding for neurofilament light polypeptide (NEFL) and neurofilament 4 (PRPH) (markers for small axons) showed significantly higher mRNA abundances in AF compared with skin (18.4 and 4.4-fold higher), muscle (6.4- and 1.3-fold higher), liver (41.2- and 2.9-fold higher) and HK (8.6- and 1.7-fold higher). Noteworthy, the gene encoding the high-affinity calcium ion-binding protein parvalbumin (PVALB7), a marker gene for large axons, was highly expressed (>1×10^5 copies/μg RNA) in skin, muscle, and brain (Figure 2).

NGFR transcripts encoding the neurotrophic receptors, which characterize types of neurons and neuron-associated glial cells, were detected at high levels (>1×10^5 copies/μg RNA) in brain, skin, muscle, and the three fin types, while it was virtually absent in liver and HK (Figure 2). Moreover, the *NGFR* copy number was >6.2-fold higher in AF compared with the copy number in skin, liver, and HK and even exceeded the values in brain samples. The glial-cell marker genes *GFAP*, *MBP*, *MPZ* (Figure 2), *PMP22*, *S100B*, *NCAM1*, and *SOX10* (Figure A1a) were highly expressed (from >2×10^4 to >5.7×10^6 copies/μg RNA) in the brain, as expected. Particularly, the gene encoding the glial fibrillary acidic protein (*GFAP*) was strongly expressed in the brain but was also detectable in AF and DF in substantial levels. The genes coding for the myelin-forming MBP, MPZ (Figure 2), and PMP22 (Figure A1a) were strongly expressed in the skin and to a lesser but still remarkable extent in the fins. The general neuron and glial-cell marker genes *S100B*, *NCAM1*, and *SOX10* (Figure 4a, Figure A1a) were strongly expressed in brain, skin, muscle, and the three fins investigated, but merely detectable in the liver.

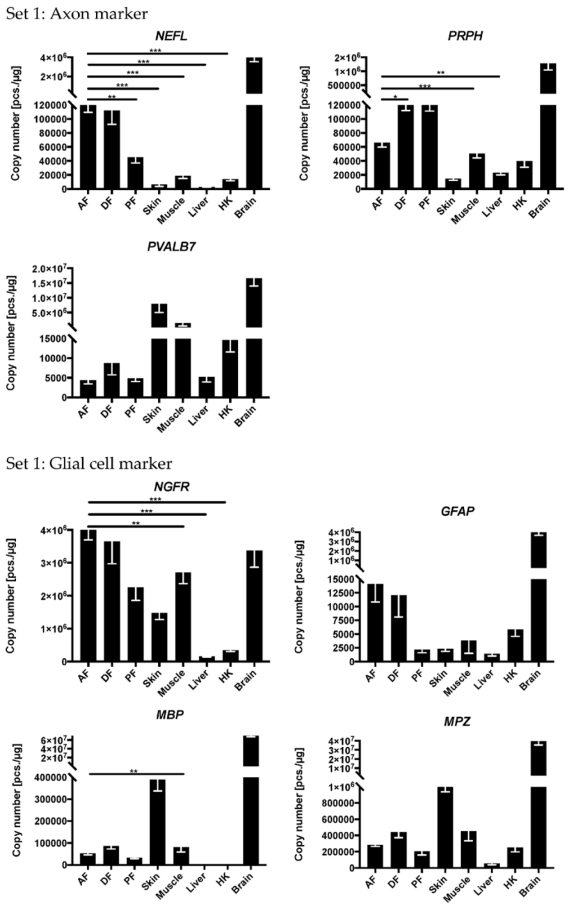

Figure 2. Expression levels of selected neuron and glial cell marker genes from Set 1 across tissues. The expression levels are given as absolute copy numbers (per 1 µg RNA) normalized against three reference genes. Statistically significant deviations are indicated only between AF and the other tissues. Expression values determined in brain were excluded from the statistical evaluation. Significance levels are indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Error bars indicate the SEM.

3.2. Genes Coding for Mechanoreceptor Proteins were Expressed in the Adipose Fins

The receptor marker genes *TRPC1*, *ASIC1*, *-2* and *-4*, *KCNK2* and -4 and *PIEZO2* showed distinct expression patterns in the investigated tissues (Figure 3 and Figure 4b). TRPC1 is a mechanoreceptive channel protein, whose mRNA level was extremely high in brain (~850,000 copies/µg RNA), followed by AF (~10,000 copies/µg RNA) and muscle (~8000 copies/µg RNA) (Figure 3). The genes coding for the mechanoreceptive potassium channel protein KCNK2, -4 and -10 were most strongly expressed in the brain (>2500 copies/µg RNA). Among the *KCNK* genes, *KCNK2* revealed the highest transcript abundance (with up to ~380,000 copies/µg RNA) in brain, AF (~33,000 copies/µg RNA), PF (~20,000 copies/µg RNA), and muscle (~13,000 copies/µg RNA) (Figure 3). In the same way, *ASIC* transcripts

were found in high amounts in the brain (>135,000 copies/µg RNA). In the remaining tissues, high levels of *ASIC2* were mainly present in AF (~14,000 copies/µg RNA), DF (~8000 copies/µg RNA), skin (~11,000 copies/µg RNA), muscle (~7000 copies/µg RNA), and HK (~18,000 copies/µg RNA) (Figure 3), while *ASIC4* levels were high in fins, muscle, and HK (Figure 4b and Figure A1b).

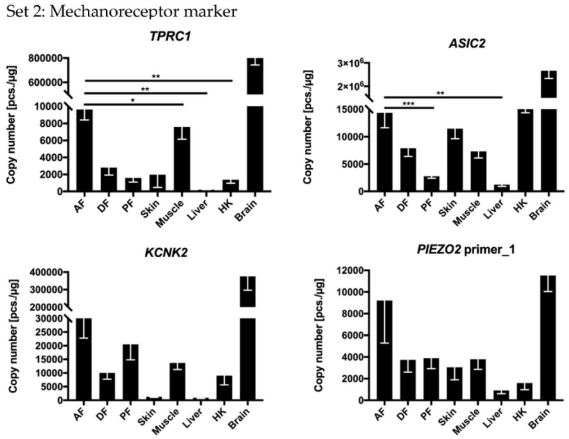

Figure 3. Expression levels of selected mechanoreceptor marker genes from Set 2 across tissues. The expression levels are given as absolute copy numbers (per 1 µg RNA) normalized against three reference genes. Statistically significant deviations are indicated only between AF and the other tissues. Expression values determined in brain were excluded from the statistical evaluation. Significance levels are indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Error bars indicate the SEM. The presentation is limited to those genes which have proven to be particularly significant and meaningful in literature research, all others are listed in Appendix Figure A1b and Figure 4b.

PIEZO2 was analyzed with three transcript variant-specific primer pairs. The primer pairs 1 and 4 are located on an alternatively spliced *PIEZO2* variant, which is specific to neurons in mammals [63]. Primer pair 3 is specific for two *PIEZO2* transcript variants including one that has not been exclusively described for neurons in mammals [63]. Transcript variant 1 was strongly expressed in AF and brain (~9200 to ~12,000 copies/µg RNA) (Figure 3). Transcript variant 4 was detectable in the AF at a level of~2000 copies/µg RNA, and to a lesser extent in the other examined tissues (>1000 copies/µg RNA in PF, skin, and muscle; <100 copies/µg RNA in DF, liver, HK, and brain) (Figure A1b). The transcript variant 3 was strongly expressed (>35,000 copies/µg RNA) in the PF, DF, brain, and, to a significantly lesser extent (~6000 copies/µg RNA), in the AF (Figure A1b).

3.3. Comparison of Gene Expression between Salmonid Species

In addition to the expression analysis of neuron- and glial-cell marker genes in *O. mykiss*, the expression of a subset of these genes was profiled in *C. maraena*. Here, a generally lower copy number level than in *O. mykiss* was found (Figure A2). *TRPC1* showed a congruent expression pattern in *C. maraena* and *O. mykiss*, but in the latter species, it was higher expressed by a factor of 10. The general neuron-marker genes *NEFL* and *NGFR* showed almost similar expression patterns between the tissues of both species, but the expression levels are higher in the adipose fin of *O. mykiss* by the factor 42.6 and 6.3, respectively, in comparison to *C. maraena*. Interestingly, the *PIEZO2* primer pair 1 generated considerably higher levels in *C. maraena*. Moreover, this transcript variant was more strongly expressed in *C. maraena* in the dorsal fin than in the adipose fin, whereas the opposite was observed in *O. mykiss*.

4. Discussion

4.1. Gene-Expression Profiling Indicates the Innervation of the Salmonid Adipose Fin

We established qPCR assays for 20 genes specific for neurons and glial cells (cf. Figures 1 and 4a). The obtained qPCR data suggest the presence of nerve fibers in the adipose fin. This is indicated by specific gene-expression patterns of glial cells that are generally absent in tissues without direct association to neurons [51]. The transcripts coding for ten mechanoreceptors (cf. Figures 1 and 4b) have been selected to cover a wide range of receptors known from vertebrates. The AF showed the most prominent expression of mechanoreceptors compared with the innervated tissues brain, skin, and muscle, all of which have well-defined mechanoreceptive potentials.

NEFL and *PRPH* are the most widely used marker genes for small axons [45,64]. The reception spectrum of fibers expressing these markers covers sensations from nociception to mechanoreception [35,65]. *NEFL* and *PRPH* transcripts were highly abundant in all three fin types analyzed. On the other hand, they were expressed only at low levels in brain, skin, and muscle, suggesting that other kinds of neurons are present there. *PVALB7* is a marker for very large, strongly myelinated neurons [66,67] and was outstandingly high-expressed in brain and skin, but showed low expression values in fins. This might indicate that large nerve trunks do not innervate the fins.

GFAP is a marker for astrocytes in the CNS and Schwann cells in the PNS [68–71]. *GFAP* was most abundantly expressed in the brain and in AF. This is in line with findings from Buckland-Nicks and colleagues [23], who identified plenty of GFAP-positive cells within the AF using antibody staining. The association of GFAP-positive cells, nerve cells, and collagen was described by Buckland-Nicks [23] as common for receptor structures.

NGFR and *SOX*10 are highly specific marker genes of innervated tissues [37,51,65,72,73]. Both were unanimously and significantly lower transcribed in the liver and HK compared with brain and fins. The *NGFR* gene was even more highly expressed in the AF than in the DF or even the brain, indicating the presence of nerve structures [51,65,72–74].

PIEZO2 is known as key mechanotransducer, particularly in sensory afferents [39]. Orthologs in mammals and fish show a high degree of conservation of the nucleotide (nt) sequences and exon

Borders (Figure A3). Accordingly, we found abundant PIEZO2 copy numbers in all fins, with varying copy numbers between the different salmonid-specific transcript variants. ASIC2, TPRC1, and KCNK2 build up channel proteins with mechanoreceptive function. ASIC2 mainly occurs in mechanoreceptive afferents. TPRC1 is responsible for mechanoreception in a tactile and contact-related manner [46]. KCNK2 is described as being physiologically important for tuning the activation of mechanoreceptive DRG neurons [75]. These three genes were expressed in the adipose fin to a much greater extent compared to all other innervated tissues except the brain.

mRNA	AF	DF	PF	Skin	Muscle	Liver	HK	Brain
(a) Set 1: Neuron- and glia-cell marker								
MAP2	33124	12908	18491	2687	12014	8410	20121	12456312
RBFOX3	18406	43525	35214	8525	23466	2790	22934	1455630
SULT4A1	6631	7181	10316	6676	4570	4714	17422	5616096
NTRK2	27940	56286	66867	55316	55342	38819	43240	2013991
NTRK3	2766	525	1479	535	431	5668	1647	898329
GFRa2	4382	4816	3330	8905	7892	347799	12926	184219
NEFL	120359	112328	45713	6537	18820	2919	13921	4403685
PRPH	66340	122596	125281	15033	50750	23187	39916	1286604
TUBB3	251761	425735	276527	171813	310475	58272	905556	2600239
PVALB7	4447	8774	4949	8051048	1498762	5283	14647	16711518
STMN2	14146	19670	36192	5038	13358	-	65185	17129857
NGFR	4102507	3657686	2258931	1478472	2713317	161672	349345	3378382
GFAP	14131	12081	2230	2359	3867	1466	5898	4040595
MBP	53143	87180	33512	390478	81057	986	1559	76995030
MPZ	285689	444722	206629	1032405	456744	57735	254839	39671358
PMP22	3356571	3991822	2663071	7827149	4340096	567409	4753848	1986666
S100B	38946	106830	20893	102177	23157	442	10495	47175113
NCAM1	95153	110986	96631	41707	174442	479	83779	791241
SOX10	35656	58836	35588	35214	79242	537	5830	273850
ALDH1L1	8058	12994	7206	1490022	259848	6135591	779326	460922
(b) Set 2: Receptor marker								
TPRC1	9608	2819	1589	1972	7579	189	1380	849036
KCNK2	33403	10014	20517	1296	13702	931	9023	375847
KCNK4	891	527	1157	72	213	424	619	2558
KCNK10	265	770	370	799	3347	37	208	34378
ASIC1	1653	2531	1170	3359	2721	1009	2895	195836
ASIC2	14345	7902	2753	11460	7330	1219	18214	2658930
ASIC4	2376	4185	2202	827	1546	352	2116	137739
PIEZO2_1	9216	3729	3885	3041	3791	902	1592	11513
PIEZO2_3	5850	78913	137594	24697	19055	3746	6488	37638
PIEZO2_4	2136	780	1014	1325	1154	253	132	878
PIEZO1	197070	348038	356132	165148	236833	31166	524734	128285
CACNA1H	12673	18608	12877	42319	34284	2484	15366	992515
TH	705	0	162	212	1085	1974	87600	51352
TPH2	2891	4279	3315	1729	5480	0	7350	154871
SLC1A2	1922	1981	949	728	6427	591	1041	9492197
SLC17A7	283	745	308	207	438	653	1482	53228
SLC17A8	954	1617	1195	477	638	16165	1156	42486
Scalebar	---	--	-			+	++	+++

Figure 4. Expression profile of (**a**) neuron and glial cell- and (**b**) mechanoreceptor-specific marker genes across all the tissues investigated in *O. mykiss*. Field numbers indicate the absolute copy number per 1 µg RNA. Color codes range from low abundance (dark blue) to high abundance (bright yellow) relative to the mean expression value of each particular gene. Expression in the brain was mostly excluded from the HeatMap illustration due to extremely high expression levels.

4.2. The Expression of Neuron- and Glial-Cell Markers is Tissue-Specific in Salmonids

We recorded tissue-specific expression patterns for most of the investigated genes, which are putatively involved in the proprioceptive machinery in the muscle.

The muscle tissue of rainbow trout expressed relatively high levels of *ASIC1*, *TRPC1*, *KCNK10*, and *CACNA1H*. Additionally, the copy numbers of all *ASIC* and *PIEZO2* variants were detected at substantially high levels. Moreover, the copy numbers of *SLC1A2*, a glutamate transporter, and *TPH2*, the rate-limiting enzyme in the serotonin synthesis [76], were at high concentrations. Glutamate has several well-known and proposed functions in the muscle tissue. On the one hand, it acts as neurotransmitter within the muscle spindles [77]. On the other hand, it might be metabolized in the muscle, and SLC1A2 is necessary for its transport [78]. TPH2 is vital for efferent γ-motor neurons using serotonin in the sensory feedback of muscle spindles [79]. ASICs are involved in mammalian muscle spindle mechanotransduction [80,81], and PIEZO2 is considered as the principle mechanotransducer in proprioception [67]. Taken together, these genes indicate the presence of mechanoreceptive muscle spindles. Confirmatory, markers for nerves and glial cells, in particular,

PRPH, *NGFR*, *GFAP*, *MBP*, *MPZ*, and *PMP22*, were also expressed in substantial levels in the muscle of rainbow trout. Furthermore, the strong expression of *NCAM1* and *SOX10* in the muscle indicates a higher density of glial cells, which are necessary for large nerve fibers. *PVALB7*, which is required in innervating muscle spindles with large neurons [67], was present in the muscle in similar high copy numbers as in brain.

The skin tissue of the rainbow trout shows a different expression pattern compared to all other tissues and appears to be interspersed with large nerve strands. This is consistent with the knowledge about the nerve supply of the skin of higher vertebrates [33–35,44]. The cutaneous low-threshold mechanoreceptors (LTMRs), responsible for touch sensitivity in vertebrates, possess large and highly myelinated neurons that require correspondingly high amounts of glial cells. In the skin of mammals, particularly high proportions of glial-cell-specific genes *MPZ*, *MBP*, *PMP22*, and *S100B*, as well as the neuron marker *PVALB7*, are expressed. This agrees with the results of this study on rainbow trout. However, only few copies were detected for the mechanoreceptive channel proteins (necessary for LTMRs), except for TPRC1, KCNK2, ASIC1, and ASIC2, and the modulator CACNA1H. We note that mechanoreception in the skin requires several other receptor proteins that have not been included in the present study.

In HK tissue, many specific nerve markers, such as *TUBB3*, *STMN2*, *MAP2*, and *SULT4A1*, revealed particularly high expression levels. The teleost HK is a lympho-myeloid compartment containing immune and endocrine cells, which secrete cortisol, thyroid hormones, and catecholamines [82], such as dopamine. Serotonin is known to stimulate the secretion of cortisol in fish [83]. In this context, we refer to two important non-immune cells with different origin, the chromaffin cells, and the interrenal cells [84]. The chromaffin cells are descendants of neural crest cells, which share many functions and secretion patterns with peripheral neurons and glial cells [85]. Above all, *ASICs* and *PIEZO1* were strongly expressed in the HK. Both gene products are known to be involved in the fluid balance of teleost cells [86]. Of note, PIEZO1 is not associated with nerve cells and was considered rather as a reference gene in this study. In addition, the genes *TH*, *TPH2*, and *SCL17A7*, coding for enzymes involved in the serotonin and dopamine synthesis and the transport of glutamate, respectively, were strongly expressed in HK. The cell adhesion molecule NCAM1 is responsible for maintaining glial-neuronal connections [87] and has vital functions in natural killer cells and dendritic cells [88]. Both immune-cell populations are abundantly present in the HK since this organ is the main hematopoietic organ in fish [84]. *TUBB3*, a microtubule-forming gene, was included in this study as another reference gene, since its transcripts are not transported to the axons [41]. This supports our observation that the HK has by far the highest concentration of *TUBB3* transcripts compared with the more innervated tissues.

In the liver, there is virtually no expression of any receptor channel protein. Only *PIEZO1* was detectable at higher levels. Besides, *SLC17A8* encoding a glutamate transporter was highly expressed. Glutamate transporters allow the uptake of glutamine and glutamate into the liver cells, where glutamate is involved in amino-acid metabolizing pathways [89].

The genes *NTRK2*, *NTRK3*, and *GFRa2* encode neurotrophic receptors and were used in this study to distinguish between the different nerve types, as previously done in studies on mammalian models [32,33,43,45,63,67]. Neurotrophins control the differentiation and survival of nerve cells, whereby different classes of neurons depend on different neurotrophins [90]. However, the present study revealed remarkably high levels of neurotrophin-encoding transcripts in the liver and, therefore, neurotrophins might have a cross-tissue function.

4.3. Mechanosensation is a Characteristic of the Salmonid Adipose Fin

The overall expression profile of the adipose fin (Figure 5) highly suggests the presence of nerve endings including mechanoreceptive channel proteins. *PRPH*, *NEFL*, and *NGFR* indicate the presence of small neurons with unmyelinated or slightly myelinated axons. This assumption is supported by the presence of the myelin-forming genes *MBP*, *MPZ* and *PMP22* in the adipose fin (compared to skin and muscle tissue, for instance), although at low levels. The suggested afferent nerve endings—defined as free nerve endings, C-fibres, C-LTMRs, and Aδ-fibers—may be coupled to collagen fibers

via GFAP-positive glial cells [23,91]. These are able to sense mechanical stimuli through movements of the fin structure. *TRPC1*, *PIEZO2*, and *KCNKs* were only recently described as markers for C-LTMRs [65]. The expression profiles of the fins of rainbow trout indicate the presence of smaller mechanoreceptive C-fibres. Besides the mechanoreceptive function, it seems moreover likely that pain signals can be perceived in the adipose fin since many smaller nerve cells are known to be nociceptors. These were, however, not included in our gene panel.

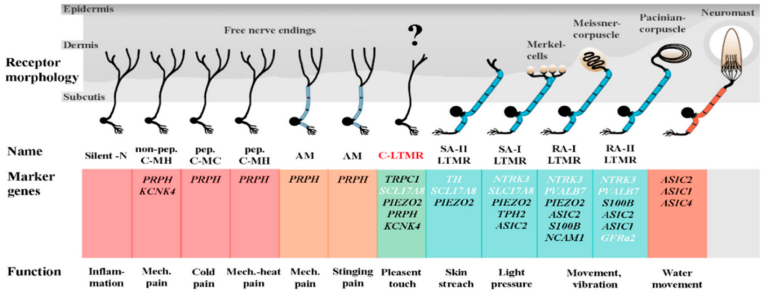

Figure 5. Summary of the possible presence of somatosensory receptors in the adipose fin of rainbow trout. Listed are the genes indicating the presence of nociceptors with free nerve endings (red, orange) and with specialized receptor structures (green) and cutaneous LTRMs with receptor corpuscles (blue) as well as neuromasts (light red). Marker genes identified in the adipose fin in ample amounts are printed in black. Marker gene names are printed in white if the expression in the AF was not outstandingly high in the comparison of the tissues analyzed. The specific cell types for which the listed genes are characteristic are labeled as follows: Non-peptideric C-fibre mechano-heat receptor (non-pep.-C-MH), peptideric mechano-cold nociceptor (pep. C-MC), peptideric C-fibre mechano-heat nociceptor (pep. C-MH), A-fibre mechanonociceptor (AM), C-fiber low-threshold mechanoreceptor (C-LTMR), Aβ-fiber slowly-adapting type I and type II LTMR (SA-I LTMR and SA-II LTMR), Aβ-fibre rapidly-adapting type I and type II LTMR (RA-I LTMR and RA-II LTMR). Marker genes were extracted from literature [35–47,65,92,93], figure is adapted from [35].

5. Conclusions

The present study suggests that the adipose fin is innervated by a high amount of small nerve fibers with, most probably, mechanoreceptive potential. In the adipose fin of rainbow trout and maraena whitefish, the entire repertoire of neurons, glial cells, and receptor proteins seems to be present on the RNA level. This supports a previous hypothesis about the adipose fin as a flow sensor [22,23], and thus its significance for the animal's locomotion in water currents. With regard to the welfare of fish, our data accelerate the discussion about the use of adipose-fin clipping for marking purposes. On the one hand, the adipose fin is a criterion for the choice of suitable sexual partners [94] and, on the other hand, contributes to the swimming efficiency [26]. Thus, the resection of the adipose fin tissue seems to be a less suitable method, particularly from an economic point of view regarding sea ranching and large-scale aquaculture in the future.

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

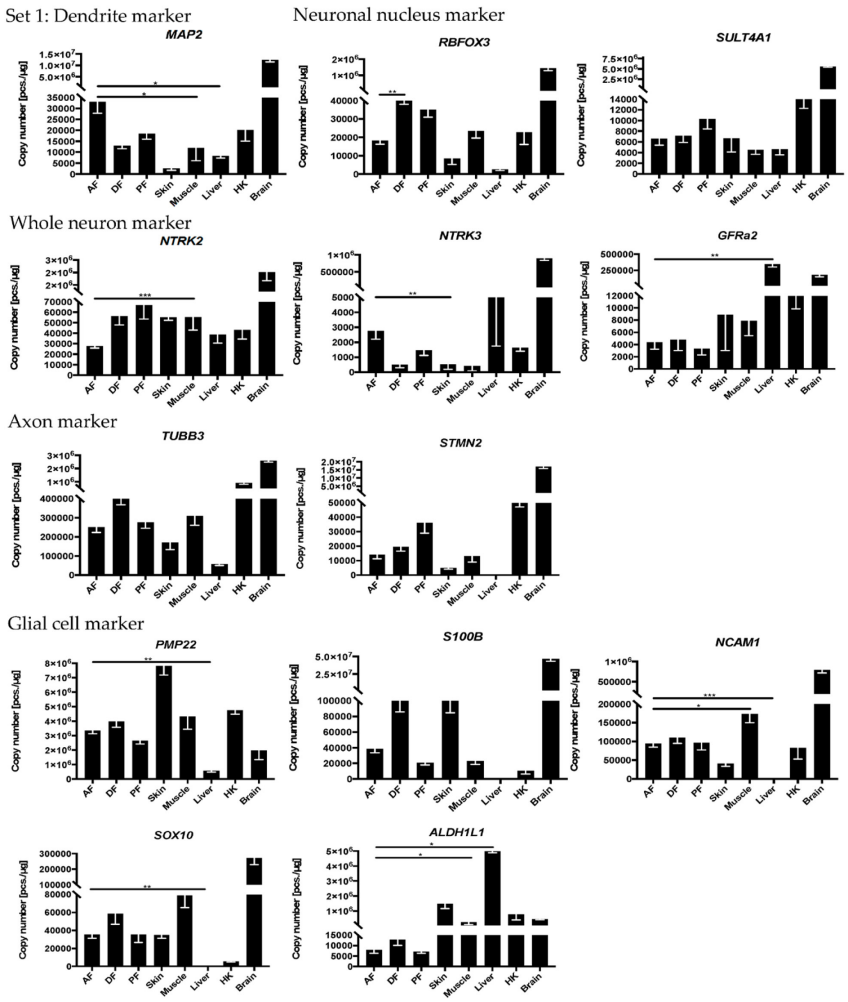
Appendix A

Table A1. Primer assays used in the present study.

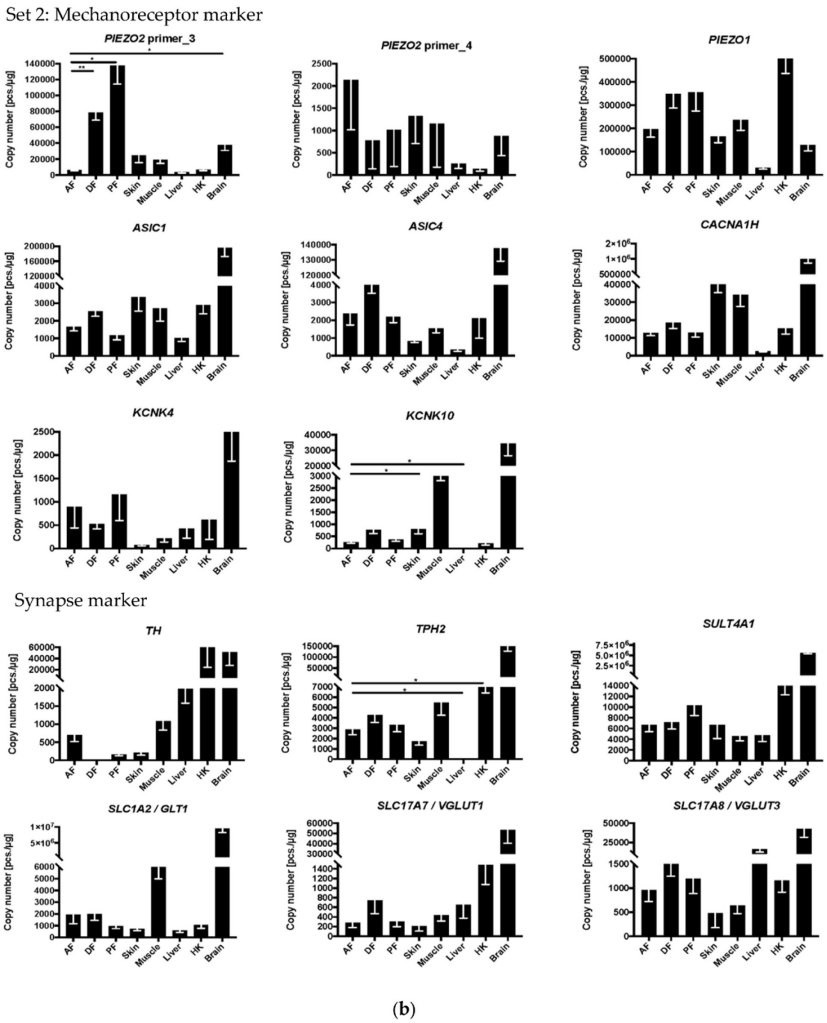
(A)	(B)	(C)	(D)	(E)	(F)	(G)
Gene Symbol	**Localization**	**Basic Function**	**Accession Code of Selected Ortholog of (A) in *O. mykiss* (Incl. Chromosome no.)**	**Accession Code of Paralogs of (D) in *O. mykiss* (Chromosome no.; % CDS Divergence to [D])**	**Sense and Antisense Primer Sequence (5′ - 3′) Derived from (D)**	**Prediction of Specificity of Primers (F) for Selected Ortholog (D)**
				Neuron marker		
NEFL	Axon	Neurofilament	XM_021605918 (6)	XM_021621429 (11; 91.4%) XM_021602316 (5; 71.1%) XM_021590833 (29; 70.6%)	CTTACAGGAAGCTGCTTGAAGG, GATGAGCTGTACATGCGTAGGT	Binding to XM_021621429 (no mismatch), but not to XM_021602316, XM_021590833 (antisense: 6 and 7 mismatches)
NEFH	Myelinated axons	Neurofilament	XM_021621725 (11)	XM_021606185 (6; 91.2%)	GTGAGTCACTAACACACTGCATA, TGTTTGTCTCCTGCTCTGCTCT	Not binding to XM_021606185 (sense: 6 mismatches)
PRPH	Unmyelinated axons	Neurofilament	XM_021610098 (7)	XM_021569035 (17; 92.2%)	ACGTGCAGGTGAGTGTCCAGA, AGGTCAGCAAACTTGGACTTGTA	Binding to both paralogs (sense: 2 mismatches)
TUBB3	Axon	Microtubule assembly	XM_021607465 (6)	XM_021586327 (26; 98.8%) XM_021580863 (23; 85.0%)	AGGCCTCATCCTCTAAGTACGT, CCTTGGCCCAGTTGTTACCAG	Binding to XM_021586327, but not to XM_021580863 (sense: 6 mismatches)
PVALB7	Myelinated axon	Calcium binding	XM_021557489 (13)	XM_021624534 (12; 97.4%)	CGCAGCGGCTGACTCTTTTGA, GAGGCAAATCCCTTCAGTACGA	Binding to both paralogs (sense: 1 mismatch)
STMN2	Axon	Microtubule dynamics	XM_021559849 (14)	XM_021598102 (3; 84.4%) XM_021585013 (3; 84.7%) XM_021572874 (18; 96.8%)	TGGCTAAAACAGCAATTGCGTAC, AGAGGCACGCTTGTTGATGGG	Not binding to XM_021598102 XM_021585013, XM_021572874 (3 to 10 mismatches per primer)
MAP2	Neuron dendrites	Microtubule assembly	XM_021597500 (3)	XM_021579611 (22; 86.9%)	CGTCAAGAAGAAAAAAGCCGTGA, ACTGTAGGTTTCCTCCTAGCAC	Binding to both paralogs (sense: 1 mismatch)
RBFOX3	Neuronal nucleus	Neuronal nucleus production	XM_021581050 (23)	XM_021576584 (20; 86.7%) XM_021625440 (12; 81.2%) XM_021556260 (13; 84.3%)	AGTATCGCAGGCAGAAGAGGTT, CCCAAACATTTGCCTGAGGTCT	Binding to XM_021576584, but not binding to XM_021625440, XM_021556260 (sense: 9-nt gap)
				Glial cell and glial cell type marker		
GFAP	Astrocytes and Schwann cells (SC)	Cell communication	XM_021558456 (13)	XM_021625581 (12; 98.9%)	TGACGGAGCTGACCCAACTGA, TCTCATCTTGCAGTCTCTGTTTG	Binding to both paralogs (no mismatch)
ALDH1L1	Astrocytes and liver cells	Energy supply	XM_021610613 (7)	————————	GAACAGCTATCTGTGATGTGTCT, TCCATCAGGTCAGCCAGCTTAT	
MBP	Myelinating SC and oligo-dendrocytes	Myelin formation	XM_021571745 (18)	XM_021594735 (?; 92.8%)	ATCAGATTAGCACGTTCTTTGG, AGAGGCTGTCACGCTCAAGCT	Not binding to XM_021594735 (antisense: 39-nt gap)
MPZ	Myelinating SC and oligo-dendrocytes	Myelin formation	XM_021588760 (28)	XM_021614027 (8; 93.3%)	ATCTACACGGGCTGGGAGCG, CCGGTGTAGTGGAAGATAGAGA	Binding to XM_021614027 (antisense: 3 mismatches)

PMP22	Myelinating SC and oligo-dendrocytes	Myelin formation	XM_021576248 (20)	XM_021581303 (23; 92.2%) XM_021559021 (13; 77.5%)	TCTTCCAGATCCTCGCCAGTC, TGACGTAGATGAGTCCGCTGAT	Binding to XM_021581303 (antisense: 1 mismatch), but not to XM_021559021 (antisense: 5 mismatches)
S100B	Glial cells and neurons	Calcium binding	XM_021608876 (7)	XM_021571442 (18; 96.5%)	ATTACAAACCACAATGACTGACCT, TGGTCCTTCACTTGCCCTGTAA	Binding to XM_021571442 (sense: 1 mismatch)
NCAM1	Glial cells and neurons	Cell contact and communication	XM_021617837 (10)	XM_021623770 (12; 91.5%) XM_021588052 (27; 74.7%) XM_021582629 (24; 75.7%)	AGAAGCTTTTACCGAACAGACAG, TTTGGAAGATTTTCACGTTGACAG	Binding to XM_021623770 (2-3 mismatches), but not to XM_021588052, XM_021582629 (sense: ≥9-nt gap)
SOX10	Glial cells and neurons	Neuron survival	XM_021567709 (17)	XM_021556808 (13; 98.7%) XM_021558042 (13; 75.4%) XM_021625106 (12; 75.2%)	CGCGTAAACAACGGGAACAAGA, ATTCAGGAGCCTCCACAGTTTG	Binding to XM_021556808 (no mismatch), but not to XM_021558042, XM_021625106 (antisense: 5-7 mismatches)
				Neuron characterization		
NGFR	Glial cells and neurons	Neuron assembly and survival	XM_021558479 (13)	XM_021625607 (12; 98.2%) XM_021565429 (16; 80.6%)	CAGTGCCTAGACAGTGAGACC, CCTCATTCAGGTAGTAGTTGTAG	Binding to XM_021625607 (no mismatch), but not to XM_021565429 (sense: 38-nt gap)
NTRK2	A-delta LTMR	Signalling and neuron survival	XM_021605433 (6)	XM_021621031 (11; 93.7%) XM_021602994 (5; 84.8%) XM_021622759 (12; 85.2%)	CCTCACGAATCTAACTGTGACTA, AGCGGGTTCCCTGAAAGAATCA	Binding to XM_021621031 (no mismatch), but not to XM_021602994, XM_021622759 (antisense: ≥ 6 mismatches)
NTRK3	Proprioceptors and LTMR	Signalling and neuron survival	XM_021591923 (1)	XM_021568341 (2; 94.9%) XM_021593111 (?; 82.3%)	CAAGAACATCACCTCAATACACAT, GGTTCTTCGATAAGTTTATGTAGC	Not binding to XM_021593111
GFRa2	C-LTMR	Signalling and neuron survival	XM_021605433 (6)	XM_021621031 (11; 93.7%) XM_021602994 (5; 84.8%) XM_021622759 (12; 85.2%)	ATTATCTCAGGGATGCACACTGT, TGGCAGCGCTTACGGTTACAC	Not binding to XM_021621031, XM_021622759, XM_021602994 (sense: ≥ 4-nt gap)
				Receptor / synapse characterization		
SLC1A2	Glial cells, neurons, receptors	Glutamate transport	XM_021600639 (1)	XM_021573109 (2; 94.2%) XM_021608174 (6; 82.2%) XM_021625950 (12; 80.4%)	AACAGATCCAAACGGTTACTAAGA, TAACACGTTCATGCCACTCTTGA	Binding to XM_021573109 (2-3 mismatches), but not to XM_021625950 (sense: 18-nt gap) and XM_021608174 (7 mismatches)
SLC17A7	Glial cells, neurons, receptors	Glutamate transport	XM_021575504 (20)	XM_021565125 (16; 94.4%) XM_021558924 (13; 80.2%)	TACGGCAGCTTTGGGATCTTCT, AAAAGGCTCTCCAAGGCGTGTT	Binding to XM_021565125 (sense: 1 mismatch), but not to XM_021558924 (sense: 12-nt gap)
SLC17A8	Glial cells, neurons, receptors	Glutamate transport	XM_021601245 (1)	XM_021574127 (2; 93.4%)	TATGGTGTATTTGGGATCATATGG, GAATTTCTCAGTGGCGCTCAATA	Binding to XM_021574127 (sense, antisense: 1 mismatch)
TH	Glial cells, neurons, receptors	Dopamine synthesis	XM_021564247 (2)	————————	TGTTCGAGACGTTTGAAGCTAAG, GTTTTGACATCCTCTGCTATCCT	
TPH2	Glial cells, neurons, receptors	Serotonin synthesis	XM_021576444 (2)	————————	GCCCTACGCCTTTTTCAGGAG, AGGCGTGTTGAAGGAGATGATAT	
SULT4A1	Neuron nucleus	Purposed neuro-transmitter synthesis	XM_021577380 (21)	XM_021564205 (15; 99.5%)	CCCAGATGAGATTGGTCTGATG, TCGCCATGTAGATCACCTTGGA	Binding to XM_021564205 (no mismatch)
				Mechanoreceptor characterization		

PIEZO2	Afferent neuron ending and receptors	Stretch-receptor channel	XM_021588681 (28)	XM_021590114 (28; 90%) XM_021614324 (8; 88.0%)/ XM_021614323 (8; 87.5%)/ XM_021614322 (8; 94.8%), XM_021622313 (11; 78.3%), XM_021564358 (15; 80.2%)	GATAGTATATCCAGTGCCTACAC, CTACTGCTGCTGTCAGTCGATT, AGAGAGGTCAAAAAGGGCAACG, TCCTGGCTCTCCATGCGATAG, AACTGTGATGTAACAACGGTAAG, ACGTCCTCTGGTGGTCTGTTTT	Pair 1 binding to XM_021588681 and XM_021614324; pair 3 binding to XM_021614324; pair 4 binding to XM_021588681; no pair binds to XM_021590114, XM_021622313, XM_021564358 (sense: > 4 mismatches or 20-nt gap)
PIEZO1	Non-sensory tissues	Stretch-receptor channel	XM_021585995 (26)	XM_021607119 (6; 97.1%)	ACTGTAGTTTGTGGGAGACGCT, TCTCTTCTTGACCAGCCGGTTA	Binding to XM_021607119 (1-3 mismatches)
ASIC1	Receptors	Mechano-receptor channel	XM_021615628 (9)	XM_021566621 (16; 94.3%) XM_021610711 (7; 82.15%) XM_021567553 (17; 79.0%)	AGACGGATGAGACCACGTTTGA, AGGGTGGGGGCAAATATATCAG	Binding to XM_021566621 (sense: 3 mismatches), but not to XM_021610711, XM_021567553 (sense, antisense: ≥ 5 mismatches)
ASIC2	Receptors	Mechano-receptor channel	XM_021558500 (13)	XM_021625621 (12; 98.7%)	CTGCCCTTGCCAAGTTGTCAAT, TGTTATCCGTGATGTATTTCTCAG	Binding to XM_021625621 (no mismatch)
ASIC4	Receptors	Mechano-receptor channel	XM_021579184 (22)	XM_021596998 (3; 96.5%)	ATATCCAACAGGACGAGTATCTC, GGTCAGCCTTTGTTCCTGACAT	Binding to XM_021596998 (sense: 1 mismatch)
TRPC1	Receptors	Mechano-receptor channel	NM_001185053 (11)	————	TAAGCCCTCCATCGCTAAACTG, GGCATTACAGAGAGTACACTCG	
KCNK2	Receptors	Mechano-receptor channel	XM_021600681 (4)	————	GTGACTTTGTGGCCGGTGAAAA, CCCCTACCTCCTCCTTGGTTT	
KCNK4	Receptors	Mechano-receptor channel	XM_021583157 (25)	XM_021561640 (14; 87.6%)	CAGCGACCTCATAAAGAGTGTG, GTCCTGGGAGAAAGGTTACCAA	Binding to XM_021561640 (sense, antisense: 2-3 mismatches)
KCNK10	Receptors	Mechano-receptor channel	XM_021574081 (19)	XM_021583031 (25; 90.5%), XM_021611349 (8; 72.7%)	GTGGAGAAGATATACAGGCAAAAA, TGATAGCGTGATGATGACAAAGTA	Not binding to XM_021583031 (sense or antisense: ≥ 4 mismatches)
CACNA1H	Action potential generation zone	Modulation of firing patterns	XM_021593500 (?)	————	CGCTAGAGTGTTGAAGCTGTTG, TCTCCTCGGAACACTCCAGTTT	


Set 1: Dendrite marker
Neuronal nucleus marker
MAP2
RBFOX3
SULT4A1
Whole neuron marker
NTRK2
NTRK3
GFRa2
Axon marker
TUBB3
STMN2
Glial cell marker
PMP22
S100B
NCAM1
SOX10
ALDH1L1
Copy number [pcs./µg]
AF
DF
PF
Skin
Muscle
Liver
HK
Brain

(**a**)



(**b**)

Figure A1. (**a**). Expression levels of the remaining neuron and glial cell marker genes from Set 1 across tissues in *O. mykiss*. The expression levels are given as absolute copy numbers (per 1 µg RNA) normalized against three reference genes. Statistically significant deviations are indicated only between AF and the other tissues. Expression values determined in the brain were excluded from the statistical evaluation. Significance levels are indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Error bars indicate the SEM. (**b**). Expression levels of mechanoreceptor-encoding and synapse marker genes from Set 2 across tissues in *O. mykiss*. The expression levels are given as absolute copy numbers (per 1 µg RNA) normalized against three reference genes. Statistically significant deviations are indicated only between AF and the other tissues. Expression values determined in brain were excluded from the statistical evaluation. Significance levels are indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Error bars indicate the SEM.

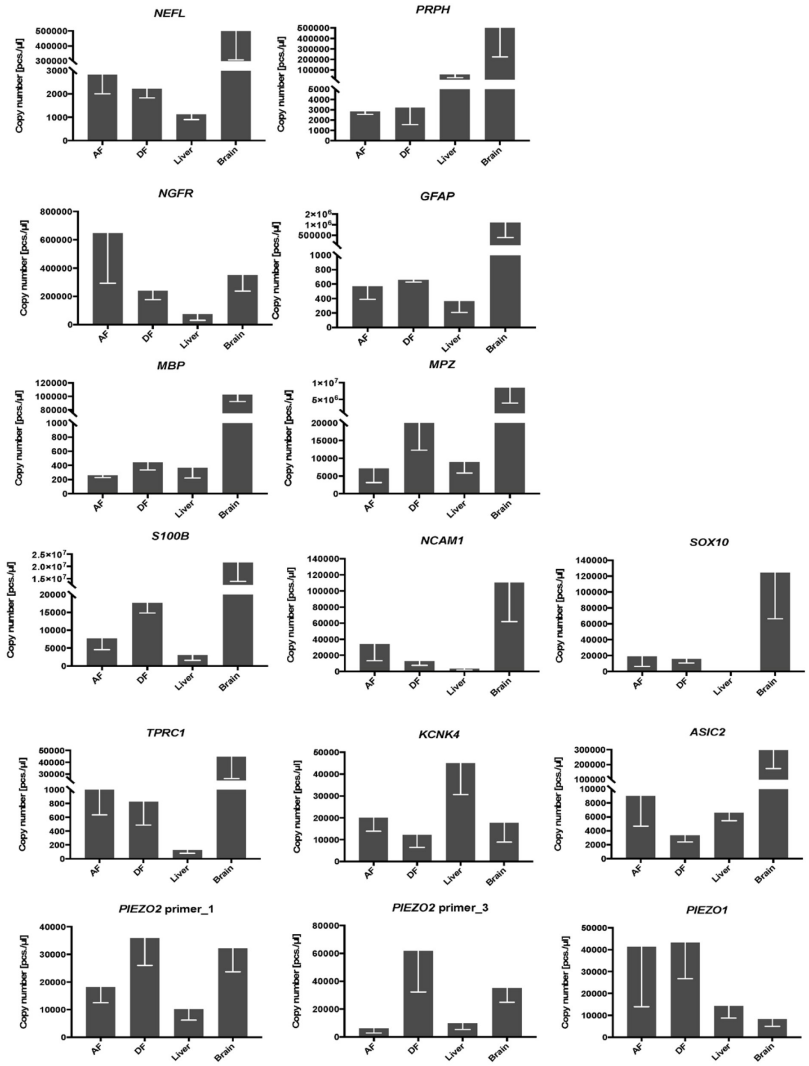


Figure A2. Expression levels of Set 1 and Set 2 marker genes across tissues in *C. maraena*. The expression levels are given as absolute copy numbers (per 1 µg RNA) normalized against three reference genes. Statistically significant deviations are indicated only between AF and the other tissues. Expression values determined in brain were excluded from the statistical evaluation. Significance levels are indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Error bars indicate the SEM.

Figure A3. Part of the Clustal Omega Alignment of mouse, zebrafish *Danio rerio* (Hamilton, 1822), and *O. mykiss* PIEZO2 CDS (European Bioinformatics Institute, 2018). Shown are exons 39 through 41 with high identity across the species. Exon-exon boundaries are labeled in blue and red. Position of primer pair 1 is shown by grey underlay. Exon 40 of the murine ortholog is alternatively spliced and deleted in non-neural tissues [63]. Sequence and exon-exon borders were provided by [63]. (Accession number *D. rerio* XM_021468270).

References

1. Macqueen, D.J.; Primmer, C.R.; Houston, R.D.; Nowak, B.F.; Bernatchez, L.; Bergseth, S.; Davidson, W.S.; Gallardo-Escárate, C.; Goldammer, T.; Guiguen, Y.; et al. Functional Annotation of All Salmonid Genomes (FAASG): An international initiative supporting future salmonid research, conservation and aquaculture. *BMC Genom.* **2017**, *18*, 484.
2. Stewart, T.A.; Smith, W.L.; Coates, M.I. The origins of adipose fins: An analysis of homoplasy and the serial homology of vertebrate appendages. *Proc. R. Soc. B Biol. Sci.* **2014**, *281*, 20133120.
3. FAO. *The State of World Fisheries and Aquaculture (SOFIA) 2014*; Fisheries and Aquaculture Department: Rome, Italy, 2014.
4. North Pacific Anadromous Fish Comission (NPAFC). ICES ICES WGBAST Report 2018. In Proceedings of the Report of the Baltic Salmon and Trout Working Group (WGBAST), Turku, Finland, 20–28 March 2018; pp. 11–20.
5. *ICES ICES WGBAST Report 2018 Report of the Baltic Salmon and Trout Working Group (WGBAST)*; Ices Advisory Committee: Turku, Finland, 2018.
6. Bourret, V.; O'Reilly, P.T.; Carr, J.W.; Berg, P.R.; Bernatchez, L. Temporal change in genetic integrity suggests loss of local adaptation in a wild Atlantic salmon (Salmo salar) population following introgression by farmed escapees. *Heredity* **2011**, *106*, 500–510.
7. Akimenko, M.A.; Marí-Beffa, M.; Becerra, J.; Géraudie, J. Old questions, new tools, and some answers to the mystery of fin regeneration. *Dev. Dyn.* **2003**, *226*, 190–201.
8. Armstrong, G.C. Mortality, rate of growth, and fin regeneration of marked and unmarked lake trout fingerlings at the provincial fish hatchery, Port Arthur, Ontario. *Trans. Am. Fish. Soc.* **1949**, *77*, 129–131.
9. Stauffer, T.M.; Hansen, M.J. Mark retention, survival, and growth of jaw-tagged and fin- clipped rainbow trout. *Trans. Am. Fish. Soc.* **1969**, *98*, 225–229.
10. Weber, D.; Wahle, R.J. Effect of Finclipping on Survival of Sockeye Salmon (*Oncorhynchus nerka*). *J. Fish. Res. Board Can.* **1969**, *26*, 1263–1271.
11. Johnsen, B.O.; Ugedal, O. Effects of different kinds of fin-clipping on over-winter survival and growth of fingerling brown trout, Salmo trutta L., stocked in small streams in Norway. *Aquac. Res.* **1988**, *19*, 305–311.
12. VKM. *Risk Assessment of Marking and Tracing Methods with Regards to the Welfare of Farmed Salmonids. Opinion of the Panel on Animal Health and Welfare, Oslo, Norway*; Norwegian Scientific Committee for Food Safety (VKM): Oslo, Norway, 2016; ISBN 9788282592574.
13. ICES. ICES WGBAST Report 2007. In Proceedings of the Report of the Baltic Salmon and Trout Working Group (WGBAST), Riga, Latvia, 27 March–4 April 2017; pp. 11–20.

14. Washington Department of Fish and Wildlife (WDFW). *Hatchery Fish. Mass-Marking*; Washington Department of Fish and Wildlife (WDFW): Takoma Park, MD, USA, 2019.
15. Saunders, R.L.; Allen, K.R. Effects of tagging and fin-clipping on the survival and growth of Atlantic salmon between smolt and adult stages. *J. Fish. Res. Board Can.* **1967**, *24*, 2595–2611.
16. Nicola, S.J.; Cordone, A.J. Effects of fin removal on survival and growth of rainbow trout (Salmo gairdneri) in a natural environment. *Trans. Am. Fish. Soc.* **1973**, *102*, 753–758.
17. O'Grady, M.F. The Effects of Fin-Clipping, Floy-Tagging and Fin-Damage on the Survival and Growth of Brown Trout (*Salmo trutta* L) Stocked in Irish Lakes. *Fish. Manag.* **1984**, *152*, 49–58.
18. Gjerde, B.; Refstie, T. The effect of fin-clipping on growth rate, survival and sexual maturity of rainbow trout. *Aquaculture* **1988**, *73*, 383–389.
19. Hansen, L.P. Effects of Carlin tagging and fin clipping on survival of Atlantic salmon (*Salmo salar* L.) released as smolts. *Aquaculture* **1988**, *70*, 391–394.
20. Vander Haegen, G.E.; Blankenship, H.L.; Hoffmann, A.; Thompson, D.A. The Effects of Adipose Fin Clipping and Coded Wire Tagging on the Survival and Growth of Spring Chinook Salmon. *N. Am. J. Fish. Manag.* **2005**, *25*, 1161–1170.
21. Bumgarner, J.D.; Schuck, M.L.; Blankenship, H.L. Returns of Hatchery Steelhead with Different Fin Clips and Coded Wire Tag Lengths. *N. Am. J. Fish. Manag.* **2009**, *29*, 903–913.
22. Reimchen, T.E.; Temple, N.F. Hydrodynamic and phylogenetic aspects of the adipose fin in fishes. *Can. J. Zool.* **2004**, *82*, 910–916.
23. Buckland-Nicks, J.A. New details of the neural architecture of the salmonid adipose fin. *J. Fish. Biol.* **2016**, *89*, 1991–2003.
24. Aiello, B.R.; Stewart, T.A.; Hale, M.E. Mechanosensation in an adipose fin. *Proc. R. Soc. B Biol. Sci.* **2016**, *283*, 20152794.
25. Garstang, W. The phyletic classification of Teleostei. *Proc. Leeds Philos. Lit. Soc. Sci. Sect.* **1931**, *2*, 240–261.
26. Temple, N.F.; Reimchen, T.E. Adipose fin condition and flow regime in catfish. *Can. J. Zool.* **2008**, *86*, 1079–1082.
27. Sneddon, L.U.; Elwood, R.W.; Adamo, S.A.; Leach, M.C. Defining and assessing animal pain. *Anim. Behav.* **2014**, *97*, 201–212**.**
28. Sneddon, L.U. Trigeminal somatosensory innervation of the head of a teleost fish with particular reference to nociception. *Brain Res.* **2003**, *972*, 44–52.
29. Ashley, P.J.; Sneddon, L.U.; McCrohan, C.R. Nociception in fish: Stimulus-response properties of receptors on the head of trout Oncorhynchus mykiss. *Brain Res.* **2007**, *1166*, 47–54.
30. Sneddon, L.U. Pain perception in fish: Indicators and endpoints. *ILAR J.* **2009**, *50*, 338–342.
31. Sneddon, L.U.; Braithwaite, V.A.; Gentle, M.J. Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system. *Proc. R. Soc. B Biol. Sci.* **2003**, *270*, 1115–1121.
32. Li, L.; Rutlin, M.; Abraira, V.E.; Cassidy, C.; Kus, L.; Gong, S.; Jankowski, M.P.; Luo, W.; Heintz, N.; Koerber, H.R.; et al. The functional organization of cutaneous low-threshold mechanosensory neurons. *Cell* **2011**, *147*, 1615–1627.
33. Victoria E.A.; David, D. Ginty Neuron The Sensory Neurons of Touch. *Neuron* **2013**, *79*, 1–44.
34. François, A.; Schüetter, N.; Laffray, S.; Sanguesa, J.; Pizzoccaro, A.; Dubel, S.; Mantilleri, A.; Nargeot, J.; Noël, J.; Wood, J.N.; et al. The Low-Threshold Calcium Channel Cav3.2 Determines Low-Threshold Mechanoreceptor Function. *Cell Rep.* **2015**, *10*, 370–382.
35. Lechner, S.G. Neue Einsichten in die spinalen und peripheren Signalwege der Schmerzentstehung. *e-Neuroforum* **2017**, *23*, 173–178.
36. Brierley, S.M. Molecular basis of mechanosensitivity. *Auton. Neurosci. Basic Clin.* **2010**, *153*, 58–68.
37. Wilson, R.I.; Corey, D.P. The Force Be With You: A Mechanoreceptor Channel in Proprioception and Touch. *Neuron* **2010**, *67*, 349–351.
38. Gumy, L.F.; Yeo, G.S.H.; Tung, Y.L.; Zivraj, K.H.; Willis, D.; Coppola, G.; Lam, B.Y.H.; Twiss, J.L.; Holt, C.E.; Fawcett, J.W. Transcriptome analysis of embryonic and adult sensory axons reveals changes in mRNA repertoire localization Transcriptome analysis of embryonic and adult sensory axons reveals changes in mRNA repertoire localization. *RNA* **2011**, *17*, 85–98.
39. Chiu, I.M.; Barrett, L.B.; Williams, E.K.; Strochlic, D.E.; Lee, S.; Weyer, A.D.; Lou, S.; Bryman, G.S.; Roberson, D.P.; Ghasemlou, N.; et al. Transcriptional profiling at whole population and single cell levels reveals somatosensory neuron molecular diversity. *Elife* **2014**, *3*, 1–32.

40. Le Pichon, C.E.; Chesler, A.T. The functional and anatomical dissection of somatosensory subpopulations using mouse genetics. *Front. Neuroanat.* **2014**, *8*, 1–18.
41. Ranade, S.S.; Syeda, R.; Patapoutian, A. Mechanically Activated Ion Channels. *Neuron* **2015**, *87*, 1162–1179.
42. Usoskin, D.; Furlan, A.; Islam, S.; Abdo, H.; Lönnerberg, P.; Lou, D.; Hjerling-Leffler, J.; Haeggström, J.; Kharchenko, O.; Kharchenko, P.V.; et al. Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. *Nat. Neurosci.* **2015**, *18*, 145–153.
43. Li, C.L.; Li, K.C.; Wu, D.; Chen, Y.; Luo, H.; Zhao, J.R.; Wang, S.S.; Sun, M.M.; Lu, Y.J.; Zhong, Y.Q.; et al. Somatosensory neuron types identified by high-coverage single-cell RNA-sequencing and functional heterogeneity. *Cell Res.* **2016**, *26*, 83–102.
44. Schlosser, G. A Short History of Nearly Every Sense—The Evolutionary History of Vertebrate Sensory Cell Types. *Integr. Comp. Biol.* **2018**, *58*, 301–316.
45. Zeisel, A.; Hochgerner, H.; Lönnerberg, P.; Johnsson, A.; Memic, F.; van der Zwan, J.; Häring, M.; Braun, E.; Borm, L.E.; La Manno, G.; et al. Molecular Architecture of the Mouse Nervous System. *Cell* **2018**, *174*, 999–1014.
46. Hockley, J.R.F.; Taylor, T.S.; Callejo, G.; Wilbrey, A.L.; Gutteridge, A.; Bach, K.; Winchester, W.J.; Bulmer, D.C.; McMurray, G.; Smith, E.S.J. Single-cell RNAseq reveals seven classes of colonic sensory neuron. *Gut* **2018**, *68*, 633–644.
47. Martinac, B.; Poole, K. Mechanically activated ion channels. *Int. J. Biochem. Cell Biol.* **2018**, *97*, 104–107.
48. Zaccone, G. Neuron-specific enolase and serotonin in the Merkel cells of conger-eel (Conger conger) epidermis—An immunohistochemical study. *Histochemistry* **1986**, *85*, 29–34.
49. Reeves, S.; Helman, L.; Allison, A.; Israel, M. Molecular cloning and primary structure of human glial fibrillary acidic protein. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 5178–5182.
50. Genever, P.G.; Maxfield, S.J.; Kennovin, G.D.; Maltman, J.; Bowgen, C.J.; Raxworthy, M.J.; Skerry, T.M. Evidence for a novel glutamate-mediated signaling pathway in keratinocytes. *J. Investig. Dermatol.* **1999**, *112*, 337–342.
51. Reinisch, C.M.; Tschachler, E. The dimensions and characteristics of the subepidermal nerve plexus in human skin—Terminal Schwann cells constitute a substantial cell population within the superficial dermis. *J. Dermatol. Sci.* **2012**, *65*, 162–169.
52. Abbate, F.; Catania, S.; Germanà, A.; González, T.; Diaz-Esnal, B.; Germanà, G.; Vega, J.A. S-100 protein is a selective marker for sensory hair cells of the lateral line system in teleosts. *Neurosci. Lett.* **2002**, *329*, 133–136.
53. Feito, J.; García-Suárez, O.; García-Piqueras, J.; García-Mesa, Y.; Pérez-Sánchez, A.; Suazo, I.; Cabo, R.; Suárez-Quintanilla, J.; Cobo, J.; Vega, J.A. The development of human digital Meissner's and Pacinian corpuscles. *Ann. Anat.* **2018**, *219*, 8–24.
54. García-Mesa, Y.; García-Piqueras, J.; García, B.; Feito, J.; Cabo, R.; Cobo, J.; Vega, J.A.; García-Suárez, O. Merkel cells and Meissner's corpuscles in human digital skin display Piezo2 immunoreactivity. *J. Anat.* **2017**, *231*, 978–989.
55. Jung, H.; Yoon, B.C.; Holt, C.E. Axonal mRNA localization and local protein synthesis in nervous system assembly, maintenance and repair. *Nat. Rev. Neurosci.* **2012**, *13*, 308–324.
56. Berthelot, C.; Brunet, F.; Chalopin, D.; Juanchich, A.; Bernard, M.; Noël, B.; Bento, P.; Da Silva, C.; Labadie, K.; Alberti, A.; et al. The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. *Nat. Commun.* **2014**, *5*, 3657.
57. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Müller, R.; Nolan, T.; Pfaffl, M.; Shipley, G.; et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **2009**, *55*, 611–622.
58. Altmann, S.; Rebl, A.; Kühn, C.; Goldammer, T. Identification and de novo sequencing of housekeeping genes appropriate for gene expression analyses in farmed maraena whitefish (Coregonus maraena) during crowding stress. *Fish. Physiol. Biochem.* **2015**, *41*, 397–412.
59. Rebl, A.; Köbis, J.M.; Fischer, U.; Takizawa, F.; Verleih, M.; Wimmers, K.; Goldammer, T. MARCH5 gene is duplicated in rainbow trout, but only fish-specific gene copy is up-regulated after VHSV infection. *Fish. Shellfish Immunol.* **2011**, *31*, 1041–1050.
60. Köbis, J.M.; Rebl, H.; Goldammer, T.; Rebl, A. Multiple gene and transcript variants encoding trout C-polysaccharide binding proteins are differentially but strongly induced after infection with Aeromonas salmonicida. *Fish. Shellfish Immunol.* **2017**, *60*, 509–519.

61. Sanger, F.; Coulson, A.R. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.* **1975**, *94*, 441–448.
62. Bookout, A.L.; Mangelsdorf, D.J. Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. *Nucl. Recept. Signal.* **2003**, *1*, nrs-01012.
63. Szczot, M.; Pogorzala, L.A.; Solinski, H.J.; Young, L.; Yee, P.; Le Pichon, C.E.; Chesler, A.T.; Hoon, M.A. Cell-Type-Specific Splicing of Piezo2 Regulates Mechanotransduction. *Cell Rep.* **2017**, *21*, 2760–2771.
64. Lariviere, R.C.; Julien, J.P. Functions of Intermediate Filaments in Neuronal Development and Disease. *J. Neurobiol.* **2004**, *58*, 131–148.
65. Li, C.; Wang, S.; Chen, Y.; Zhang, X. Somatosensory Neuron Typing with High-Coverage Single-Cell RNA Sequencing and Functional Analysis. *Neurosci. Bull.* **2018**, *34*, 200–207.
66. de Nooij, J.C.; Doobar, S.; Jessell, T.M. Etv1 Inactivation Reveals Proprioceptor Subclasses that Reflect the Level of NT3 Expression in Muscle Targets. *Neuron* **2013**, *77*, 1055–1068.
67. Woo, S.H.; Lukacs, V.; De Nooij, J.C.; Zaytseva, D.; Criddle, C.R.; Francisco, A.; Jessell, T.M.; Wilkinson, K.A.; Patapoutian, A. Piezo2 is the principal mechanotransduction channel for proprioception. *Nat. Neurosci.* **2015**, *18*, 1756–1762.
68. Zhang, Y.; Chen, K.; Sloan, S.A.; Bennett, M.L.; Scholze, A.R.; O'Keeffe, S.; Phatnani, H.P.; Guarnieri, P.; Caneda, C.; Ruderisch, N.; et al. An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and Vascular Cells of the Cerebral Cortex. *J. Neurosci.* **2014**, *34*, 11929–11947.
69. Sun, W.; Cornwell, A.; Li, J.; Peng, S.; Osorio, M.J.; Aalling, N.; Wang, S.; Benraiss, A.; Lou, N.; Goldman, S.A.; et al. SOX9 Is an Astrocyte-Specific Nuclear Marker in the Adult Brain Outside the Neurogenic Regions. *J. Neurosci.* **2017**, *37*, 4493–4507.
70. Weil, M.-T.; Heibeck, S.; Töpperwien, M.; tom Dieck, S.; Ruhwedel, T.; Salditt, T.; Rodicio, M.C.; Morgan, J.R.; Nave, K.-A.; Möbius, W.; et al. Axonal Ensheathment in the Nervous System of Lamprey: Implications for the Evolution of Myelinating Glia. *J. Neurosci.* **2018**, *38*, 6586–6596.
71. Jessen, K.R.; Morgan, L.; Stewart, H.J.; Mirsky, R. Three markers of adult non-myelin-forming Schwann cells, 217c(Ran-1), A5E3 and GFAP: Development and regulation by neuron-Schwann cell interactions. *Development* **1990**, *109*, 91–103.
72. Bhatheja, K.; Field, J. Schwann cells: Origins and role in axonal maintenance and regeneration. *Int. J. Biochem. Cell Biol.* **2006**, *38*, 1995–1999.
73. Wewetzer, K.; Verdú, E.; Angelov, D.N.; Navarro, X. Olfactory ensheathing glia and Schwann cells: Two of a kind? *Cell Tissue Res.* **2002**, *309*, 337–345.
74. Hartmann, M. Truncated TrkB receptor-induced outgrowth of dendritic filopodia involves the p75 neurotrophin receptor. *J. Cell Sci.* **2004**, *117*, 5803–5814.
75. Brohawn, S.G.; Su, Z.; MacKinnon, R. Mechanosensitivity is mediated directly by the lipid membrane in TRAAK and TREK1 K^+ channels. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 3614–3619.
76. Berman, N.E.J.; Puri, V.; Chandrala, S.; Puri, S.; Macgregor, R.; Liverman, C.S.; Klein, R.M. Serotonin in trigeminal ganglia of female rodents: Relevance to menstrual migraine. *Headache* **2006**, *46*, 1230–1245.
77. Lund, J.P.; Sadeghi, S.; Athanassiadis, T.; Salas, N.C.; Auclair, F.; Thivierge, B.; Arsenault, I.; Rompré, P.; Westberg, K.G.; Kolta, A. Assessment of the potential role of muscle spindle mechanoreceptor afferents in chronic muscle pain in the rat masseter muscle. *PLoS ONE* **2010**, *5*, e11131.
78. Berger, U.V.; Hediger, M.A. Distribution of the glutamate transporters GLT-1 (SLC1A2) and GLAST (SLC1A3) in peripheral organs. *Anat. Embryol.* **2006**, *211*, 595–606.
79. Enjin, A.; Leão, K.E.; Mikulovic, S.; Le Merre, P.; Tourtellotte, W.G.; Kullander, K. Sensorimotor function is modulated by the serotonin receptor 1d, a novel marker for gamma motor neurons. *Mol. Cell. Neurosci.* **2012**, *49*, 322–332.
80. Chen, W.N.; Lee, C.H.; Lin, S.H.; Wong, C.W.; Sun, W.H.; Wood, J.N.; Chen, C.C. Roles of ASIC3, TRPV1, and NaV1.8 in the transition from acute to chronic pain in a mouse model of fibromyalgia. *Mol. Pain* **2014**, *10*, 1744–8069.
81. Simon, A.; Shenton, F.; Hunter, I.; Banks, R.W.; Bewick, G.S. Amiloride-sensitive channels are a major contributor to mechanotransduction in mammalian muscle spindles. *J. Physiol.* **2010**, *588*, 171–185.
82. Geven, E.J.W.; Klaren, P.H.M. The teleost head kidney: Integrating thyroid and immune signalling. *Dev. Comp. Immunol.* **2017**, *66*, 73–83.
83. Lim, J.E.; Porteus, C.S.; Bernier, N.J. Serotonin directly stimulates cortisol secretion from the interrenals in goldfish. *Gen. Comp. Endocrinol.* **2013**, *588*, 171–185.

84. Abdel-Aziz, E.S.H.; Ali, T.E.S.; Abdu, S.B.S.; Fouad, H.F. Chromaffin cells and interrenal tissue in the head kidney of the grouper, Epinephilus tauvina (Teleostei, Serranidae): A morphological (optical and ultrastructural) study. *J. Appl. Ichthyol.* **2010**, *26*, 522–527.
85. Schreck, C.B., Tort, L., Farrell, A.P., Brauner, C.B. Biology of Stress in Fish. In *Biology of Stress in Fish*; Academic press: London, UK, 2016; ISBN 9780128027288.
86. Dymowska, A.K.; Boyle, D.; Schultz, A.G.; Goss, G.G. The role of acid-sensing ion channels in epithelial Na+ uptake in adult zebrafish (*Danio rerio*). *J. Exp. Biol.* **2015**, *218*, 1244–1251.
87. Maness, P.F.; Schachner, M. Neural recognition molecules of the immunoglobulin superfamily: Signaling transducers of axon guidance and neuronal migration. *Nat. Neurosci.* **2007**, *10*, 19–26.
88. Van Acker, H.H.; Capsomidis, A.; Smits, E.L.; Van Tendeloo, V.F. CD56 in the immune system: More than a marker for cytotoxicity? *Front. Immunol.* **2017**, *8*, 892.
89. Ferrara, C.T.; Wang, P.; Neto, E.C.; Stevens, R.D.; Bain, J.R.; Wenner, B.R.; Ilkayeva, O.R.; Keller, M.P.; Blasiole, D.A.; Kendziorski, C.; et al. Genetic networks of liver metabolism revealed by integration of metabolic and transcriptional profiling. *PLoS Genet.* **2008**, *4*, e1000034.
90. Yuan, J.; Yankner, B.A. Apoptosis in the nervous system. *Nature* **2000**, *407*, 802–809.
91. Djouhri, L.; Lawson, S.N. Aβ-fiber nociceptive primary afferent neurons: A review of incidence and properties in relation to other afferent A-fiber neurons in mammals. *Brain Res. Rev.* **2004**, *46*, 131–145.
92. Abbate, F.; Madrigrano, M.; Scopitteri, T.; Levanti, M.; Cobo, J.L.; Germanà, A.; Vega, J.A.; Laurà, R. Acid-sensing ion channel immunoreactivities in the cephalic neuromasts of adult zebrafish. *Ann. Anat.* **2016**, *207*, 27–31.
93. Buckland-Nicks, J.A.; Gillis, M.; Reimchen, T.E. Neural network detected in a presumed vestigial trait: Ultrastructure of the salmonid adipose fin. *Proc. R. Soc. B Biol. Sci.* **2012**, *279*, 553–563.
94. Westley, P.A.H.; Carlson, S.M.; Quinn, T.P. Among-population variation in adipose fin size parallels the expression of other secondary sexual characteristics in sockeye salmon (*Oncorhynchus nerka*). *Environ. Biol. Fishes* **2008**, *81*, 439–446.

6. References

Aiello, B. R., Stewart, T. A., and Hale, M. E. (2016). Mechanosensation in an adipose fin. *Proc. R. Soc. B Biol. Sci.* 283, 20152794. doi:10.1098/rspb.2015.2794.

Altmann, S., Korytář, T., Kaczmarzyk, D., Nipkow, M., Kühn, C., Goldammer, T., et al. (2016). Toll-like receptors in maraena whitefish: Evolutionary relationship among salmonid fishes and patterns of response to Aeromonas salmonicida. *Fish Shellfish Immunol.* 54, 391–401. doi:10.1016/j.fsi.2016.04.125.

Aluru, N., and Vijayan, M. M. (2009). Stress transcriptomics in fish: A role for genomic cortisol signaling. *Gen. Comp. Endocrinol.* 164, 142–150. doi:10.1016/j.ygcen.2009.03.020.

Angata, T., Tabuchi, Y., Nakamura, K., and Nakamura, M. (2007). Siglec-15: An immune system Siglec conserved throughout vertebrate evolution. *Glycobiology* 17, 838–846. doi:10.1093/glycob/cwm049.

Backström, T., and Winberg, S. (2017). Serotonin coordinates responses to social stress-What we can learn from fish. *Front. Neurosci.* 11, 1–10. doi:10.3389/fnins.2017.00595.

Barcellos, L. J. G., Volpato, G. L., Barreto, R. E., Coldebella, I., and Ferreira, D. (2011). Chemical communication of handling stress in fish. *Physiol. Behav.* 103, 372–375. doi:10.1016/j.physbeh.2011.03.009.

Bardach, J. E., and Case, J. (1965). Sensory Capabilities of the Modified Fins of Squirrel Hake (Urophycis chuss) and Searobins (Prionotus carolinus. *Copeia* 1965, 194–206.

Barnes, N. M., and Sharp, T. (1999). A review of central 5-HT receptors and their function. *Neuropharmacology* 38, 1083–1152. doi:10.1016/S0028-3908(99)00010-6.

Barton, B. A. (2002). Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integr. Comp. Biol.* 42, 517–525. doi:10.1093/icb/42.3.517.

Barton, B., Schreck, C., and Barton, L. (1987). Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. *Dis. Aquat. Organ.* 2, 173–185. doi:10.3354/dao002173.

Basu, M., Paichha, M., Lenka, S. S., Chakrabarty, R., and Samanta, M. (2016). Hypoxic stress: impact on the modulation of TLR2, TLR4, NOD1 and NOD2 receptor and their down-stream signalling genes expression in catla (Catla catla). *Mol. Biol. Rep.* 43, 1–9. doi:10.1007/s11033-015-3932-4.

Bayne, C. J., and Gerwick, L. (2001). The acute phase response and innate immunity of fish. *Dev. Comp. Immunol.* 25, 725–743. doi:10.1016/S0145-305X(01)00033-7.

Bermejo-Nogales, A., Calduch-Giner, J. A., and Pérez-Sánchez, J. (2014). Tissue-specific gene expression and functional regulation of uncoupling protein 2 (UCP2) by hypoxia and nutrient availability in gilthead sea bream (Sparus aurata): Implications on the physiological significance of UCP1-3 variants. *Fish Physiol. Biochem.* 40, 751–762. doi:10.1007/s10695-013-9882-7.

Bertorelli, G., Bocchino, V., and Olivieri, D. (1998). Heat Shock Protein Interactions with the Glucocorticoid Receptor. *Pulm. Pharmacol. Ther.* 11, 7–12. doi:10.1006/pupt.1998.0119.

Boltaña, S., Rey, S., Roher, N., Vargas, R., Huerta, M., Huntingford, F. A., et al. (2013). Behavioural fever is a synergic signal amplifying the innate immune response. *Proc. R. Soc. B Biol. Sci.* 280. doi:10.1098/rspb.2013.1381.

Bornhöfft, K. F., Goldammer, T., Rebl, A., and Galuska, S. P. (2018). Siglecs: A journey through the evolution of sialic acid-binding immunoglobulin-type lectins. *Dev. Comp. Immunol.* 86, 219–231. doi:10.1016/j.dci.2018.05.008.

Brietzke, A., Korytář, T., Jaros, J., Köllner, B., Goldammer, T., Seyfert, H.-M., et al. (2015). Aeromonas salmonicida Infection Only Moderately Regulates Expression of Factors Contributing to Toll-Like Receptor Signaling but Massively Activates the Cellular and Humoral Branches of Innate Immunity in Rainbow Trout (Oncorhynchus mykiss). *J. Immunol. Res.* 2015, 1–16. doi:10.1155/2015/901015.

Broom, D. M. (2006). Behaviour and welfare in relation to pathology. *Appl. Anim. Behav. Sci.* 97, 73–83. doi:10.1016/j.applanim.2005.11.019.

Broom, D. M., and Corke, M. J. (2002). Effects of disease on farm animal welfare. *Acta Vet. Brno* 71, 133–136. doi:10.2754/avb200271010133.

Bshary, R., and Brown, C. (2014). Fish cognition. *Curr. Biol.* 24, R947–R950. doi:10.1016/j.cub.2014.08.043.

Bücheler, M. ., Hadamek, K., and Hein, L. (2002). Two α2-adrenergic receptor subtypes, α2A and α2C, inhibit transmitter release in the brain of gene-targeted mice. *Neuroscience* 109, 819–826. doi:10.1016/S0306-4522(01)00531-0.

Buckland-Nicks, J. A. (2016). New details of the neural architecture of the salmonid adipose fin. *J. Fish Biol.* 89, 1991–2003. doi:10.1111/jfb.13098.

Campbell, P. M., Pottinger, T. G., and Sumpter, J. P. (1992). Stress reduces the quality of gametes produced by rainbow trout. *Biol. Reprod.* 47, 1140–1150. doi:10.1095/biolreprod47.6.1140.

Campbell, P. M., Pottinger, T. G., and Sumpter, J. P. (1994). Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. *Aquaculture* 120, 151–169. doi:10.1016/0044-8486(94)90230-5.

Carruth, L. L., Jones, R. E., and Norris, D. O. (2002). Cortisol and Pacific salmon: A new look at the role of stress hormones in olfaction and home-stream migration. *Integr. Comp. Biol.* 42, 574–581. doi:10.1093/icb/42.3.574.

Castanheira, M. F., Conceição, L. E. C., Millot, S., Rey, S., Bégout, M. L., Damsgård, B., et al. (2017). Coping styles in farmed fish: consequences for aquaculture. *Rev. Aquac.* 9, 23–41. doi:10.1111/raq.12100.

Castillo, J., Teles, M., Mackenzie, S., and Tort, L. (2009). Stress-related hormones modulate cytokine expression in the head kidney of gilthead seabream (Sparus aurata). *Fish Shellfish Immunol.* 27, 493–499. doi:10.1016/j.fsi.2009.06.021.

Céspedes, H. A., Zavala, K., Vandewege, M. W., and Opazo, J. C. (2017). Evolution of the α2-adrenoreceptors in vertebrates: ADRA2D is absent in mammals and crocodiles. *Gen. Comp. Endocrinol.* 250, 85–94. doi:10.1016/j.ygcen.2017.06.006.

Chabbi, A., and Ganesh, C. B. (2015). Evidence for the involvement of dopamine in stress-induced suppression of reproduction in the cichlid fish oreochromis mossambicus. *J. Neuroendocrinol.* 27, 343–356. doi:10.1111/jne.12269.

Chang, Y. C., and Nizet, V. (2014). The interplay between Siglecs and sialylated pathogens. *Glycobiology* 24, 818–825. doi:10.1093/glycob/cwu067.

Chappell, C. P., Draves, K. E., and Clark, E. A. (2017). CD22 is required for formation of memory B cell precursors within germinal centers. *PLoS One* 12, 1–10. doi:10.1371/journal.pone.0174661.

Chettri, J. K., Raida, M. K., Holten-Andersen, L., Kania, P. W., and Buchmann, K. (2011). PAMP induced expression of immune relevant genes in head kidney leukocytes of rainbow trout (Oncorhynchus mykiss). *Dev. Comp. Immunol.* 35, 476–82. doi:10.1016/j.dci.2010.12.001.

Clark, E. A., and Giltiay, N. V. (2018). CD22: A Regulator of Innate and Adaptive B Cell Responses and Autoimmunity. *Front. Immunol.* 9, 2235. doi:10.3389/fimmu.2018.02235.

Cockrem, J. F. (2013). Individual variation in glucocorticoid stress responses in animals. *Gen. Comp. Endocrinol.* 181, 45–58. doi:10.1016/j.ygcen.2012.11.025.

Conte, F. S. (2004). Stress and the welfare of cultured fish. *Appl. Anim. Behav. Sci.* 86, 205–223. doi:10.1016/j.applanim.2004.02.003.

Crocker, P. R. (2005). Siglecs in innate immunity. *Curr. Opin. Pharmacol.* 5, 431–437. doi:10.1016/j.coph.2005.03.003.

Crocker, P. R., Paulson, J. C., and Varki, A. (2007). Siglecs and their roles in the immune system. *Nat. Rev. Immunol.* 7, 255–266. doi:10.1038/nri2056.

Crocker, P. R., and Varki, A. (2001). Siglecs in the immune system. *Immunology* 103, 137–145. doi:10.1046/j.0019-2805.2001.01241.x.

Cui, M., Zhang, Q., Yao, Z., Zhang, Z., Zhang, H., and Wang, Y. (2010). Immunoglobulin M gene expression analysis of orange-spotted grouper, Epinephelus coioides, following heat shock and Vibrio alginolyticus challenge. *Fish Shellfish Immunol.* 29, 1060–1065. doi:10.1016/j.fsi.2010.08.018.

Delaney, M. A., and Klesius, P. H. (2004). Hypoxic conditions induce Hsp70 production in blood, brain and head kidney of juvenile Nile tilapia Oreochromis niloticus (L.). *Aquaculture* 236, 633–644. doi:10.1016/j.aquaculture.2004.02.025.

Demers, N. E., and Bayne, C. J. (1997). The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. *Dev. Comp. Immunol.* 21, 363–373. doi:S0145-305X(97)00009-8 [pii].

Dhabhar, F. S. (2002). Stress-induced augmentation of immune function - The role of stress hormones, leukocyte trafficking, and cytokines. *Brain. Behav. Immun.* 16, 785–798. doi:10.1016/S0889-1591(02)00036-3.

Dror, M., Sinyakov, M. S., Okun, E., Dym, M., Sredni, B., and Avtalion, R. R. (2006). Experimental handling stress as infection-facilitating factor for the goldfish ulcerative disease. *Vet. Immunol. Immunopathol.* 109, 279–287. doi:10.1016/j.vetimm.2005.08.022.

Duval, T., Saliani, A., Nami, H., Nanci, A., Stikov, N., Leblond, H., et al. (2019). Axons morphometry in the human spinal cord. *Neuroimage* 185, 119–128. doi:10.1016/j.neuroimage.2018.10.033.

Estrada, L. D., Ağaç, D., and Farrar, J. D. (2016). Sympathetic neural signaling via the β2-adrenergic receptor suppresses T-cell receptor-mediated human and mouse CD8+ T-cell effector function. *Eur. J. Immunol.* 46, 1948–1958. doi:10.1002/eji.201646395.

Fabbri, E., and Moon, T. W. (2016). Adrenergic signaling in teleost fish liver, a challenging path. *Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol.* 199, 74–86. doi:10.1016/j.cbpb.2015.10.002.

FAO (2020). *The State of World Fisheries and Aquaculture 2020*. FAO doi:10.4060/ca9229en.

Feldman, S., Conforti, N., and Weidenfeld, J. (1995). Limbic pathways and hypothalamic neurotransmitters mediating adrenocortical responses to neural stimuli. *Neurosci. Biobehav. Rev.* 19, 235–240. doi:10.1016/0149-7634(94)00062-6.

Fierro-Castro, C., Barrioluengo, L., López-Fierro, P., Razquin, B. E., and Villena, A. J. (2013). Fish cell cultures as invitro models of inflammatory responses elicited by immunostimulants. Expression of regulatory genes of the innate immune response. *Fish Shellfish Immunol.* 35, 979–987. doi:10.1016/j.fsi.2013.07.015.

Flory, C. M. (1989). Autonomic innervation of the spleen of the coho salmon, Oncorhynchus kisutch: A histochemical demonstration and preliminary assessment of its immunoregulatory role. *Brain Behav. Immun.* 3, 331–344. doi:10.1016/0889-1591(89)90032-9.

Flory, C. M. (1990). Phylogeny of neuroimmunoregulation: Effects of adrenergic and cholinergic agents on the in vitro antibody response of the rainbow trout, Onchorynchus mykiss. *Dev. Comp. Immunol.* 14, 283–294. doi:10.1016/0145-305X(90)90019-B.

Flory, C. M., and Bayne, C. J. (1991). The influence of adrenergic and cholinergic agents on the chemiluminescent and mitogenic responses of leukocytes from the rainbow trout, Oncorhyncus mykiss. *Dev. Comp. Immunol.* 15, 135–142. doi:10.1016/0145-305X(91)90004-I.

Flügge, G. (1999). "Regulation of monoamine receptors in the brain: dynamic changes during stress," in *International Review of Cytology*, 145–213. doi:10.1016/S0074-7696(08)62705-9.

Freyhof, J., and Brooks, E. (2011). *European Red List of Freshwater Fishes*. doi:https://doi.org/10.2779/85903.

Fröhlich, F. (2016). "Neuromodulators," in *Network Neuroscience* (Elsevier), 59–71. doi:10.1016/B978-0-12-801560-5.00005-7.

Fuller, R. W. (1992). The involvement of serotonin in regulation of pituitary-adrenocortical function. *Front. Neuroendocrinol.* 13, 250–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1334001.

Gahete, M. D., Durán-Prado, M., Luque, R. M., Martínez-Fuentes, A. J., Quintero, A., Gutiérrez-Pascual, E., et al. (2009). Understanding the multifactorial control of growth hormone release by somatotropes: Lessons from comparative endocrinology. *Ann. N. Y. Acad. Sci.* 1163, 137–153. doi:10.1111/j.1749-6632.2008.03660.x.

Gamperl, A. K., Vijayan, M. M., and Boutilier, R. G. (1994). Experimental control of stress hormone levels in fishes: techniques and applications. *Rev. Fish Biol. Fish.* 4, 215–255. doi:10.1007/BF00044129.

Garstang, W. (1931). The phyletic classification of Teleostei. *Proc. Leeds Philos. Lit. Soc. Sci. Sect* 2, 240–261.

Gaspar, P., and Lillesaar, C. (2012). Probing the diversity of serotonin neurons. *Philos. Trans. R. Soc. B Biol. Sci.* 367, 2382–2394. doi:10.1098/rstb.2011.0378.

Gazola, R., Borella, M. I., Val-Sella, M. V, Moraes, F. F., and Bernardino, G. (1995). Histophysiological aspects of the interrenal gland of the pacu female, Piaractus mesopotamicus (Telostei, Cyprinidae). *Bol. Tec. do CEPTA.Pirassununga [BOL.TEC.CEPTA]* 8, 1–12.

Gesto, M., López-Patïo, M. A., Hernández, J., Soengas, J. L., Míguez, J. M., Lopez-Patino, M. A., et al. (2013). The response of brain serotonergic and dopaminergic systems to an acute stressor in rainbow trout: a time course study. *J. Exp. Biol.* 216, 4435–4442. doi:10.1242/jeb.091751.

Geven, E. J. W., and Klaren, P. H. M. (2017). The teleost head kidney: Integrating thyroid and immune signalling. *Dev. Comp. Immunol.* 66, 73–83. doi:10.1016/j.dci.2016.06.025.

Gornati, R., Gualdoni, S., Cavaliere, R., Terova, G., Saroglia, M., and Bernardini, G. (2005). Molecular biology and fish welfare: A winning combination. *Aquac. Int.* 13, 51–55. doi:10.1007/s10499-004-9034-2.

Greenwood, A. K., Butler, P. C., White, R. B., Demarco, U., Pearce, D., and Fernald, R. D. (2003). Multiple corticosteroid receptors in a teleost fish: Distinct sequences, expression patterns, and transcriptional activities. *Endocrinology* 144, 4226–4236. doi:10.1210/en.2003-0566.

Grisanti, L. A., Evanson, J., Marchus, E., Jorissen, H., Woster, A. P., DeKrey, W., et al. (2010). Pro-inflammatory responses in human monocytes are β1-adrenergic receptor subtype dependent. *Mol. Immunol.* 47, 1244–1254. doi:10.1016/j.molimm.2009.12.013.

Groh, J., Ribechini, E., Stadler, D., Schilling, T., Lutz, M. B., and Martini, R. (2016). Sialoadhesin promotes neuroinflammation-related disease progression in two mouse models of CLN disease. *Glia* 64, 792–809. doi:10.1002/glia.22962.

Hartmann, M., Brigadski, T., Erdmann, K. S., Holtmann, B., Sendtner, M., Narz, F., et al. (2004). Truncated TrkB receptor-induced outgrowth of dendritic filopodia involves the p75 neurotrophin receptor. *J. Cell Sci.* 117, 5803–5814. doi:10.1242/jcs.01511.

Höglund, E., Moltesen, M., Castanheira, M. F., Thörnqvist, P.-O., Silva, P. I. M., Øverli, Ø., et al. (2020). Contrasting neurochemical and behavioral profiles reflects stress coping styles but not stress responsiveness in farmed gilthead seabream (Sparus aurata). *Physiol. Behav.* 214, 112759. doi:10.1016/j.physbeh.2019.112759.

Höglund, E., Weltzien, F.-A., Schjolden, J., Winberg, S., Ursin, H., and Døving, K. B. (2005). Avoidance behavior and brain monoamines in fish. *Brain Res.* 1032, 104–110. doi:10.1016/j.brainres.2004.10.050.

Huntingford, F. A., Adams, C., Braithwaite, V. A., Kadri, S., Pottinger, T. G., Sandøe, P., et al. (2006). Current issues in fish welfare. *J. Fish Biol.* 68, 332–372. doi:10.1111/j.0022-1112.2006.001046.x.

Huntingford, F. A., Andrew, G., Mackenzie, S., Morera, D., Coyle, S. M., Pilarczyk, M., et al. (2010). Coping strategies in a strongly schooling fish, the common carp Cyprinus carpio. *J. Fish Biol.* 76, 1576–1591. doi:10.1111/j.1095-8649.2010.02582.x.

ICES WGBAST Report (2007). ICES WGBAST Report 2007 Report of the Baltic Salmon and Trout Working Group (WGBAST). Available at: https://www.ices.dk/community/groups/Pages/WGBAST.aspx.

Iliev, D. B., Liarte, C. Q., MacKenzie, S., and Goetz, F. W. (2005). Activation of rainbow trout (Oncorhynchus mykiss) mononuclear phagocytes by different pathogen associated molecular pattern (PAMP) bearing agents. *Mol. Immunol.* 42, 1215–1223. doi:10.1016/j.molimm.2004.11.023.

Irwin, M. R., and Cole, S. W. (2011). Reciprocal regulation of the neural and innate immune systems. *Nat. Rev. Immunol.* 11, 625–632. doi:10.1038/nri3042.

Iwama, G. K., Pickering, A. D., Sumpter, J. P., and Schreck, C. B. (1998). Fish Stress and Health in Aquaculture. *Estuaries* 21, 501. doi:10.2307/1352849.

Jandus, C., Simon, H. U., and Von Gunten, S. (2011). Targeting Siglecs-A novel pharmacological strategy for immuno- and glycotherapy. *Biochem. Pharmacol.* 82, 323–332. doi:10.1016/j.bcp.2011.05.018.

Jansen, W., Jennerich, H.-J., Wenzel, H.-J., and Schulz, S. (2008). Zur Haltung von Ostseeschnäpeln in einem Kaltwasserkreislauf in Hohen Wangelin. *Fischerei Fischmarkt M-V* 4, 36–39.

Janzen, W. J., Duncan, C. A., and Riley, L. G. (2012). Cortisol treatment reduces ghrelin signaling

and food intake in tilapia, Oreochromis mossambicus. *Domest. Anim. Endocrinol.* 43, 251–259. doi:10.1016/j.domaniend.2012.04.003.

Jentoft, S., Aastveit, A. H., Torjesen, P. A., and Andersen, Ø. (2005). Effects of stress on growth, cortisol and glucose levels in non-domesticated Eurasian perch (Perca fluviatilis) and domesticated rainbow trout (Oncorhynchus mykiss). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 141, 353–358. doi:10.1016/j.cbpb.2005.06.006.

Jeswin, J., Joo, M.-S., Jeong, J.-M., Bae, J.-S., Choi, K.-M., Cho, D.-H., et al. (2018). The first report of siglec-3/CD33 gene in a teleost (rock bream, Oplegnathus fasciatus): An analysis of its spatial expression during stimulation to red seabream iridovirus (RSIV) and two bacterial pathogens. *Dev. Comp. Immunol.* 84, 117–122. doi:10.1016/j.dci.2018.02.005.

Johansen, R., Needham, J. R., Colquhoun, D. J., Poppe, T. T., and Smith, A. J. (2006). Guidelines for health and welfare monitoring of fish used in research. *Lab. Anim.* 40, 323–340. doi:10.1258/002367706778476451.

Jørgensen, J. B., Lunde, H., Jensen, L., Whitehead, A. S., and Robertsen, B. (2000). Serum amyloid A transcription in Atlantic salmon (Salmo salar L.) hepatocytes is enhanced by stimulation with macrophage factors, recombinant human IL-1β, IL-6 and TNFα or bacterial lipopolysaccharide. *Dev. Comp. Immunol.* 24, 553–563. doi:10.1016/S0145-305X(00)00022-7.

Jørgensen, J. B., Sharp, G. J. E., Secombes, C. J., and Robertsen, B. (1993). Effect of a yeast-cell-wall glucan on the bactericidal activity of rainbow trout macrophages. doi:10.1006/fsim.1993.1026.

Kaslin, J., and Panula, P. (2001). Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (Danio rerio). *J. Comp. Neurol.* 440, 342–377. doi:10.1002/cne.1390.

Kawasaki, T., and Kawai, T. (2014). Toll-like receptor signaling pathways. *Front. Immunol.* 5, 1–8. doi:10.3389/fimmu.2014.00461.

Khansari, A. R., Parra, D., Reyes-López, F. E., and Tort, L. (2017a). Cytokine modulation by stress hormones and antagonist specific hormonal inhibition in rainbow trout (Oncorhynchus mykiss) and gilthead sea bream (Sparus aurata) head kidney primary cell culture. *Gen. Comp. Endocrinol.* 250, 122–135. doi:10.1016/j.ygcen.2017.06.005.

Khansari, A. R., Parra, D., Reyes-López, F. E., and Tort, L. (2017b). Modulatory in vitro effect of stress hormones on the cytokine response of rainbow trout and gilthead sea bream head kidney stimulated with Vibrio anguillarum bacterin. *Fish Shellfish Immunol.* 70, 736–749. doi:10.1016/j.fsi.2017.09.009.

Kimani, J. K. (1995). Elastin and Mechanoreceptor Mechanisms with Special Reference to the Mammalian Carotid Sinus. *Mol. Biol. Pathol. Elastic Tissues* 117, 215–236. doi:10.1002/9780470514771.ch12.

Korytář, T., Jaros, J., Verleih, M., Rebl, A., Kotterba, G., Kühn, C., et al. (2013). Novel insights into the peritoneal inflammation of rainbow trout (Oncorhynchus mykiss). *Fish Shellfish Immunol.* 35, 3–10. doi:10.1016/j.fsi.2013.07.032.

Korytář, T., Nipkow, M., Altmann, S., Goldammer, T., Köllner, B., and Rebl, A. (2016). Adverse husbandry of maraena whitefish directs the immune system to increase mobilization of myeloid cells and proinflammatory responses. *Front. Immunol.* 7, 1–14. doi:10.3389/fimmu.2016.00631.

Kottelat, M., and Freyhof, J. (2007). *Handbook of European freshwater fishes*. Publications Kottelat.

Krasnov, A., Koskinen, H., Pehkonen, P., Rexroad, C. E., Afanasyev, S., and Mölsä, H. (2005). Gene

expression in the brain and kidney of rainbow trout in response to handling stress. *BMC Genomics* 6, 3. doi:https://doi.org/10.1186/1471-2164-6-3.

Kubokawa, K., Watanabe, T., Yoshioka, M., and Iwata, M. (1999). Effects of acute stress on plasma cortisol, sex steroid hormone and glucose levels in male and female sockeye salmon during the breeding season. *Aquaculture* 172, 335–349. doi:10.1016/S0044-8486(98)00504-3.

Kuçceukgul Guleç, A., and Cengizler, I. (2012). Determination of acute phase proteins aft er experimental Streptococcus iniae infection in tilapia (Oreochromis niloticus L.). *Turkish J. Vet. Anim. Sci.* 36, 380–387. doi:10.3906/vet-1011-2.

Lariviere, R. C., and Julien, J. P. (2004). Functions of Intermediate Filaments in Neuronal Development and Disease. *J. Neurobiol.* 58, 131–148. doi:10.1002/neu.10270.

Leal, E., Fernández-Durán, B., Guillot, R., Ríos, D., and Cerdá-Reverter, J. M. (2011). Stress-induced effects on feeding behavior and growth performance of the sea bass (Dicentrarchus labrax): A self-feeding approach. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 181, 1035–1044. doi:10.1007/s00360-011-0585-z.

Lehmann, F., Gäthje, H., Kelm, S., and Dietz, F. (2004). Evolution of sialic acid-binding proteins: Molecular cloning and expression of fish siglec-4. *Glycobiology* 14, 959–968. doi:10.1093/glycob/cwh120.

Li, C., Wang, S., Chen, Y., and Zhang, X. (2018). Somatosensory Neuron Typing with High-Coverage Single-Cell RNA Sequencing and Functional Analysis. *Neurosci. Bull.* 34, 200–207. doi:10.1007/s12264-017-0147-9.

Lillesaar, C., Stigloher, C., Tannhäuser, B., Wullimann, M. F., and Bally-Cuif, L. (2009). Axonal projections originating from raphe serotonergic neurons in the developing and adult zebrafish, Danio Rerio, using transgenics to visualize raphe-specific pet1 expression. *J. Comp. Neurol.* 512, 158–182. doi:10.1002/cne.21887.

Lőrincz, M. L., and Adamantidis, A. R. (2017). Monoaminergic control of brain states and sensory processing: Existing knowledge and recent insights obtained with optogenetics. *Prog. Neurobiol.* 151, 237–253. doi:10.1016/j.pneurobio.2016.09.003.

Lowenstein, O. (1956). Pressure receptors in the fins of the dogfish. *J. Exp. Biol.* 33, 417–421.

Lu, K. D., Radom-Aizik, S., Haddad, F., Zaldivar, F., Kraft, M., and Cooper, D. M. (2017). Glucocorticoid receptor expression on circulating leukocytes differs between healthy male and female adults. *J. Clin. Transl. Sci.* 1, 108–114. doi:10.1017/cts.2016.20.

Macauley, M. S., Crocker, P. R., and Paulson, J. C. (2014). Siglec-mediated regulation of immune cell function in disease. *Nat. Rev. Immunol.* 14, 653–666. doi:10.1038/nri3737.

Madison, B. N., Tavakoli, S., Kramer, S., and Bernier, N. J. (2015). Chronic cortisol and the regulation of food intake and the endocrine growth axis in rainbow trout. *J. Endocrinol.* 226, 103–119. doi:10.1530/JOE-15-0186.

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish Shellfish Immunol.* 20, 137–151. doi:10.1016/j.fsi.2004.09.006.

Marino, F., and Cosentino, M. (2013). Adrenergic modulation of immune cells: An update. *Amino Acids* 45, 55–71. doi:10.1007/s00726-011-1186-6.

Martorell-Ribera, J., Nipkow, M., Viergutz, T., Brunner, R. M., Bochert, R., Koll, R., et al. (2020). Early response of salmonid head-kidney cells to stress hormones and toll-like receptor ligands. *Fish Shellfish Immunol.* 98, 950–961. doi:10.1016/j.fsi.2019.11.058.

Martos-Sitcha, J. A., Bermejo-Nogales, A., Calduch-Giner, J. A., and Pérez-Sánchez, J. (2017). Gene

expression profiling of whole blood cells supports a more efficient mitochondrial respiration in hypoxia-challenged gilthead sea bream (Sparus aurata). *Front. Zool.* 14, 1–12. doi:10.1186/s12983-017-0220-2.

Maximino, C., and Herculano, A. M. (2010). A review of monoaminergic neuropsychopharmacology in zebrafish. *Zebrafish* 7, 359–378. doi:10.1089/zeb.2010.0669.

Mayeux, R. (2004). Biomarkers: Potential uses and limitations. *NeuroRX* 1, 182–188. doi:10.1602/neurorx.1.2.182.

Mazur, C. F., and Iwama, G. K. (1993). Handling and Crowding Stress Reduces Number of Plaque-Forming Cells in Atlantic Salmon. *J. Aquat. Anim. Health* 5, 98–101. doi:10.1577/1548-8667(1993)005<0098:HACSRN>2.3.CO;2.

McCormick, S. D. (2012). "Smolt Physiology and Endocrinology," in *Fish Physiology*, 199–251. doi:10.1016/B978-0-12-396951-4.00005-0.

Medeiros, L. R., Mager, E. M., Grosell, M., and McDonald, M. D. (2010). The serotonin subtype 1A receptor regulates cortisol secretion in the Gulf toadfish, Opsanus beta. *Gen. Comp. Endocrinol.* 168, 377–387. doi:10.1016/j.ygcen.2010.05.004.

Medeiros, L. R., and McDonald, M. D. (2013). Cortisol-mediated downregulation of the serotonin 1A receptor subtype in the Gulf toadfish, Opsanus beta. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 164, 612–621. doi:10.1016/j.cbpa.2013.01.014.

Mileva, V. R., Gilmour, K. M., and Balshine, S. (2011). Effects of maternal stress on egg characteristics in a cooperatively breeding fish. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 158, 22–29. doi:10.1016/j.cbpa.2010.08.017.

Mishra, A., Singh, S., and Shukla, S. (2018). Physiological and functional basis of dopamine receptors and their role in neurogenesis: Possible implication for parkinson's disease. *J. Exp. Neurosci.* 12, 117906951877982. doi:10.1177/1179069518779829.

Mold, C., Gewurz, H., and Du Clos, T. W. (1999). Regulation of complement activation by C-reactive protein. *Immunopharmacology* 42, 23–30. doi:10.1016/S0162-3109(99)00007-7.

Moltesen, M., Laursen, D. C., Thörnqvist, P.-O., Andersson, M. Å., Winberg, S., and Höglund, E. (2016). Effects of acute and chronic stress on telencephalic neurochemistry and gene expression in rainbow trout (Oncorhynchus mykiss). *J. Exp. Biol.* 219, 3907–3914. doi:10.1242/jeb.139857.

Mommsen, T. P., Vijayan, M. M., and Moon, T. W. (1999). Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fish.* 9, 211–268. doi:https://doi.org/10.1023/A:1008924418720.

Mott, R. T., Ait-Ghezala, G., Town, T., Mori, T., Vendrame, M., Zeng, J., et al. (2004). Neuronal expression of CD22: Novel mechanism for inhibiting microglial proinflammatory cytokine production. *Glia* 46, 369–379. doi:10.1002/glia.20009.

Mueller, T. (2012). What is the thalamus in zebrafish? *Front. Neurosci.* 6, 1–14. doi:10.3389/fnins.2012.00064.

Mylonas, C. C., and Robles, R. (2019). New species for EU aquaculture. *Food Sci. Technol.* 33, 22–26. doi:10.1002/fsat.3302_6.x.

Nakane, T., Szentendrei, T., Stern, L., Virmani, M., Seely, J., and Kunos, G. (1990). Effects of IL-1 and cortisol on β-adrenergic receptors, cell proliferation, and differentiation in cultured human A549 lung tumor cells. *Lung Cancer* 7, 186–187. doi:10.1016/0169-5002(91)90123-n.

Nichols, D. E., and Nichols, C. D. (2008). Serotonin receptors. *Chem. Rev.* 108, 1614–1641. doi:10.1021/cr078224o.

Nilsson, J., Stien, L. H., Fosseidengen, J. E., Olsen, R. E., and Kristiansen, T. S. (2012). From fright to anticipation: Reward conditioning versus habituation to a moving dip net in farmed Atlantic cod (Gadus morhua). *Appl. Anim. Behav. Sci.* 138, 118–124. doi:10.1016/j.applanim.2012.02.014.

Nitschke, L. (2005). The role of CD22 and other inhibitory co-receptors in B-cell activation. *Curr. Opin. Immunol.* 17, 290–297. doi:10.1016/j.coi.2005.03.005.

Noble, C., Gismervik, K., Iversen, M. H., Kolarevic, J., Nilsson, J., Stien, L. H., et al. (2018). *Welfare Indicators for farmed Atlantic salmon: tools for assessing fish welfare*. Available at: www.nofima.no/fishwell/english.

Noble, C., Gismervik, K., Iversen, M. H., Kolarevic, J., Nilsson, J., Stien, L. H., et al. (2020). *Welfare Indicators for farmed rainbow trout: tools for assessing fish welfare*. Available at: www.nofima.no/fishwell/english.

Noble, C., Jones, H. A. C., Damsgård, B., Flood, M. J., Midling, K. O., Roque, A., et al. (2012). Injuries and deformities in fish: Their potential impacts upon aquacultural production and welfare. *Fish Physiol. Biochem.* 38, 61–83. doi:10.1007/s10695-011-9557-1.

O'Reilly, M. K., and Paulson, J. C. (2009). Siglecs as targets for therapy in immune-cell-mediated disease. *Trends Pharmacol. Sci.* 30, 240–248. doi:10.1016/j.tips.2009.02.005.

Olesen, I., Gjedrem, T., Bentsen, H. B., Gjerde, B., and Rye, M. (2003). Breeding Programs for Sustainable Aquaculture. *J. Appl. Aquac.* 13, 179–204. doi:10.1300/J028v13n03_01.

Otten, W., Kanitz, E., Couret, D., Veissier, I., Prunier, A., and Merlot, E. (2010). Maternal social stress during late pregnancy affects hypothalamic-pituitary-adrenal function and brain neurotransmitter systems in pig offspring. *Domest. Anim. Endocrinol.* 38, 146–156. doi:10.1016/j.domaniend.2009.09.002.

Øverli, Ø., Harris, C. A., and Winberg, S. (1999). Short-term effects of fights for social dominance and the establishment of dominant-subordinate relationships on brain monoamines and cortisol in rainbow trout. *Brain. Behav. Evol.* 54, 263–275. doi:10.1159/000006627.

Øverli, Ø., Korzan, W. J., Höglund, E., Winberg, S., Bollig, H., Watt, M., et al. (2004). Stress coping style predicts aggression and social dominance in rainbow trout. *Horm. Behav.* 45, 235–241. doi:10.1016/j.yhbeh.2003.12.002.

Øverli, Ø., Pottinger, T. G., Carrick, T. R., Øverli, E., and Winberg, S. (2001). Brain monoaminergic activity in rainbow trout selected for high and low stress responsiveness. *Brain. Behav. Evol.* 57, 214–224. doi:10.1159/000047238.

Pankhurst, N. W. (2011). The endocrinology of stress in fish: An environmental perspective. *Gen. Comp. Endocrinol.* 170, 265–275. doi:10.1016/j.ygcen.2010.07.017.

Panula, P., Chen, Y.-C., Priyadarshini, M., Kudo, H., Semenova, S., Sundvik, M., et al. (2010). The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases. *Neurobiol. Dis.* 40, 46–57. doi:10.1016/j.nbd.2010.05.010.

Qin, Y., Pan, J., Cai, M., Yao, L., and Ji, Z. (2016). Pattern Genes Suggest Functional Connectivity of Organs. *Sci. Rep.* 6, 1–7. doi:10.1038/srep26501.

Quarles, R. H. (2007). Myelin-associated glycoprotein (MAG): Past, present and beyond. *J. Neurochem.* 100, 1431–1448. doi:10.1111/j.1471-4159.2006.04319.x.

Ramsay, D. S., and Woods, S. C. (2014). Clarifying the roles of homeostasis and allostasis in physiological regulation. *Psychol. Rev.* 121, 225–247. doi:10.1037/a0035942.

Ravetch, J. V., and Lanier, L. L. (2000). Immune inhibitory receptors. *Science (80-.).* 290, 84–89. doi:10.1126/science.290.5489.84.

Rebl, A., and Goldammer, T. (2018). Under control: The innate immunity of fish from the inhibitors' perspective. *Fish Shellfish Immunol.* 77, 328–349. doi:10.1016/j.fsi.2018.04.016.

Rebl, A., Goldammer, T., Fischer, U., Köllner, B., and Seyfert, H.-M. H.-M. (2009). Characterization of two key molecules of teleost innate immunity from rainbow trout (Oncorhynchus mykiss): MyD88 and SAA. *Vet. Immunol. Immunopathol.* 131, 122–6. doi:10.1016/j.vetimm.2009.03.006.

Rebl, A., Verleih, M., Nipkow, M., Altmann, S., Bochert, R., and Goldammer, T. (2018). Gradual and acute temperature rise induces crossing endocrine, metabolic, and immunological pathways in maraena whitefish (Coregonus maraena). *Front. Genet.* 9. doi:10.3389/fgene.2018.00241.

Reid, S. G., Bernier, N. J., and Perry, S. F. (1998). The adrenergic stress response in fish: control of catecholamine storage and release. *Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol.* 120, 1–27. doi:10.1016/S0742-8413(98)00037-1.

Reimchen, T. E., and Temple, N. F. (2004). Hydrodynamic and phylogenetic aspects of the adipose fin in fishes. *Can. J. Zool.* 82, 910–916. doi:10.1139/z04-069.

Reinisch, C. M., and Tschachler, E. (2012). The dimensions and characteristics of the subepidermal nerve plexus in human skin - Terminal Schwann cells constitute a substantial cell population within the superficial dermis. *J. Dermatol. Sci.* 65, 162–169. doi:10.1016/j.jdermsci.2011.10.009.

Rey, S., Huntingford, F. A., Boltaña, S., Vargas, R., Knowles, T. G., and Mackenzie, S. (2015). Fish can show emotional fever: Stress-induced hyperthermia in zebrafish. *Proc. R. Soc. B Biol. Sci.* 282, 0–6. doi:10.1098/rspb.2015.2266.

Sadoul, B., and Geffroy, B. (2019). Measuring cortisol, the major stress hormone in fishes. *J. Fish Biol.* 94, 540–555. doi:10.1111/jfb.13904.

Sanders, V. M., and Kavelaars, A. (2007). *Adrenergic Regulation of Immunity AND IMMUNE SYSTEMS COMMUNICATE WITH EACH OTHER*. Fourth Edi. Elsevier Inc. doi:10.1016/B978-0-12-088576-3.50006-X.

Sangkuhl, K., Klein, T. E., and Altman, R. B. (2009). Selective serotonin reuptake inhibitors pathway. *Pharmacogenet. Genomics* 19, 907–909. doi:10.1097/FPC.0b013e32833132cb.

Sauter, S. T., Crawshaw, L. I., and Maule, A. G. (2001). Behavioral Thermoregulation by Juvenile Spring and Fall Chinook Salmon, Oncorhynchus Tshawytscha, during Smoltification. *Environ. Biol. Fishes* 61, 295–304. doi:10.1023/A:1010849019677.

Schreck, C. B., Tort, L., Farrell, A. P., and Brauner, C. J. (2016). "Biology of stress in fish," in, iii. doi:10.1016/B978-0-12-802728-8.00016-3.

Segner, H., Sundh, H., Buchmann, K., Douxfils, J., Sundell, K. S., Mathieu, C., et al. (2012). Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. *Fish Physiol. Biochem.* 38, 85–105. doi:10.1007/s10695-011-9517-9.

Seibel, H., Baßmann, B., and Rebl, A. (2021). Blood Will Tell: What Hematological Analyses Can Reveal About Fish Welfare. *Front. Vet. Sci.* 8, 1–21. doi:10.3389/fvets.2021.616955.

Seppola, M., Larsen, A. N., Steiro, K., Robertsen, B., and Jensen, I. (2008). Characterisation and

expression analysis of the interleukin genes, IL-1beta, IL-8 and IL-10, in Atlantic cod (Gadus morhua L.). *Mol. Immunol.* 45, 887–97. doi:10.1016/j.molimm.2007.08.003.

Siew, J. J., and Chern, Y. (2018). Microglial lectins in health and neurological diseases. *Front. Mol. Neurosci.* 11, 1–17. doi:10.3389/fnmol.2018.00158.

Silbergeld, E. K. (1974). Blood glucose: A sensitive indicator of environmental stress in fish. *Bull. Environ. Contam. Toxicol.* 11, 20–25. doi:10.1007/BF01685023.

Singh, C., Oikonomou, G., and Prober, D. A. (2015). Norepinephrine is required to promote wakefulness and for hypocretin-induced arousal in zebrafish. *Elife* 4, 1–22. doi:https://doi.org/10.7554/eLife.07000.001.

Son, C. G., Bilke, S., Davis, S., Greer, B. T., Wei, J. S., Whiteford, C. C., et al. (2005). Database of mRNA gene expression profiles of multiple human organs. *Genome Res.* 15, 443–450. doi:10.1101/gr.3124505.

Stewart, T. A., and Hale, M. E. (2013). First description of a musculoskeletal linkage in an adipose fin: innovations for active control in a primitively passive appendage. *Proc. R. Soc. B Biol. Sci.* 280, 20122159. doi:10.1098/rspb.2012.2159.

Stolte, E. H., de Mazon, A. F., Leon-Koosterziel, K. M., Jesiak, M., Bury, N. R., Sturm, A., et al. (2008a). Corticosteroid receptors involved in stress regulation in common carp, Cyprinus carpio. *J. Endocrinol.* 198, 403–417. doi:10.1677/JOE-08-0100.

Stolte, E. H., Nabuurs, S. B., Bury, N. R., Sturm, A., Flik, G., Savelkoul, H. F. J., et al. (2008b). Stress and innate immunity in carp: Corticosteroid receptors and pro-inflammatory cytokines. *Mol. Immunol.* 46, 70–79. doi:10.1016/j.molimm.2008.07.022.

Sumpter, J. P., Dye, H. M., and Benfey, T. J. (1986). The effects of stress on plasma ACTH, α-MSH, and cortisol levels in salmonid fishes. *Gen. Comp. Endocrinol.* 62, 377–385. doi:10.1016/0016-6480(86)90047-X.

Sun, J., Shaper, N. L., Itonori, S., Heffer-Lauc, M., Sheikh, K. A., and Schnaar, R. L. (2004). Myelin-associated glycoprotein (Siglec-4) expression is progressively and selectively decreased in the brains of mice lacking complex gangliosides. *Glycobiology* 14, 851–7. doi:10.1093/glycob/cwh107.

Sunyer, J. O., and Tort, L. (1995). Natural hemolytic and bactericidal activities of sea bream Sparus aurata serum are effected by the alternative complement pathway. *Vet. Immunol. Immunopathol.* 45, 333–345. doi:10.1016/0165-2427(94)05430-Z.

Sveen, L. R., Timmerhaus, G., Krasnov, A., Takle, H., Stefansson, S. O., Handeland, S. O., et al. (2018). High fish density delays wound healing in Atlantic salmon (Salmo salar). *Sci. Rep.* 8, 16907. doi:10.1038/S41598-018-35002-5.

Švob Štrac, D., Pivac, N., and Mück-Šeler, D. (2016). The serotonergic system and cognitive function. *Transl. Neurosci.* 7, 35–49. doi:10.1515/tnsci-2016-0007.

Talbot, A. T., Pottinger, T. G., Smith, T. J., and Cairns, M. T. (2009). Acute phase gene expression in rainbow trout (Oncorhynchus mykiss) after exposure to a confinement stressor: A comparison of pooled and individual data. *Fish Shellfish Immunol.* 27, 309–17. doi:10.1016/j.fsi.2009.05.016.

Tedder, T. F., Poe, J. C., and Haas, K. M. (2005). CD22: A multifunctional receptor that regulates B lymphocyte survival and signal transduction. *Adv. Immunol.* 88, 1–50. doi:10.1016/S0065-2776(05)88001-0.

Teles, M. C., Dahlbom, S. J., Winberg, S., and Oliveira, R. F. (2013). Social modulation of brain monoamine levels in zebrafish. *Behav. Brain Res.* 253, 17–24.

doi:10.1016/j.bbr.2013.07.012.

Temple, N. F., and Reimchen, T. E. (2008). Adipose fin condition and flow regime in catfish. *Can. J. Zool.* 86, 1079–1082. doi:10.1139/Z08-086.

Tort, L. (2011). Stress and immune modulation in fish. *Dev. Comp. Immunol.* 35, 1366–1375. doi:10.1016/j.dci.2011.07.002.

van den Heuvel, L. L. (2014). Stahl's essential psychopharmacology: Neuroscientific basis and practical applications (4th edition). *J. Child Adolesc. Ment. Heal.* 26, 157–158. doi:10.2989/17280583.2014.914944.

van Treeck, R., Van Wichelen, J., and Wolter, C. (2020). Fish species sensitivity classification for environmental impact assessment, conservation and restoration planning. *Sci. Total Environ.* 708, 135173. doi:10.1016/j.scitotenv.2019.135173.

Varki, A. (2011). Letter to the Glyco-Forum: Since there are PAMPs and DAMPs, there must be SAMPs? Glycan "self-associated molecular patterns" dampen innate immunity, but pathogens can mimic them. *Glycobiology* 21, 1121–1124. doi:10.1093/glycob/cwr087.

Varki, A. (2017a). Are humans prone to autoimmunity? Implications from evolutionary changes in hominin sialic acid biology. *J. Autoimmun.* 83, 134–142. doi:10.1016/j.jaut.2017.07.011.

Varki, A. (2017b). Biological roles of glycans. *Glycobiology* 27, 3–49. doi:10.1093/glycob/cww086.

Varki, A., and Angata, T. (2006). Siglecs — the major subfamily of I-type lectins. 16, 1–27. doi:10.1093/glycob/cwj008.

Varsamos, S., Flik, G., Pepin, J. F., Bonga, S. E. W., and Breuil, G. (2006). Husbandry stress during early life stages affects the stress response and health status of juvenile sea bass, Dicentrarchus labrax. *Fish Shellfish Immunol.* 20, 83–96. doi:10.1016/j.fsi.2005.04.005.

Villarroel, F., Casado, A., Vásquez, J., Matamala, E., Araneda, B., Amthauer, R., et al. (2008). Serum amyloid A: A typical acute-phase reactant in rainbow trout? *Dev. Comp. Immunol.* 32, 1160–1169. doi:10.1016/J.DCI.2008.03.004.

Viltart, O., and Vanbesien-Mailliot, C. C. A. (2007). Impact of prenatal stress on neuroendocrine programming. *Sci. World J.* 7, 1493–1537. doi:10.1100/tsw.2007.204.

Virion, Z., Doly, S., Saha, K., Lambert, M., Guillonneau, F., Bied, C., et al. (2019). Sialic acid mediated mechanical activation of β2 adrenergic receptors by bacterial pili. *Nat. Commun.* 10, 4752. doi:10.1038/s41467-019-12685-6.

Wachstein, J., Tischer, S., Figueiredo, C., Limbourg, A., Falk, C., Immenschuh, S., et al. (2012). HSP70 Enhances Immunosuppressive Function of CD4+CD25+FoxP3+ T Regulatory Cells and Cytotoxicity in CD4+CD25- T Cells. *PLoS One* 7. doi:10.1371/journal.pone.0051747.

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol. Rev.* 77, 591–625. doi:10.1152/physrev.1997.77.3.591.

Weyts, F. A. A., Cohen, N., Flik, G., and Verburg-van Kemenade, B. M. L. (1999). Interactions between the immune system and the hypothalamo-pituitary-interrenal axis in fish. *Fish Shellfish Immunol.* 9, 1–20. doi:10.1006/fsim.1998.0170.

Weyts, F. A. A., Flik, G., Rombout, J. H. W. M., and Verburg-Van Kemenade, B. M. L. (1998). Cortisol induces apoptosis in activated B cells, not in other lymphoid cells of the common carp, Cyprinus carpio L. *Dev. Comp. Immunol.* 22, 551–562. doi:10.1016/S0145-305X(98)00033-0.

White, A., and Fletcher, T. C. (1985). The influence of hormones and inflammatory agents on c-

reactive protein, cortisol and alanine aminotransferase in the plaice (Pleuronectes platessa L.). *Comp. Biochem. Physiol. Part C, Comp.* 80, 99–104. doi:10.1016/0742-8413(85)90138-0.

Williams IV, R., Neubarth, N., and Hale, M. E. (2013). The function of fin rays as proprioceptive sensors in fish. *Nat. Commun.*, 1–6. doi:10.1038/ncomms2751.

Winberg, S., and Nilsson, G. E. (1993). Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. *Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol.* 106, 597–614. doi:10.1016/0742-8413(93)90216-8.

Winberg, S., Nilsson, G. E., and Olsén, K. H. (1992). Changes in brain serotonergic activity during hierarchic behavior in Arctic charr (Salvelinus alpinus L.) are socially induced. *J. Comp. Physiol. A* 170, 93–99. doi:10.1007/BF00190404.

Wojtaszek, J., Dziewulska-Szwajkowska, D., Lozińska-Gabska, M., Adamowicz, A., and Dugaj, A. (2002). Hematological effects of high dose of cortisol on the carp (Cyprinus carpio L.): Cortisol effect on the carp blood. *Gen. Comp. Endocrinol.* 125, 176–183. doi:10.1006/gcen.2001.7725.

World Health Orgnization (1993). Biomarkers and risk assessment: concepts and principles. Environmental Health Criteria 155. 82. Available at: http://www.inchem.org/documents/ehc/ehc/ehc155.htm#SectionNumber:1.1.

Wulff, S., Vandergaw, A., Lopez, P., and Storie, I. (2006). *Dako Flow Cytometry Educational Guide 2nd Edition Guide to Flow Cytometry*. 2nd ed. , ed. S. Wulff Carpinteria, California, USA: Dako Cytomation.

Yada, T. (2007). Growth hormone and fish immune system. *Gen. Comp. Endocrinol.* 152, 353–358. doi:10.1016/j.ygcen.2007.01.045.

Zeisel, A., Hochgerner, H., Lönnerberg, P., Johnsson, A., Memic, F., van der Zwan, J., et al. (2018). Molecular Architecture of the Mouse Nervous System. *Cell* 174, 999-1014.e22. doi:10.1016/j.cell.2018.06.021.

Statement of contributions

I hereby declare that my share of the publications summarized in this thesis is as follows:

Publication I

Time-Dependent Effects of Acute Handling on the Brain Monoamine System of the Salmonid *C. maraena*.
Martorell-Ribera, J., Venuto, M. T., Otten, W., Brunner, R. M., Goldammer, T., Rebl, A., & Gimsa, U.
Frontiers in Neuroscience (2020), 14.

- 90% Planning and execution of handling stress experiments
- 90% Fish rearing and sampling
- 80% Molecular and genetic analysis
- 80% Statistical evaluation of the data
- 90% Discussion and interpretation of the results
- 100% Writing of the manuscript, tables, figures and literature search

Publication II

Early response of salmonid head-kidney cells to stress hormones and toll-like receptor ligands.
Martorell-Ribera, J. & Nipkow, M., Viergutz, T., Brunner, R. M., Bochert, R., Koll, R., Goldammer, T., Gimsa, U. & Rebl, A.
Fish and Shellfish Immunology (2020), 98, 950–961.

- 75% Fish rearing and sampling
- 80% Planning and execution of *in vitro* experiments
- 90% Sample preparation for cell sorting and gene expression analysis
- 90% Statistical evaluation of the data
- 75% Discussion and interpretation of the results
- 75% Writing of the manuscript, tables, figures and literature search

Publication III

Characterization of Sialic Acid-Binding Immunoglobulin-Type Lectins in Fish Reveals Teleost-Specific Structures and Expression Patterns.
Bornhöfft, K. F. & **Martorell-Ribera, J**., Viergutz, T., Venuto, M. T., Gimsa, U., Galuska, S. P., & Rebl, A.
Cells (2020), 9(4), 836.

- 90% Fish rearing and sampling
- 90% Planning and execution of handling stress experiments
- 80% Sample preparation for cell sorting and gene expression analysis
- 75% Statistical evaluation of the data
- 50% Discussion and interpretation of the results
- 50% Writing of the manuscript, tables, figures and literature search

Publication IV

Gene Profiling in the Adipose Fin of Salmonid Fishes Supports Its Function as a Flow Sensor.
Koll, R., **Martorell-Ribera, J.**, Brunner, R. M., Rebl, A., & Goldammer, T.
Genes (2020), 11(1), 21.

- 80% Fish rearing and sampling
- 50% Discussion and interpretation of the results
- 50% Review and correction of the manuscript

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Publications and conferences

Peer-reviewed works (*part of the doctoral thesis)

1. ***Martorell-Ribera, J.**, Venuto, M. T., Otten, W., Brunner, R. M., Goldammer, T., Rebl, A., & Gimsa, U. (2020). Time-Dependent Effects of Acute Handling on the Brain Monoamine System of the Salmonid *Coregonus maraena*. Frontiers in neuroscience, 14. https://doi.org/10.3389/fnins.2020.591738

2. ***Martorell-Ribera, J**. & Nipkow, M., Viergutz, T., Brunner, R. M., Bochert, R., Koll, R., Goldammer, T., Gimsa, U., & Rebl, A. (2020). Early response of salmonid head-kidney cells to stress hormones and toll-like receptor ligands. Fish Shellfish Immunol. 98, 950–961. https://doi.org/10.1016/j.fsi.2019.11.058

3. *Bornhöfft, K. F. & **Martorell-Ribera, J.**, Viergutz, T., Venuto, M. T., Gimsa, U., Galuska, S. P., & Rebl, A. (2020). Characterization of Sialic Acid-Binding Immunoglobulin-Type Lectins in Fish Reveals Teleost-Specific Structures and Expression Patterns. Cells, 9(4), 836. https://doi.org/10.3390/cells9040836

4. *Koll, R., **Martorell-Ribera, J.**, Brunner, R. M., Rebl, A., & Goldammer, T. (2020). Gene Profiling in the Adipose Fin of Salmonid Fishes Supports Its Function as a Flow Sensor. Genes, 11(1), 21. https://doi.org/10.3390/genes11010021

5. Venuto, M. T., **Martorell-Ribera, J.**, Bochert, R., Harduin-Lepers, A., Rebl, A., & Galuska, S. P. (2020). Characterization of the Polysialylation Status in Ovaries of the Salmonid Fish *Coregonus maraena* and the Percid Fish Sander lucioperca. Cells, 9(11), 2391. https://doi.org/10.3390/cells9112391

6. Venuto, M.T., Decloquement, M., **Martorell-Ribera, J.**, Noel, M., Rebl, A., Cogez, V., Petit, D., Galuska, S.P., & Harduin-Lepers, A. (2020). Vertebrate Alpha2, 8-Sialyltransferases (ST8Sia): A Teleost Perspective. International journal of molecular sciences, 21(2), 513. https://doi.org/10.3390/ijms21020513

7. Frenzilli, G., **Martorell-Ribera, J.**, Bernardeschi, M., Scarcelli, V., Bergman, E. J., Diano, N., Moggio, M., Guidi, P., Sturve, J., & Asker, N. (2021). Bisphenol A and Bisphenol S Induce Endocrine and Chromosomal Alterations in Brown Trout. Frontiers in Endocrinology, 12, 161. https://doi.org/10.3389/fendo.2021.645519

Conference Articles

1. **Martorell-Ribera, J.**, Nipkow, M., Viergutz, T., Goldammer, T., Gimsa, U., & Rebl, A. (2019). Stress hormones modulate early immune activities in the head kidney of *Coregonus maraena.* Fish & Shellfish Immunology, 91, 425-426.
https://doi.org/10.1016/j.fsi.2019.04.166

Microarray data in public databases

NCBI Gene expression omnibus (GEO), accession number GSE183125. Microarray-predicted marker genes indicating handling stress in maraena whitefish (Coregonus maraena). Martorell Ribera J, Koczan D, Tindara Venuto M, Viergutz T, Brunner RM, Goldammer T, Gimsa U, Rebl A (2021)

Articles in preparation

1. **Martorell-Ribera, J.**, Koczan, D., Venuto, M. T., Verleih, M., Viergutz, T., Brunner, R., Goldammer, T., Gimsa, U. & Rebl, A. Head Kidney Gene Regulation Has a Late Response After a Single Episode of Handling and Impairs Myeloid Phagocytic Capacities in the Salmonid *Coregonus maraena.*

2. Koll, R., **Martorell-Ribera, J.**, Otten, W., Gimsa, U., Brunner, R. M., Rebl, A., & Goldammer, T. Adipose fin clipping as negative influence for aquaculture and stocking programs: Multiparametric assessment of stress response in salmonid fish.

3. Magray, A. R., **Martorell-Ribera, J.**, Isernhagen, Lisa., Galuska, S.,Günther, J., Verleih, M., Viergutz, T., Brunner, R. M., Ganai, B. A., Ahmad, F., Zlatina, K. & Rebl, A. Evaluation of the blood cell viability rate, gene expression, and *O*-GlcNAcylation profiles as indicative signatures for fungal stimulation of salmonid cell models.

4. Gebauer, T., **Martorell-Ribera, J.**, Verleih, M., Plinski, C., Brunner, R., Stejskal, V., Korytář, T., Rebl, A. Development of *in vitro* model for studies of inflammatory pathways in European perch, *Perca fluviatilis*.

Contributions to international conferences

1. **Martorell-Ribera**, J., Nipkow, M., Viergutz, T., Goldammer, T., Brunner, R. M., Gimsa, U., & Rebl, A. (2018). Characterization of the stress-modulated immune response in head-kidney leukocytes of *Coregonus maraena*. **Fish immunology workshop**, Wageningen, Netherlands (**Poster & Presentation**)

2. **Martorell-Ribera, J.**, Nipkow, M., Viergutz, T., Goldammer, T., Gimsa, U., & Rebl, A. (2019). Stress hormones modulate early immune activities in the head kidney of *Coregonus maraena*. **3rd Conference of the International Society of Fish & Shellfish Immunology** (2019), Las Palmas de Gran Canaria, Spain (**Presentation**)

3. **Martorell-Ribera, J.**, Nipkow, M., Viergutz, T., Goldammer, T., Gimsa, U., & Rebl, A. (2019). Stress-related neuro-immune modulation in *Coregonus maraena*. An *in vitro* and *in vivo* approach.
Aquaculture Europe 2019, Berlin, Germany (**Presentation**)

In-house presentations

1. **Martorell-Ribera, J**., Goldammer, T., Verleih, M., Brunner, R. M., Viergutz, T., Tuchscherer, A., Gimsa, U., & Rebl, A. (2018). Neuroimmunomodulation by handling stress in maraena whitefish. **Day of the Doctoral Student (Beginners)**, Dummerstorf, Germany (**Presentation**)

2. **Martorell-Ribera, J**., Venuto, M.T., Plinski, C., Viergutz, T., Otten, W., Tuchscherer, A., Goldammer, T., Gimsa, U., & Rebl, A. (2019). Neuro-immune modulation by handling stress in maraena whitefish. **Day of the Doctoral Student (Advanced)**, Dummerstorf, Germany (**Presentation**)

3. **Martorell-Ribera, J**., Nipkow, M., Viergutz, T., Goldammer, T., Gimsa, U., & Rebl, A. (2019). Stress hormones modulate early immune activities in the head kidney of *Coregonus maraena*. **PhD Seminar**, Dummerstorf, Germany (**Presentation)**

4. Rebl, A., Galuska, S.P., Verleih, M., Brunner, R. M., Viergutz, T., **Martorell-Ribera, J**., Sarais, F., Schäfer, N., Bornhöfft, K. F., Venuto, M.T., Nipkow, M., Tuchscherer, A., Gimsa, U., & Goldammer, T. Immune inhibitors in salmonid fish-poorly explored parameters contributing to vaccination success and immunocompetence. **Leibniz association evaluation (2019)**, Dummerstorf, Germany (**Poster & Presentation**)

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Declaration of authorship

I hereby certify that I have written this thesis independently and without outside help, that I have not used any aids or sources other than those indicated by me, and that I have marked the content and literal passages taken from the works used as such.

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Selbständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

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Curriculum vitae

July 2017 - June 2021	**PhD studies** *Institute of Genome Biology & Institute of Behavioral Physiology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany* Project: Study of the stress-modulated neuro-immune crosstalk in *Coregonus maraena* Supervisors: Prof. Dr. Ulrike Gimsa, Dr. Alex Rebl
February 2020 - October 2020	**Project coordination** *Institute of Genome Biology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany* Project: Characterization of the sensory functions of the adipose fin in salmonids Supervisor: Prof. Dr. Tom Goldammer
October 2018	**FELASA (A, B, C)** *Autonomous University of Barcelona, Cerdanyola del Valles, Spain* Training course in Laboratory Animal Science for scientists responsible for the design or conduct of animal experiments.
April 2018	**Fish immunology workshop** *Wageningen Institute of Animal Sciences, University of Wageningen, Wageningen, Netherlands*
November 2016 - June 2017	**Project assistant** *Institute for Hydrobiology and Fisheries Science, University of Hamburg, Hamburg, Germany* Project: Thermal Impacts on the Ontogeny of Routine Swimming and Foraging Behavior in Atlantic Herring Larvae Supervisor: Prof. Dr. Myron Peck, Dr. Marta Moyano
September 2013 - June 2015	**Master of Science in Biology** *Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg, Sweden* Master project: Endocrine disrupting effects of bisphenol A, bisphenol S and benzyl butyl-phthalate on the thyroid system of juvenile brown trout (*Salmo trutta*) Supervidor: Dr. Elisabeth Jönsson Bergman
February 2011- Mayo 2011	**Internship - Genetic Ichthyology Laboratory** *Science faculty, University of Girona, Girona, Spain* Supervisor: Dr. Jordi Viñas
September 2006 - January 2013	**Biology Degree** *Science Faculty, University of Girona, Girona, Spain*

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