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Characterisation of Potentially Adverse Rearing Conditions and Identification of Reliable Indicators for Monitoring the Health Status in Intensively Farmed Pikeperch

Thesis of Cumulative Dissertation

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“Über den Wolken muss die Freiheit wohl grenzenlos sein…”

Reinhard Mey

To my mother and my brother

Contents

List of figures & tables

Abbreviations

°C	degree Celsius
%	percentage
AQUAEXCEL^{2020}	Aquaculture Infrastructures for Excellence in European fish research towards 2020
Bio-FISH M-V	Biologisches Funktionales & Immunologisches Screening zur Charakterisierung regionaler selektierter Nutzfischarten in M-V
BSC	brain-sympathetic-chromaffin
CABI	Centre for Agriculture and Bioscience International
cm	centimetre
D	dark phase
DD	degree days
DNA	deoxyribonucleic acid
DO	dissolved oxygen
dpf	days post fertilisation
dph	days post hatch
e.g.	exempli gratia (lat.), for example
ELISA	enzyme-linked immunosorbent assay
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FBN	Forschungsinstitut für Nutztierbiologie/Research Institute for Farm Animal Biology
g	gram
h	hours
HK	head kidney
HPI	hypothalamic-pituitary-interrenal
HSI	hepato-somatic index

HSP	heat shock proteins
i.e.	id est (lat.), which is
kg	kilogram
l	litre
L	light phase
L.	Linnaeus
mg	milligram
mmol	millimole
mRNA	messenger ribonucleic acid
Mya	million years ago
n.a.	not applicable
NCBI	National Center for Biotechnology Information
ng	nanogram
nmol	nanomole
PCR	polymerase chain reaction
ppt	parts per thousand
RT-qPCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
SSI	spleno-somatic index
t	tonnes
TNA	Transnational Access
UV	ultraviolet

Summary

The pikeperch (*Sander lucioperca* L., 1758) is a highly valued food fish with increasing economic importance for the desired diversification of the local European market. It is considered an adequate candidate for intensive rearing in land-based closed recirculation systems. However, increased losses, especially during the early ontogenesis and rearing phase, are a persistent problem in this regard. This doctoral thesis investigates the effects of current husbandry conditions as well as potential stressors on the health status of pikeperch in intensive aquaculture. Potentially suitable genes for future monitoring of physiological processes and possible disturbance during the early developmental phase of the pikeperch could be characterised. The genes involved are important e.g. for lipid metabolism, organogenesis/morphogenesis and the immune system. Furthermore, non-transcript-based (i.e. plasma cortisol, glucose and lactate levels in blood, composition of leucocytes in peripheral blood and important immune organs as well as organosomatic indices) and transcript-based indicators were used to characterise a possible stress response in juvenile pikeperch by increasing temperatures (15°C - 25°C; 1°C per day) as well as reduced oxygen levels (40 % DO/± 3.2 mg/l DO for 28 days; additional immunostimulation with inactivated *Aeromonas hydrophila* on day 8). It was shown that these conditions do not represent a pronounced stress situation for the fish. By means of gene expression analyses, potential indicators for the detection of an incipient response process could be identified, with a correlating *SERPINH1* expression in liver with increasing water temperatures and a significantly reduced expression of *HIF1A* detectable in the head kidney after oxygen reduction. Overall, the present work provides indications that each gene set used should be optimally adapted to the conditions to be investigated as well as to the tissues sampled. Thus, a close examination of certain husbandry parameters using non-transcript-based together with transcript-based indicators is advisable in order to identify potential stressors during intensive pikeperch farming. *RPL32* and *RPS5* could be verified as suitable reference genes for single as well as multiplex RT-qPCR analyses. In summary, the pikeperch appears to be significantly more robust to the investigated challenging conditions than previously assumed. The results contribute to the understanding of its stress physiology and aim to identify and counteract potential stressors to overcome obstacles during the breeding process.

Zusammenfassung

Der Zander (*Sander lucioperca* L., 1758) ist ein hoch geschätzter Speisefisch mit zunehmender wirtschaftlicher Bedeutung für die angestrebte Diversifizierung des lokalen europäischen Marktes. Er gilt als geeigneter Kandidat für die intensive Aufzucht in geschlossenen Kreislaufsystemen an Land. Erhöhte Verluste, vor allem in der frühen Ontogenese- und Aufzuchtphase, sind dabei jedoch ein anhaltendes Problem. In dieser Dissertation werden die Auswirkungen aktueller Haltungsbedingungen sowie potenzieller Stressoren auf den Gesundheitszustand des Zanders in intensiver Aquakultur untersucht. Für die zukünftige Überwachung physiologischer Prozesse und möglicher Störungen während der frühen Entwicklungsphase des Zanders konnte ein potentielles Gen-Set charakterisiert werden. Dieses enthält unter anderem Gene, welche für den Lipid-Metabolismus, die Organo- und Morphogenese sowie das Immunsystem eine wichtige Rolle spielen. Darüber hinaus wurden sowohl nicht-transkriptbasierte (i.e. Plasmacortisol, Glukose- und Laktatspiegel im Blut, Zusammensetzung der Leukozyten im peripheren Blut und wichtigen Immunorgane sowie organosomatische Indizes) als auch transkriptbasierte Indikatoren verwendet, um eine mögliche Stressreaktion beim juvenilen Zander durch steigende Temperaturen (15°C - 25°C; 1°C pro Tag) sowie reduzierte Sauerstoffwerte (40 % DO/± 3.2 mg/l DO für 28 Tage; zusätzliche Immunstimulation mit inaktiviertem *Aeromonas hydrophila* an Tag 8). Es konnte gezeigt werden, dass diese Bedingungen keine ausgeprägte Stresssituation für die Fische darstellen. Mittels Genexpressionsanalysen konnten potentielle Indikatoren für den Nachweis eines beginnenden Reaktionsprozesses identifiziert werden, wobei eine mit steigenden Wassertemperaturen korrelierende *SERPINH1*-Expression in der Leber und eine signifikant reduzierte Expression von *HIF1A* in der Kopfniere nach Sauerstoffreduktion nachweisbar waren. Insgesamt liefert die vorliegende Arbeit Hinweise darauf, dass jedes verwendete Gen-Set optimal an die zu untersuchenden Bedingungen sowie an die beprobten Gewebe angepasst werden sollte. So ist, nach derzeitigem Stand, eine genaue Untersuchung bestimmter Haltungsparameter unter Verwendung nicht-transkriptbasierter sowie transkriptbasierter Indikatoren ratsam, um potenzielle Stressoren bei der intensiven Zanderzucht zu identifizieren. *RPL32* und *RPS5* konnten als geeignete Referenzgene sowohl für Einzel- als auch für Multiplex-RT-qPCR-Analysen verifiziert werden. Zusammenfassend lässt sich

sagen, dass der Zander gegenüber den untersuchten kritischen Bedingungen deutlich robuster zu sein scheint als bisher angenommen. Die Ergebnisse tragen zum Verständnis seiner Stressphysiologie bei und zielen darauf ab, potenzielle Stressoren zu identifizieren und ihnen entgegenzuwirken, um Hindernisse während des Zuchtprozesses zu überwinden.

1. General Introduction

1.1. Taxonomic Classification, Morphology, and Biology of *Sander lucioperca*

Sander lucioperca (Linnaeus 1758), commonly named “pikeperch” in English or “Zander” in German, is a teleost fish and belongs to the family of the Percidae (containing all “perches”) within the order of Perciformes (= perch-like fishes) [1]. The genus *Sander* (formerly *Stizostedion*) includes five species, with *S. lucioperca*, *S. volgensis* and *S. marinus* distributed in Eurasia and *S. canadensis* together with *S. vitreus* found in North America [1–4]. *S. lucioperca* is a rather young fish species. Its diversion into these two clades took place around 21 million years ago (Mya) and the contemporary genotypes of *S. lucioperca* differentiated from each other approximately 5 Mya [5].

The morphology of pikeperch is characterised by an elongated and laterally compressed body shape with a maximum total length of 130 cm and up to 18 kg and a pointy snout comprising an upper jaw reaching past the eye level (Figure 1) [1,6]. It appears in an either greenish-grey, brown, silver-grey or gold-yellow colour depending on the strain, and further comprises a white belly, dark spotted dorsal and caudal fins and 8-10 dark stripes on the side that fade with age [6]. A few enlarged canine teeth are located in the front to catch the prey, and supplementary rows of small teeth stretch across the jaws and the palatine [7]. Typical for percids, pikeperch comprise a split dorsal fin, with spiny rays on the first fin and branched soft rays on the second fin [6]. To increase the sensitivity to low light conditions within a murky environment, the eyes include a highly reflective layer behind the retina called “tapetum lucidum” [8,9].

Figure 1: ***Sander lucioperca*** **morphology**

Morphology of pikeperch reared in intensive aquaculture system. **a** Three-months-old juvenile with 13.3 g of weight and a total length of 10.8 cm. **b** One-year-old juvenile with 180.4 g of weight and a total length of 31.3 cm. Copyright by Dr. R. M. Brunner (FBN).

As a key top piscivore, pikeperch actively selects favourite prey types and forages preferably under low light conditions during dusk and nighttimes [10,11]. Habitats include turbid rivers, eutrophic lakes and reservoirs as well as brackish coastal waters with adults tolerating salinities of up to ~ 10 ppt in the Baltic Sea [6,12,13]. During the process of early ontogenesis, the feeding ecology transforms from initially zooplanktivory to primarily benthivory and finally to obligatory piscivory [14]. Pikeperch become sexually mature mainly within 2-6 years, depending on the geographic region, with males usually earlier than females. *S. lucioperca* inhabits a considerable geographic area which spans from Northern, Central and Eastern Europe to the Near East and North Asia (Figure 2) [15–18]. It was further introduced to areas in Western and Southern Europe, North Africa, North America and China [19–23].

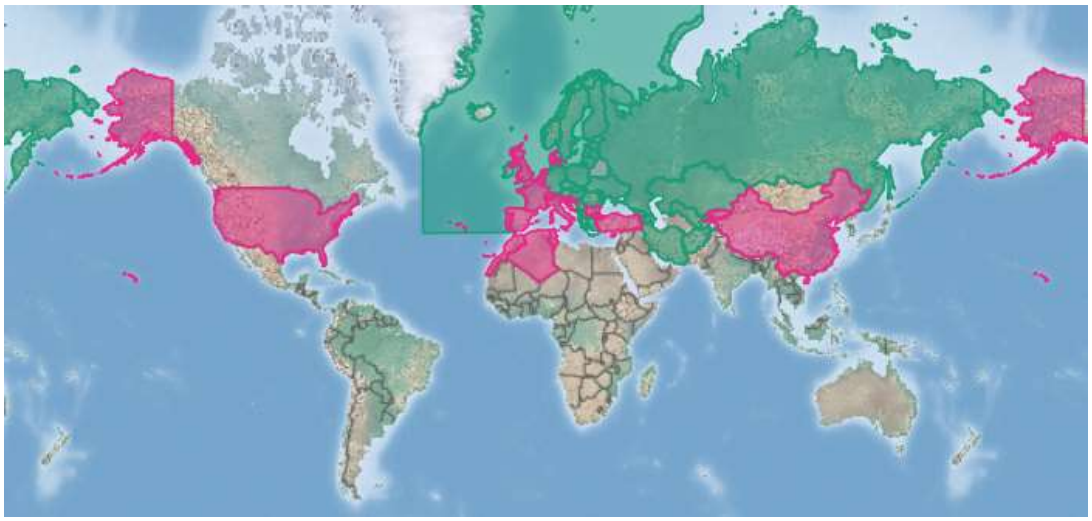

Figure 2: **Geographical distribution of *Sander lucioperca***

The distribution of pikeperch includes the Aral, Azov, Baltic, Black, Caspian, Mediterranean and the North Sea basins. Native habitats are highlighted in green, areas of additional introduction are highlighted in pink. Figure adapted from CABI – Invasive Species Compendium [24].

1.2. Pikeperch as Noble Food Fish in European Aquaculture

Our continuously growing population and a change in the consumer's behaviour towards a healthier lifestyle requests an alternative to meat as source of proteins and vitamins. A diversification of the European freshwater aquaculture has been the aim in recent years, to cover a steadily increasing demand for food fish and similarly to decrease imports to the EU. Pikeperch belongs to the noble food fish important to Europe, with an approximate inland production of 894 t, compared to ~ 3.200 t worldwide in the year 2019 [25]. In Germany a total of 212 aquaculture facilities, primarily by extensive pond systems in Bavaria, Lower Saxony, Saxony and Thuringia, produced ~ 56.6 t of pikeperch [26]. Pond aquaculture is the oldest and still the most widely used form of aquatic animal farming worldwide. Although, it is characterised by low production and investment costs, this farming method is spatially restricted, depends on environmental factors (e.g. water supply and seasonal water & light conditions) and affects its environment. Especially for temperate fish species, like pikeperch, the dependence on natural conditions restricts its production to one natural spawning event during the year [27]. In order to achieve increased production levels of high-quality finfish local to Europe, pikeperch was introduced to

intensive aquaculture in the 21st century, since stable quantities with corresponding quality cannot be served by capture fisheries [28,29].

Within the EU-supported project called “Diversify” *S. lucioperca* was selected as the only freshwater species with great potential for being farmed in the EU with focus on recirculating aquaculture systems (RAS) [30]. RAS is a highly ecological rearing system for intensively farming aquatic animals by reusing the purified production water (Figure 3). Hereby, a mechanical filter system ensures the removal of accumulated debris (e.g. faeces and feed residues) and a biological filtering unit provides a settlement area for nitrifying bacteria (converting harmful ammonia). Before re-introduction of the water into the fish holding tank, it is further enriched with oxygen, degassed (e.g. removal of CO_2) and disinfected by UV irradiation. Main advantages of RAS are the highly reduced volume of fresh water introduced into the system per day (< 35 %), the absence of natural water eutrophication due to the applied water purification, the independence of external conditions (i.e. its location), the prevention from the use of wild caught broodstock, the possibility to precisely set the conditions as well as the possibility to reuse the nutrients from the farmed fish for biogas production or to fertilise agricultural farming land [31,32].

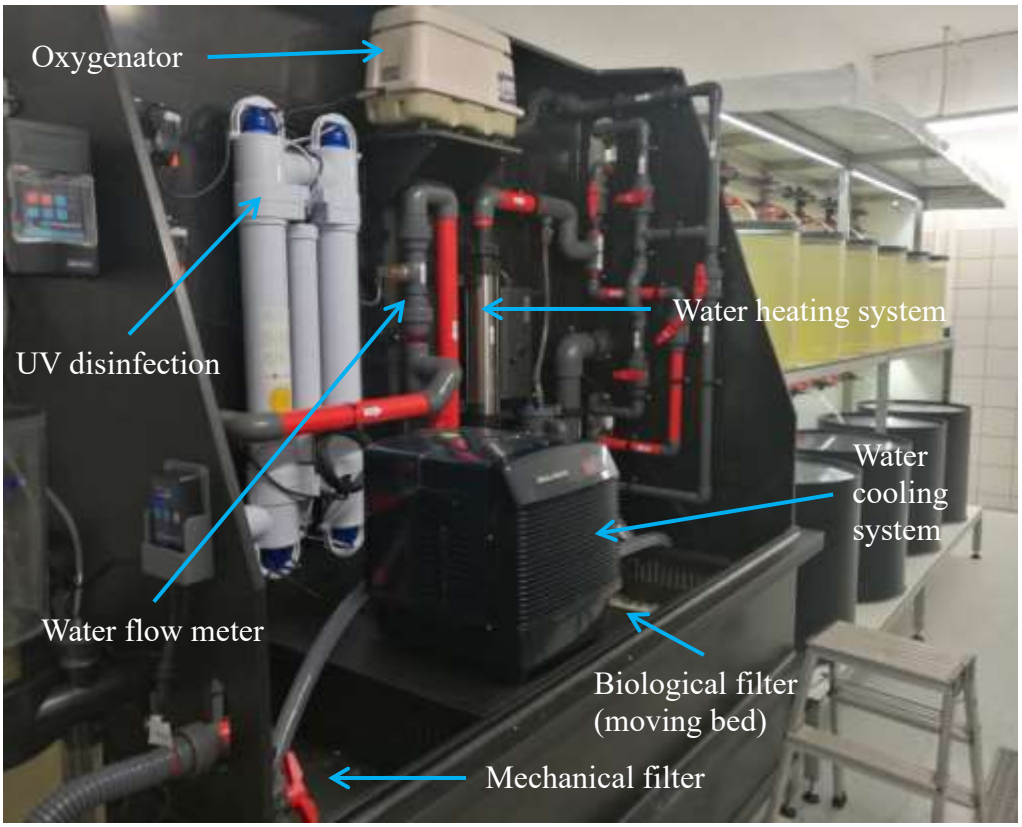

Figure 3: **Recirculating aquaculture system (RAS)-facility**

Illustration of the Experimental Animal Facility Aquaculture of the Research Institute for Farm Animal Biology (FBN, Dummerstorf, Germany) for intensive pikeperch farming. Copyright by Dr. R. M. Brunner (FBN).

Intensive pikeperch farming under conditions of RAS have been more and more applied in Northern, Western, and Central Europe (e.g. France, the Netherlands, Denmark, Finland, Czech Republic) [33]. Its high-quality meat with natural low fat content (~ 1-2 %) and small amount of bones achieves prices up to ~ 18 €/kg in German aquaculture facilities by direct marketing [26,34,35]. These prices are important to counterbalance the high investment and maintenance costs accompanied by RAS. Optimal production conditions all year round guarantee a fast growth rate to ensure the achievement of the market size in 13 to 15 months, compared to three to five years in pond aquaculture [36]. In addition, constantly high-water temperatures suppress the natural reproduction and thus prevent the use of energy other than for growth. An artificial simulation of seasons in separate brood chambers, concerning an adapted day-night light regime (~ 12L:12D–14L:10D) and lower temperatures (< 15°C), enables a year-round production [37–42]. Naturally living and hunting in groups is another advantage of pikeperch to be

farmed in enclosed aquaculture systems with a high stocking density (on-growing phase 80 kg/m^3) [6]. However, a prerequisite for successful year-round reproduction of pikeperch in RAS is the detailed knowledge of its specific requirements for an optimal rearing process, including husbandry and environmental conditions.

1.3. Challenges in the Farming Process of Pikeperch

The intensive farming of pikeperch in RAS is currently characterised by a low success rate of the production cycle. Former investigations describe pikeperch as quite vulnerable to stressors associated with an artificial environment [43,44]. Typical stressful stimuli in intensive aquaculture are either related to husbandry and management procedures (e.g. nutrition, confinement, handling, and stocking density) or environmental conditions (e.g. inadequate water quality and light regime) [45–47]. The conditions for the rearing process are still under development with high losses, particularly in the early phase of development. To counteract this situation and make pikeperch farming in European intensive aquaculture facilities profitable and successful, two crucial conditions must be met:

(i) a production of healthy and sufficient stocking material (up to the early juvenile stage)

(ii) the maintenance of optimal animal welfare and health status during the complete production cycle.

The early ontogenesis is a phase which involves many critical processes like morphological changes, acclimatisation to the new surrounding environment as well as adaptation to a new energy source. During this phase, different stages show altered responsiveness to stressors, e.g. a reduced stress resistance during hatching as well as at the beginning of exogenous feeding [48]. The diversity of the individual development among the different fish species require specific adaptation of rearing conditions to avoid possible impairments of the fish's health status. Swim bladder infiltration between 5 dph and 20 dph, conversion from live to formulated feed, called weaning, beginning at 15-19 days post hatch, and severe cannibalism between 18 dph and 39 dph are main bottlenecks of the present larval and early juvenile pikeperch farming [40,49–52]. Possible negative outcomes are malformations, impaired growth and a lowered overall survival rate (50 % after 5 weeks) [40,53–55]. It is particularly important that the

husbandry parameters are optimally set up to meet the needs of this fish species. The feeding regime as well as the quality of the feed does not only influence the nutritional status of pikeperch, but also plays an important role in the onset of cannibalism [56–60]. The optimal composition of the final artificial feed is still a major topic in research, and adaptations seem to affect the stress susceptibility of pikeperch [49,61–63]. Since the production of larval and early juvenile stages of stable quality and quantities are a major obstacle within the intensive pikeperch farming, it is important to further optimise the applied husbandry settings. Especially, fish with malformations are not well accepted on the market and will be sorted out and thus, represent a high loss for the fish farms (Figure 4). Improving the health status and well-being of pikeperch within artificial environments will result in higher survival rates and quality and thus, in a higher production efficiency.

Figure 4: **Malformation in intensively reared *S. lucioperca***

Juvenile pikeperch with prominent scoliosis and a reduced caudal fin due to cannibalism. Copyright by Dr. R. M. Brunner (FBN).

Environmental stressors, like inadequate water temperatures and oxygen saturations, have already been demonstrated to interfere with the health status of fish and to provoke a stress response [64–67]. The majority of fishes are poikilothermic organisms, with their aerobic metabolisms being highly affected by the surrounding temperature and oxygen saturation. The level of dissolved oxygen (DO) as well as the water temperature are thus major restrictive parameters in the rearing process regulating the fish's health status. Within their natural habitat, fish will avoid areas of inadequate water temperatures and oxygen saturation by simply migrating to regions with preferred water quality. Yet, in the artificial environment of intensive fish farming facilities, this opportunity is not given due to a restricted space capacity. It is of high importance, that the stress susceptibility highly depends on the particular fish

species as well as the type of stressor together with its intensity and duration [68,69]. The effects of major environmental stressors in intensive aquaculture systems need to be defined for every newly introduced species, like *Sander lucioperca*. A depletion of oxygen saturation that negatively affects essential life functions and the well-being of the fish is defined as hypoxia [64]. Possible reasons for hypoxic conditions in fish farming systems are inadequate water circulation, high stocking densities and high water temperatures [70–72]. An oxygen saturation of around 100 % DO (equivalent to ~ 7-9 mg/l DO at 20-25°C) is suggested for intensive pikeperch farming [73–75]. This concentration is equivalent to the situation when water is saturated with air and matches the range of oxygen levels in its natural habitat (5.5 mg/l to 12.9 mg/l) [76,77]. For aquaculture facilities, DO saturations below 40 % (equivalent to ~ 3-4 mg/l at 20-25°C) were classified as inadequate to rear fish in general and DO < 4 mg/l is considered critical for perciforms reared in ponds [31,78]. However, long-lasting oxygen saturations of 50 %-60 % DO already negatively affect the feed intake and growth rate as well as significantly increase the plasma-complement activity of intensively farmed pikeperch [44,79]. It therefore remains to be seen to what extent the health status of intensively farmed pikeperch is influenced by the "general" limit of 40 % DO. Temperatures above 35°C are lethal to juvenile pikeperch and define the upper limit of its tolerance range [80]. At temperatures below 15°C the growth performance of pikeperch is majorly compromised and a drop below 10°C results in a complete growth stagnation. The lower temperature limit of pikeperch lies below 1°C [81]. Typical rearing temperatures for pikeperch in RAS range between 22°C and 24°C [75,82]. The applied temperatures above 20°C are chosen to optimise the growth performance and suppress the allocation of energy to gonad maturation but not for welfare reasons [39,81]. The temperatures chosen could therefore have an effect on the well-being and health status of pikeperch farmed in intensive aquaculture facilities.

1.4. The Stress Response in Teleost Fishes

Schreck and Tort defined stress in fish as "the physiological cascade of events that occurs when the organism is attempting to resist death or re-establish homeostatic norms in the face of insult" [83]. The outcome, i.e. the magnitude and duration of the initiated stress response, depends on the type, intensity, and period of the perceived stressor [69]. While mild and acute stress stimuli usually result in a full

recovery of the fish, very severe acute and non-severe repeated or chronic stressors either lead to compensation mechanisms or even to final exhaustion and thus, to death [84,85]. The response of fish to challenges are defined as primary, secondary, and tertiary [45].

The head kidney (HK) is the central organ in fish controlling the primary response process based on a neuroendocrine adaptation. It unites the endocrine functionality of the adrenal gland and the haematopoietic-lymphoid functionality of the bone marrow of mammals. Three different sets of cell types comprise catecholamine-producing chromaffin cells, cortisol-producing interrenal cells and surrounding haematopoietic tissue in which immune cells secrete cytokines and antibodies [86]. This structure enables a close communication between the three regulatory systems, i.e. neural, endocrine and immune, by integrating the stress response via the brain-sympathetic-chromaffin (BSC) and hypothalamic-pituitary-interrenal (HPI) axis (Figure 5). The BSC is activated first and results in the release of catecholamines, like adrenaline, within seconds to minutes. The stimulated HPI initiates the release of cortisol, the main corticosteroid in fishes, after minutes [87,88].

The secondary stress reaction comprises the effects of the released hormones [68]. The glycogenolytic adrenaline quickly provides energy for the action of an initiated stress response and increases the oxygen supply to tissues by the initiation of cardio-respiratory actions [89–91]. Cortisol subsequently ensures a stable supply of metabolic energy throughout minutes and hours for target tissues to cope with the stressor and restore pre-stress conditions. Cortisol further influences the osmoregulation to restore the hydromineral balance [92]. The released endocrines of the stress response additionally influence the immunity. During the activation phase of an acute stress stimulus, an innate immune response is initiated, mainly by catecholamines but partly by cortisol. This includes the production of acute phase proteins, heat-shock proteins (HSPs) as well as the release of certain cytokines and a rise in numbers of circulating leukocytes, in particular neutrophils [69,88]. If the stress response continues and cortisol levels stay high, the animal does not fully recover and suffer from persistent disturbance of its prestress condition. Hereby, highly energy-consuming processes like the proliferation and migration of lymphocytes, the cytokine release and the production of antibodies can be transiently inactivated or

delayed. A chronic stressor therefore potentially leads to a suppression of the immune defence and thus compromising the resistance to pathogens [69].

The tertiary response to stressor perception includes all organs and modifies the whole performance of the organism [45,69]. A persistent allocation of energy and metabolites towards a chronically activated stress pathway finally affects essential life functions like growth and reproduction but also health and disease resistance [69,93,94].

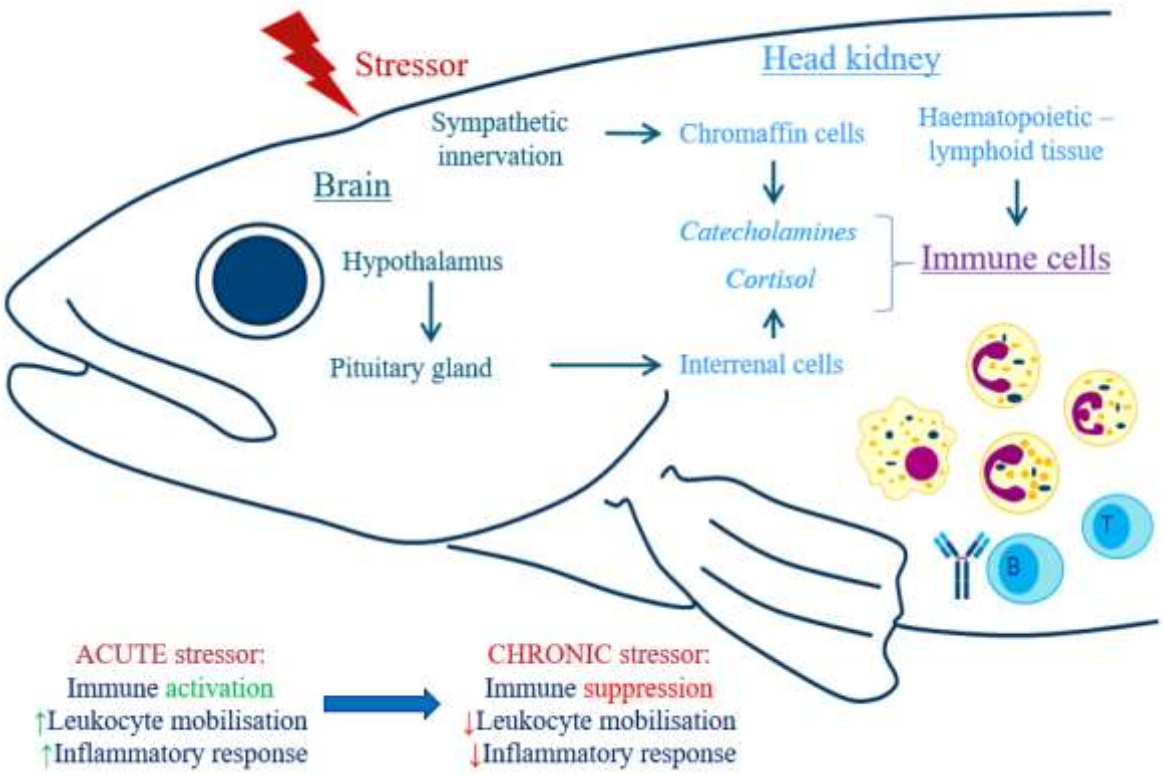

Figure 5: **Stress response pathway in teleost fishes**

Schematic drawing of the response pathway of teleost fishes after stressor perception, with head kidney as central organ. Figure modified from Khansari et al. 2017 & Tort 2011 [69,95].

The pikeperch is considered as fish species with enhanced susceptibility to stressors commonly found in the artificial environment of aquaculture facilities [43,44]. However, detailed information about its stress physiology, especially regarding its early phase of development and specific stress stimuli like rising water temperatures and extensively lowered oxygen saturation, is still missing.

1.5.How to Measure a Prevailing Stress

Breeding and farming of pikeperch in intensive aquaculture is still very much characterised by optimisation of the rearing conditions. In order to determine whether husbandry conditions are optimal or need to be adjusted, it is important to precisely and promptly detect and characterise a possible induced stress response.

Commonly investigated non-transcript-based indicators for stressful situations in farmed fish include the quantification of the primary stress response (by measuring the stress response itself, i.e. stress hormones) or by quantification of secondary and tertiary stress responses (by measuring physiological changes). Plasma cortisol is the major stress hormone and an established bioindicator for stress stimuli in fish [87]. After stress perception, blood glucose levels are elevated by hepatic gluconeogenesis and mobilizing skeletal glycogen stores, mainly due to cortisol [96,97]. Further, circulating lactate is increased during the stress response, which is an energy source for anaerobic metabolism, e.g. in muscle tissue [98]. The increase of both metabolic parameters has been linked to stress in many fish studies [99]. When evaluating the results obtained, it needs to be considered that, blood parameters can be influenced by physiological and environmental factors. The baseline level of cortisol, for example, is dependent on the sex (specific for fish species), developmental stage, food intake, social status of the animals being examined as well as diurnal or seasonal fluctuations and water temperature [100–103]. Glucose and lactate levels are affected by metabolic processes and thus, can vary due to the applied feed and feeding regime [104,105]. It is therefore, important that results are carefully interpreted, especially if prolonged treatment of potentially stressful conditions are under examination. Stressors can affect the function or magnitude of the immune response. To evaluate the effect of stress stimuli on the immunity of a fish, the proportion of myeloid to lymphoid cells in circulating leukocytes is an additional well accepted indicator in fish. Changes within the immune cell proportion in peripheral blood or immune organs are detectable for a much longer time span, which can be advantageous, especially, when measuring persistent environmental stressors [99]. The challenge in interpreting the composition of leukocytes is that they can be altered by stress but also by infection. The evaluation is thus, especially challenging in multi-stressor approaches [106]. Organosomatic indices are accepted as general

indicators for the health status of an animal, with spleno-somatic index (SSI) related to the immune status and hepato-somatic index (HSI) representing the energy status [107,108]. Changes within these values are commonly referred to a redistribution of energy resources within the animal towards ongoing processes initiated by a stress response [109]. Organosomatic indices have the advantage to be able to detect chronic stress [110]. It should be noted that these indices, however, can be influenced by, for example, gender and season [111–113].

The present doctoral thesis partly deals with specific changes within the husbandry conditions of juvenile pikeperch in intensive aquaculture. In this context, non-transcript-based indicators were evaluated to characterise a possibly initiated stress response. Cortisol was measured as indicator for a primary stress response either in whole blood samples via ELISA assay (**Study II**) or by liquid chromatography/mass spectrometry in whole animals (**Study III**). Secondary stress indicators were further examined, with blood glucose and lactate levels determined by reflectance photometric measurement and immune cell proportions in peripheral blood and relevant immune organs identified by flow cytometry (**Study II**). SSI and HSI were additionally evaluated as indicators for a probable tertiary stress response in juvenile pikeperch (**Study II**). These indices are calculated by dividing the weight of the corresponding organ by the total body weight of the fish, and subsequently multiply the result by 100.

With the establishment of intensive rearing facilities, the application of molecular DNA-based methods, e.g. PCR and sequencing techniques, gained more and more importance. They provide a significant opportunity for routine examinations in intensive aquaculture facilities to increase production efficiency and improve product quality as well as animal health [114]. One important area of application for PCR techniques is the identification of aquaculture-specific stressful conditions, e.g. inadequate water qualities, handling, and confinement stress. Hereby, expression analysis of particular key genes helps to comprehend physiological processes which are involved in stress- and/or immune-response pathways of the reared fish species and thus, supports the improvement of animal welfare [115,116].

A main part of this dissertation was the characterisation of a selected set of candidate genes to determine the welfare status of various developmental stages of pikeperch during prevailing or potentially adverse

farming conditions. Generally applied non-transcript-based indicators show partially limited capacities to accurately reflect the animal's welfare or health status of farmed fishes as discussed above [99,117]. An established gene panel gives the possibility to further standardise an assay for a species-specific and highly sensitive detection of impaired well-being in intensively farmed pikeperch. Examined candidate genes were selected based on literature relevant to the topic, previously conducted studies on maraena whitefish, rainbow trout and Atlantic salmon at the Fish Genetics Unit of the FBN as well as their distinctive function during the conditions under study [118–126]. In total, 70 genes belonging to eight categories were examined within the three included studies (Table 1). Quantitative assays were applied to analyse the specific expression of candidate genes under the different husbandry conditions examined. Transcript levels were characterised either in whole early ontogenetic stages or certain organs representative for the expected stress response in juvenile pikeperch by single (**Study I**, **Study II** and specific genes of **Study III**) or multiplex RT-qPCR assay (**Study III**). The identified transcript patterns will allow a first characterisation of a possible functional correlation between the expression of specific candidate genes and the stress susceptibility of the pikeperch during the tested farming environment.

1.6. Working Hypothesis and Objectives

The present work is part of the Campus bioFISCH-MV project and was funded by the European Maritime and Fisheries Fund (EMFF) together with the Ministry of Agriculture and the Environment in Mecklenburg-Western Pomerania (Grant #: MV-II.1-RM-001), by the Union's Horizon 2020 research and innovation program under grant agreement No 65281 (AQUAEXCEL^{2020}; TNA project ID number: AE080004) as well as the Ministry of Education, Youth and Sports of the Czech Republic – project: Sustainable production of healthy fish in various aquaculture systems; PROFISH (CZ.02.1.01/0.0/16_019/0000869).

Pikeperch is a promising candidate for the diversification of the European aquaculture, in which local finfish species are applied for the aim of sustainability. This thesis addresses the stress vulnerability of the local fish species *Sander lucioperca* under conditions of intensive aquaculture. Our central hypothesis is:

Pikeperch show an increased susceptibility to husbandry and environmental stressors during the rearing process due to a rather low adaptation while recently introduced to intensive aquaculture systems. This increased susceptibility can be detected on the molecular level.

The studies presented as part of the doctoral dissertation address various rearing conditions in different developmental stages of the pikeperch. The main subject of this dissertation is the characterisation of presumably adverse rearing conditions. Together with the evaluation of the applicability of molecular genetic indicators for future monitoring of the response to typical aquaculture-related stressors in intensively farmed pikeperch (Figure 6). For this achievement, the transcript patterns of genes involved in specific developmental processes as well as stress and immune response pathways were investigated. Moreover, selected physiological and molecular health parameters were investigated to further describe the stress physiology of the pikeperch. In particular, the two following main objectives were addressed in the three publications summarised in this dissertation:

1) Characterisation of basal expression patterns of genes important during critical processes of the early ontogenesis and early juvenile growth phase of pikeperch within the artificial environment of intensive aquaculture (**Study I**)

2) Evaluation of modifications in tissue-specific expression of stress- and immune-related candidate genes together with blood parameters (**Study II** and **Study III**), organosomatic indices and immune cell mobilisation (**Study II**) in juvenile pikeperch following probable inadequate water conditions:

    - Low oxygen saturation (with additional intraperitoneal immune stimulation) (**Study II**)
    - Increasing water temperatures (**Study III**)

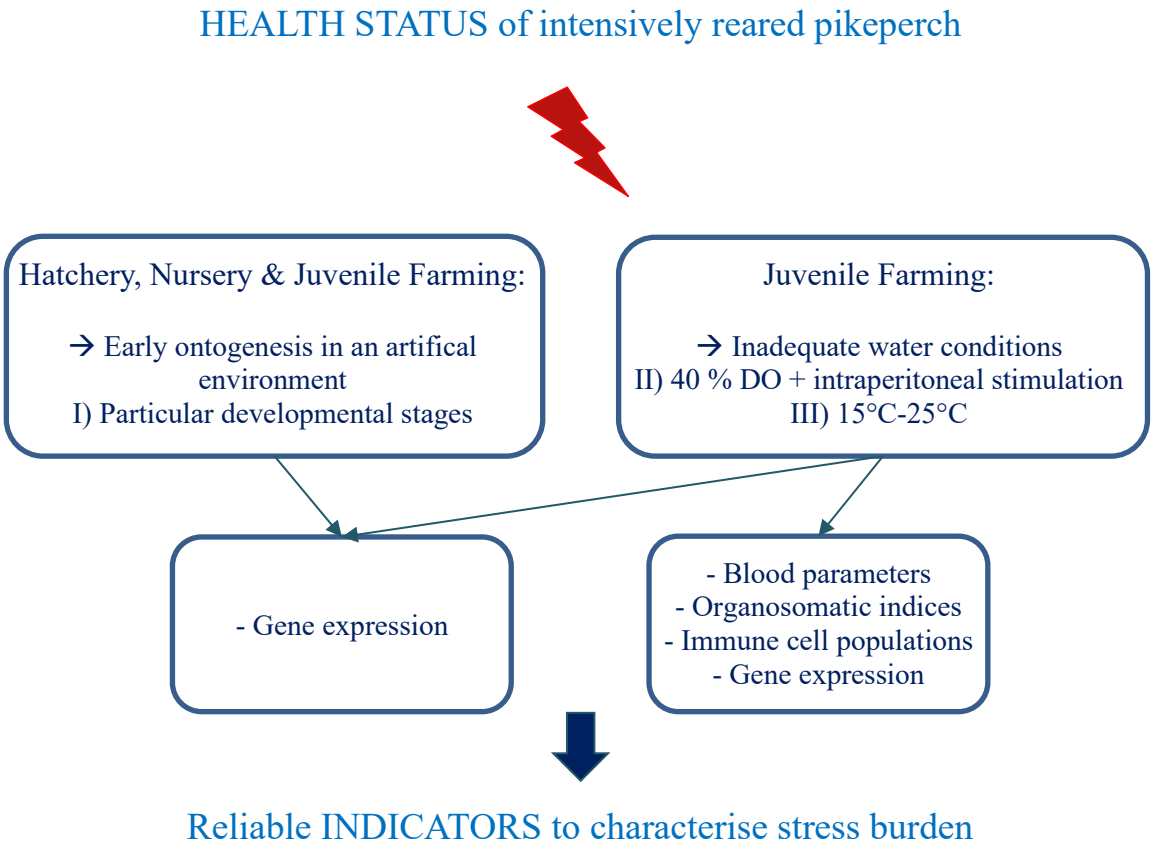

Figure 6: **Schematic summary of the intended objectives**

The current dissertation aims at the establishment of reliable indicators for assessing the health and immune status of intensively farmed pikeperch. The three studies included address the challenges of an artificial environment during early ontogenetic processes and potentially inadequate water conditions. I) Schaefer et al. 2021 *Fish Physiology & Biochemistry* II) Schaefer et al. 2021 *Biology* III) Swirplies et al. 2019 *Aquaculture*; DO = dissolved oxygen.

The studies conducted will help to elucidate the stress physiology of different pikeperch stages during intensive rearing. Understanding these mechanisms is the basis for successful pikeperch breeding under intensive farming conditions and will contribute to significantly improve the animal welfare concepts currently applied. The long-term objective of this study is to identify reliable indicators for monitoring stressful situations and situations affecting the welfare of the animals. These indicators will help to adapt the current rearing conditions, increase the health status and thus survival rate of intensively reared larval and juvenile pikeperch.

2. Summary of Included Studies

Study I:

Schäfer N, Kaya Y, Rebl H, Stüeken M, Rebl A, Nguinkal JA, Franz GP, Brunner RM, Goldammer T, Grunow B and Verleih M (2021). Insights into Early Ontogenesis: Characterization of Stress and Developmental Key Genes of Pikeperch (*Sander lucioperca*) in vivo and in vitro. *Fish Physiology and Biochemistry* 47,515-532. doi: 10.1007/s10695-021-00929-6.

Background:

Given the low survival rate of early pikeperch stages, especially during the steps of hatchery, nursery and early juvenile farming. The improvement of the animal's health status and thus the production of stocking material for intensive aquaculture purposes is of high importance. Reliable genetic indicators for the characterisation of intrinsic and extrinsic stimuli that influence the life cycle of pikeperch may help to identify and avoid possible stress burden.

Summary:

This study addresses the analysis of basal expression levels of 21 genes involved in physiological processes (i.e. nutritional status, energy and lipid metabolism, growth, cell homeostasis, maturation and organogenesis) during early development and key stress and immune genes in pikeperch bred under intensive farming conditions and reared in RAS. For this purpose, the following early ontogenetic stages were examined: eyed eggs, yolk-sac larvae, larvae fed with live prey and larvae fed with formulated feed, as well as 125 days and 175 days-old juvenile stages stated as “fingerlings”. mRNA was extracted either from whole animals (egg and larval stages) or from liver tissues of juvenile stages.

Most of the determined expression patterns could be assigned to certain developmental phases within the early ontogenesis of unchallenged pikeperch. Within eyed-eggs and larval stages, high mRNA abundance was observable for genes involved in cell homeostasis, energy metabolism, nutritional status, organogenesis and stress response. In liver tissues of juveniles, genes important for growth, immune response, nutritional status and organogenesis/lipid metabolism were highly expressed. Changes in the

expression of the genes considered here could be used in future studies to monitor possible effects on the developmental process of the pikeperch during adaptation of husbandry conditions.

Study II:

Schäfer N, Matoušek J, Rebl A, Stejskal V, Brunner RM, Goldammer T, Verleih M and Korytář T (2021). Effects of Chronic Hypoxia on the Immune Status of Pikeperch (*Sander lucioperca* Linnaeus, 1758). *Biology* 10(7), 649. doi: 10.3390/biology10070649.

Background:

Although the most critical phase of early development has already been completed, juvenile pikeperch still seem to be particularly vulnerable to stressors connected to confinement. Especially in closed systems, as is common in intensive aquaculture systems, water conditions must be precisely regulated to avoid adverse rearing conditions affecting the animal's well-being. Inadequately low oxygen saturations initiate stress response pathways in fishes, and prolonged situations may result in a compromised immune system and thus in an increased susceptibility to pathogenic infections. Therefore, it is important to investigate major environmental stressors to ensure the maintenance of animal welfare and thus, improve the health status of the intensively farmed fish.

Summary:

This publication focuses on the effects of long-term low oxygen saturations (40 % DO/± 3.2 mg/l) on the health status and stress response of juvenile pikeperch reared in RAS at ± 23°C for up to 28 days. To further examine the reactivity of the immune system during the applied challenge, fish were in addition intraperitoneally stimulated with inactivated *Aeromonas hydrophila* at day eight of the experiment. Besides the analysis of the expression of common stress- and immune genes in important metabolic and immune tissues (i.e. head kidney, liver, and spleen), blood and health parameters as well as the proportion of immune cells within examined tissues (i.e. blood, head kidney, peritoneal cavity, and spleen) were investigated.

The results of this study suggest that 40 % DO does not provoke a severe stress response or severe immunosuppression in pikeperch. Examined non-transcript-based indicators did not reveal significant changes between control fish and fish kept at low DO levels. However, transcript patterns of certain

genes suggest an ongoing adaptation response due to 40 % DO saturation. In pikeperch treated with low DO levels, the reaction of the adaptive immunity but not the innate immune response was impaired during additional acute peritoneal inflammation.

Study III:

Swirplies F, Wuertz S, Baßmann B, Orban A, **Schäfer N**, Brunner RM, Hadlich F, Goldammer T and Rebl A (2019). Identification of Molecular Stress Indicators in Pikeperch *Sander lucioperca* Correlating with Rising Water Temperatures. *Aquaculture* 501, 260-271. doi: 10.1016/j.aquaculture.2018.11.043.

Background:

Another crucial factor influencing the developmental process as well as the stress burden within the farming process is the prevailing water temperature. Since fish are ectothermic aquatic animals, long-lasting elevated and rapidly changing water temperatures can have a major impact on their well-being and can initiate stress response pathways and even lead to their death. Distinctive temperatures are applied for the individual rearing steps, with settings usually above 20°C for juvenile pikeperch to achieve the largest possible growth rate. Nevertheless, these conditions may interfere with the welfare concept within intensive aquaculture of pikeperch.

Summary:

The effect of continuously increasing water temperatures from 15°C to 25°C (with DO levels of > 4 mg/l) within eleven days on the health status of farmed juvenile pikeperch is the content of this study. It contains the expression analysis of 38 genes involved in stress, immune and metabolic pathways together with the determination of the total level of glucocorticoid hormones, an accepted parameter of the acute stress response. The findings indicate that no prominent acute stress response was induced. No significant differences were determined for whole-body cortisol at any day of the experiment. However, a rather gradual increase in mRNA transcripts of certain candidate genes seem to indicate that temperatures of 22°C or lower might be preferable for intensive pikeperch rearing.

3. General Discussion

The pikeperch is thought to be particularly susceptible to typical stress factors in intensive aquaculture due to its relatively recent intensive-farming history. Since pikeperch has a great potential for the diversification of European aquaculture, this thesis focussed on its stress susceptibility and the accompanying challenges in intensive farming systems. It aimed to contribute to fish welfare by identifying and characterising potentially stressful and health-compromising situations to subsequently establish reliable indicators for a continuous monitoring of its actual health status. The dissertation is divided into three main parts to successfully achieve these aims: (i) to assess basal expression patterns of developmentally relevant genes during intensive pikeperch rearing in various developmental stages (**Study I**); (ii) to characterise the molecular and physiological stress response of juvenile pikeperch when exposed to hypoxic and thermal challenge (**Study II** and **Study III**); and (iii) to identify possible candidate genes as reliable indicators for challenging and adverse situations in intensive pikeperch aquaculture (**Study I, Study II** and **Study III**).

3.1. Comparability of the Included Studies

Due to the different questions and objectives of the individual included studies, the comparability of the studies with regard to their results is not uniform.

For all three studies (**Study I, Study II** and **Study III**), promising reference genes could be identified for the gene expression studies conducted here as well as for future studies with regard to possible inadequate husbandry conditions (see chapter 3.3.2). A particular challenge was the selection of the candidate genes to be studied. In **Study I**, mainly genes that could play a direct role in physiological processes during early ontogenesis were included, except for a few specific stress genes (Table 1). In **Study II** and **Study III**, the main focus was on a possible induced stress response due to environmental factors, with **Study II** further focusing on an induced immune response. **Study II** investigated genes associated with the response pathway after oxygen deprivation. **Study III** mainly looked at genes that already showed significantly altered transcription after heat stress in other vertebrates (Table 1). In the course of this, the transcription of the gene *NR3C1* was examined as a “general stress indicator” in all

three studies. However, it became apparent that certain genes could also be suitable candidate genes for the other conditions investigated (see chapter 3.3.2). With regard to the tissues examined, in **Study I** whole eggs as well as larval stages were used for nucleic acid extraction in order to obtain sufficient starting material for a subsequent gene expression study. Within the examined juvenile fish (**Study I, Study II** and **Study III**), the liver was chosen as assessed organ, due to its important relevance in stress response pathways as main metabolic organ [127]. In **Study II**, the head kidney was also included, as an essential organ regulating the stress signalling cascade in teleost fishes [86,88]. Together with the HK, the spleen is a main lymphoid organ in teleost fish and was further included in **Study II** [128]. In addition, the composition of leukocytes in the peritoneal cavity was examined in **Study II** to investigate the immune response to acute inflammation. Gills were selected in **Study III** for gene expression analysis because of their particular susceptibility to environmental modifications due to their direct contact to the water. Within **Study II** and **Study III** peripheral blood was further evaluated for non-transcript-based indicators (exclusively cortisol in **Study III**). Since **Study I** aimed to determine basal transcript levels of selected genes during early ontogeny, and thus did not involve a treatment experiment, the detection of non-transcript-based indicators was not included. For future studies in the field of adaptation of husbandry conditions, it would be interesting to investigate other tissues and organs to obtain more detailed information on the possible effects on the welfare and health status of the pikeperch. For example, the inclusion of gills tissue is certainly interesting for the assessment of hypoxic conditions as already performed in fish species like ruffe (*Gymnocephalus cernua*), European flounder (*Platichthys flesus*), Nile tilapia (*Oreochromis niloticus*) and Korean rockfish (*Sebastes schlegelii*) [129–131]. Additional examination of the HK of pikeperch may well provide further information for the overall picture of response pathways to rising temperatures, as demonstrated in rainbow trout (*Oncorhynchus mykiss*) [122,132,133].

For animal welfare reasons, no extreme conditions were considered in more detail, but critical or even close-to-practice situations were examined in this dissertation. Water temperatures above 25°C are already defined as critical for pikeperch farming due to the negative correlation of oxygen saturation with rising water temperatures [71]. Oxygen saturation of 40 % DO have been set as critical limit for fish rearing in general [31].

The three included studies (**Study I, Study II** and **Study III**) provide a first insight into basal gene expression under currently applied conditions of intensive farming conditions in pikeperch as well as into altered gene expression under changed environmental conditions. Particularly **Study II** allows conclusions to be drawn about the significance of non-transcript-based indicators compared to transcript-based indicators.

3.2.Potentially Challenging Conditions During Intensive Pikeperch Farming

The artificial environment of intensive aquaculture systems is likely to create a stress burden on the developing fish not only compromising its well-being but also, in the case of a persistent situation, affecting its immune competence. The present thesis contributes primarily to the elucidation of the stress response during commonly applied water temperatures and reduced oxygen saturation as potential stressors. Further, this dissertation contributes to the characterisation of candidate genes to examine physiological processes during early pikeperch development under prevailing confinement conditions.

3.2.1. Monitoring of the Early Ontogenesis Under Confinement Conditions

To the time of **Study I**, insufficient information of basal transcript levels for genes involved in the developmental process of pikeperch have been available for comparative studies. In this thesis, transcript patterns for 21 genes were to be determined from several ontogenetic stages (Table 1) (**Study I**). Eggs and larvae examined in this dissertation were reared in a commercial aquaculture facility (State Research Centre for Agriculture & Fisheries Mecklenburg-Vorpommern – Hohen Wangelin) during an intensive production cycle. Juvenile pikeperch were reared in a new experimental RAS facility at the Research Institute for Farm Animal Biology (FBN) and sorted by size to prevent growth-rate-related cannibalism. The aim was to record the physiological state of the different developmental stages at the genetic level under the given farming conditions by answering the following question: Are the determined expression patterns to be assigned to certain characteristics of the early developmental phase under the prevailing rearing conditions?

The majority of the genes studied could indeed be associated with certain developmental stages (**Study I**). With regard to the genes and developmental stages/tissues studied, eight genes seem to be

particularly interesting with respect to the effects of the applied feeding regime on pikeperch ontogenesis.

BMP4 and *BMP7* (encoding for bone morphogenetic proteins four or seven) were highly expressed in eyed-eggs and larval stages, when various morphological changes take place. For *PPARA*, *PPARD* (encoding for peroxisome proliferated activated receptors either alpha or delta) and *RXRA* (encoding for retinoid x receptor alpha) the highest transcript levels were observed in liver tissues of juvenile stages. *BMP4* and *BMP7* are involved in processes of chondro- and skeletogenesis, but also in organogenesis. *PPARA*, *PPARD* together with *RXRA* are important for the lipid metabolism [134–136]. Since development of feeding fish larvae and juveniles depends, amongst other factors, on the nutritional status, effects of the feeding regime on the ontogenetic process might be detectable at the genetic level with the help of these genes. For example, inadequate diet composition can result in malformations of the skeleton of fish larvae [137]. For the crucial transition from live-prey feeding larvae to larvae fed with formulated feed, no significantly changed expression for any of the examined genes was detectable. Changes in the feeding regime are a central part of the adjustment of rearing parameters in commercial aquaculture to achieve a better nutritional status of pikeperch larvae and early juveniles and thus, an increased production success [138,139]. The transcription patterns of further genes should be investigated explicitly for this period of feed conversion in future research studies.

The genes *LYZ* (encoding for the antibacterial enzyme lysozyme), *EPAS1* (encoding for endothelial PAS domain protein 1; involved in developmental processes and stress response to hypoxic conditions) and *tOSTF1* (teleost-specific osmotic stress transcription factor; important role in osmoregulation) were highly expressed in juvenile stages [140–142]. It is important to mention, that water parameters were in the range of suggested “adequate values” for intensive pikeperch farming and no pathogenic water contamination were detectable at any time of **Study I**. As the stress susceptibility of pikeperch can be influenced by the applied artificial feed, changes in the expression of these genes might be promising indicators for future adaptation processes with regard to an optimal feeding regime [49,61–63].

The identification and characterisation of candidate genes involved in physiological processes during early ontogeny is of high importance in terms of future studies related to the improvement of husbandry

conditions and thus the production success. For example, these values could be compared with other intensive aquaculture rearing programmes for pikeperch. To monitor the developmental process of early ontogenesis in pikeperch, a first promising gene set could be characterised in this dissertation. Future studies should be conducted with altered husbandry parameters to confirm the importance of the candidate genes for the corresponding challenges during pikeperch farming. In addition, other tissues of juvenile stages need to be examined, such as muscles for genes involved in energy metabolism, head kidney and spleen for immune specific genes and gills for stress responsive genes.

3.2.2. Welfare Compromise due to Environmental Stressors

At the time of **Study II** and **Study III**, neither the optimal husbandry temperature in terms of animal welfare nor the tolerance capacity to hypoxic conditions was yet known for pikeperch. The aim was therefore to answer the following research question: Does moderately low DO levels and applied temperatures above 20°C impair the animal health status while reared under intensive aquacultures conditions?

In this thesis, it could be demonstrated that chronically reduced oxygen saturation and increasing water temperatures have no significant adverse effect on juvenile pikeperch. Neither 40 % DO (± 3.2 mg/l) at 23°C for 28 days (**Study II**) nor an increment of 1°C/day from 15°C to 25°C and DO levels above 4 mg/l (**Study III**) resulted in an expected severe stress response. These data are not in accordance with increased blood parameters associated with stressful conditions or changed transcription patterns of stress genes formerly seen in rainbow trout under heat stress and in European sea bass (*Dicentrarchus labrax*) or Atlantic salmon (*Salmo salar*) under long-term hypoxia [132,143–145]. The results of the two studies (**Study II** and **Study III**) rather indicate an adaptation process during the conditions studied, especially seen at the molecular level. According to certain gene expression patterns (e.g. *HIF1A* in **Study II**, *SERPINH1* and *C3* in **Study III**) DO saturations of 40 % and water temperatures above 22°C might be less favourable for intensive farming, since an ongoing energy-demanding adaptation process possibly interferes with e.g. an optimal growth rate of juvenile pikeperch. This outcome was unexpected, since pikeperch is considered particularly stress susceptible during the intensive farming process. However, this assumption is mainly based on one publication [43]. The pikeperch seems to be similarly

tolerant to temperatures up to ~ 25°C as yellow perch (*Perca flavescens*), Eurasian perch (*Perca fluviatilis*) and European sea bass [146–148]. Pikeperch further seems to have a tolerance spectrum to intermediate hypoxic conditions (~ 3 mg/l DO) similar to Eurasian perch and golden perch (*Macquaria ambigua*) [149,150].

In commercial aquaculture facilities, fish are usually exposed to several potential stressors rather than a single stimulus. Previous experience to stress exposure determines the ability of developing a further response and therefore the efficiency of the regulatory systems including the immune activity [151,152]. Former observations suggest a negative impact of hypoxic conditions on the immune system of fish [153–155]. An additional immune challenge by inactivated bacteria (*Aeromonas hydrophila*) during reduced DO saturation of 40 % (± 3.2 mg/l) non-significantly affected the adaptive immune response in juvenile pikeperch (**Study II**). These results seem to support the absence of a persistent severe stress response due to the applied oxygen saturation, since even an additional pathogenic stressor does not result in a critical immunosuppression. Although, there was a moderately affected adaptive immune response, the acute inflammation subsided after three days. This outcome is comparable to former studies in rainbow trout without additional pre-challenging conditions [156].

Overall, juvenile pikeperch appear to have a higher tolerance and adaptation potential to the investigated environmental stressors than expected. Although it is formerly described as skittish, shy and easily stressed, pikeperch does seem to adapt to DO saturations down to 40 % (± 3.2 mg/l) as well as to conventionally used rearing temperatures of up to 25°C in intensive aquaculture facilities [157]. Both examined conditions do not seem to represent an extreme situation for juvenile pikeperch, but rather indicate a challenging condition with induced adaptation processes. This result suggests that pikeperch in itself has a high potential for intensive aquaculture farming. With additional peritoneal stimulation during reduced oxygen concentrations, an impairment in cell mobilisation of the adaptive immune system could be detected, albeit only in a moderate range. Nevertheless, situations provoking energy-demanding adaptation processes should be avoided as far as possible in aquaculture to achieve optimal rearing conditions for pikeperch farming. Therefore, the husbandry conditions should be chosen in the best possible way to not exceed any limit values and to avoid serious fluctuations in the underlying

parameters. For the future, further studies are necessary, with the focus on characterising possible multi-stressors events affecting the well-being of pikeperch reared in intensive aquaculture facilities. It still remains to be seen, if critical fluctuations in oxygen saturation may actually severely affect its health status. With regard to rearing temperatures, the effect of additional stressors (e.g. pathogenic) needs to be further investigated, especially towards immunosuppression.

3.3. Identification of Reliable Health-Status Indicators

In this thesis, a variety of tools has been used to examine the physiological and molecular response of juvenile pikeperch to applied rearing settings as well as potentially adverse conditions common in intensive rearing systems. An assessment of possible stressful conditions and their effects on the farmed fish play an important role in ensuring animal welfare and a good health status under the prevailing farming conditions, and thus contribute to an improved production success. The use of transcript-based together with established non-transcript-based indicators in this doctoral thesis is intended to clarify the question: to what extent can expression analyses contribute to the quantification of possible health-challenging rearing conditions?

3.3.1. Non-Transcript-Based Indicators

As indicators for a primary stress response to the prevailing conditions, either the level of plasma cortisol (**Study II**) or amount of whole-body cortisol (**Study III**) was recorded in this dissertation. The difference in methodology is due to the diverse size of juvenile pikeperch to be examined, with less collectable blood in smaller fishes. Changes in cortisol are a well-accepted indicator for acute, severe stress in fishes [87]. In the current thesis, no significant increase in cortisol levels was detectable in juvenile pikeperch already 24 h after rearing at 40 % DO saturation (**Study II**) or after a rise in temperature of 1°C (**Study III**). Whether clearance had already taken place at the time of evaluation or the challenge was not severe enough for a pronounced release of cortisol, cannot be clarified without further investigations. In addition, the majority of the fish examined during hypoxic challenge were below the detection limit of 6.9 nmol/l (**Study II**). Examined indicators for an initiated secondary stress response to prolonged hypoxic challenge included glucose and lactate levels (**Study II**). The detected

levels revealed inter-individual differences in control and treatment groups (Table 2). In accordance with these results, a recent study in maraena whitefish (*Coregonus maraena*) revealed inter-individual levels in blood glucose during temperature challenge [121]. Regarding the absence of an acutely, severely stressful condition, the examined blood parameters may not be recommended as a stand-alone indicator evaluating a possible stress response in pikeperch during the critical conditions investigated here. Studies in pikeperch demonstrated a time-limited significant change in levels of cortisol, glucose, and lactate after short-term NaCl exposure and handling stress [158,159]. The results of **Study II** and **Study III** seem to support a missing severe stress response of juvenile pikeperch farmed at low DO levels or during a temperature increment, since no significant changes within these indicators have been detected. The outcome further contributes to former studies which indicate that the particular level or amount of cortisol does not seem to be generally representative for moderate long-lasting stress stimuli possibly due to a de-sensitisation of the HPI axis [160]. Detecting cortisol in scales has been shown to be a possible alternative to determine cortisol levels during chronic stressful conditions and would be interesting to include in future studies examining the stress response of pikeperch [160].

For the assessment of possible suppressive effects of reduced oxygen saturations on the immune system, the leukocyte profiles in peripheral blood and main immune organs as well as organosomatic indices were evaluated after additional immune stimulation (**Study II**). SSI together with immune cell proportions indicate a missing pronounced effect on the immune reaction to acute inflammation after intraperitoneal stimulation with inactivated *Aeromonas hydrophila*. However, both indicators suggest a moderate alteration within the adaptive immunity following an acute intraperitoneal inflammation after eight days of hypoxic conditions. Since persistent inadequate conditions may lead to a tertiary stress response affecting energy-demanding processes like the immunity, these results may indicate an ongoing adaptation process [69]. The data confirm a former study in which pikeperch showed adaptation to persistent stress stimuli by moderately reduced numbers of circulating lymphocytes [161]. Especially for long-term adverse conditions, indicators for a secondary and tertiary stress response seem to be more adequate. Their changes are slower compared to cortisol levels and their possibility to show an animal's ability to cope with a certain stressor [99].

After considering the outcome of all measured non-transcript-based indicators, a severe stress in juvenile pikeperch reared under low DO levels (**Study II**) and increasing temperatures (**Study III**) could most likely be excluded. Nonetheless, a missing physiological stress response does not always mean that the animals' welfare status has not been affected by the prevailing conditions after all [99]. Especially when investigating the effect of non-severe chronic challenges, an adaptation response interfering with the well-being of fish may be the possible outcome. The next step was to clarify whether the use of transcript-based indicators can provide further information on the health status, or even replace non-transcript-based indicators for studies in juvenile pikeperch under potentially inadequate husbandry conditions.

3.3.2. Transcript-Based Indicators

At the time of **Study III** (conducted before **Study I** and **Study II**), insufficient information was available on appropriate reference genes to normalise gene expression data in pikeperch. The aim was therefore to establish generally suitable and applicable reference genes independent of the sample material, the applied husbandry condition and the detection technique. The reference genes considered included genes of previous studies with stable transcript levels unrelated to the particular treatment of fish and already known “house-keeping genes” (*ACTB*, *GADPH*, *RNA18S*) [124,162–165] (Table 1). Each individual gene was analysed for its suitability within the three included studies by applying the qBase^{+} software. In particular, for two out of the potentially eight reference genes, stable mRNA abundances could be detected for any examined tissue under any husbandry condition in every single study. Transcript levels of *RPL32* and *RPS5* were stable in HK (**Study II**), liver (**Study I**, **Study II**, **Study III**), spleen (**Study II**) and gills (**Study III**) tissues as well as whole organisms, i.e. eyed-eggs, yolk-sac larvae and feeding larvae (**Study I**). Moreover, both genes were suitable references to normalise data established with single RT-qPCR (**Study I**, **Study II** and **Study III**) and multiplex RT-qPCR (**Study III**) during intensive husbandry conditions (**Study I**) and potential environmental challenges (**Study II** and **Study III**). Stable transcript values were demonstrated for *EEF1A1* in single RT-qPCR experiments with mRNA extracted from whole organisms (**Study I**) as well as liver (**Study I**, **Study II** and **Study III**), spleen and HK (**Study II**) tissues of juvenile pikeperch. The only exception

was gills tissues of juvenile pikeperch for which the gene *MRTRN2* was identified as suitable reference (**Study III**). *EEF1A1*, *RPL32* and *PRS5* were applicable for candidate genes with expression levels ranging from 1×10^1 to 1×10^8 copies per 100 ng RNA, while *MTRNR2* was tested up to 1×10^5 copies per 100 ng RNA. The MIQUE guidelines suggest that at least two reference genes should be used for normalisation of RT-qPCR data but also, that the necessity of further references should be determined for any implemented experiment [166]. Considering the results of the current dissertation three reference genes, i.e. *RPL32*, *RPS5* and either *EEF1A1* or *MTRNR2* (depending on the tissue), seem to suitable for data normalisation of single RT-qPCR analyses performed on pikeperch. The outcome further indicates that the two genes *RPL32* and *RPS5* seem to be adequate and sufficient for a reliable normalisation of multiplex RT-qPCR data established in juvenile pikeperch. These data are in accordance with successful implementation of the examined genes as reference in gene expression studies on teleost [124,167,168].

The set of candidate genes for characterising possibly adverse rearing conditions in this thesis included, amongst others, selected genes being considered "general" indicators of an activated stress response in vertebrates (Table 1). The gene "nuclear receptor subfamily 3 group c member 1" encodes a glucocorticoid receptor which plays a major role in the stress response pathway [169,170]. Significantly increased transcription levels of *NR3C1* were observed in yolk-sac larvae at 4 dph at which larvae might still adapt to their new surrounding environment. Whereas, no significantly altered expression levels of *NR3C1* were detectable in larvae either exposed to first exogenous feeding (on live prey) or weaning (to formulated feed) (**Study I**). This might be due to the fact that the current feeding and weaning regime may not result in a severe stress burden. To verify this outcome, comparative studies with different conditions will be necessary in the future. Matching these results, *NR3C1* was not differentially regulated in liver or gills of juvenile pikeperch during a temperature increment (**Study III**). In contrast, a challenge due to moderately reduced oxygen saturation resulted in a significant decrease in *NR3C1* transcript level in sampled HK and liver of juvenile pikeperch (**Study II**). Due to its challenge-dependent transcriptional regulation, *NR3C1* does not seem to be a suitable indicator to characterise the health status of pikeperch in general. The transcript levels of *HMX1*, the gene which encodes the omnifunctional "H6-family homeobox protein 1", were strongly dependent on temperature in sampled gills of juvenile pikeperch (**Study III**). *HMX1* seems to be a suitable candidate to study the effects of

rising temperatures in the gills of pikeperch. This gene is especially known for its role in fish development, but has received marginal attention as a stress indicator in fish so far [171,172]. As the oxygen saturation decreases with increasing water temperature, an examination of *HMX1* expression at moderately reduced or even declining oxygen levels could be of future interest. To make a more detailed statement regarding particular stress stimuli, the transcription of additional specific genes was examined, including "hypoxia inducible factor 1A", the main regulator of the response pathway initiated by hypoxic conditions [140]. *HIF1A* reached significant lower transcript numbers in HK tissues. While in liver of juvenile pikeperch, significantly increased transcript levels were observable (**Study II**). *HIF1A* seems to be a possible candidate gene for studies investigating the effects of long-lasting reduced oxygen levels of juvenile pikeperch. In contrast to these results, chronically reduced oxygen saturation induced an increase in *HIF1A* transcript levels in liver and muscle of European sea bass [144]. In yellow perch, low-oxygen saturations did not affect *HIF1A* gene expression in juveniles [173]. The findings in **Study II** further contribute to the assumption, that *HIF1A* expression during reduced oxygen levels changes in a tissue- and species-specific manner. Acute hypoxia induced generally elevated *HIF1A* transcription in several tissues of channel catfish (*Ictalurus punctatus*), while chronic hypoxic conditions increased *HIF1A* expression in muscle of Eurasian perch [174]. It would be interesting to know if the expression of *HIF1A* in different tissues of pikeperch correlate with decreasing oxygen saturations. In Atlantic salmon, *HIF1A* transcript levels significantly decreased due to short- and long-term elevated water temperatures [175,176]. It would have been interesting to see how the transcript pattern of *HIF1A* changes during increasing temperatures in pikeperch. Unfortunately, due to the chronological order of the included studies, there was no established primer pair for *HIF1A* at the time of **Study III**. For the observation of physiological processes during the early development of pikeperch, *HIF1A* does not seem to be a suitable candidate for specific processes, since a biphasic expression in early and late ontogenesis stages were observable (**Study I**). This result is in accordance with a dynamic *HIF1A* expression during the development of zebrafish (*Danio rerio*) [177]. In order to assess the stress response of juvenile pikeperch to possibly inadequate thermal conditions, the expression of so-called "heat-shock proteins" (HSPs) was examined [178]. It is important to mention, that HSPs are a vast group of various proteins that respond not only to the condition of heat shock, but also to osmotic stress stimuli

and xenobiotic compounds [179]. The genes *HSP90AA1* (encoding for "heat shock protein family 90 alpha family class A member 1"), *HSPA8b* (encoding for "heat shock protein family A (HSP70) member 8b") and *SERPINH1* (alias *HSP47*, encoding for "serpin peptidase inhibitor, clade H, member 1") are typically heat-inducible HSPs in vertebrates and also well-known indicators in fish [117,122,180]. *HSP90AA1* mRNA abundance was upregulated in liver and gills of juvenile pikeperch during increasing water temperatures. However, only in gills, the transcript levels were correlated to a temperature increment. The results of the current doctoral thesis confirm the temperature-related inducibility of *HSP90AA1* in liver of teleost including maraena whitefish, rainbow trout and Atlantic salmon [121,130,175]. The transcript pattern of *HSP90AA1* was in addition investigated under hypoxic conditions. However, this revealed a rather dynamically regulated transcription in HK and a stable expression in liver of juvenile pikeperch (**Study II**). *HSP90AA1* seems to be a suitable candidate for studying the effects of thermal changes, especially in gills, but may not be suitable for the examination of moderately reduced oxygen saturations. In order to be able to make further statements on this, however, the gills of pikeperch should also be examined during hypoxic conditions. Transcript levels of *HSPA8b* were downregulated in liver tissues and upregulated in gills tissues during temperature increment. In both tissues its mRNA abundance was moderately high correlated to increasing temperatures (**Study III**). These results are in accordance with studies in rainbow trout gills, in which *HSPA8* expression was moderately induced during thermal stress [119]. *HSPA8b* may further be suggested as a suitable candidate for investigating adaptation responses to inadequately increasing water temperatures in pikeperch. The most promising candidate for characterising responses to a temperature increment was *SERPINH1*. In liver as well as gills tissues of juvenile pikeperch, a cumulative increasing transcript abundance was detectable. These data are in accordance with current and former studies on heat-inducibility of *SERPINH1* in liver and gills of rainbow trout and Atlantic salmon [119,175]. Due to its correlating mRNA abundance with rising temperatures, *SERPINH1* might also be an interesting candidate to examine a stress response in gills due to decreasing oxygen saturations. Moderate persistent stressors may result in an immunosuppression in fish. Several genes, particularly involved in innate immunity, have further been included in the transcript pattern analysis. The transcript abundance of the genes encoding for acute phase proteins "complement component 3" (C3) and "haptoglobin" (HP) in

liver tissues was upregulated with increasing water temperatures and showed either a moderately high or strong correlation with temperature (**Study III**) [181]. The outcome is in accordance with an increased *C3* expression in liver of Atlantic salmon due to heat stress [175]. Both genes might further be interesting candidates for the evaluation of an impairment of the well-being due to reduced oxygen levels. Within **Study II** the transcript level of *FTH1*, encoding the iron-storage protein "ferritin heavy chain 1" which maintains an important role in immunoregulation, were significantly downregulated in HK and liver tissues due to hypoxic conditions [182]. The significant change in transcript levels in juvenile pikeperch is in accordance with *FTH1* being listed as hypoxia-responsive gene in fish and an increased expression in liver of rainbow trout due to rising water temperatures [183,184]. Since an upregulation in *FTH1* levels during reoxygenation in gills of silver sillago (*Sillago sihama*) was observable, investigations in gills might be of further interest for future studies in pikeperch [185]. During an incremental temperature increase, no detectable change of *FTH1* gene expression was observable in liver or gills (**Study III**). This contradicts the outcome of former studies in rainbow trout and catfish (*Ictalurus furcatus*) in which changes in the transcript levels of *FTH1* were observable in liver and gills [119,186]. With regard to the additional immune stimulation during reduced oxygen concentrations, the gene expression of immune-relevant genes (e.g. *CXCL8*, *IL1B*, *TNF* and *MPO*) could confirm the lack of a severe immunosuppression.

HSP90AA1, *HSPA8b* and *HP* showed dynamically regulated mRNA levels in juvenile pikeperch. Therefore, the most suitable candidate genes for temperature-relevant studies in juvenile pikeperch seem to be *SERPINH1* and *HMX1* in gills tissues, together with *C3* in liver tissues. The most promising genes, out of the list of candidates, for the investigation of hypoxic conditions seem to be *HIF1A* in HK and *FTH1* in liver tissues. In principle, the observation of gene expression enables the determination of externally invisible impaired well-being. In the context of this dissertation, gene expression analyses could help to detect an induced adaptation process even if no changes in physiological parameters were present. However, this detection was only achievable with a small selection of genes. A possible reason might be that only critical and not acutely severe stressful conditions were tested. The results of this dissertation indicate that no single gene set can be established for the evaluation of the health status in pikeperch under intensive husbandry conditions. Rather, it seems that future approaches will consist of

tissue-specific indicators that should be selected depending on the type of stressor. In future studies concerning hypoxic conditions and their effects, the gills seem to be an adequate tissue to be examined. Whereas the head kidney is certainly an interesting organ for temperature-related studies. In order to be able to actually define the genes selected here as indicators for certain stress stimuli, they would still have to be confirmed for their suitability by means of severe stressful conditions. The data generated suggest that only a joint consideration of non-transcript-based and transcript-based indicators is meaningful for the overall assessment of whether a particular condition is actually stressful or rather a challenge for the fish. For a future expansion of possible candidate genes, an all-encompassing transcriptome analysis could bring decisive advantages. The Fish Genetics Unit (FBN) recently demonstrated the advantage of transcriptomic analyses in the field of multi-stressor approaches in rainbow trout [117].

3.4.Conclusion & Implications for Intense Pikeperch Farming

The three studies included in this dissertation contribute to a further elucidation of the stress susceptibility of pikeperch under intensive husbandry conditions. They further contribute to evaluate the applicability of gene expression analyses with regard to identification and characterisation of an induced stress response in fish under critical rearing conditions.

Our central hypothesis that pikeperch shows an overall increased susceptibility to stress when reared in intensive aquaculture facilities could not be confirmed in the context of this thesis. The results indicate that juvenile pikeperch are less stress susceptible to reduced water quality, than previously thought. No severe stress response was recorded during increasing water temperatures or reduced oxygen saturations. In commercial aquaculture farms, juvenile pikeperch are kept at temperatures above 20°C in order to achieve an increased growth rate and thus an economic advantage. The outcome suggests, that temperatures up to 22°C are favourable for juvenile pikeperch farming. The results further indicate, that there might be a greater scope for oxygen levels still being adequate for rearing juvenile pikeperch in RAS. An oxygen saturation close to 100 % DO is the standard in intensive pikeperch aquaculture. Even though, a severe stress response was missing, the application of transcript-based indicators enabled the detection of significant and/or moderate changes within transcript patterns of certain candidate genes mainly involved in stress- and immune response pathways. These promising genes, i.e. *C3*, *FTH1*, *HIF1A*, *HMX1* and *SERPINH1*, can be used in the future as indicators for the early detection of an incipient welfare compromise of farmed pikeperch as well as for the detection of a possible exceeding of its adaptation range. However, a mix of non-transcript-based as well as transcript-based indicators seems to be best suited for the first basic assessment of a potential stressor, according to the results. A suitable gene set for monitoring the developmental processes in different ontogenetic stages of pikeperch could further be characterised within this dissertation including the genes *BMP4, BMP7*, *EPAS1*, *LYZ*, *tOSTF1*, *PPARA, PPARD* and *RXRA*. This set will give the possibility to evaluate whether certain changes in the rearing process, such as the feeding regime, impact the proper ontogenesis or stress resistance in pikeperch by characterising differences at the transcriptional level. The results of the current dissertation contribute to the establishment of reliable and accurate indicators to regularly

monitor the stress state of farmed pikeperch. These selected indicators can further be applied to adjust husbandry conditions in order to maintain optimal animal health and welfare in intensive pikeperch aquaculture. As a long-term goal, the indicators identified in this doctoral thesis will contribute to the establishment of generally applicable husbandry guidelines for pikeperch farming.

To verify the importance of the characterised candidate genes in regard to particular stressful conditions, further detailed studies need to be performed. In particular, a multi-stressor setup, as likely in intensive aquaculture systems, could further determine the suitability of individual genes for characterisation and determination of inadequate conditions. Furthermore, future studies should include additional possibly relevant tissues to get a more detailed overview of the impact of the environmental challenges considered here. On a future perspective, it would be highly valuable to expand the focus on other probable stressors, to further characterise the stress physiology and susceptibility of intensively farmed pikeperch at the transcriptional level, e.g. changes in feeding regime and feed composition.

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5. Publications

5.1. Declaration of Own Share in the Publications

I hereby declare that my own share in the three included publications in this doctoral thesis is as follows:

Publication I

Insights into Early Ontogenesis: Characterization of Stress and Developmental Key Genes of Pikeperch (*Sander lucioperca*) in vivo and in vitro. **Schäfer N**, Kaya Y, Rebl H, Stüeken M, Rebl A, Nguinkal JA, Franz GP, Brunner RM, Goldammer T, Grunow B, Verleih M *Fish Physiology and Biochemistry* (2021), 47, 515-532. doi: 10.1007/s10695-021-00929-6.

1) Sampling of the individual developmental stages and liver tissues of the juvenile fish
2) Selection of candidate genes
3) Primer design and evaluation of the sequencing data for quality control purposes
4) Experimental procedures including RNA isolation, quantitative real-time PCR and cell culture procedures (WF2 cell line)
5) Measurement of gene expression data
6) Statistical evaluation of the gene expression data
7) Discussion and interpretation of the gene expression data
8) Drafting, writing, editing and reviewing the manuscript including literature research and preparation of tables and figures

Publication II

Effects of hypoxic challenge with additional intraperitoneal stimulation on the immune status of pikeperch (*Sander lucioperca* L., 1758). **Schäfer N**, Matoušek J, Rebl A, Stejskal V, Brunner RM, Goldammer T, Verleih M, Korytář T *Biology* (2021), 10(7), 649. doi: 10.3390/biology10070649.

1) Research design of the stress experiments (hypoxic conditions)

2) Funding acquisition
3) Sampling including peripheral blood samples, tissue samples and cell isolation procedures
4) Experimental procedures including ELISA for cortisol quantification, RNA isolation, quantitative real-time PCR, flow cytometric analyses and intraperitoneal stimulation
5) Selection of candidate genes
6) Primer design and evaluation of the sequencing data for quality control purposes
7) Measurement of blood parameters, health parameters and gene expression data
8) Statistical evaluation of the data obtained
9) Discussion and interpretation of the data obtained
10) Drafting, writing, editing and reviewing the manuscript including literature research and preparation of tables and figures

Publication III

Identification of molecular stress indicators in pikeperch *Sander lucioperca* correlating with rising water temperatures. Swirplies F, Wuertz S, Baßmann B, Orban A, **Schäfer N**, Brunner RM, Hadlich F, Goldammer T, Rebl A *Aquaculture* (2019), 501, 260-271. doi: 10.1016/j.aquaculture.2018.11.043.

1) Sampling including in vitro cell isolation (WF2 cell line)
2) Experimental procedures including the in vitro experiment, RNA isolation and (multiplex) quantitative real-time PCR
3) Execution of the in vitro experiment
4) Measurement and evaluation of gene expression data from the in vitro experiment
5) Generation of an in vitro data set to test the applicability of the selected genes for the detection of transcriptional changes after hyperthermia in *Sander* spp.
6) Participation in the interpretation and discussion of the collected data (single as well as multiplex quantitative real-time PCR)
7) Contribution to the writing and editing of the manuscript
8) Revision of the manuscript

5.2. Insights into Early Ontogenesis: Characterization of Stress and Development Key Genes of Pikeperch (*Sander lucioperca*) in vivo and in vitro.

Schäfer N, Kaya Y, Rebl H, Stüeken M, Rebl A, Nguinkal JA, Franz GP, Brunner RM, Goldammer T, Grunow B and Verleih M (2021) in *Fish Physiology and Biochemistry* 47,515-532.

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Insights into early ontogenesis: characterization of stress and development key genes of pikeperch (*Sander lucioperca*) in vivo and in vitro

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Abstract There are still numerous difficulties in the successful farming of pikeperch in the anthropogenic environment of various aquaculture systems, especially during early developmental steps in the hatchery. To investigate the physiological processes involved on the molecular level, we determined the basal expression patterns of 21 genes involved in stress and immune responses and early ontogenesis of pikeperch between 0 and 175 days post hatch (dph). Their transcription patterns most likely reflect the challenges of growth and feed conversion. The gene coding for apolipoprotein A (*APOE*) was strongly expressed at 0 dph, indicating its importance for yolk sac utilization. Genes encoding bone morphogenetic proteins 4 and 7 (*BMP4*, *BMP7*), creatine kinase M (*CKM*), and SRY-box transcription factor 9 (*SOX9*) were highly abundant during the peak phases of morphological changes and acclimatization processes at 4–18 dph. The high expression of genes coding for peroxisome proliferator-activated receptors alpha and delta (*PPARA*, *PPARD*) at 121 and 175 dph, respectively, suggests their importance during this strong growth phase of juvenile stages. As an alternative experimental model to replace further in vivo investigations of ontogenetically important processes, we initiated the first approach towards a long-lasting primary cell culture from whole pikeperch embryos. The present study provides a set of possible biomarkers to support the monitoring of pikeperch farming and provides a first basis for the establishment of a suitable cell model of this emerging aquaculture species.

Keywords Aquaculture · Animal welfare · Fish cell line · Early ontogenesis · Pikeperch · Stress response

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Introduction

Pikeperch (*Sander lucioperca* L., 1758) is an important food fish in Europe. Due to its exceptionally soft flesh, rapid growth, and positive market acceptance, pikeperch

is traded as an outstandingly high-quality fish. Consequently, it became an increasingly attractive freshwater species for European aquaculture (FAO 2018). For more than two decades, efforts have been made to improve the intensive production of pikeperch in recirculating aquaculture systems (RAS) (Hilge and Steffens 1996; Steenfeldt 2015; Policar et al. 2016). Nevertheless, pikeperch farming and especially hatcheries are hampered by difficulties during early ontogenesis, which comprises the central developmental stage of organogenesis, including myo-, skeleto-, and neurogenesis, as well as the phase of growth and the development of the immune system (Zapata et al. 2006; Alix et al. 2015). The embryonic phase of pikeperch ends with hatching at approximately 4 to 6 days post fertilization (dpf). It spans from shortly after the mouth opens and the mixotrophic feeding phase begins until the complete resolution of the yolk sac up to 7 dph. This is followed by the gradual adaptation to pelleted feed and transition to the juvenile stage (Ott et al. 2012a; Güralp et al. 2017). The three main bottlenecks of pikeperch farming are the conversion from endogenous to exogenous feed, the inflation of the swim bladder, and cannibalism (summarized in Steenfeldt 2015). This results in general problems such as animal malformations, impaired growth, and high mortality rates (Kestemont et al. 2007; Szkudlarek and Zakś 2007; Policar et al. 2016; Schaefer et al. 2017; Baekelandt et al. 2018; Schaefer et al. 2018).

Therefore, basic knowledge of the physiological processes that occur during pikeperch rearing is of major importance for improving aquaculture production. This includes the profiling of the basal gene expression pattern during the early developmental stages. Although pikeperch is considered to be highly stress susceptible (Németh et al. 2013; Baekelandt et al. 2018), the stress physiology of the species has received little attention thus far (Milla et al. 2015; Swirplies et al. 2019; Wang et al. 2019), especially in the early phases of development. This also applies to the immune response, which is well investigated in percid fishes such as yellow perch (*Perca flavescens*) or Eurasian perch (*Perca fluviatilis*) but is poorly documented in pikeperch. Current studies mainly focus on humoral stress and immune markers under certain husbandry conditions (Baekelandt et al. 2019; Baekelandt et al. 2020; Żarski et al. 2020), but alterations within gene expression during physiological changes of early ontogenesis have not been reported so far. In the present study, we investigated the transcription patterns of selected genes involved in stress and immune responses as well as the growth phase and the previously mentioned stages of early development. During organogenesis, the retinoid X receptor alpha (RXRA) is important for the development of the posterior brain, neural crest, and tail bud (He et al. 2009). Bone morphogenetic proteins such as BMP4 and BMP7 have been shown to be involved in skeletogenesis and the development of the immune system (Zapata et al. 2006). The growth phase is represented among others by the growth hormone receptor (GHR) (Calduch-Giner et al. 2003). Since pikeperch is particularly sensitive during the phase of feed conversion, genes important for lipid metabolism and the mobilization of energy reserves such as *APOE* (Otis et al. 2015) have been included. Common markers for stress such as the glucocorticoid receptor (NR3C1) have further been applied regarding the high impact of stressful events on the welfare status of pikeperch in early stages.

In addition to the in vivo studies, it is also our concern to advance the in vitro studies in fish research, as the efforts of the 3Rs (replacement, reduction, and refinement) have so far been highly limited in fish research. This statement is underlined by high numbers of experimental animals (2017: ~ 1,220,000 fish within the European Union [European Commission 2020]), which suggests that the establishment of alternative experimental models such as cell culture systems of the respective fish species are necessary. These 3R cell models can be used as an essential tool for detailed research purposes, such as for studying ontogenetically relevant processes or the effects of stress under controlled exogenous conditions. There are several commercially available cell lines from different teleost fishes (ATCC and ExPASy databases, reviewed by Lakra et al. 2011), including the cell lines established from walleye (*Sander vitreus*): WF1 (dermal sarcoma; BS TCL 65) and WF2 (whole fry (Wilensky, C and Bowser 2005); BS CI 88) (IZSLER Brescia, Italy). A cell line from *S. lucioperca* is currently not available.

Therefore, the main goals of this study were (i) to characterize and evaluate key genes for development as well as stress response in the early ontogenesis of pikeperch, (ii) to initiate the first approach to generating a cell model from pikeperch derived out of whole embryos, and (iii) to analyze the suitability of an in vitro model for studying developmental processes in pikeperch.

Materials and methods

Fish sample material

Pikeperch (strain "Sachsen," Germany) were bred and reared in RAS at the State Research Centre for Agriculture and Fisheries Mecklenburg-Vorpommern (Hohen Wangelin, Germany) within their normal production cycle from June 2018 until their transfer as fingerlings (108 dph) to the Experimental Animal Facility Aquaculture of the Leibniz Institute for Farm Animal Biology (FBN, Dummerstorf, Germany).

Seven matings of pikeperch were generated with a sex ratio of 2:1 or 1:1. The progeny was mixed and reared in a RAS system with a total volume of 9 m^3. A pump with a capacity of 9 m^3/h supplies eight fish tanks with a volume of 0.5 m^3 each with a water exchange rate of 20%/h/tank. The system also consists of a drum filter (72-qm gauze), a heater, an electrically controlled water supply, a moving bed biofilter (50% of the total volume, granulate surface of 850 m^2/m^3), and pressure sensors for pump operation. The initial stocking density was 100 larvae/l. The water quality was ensured by continuous purification, UV disinfection, and daily monitoring of temperature, oxygen saturation, and pH value. Concentrations of NH^{4+}, NO^2, and NO^{3-} were determined twice per week in circulating systems of larvae and fingerlings. The photoperiod during hatchery was set at 24L:0D until day 45 and subsequently at 17L:7D (+ 1.5 h dusk and dawn). The feed included a copepod mix from 5 dph (Aquacopa, Germany), followed by *Artemia* nauplii from 7 dph, and enriched *Artemia* from 9 dph (both Inve, Belgium). From 15 dph onwards, dry feed was added until the complete conversion to dry feed (Otohime B1, PTAqua, Ireland) had taken place. Fish were transported to the Leibniz Institute for Farm Animal Biology in a transport box with an additional oxygen provision and in small groups for animal welfare reasons. The transport was followed by an acclimation period of at least two weeks to the local RAS system before sampling.

Eyed eggs were kept at ~ 15.5 °C, ~ 12.9 mg/l dissolved oxygen (DO), and a pH of ~ 8.0. To ensure clean water, approximately 20–30% of the water was renewed every day. Larvae were kept at ~ 15.7 °C, ~ 9.2 mg/l DO, ~ 0.1 mg/l NH^{4+}, ~ 15.7 mg/l NO^{3-}, ~ 0.2 mg/l NO^{2-}, and a pH of ~ 8.7. Fingerlings were kept at ~ 21.1 °C, ~ 8.1 mg/l DO, < 0.001 mg/l NH^{4+}, ~ 38.8 mg/l NO^{3-}, ~ 0.07 mg/l NO^{2-}, and a pH of ~ 8.5. For gene expression analysis, we sampled eyed eggs (0 dph/78 degree days (DD); three pools of n = 20), yolk sack larvae (4 dph/137 DD; three pools of n = 20), larvae fed with *Artemia* spp. (7 dph/252 DD; three pools of n = 30), larvae fed with dry feed (18 dph/481 DD; three pools of n = 30), and liver tissues from 121- to 175-dph-old fingerlings (each n = 3 individuals). Prior to tissue sampling, we anesthetized fingerlings with 2-phenoxyethanol (50 mg/l). According to the recommendations of the German Animal Welfare Act (§ 4(3) TierSchG), fishes were then stunned by a blow on the head and killed directly by a bleed cut in the heart as well as cutting of the spinal cord posterior to the head. Collected material was snap-frozen in liquid nitrogen and stored at − 80 °C until further investigation.

Cell isolation

By generating an embryonic cell line, we aimed to create an alternative to the use of embryos for experimental purposes. Nine days after fertilization and at an age of 125 DD, 12 embryos from pikeperch, with a length of about 4.7 ± 0.07 mm, were used for cell isolation. At 125 DD, the embryos were at eyed egg stage (= 0 dph) which was confirmed by observing them under the microscope. Based on the trypsinization technique, which we had already applied for the Atlantic sturgeon (*Acipenser oxyrinchus*) cell line AOXlar7 (Grunow et al. 2011a), we isolated the specimen from the eggshell and decapitated it using forceps. After washing three times with 1× DPBS (Dulbecco's Phosphate-Buffered Saline; PAN-Biotech), we transferred whole embryos into a 1.5-ml tube and dissociated each with scissors and 100 µl of 0.1% trypsin/EDTA solution (Gibco Life Technologies) for one to two minutes. Digestion was terminated by adding triple the volume of Dulbecco's modified Eagle medium (DMEM, with 4.5 g/l glucose and L-glutamine; Lonza BioWhittaker) supplemented with 20% FBS (fetal bovine serum, PAN-Biotech) and a 1% (v/v) penicillin/streptomycin solution (Gibco). After centrifugation for 5 min at 130*g*, cells were resuspended in cell culture medium supplemented with additional antibiotics (Gentamycin: 0.1 mg/ml and Kanamycin: 0.1 mg/ml; Biochrom AG). Additionally, an antimycotic agent (Amphotericin: 250 µg/ml, Biochrom AG) was added. Cells were placed into 6-well culture plates (TPP) and incubated at 20 °C and 2.5% CO_2. In the following, this long-term cell culture is called SLUlar1.

Cell culture

For SLUlar1 cells, the medium was exchanged every 2 days for the first 2 weeks. Afterwards, half the medium was exchanged once or twice per week, without the use of additional antibiotics. Cells were sub-cultured at a ratio of 1:2, when confluence of 80 to 90% was reached. Therefore, the cells were washed with DPBS and incubated with 0.1% trypsin/EDTA solution at 37 °C for 1 to 2 min. Trypsinization was stopped by adding double the volume of the cell culture medium. Cells were centrifuged for 5 min at 130 g, and the cell pellet was resuspended in a new culture medium and transferred into new culture dishes. From passage two onwards, T25 flasks (TPP) were applied. Cell attachment and cell morphology were visualized under the inverted phase-contrast microscope (Motic AE2000), and pictures were taken with Motic Images Plus 3.0 Software. Images were optimized with Adobe Photoshop CS 4 (Adobe Inc.).

We tested the freezing and thawing of SLUlar1 cells as follows: After trypsinization, cells were resuspended in precooled (+ 4 °C) freezing medium (9:1 ratio of precooled FBS:DMSO [Dimethylsulfoxid; Roth]), placed into 1.2-ml cryogenic vials (Roth; ~ $7.5 \times 10^5 \pm 0.4 \times 10^3$ cells/ml), and transferred into a freezing container filled with isopropanol (Thermo Scientific) for a freezing step at − 80 °C until long-term storage in liquid nitrogen. To thaw frozen cells, cryogenic vials were warmed up at room temperature (22–23 °C) until the ice crystals had nearly disappeared. Cell suspension was mixed with triple the volume of culture medium, centrifuged at 130*g* for 5 min, resuspended in cell culture medium, and placed in a T25 flask (TPP) for incubation at 20 °C and 2.5% CO_2. After the freezing and thawing process, the total number of cells and percentage of viable cells was determined according to the manufacturer's instructions applying the Countess Automated Cell Counter using trypan blue staining.

Commercially available WF2 cells (IZSLER Brescia) were incubated in 100 mm culture dishes (Sarstedt) with MEM Eagle medium, including Earle's salts (Sigma) at 20 °C and 3% CO_2. Medium was complemented with 10% fetal bovine serum (Gibco), 10 mM non-essential amino acids (Merck), 40 mM L-glutamine (Merck), and penicillin/streptomycin (Sigma). Cells were grown until a confluency of ~90% was reached, followed by harvesting for RNA extraction.

Immunofluorescence

The morphology of SLUlar1 cells at passage six was evaluated by immunofluorescence labeling. 300,000 cells were cultivated in 35-mm µ-dishes (ibidi GmbH) for 24 h, washed three times with PBS, fixed with paraformaldehyde (4%, 10 min, Merck), and permeabilized with Triton X-100 (0.1%, 10 min, Sigma-Aldrich). Actin staining was performed using Bodipy FL Phallacidin (1:40, 30 min, Molecular Probes, Eugene). Focal adhesions were stained using vinculin-Alexa 647 (1:100, 30 min; Abcam, Cambridge, UK), and nuclei were stained by Hoechst 33342 dye (1 µg/ml, Sigma-Aldrich). Finally, cells were analyzed with a confocal laser scanning microscope (LSM) 780 (Carl Zeiss), using a × 63 oil objective via the software ZEN2.3.

Nucleic acid isolation

For total RNA extraction from different developmental stages of pikeperch, sampled material was homogenized individually in 1 ml TRIzol Reagent (Thermo Fisher Scientific), based on the manufacturer's protocol. SLUlar1 cells (passage six) and WF2 cells (passage 17) were resuspended in 350-µl RLY lysis buffer (ISOLATE II RNA Mini Kit; Bioline) with an additional 3.5 µl of 2-mercaptoethanol (Sigma) following a washing step with phosphate-buffered saline solution (PBS, Biochrom) and centrifugation at 300*g* for 5 min at 15 °C. All samples were subsequently purified with the RNeasy Mini Kit (Qiagen) including DNase treatment. The quality and quantity of isolated RNA were analyzed by agarose gel electrophoresis and spectrophotometry in repeated measurements (ND 1000; NanoDrop Technologies/Thermo Fisher Scientific). RNA was stored at − 80 °C until further use.

Gene selection and primer design

To establish a screening panel for key steps of the developmental process, we included 21 genes involved in stress (*NR3C1*, endothelial PAS domain protein 1 [*EPAS1*], hypoxia inducible factor 1 subunit alpha [*HIF1A*], heat shock transcription factor 1 [*HSF1*], heat shock transcription factor 2 [*HSF2*], *teleost-specific* osmotic stress transcription factor 1 [*tOSTF1*]; Le Goff et al. 2004; Deane and Woo 2011; Tse 2014; Malandrakis et al. 2016; Pelster and Egg 2018), and immune response (interleukin 1 beta [*IL1B*], lysozyme

[*LYZ*]; Saurabh and Sahoo 2008; Zou and Secombes 2016) as well as cell homeostasis (transcription factor EB [*TFEB*]; Settembre and Ballabio 2011; Raben and Puertollano 2016), nutritional status (*APOE*, *PPARA*, *PPARD*; Poupard et al. 2000; Leaver et al. 2005; Napolitano and Ballabio 2016), growth (insulin-like growth factor 2 [*IGF2*], *GHR*; Bergan-Roller and Sheridan 2018; Nipkow et al. 2018), energy metabolism (*CKM*, glycine amidinotransferase [*GATM*]; Borchel et al. 2019), the process of gonadal maturation (*SOX9*; Leet et al. 2011; Bhat et al. 2016), and the organogenesis of the early life stages (*BMP4*, *BMP7*, myosin heavy chain [*MYH6*], *RXRA*; He et al. 2009; Ahi 2016; Bloomquist et al. 2017; Tang et al. 2018) (Table 1). For the stress screening of SLUlar1 and WF2 cells, the additional immune markers interleukin 8 (*CXCL8*) and interleukin 10 (*IL10*) were included. No cDNA sequences were publicly available for the selected candidate genes at the beginning of the study, except for *CXCL8*, and *NR3C1* (Swirplies et al. 2019). Therefore, we identified orthologous gene sequences from the order Perciformes (*Acanthochromis*, *Dicentrarchus*, *Epinephelus*, *Gasterosteus*, *Notothenia*, *Oreochromis*, *Perca*, *Sebastes* spp.) from the NCBI (National Centre for Biotechnology Information) GenBank (GB) database. Using BLAST searches against our recently published genome of *S. lucioperca* (RefSeq NCBI: GCA_008315115.1), obtained with Illumina technology and PacBio Sequel System (Nguinkal et al. 2019), we identified the corresponding sequences. To verify the identified sequence fragments, a reciprocal BLAST against the NCBI nucleotide database was performed. Optimal pikeperch-specific oligonucleotide primers (Sigma-Aldrich) were derived using the Pyrosequencing Assay Design software (version 1.0.6; Biotage; Table 1). For primer validation, all PCR products were sequenced on an Applied Biosystems 3500 Genetic Analyzer (Life Technologies).

Real-time quantitative PCR

For gene expression profiling in vivo and in vitro, similar as in our previous study (Swirplies et al. 2019), real-time quantitative PCR (RT-qPCR) was performed with a LightCycler96 system (Roche Diagnostics) and the SensiFAST™ One-Step qPCR kit (Bioline), in line with the manufacturer's instructions. Therefore, cDNA synthesis from 1.5 µg (in vivo samples) or 0.03–0.1 µg (in vitro samples) of total RNA was performed using the SuperScript II Reverse Transcriptase Kit (Thermo Fisher Scientific) based on the manufacturer's instructions. The resulting cDNA was stored at − 20 °C until further use. An initial denaturation step (95 °C, 5 min) was followed by 40 cycles of denaturation (95 °C, 5 min), 15 s of annealing (60 °C), 15 s of elongation (72 °C), and a fluorescence measurement step for 10 s (72 °C). Standard curves were established for all genes to calculate the copy numbers using linear regression analysis ($R_2 > 0.998$). These were based on the C_q values of tenfold dilutions of the generated fragments (1×10^3 –1×10^8 copies). C_q values < 35 were considered detectable. For data normalization, three reference genes (*EEF1A1*, *RPL32*, *RPS5*) which were already established for data normalization in pikeperch (Swirplies et al. 2019) were included and evaluated for each sample (Table 1). For quality control, PCR products were verified via gel electrophoresis and melting curve analysis.

Statistics

RT-qPCR data were analyzed with the LightCycler 96 analysis software v.1.1, and the suitability of reference genes was assessed using the qBase+ software (Biogazelle, with CV ≤ 0.3). The statistical significances of different ontogenesis stages of pikeperch were calculated using the one-way analysis of variance (ANOVA) followed by parametric Tukey's multiple comparison test using the GraphPad Prism 8 software, version 8.3.0.538. To analyze cell number and viability, mean and S.E.M. were calculated for all cell passages of SLUlar1.

Results

Ontogenetic stage-specific expression profiles of unchallenged pikeperch

In the current study, we defined the basal mRNA abundance of the 21 genes listed in Table 1 at 0 dph, 4 dph, 7 dph, 18 dph, 121 dph, and 175 dph of farmed pikeperch through RT-qPCR analysis (Fig. 1). The average transcript numbers of the analyzed genes ranged from around 1×10^1 (*BMP7* at 121 dph) to 1×10^8 (*LYZ* at 121 dph) copies per 100 ng RNA. We detected ontogenesis-specific transcription patterns for all genes analyzed with the exception of *HSF1*, which was

Table 1 Gene-specific primer set used in this study

Gene symbol	Official names	Sense primer (5'–3')	Antisense primer (3'–5')	Primer efficiency [%]	Fragment length [bp]
Reference genes					
EEF1A1	Elongation factor 1 alpha	ATGGACAGACCCGT GAGCATG	TTCTTGATGTAGGT GCTCACTTC	105	151
RPL32	Ribosomal protein L32	GGCGTAAACCCAGA GGTATTGA	ACCTCGAGCTCCTT GACATTGT	105	157
RPS5	Ribosomal protein S5	GCAGGATTACATTG CTGTGAAAG	TCATCAGCTTCTTG CCATTGTTG	101	161
Target genes					
Stress response					
EPAS1	Endothelial PAS domain protein1	AGTGCAGAGGACGC ACAGATG	TCATGTTCACCTGC GTGAGCC	100	139
HIF1A	Hypoxia inducible factor 1 subunit alpha	CCAGTCGAATCCCT TGAGAGTT	CTGTGGGGTCCTCT TAGCAAC	97	156
HSF1	Heat shock transcription factor 1	TGTGTCTTGTGCAG AGTGGAAC	GCTGGCCATGTTGT TGTGTTTG	111	101
HSF2	Heat shock transcription factor 2	AGCCGTCCCGCAGC TCCCT	CGGGACTCAGTTCG CACAGG	91	93
tOSTF1	*Teleost-specific* osmotic stress transcription factor 1	CTCCCTTGAATCGG TGGTGAG	GACACTGTGAAAGA AGAGCAGTA	102	109
NR3C1	Nuclear receptor subfamily 3 group c member 1	CCAGTCCTGCATGG ATTCACTT	AGGTCCATAGTGTT GTCACTGAA	100	180
Immune response					
CXCL8†	Interleukin 8	AACAGGGATGAGTC TGAGAAGC	GCTTGGAAATGAAG TCTTACATGA	98	158
IL1B	Interleukin 1 beta	TCGACCTACTTGCA CCCTACA	TCTGCCTCCACAAC CTGAA	101	137
IL10†	Interleukin 10	TTTGCCTGCCACGC CATGAAC	AGGCTTTAAGTCAT TGGTCTCCT	95	102
LYZ	Lysozyme	TTTGGCCAACGCCA GGGTCTA	TCCGTCTGTGTTGT GGTTGATG	98	160
Cell homeostasis					
TFEB	Transcription factor EB	AGTGATGTGCGCTG GAACAAAG	CCTGTTACCTGGAT GCGTAGC	95	158
Nutritional status					
APOE	Apolipoprotein E	GCTAGAGCACTCTG ATCTCTGA	TTGGCATCCAGCAT GTCCTTCT	99	160
PPARA	Peroxisome proliferator-activated receptor alpha	ATCTGAATGATCAG GTGACTCTC	TTGGGCTCCATCAT GTCGCTAA	96	172
PPARD	Peroxisome proliferator-activated receptor delta	CTTTGTGACCAGGG AGTTCCTT	AGGACGATCTGGAC AGAGAATAA	99	157
Growth					
GHR	Growth hormone receptor	ACCACAAACTGGGA AGCATTGGA	CCTTTGCTGGGAAT CTCAGTCA	96	173
IGF2	Insulin-like growth factor 2	GAGGCTTCTATTTC AGGTAGGC	ACGGGTATGACCTG CAGAGAG	108	179
Energy metabolism					
CKM	Ceratine kinase, M	AGTACTACCCCCTG AAGTCCAT	TCTTGCTGTCGTTG TGCCAGAT	98	156
GATM	Glycine amidinotransferase	ATCCTTCTGGTTGT CGGGAATG	GGATGGGGTAGTCC TGAACATA	92	178
Gonadal maturation					
SOX9	SRY-box transcription factor 9 c	CGCGTTAACGGCTC AAGTAAAAA	TTCGTTGAGCAATC TCCAAAGTTT	94	165
Organogenesis					

Table 1 (continued)

Gene symbol	Official names	Sense primer (5'–3')	Antisense primer (3'–5')	Primer efficiency [%]	Fragment length [bp]
BMP4	Bone morphogenetic protein 4	CCGTAAACGCAACC GCAACTG	TGAGTTCAGATGAT CCGCCAGA	94	151
BMP7	Bone morphogenetic protein 7	TGTTTCTGCTGGAC TCTCGGG	TTGATGCTCTCTCC GTTTGTGC	98	151
MYH6	Myosin heavy chain	GGGAAGACTGTGAA CACCAAGA	TCCCGAAGCGAGAC GAGTTGT	98	175
RXRA	Retinoid x receptor alpha	CATGAAGAGAGAAG CCGTTCAG	GTATGTCTCGGTTT TGGGTTCC	98	151

† Genes applied exclusively for in vitro analysis

constitutively expressed. For *MYH6*, we did not obtain valid data at 0 dph.

For 10 genes, we observed the highest copy numbers in early developmental stages (0–18 dph). *APOE* reached the highest transcript levels at 0 dph. The transcript levels of *BMP4*, *BMP7*, *CKM*, *MYH6*, and *NR3C1* were highest at 4 dph. While the expression of *BMP4* significantly decreased from early (4 and 7 dph) to late larval (18 dph) and fingerling stages (121 and 175 dph), that of *BMP7*, *CKM*, and *NR3C1* significantly increased from 0 dph to either 4 dph (*BMP7* and *NR3C1*) or all larval stages (4–18 dph; *CKM*). The four genes *GATM*, *HSF2*, *SOX9*, and *TFEB* were most strongly expressed at 7 dph, whereby the transcript levels of *HSF2*, *SOX9*, and *TFEB* significantly increased between 0 and 7 dph. *GATM* significantly decreased between the early (0–18 dph) and late developmental stages (121 and 175 dph).

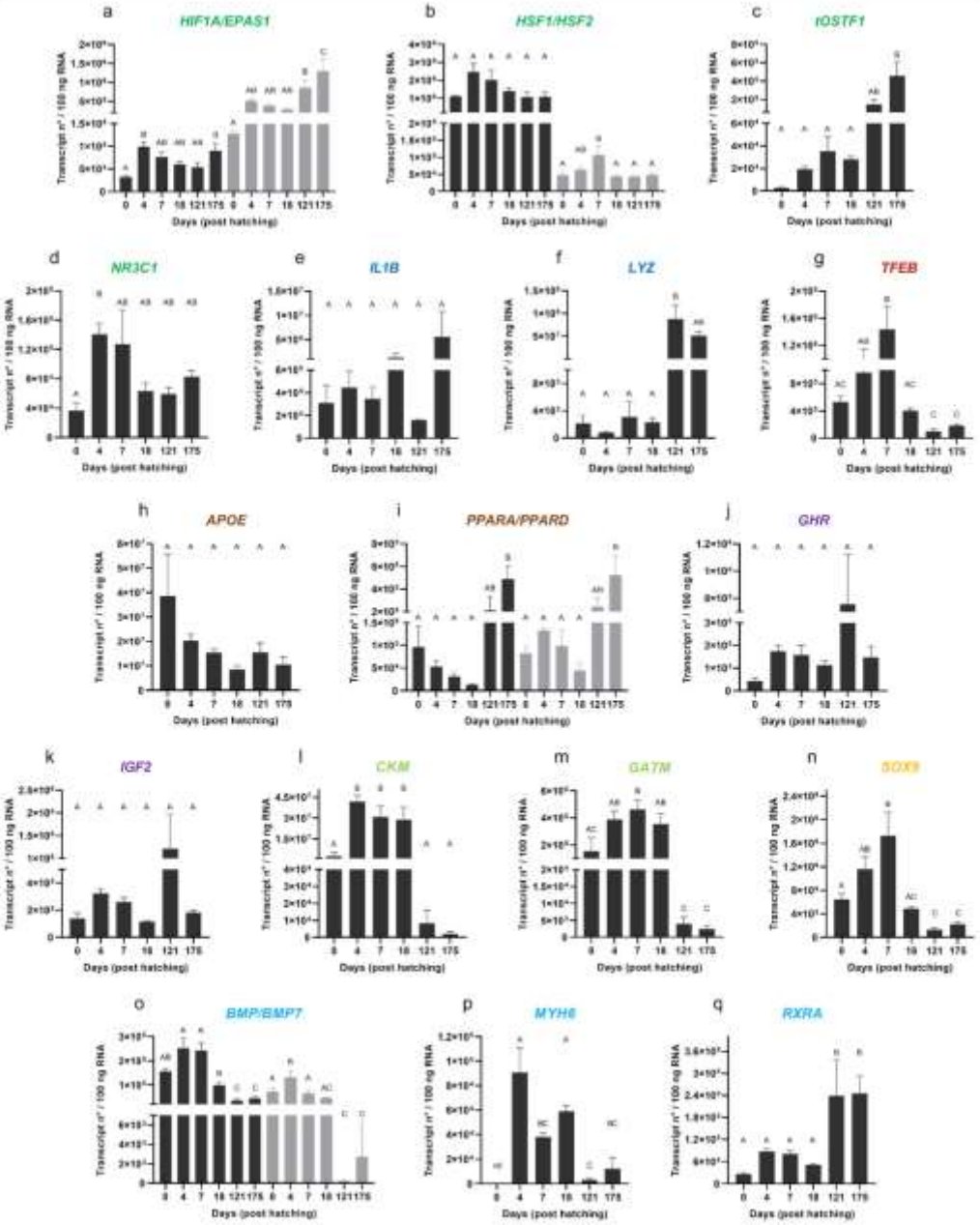
The genes *GHR*, *IGF2*, and *LYZ* were most strongly expressed at 121 dph, whereby the expression of *LYZ* was highly significantly ($p = 0.003$) increased from early (0–18 dph) to late developmental stages (121 dph). *EPAS1*, *IL1B*, *tOSTF1*, *RXRA*, *PPARA*, and *PPARD* revealed the highest transcript levels at the fingerling stage of 175 dph. Thereby, *EPAS1* and *RXRA* were significantly increased between the early (0–18 dph) and late developmental stages (121 and 175 dph). The expression of *tOSTF1*, *PPARA*, and *PPARD* was enhanced compared with the early developmental stages but only significant at 121 dph.

HIF1A significantly increased in transcript number from 0 to 4 dph as well as the late fingerling stage (175 dph), with similarly high copy numbers for each.

Characterization of the basal stress level of a long-lasting primary cell culture

As the first approach towards a cell model from pikeperch, the long-lasting primary cell culture SLUlar1 out of isolated embryos was established (Fig. 2a, b). Following cell isolation, single cells and tissue fragments attached to the culture dish within 48 h. With increasing numbers of passages, the cells grew in monolayer, and from the third passage onwards, tissue fragments were no longer present. Cell size increased from 10 to 13 µm in passage two to 15 to 17 µm in passage six. Cells exhibited a density of $7.5 \times 10^5 \pm 0.4 \times 10^3$ cells/ml with a vitality of 91 ± 2% after trypsinization. After thawing, cell vitality was around 89 ± 0.7%. However, even with a vitality of 90%, only 60 to 70% of the cells attached to the bottom after 2 days. Therefore, cells needed up to 4 weeks for recovery and to reach a confluency of 80 to 90%. Although we tested various cultivation conditions, including different temperatures (16 °C, 20 °C, and 25 °C), different cell culture media (DMEM and Leibowitz-15), as well as gas mixtures (with or without CO_2), cells stopped proliferating at passage eight and remained in the stagnation phase but without signs of cell death.

The morphology of SLUlar1 cells at passage six was evaluated by phase-contrast microscopy (Fig. 2c, d) and immunofluorescence labeling of β-actin and vinculin (Fig. 2e–g). A high proportion of stress fiber formations and cross-linked actin networks (CLANs) was observable. Regarding the location of β-actin, three different population types were determined: (i) cells with well-established cortical actin rings in the periphery and rather thin actin fibers spanning the cell body (Fig. 2e,


a
HIF1A/EPAS1
b
HSF1/HSF2
c
tOSTF1
d
NR3C1
e
IL1B
f
LYZ
g
TFEB
h
APOE
i
PPARA/PPARD
j
GHR
k
IGF2
l
CKM
m
GATM
n
SOX9
o
BMP/BMP7
p
MYH6
q
RXRA
Transcript n° / 100 ng RNA
Days (post hatching)

◀ **Fig. 1** Transcription patterns of candidate genes in developing pikeperch. Genes categorized in stress response (dark green), immune response (dark blue), cell homeostasis (red), nutritional status (brown), growth (purple), energy metabolism (light green), maturation (yellow), and organogenesis (light blue). Columns represent normalized mean (+SEM), calculated per 100 ng of total RNA of each three pools of eyed eggs (0 days post hatch (dph); $n = 20$/pool), yolk sac larvae (4 dph; n = 20/pool), larvae fed with *Artemia* spp. (7 dph; $n = 30$/pool), larvae fed with dry feed (18 dph; n = 30/pool), and three individual samples of liver tissue from fingerlings (121 and 175 dph). Different letters (A–D) indicate significant changes in transcript numbers ($p < 0.05$); in the case of two represented genes: first depicted in black and second depicted in grey. nd: no data detectable

e1), (ii) cells with strong stress fiber formation throughout the cell body (Fig. 2f, f1), and, frequently observed, (iii) cells with actin arranged in a net-like shape as it is described for CLANs (Fig. 2 g, g1). The focal adhesions (represented by vinculin staining) of cells with cortical actin were located in the cell margins and larger than cells with stress fibers. In contrast, cells with CLANs exhibited high numbers of rather small focal adhesion spots throughout the entire cell.

Since this altered cell morphology indicates stress, we further investigated the stress level of the cells and compared it to that of the established cell line WF2 by determining the transcript level of 10 marker genes for stress (*HIF1A*, *EPAS1*, *HSF1*, *HSF2*, *tOSTF1*, and *NR3C1*) and immunity (*LYZ*, *IL1B*, *CXCL8*, and *IL10*) at cell passage six (Fig. 3). In SLUlar1 cells, we detected high transcript levels of *HSF1* (1×10^7) and *IL1B* (1×10^6), moderate mRNA levels of *HIF1A* (7×10^5), *NR3C1* (6×10^5), *HSF2* (1×10^5), and *tOSTF1* (2×10^4), as well as *EPAS1* (1×10^4), and low transcript numbers for *CXCL8* (8×10^2) and *LYZ* (9×10^1). In WF2 cells, the highest copy numbers were detected for *tOSTF1* (4×10^5), *HSF1* (1×10^5), and *HSF2* (5×10^4), while *EPAS1* (8×10^2), *NR3C1* (5×10^2), *CXCL8* (1×10^2), and *LYZ* (6×10^1) were only marginally expressed. Transcript levels of *HIF1A*, *IL1B*, and *IL10* were not detectable.

Expression profiles of unchallenged WF2 cell line

Due to the above described difficulties in establishing a specific cell line from *S. lucioperca*, we investigated the

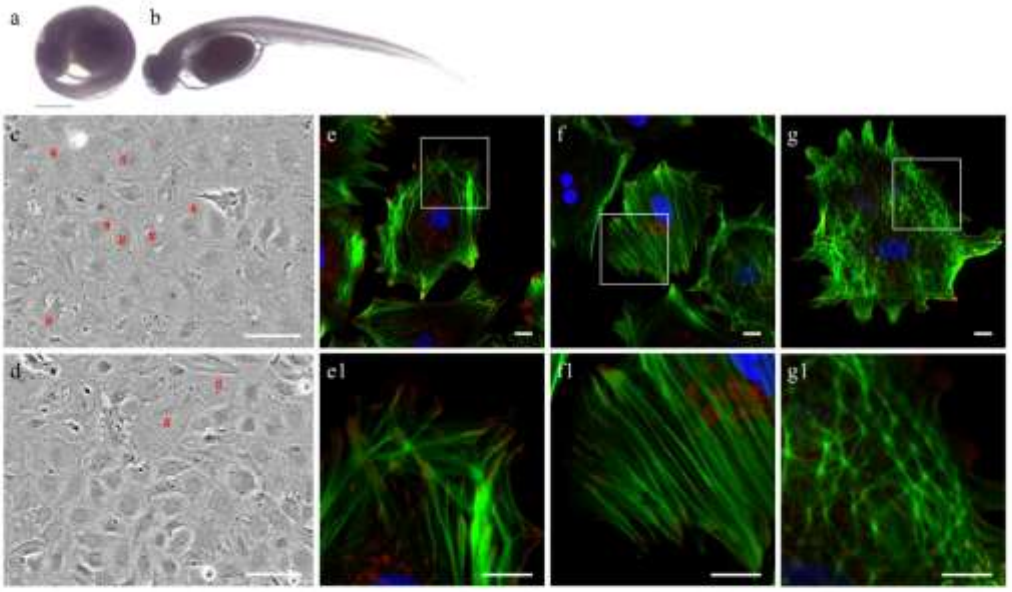


Fig. 2 Source and morphology of SLUlar1 cells from *Sander lucioperca*. Larvae at an age of 125 degree days (DD; nine days after fertilization) in (a) the egg and (b) isolated from the eggshell, phase contrast microscopy of isolated SLUlar1 cells from (c) passage five and (d) passage six. High concentrations of actin filaments are marked with #. Immunofluorescence: Cells with cortical actin rings (e) clearly differ from cells with stress fibers throughout the cell body (f) and cells with cross-linked actin networks (g). ß-actin: green; vinculin: red; nuclei: Hoechst 33342 dye. Scale bars: (a–d) 100 µm, (e–g) 10 µm

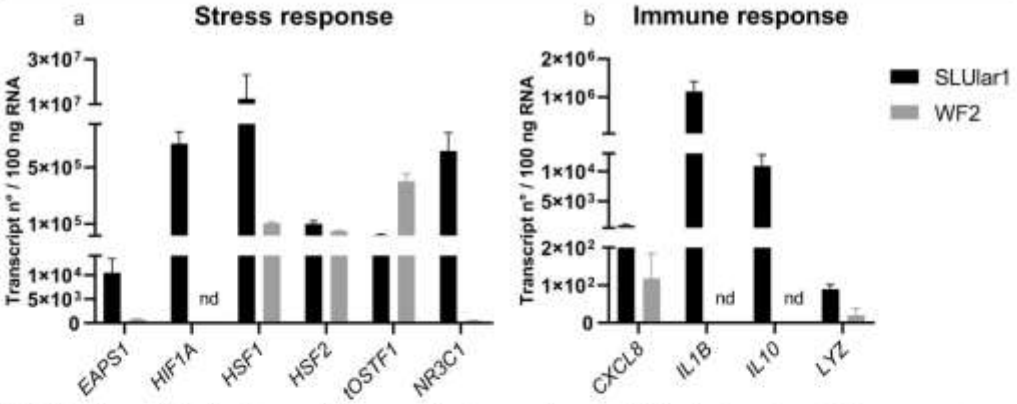


Fig. 3 Basal transcript levels of stress and immune marker in unchallenged cell models. Transcription patterns of genes important for stress (a) and immune response (b) in SLUlar1 (black columns) and WF2 cells (grey columns). Columns represent normalized means of three individual samples (+ SEM), calculated per 100 ng of total RNA. nd: no data detectable

suitability of the existing cell line WF2 from *S. vitreus* to examine the expression of the 21 ontogenetic target genes from the in vivo study (compare Table 1). For 16 of these genes, we detected relevant transcript numbers in WF2 cells. However, we did not for *CKM*, *IGF2*, *HIF1A*, *IL1B*, and *GATM*. We recorded average transcript numbers from 6×10^1 (*LYZ*) to 2×10^6 (*BMP4*) copies per 100 ng RNA (Table 2). Gene expression in the WF2 cell line was similar compared with that in pikeperch at 0 dph in the case of *GHR*, at 4 dph in the case of *PPARD*, and fingerlings in the case of *MYH6* (175 dph), *PPARA* (175 dph), *RXRA* (121 and 175 dph), and *tOSTF1* (121 and 175 dph). Moreover, transcript levels of *HSF2* in the cells were similar to several developmental stages of whole pikeperch (0, 18, 121, 175 dph). In contrast, *APOE*, *TFEB*, *GATM*, *EPAS1*, *HSF1*, *NR3C1*, *LYZ*, and *SOX9* were expressed slightly less, while *BMP4* and *BMP7* were more strongly expressed in WF2 cells than in vivo.

Discussion

The farming of pikeperch in intensive aquaculture systems such as RAS is continuously growing in importance for European aquaculture. The strategies for intensive pikeperch rearing generally focus more on economic considerations than on welfare concerns. However, optimizations of intensive aquaculture techniques are necessary to improve animal welfare as well as to increase the production of fingerlings for stocking considering the determination of optimal breeding parameters as well as the definition of limit values. The current study evaluated key genes involved in the early steps of ontogenesis to improve basic knowledge of the physiological processes during pikeperch rearing and the associated challenges.

Transcription factors *HSF1* and *HIF1A* with uniform or biphasic expression patterns

While the majority of the examined genes displayed an ontogenesis-specific transcription pattern with a peak expression at a specific developmental stage, *HSF1* was constitutively expressed. This transcription factor is the major regulating factor involved in the response to environmental stressors in vertebrates (Morimoto 1998; Buckley and Hofmann 2002; Padmini and Usha Rani 2009). It coordinates the transcriptional activation of heat shock proteins (HSPs), protects protein and lipid metabolism during stress conditions, and contributes to distinct immune processes (Deane and Woo 2004; Roberts et al. 2010). The constant expression of *HSF1* in the present study indicates that no specific stress response was induced by *HSF1* at any time point.

For *HIF1A*, we noticed a biphasic expression pattern with a significant increase of copy numbers at 4 and 175 dph compared with the transcript level at 0 dph. During organogenesis as well as during the period of growth in general, the organs require higher levels of oxygen

Table 2 Expression profiles of candidate genes in the cell line WF2

	EPAS1	*HSF1*	*HSF2*	*tOSTF1*	*NR3C1*	*LIZ*	*TFEB*	*APOE*
n°	7.76E+02	1.03E+05	4.77E+04	4.00E+05	4.89E+02	5.92E+01	4.05E+04	1.80E+04
SEM	1.02E+02	7.40E+03	4.86E+03	4.54E+04	7.78E+01	1.61E+01	5.92E+03	1.24E+03
	PPARA	*PPARD*	*GHR*	*SOX9*	*BMP4*	*BMP7*	*MYH6*	*RXRA*
n°	5.29E+05	1.57E+05	6.67E+04	2.00E+04	1.54E+06	7.26E+05	1.60E+04	2.32E+05
SEM	1.09E+04	8.96E+03	3.11E+03	1.36E+03	1.56E+05	5.34E+04	6.02E+03	1.71E+04

n°, transcript number per 100 ng of total RNA; *SEM*, standard error of the mean; not detectable: *CKM*, *GATM*, *HIF1A*, *IGF2*, *IL1B*

(Anderson and Podrabsky 2014). *HIF1A* is the major regulator of the response to hypoxia (Rytkönen et al. 2007; Geng et al. 2014). Additionally, *HIF1A* is involved in several physiological processes during vertebrate development, such as angiogenesis, glucose uptake and metabolism, and cellular proliferation, as well as apoptosis (Gracey et al. 2001; Vuori et al. 2004; Rojas et al. 2007; Liu et al. 2017; Tan et al. 2017). The observed constitutive *HIF1A* expression pattern in pikeperch resembles observations during the early ontogenesis of zebrafish (*Danio rerio*), Wuchang bream (*Megalobrama amblycephala*), and lake whitefish (*Coregonus clupeaformis*) (Rojas et al. 2007; Shen et al. 2010; Whitehouse and Manzon 2019). Nevertheless, *HIF1A* appears to underly species-specific modulations during the different developmental stages, as exhibited in zebrafish and lake whitefish (Rojas et al. 2007; Whitehouse and Manzon 2019).

Gene expression of *APOE*, *BMP4*, *BMP7*, *CKM*, *GATM*, and *TFEB* reflect challenges in feed conversion

At 0 dph, we uncovered high *APOE* transcript levels. The encoded apolipoprotein E is involved in the vertebrate lipid metabolism, where it is crucial for the internalization of plasma lipoproteins into the cell (Mahley 1988; Babin et al. 1997). Fish egg yolk contains high amounts of lipoproteins (Wiegand 1996). Poupard et al. (2000) determined high levels for *APOE* transcripts in the yolk sac of embryonic and larval turbot (*Scophthalmus maximus*). *APOE* is highly expressed in the yolk syncytial layer and appears to control its utilization during the early steps of ontogenesis (Otis et al. 2015). Thus, our findings in pikeperch embryos are in line with this study. Furthermore, our data indicate a general decline in the expression levels from 0 dph to the later stages, suggesting the necessity of *APOE* for the continuous consumption of the yolk sac reserves from egg to larvae as well as the final basal expression level for lipid metabolism after entering the exotrophic feeding phase.

The examined yolk sac larvae at 4 dph demonstrated the highest expression levels for genes involved in organogenesis (*BMP4*, *BMP7*, and *MYH6*), general stress response (*NR3C1*), and energy metabolism (*CKM*). *BMP4* and *BMP7* encode for bone morphogenetic proteins 4 and 7. These proteins are involved in the process of chondro- and skeletogenesis as well as in the morphogenesis of several vertebrate organs (Streelman et al.

2003; Hoffman et al. 2006; Adams et al. 2007; Bonilla-Claudio et al. 2012). *MYH6* is expressed in the heart muscles of vertebrates (Dhillon et al. 2009; López-Unzu et al. 2019). The myosin heavy chain is the major component of the motor protein myosin in eukaryotic cells (Vikstrom et al. 1997).

Perciformes such as pikeperch and Eurasian perch undergo several morphological changes, including skull and jaw adaptation as well as fin and teeth development around hatching time and until the start of the first uptake of exogenous feed (Löffler et al. 2008; Ott et al. 2012b; Alix et al. 2015). Güralp et al. (2017) determined the beginning of pectoral fin formation at 1 dph and mouth opening at 5 dph in pikeperch reared at 15 °C (Güralp et al. 2017). Before mouth opening, Ostaszewska et al. (2005) observed changes within the larval intestines of pikeperch, including the length, the lumen, and the appearance of the mucosal lining. Another aspect of organogenesis is the growth of the developing organs (Ostaszewska 2005). The first heart beating in pikeperch was observed at the 34-somite stage (around 1.5 h post fertilization [hpf]) and the beginning of blood circulation at the 50-somite stage, right before the start of hatching (equal to around 2 hpf) (Güralp et al. 2017). In our study, the highest expression of the *BMP4* and *MYH6* at 4 dph might reflect these processes. In line with this, the subsequent stages of fed larvae and fingerlings were characterized by a significant decrease in copy numbers (except for *BMP4* at 7 dph).

The process of hatching and adapting to new environmental conditions is stressful and therefore energy-consuming. The glucocorticoid receptor, encoded by *NR3C1*, is the main regulator of the general stress response in vertebrates, including developing fish (Pavlidis et al. 2011; Tsalafouta et al. 2018). We recorded a significant *NR3C1* increase from 0 to 4 dph, with a stable expression at later stages of ontogenesis. CKM serves as an energy buffer in mammals and fish (Wyss and Kaddurah-Daouk 2000; Borchel et al. 2014; Borchel et al. 2019). It is responsible for the dephosphorylation of creatine in muscle cells, which is further used for the regeneration of ADP to ATP within the target tissue (Wyss and Kaddurah-Daouk 2000). Our results for *NR3C1* and *CKM* seem to reflect the restructuring programs of the developing body of freshly hatched larvae and the challenges of the new environment such as oxygen and carbon dioxide gas exchange or the acclimatization to new energy resources due to exogenous feed intake (*Artemia* spp. followed by dry feed).

At day 5 post hatching, the mouth opens and the mixotrophic feeding phase begins, until complete yolk sac resolution (up to 14 dph) (Güralp et al. 2017). Due to the small size of freshly hatched pikeperch (4–5-mm total length) (Schlumberger and Proteau 1996) and the incompletely developed intestine, the initial feeding requires live prey (Hamza et al. 2007). The phase of conversion from endo- to exotrophic feeding is highly critical, since this is when fish react strongly to chemical or physical stimuli (Woltering 1984). During this important phase of development at 7 dph, genes involved in energy metabolism (*GATM*), nutrition (*TFEB*), gonadal maturation (*SOX9*), and stress response (*HSF2*) were most strongly expressed.

GATM contributes to the creatine energy system in mammals and fish (Borchel et al. 2014; Borchel et al. 2019). For pikeperch, we detected similar expression levels in all larval stages at 4–18 dph, with the highest copy numbers at 7 dph. At that stage, larvae experience exogenous feed intake including digestion for the first time. Moreover, the complete yolk sac resorption must be compensated to reach homeostasis of the energy metabolism. Thus, high expression of *GATM* is concordant with the required new energy levels. In line with this, we found that the expression of the gene encoding transcription factor EB (*TFEB*) gradually increased from 0 dph to larvae fed with *Artemia* spp at 7 dph. and then decreased in later developmental stages. In vertebrates, TFEB is important for cell homeostasis and is involved in several cellular processes, such as lipid metabolism, bone resorption, and immune response (Ferron et al. 2013; Settembre et al. 2013; Tiller and Garsin 2014). According to Settembre et al. (2013), TFEB is the main factor coordinating the metabolic response to the process of starvation in the nematode *Caenorhabditis elegans*. The first oral feeding most likely provoked an immune response due to the first contact with non-self molecules. Furthermore, the final resorption of the endogenous yolk sac energy resources might lead to a state similar to starvation (until first feeding), while the exogenous feed intake delivers energy. All three conditions might modulate the observed transcription of *TFEB*.

SOX9 is critical for the sex determination, gonad formation, and development of vertebrates (Yokoi et al. 2002; Chaboissier et al. 2004). In Nile tilapia (*Oreochromis niloticus*), *SOX9* is highly expressed during the early

ontogenesis of both sexes, but the concentration decreases in later stages (Ijiri et al. 2008). In pikeperch, we found a similar transcription pattern with the highest expression of *SOX9* at 7 dph, followed by a decline in later developmental stages. This pattern was also observed for *HSF2*, which is involved in the development of embryos in vertebrates (Eriksson et al. 2000). This pattern might reflect the contribution of both factors to early developmental processes.

Subsequent to the transition from endo- to exotrophic feeding, the conversion to artificial feed is another challenging phase that influences growth and mortality (Kestemont et al. 2007; Hubenova et al. 2015). Several studies have demonstrated that the transversion from endo- to exogenous feed is a critical step in the farming of fish larvae (Hamza et al. 2007; Kestemont et al. 2007). Unexpectedly, none of the evaluated genes displayed a peak expression in the stage of larvae fed with dry feed (18 dph).

The genes *GHR*, *IGF2*, and *LYZ* were highly expressed during the growth phase of juvenile stages

Within fingerlings at 121 and 175 dph, genes of stress (*tOSTF1*, *EPAS1*) and immune response (*LYZ*, *IL1B*), growth (*GHR*, *IGF2*), organogenesis (*RXRA*), and nutritional status (*PPARA*, *PPARD*) were highly expressed. The innate immune genes *LYZ* and *IL1B* are part of the first line of defense. The transcript levels of c- and g-type lysozyme were found to be low in olive flounder (*Paralichthys olivaceus*) from hatching until 20 dph, followed by a significant increase to 50 dph (Lee et al. 2014). Our results indicate a similar significant increase in the mRNA levels of *LYZ* from early stages at 0–18 dph to the stage of juveniles at 121 dph.

The two genes, *GHR* and *IGF2*, are involved in the growth of fish (Schlueter et al. 2007; Besseau et al. 2013; Claudino da Silva et al. 2019). Investigations of gilthead sea bream (*Sparus aurata*) demonstrated a positive correlation between the expression of *GHR* and a growth spurt during summer months (Calduch-Giner et al. 2003). Nipkow et al. (2018) detected increased *IGF2* transcript levels in maraena whitefish (*Coregonus maraena*) at the onset of oral feeding and during development into fingerlings. We found similar patterns in pikeperch with an increase in *IGF2* and *GHR* transcript levels from 0 dph to 4 and 7 dph and the highest levels at 121 dph, reflecting the strong phase of growth of fingerlings.

The long-lasting primary cell culture SLUlar1 shows prominent sensitivity including stress fiber formation and the high expression of stress-related genes

To establish optimal pikeperch farming conditions, detailed research regarding its physiological needs is indispensable. Especially in basic research, in vitro analyses can be a suitable replacement for animal experiments. Currently, no specific cell model of pikeperch is available.

Here, we present an approach to derive a cell model from pikeperch. However, embryonal *Sander* cells are apparently more sensitive to the standard handling process compared with other primary fish cells from, for example, Atlantic sturgeon, Atlantic salmon (*Salmo salar*), Siberian sturgeon (*Acipenser baerii*), maraena whitefish, rainbow trout (*Oncorhynchus mykiss*), and zebrafish (Ciba et al. 2008; Grunow et al. 2011b; Grunow et al. 2011a; Grunow et al. 2015). The cells stopped to proliferate and remained in the stagnation phase from passage eight onward. Furthermore, they accumulated actin filaments in the cytoskeleton, which are well-known indicators of stress. We identified three different actin populations at cell passage six; one had cortical actin rings and low stress fiber formation, which might be derived from cells of the epithelial or endothelial lineage. These cells grew firmly together and formed a stable cell architecture by using their neighboring cells as mechanical support. In another population we observed significant stress fibers, which characterizes mechanically stretched cells like muscle cells or bone cells. These cells transmit forces through their cell body and therefore have a strong actin network. Moreover, we observed a star-shaped formation of actin, which has not been described in unstimulated cells thus far. However, CLANs (cross-linked actin networks) can occur under stress or by stimulating different integrin signaling pathways (i.e., via different extracellular matrix proteins) (Filla et al. 2009; Job et al. 2010).

On the transcriptional level, high transcript numbers were detected for immune and stress marker genes *IL1B*, *NR3C1*, *HIF1A*, *HSF1*, and *HSF2* at the sixth cell passage. *IL1B* is a well-established marker for in vitro stimulation with pathogen-associated molecular patterns (PAMPs) in the primary cells of different fish species (Chaves-Pozo et al. 2004; Martorell Ribera et al. 2020). Compared with the cell line WF2, SLUlar1 cells showed prominent higher transcript levels for the examined genes, except for *tOSTF1*. Along with high expression

levels of either general cellular and environmental (*NR3C1*) or specific environmental stress response genes (*HIF1A*, *HSF1*, *HSF2*), we conclude that the current culturing conditions lead to induced stress within the SLUlar1 cells.

The WF2 cell line is currently the most suitable cell model, but does not correspond to a specific ontogenetic stage of pikeperch

As a substitution for a cell model from *S. lucioperca*, we initially tested the applicability of the in vitro system WF2 (*S. vitreus*) for investigating developmental processes of pikeperch. The expression for most of the genes could be verified, although a clear assignment to a certain developmental stage of the investigated pikeperch samples could not be detected. However, we must be aware of the dissimilarity between an in vitro model and the complexity of a whole organism. Moreover, no further detailed information about the exact ontogenetic stage of the source material is available.

Conclusion

The process of ontogenesis is accompanied by the continuous adaptation to changing physiological and environmental conditions. In the present study, we determined basal expression patterns of promising molecular markers for monitoring the developmental process of early ontogenesis in pikeperch under current farming conditions. We identified promising candidates representing the challenging steps of feed conversion (*APOE*, *BMP4*, *BMP7*, *CKM*, *GATM*, and *TFEB*) and the growth phase of juvenile pikeperch (*GHR*, *IGF2*, *RXRA*, *PPARA*, and *PPARD*), which can be used to accompany the development process of pikeperch farming in future studies. A first approach to establishing a long-lasting primary cell culture from whole pikeperch embryos was achieved. However, the importance of establishing a suitable cell line has been demonstrated, since it remains a major challenge to yield reproducible results.

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Data availability Not applicable.

Code availability Not applicable.

Declarations

Ethics approval 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: 7221.3-1-009/19).

Consent to participate Not applicable.

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5.3. Effects of Chronic Hypoxia on the Immune Status of Pikeperch (*Sander lucioperca* Linnaeus, 1758).

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Effects of Chronic Hypoxia on the Immune Status of Pikeperch (*Sander lucioperca* Linnaeus, 1758)

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Simple Summary: Inadequate oxygen saturation, or hypoxia, belongs to one of the critical stress factors in intensive aquaculture. Exposure of fish to low oxygen levels over prolonged periods substantially affects their well-being and immune competence, resulting in increased disease susceptibility and consequent economic losses. In this interdisciplinary research, we aimed to provide a deeper understanding of the effect of chronic low oxygen saturation on pikeperch farmed in recirculating aquaculture systems. The obtained data offer unprecedented insights into the changes in the immunocompetence of studied fish and suggest high robustness of this new aquaculture species to the stress factors of intensive aquaculture.

Abstract: Inadequate oxygen saturation can induce stress responses in fish and further affect their immunity. Pikeperch, recently introduced in intensive aquaculture, is suggested to be reared at nearly 100% DO (dissolved oxygen), yet this recommendation can be compromised by several factors including the water temperature, stocking densities or low circulation. Herein, we aimed to investigate the effect of low oxygen saturation of 40% DO (±3.2 mg/L) over 28 days on pikeperch farmed in recirculating aquaculture systems. The obtained data suggest that—although the standard blood and health parameters did not reveal any significant differences at any timepoint—the flow cytometric analysis identified a slightly decreased proportion of lymphocytes in the HK (head kidney) of fish exposed to hypoxia. This has been complemented by marginally downregulated expression of investigated immune and stress genes in HK and liver (including *FTH1*, *HIF1A* and *NR3C1*). Additionally, in the model of acute peritoneal inflammation induced with inactivated *Aeromonas hydrophila*, we observed a striking dichotomy in the sensitivity to the low DO between innate and adaptive immunity. Thus, while the mobilization of myeloid cells from HK to blood, spleen and peritoneal cavity, underlined by changes in the expression of key proinflammatory cytokines (including *MPO*, *IL1B* and *TNF*) was not influenced by the low DO, hypoxia impaired the influx of lymphocytes to the peritoneal niche in the later phases of the immune reaction. Taken together, our data suggest high robustness of pikeperch towards the low oxygen saturation and further encourage its introduction to the intensive aquaculture systems.

Keywords: pikeperch; hypoxia; intraperitoneal stimulation; immune response; stress response; *Aeromonas hydrophila*

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1. Introduction

Low dissolved oxygen (DO) levels induce primary, secondary and tertiary stress responses in fish [1]. Optimal oxygen saturation is a vital parameter for animals and, therefore, tightly controlled in intensive aquaculture facilities. Hypoxia is defined as depletion of oxygen concentration, which can substantially affect the fish's well-being and immune status, resulting in increased susceptibility to stressors and reduced resistance to pathogens [2]. Bregnballe (2015) defined oxygen saturations below 40% (equivalent to ~3–4 mg/L at 20–25 °C) as inadequate for aquaculture facilities in general [3]. The required concentration of dissolved oxygen is dependent on the fish species and the corresponding water parameters. Furthermore, the animal-specific sensitivity to low oxygen saturation and the duration and the intensity of hypoxic conditions influence the outcome of the triggered response [4,5]. There are several reasons for low DO levels in aquaculture systems, including inadequate water circulation [6], high water temperatures [7] and high stocking densities [8]. Percids seem to be relatively tolerant to low DO levels [9,10]. For instance, feeding rate of yellow perch (*Perca flavescens*) consumption is negatively regulated only with oxygen levels of 3.5 mg/L and lower [11]. The growth of walleye (*Sander vitreus*) and yellow perch is affected by oxygen levels only below 2 mg/L [12–14]. Nevertheless, levels below 5 mg/L DO can induce significant stress responses in Eurasian perch (*Perca fluviatilis*) [15].

The pikeperch (*Sander lucioperca*) is native to fresh and brackish waters of the northern hemisphere [16], and due to its high-quality flesh and high market acceptance, it became a significant food fish for regional aquaculture in Europe [17]. Within its natural habitat, the DO levels range from 5.5 to 12.9 mg/L [18,19]. Thus far, nearly 100% DO (equivalent to ~7–9 mg/L DO at 20–25 °C) has been suggested as the optimal oxygen saturation for pikeperch aquaculture [20–22]. However, the lower limit of the pikeperch's tolerance to low DO levels under intensive farming, and its impact on physiology and immunity remains vague. Previously, Stejskal et al. (2012) observed that a 50–60% oxygen saturation (equivalent to ~4–6 mg/L DO at 20–25 °C) correlates with lower feed intake and a reduced growth rate in intensive pikeperch farming [23], while in independent study similar oxygen saturation for 36 days led to a significant increase in the complement activity of the sera [24]. Nevertheless, a more detailed understanding of the effect of chronic low oxygen saturation on immune system of pikeperch is absent. In this study, we aimed to fill this gap and elucidate how low oxygen saturation of 40% (±3.2 mg/L DO) for 28 days influences the standard health and immune system parameters and expression of selected genes involved in the regulation of immune and stress responses. Furthermore, to gain further insights into the capacity of the immune system to induce inflammatory responses under low DO, we employed a previously established model of acute peritonitis with *Aeromonas hydrophila*, Gram-negative bacteria associated with mortality of fish kept under adverse environmental conditions [25,26].

2. Materials and Methods

2.1. Fish Rearing and Experimental Design

Ninety-eight juvenile pikeperch (obtained from Anapartners, s.r.o., Prague, Czech Republic), with an average length of 29.51 ± 0.47 cm and body weight of 219.39 ± 9.79 g, were reared in a recirculating aquaculture system (RAS) at the Institute of Aquaculture and Protection of Waters (IAPW; České Budějovice, Czech Republic) from June to August 2018. Fish were randomly assigned in six identical black plastic 300 L tanks and acclimated for two weeks at 23.1 ± 1.0 °C with 12:12 h day/night light period and a light intensity of 20–35 Lx. Water was pre-treated by drum and moving bed filters in complement with UV disinfection and aeration. In all tanks, the inflow-outflow rate was 6 L/min, generated through mixing towers, and in-tank oxygen saturation was monitored online using the controller HACH SC 1000 (HACH Lange, Düsseldorf, Germany). Feeding was performed with a commercial extruded diet (EFICO Sigma 970, 3 mm, BioMar A/S, Brande, Denmark) by automatic feeders (EHEIM Twins, Deizisau, Germany; 6 meals per day) and one hand

feeding. Fish were fed ad libitum. The experimental design is illustrated in Figure 1. The experiment was performed in triplicate tanks for up to 28 days, with ±3.2 mg/L DO levels (40% oxygen saturation) for "low DO group" (total n = 49; 12–24 per tank) and ±8.3 mg/L DO levels (>95% oxygen saturation) for the control group (total n = 49; 12–24 per tank). Low DO conditions were established by additional nitrogen administration according to the oxygen depletion system generated by Pichavant et al. (2000) [27], including individual modifications. Water quality parameters (NH_3, NH^{4+}, NO^{2-} and NO^{3-}) were monitored throughout the experiment in the two-day interval using commercial kits (LCK 304, LCK 341, LCK 339 and HACH Lange, Düsseldorf, Germany) and spectrophotometric analyses (DR 3900 and HACH Lange, Düsseldorf, Germany). The concentration of ammonia, nitrite, and nitrate was 0.24 ± 0.11, 0.21 ± 0.13 and 4.54 ± 4.18, respectively. Temperature and pH were monitored daily with HACH HQ40 multimeter and reached 23.5 ± 0.7 °C and 7.38 ± 0.25, respectively.

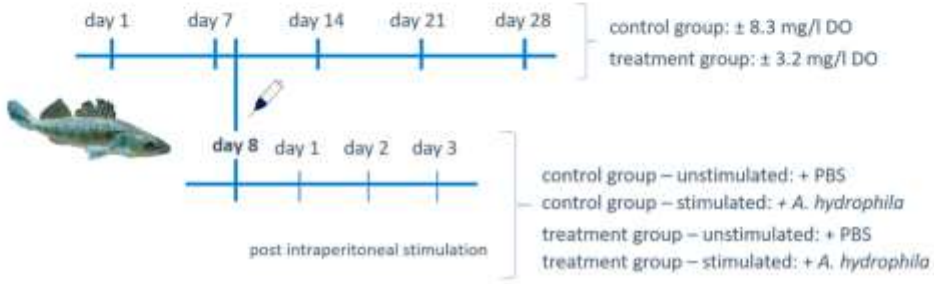

Figure 1. Outline of the experimental design. The 98 adult pikeperch were kept either under normoxic water conditions (±8.3 mg/L dissolved oxygen (DO) level) or low DO saturation (±3.2 mg/L DO level) for up to 28 days. Peripheral blood, head kidney (HK) and liver tissues were sampled from five fish of both groups. Additionally, at day 8 of the experiment, 48 fish were intraperitoneally injected with either 1×10^7 inactivated *Aeromonas hydrophila* cells in 100 µL sterile phosphate-buffered saline solution (PBS) or exclusively 100 µL PBS. At three following days, peritoneal leukocytes, peripheral blood, HK and spleen were collected from four fish per group.

Before sampling, fish were anesthetized with 30 µL/L clove oil and stunned in compliance with the terms of the Czech legislation (Section 29 of Act No.246/1992 Coll., on Protection of animals against cruelty, as amended by Act No. 77/2004 Coll.). All animal experiments have been approved by the Ministry of Education, approval ID: MSMT-18301/2018-2.

On days one, seven, fourteen, twenty-one and twenty-eight of the treatment, we sampled peripheral blood, head kidney (HK) and liver of five fish per control and low DO group. Additionally, to elucidate the impact of hypoxia on acute inflammation, we performed peritoneal stimulation described below. Upon induction of peritoneal inflammation, peripheral blood, HK, spleen and the peritoneal leukocytes were sampled at day one, two and three post-stimulation at days nine, ten and eleven of the hypoxia experiment. Peripheral blood was collected from the caudal vein with heparinized (Sigma-Aldrich, Taufkirchen, Germany) syringes. Parts of the HK, liver, and spleen were snap-frozen in liquid nitrogen and stored at −80 °C until RNA isolation. The peritoneal leukocytes were obtained from peritoneal lavage as described previously [28].

2.2. Intraperitoneal Stimulation with Aeromonas hydrophila

To evaluate the impact of low DO on the acute inflammatory response, fish were stimulated with 1.5% paraformaldehyde (PFA) inactivated *A. hydrophila*. To this end, 48 pikeperch (24 each per control and low DO group, 208.85 ± 6.58 g) were intraperitoneally injected at day eight of the experiment (Figure 1) with either a total of 1×10^7 *A. hydrophila* in 100 µL sterile phosphate-buffered saline solution (PBS) or exclusively with 100 µL PBS.

2.3. Cell Isolation

The blood samples were diluted 1:150 in DMEM (Dulbecco's modified eagle medium; Gibco/Life Technologies, Carlsbad, CA, USA) on ice. The remaining HK and spleen were homogenized by cell strainer (100 µm; Corning Inc., Corning, NY, USA) and resuspended in 4 mL DMEM (Gibco) on ice. Collected cells were further 1:1 diluted in DMEM (Gibco) on ice, layered onto an isotonic Percoll™ (Ge Healthcare, Uppsala, Sweden) gradient (34% plus 51%; r = 1.075 g/mL) on ice and centrifuged at 800× *g* for 15 min at 8 °C. We collected the HK leukocytes at the interphase of the different Percoll™ concentrations, washed the cell suspension and, finally, resuspended it in 500 µL DMEM (Gibco) on ice. Cells were further 1:2 diluted in DMEM (Gibco) on ice and applied for flow cytometry.

2.4. Flow Cytometry

To investigate the cell composition of peripheral blood, HK and spleen samples we applied flow cytometry analysis using the BD FACSCanto™ II system (BD Biosciences, Prague, Czech Republic) in medium flow rate (1 µL/s). Briefly, the leukocytes from HK and spleen were diluted to a concentration 1×10^6/mL and 100 µL of the cell suspension was used for the measurement. Furthermore, 2 µL of whole blood were diluted in 200 µL of DMEM containing the DIOC6 dye and recorded for 20 s at a constant flow of 1 µL/second. To determine the total number of peritoneal leukocytes, we employed the flow cytometry protocol described previously [28]. The cell morphology was evaluated using the FSC-SSC parameters, with lymphocytes being FSC^{lo}-SSC^{lo} and myeloid cells being defined as FCS^{hi}-SSC^{hi}.

2.5. Health Parameters

We measured traditional health indicators, including total length, weight, spleno- (SSI) and hepato-somatic indices (HSI), as well as concentrations of glucose, lactate and plasma cortisol levels. The SSI was calculated by the formula: spleen weight (g)/body weight (g) × 100; the HSI was calculated by the formula: liver weight (g)/body weight (g) × 100. Whole blood glucose and lactate levels were analyzed using the Accutrend® Plus device (Cobas; Roche Diagnostics, Mannheim, Germany). Plasma cortisol levels were measured by the Cortisol ELISA assay (DRG Instruments, Marburg, Germany) according to the manufacturer's instructions.

2.6. Gene Selection and Primer Design

To evaluate the transcriptional response to low DO saturation and the capacity of fish to induce inflammatory responses under stress, we established a screening panel with 15 genes involved in stress and immune response (listed in Table 1).

The genes, elongation factor 1 alpha (*EEF1A1*), ribosomal protein L32 (*RPL32*) and ribosomal protein S5 (*RPS5*) were applied as reference according to Swirplies et al. (2019) [29]. For the candidate genes interleukin 8 (*CXCL8*), hypoxia-inducible factor 1 subunit alpha (*HIF1A*), heme oxygenase 1 (*HMOX1*), heat shock transcription factor 1 (*HSF1*), heat shock transcription factor 2 (*HSF2*), heat shock protein 90 alpha family class A member 1 (*HSP90AA1*), interleukin 1 beta (*IL1B*), nuclear receptor subfamily 3 group c member 1 (*NR3C1*) and tumor necrosis factor (*TNF*) pikeperch-specific oligonucleotide sequences were already available from our former studies [29,30]. Using our recently published pikeperch genome (RefSeq NCBI: GCA_008315115.1) [31], we identified the remaining orthologs for *S. lucioperca*. The Pyrosequencing Assay Design software (version 1.0.6; Biotage, Uppsala, Sweden) was applied to derive optimal oligonucleotide primers. We purchased all primers from Sigma-Aldrich, Taufkirchen, Germany) and validated them by sequencing their PCR products (Applied Biosystems 3130 Genetic Analyzer; Life Technologies, Carlsbad, CA, USA).

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

Gene Symbol	Official Names	Sense Primer (5′-3′)	Antisense Primer (5′-3′)	Primer Efficiency [%]	Fragment Length [bp]
	Reference genes:				
EEF1A1	Elongation factor 1 alpha	ATGGACAGACCCGTGAGCATG	TTCTTGATGTAGGTGCTCACTTC	105	151
RPL32	Ribosomal protein L32	GGCGTAAACCCAGAGGTATTGA	ACCTCGAGCTCCTTGACATTGT	105	157
RPS5	Ribosomal protein S5	GCAGGATTACATTGCTGTGAAAG	TCATCAGCTTCTTGCCATTGTTG	101	161
	Target genes:				
Stress response					
EPAS1	Endothelial PAS domain protein1	AGTGCAGAGGACGCACAGATG	TCATGTTCACCTGCGTGAGCC	100	139
HIF1A	Hypoxia inducible factor 1 subunit alpha	CCAGTCGAATCCCTTGAGAGTT	CTGTGGGGTCCTCTTAGCAAC	97	156
HMOX1	Heme oxygenase 1	GCTCGCTGTATGAGGTCTACC	TCTCTCCAGTCCTGGCCATAG	101	154
HSP90AA1	Heat shock protein 90 alpha family class A member 1	AGATACTACACCTCGGCTTCTG	TCACCAGTGATGTAGTAGATGTG	100	101
HSF1	Heat shock transcription factor 1	TGTGTCTTGTGCAGAGTGGAAC	GCTGGCCATGTTGTTGTGTTTG	111	101
HSF2	Heat shock transcription factor 2	AGCCGTCCCGCAGCTCCCT	CGGGACTCAGTTCGCACAGG	91	93
NR3C1	Nuclear receptor subfamily 3 group c member 1	CCAGTCCTGCATGGATTCACTT	AGGTCCATAGTGTTGTCACTGAA	100	180
Immune response					
CSF2 *	Colony stimulating factor 2	CCAGCAGGAATACACAGAAATCT	CGCAGATAGAGACAATGATGAAG	95	164
CXCL8 *	Interleukin 8	AACAGGGATGAGTCTGAGAAGC	GCTTGGAAATGAAGTCTTACATGA	98	158
FTH1	Ferritin heavy chain 1	AGAACTGGCAGACTGGGTGAC	CTGCTTTCTTTGCCCAGGGTG	99	102
IL1B *	Interleukin 1 beta	TCGACCTACTTGCACCCTACA	TCTGCCTCCACAACCTGAA	101	137
MHC II alpha *	Major histocompatibility complex II alpha	TGGACCAACCACTGACCAGAAT	CATCATCAGTCCCAGCCAATCA	99	168
MPO *	Myeloperoxidase	GTTTGATCGGCCGTCCTGCTA	ATTACCAGCCAAGCCATGGTCA	98	152
RAG1 *	Recombination activating 1	CTCAGGCTTCAGTGTCATGATC	AACCTCTTTCTCCTCCTCGTCT	95	157
TNF *	Tumor necrosis factor	GTCTTTGGAACCAGGCTATTTAC	TTTATGCCTCAGGCTTGACTGG	89	157

* Genes applied exclusively for stimulation experiment.

2.7. RNA/cDNA Preparation

Total RNA was extracted from collected samples by homogenizing tissues (HK, liver and spleen) separately within 1 mL TRIzol Reagent (Invitrogen/Thermo Fisher Scientific, Karlsruhe, Germany), as stated in the manufacturer's instructions. Subsequently, we purified all samples with the RNeasy Mini Kit (Qiagen, Hilden, Germany), including DNAse treatment. For isolated HK leukocytes, 3.5 µL of 2-mercaptoethanol (Sigma-Aldrich, Taufkirchen, Germany) was added, and samples were purified with the ISOLATE II RNA Mini Kit (Bioline/Meridian Bioscience, London, UK). Applying gel electrophoresis and spectrophotometry analysis in repeated measurements (ND 1000; NanoDrop Technologies/Thermo Fisher Scientific, Waltham, MA, USA), the quantity and quality of the isolated nucleic acids were determined. Collected RNA was stored at −80 °C until further application.

Synthesis of cDNA was performed from 1.0–1.5 µg of total RNA using the SuperScript II Reverse Transcriptase Kit (Thermo Fisher Scientific, Karlsruhe, Germany) according to the manufacturer's protocol, and cDNA was stored at −20 °C.

2.8. Real-TIME Quantitative PCR (rt-qPCR)

The gene expression during low DO levels was evaluated by real-time quantitative PCR (rt-qPCR). Therefore, we implemented the SensiFAST™ SYBR No-ROX Kit (Bioline, Luckenwalde, Germany) and the LightCycler96 system (Roche, Basel, Switzerland). PCR conditions were as follows: the initial denaturation step (95 °C, 5 min) was followed by 40 cycles of denaturation (95 °C, 15 s), annealing (60 °C, 10 s), elongation (72 °C, 20 s) and a fluorescence measurement step for 10 s (75 °C). For the copy-number calculation by linear regression analysis ($R^2 > 0.999$), standard curves based on Cq values of tenfold dilutions of the generated fragments (1×10^3–1×10^8 copies) were generated. Cq values > 35 were considered as not detectable. For confirmation of the quality of all PCR products, we conducted melting curve analysis and gel electrophoresis.

Three reference genes (*EEF1A1*, *RPL32* and *RPS5*) for *S. lucioperca* [29] were evaluated for each sample and applied for data normalization.

2.9. Statistics

Rt-qPCR data were evaluated with the LightCycler 96 software v. 4.0.1. Flow cytometry data were analyzed by using the BD FACSDiva software FlowJow10. Statistically significant differences in blood parameters, gene expression and cell composition between control and low DO group were determined per day using the multiple *t*-test (Holm–Šidák corrected, $\alpha = 0.05$). For the additional stimulation experiment data, statistical significances were calculated with one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

3. Results

3.1. Reduced Oxygen Saturation Induces Marginal Changes in the Process of Adaptation

3.1.1. Blood and Health Parameters of Challenged Pikeperch

To evaluate the physiological responses of the fish challenged with the low DO levels, we recorded standard health parameters including blood glucose, lactate and plasma cortisol levels, as well as SSI and LSI throughout the experiment. Notably, these parameters did not reveal any significant differences between the "control" (±8.3 mg/L DO) and "low DO group" (±3.2 mg/L DO) at any time point of the experiment (Supplementary Table S1).

3.1.2. Composition of HK and Peripheral Blood upon Low DO Exposure

We hypothesized that inadequate oxygen saturation would affect the immune status of the fish, reflected by a change in the proportion of immune cells. Therefore, we employed flow cytometry to analyze the ratio between myeloid and lymphoid cells in HK and peripheral blood. (Figure 2).

Throughout the experiment, the average cell composition of the peripheral blood leukocytes remained almost unchanged between the control and the low DO group, with a proportion of approximately 95% lymphocytes to 5% myeloid cells (Figure 2A). Conversely,

the composition of HK exhibited notable differences between both groups. Thus, while the ratio between myeloid cells and lymphocytes in the control group underwent only mild fluctuations ranging from 63% to 79% of lymphocytes and 21% to 37% of myeloid cells, we observed more substantial changes in the group exposed to low DO. Particularly at the early time points (day one, seven and fourteen), we witnessed an increase in the proportion of myeloid cells, reaching to 46%, reflected by a 1.4× decrease in lymphocyte proportion (Figure 2B).

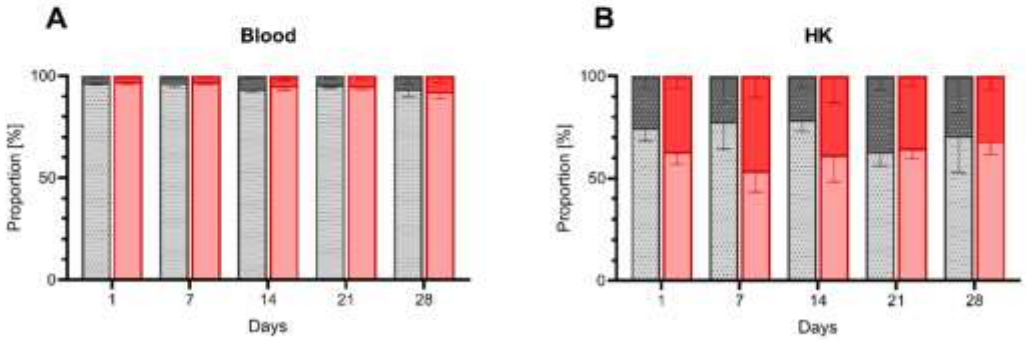

Figure 2. Proportion of myeloid and lymphoid cells within blood and HK of pikeperch challenged with low DO conditions. Proportion (%) of lymphoid (bright color) and myeloid cells (dark color) in collected blood (**A**) and head kidney (HK; (**B**)) samples of pikeperch. HK was additionally purified by Percoll-gradient. Columns represent mean of five individual samples (+SEM) from control (grey/patterned) and hypoxia group (red) after one, seven, fourteen, twenty-one and twenty-eight days of the experiments. Statistical significance per cell type per each day was determined using the multiple t-test (Holm–Šídák corrected), with alpha = 0.05.

3.1.3. Gene Expression Analysis in HK and Liver of Challenged Pikeperch

To provide deeper insights into the molecular mechanisms underlying the changes induced by low DO levels, we evaluated the expression of eight selected genes involved in response to hypoxia. The transcript numbers ranged from approximately 2×10^1 (*HSP90AA1*) to 2.5×10^6 (*HSF2*) copies per 100 ng RNA in HK and liver of individual fish (Figures 3A–C and 4A–C). We detected transcript numbers only below 2×10^3 transcripts/100 ng RNA for the genes *HMOX1* and *HSP90AA1* in HK and liver (Figures 3A and 4A). The highest copy numbers with 1.5×10^5 transcripts/100 ng RNA and above were observed for the genes *EPAS1*, *FTH1* and *HSF1* in HK and liver (Figures 3C and 4C).

In general, few genes were lower expressed under low DO levels in liver and HK than in the control group. Three of the analyzed genes (*EPAS1* in HK; *FTH1* and *NR3C1* in the liver) shared similar transcript patterns. Here, the expression levels of both groups are similar at the beginning and the end of the experiment, but at days seven and fourteen the low DO group showed lower transcript levels.

Four genes showed statistically different copy numbers between the control and the low DO group. For *HSP90AA1*, a change from significantly lower to significantly higher copy numbers during the treatment was observed in HK. *HIF1A* showed higher transcript levels during the low DO exclusively at day 2 in the liver, but lower copy numbers from day 14 till day 28 in HK. Another two genes (*FTH1* and *NR3C1*: HK and liver) showed lower transcript levels during the low DO challenge.

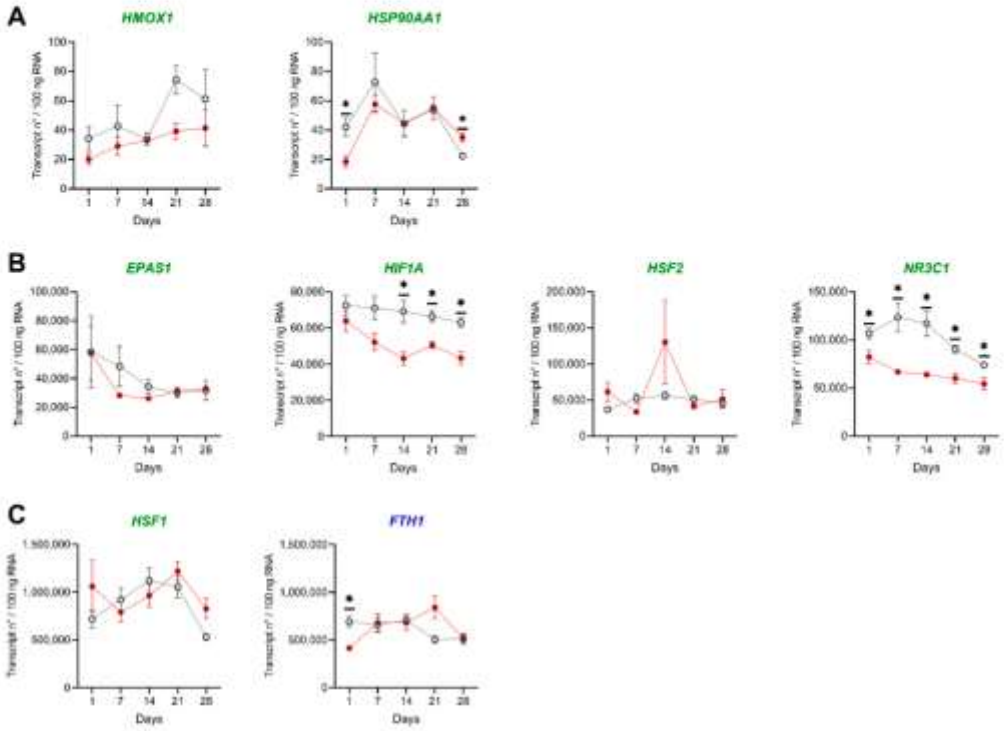

Figure 3. Stress and immune marker expression in HK of pikeperch exposed to low oxygen saturation. Gene expression of candidate genes in collected head kidney (HK) samples of pikeperch. Normoxic (grey/empty circles) and low DO (red/filled circles) groups after one, seven, fourteen, twenty-one and twenty-eight days of the experiment. Genes involved in stress (green) or immune response (blue) were either lowly (**A**), moderately (**B**) or highly (**C**) expressed. Data points represent the mean of five individual samples (+SEM), calculated per 100 ng of total RNA. Statistical significance was determined between groups for each day using the multiple t-test (Holm–Šídák corrected), with alpha = 0.05; * < 0.05.

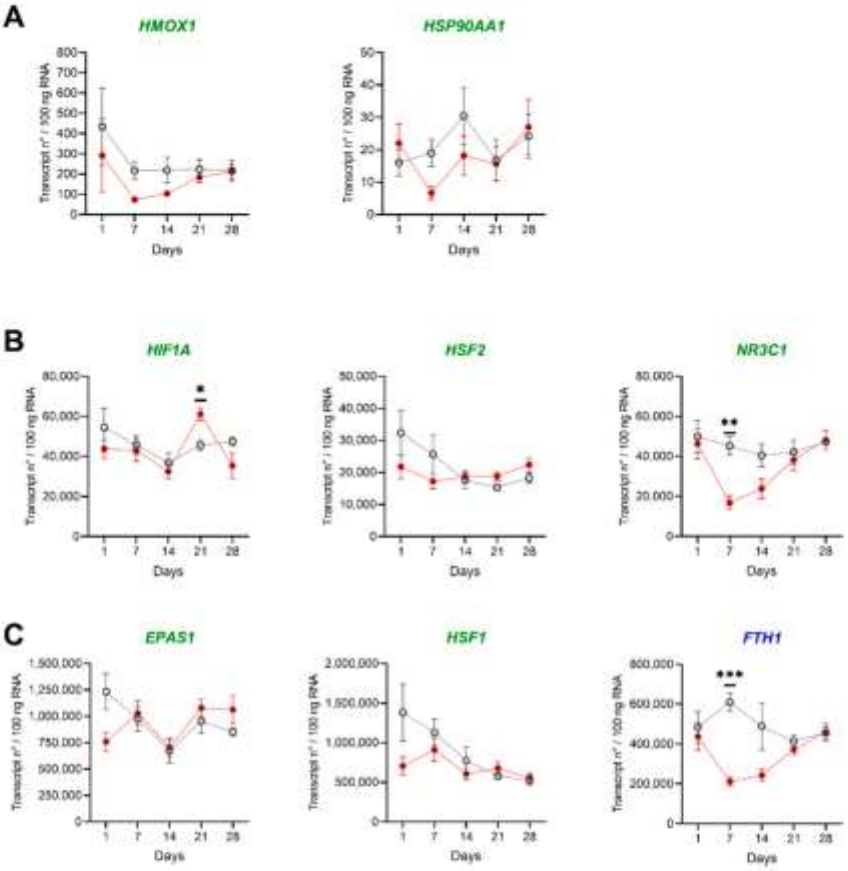

Figure 4. Stress and immune marker genes in liver of pikeperch challenged with low oxygen saturation. Gene expression of candidate genes in collected liver samples of pikeperch. Normoxic (grey/empty circles) and low DO (red/filled circles) group after one, seven, fourteen, twenty-one and twenty-eight days of the experiment. Genes involved in stress (green) or immune response (blue) are either lowly (**A**), moderately (**B**) or highly (**C**) expressed. Data points represent the mean of five individual samples (+SEM), calculated per 100 ng of total RNA. Statistical significance was determined between groups for each day using the multiple t-test (Holm–Šídák corrected), with alpha = 0.05; * < 0.05, ** < 0.01 and *** < 0.001.

3.2. *Induction of Peritoneal Inflammation under Low DO Levels*

3.2.1. SSI after Intraperitoneal Stimulation

To elucidate to which extent is the immune response of the host compromised by the reduced levels of DO, on day eight (Figure 1), we employed an adapted model of peritoneal inflammation established previously [28]. Upon the stimulation, we determined the SSI in all tested groups, the control group and low DO group, both either unstimulated (PBS as control) or stimulated with inactivated *A. hydrophila* (Figure 5).

The average spleno-somatic indices ranged from 0.040 to 0.077. The injection of inactivated bacteria resulted in an increase of the SSI in both the control and the low DO group at day one to three post-stimulation, with the most prominent and significant changes seen between unstimulated and stimulated fish of the low DO group at day

one. Furthermore, we noticed a slight, albeit nonsignificant, decrease in the SSI in the PBS-injected fish in the low DO group compared to normoxia.

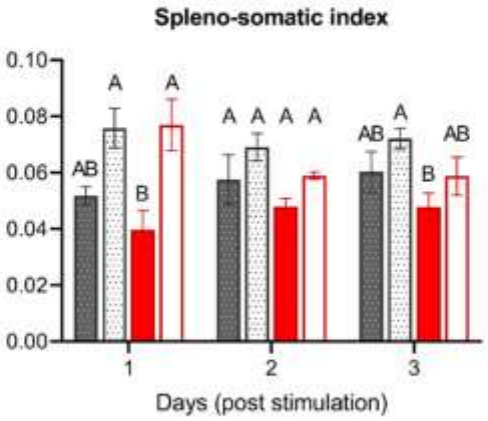

Figure 5. Spleno-somatic index of pikeperch during acute peritoneal inflammation. Spleno-somatic indices of pikeperch after one, two and three days post intraperitoneal stimulation with inactivated *Aeromonas hydrophila* cells. The graph shows differences between normoxic control groups, either unstimulated (dark grey/patterned) or stimulated (bright grey/patterned), and low DO groups, either unstimulated (dark red) or stimulated (bright red). Columns represent mean of four individual samples (+SEM). Statistical significance per each day was determined using the one-way ANOVA followed by Tukey´s multiple comparison test ($p = 0.05$); different letters (A, B) represent significant changes between different control and low DO groups.

3.2.2. Leukocyte Migration upon Intraperitoneal Stimulation

To further evaluate the impact of low DO on acute inflammation, we analyzed the cell composition in the peritoneal cavity, blood, spleen and head kidney upon intraperitoneal injection with *A. hydrophila* (Figure 6A–C and Figure 7A–C).

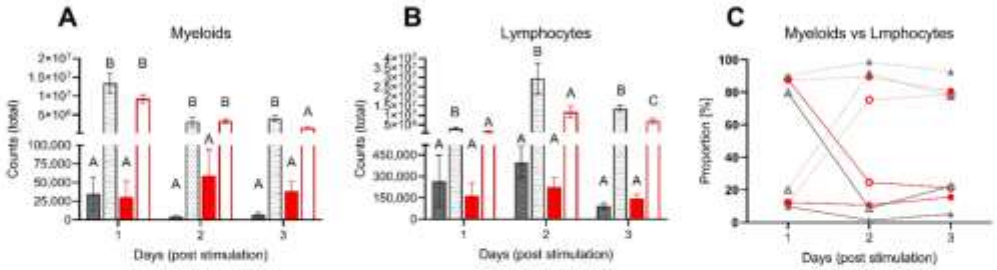

Figure 6. Kinetics of peritoneal leukocytes upon acute peritoneal inflammation. Total counts of myeloid cells (**A**), lymphocytes (**B**) and proportion of both cell types (**C**) after one, two and three days post intraperitoneal stimulation with inactivated *Aeromonas hydrophila* cells. Graphs A and B show differences between normoxic control groups, either unstimulated (dark grey/patterned) or stimulated (bright grey/patterned), and low DO groups, either unstimulated (dark red) or stimulated (bright red). Columns represent mean of four individual samples (+SEM). Statistical significance per each day was determined using the one-way ANOVA followed by Tukey´s multiple comparison test ($p = 0.05$); different letters (A–C) represent significant changes between different groups. Graph C shows differences between control groups, either unstimulated (grey, filled triangle) or stimulated (grey, open triangles), and the low DO groups, either unstimulated (red, filled circles) or stimulated (red, open circles). Dotted lines represent lymphocytes, filled lines represent myeloid cells.

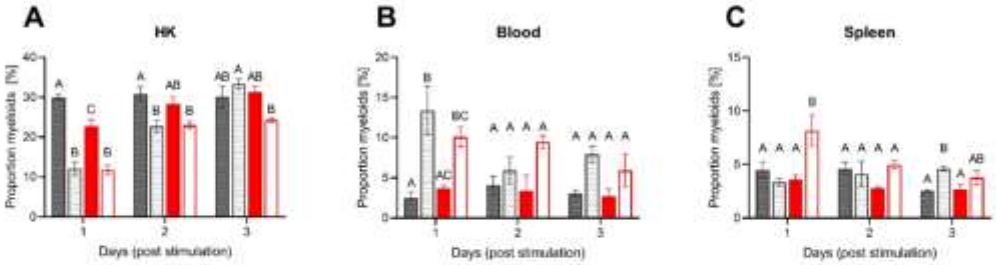

Figure 7. Proportion of myeloid cells within HK, peripheral blood and spleen of pikeperch upon intraperitoneal stimulation. Proportion (%) of myeloid cells in head kidney (HK; **A**), peripheral blood (**B**) and spleen (**C**) samples of pikeperch after one, two and three days post intraperitoneal stimulation with inactivated *Aeromonas hydrophila* cells. The graph shows differences between normoxic control groups, either unstimulated (dark grey/patterned) or stimulated (bright grey/patterned), and low DO groups, either unstimulated (dark red) or stimulated (bright red). Columns represent mean of four individual samples (+SEM). Statistical significance per each day was determined using the one-way ANOVA followed by Tukey´s multiple comparison test ($p = 0.05$); different letters (A–C) represent significant changes between different groups.

While the PBS-injected fish did not undergo any remarkable changes in the number of cells in the peritoneal cavity and retained their original composition with 80–90% of lymphocytes and 10–20% of myeloid cells throughout the experiment, the injection of inactivated *A. hydrophila* led to rapid recruitment of myeloid cells to the peritoneal cavity, resulting in a complete change in its profile (Figure 6A–C). As soon as one day after the injection, the myeloid cells comprised over 80–90% of all peritoneal leukocytes (Figure 6C), reaching the total number of 1.4×10^7 and 9×10^6 in control and DO low groups, respectively (Figure 6A). In the following 24 h, the peritoneal niche underwent the second change in its composition, and the growing number of lymphocytes substituted the peak of myeloid cells. Their total number reached approximately 3×10^7 cells in the control group (Figure 6B), representing over 80% of all peritoneal leukocytes (Figure 6C). Notably, the recruitment of lymphocytes to the peritoneum of low DO fish was three times lower than in fish kept at normal oxygen saturation. On the third day, we observed a resolution of inflammation in both stimulated groups.

The recruitment of leukocytes to the peritoneal cavity was mirrored by the changes in the composition of blood and both systemic lymphoid organs. The changes were most prominent on the first two days when the stimulation with inactivated *A. hydrophila* decreased the proportion of myeloid cells in the HK from 30% observed in PBS injected controls to ~12% and ~23%, respectively (Figure 7A). In addition, we observed a significant decrease in the myeloid proportion of the low DO group (~23%) after the first day (without additional stimulation) compared to the control group (~30%) (Figure 7A). This decrease was complemented by the increased mobilization of myeloid cells to the peripheral blood, which increased from ~3% in PBS injected groups up to 13% in stimulated fish 24 h post-injection (Figure 7B). This has been further reflected by the increased ratio of myeloid cells in the spleen of low DO fish but not in the fish kept at standard oxygen saturation (Figure 7C). With the ensuing resolution of the inflammation in the peritoneal cavity, the proportion of myeloid cells in the blood decreased gradually to ~8%.

3.2.3. Gene Profiling in HK and Spleen during Acute Inflammation

We further aimed to evaluate the transcriptomic changes orchestrating the acute inflammation using established rt-qPCR analysis (Figure 8A–C and Figure 9A–C). Ten genes (*CSF2*, *EPAS1*, *FTH1*, *HIF1A*, *HMOX1*, *HSF1*, *HSF2*, *HSP90AA1*, *NR3C1* and *RAG1*) were exclusively determined in HK with additional five genes (*CXCL8*, *IL1B*, *MHC II alpha*, *MPO* and *TNF*) in both tissues. *TNF* was exclusively detectable in the spleen, with numbers only below 10 transcripts per 100 ng RNA in HK (data not shown).

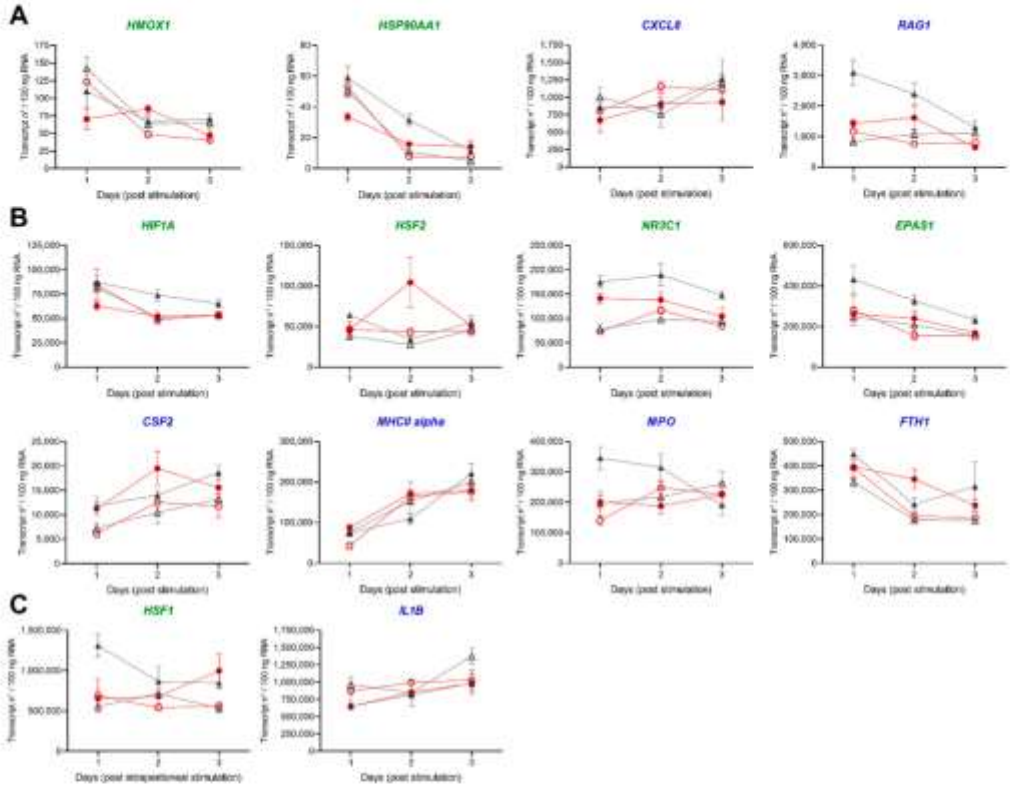

Figure 8. Expression of stress and immune markers in HK of pikeperch upon acute peritoneal inflammation. Expression of candidate genes in head kidney (HK) of pikeperch. Normoxic control group (grey), either unstimulated (grey/filled triangles) or stimulated (grey/empty triangles), and low DO treatment groups, either unstimulated (red/filled circles) or stimulated (red/empty circles) after one, two and three days post intraperitoneal stimulation with inactivated *Aeromonas hydrophila* cells. Genes involved in stress (green) or immune response (blue) are either lowly (**A**), moderately (**B**) or highly (**C**) expressed. Data points represent mean of four individual samples (+SEM), calculated per 100 ng of total RNA. Statistically significant differences were calculated via one-way ANOVA followed by Tukey's multiple comparison test ($p = 0.05$).

We detected the lowest copy numbers with transcripts only below 4×10^3 transcripts/100 ng RNA for five genes (*CXCL8*, *TNF*, *HMOX1*, *HSP90AA1* and *RAG1*) in HK and spleen of the individual fish (Figures 8A and 9A). For the three genes *HSF1*, *IL1B* and *MHCII alpha*, we determined copy numbers above 5×10^5 per 100 ng RNA in HK and spleen (Figures 8C and 9C).

In HK, we observed highly dynamic expression profiles of the selected genes. An intraperitoneal stimulation decreased the transcript levels of *FTH1*, *HMOX1*, *HIF1A* and *HSP90AA1* after two days of the experiment in the control and low DO group. *EPAS1*, *NR3C1* and *RAG1* showed lower copy numbers in stimulated control and low DO fish than the unstimulated control group on any day of the experiment. *CXCL8* transcript numbers decreased after stimulation in the control group and increased in the low DO group after two days. The transcript numbers of *MHCII alpha* increased two days post-stimulation in both groups (control and low DO).

In the spleen, the intraperitoneal stimulation led to a remarkable increase in expression of the genes coding for the inflammatory cytokines CXCL8 and IL1B, which reduced in

comparison to the levels seen in unstimulated fish two days post-stimulation. In contrast, *MHCII alpha*, *MPO* and *TNF* transcript levels increased in both groups (control and low DO) two days post-stimulation.

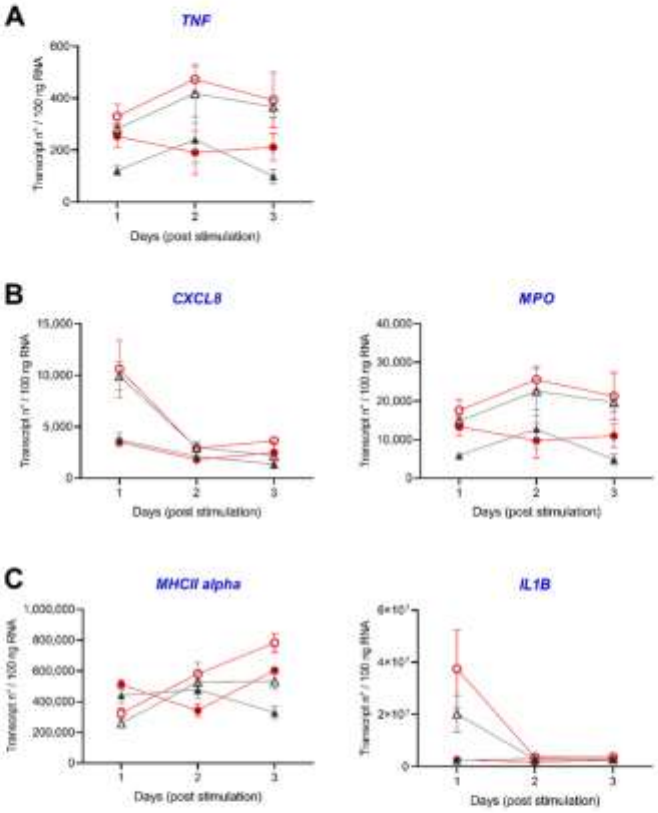

Figure 9. Expression of stress and immune markers in spleen of pikeperch upon acute peritoneal inflammation. Relative expression of candidate genes in spleen of pikeperch. Normoxic control group (grey), either unstimulated (grey/filled triangles) or stimulated (grey/empty triangles), and low DO groups, either unstimulated (red/filled circles) or stimulated (red/empty circles) after one, two and three days post intraperitoneal stimulation with inactivated *Aeromonas hydrophila* cells. Genes involved in stress (green) or immune response (blue) are either lowly (**A**), moderately (**B**) or highly (**C**) expressed. Data points represent mean of four individual samples (+SEM), calculated per 100 ng of total RNA. Statistically significant differences were calculated via one-way ANOVA followed by Tukey's multiple comparison test ($p = 0.05$).

4. Discussion

4.1. The Hypoxic Challenge of 40% DO Does Not Induce Substantial Changes in Major Health Parameters

Exposure to stress stimuli initiates a cascade of physiological mechanisms, allowing the mobilization of energy to cope with stressors and restore homeostasis. In teleost fish, like in other vertebrates, several markers, including blood parameters and health indices, are frequently applied as surrogates of the adaptation response. Apart from the spleno-somatic index, reflecting the immune status, or hepato-somatic index, representing the

organism's metabolic rate, the blood cortisol serves as the primary indicator of the ongoing stress response. Its increasing levels regulate a vast array of processes directing the energy metabolism toward the mobilization of hepatic glycogen and an increased availability of glucose to facilitate the successful adaptation to the stressor [32–35]. Unexpectedly, none of the examined parameters showed levels outside the physiological range or significant differences between the low DO group and the control group in the current study. Cortisol is an excellent marker for acute stress, including low DO levels, but its reliability under chronic conditions is uncertain due to its fast release and clearance [36–39].

The levels of free glucose are in line with former studies in pikeperch, whereas observed lactate levels were lower [40,41]. Both physiological parameters have been shown to increase after an acute short-term low DO event [42]. However, our findings are concordant with the study of O'Connor et al. (2011), in which different populations of three-spined stickleback (*Gasterosteus aculeatus*) showed no significant changes in whole-body cortisol glucose and lactate levels after a week of low oxygen conditions (2.2 mg/L DO) [43]. Similarly, Douxfils et al. (2014) reported a fast return to basal cortisol and glucose levels after a response to low oxygen saturation in juvenile Eurasian perch [44]. In common carp (*Cyprinus carpio*), unaltered HSI was detected after long-lasting low DO levels [45]. Overall, the absence of significant changes in the studied health parameters indicate relatively high tolerance of pikeperch to low levels of DO and high pace of adaptation responses preserving the homeostasis even at ±3.2 mg/L DO.

4.2. Effects of Low DO on Cell Distribution and Gene Expression

Previous studies have shown that inadequately low oxygen saturation results in modifications of the innate and adaptive immunity in fish and alters the cell composition in main lymphoid organs [46–52]. In the presented study, we employed flow cytometry to elucidate the changes induced by the chronic exposure to low DO in blood and head kidney. Interestingly, our results revealed an increased proportion of myeloid cells, both in circulation and in the lymphoid organs of low DO fish, reflecting a potentially higher mobilization of the immune system. This observation belongs to one of the hallmarks of the conserved transcriptional response to adversity and is in accord with an increased rate of circulating myeloid cells in the blood of maraena whitefish (*Coregonus maraena*) exposed to crowding stress [53], higher mobilization of myeloid cells to the blood of gilthead seabream (*Sparus aurata*) after exposure to short-term stress [54] or increased number of circulating myeloid cells observed in mammalian models of stress responses [55,56]. Induction of erythropoiesis at low DO levels, which increases oxygen transport in the blood, has been observed previously in teleosts [57]. Nevertheless, we did not observe any substantial increase in the number of circulating erythrocytes throughout the experiment (data not shown). Therefore, oxygen levels of ±3.2 mg/L DO may be classified as a hypoxic condition for pikeperch, but not as a situation of severe hypoxia.

The detected transcript patterns for the eight examined stress- and immune-relevant genes further illustrate the weak response to low DO. Although the examined genes were previously demonstrated to be responsive to hypoxic conditions or belong to downstream targets of hypoxia-inducible factor 1 alpha (HIF1A), which regulates the hypoxia response pathway, our analysis revealed only slight downregulation of their expression. More specifically, HIF1A is essential for the response of hypoxic fish with complex physiological and biochemical modifications involving the immune system [58,59]. In large yellow croaker (*Larimichthys crocea*), severe hypoxic conditions (1.6 ± 0.2 mg/L DO) for two days resulted in an upregulation of most immune genes, as well as *HIF1A*, in HK [60]. According to these investigations, we expected significant changes within the transcription in hypoxic challenged pikeperch, with increased expression of *HIF1A* and other stress marker genes such as *NR3C1* and *HSP90AA1*. However, we detected a prominent down-regulation of *HIF1A* transcript numbers in the head kidney of pikeperch, with significant differences at days 14 to 28 of the experiment, but relatively stable transcript levels in the liver. In European bass (*Dicentrarchus labrax*), acute hypoxic conditions of 1.9 mg/L DO for 4 h, and

chronic conditions of 4.3 mg/L DO for 15 days cause an elevated *HIF1A* transcription in the liver [61]. The hypoxia-sensitive percid species Eurasian perch showed up-regulated HIF1A transcription in the liver after an acute hypoxic oxygen saturation of 0.4 ± 0.1 mg/L DO for 1 h, but not after 15 days of 2.8 ± 0.3 mg/L DO [62]. Mohindra et al. (2013) observed in the hypoxia-tolerant Indian catfish (*Clarias batrachus*) a significantly up-regulated expression of HIF1A in the liver and down-regulation in the head kidney after 1 h of 0.98 mg DO per liter. Whereas, after another 5 h HIF1A was significantly up-regulated in the head kidney [63]. A down-regulation of HIF1A transcription in response to hypoxia stress was suggested to be the outcome of a hypoxia shock [64,65]. They concluded that the transcriptional regulation of HIF1A is a complex and tissue- and species-dependent process. This further suggest that the range of tolerance of pikeperch reared in intensive aquaculture is hardly impacted at DO saturations of 40%. It did not establish a severe stress response or severe immune suppression within 28 days. Collected data rather indicate an ongoing adaptation process already after 24 h lasting till day 21. Nevertheless, the obtained gene expression data are based on preselected candidate genes and global gene expression analyses, such as RNAseq or microarray-based analyses, might uncover yet not considered but regulated genes and pathways after DO decrease that influence or could affect homeostasis and fish welfare.

4.3. Acute Inflammation Is Moderately Influenced by Low DO

Previously, several observations suggested a negative impact of hypoxic conditions on the fish immune system [48,66,67]. To provide a deeper understanding of this phenomenon, we used a previously established model of acute peritoneal inflammation to evaluate how, and to what extent, acute inflammation is impaired by low DO. Generally, the processes driving the acute inflammation followed a pattern described previously in other fish species [28,68]. The injection of inactivated *A. hydrophila* induced a rapid mobilization of myeloid cells from head kidney and their release into the circulation [69,70]. Consequently, within 24 h post-injection, we observed increased SSI in both stimulated groups and a considerably increased expression of the myeloperoxidase (MPO), a key marker of granulocytes in the spleen [50]. In spleen, the detected increase of MPO lasted till the end of the experiment at 72 h. In the head kidney, a stimulation resulted in reduced transcript levels of MPO in both groups reflecting the efflux of granulocytes into the circulation. A depletion of neutrophils in head kidney after peritoneal inflammation has been detected in the goldfish (*Crassius auratus*) by Bielek et al. (1999) [71]. Simultaneously, we observed a dramatic increase in the number of myeloid cells in the peritoneal niche. Notably, in line with the aforementioned results, only marginal differences in the number of recruited cells were seen between the normal and low DO fish. On a molecular level, the rapid recruitment of myeloid cells into the peritoneal cavity was complemented by the increased production of proinflammatory cytokines (particularly CXCL8 and IL1B) in the spleen and head kidney of studied fish [72,73]. For both cytokines, the increase in gene expression was more pronounced in the stimulated spleen. Within HK the production of the major pro-inflammatory cytokine TNF was independent of the treatment marginally low (data not shown) [74]. While in spleen, higher expression was detectable for both groups one day post-injection and this increase persisted until the third day of the stimulation. The primary source of TNF is activated macrophages which, therefore, might also be involved in the detectable increased SSI [72].

Within the following 24 h, we witnessed a resolution of the acute inflammation, manifested by decreasing expression of inflammatory cytokines, reduced presence of myeloid cells in the circulation and an influx of lymphocytes into the peritoneal cavity. Strikingly, although we did not observe a strong influence of hypoxia on the recruitment of myeloid cells, the number of lymphocytes differed considerably between both groups, reaching almost three times lower numbers in the low DO group.

Taken together, these findings support the notion of dichotomy in the effect of hypoxia on the innate and adaptive arm of immunity, suggested by previous findings from mammalian models. Thus, while the innate immune cells, and granulocytes in particular,

are better equipped to maintain viability and functionality under hypoxic conditions, the lymphocytes require high energy metabolism coupled with sufficient oxygen availability for their survival and effective development of effector functions [75,76]. Similarly, in the present study, the recruitment, and the effector functions of the myeloid during the acute inflammation were comparable between both groups, while the influx of lymphocytes was impaired by the low DO, suggesting high evolutionary conservation of these processes in the tree of life.

5. Conclusions

In their natural habitats, fishes would avoid low oxygen levels by simply escaping the current situation. However, this option is not available in rearing tanks of intensive aquaculture facilities. In the present study, we evaluated the effect of hypoxic conditions (±3.2 mg/L DO) on the health and immune status of pikeperch reared in RAS. We defined stable blood parameters, slightly downregulated gene expression (*FTH1*, *HIF1A* and *NR3C1*) and a functional acute inflammatory response towards bacterial stimulation. Our results confirmed that pikeperch do not develop severe responses or immunosuppression at hypoxic conditions and together with our previous study investigating the challenge of rising water temperatures in pikeperch [29], indicates that pikeperch in aquaculture may not be as sensitive to common environmental stressors as previously thought.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biology10070649/s1, Table S1: Individual values of blood and health parameters measured in this study.

Author Contributions: A.R.: Methodology, Validation, Writing—Review & Editing, Supervision. J.M.: Investigation, Resources. M.V.: Methodology, Investigation, Validation, Writing—Review & Editing, Supervision. N.S.: Conceptualization, Project administration, Methodology, Funding acquisition, Investigation, Formal analysis, Validation, Writing—Original, Writing—Draft, Review & Editing, Visualization. R.M.B.: Resources. T.G.: Conceptualization, Funding acquisition, Writing—Review & Editing, Supervision. T.K.: Conceptualization, Project administration, Methodology, Investigation, Formal analysis, Validation, Writing—Review & Editing, Supervision, Resources. V.S.: Funding acquisition, Writing—Review & Editing, Resources. All authors have read and agreed to the published version of the manuscript.

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Table S1. Individual values of blood and health parameters measured in this study.

A		**Day1 Mean ± S.E.M**	**Day7 Mean ± S.E.M**	**Day14 Mean ± S.E.M**	**Day21 Mean ± S.E.M**	**Day28 Mean ± S.E.M**
Cotrisol (nmol/L)	Control	n.a.	n.a.	n.a.	n.a.	n.a.
	Treatment	n.a.	n.a.	n.a.	n.a.	n.a.
Glucose (mmol/L)	Control	5.80 ± 1.55	4.76 ± 0.85	3.72 ± 0.99	4.74 ± 0.81	6.01 ± 1.92
	Treatment	5.68 ± 0.48	6.07 ± 0.77	5.91 ± 1.00	3.12 ± 0.21	4.22 ± 1.11
Lactate (mmol/L)	Control	2.62 ± 0.68	0.68 ± 0.42	0.65 ± 0.22	0.60 ± 0.25	1.70 ± 0.44
	Treatment	3.08 ± 2.89	0.76 ± 0.37	0.92 ± 0.41	0.00 ± 0.00	1.34 ± 0.22
HSI	Control	1.40 ± 0.11	1.43 ± 0.11	1.29 ± 0.07	1.48 ± 0.13	1.38 ± 0.09
	Treatment	1.23 ± 0.11	1.39 ± 0.04	1.31 ± 0.08	1.27 ± 0.07	1.16 ± 0.06
SSI	Control	0.04 ± 0.00	0.03 ± 0.01	0.05 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
	Treatment	0.03 ± 0.00	0.12 ± 0.59	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00
B		Day 1 p.s.	Day 2 p.s.	Day 3 p.s.		
SSI	Control unstimulated	0.05 ± 0.00	0.06 ± 0.01	0.06 ± 0.01		
	Treatment unstimulated	0.04 ± 0.01	0.05 ± 0.00	0.05 ± 0.00		
	Control stimulated	0.08 ± 0.01	0.07 ± 0.00	0.07 ± 0.00		
	Treatment stimulated	0.08 ± 0.01	0.06 ± 0.00	0.06 ± 0.01		

HSI = hepato-somatic index, p.s. = post stimulation; S.E.M. =standard error of the mean; SSI = spleen somatic index;
A: n = 5 fish per group and day, B: n = 4 fish per group and day.

5.4. Identification of Molecular Stress Indicators in Pikeperch *Sander lucioperca* Correlating with Rising Water Temperatures.

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Identification of molecular stress indicators in pikeperch *Sander lucioperca* correlating with rising water temperatures

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Welfare

ABSTRACT

Water temperature is doubtlessly one of the most important factors affecting the well-being of fish. For pikeperch *Sander lucioperca* an ambient water temperature of > 20 °C has been suggested to achieve optimal growth in aquaculture farms, although natural habitats of pikeperch seldom reach such temperatures. We used multiplex qPCR arrays to assess the impact of temperature between 15 °C and 25 °C based on the expression of 38 potential biomarker genes characterising heat shock and hypoxia response, immune activation, energy supply and development in the liver and gills of pikeperch. In addition, liquid chromatography/mass spectrometry was used to assess the individual levels of glucocorticoids. Our data revealed that pikeperch do not develop severe responses to ambient temperatures between 15 °C and 25 °C, although specific parameters indicated a phased restriction of optimal conditions, above all the heat-shock genes *SERPINH1*, *HSP90AA1* and *HSPA8b*; the acute-phase genes *HP*, *C3* and *SAA*; and the transcription factor-encoding gene *HMX1*. In contrast to these *in vivo* observations, a temperature rise up to 37 °C led to an acute response in WF2-*Sander* cells characterised by the pronounced upregulation of *HSP90AA1*, *HSP1A1*, *SERPINH1*, *HSPB1*, *HSPA8b* and *HSP88*. This report paves the ground for future assessment studies on the imbalanced homeostasis of pikeperch by means of molecular tools.

1. Introduction

The pikeperch (*Sander lucioperca* L., 1758; NCBI taxonomy ID: 283035) is a highly attractive species for aquaculture due to its relatively rapid weight gain and its excellent flesh quality. Most importantly, the control of rearing conditions including temperature and light supports multiple out-of-season reproductions in recirculating aquaculture systems (RAS) (Hermelink et al., 2011, 2013, 2017). Still, the future development of the industry is impeded by an unreliable supply of stocking material. In commercial aquaculture, the variable quality of gametes has been associated with handling and rearing stress (Schaefer et al., 2016). The pikeperch is considered a highly stress-susceptible domestic species (Baekelandt et al., 2018; Németh et al., 2013), but little information has been published on its stress physiology (Milla et al., 2015), specifically with regard to temperature stress.

The natural habitats of pikeperch rarely reach temperatures above 20 °C (Łożys, 2004). Congruently, reproduction requires a wintering period to induce puberty (Hermelink et al., 2011) and slightly higher temperatures that are still well below 20 °C to ideally progress vitellogenesis (Hermelink et al., 2013). In aquaculture, gonad maturation can be blocked completely at temperatures above 20 °C, preventing energy allocation to the gonad and increasing growth in farmed fish. Also, optimal growth rates in fingerlings and juveniles have been reported at temperatures up to 28 °C (Frisk et al., 2012; Hilge and Steffens, 1996; Rónyai and Csengeri, 2008; Wang et al., 2009). The optimal temperature for a given species has often been considered as the temperature range where at least juvenile fish maximize energy capacity and exhibit maximum growth rates. Here, energy allocation to maintain homeostasis is optimal, allowing maximal energy to be allocated to growth. However, this is more focused on economic considerations than on welfare concerns. The growth performance of pikeperch is strongly retarded at temperatures below 15 °C and stagnates at temperatures below 10 °C (Ziemert and Heidrich, 2005). Ambient water temperatures below 1 °C (Ziemert and Heidrich, 2005) and above 35 °C (Horoszewicz, 1973) have proved to be lethal and stressful conditions further narrow the tolerance range. In addition, the concentration of dissolved oxygen

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A

Reference	Heat-shock response		Stress response	Immune response	Metabolic response
ACTB	DNAJA1*	HSPA8a1*	CYSTM1	C3*	CS
EEF1A1	DNAJA3*	HSPA8a2*	HMOX1	CXCL8†	ENO1
GAPDH	DNAJB11*	HSPA8b*	HMX1	FGL2†	IGF1
MTRNR2	HSP90AA1*	HSPB1*	NR3C1	FKBP4*	PGM3
RNA18S	HSP90AB1°	HSPB6°	OSER1	FTH1†	UCP2
RPL32	HSPA1A*	HSPB8*	OSGIN2	HP†	
RPS5	HSPA4°	HSPD1*	STK39	SAA†	Development
RPS9	HSPA6*	SERPINH1*	TNF	TCRG*	MUSTN1

B

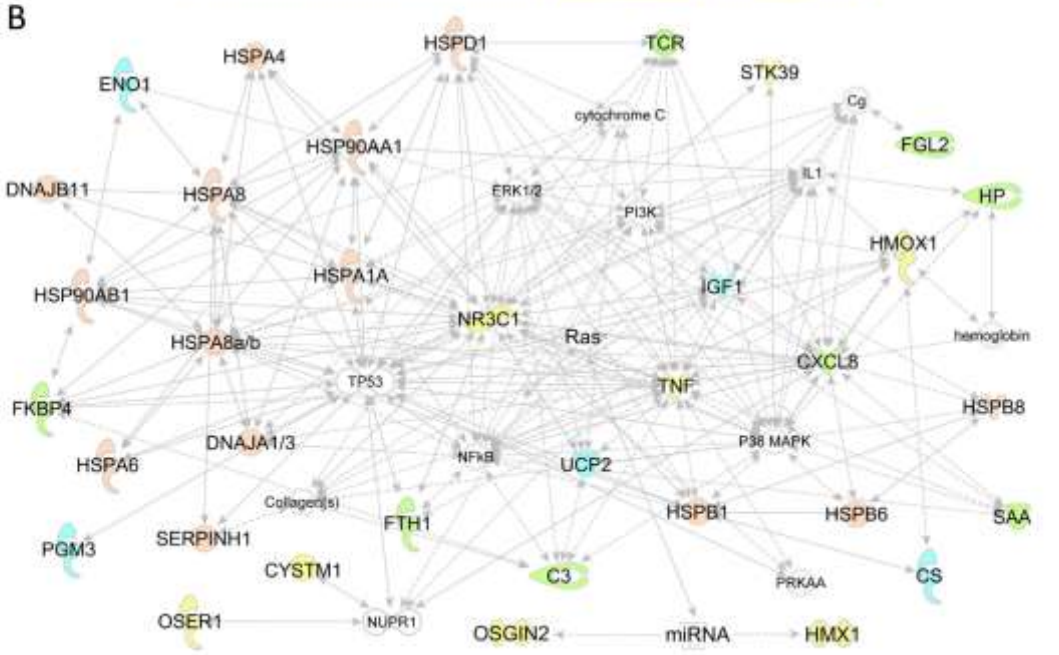

Fig. 1. Gene-profiling panel for detecting stress in pikeperch.
(A) Illustrative list of potential biomarker genes (given as official gene symbols, lower case letters indicate gene variants present in pikeperch) involved in heat-shock response (reddish underlay; circles indicate putatively constitutive genes, plus signs indicate inducible genes); stress response (yellow underlay); immune defence (green underlay; cross and asterisk symbols indicate innate and adaptive immune genes, respectively); metabolism (blue underlay) and development (grey underlay). Potential reference genes for data normalization are listed in the first column. Note that individual genes may have pleiotropic functions and could thus be assigned to multiple categories (above all *NR3C1*, *TNF* and *CXCL8*). The intense interconnection of the selected genes is illustrated in (B). The gene network has been plotted using Ingenuity Pathway Analysis software (Qiagen). Arrows specify the direction and direct (solid line) or indirect (dashed line) influence between genes. Symbol colours correspond to the categories listed in (A). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

negatively correlates with increasing water temperatures, leading to significantly reduced growth rates. For this reason, temperatures above 25 °C are rated as critical (Frisk et al., 2012). Pikeperch are considered eurythermal due to their spatial distribution in its natural habitat as well as small effects of temperature change on the standard metabolic rate within a broad thermal comfort zone (13–25 °C). According to a bioenergetic model, fish with larger body size prefer cooler water (Morita et al., 2010), suggesting an ontogenetic shift of temperature optima towards lower temperatures during the life cycle of pikeperch.

Like all vertebrates, fish have developed several sophisticated response features to cope with thermal stress. The hypothalamic-pituitary-interrenal axis and the hypothalamic-sympathetic-chromaffin axis initiate the release of certain peptides and steroid hormones (such as the characteristic stress hormone cortisol) as a primary response (Aluru and Vijayan, 2009; Tort, 2011). These hormones mobilise the energetic reserves (Hemre and Krogdahl, 1996; Silbergeld, 1974) that are required for 'whole-animal performance' covering behaviour, disease resistance or growth (Barton, 2002; Harper and Wolf, 2009; Martins et al., 2012). For instance, the expression of a large and diverse group of molecules termed "heat-shock proteins" (HSPs) have been proven to allow adaptation to thermal or osmotic stress or xenobiotic compounds, which is reflected in their alternative naming as "stress proteins" (Iwama et al., 1998). While the expression of particular HSPs is dramatically upregulated upon exposure to several stressful stimuli, there

Table 1
Gene-specific primers used in this study.

Gene symbol	Sense primer (5'-3')	Antisense primer (5'-3')	Primer efficiency [%]	Fragment length [bp]
Reference genes				
ACTB	AGGGAGTGATGGTGGGTATGG	CAGCTCGTTGTAGAAGGTGTGA	101	161
EEF1A1	ATGGACAGACCCGTGAGCATG	TTCTTGATGTAGGTGCTCACTTC	105	151
GAPDH	AGAGCACAGTTCATGCCATCAC	TTGAGCTCAGGGATGACTTTGC	102	151
MTRNR2	AGCACCCCTCCTACAACTAAGA	TGATCCAACATCGAGGTCGTAAA	103	164
RNA18S	GCGGTAATTCCAGCTCCAATAG	GCGGGACACTCAGTTAAGAGC	94	165
RPL32	GGCGTAAACCCAGAGGTATTGA	ACCTCGAGCTCCTTGACATTGT	105	157
RPS5	GCAGGATTACATTGCTGTGAAAG	TCATCAGCTTCTTGCCATTGTTG	101	161
RPS9	TACTGTCCCATCTTCTTTCAAGG	TTTTTGCATACCTTCAAACAGACG	102	156
Target genes				
C3	AGCTTAGTGGAGTGATGCATAGA	GGTGTTAAAGAACATAAGGCTTTG	100	163
C5	GGGCCTACTCTGAGGGTGTC	CCAGTCCAGGTTCGAGTCGAT	100	161
CXCL8	AACAGGGATGAGTCTGAGAAGC	GCTTGGAAATGAAGTCTTACATGA	98	158
CYSTM1	AACCACAGTTTGGCTGGCAGG	CACCTGTGTTTTTGGGAGCTTC	100	72
DNAJA1	GACCCAAAGTTTAGATCGTAGTG	AACCTAAACGCTTCTGTTACTCC	103	176
DNAJA3	GCAGCAAAGGGAGTCAACAAAG	CTGGTGGCCCTTACCATCACA	106	78
DNAJB11	ACAGGAACATCCCCAGAGGAAA	CTCATCTCCTGTCTGCAGTTAC	99	154
ENO1	AGGCAACAAGCTGGCCATGCA	TCCCTCGTCTCCCACGTTAGT	100	157
FGL2	ACTTTGAGGGTGTTCGGGAGTA	ACATATCGTTGTCGCGGTCGG	86	180
FKBP4	ACTTGTAGGTGGAACTGTTTGAAT	AAAAAGCTGTGTCTGGATGTGTTA	103	156
FTH1	ATTGAGACACACTACCTGGATGA	ACGGATTTAGCTGCTTTCTTTGC	99	153
HMOX1	GCTCGCTGTATGAGGTCTACC	TCTCTCCAGTCCTGGCCATAG	101	154
HMX1	AGGTGTTCCAGCTCGAGTCCA	TGTCTGCAGCGATCTGCCTCT	84	156
HP	GCTGAAACTGGGGACATTTACG	GAGCGCAGAGCAGACGATTTC	99	155
HSP90AA1	AGATACTACACCTCGGCTTCTG	TCACCAGTGATGTAGTAGATGTG	100	101
HSP90AB1	AAGAAGATTGCCGAGGAGCATTA	TCCTTCATGCGCTCCACATACT	100	182
HSPA1A	GTCCAACGCAGTGGTCACAGT	TGCGTTCTCCCGACTTGCTTTT	101	164
HSPA4	TAAGATGATCATGCAGGACAAGC	AGCATCCCATGTAGTTTGTCCC	99	102
HSPA6	GTCACAGTCCCCGCCTACTTT	GTTGCGTTCTCCCGACTTGCT	103	153
HSPA8a1	ATTCGGTAACATGTCTAAGGGAC	AGAGTCAGTGAAGGCAACGTAG	102	151
HSPA8a2	CACTTCATCGCAGAGTTCAAGC	AGTCAACTCCCTCGTACAGAGA	101	160
HSPA8b	TCCACACGTATCCCCAAGATTC	ACCATAAGCCACAGCCTCATCA	103	99
HSPB1	GTCCTTGGATGTCAACCACTTC	CAGTGATTTCCACCACACCATC	99	74
HSPB6	AAGGATGGTCCAGGGGTTACAA	AGGTTATTTGGTCAGGATTCTGG	117	157
HSPB8	CTCAGCTCGCGCCTCAGTTC	CGCAAACTTTCCACGGTTCCC	102	163
HSPD1	GAACATCGGTGCCAAGCTGGT	ATGACTCCCCGGCGGATCTC	102	162
IGF1	TGTGTGGAGAGAGAGGCTTTTAT	AGCGAGCAGCCTTGCTAGTCT	104	159
MUSTN1	TATTGCCCCAAAGCGCTACAAG	TTGATGGGCGACATCTTGGTAG	103	153
NR3C1	CCAGTCCTGCATGGATTCACTT	AGGTCCATAGTGTTGTCACTGAA	102	180
OSER1	TACGTGGACGACATCACGATAG	CGGCCATGTGTGACATCTTCTT	98	85
OSGIN2	CGCAGGTACTAAGAGAGGTTGA	GAGGCCCTTAAGCTTCACATAG	99	175
PGM3	AAGAAGATTGCCGAGGAGCATTA	TCCTTCATGCGCTCCACATACT	97	182
SAA1	CTGAAGGAGCTGGTGATATGTG	CTACTCTTTGCTTTTCACCTGATA	104	163
SERPINH1	GAGACAAGAGGAGTGCAGTCAA	ATCTGGGTTCTGCACATCCTTG	103	98
STK39	GAAACTCCGGCTCCTATCCTC	TGGGGACGCGATCCTGTCTG	103	154
TCRG	GTAATGTCTCTGTTGTGCCATATT	TCTCAGAGCAAATGCCATGGTC	103	177
TNF	GTCTTTGGAACCAGGCTATTTAC	TTTATGCCTCAGGCTTGACTGG	89	157
UCP2	ATTGGTCTCTACGACTCTGTTAAA	CTGGAAGCGAACTTTCACCACA	95	152

are also other HSP family members constitutively expressed to continuously maintain protein folding and export. HSPs are generally classified in several families based on their molecular weight such as HSP90 (alias HSPC with 85–90 kDa), HSP70 (HSPA, 68–73 kDa), HSP60 (HSPD), HSP47 (SERPINH1), HSP40 (DNAJ) and small HSPs (HSPB, 12–43 kDa) (Kampinga et al., 2009). The counterparts of nearly all of these factors have been identified in teleost fish (Campbell and Narum, 2009) and it is well documented that the activities of teleostean HSPs cover a wide functional spectrum (Roberts et al. 2010). Apart from HSPs, several other proteins that are involved in hypoxia response, immune activation and energy supply have been described as playing important roles during stress responses (Alves et al., 2010; Baekelandt et al., 2018; Forgati et al., 2017; Padgett and Glaser, 2003; Webster and Marketon, 2008). These homeostatic perturbations are detectable on the molecular level. A gene whose expression responds to an environmental stressor is categorized as "inducible" as opposed to a "constitutively expressed" gene which is required to maintain basic cellular functions. The high-throughput detection of inducible biomarkers in farmed fish, and thus the instant querying of their welfare status, will soon have an important role in aquaculture production. Hence, the major goal of this study was to select an informative panel of such inducible genes that are presumably involved in the pikeperch's response to temperature changes in order to standardise a multiplex assay for stress detection in finfish aquaculture.

Due to the scarcity of fish-specific antibodies and protein-analysis tools, quantitative PCR (qPCR) is still the dominant and highly sensitive method for the detection of the smallest changes in gene-expression dynamics (Kralik and Ricchi, 2017). Therefore, we combined liquid chromatography (LC)/mass spectrometry (MS) to analyse cortisol levels with two qPCR techniques to profile the expression of a panel of potential stress-related genes in pikeperch maintained at temperatures between 15 °C and 25 °C. Gene profiling was conducted in two cortisol-responsive organs that are referred to as 'frequent targets for stress responses' (Harper and Wolf, 2009). The liver is the dominant metabolic organ of stress responses (Rui, 2014), and thus has been intensively researched for decades and in numerous animal and cell models. Gills are also particularly sensitive to changes in the aquatic environment and have been addressed in several investigations of stress in bony fish (Forgati et al., 2017; Johansson et al., 2016; Mino et al., 2018; Narum and Campbell, 2015; Rebl et al., 2013).

2. Materials and methods

2.1. Gene selection and primer design

We collated genes that are well-known responders to stress in general and to temperature stress in particular (Supplementary Data; Fig. 1) based on our previous studies on stress in fish (Korytář et al., 2016; Rebl et al., 2013, 2017, 2018; Verleih et al., 2015) in complement with the Ingenuity Pathway Analysis (IPA) software (Qiagen). The IPA database incorporates research results from investigations into mammalian, but not teleostean, *in vivo* and *in vitro* systems, so all extracted genes and gene interactions were carefully checked and compared with reports that are relevant to fish physiology.

BLAST searches against the GenBank database outputted respective homolog sequence from percid fish species, mainly *Notothenia*, *Sebastes*, *Gymnocephalus* and *Perca* spp. No sequence information was available for the chosen candidate genes in pikeperch, so we searched for the respective homologs in our unpublished pikeperch genome that we obtained with Illumina RNA-seq technology. To confirm the identity of the obtained sequence fragments, we performed a reciprocal BLAST to the NCBI nucleotide database.

The verified sequence fragments were imported into the Pyrosequencing Assay Design software (version 1.0.6; Biotage, Uppsala, Sweden) to predict optimal pikeperch-specific primer oligonucleotides with a primer score ≥ 90 and primer efficiency between 84% and 106% for the amplification of target fragments between 72 and 182 bp (Table 1). The eventual PCR products were sequenced (Applied Biosystems 3130 Genetic Analyzer; Life Technologies) to validate their correctness. Subsequently, standard curves for all 46 genes were performed individually based on the CP values of tenfold dilutions of the respective PCR-generated fragments (1×10^3–1×10^6 copies) using the LightCycler 96 System (Roche, Mannheim, Germany) as described below.

2.2. Preliminary study and in vivo temperature experiments

In a preliminary study we recorded the basic expression of these genes that we compiled in the qPCR-primer set. Juvenile pikeperch were reared in aquaculture raceways of the Fischerei Müritz-Plau GmbH (Waren/Müritz, Germany) from June–September 2016 at temperatures between 17.2 °C and 24.1 °C, and oxygen content between 5.2 mg/l and 12.2 mg/l. At an age of 14 weeks, 20 pikeperch averaging 13.0 g ± 0.3 g in weight were randomly selected and sampled. The gills and liver were isolated and immediately snap-frozen until nucleic acid preparation.

The temperature experiment was also conducted with juvenile pikeperch reared at the State Research Institute for Agriculture and Fisheries Mecklenburg-Western Pomerania (Hohen-Wangelin, Germany) since September 06, 2016 (date of hatching) for 8 weeks (56 days). Six hundred individuals were transferred to the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (Berlin, Germany) and distributed randomly into 12 50-L freshwater tanks. After an acclimation period of 13 days, fish with an average weight of 8.3 g ± 3.0 g were subjected to the experimental procedures illustrated in Fig. 2A. Three of the 12 experimental tanks served as control tanks and were kept at constant temperature of 15 °C for the entire duration of the experiment (Fig. 2A, tanks I, II, III). The water temperature in the other nine tanks (1a–c; 2a–c; 3a–c) was gradually elevated from 15 °C to 25 °C by 1 °C every day for a period of 11 days (until the end of experiment). The water temperature and other relevant parameters including inorganic compounds such as NH_4^+, NO_2^-, NO_3^- (in each case far below critical values), pH (between 7.3 and 8.5) and dissolved O_2 were recorded throughout the experiment. While the O_2 level was in the optimal range in the control tanks, it decreased in the experimental tanks with rising temperatures and reached its lowest value at the last day of the experiment (Fig. 2B), although it did not meet the critical threshold of 3 mg/l (Blanck et al., 2007). Every day, three fish from each experimental group were anaesthetised with eugenol (60 mg/l) and sampled 12 h after temperature increase on a rotating basis (as indicated in Fig. 2A). Three fish from each of the three 15-°C control tanks were sampled at the beginning on day 1, in the middle on day 6 and at the end of the experiment on day 11. At the end of the experiment, pikeperch had reached an average weight of 10.1 g ± 2.5 g. The gills and livers were isolated and immediately snap-frozen and the remaining fish were used for cortisol analysis. This experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Berlin State (Landesamt für Gesundheit und Soziales Berlin, Germany; approval ID: G0150/16).

2.3. In-vitro heat-shock experiment

The fibroblastic cell line WF2 (walleye fry 2; RRID: CVCL_4556) from *Sander vitreus* (IZSLER Biobank, Brescia, Italy) was used to assess the heat-shock response *in vitro*. Cells were cultured at 22 °C and 5% CO_2 in 75 cm^2 cell culture flasks (Sarstedt) with MEM Eagle medium including Earle's salts (Sigma). The medium was complemented with 10% foetal bovine serum (Gibco), 10 mM non-essential amino acids (Merck), 40 mM L-glutamine (Merck) and antibiotics.

Cells were grown at a confluency of 80%–90%. After rinsing with phosphate-buffered saline (Biochrom), the cells were detached with cell scrapers and centrifuged at 233*g* for 10 min at 15 °C. For the stress experiment, the cell concentration was adjusted at ~400,000 cells per well of a 6-well plate (TPP) using the cell viability counter Cellometer Auto 2000 (Nexcelom). Cells were then incubated in 2 ml medium at 22 °C and 5% CO_2, until a cell confluency of approximately 90% was reached. The heat stress was induced in triplicate for 1 h at 37 °C and 5% CO_2 and was compared to the control cells incubated at the optimal culture conditions of 22 °C and 5% CO_2.

2.4. RNA preparation

RNA isolation from the gills and liver of sampled pikeperch was carried out separately using TRIzol (Invitrogen/Thermo Fisher Scientific) according to the manufacturer's instructions. For RNA extraction from WF2, cells were detached with 1.5 ml 5× passive lysis buffer (Promega) while incubating for 20 min at room temperature on a horizontal shaker. After centrifugation at 21,000*g* for 5 min, 15 µl of 2-mercaptoethanol was added to the supernatant. RNA extracted from tissues or cells was subsequently purified using the RNeasy Mini Kit (Qiagen). Nucleic acid concentrations were quantified with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies/Thermo Fisher Scientific).

2.5. Real-time qPCR (RT-qPCR)

Multiplex RT-qPCR was conducted using the integrated fluidic circuit (IFC) technology of the Fluidigm Gene Expression biochips, to assess the mRNA abundance of 46 genes (including eight reference genes) during the thermal stress response.

RNA samples from three fish per day were reverse-transcribed into cDNA using the Reverse Transcription Master Mix (Fluidigm), pre-amplified in 10 cycles using the PreAmp Master Mix (Fluidigm) and subsequently treated with exonuclease I (New England BioLabs) following the manufacturer's instructions. After the addition of the primer assays to the 48.48 IFC inlets, the IFC chip was primed in the MX IFC Controller (Fluidigm). Then, the preamplified cDNA samples were loaded on the primed 48.48 IFC chip and analysed with the Biomark HD system using the manufacturer's thermal protocol 'GE Fast 48x48 PCR + Melt v2.pcl'. EvaGreen fluorescence dyes (Bio-Rad) served as DNA-binding reporter molecules to allow the quantification of the amplified target fragments. The qBase$^+$ software (Biogazelle, Ghent University, Belgium) evaluated the suitability of eight potential pikeperch-specific

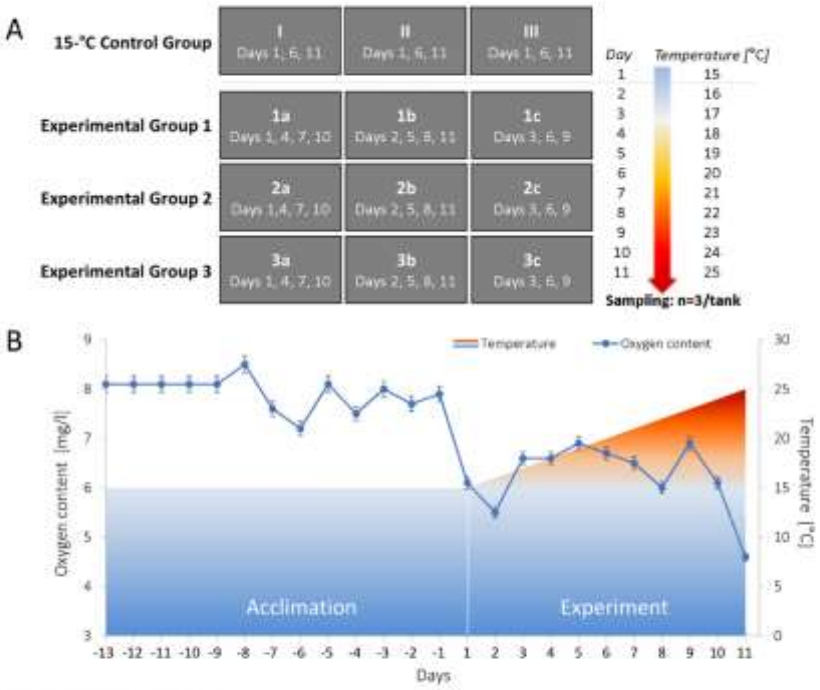

Fig. 2. Setup of the temperature-stress experiment.
(A) Juvenile pikeperch were acclimated for 13 days prior to the start of the experiments in 12 tanks, providing control tanks (I, II, III) that remained at 15 °C throughout the experiment and treatment tanks where temperature was increased by 1 °C/d until 25 °C was reached. Each group was assessed in triplicate (1, 2, 3). Thereby, sampling three individuals per day in triplicate was carried out in three-day intervals to reduce stress during sampling (a, b, c). (B) Oxygen content [mg/l] (left ordinate, solid blue line) and water temperature [°C] (right ordinate, blue/red coloured area) were recorded in all experimental tanks during the acclimatisation and experimental phases (abscissa). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reference genes (*ACTB*, *EEF1A1*, *GAPDH*, *MTRNR2*, *RNA18S*, *RPL32*, *RPS5*, and *RPS9*) based on averaged GeNorm stability values (M) and coefficients of variation (CV) (exemplarily shown in Fig. 3A, B). Fluidigm data were analysed using the Fluidigm Real-Time PCR analysis software v. 4.0.1 and were evaluated based on a linear regression of standard curves for each individual amplicon.

Single-gene RT-qPCR analyses were performed with the LightCycler 96 System (Roche) using the Sensi-FAST SYBR No-ROX Kit (Bioline). RNA samples from nine fish per time point (Fig. 2A) were individually reverse-transcribed using the Super Script II kit (Thermo Fisher Scientific) and subsequently, a cDNA equivalent of 75 ng RNA was used for the copy-number analysis. 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). The quality of the PCR products was assessed based on gel electrophoresis and melting-curve analysis. Light-Cycler data were analysed using the LightCycler 96 analysis software v. 1.1 and the qBase+ software (Biogazelle). C_q values < 35 were considered detectable. We used one-way analyses of variance (ANOVA) followed by parametric Tukey or non-parametric Dunnett multiple comparisons for our statistical analyses using the IBM SPSS Statistics software, version 22.0. The Spearman's Rho correlation coefficients were calculated using the Social Science Statistics tool (http://www.socscistatistics.com/tests/) to evaluate the concordance between the experimental temperature and the transcript number, and a two-tailed *t*-test was applied to assess statistical significance.

2.6. LC-MS/MS of glucocorticoid fraction

For the extraction, whole fish were chopped in pieces, weighed in glass centrifuge tubes and 50 µl cortisol-D_4 solution (100 ng ml^{-1} in methanol) was added as an internal standard. The tubes were kept on ice, 5 ml methanol was added and the samples were homogenized (VDI 12 homogenizer, VWR). All samples were continuously agitated with an overhead shaker (Reax 2, Heidolph Instruments) at 20 rpm and room temperature for 1 h. Then, samples were incubated at −80 °C for 1 h to precipitate fats and proteins. After centrifugation for 5 min at −9 °C and 3500 g, 800 µl of the supernatant was transferred to a HPLC vial using a syringe with a filter (0.2 µm).

Chromatographic separation was performed on an Agilent Zorbax RRHD Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm) using the 1290 Infinity II LC system from Agilent technologies (Agilent) equipped with a G7167B Multisampler. The mobile phase was composed of solution A (H_2O containing 0.1% formic acid) and solution B (acetonitrile containing 0.1% formic acid) with a flowrate of 0.4 ml min^{-1}, using the following gradient elution: initial 80% A, 20% B; 0–20 min from 20%–100% B; 20–25 min from 100%–20% B; 25–40 min 20% B. The mass spectrometer was operated using the Agilent 6470A Triple Quadrupole LC/MS System equipped with an Agilent Jet Stream 'electrospray ionization' source in positive mode. For best selectivity and sensitivity, the 'multiple reaction monitoring' mode was used for detection. The sheath gas temperature was set to 400 °C with a flow of 12 l min^{-1}, nebulizer at 35 psi and capillary voltage at 4000 V.

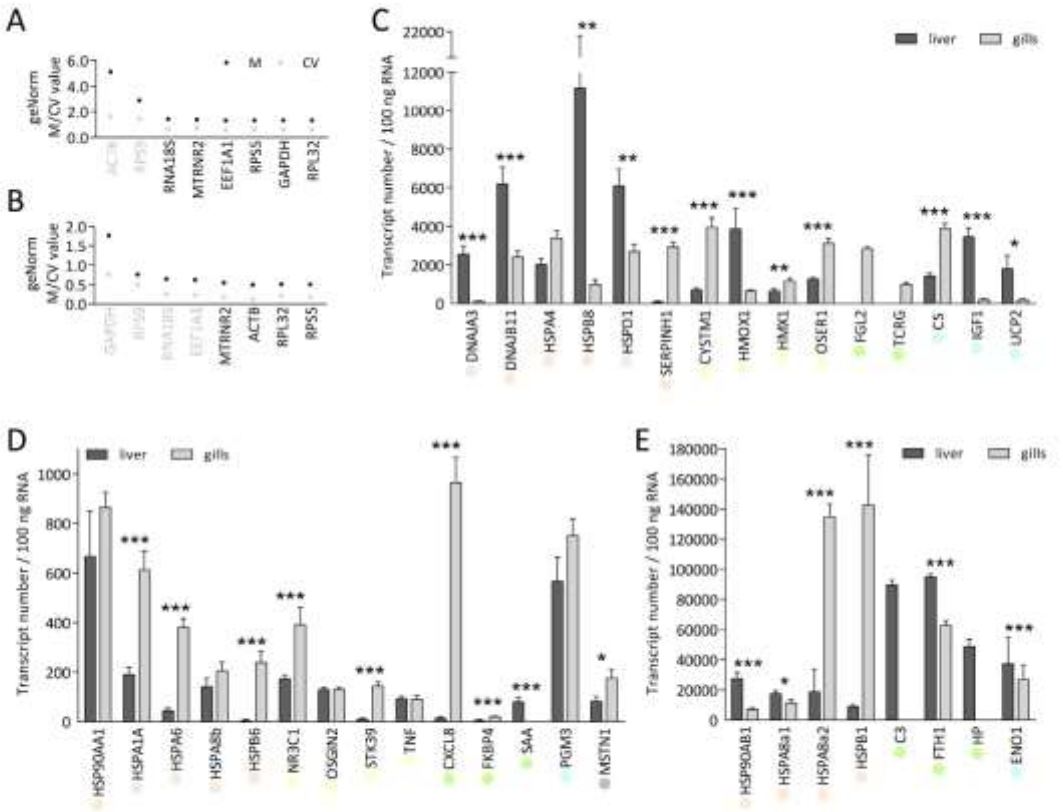

Fig. 3. Profiling of temperature stress-related genes in unchallenged pikeperch.
Reference genes were evaluated for each particular set of samples from (A) liver and (B) gills based on the geNorm parameters M (black dots) and CV (grey dots). Reference genes that passed the quality check are indicated by black gene symbols; others are indicated by grey gene symbols. (C) Moderately expressed, (D) lowly expressed and (E) highly expressed stress-related target genes (indicated as gene symbols together with circles coloured according to the categories listed in Fig. 1A) were quantified in liver (dark grey) or gill tissue (light grey) of juvenile pikeperch. Columns represent the mean values calculated per 100 ng of total RNA and normalised against the reference genes as indicated in (A) and (B). Error bars indicate standard error of the mean (SEM). Asterisks mark significant differences (* $p < .05$; ** $p < .01$; *** $p < .001$).

3. Results

3.1. A gene-profiling panel for detecting stress in pikeperch

At the beginning of the present study, no cDNA sequence encoding a stress-relevant factor from pikeperch was freely accessible. Therefore, we performed a BLAST search of a set of potential genes involved in the piscine stress response against 826 million high-quality sequencing reads representing the genome of pikeperch muscle (generated in an unpublished study of our working group). Based on the verified gene fragments, we designed real-time quantitative PCR (RT-qPCR) assays for profiling studies across 38 factors constituting the response to critical temperatures in pikeperch (Fig. 1A). The resulting set comprises genes encoding the heat-shock proteins *DNAJ* (members *A1*, *A3*, *B11*), *HSPA* (members *1A*, *4*, *6*, *8a1*, *8a2*, *8b*), *HSPB* (members *1*, *6*, *8*), *HSPC* (*HSP90AA1*, *HSP90AB1*), *HSPD* (member *1*) and *SERPINH1* (alias *HSP47*) as well as genes involved in stress signalling (*CYSTM1*, *HMX1*, *NR3C1*, *STX39*, *TNF*) including hypoxia-induced genes (*HMOX1*, *OSER1*, *OSGIN2*), acute-phase and associated genes (*C3*, *CXCL8*, *FGL2*, *FTH1*, *HP*, *SAA*) and genes encoding key factors of the adaptive immune system (*FKBP4*, *TCRG*). Moreover, we included genes coding for key metabolic enzymes (*CS*, *ENO1*, *PGM3*), carriers (*UCP2*) and hormones (*IGF1*) that ensure sufficient energy supply during stress responses. Most of these selected factors are well-known biomarkers involved and interconnected in the cellular response to (temperature) stress (Fig. 1B). In addition, we chose *MUSTN1* (Gersch and Hadjiargyrou, 2009) to quantify a further developmental parameter of pikeperch during the experiment, as well as the omnifunctional *HMX1*.

For data normalization, we selected traditionally chosen "housekeeping genes" (*ACTB*, *GAPDH*, *RNA18S*) (Chapman et al., 2015) and genes that proved stable mRNA abundances across different treatment groups in previous investigations on fish (*EEF1A1*, *MTRNR2*, *RPL32*, *RPS5*, and *RPS9*) (Altmann et al., 2015; Amer et al., 2016; Mitter et al., 2009; Ye et al., 2010). Nevertheless, we evaluated the suitability of each reference gene using the qBase $^+$ software, regardless of published recommendations.

3.2. Distinct and common tissue-specific expression profiles of stress-related genes in unchallenged pikeperch

In a pilot study, the set of primer pairs was evaluated and we determined the basal mRNA abundance of 38 potential stress genes as well as 8 reference genes in 14-week-old unchallenged fish (Fig. 3). The geNorm algorithm was used to identify those reference genes, which were suitable for the normalisation of data recorded in liver (Fig. 3A) and gill tissue (Fig. 3B). For most genes, we detected between approximately 1×10^3 and 1×10^4 transcripts per 100 ng RNA (Fig. 3C). *DNAJA1* was the only transcript that was measured beyond the limit of quantification (set at C_q = 35/40) in both tissues. For about a third of the measured genes (n = 14 including *HSP90AA1*, *HSPA1A*, *HSPA6*, *HSP8b* and *HSPB6*), we detected copy numbers only below 10^3 transcripts/100 ng RNA from both tissues (Fig. 3D). In contrast, > 10^4 transcripts/100 ng RNA were measured in both tissues for four target genes (*HSPA8a1*, *HSPA8a2*, *FTH1* and *ENO1*; Fig. 3E), thus reaching as high of concentrations as determined for each of the three reference genes *EEF1A1*, *GAPDH* and *MTRNR2*.

Notably, the copy numbers of 11 genes were rather similar (≥ 2-fold different) when comparing the two tissues with each other, *i.e.* *HSP90AA1*, *HSPA4*, *HSPA8a1*, *HSPA8b*, *HMX1*, *OSGIN2*, *TNF*, *FTH1*, *ENO1*, *PGM3*, and *MUSTN1*. On the other hand, we also found tissue-specific expression patterns; *FGL2* and *TCRG* transcripts were only detectable in gills and not in the liver (Fig. 3C), while the transcript levels of the immune-relevant genes *HP* and *C3* were negligibly low in gills, but significantly high in the liver (Fig. 3E).

3.3. Acute temperature response in pikeperch-model cells

Previous studies in fish revealed that acute temperature stress leads to the fast and strong upregulation of HSPs and related factors (Campbell and Narum, 2009; Quinn et al., 2011; Rebl et al., 2018). Therefore, we assumed that a second pre-test *in vitro* could assess which genes included in the profiling panel would be suitable as detectors for temperature-induced responses. To this end, we used fibroblastic WF2 cells as the model-cell line from *Sander vitreus* (Wilensky and Bowser, 2005) and, together with the WC1 line from *S. vitreus*, the only available cell line from the *Sander* genus. WF2 cells were exposed for a short period of time to an environment that was 15 °C warmer, after which they displayed a clear transcriptional signature in comparison with control cells (Fig. 4A). *HSP90AA1* (105.5-fold), *HSP1A1* (25.3-fold) and *SERPINH1* (13.2-fold) were the most strongly upregulated genes, followed by *HSPB1*, *HSPA8b*, *HSPB8* and *HSPA8a1* (between 4.8- and 2.9-fold upregulated), whereas *IGF1* (−2.6-fold) was the most strongly downregulated gene. Among the HSP-encoding genes, the transcript levels of *DNAJA3*, *−11*, *HSP90AB1*, *HSPA4* and *HSPA8a2* were obviously not modulated by temperature rise. Lastly, it must be mentioned that few genes (*FKBP4*, *C3*, *HP*, *SAA*) were not detectable in this fibroblastic cell line.

3.4. Temperature-dependent expression profiles of stress-related genes in pikeperch

The main experiment was conducted with 8-week-old pikeperch (Fig. 2A). The temperature in the three experimental groups was elevated gradually from 15 °C to 25 °C within 11 days, which mimicked at natural gradual temperature increase. To gain an initial overview of the transcript abundance of the selected stress-related genes, we conducted multiplex RT-qPCRs on liver and gill samples from initially only three individuals per time point. In addition, we included samples from three individuals each from the 15-°C control groups that were sampled at the beginning, in the middle and at the end of the experiment. The profiling data confirmed no significant differences ($p < .05$) between individuals from the 15-°C control groups and individuals from the experimental groups sampled at day 1 (15 °C). Similar to the pilot study (Fig. 3), no or negligibly low copy numbers were detected for specific genes in the liver (*CXCL8*, *DNAJA1*, *FGL2*, *FKBP4*, *HMX1*, *HSPB6*, *STK39*, *OSGIN2*) and gills (*C3*, *DNAJA1*, *HP*, *OSGIN2*, *SAA*) throughout the experiment.

The comparison among the different time points revealed that relatively few genes were differentially regulated by > 3-fold, and even fewer of those showed a consistent perturbation over several days, in the liver (up: *DNAJA3*, *HSP90AA1*, *SERPINH1*, *C3*, *HP* and *SAA*; down: *HSP1A1*, *HSPA6*, *HSPA8b*, *CYSTM1*, *HMOX1*, *TNF*, *UCP2* and *MSTN1*) and in gills (up: *HSP90AA1*, *HSPA8b*, *SERPINH1* and *HMX1*; down: *HSPB6* and *HMOX1*) of pikeperch during the temperature-stress experiment (Fig. 4B, C). An IPA-assisted database analysis revealed that an interaction network consisting of seven transcriptional activators most likely coordinated the transcription of most of the selected genes regulated at 25 °C (Fig. 4D, E). In the liver, hepatocyte nuclear factor 4-alpha (HNF4A), deactivated heat-shock factor 1 (HSF1), activated bHLH transcription factor MYC, activated erythroid 2-like nuclear factor 2 (NFE2L2), nuclear factor kappa B (NF-κB), deactivated tumour protein p53 (TP53) and the activated subunit alpha of hypoxia-inducible factor 1 (HIF1A) have been predicted to control the expression of the majority of the above genes, at least in mammals (Fig. 4D). In the gills, the same transcriptional activators, partly in a different activation state, modulate the expression of the investigated genes in a different fashion (Fig. 4E).

Based on the multiplex-qPCR data comprising three initial data points, we selected eight dynamic gene-expression profiles for validation with an alternative qPCR technique in a total of nine individuals per experimental group. These genes were chosen as they represent HSP-encoding genes that are either significantly up- (*HSP90AA1*, *HSPA8b*, *SERPINH1*; Fig. 5 A-C) or downregulated (*HSPA6*; Fig. 5 D) and immune genes that are predominantly expressed in the liver (*C3*, *HP*, *SAA*; Fig. 5 E-G) or gill tissue (*HMX1*; Fig. 5 H). Our additional single-gene RT-qPCR analyses validated the previous observations based on the multiplexing assays. We also noticed that the mRNA levels of *HSP90AA1*, *HSPA8b*, *HSPA6*, *HP* and *SAA* were dynamically regulated, in part with strong interindividual variance, while *SERPINH1* showed cumulatively increasing mRNA abundances in both the gills and liver, correlating very strongly (correlation coefficient $R > 0.9$, $p \leq 4 \times 10^{-5}$) with the rising temperatures. Similarly strong ($R > 0.9$, $p \leq 4 \times 10^{-4}$) is the temperature-dependence of *HP* and *HMX1* transcript abundance in either the liver or gills, respectively. The correlation between temperature and transcript abundance of *C3* and *SAA* in the liver, *HSP90AA1* in the gills, and *HSPA8b* in the liver and gills were still moderately high ($R > 0.6$, $p < .05$).

3.5. Systemic stress as evaluated by glucocorticoid hormones

We established a LC-MS/MS method which allows for the normalization of the extraction efficiency making use of an internal isotope standard (cortisol-D_4). After 10 days of incremental temperature increases (1 °C/d), at a temperature of 25 °C, there was no increase observed in glucocorticoids. At the end of the experiment, 11.8 ± 13.0 ng/g wet-weight cortisol was observed in the treatment compared to 25.6 ± 14.6 ng/g wet weight. Congruently, 4.5 ± 5.0 ng/g wet-weight cortisone was observed in the treatment group, compared to 3.9 ± 3.8 ng/g wet weight.

4. Discussion

Temperature is among the most tightly controlled parameters in aquaculture, usually fluctuating by only 2 °C. From a farmer's perspective, temperature is selected to assure maximal growth since its effects on animal welfare are widely unknown. Pikeperch is commonly reared between 23 °C and 25 °C in RAS, though they hardly face temperatures above 20 °C in their natural habitat. Here, we studied the stress response of pikeperch at an incremental temperature increase of

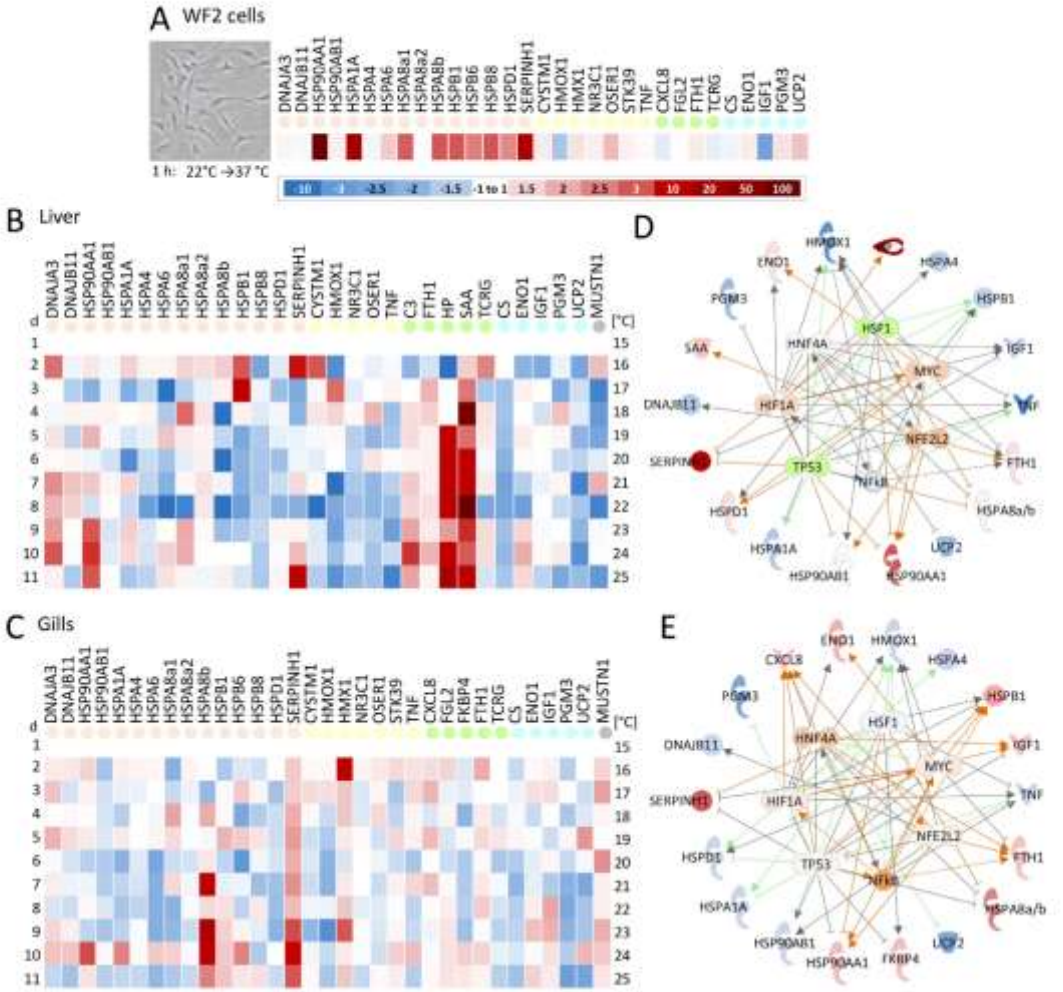

Fig. 4. Multiplex profiling of stress-related genes following *in-vitro* and *in-vivo* temperature challenge experiments.
HeatMap of gene expression values as determined (A) *in vitro* and (B, C) *in vivo* following the experimental temperature increase. Quantified genes are indicated as gene symbols above the HeatMaps, together with circles coloured according to the categories listed in Fig. 1A. (A) WF2 cells were heat shocked at 37 °C for 1 h (treatment group, $n = 3$) and compared to a control incubated constantly at 22 °C (control group, $n = 3$). The modulation of mRNA expression of the selected genes in treated *versus* control cells is displayed as fold-change values (coloured according to the given scale). During the *in-vivo* experiment, temperature was increased gradually from 15 °C to 25 °C (listed on the right margin) within 11 days (left margin) and gene expression was profiled (B) in the liver and (C) gills of three pikeperch (one per experimental group). The mean expression of individuals from control groups and day 1 of experimental groups was set as 1.0 and the means from the other time points were expressed in relation to this reference and coloured according to the given scale. *RPL32* and *RPS5* served as reference genes recommended to normalize the data. (C, D) The "Upstream analysis" of the IPA program plotted putative transcription factors, their expected activation status (active in orange; inhibited in green; indifferent/unknown in grey) and their impact on the expression of genes (coloured according to the HeatMap) based on the expression data (D) in the liver or (E) gills on day 11 of the experiment. Full and broken lines indicate direct and indirect impacts on gene expression, respectively (activation in orange; inhibition in green; inconsistent or no prediction in grey). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1 °C per day starting at an ambient temperature of 15 °C.

The response to temperature stress is generally characterised by increased levels of circulating hormones and transcripts coding for HSPs to cope with harmful conditions including hyperthermia, hypoxia, and/or oxidative stress. Moreover, stress modulates the concentration of several immune and metabolic transcripts. The technical requirements for a fast, efficient and cheaper recording of such transcript-based parameters have also been developed, for use in farm-animal production including aquaculture. Since the parallel quantification of multiple parameters allows more detailed insights into physiological

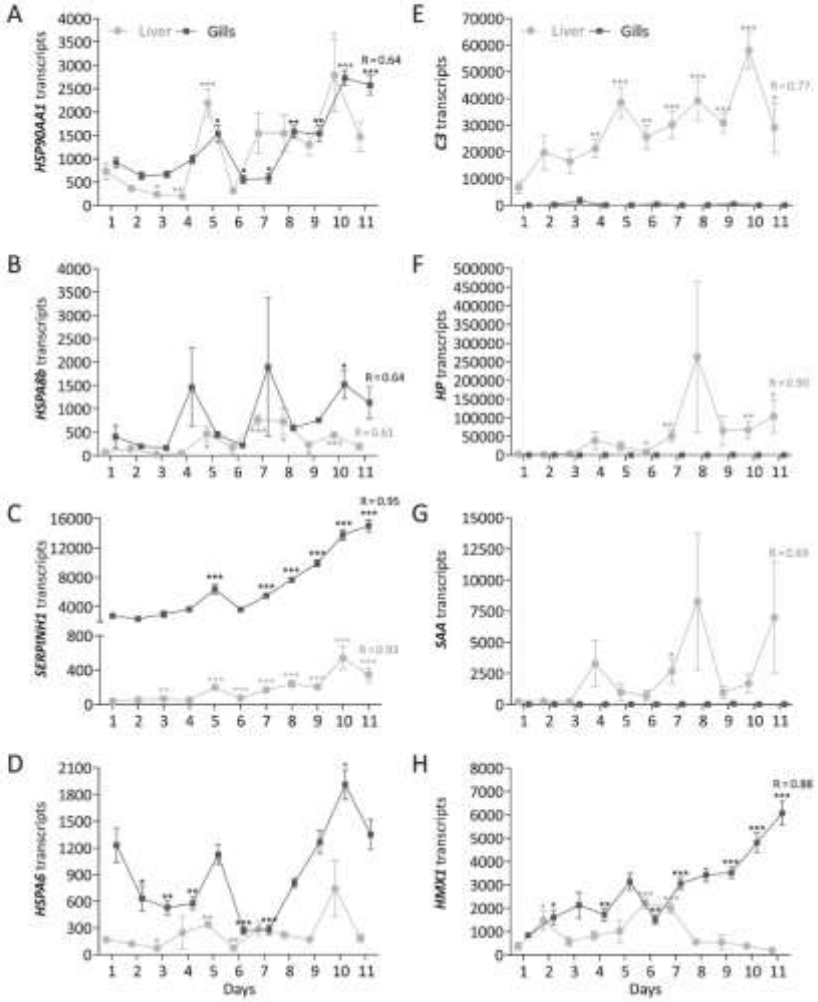

Fig. 5. Single-qPCR profiling of stress-related genes in thermally challenged pikeperch. Transcript amounts were calculated per 100 ng of total RNA of liver (grey) and gills (black) from pikeperch ($n = 9$) and normalized to the mRNA concentrations of the reference genes *MTRNR2*, *EEF1A1*, *RPL32* and *RPS5*. Data are expressed as means ± SEM; asterisks indicate significant differences (*$p < .05$; **$p < .01$; ***$p < .001$). Spearman's Rho (R) correlation is indicated at the end point of each graph in cases where the association between the transcript number and temperature is considered statistically significant (two-tailed $p < .05$).

alterations, we compiled a pikeperch-specific panel composed of 38 genes potentially indicating stress and 8 reference genes. This panel was evaluated on heat-stressed *Sander* cells, which revealed the joint strong upregulation of several HSP-encoding genes such as *HSP90AA1*, *HSP1A1* and *SERPINH1* representing well-known marker genes in fish. In contrast, other HSP members, including *HSP90AB1*, *HSPA4* and *HSPA8a2*, were unaffected by heat as were most of the general-stress, immune and metabolic genes. The reason for this may be found in the short challenge period that gave just enough time to develop an early warning and an acute supply with chaperones.

To answer our research question of whether pikeperch suffer from inadequate water temperatures in closed tank systems, our *invivo* experiment focussed on a significantly longer time frame to detect dynamics in the transcriptional regulation of stress responses in the liver and gills, which are two tissues with major relevance for stress physiology in fish. Our investigation revealed that pikeperch do not develop severe responses to ambient temperatures between 15 °C and 25 °C if they are confronted with mild temperature increments because the expression levels for most of the investigated genes were only modestly altered. This is attributable to the fact that some of the analysed genes are known as non-inducible and/or constitutively expressed features in mammals (*HSP90AB1*, *HSPA4*) and their piscine orthologs behave obviously in a similar way. Additionally, our *in vitro* analysis confirmed the low transcriptional activity of other HSPs (*DNAJA3*, *– 11*) and distinct isoforms (*HSPA8a2*). Nevertheless, we detected a few interesting profiles of promising biomarker candidates, and their

remarkably modulated changes in expression might indicate that they could exceed a critical threshold under different conditions.

Among the investigated HSP-encoding genes, *SERPINH1* (alias *HSP47*) carries the most obvious potential for a diagnostic marker gene indicating cumulative temperature stress in both of the tissues investigated from pikeperch (as assessed by correlation analysis). Similar studies reported previously the outstanding expression profile of *SERPINH1* in various salmonid species (Jeffries et al., 2014; Jeffries et al., 2012; Li et al., 2017; Rebl et al., 2013; Stefanovic et al., 2016; Tomalty et al., 2015; Verleih et al., 2015; Wang et al., 2016) as well as in cyprinid species (Lele et al., 1997; Long et al., 2012; Mahanty et al., 2017) upon exposure to thermal stress. SERPINH1 is involved in the biosynthesis of collagen (Nagata et al., 1988; Nakai et al., 1992), which is a major component of the extracellular matrix. It is worth noting here that we observed a stronger basal expression in the gills compared to the liver. This is most likely due to the fact that branchial lamella are composed of collagen columns (Kudo et al., 2007; Sardet et al., 1979) that require the presence of the collagen chaperone SERPINH1. It also functions as a binding partner of the amyloid-precursor protein (Bianchi et al., 2011) and seems to play a key role in the restoration of homeostasis during heat stress, including the detoxification of "reactive oxygen species", which occur as pathophysiologic side-effects of thermal stress responses (Wang et al., 2016).

Together with *SERPINH1*, the transcripts that encode the H6-family homeobox protein 1 (HMX1) correlated highly with rising temperatures in the gills of pikeperch, which was in stark contrast to the HMX1-transcript number in the liver. Homeobox-transcription factors have been thoroughly investigated regarding their vital role in the developing central nervous system (Heuer and Kaufman, 1992). Moreover, HMX1 plays critical roles during stress responses as they modulate the transition of noradrenergic/cholinergic phenotypes (Furlan et al., 2013) and control the activity of protein-tyrosine phosphatases, which are involved in various signal-transduction pathways (Boulling et al., 2013).

Similar as *SERPINH1*, *HSP90AA1* has frequently been proven as a universal marker gene in studies on thermal stress in fish (Buckley et al., 2006; Jeffries et al., 2014; Rebl et al., 2013; Tomalty et al., 2015; Verleih et al., 2015). In line with this, the inducible expression of *HSP90AA1* could be demonstrated once again in the WF2-cell line and the investigated tissues of pikeperch peaking at 19 °C and 24 °C, contrary to the relatively stable expression of its paralog *HSP90AB1*.

It is well documented that *HSPA1A* and *HSPA6* are the most heat-inducible members of the HSPA (HSP70) gene family (Kampinga et al., 2009) and our *in vitro* test also reflected its strong inducibility. Surprisingly, *HSPA1A* showed a similarly stable expression in the liver and gills of pikeperch as other HSPs that are generally known as not or only modestly heat-responsive, such as *HSP90AB1* (Subbarao Sreedhar et al., 2004) and *HSPA4* (Nonoguchi et al., 1999). The transcript number of *HSPA6* also did not correlate significantly with rising water temperature. In this context, it should be noted that we failed to detect DNAJA1-encoding transcripts in the liver and gills at any temperature, which, together with the hardly altered mRNA level of *DNAJB11*, strongly indicates the obvious absence of a significant heat-stress response.

HSPA8 was previously reported as an inducible, stress-responsive factor in fish (Boone and Vijayan, 2002) and supports our finding that isoform b of *HSPA8* from pikeperch might be considered a marker for a developing stress response in gills. In contrast, isoform *HSPA8a2* remained on a constant level in model cells as well as in the liver and gills of challenged pikeperch. Notably, *HSPA8b* copy numbers were detected on a comparably low level suggesting that this HSP isoform may act as an upstream regulator, which triggers downstream signalling cascades. Consistent with this, it has been documented that HSPA8 stimulates the synthesis of TNF *in vitro* (Ihara et al., 2010), but other HSPs also induced several cytokines, such as TNF and interleukins (Srivastava, 2002). We also did not detect significantly altered transcript numbers of *TNF* or *CXCL8*, but we found an increased expression of acute-phase genes such as *HP*, *SAA* and complement *C3*. Acute-phase genes are well known for their remarkable inducibility upon immune stimulation (Cray, 2012; Jensen et al., 1997; Köhis et al., 2015; Magnadottir et al., 2011), but they respond also to temperature stress (Nikoskelainen et al., 2004; Rebl et al., 2013) and the interlinked expression of HSPs and APPs has been characterised (Gruys et al., 2005). Moreover, *C3* expression was impacted in pikeperch exposed to changing light regimes (Baekelandt et al., 2018). Also, confinement stress has been identified to trigger the expression of *SAA* and *HP* (Talbot et al., 2009), but the authors still detected large interindividual variations in *SAA* expression that are comparable to those seen in the present study, which disqualifies SAA as a reliable biomarker.

Among the downregulated features, *UCP2* is noteworthy. *UCP2* transcript levels commonly decreased in a moderate fashion in both tissues of stressed fish almost throughout the experiment. It has been suggested that UCPs have a thermogenic function (Brand and Esteves, 2005), which provides a plausible indication for its downregulation in an increasingly warmer environment.

Previous reports documented that yellow perch *Perca flavescens* and Eurasian perch *Perca fluviatilis* can be held at ranges between ~10 °C and 25 °C (Grasset et al., 2016; Overton et al., 2008), even in combination with salinity changes, but a temperature of 31 °C has been determined as the upper thermal tolerance limit of Eurasian perch (Jensen et al., 2017). Taken together with the present study, several perciform species including pikeperch tolerate temperatures around 25 °C. However, we interpret distinct gene profiles recorded in the present study as early indicators of compromised well-being.

5. Conclusion

Temperature controls important physiological processes (Brett, 1971; Brett et al., 1969; Kestemont and Baras, 2001). Recording multiple, informative stress-biomarker genes gains more and more importance for the aquaculture industry with regard to fish welfare and consumer opinion. The present study determined the transcript levels of 38 potential stress-biomarker genes in two tissues relevant for the teleostean stress response. We identified only a few apparently temperature-sensitive genes whose transcript abundances correlate with increasing ambient water temperatures, above all *SERPINH1*, followed by *HP*, *HMX1*, *C3*, *HSP90AA1* and *HSPA8b*. The mass spectrometric detection of cortisol levels confirmed that pikeperch do not develop severe responses at ambient temperatures between 15 °C and 25 °C. Altogether, the present study provides no indications for the need to reconsider and adapt the conventional breeding of pikeperch at temperatures between 23 °C and 25 °C in RAS, as long as temperature fluctuations are avoided.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2018.11.043.

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Supplementary Table 1: List of potential biomarker genes investigated in the present study along with gene symbols and annotated function

Official gene symbol	Alternative gene name	Gene product	Function
Reference genes:			
ACTB	*BRWS1*	Beta-actin	Cytoskeletal composition
EEF1A1	*EF1A, CCS3*	Eukaryotic elongation factor 1 alpha 1	Translation
GAPDH	*G3P*	Glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate metabolism
MTRNR2	*16S RNA*	Mitochondrially encoded 16S RNA	Cell survival
RNA18S	*RN18S*	18S ribosomal RNA	Protein synthesis
RPL32	*L32, PP9932*	Ribosomal protein L32	Protein synthesis
RPS5	*RS5*	Ribosomal protein S5	Protein synthesis
RPS9	*RS9*	Ribosomal protein S9	Protein synthesis
Target genes:			
C3	*ASP*	Complement component C3	Innate immune response
CS	*cisY*	Citrat synthase	Oxidative metabolism
CXCL8	*IL-8*	Interleukin 8	Inflammation
CYSTM1	*C5orf32*	Cystein rich transmembrane module-containing protein 1	Stress and immune response
DNAJA1	*DJ-2*	DnaJ heat shock protein family (Hsp40) member A1	Regulation of ATP hydrolysis activity, recruiting of substrates
DNAJA3	*TID1*	DnaJ heat shock protein family (Hsp40) member A3	Protein folding, regulation of proliferative and apoptotic processes
DNAJB11	*CB954*	DnaJ heat shock protein family (Hsp40) member B11	Protein folding
ENO1	*EH28_01742*	Enolase 1	Glycolysis
FGL2	*T49*	Fibrinogen-like protein 2	Regulation of immune responses
FKBP4	*FB34f09*	FK506 binding protein 4	Protein folding, regulation of immune responses
FTH1	*FHC*	Ferritin heavy chain 1	Iron storage
HMOX1	*EH28_20803*	Heme oxygenase 1	Haeme catabolism
HMX1	*Nkx5-3*	H6 homeobox 1	Morphogenesis, response to stress
HP	*BP*	Haptoglobin	Plasma haemoglobin-binding

HSP90AA1	*HSP90(A)*	Heat shock protein 90 alpha family class A member 1	Protein folding, response to stress
HSP90AB1	*HSP90B*	Heat shock protein 90 alpha family class B member 1	Protein folding, signal transduction
HSPA1A	*HSP70-1*	Heat-shock protein family A (Hsp70) member 1A	Protein folding, response to stress
HSPA4	*HSP70RY*	Heat-shock protein family A (Hsp70) member 4	Protein folding, response to stress
HSPA6	*HSP70B*	Heat-shock protein family A (Hsp70) member 6	Protein folding, response to stress
HSPA8a1	*HSC70-1*	Heat-shock protein family A (Hsp70) member 8a1	Protein folding, response to stress
HSPA8a2	*HSC70-1*	Heat-shock protein family A (Hsp70) member 8a2	Protein folding, response to stress
HSPA8b	*HSC70-1*	Heat-shock protein family A (Hsp70) member 8b	Protein folding, response to stress
HSPB1	*HSP27*	Heat-shock protein family B (small) member 1	Protein folding, response to stress
HSPB6	*HSP20*	Heat-shock protein family B (small) member 6	Protein folding, vasodilation, platelet function, insulin resistance
HSPB8	*FC09C11*	Heat-shock protein family B (small) member 8	Cell proliferation, apoptosis and carcinogenesis
HSPD1	*HSP60*	Heat-shock protein 60	Protein folding, signalling in innate immune system
IGF1	*MGF*	Insulin-like growth factor 1	Growth regulation, Anabolism
MUSTN1	*MSTN1*	Musculoskeletal embryonic nuclear protein 1	Growth regulation
NR3C1	*GR*	Nuclear receptor subfamily 3 group C member 1	Regulation of inflammatory responses, cellular proliferation, and differentiation
OSER1	*Perit1*	Oxidative stress responsive serine-rich protein 1	Response to oxidative stress
OSGIN2	*hT41*	Oxidative stress-induced growth inhibitor family member 2	Regulation of cell growth
PGM3	*AGM1*	Phosphoglucomutase 3	Glycolysis
SAA1	*PIG4*	Serum amyloid A1	Acute-phase response, tissue protection
SERPINH1	*HSP47*	Serpin family H member 1	Collagen biosynthesis
STK39	*DCHT*	Serin/threonin kinase 39	Response to cellular stress
TCRG	*TCRGV1S1*	T-cell receptor gamma chain	Adaptive immune response
TNF	*DIF*	Tumor necrosisfactor	Innate immune response
UCP2	*SLC25A9*	Uncoupling protein 3	ATP synthesis, protection from oxidative stress

6. Appendix

6.1. Tables

Table 1: **Candidate genes included in all three studies**

Genes examined in the included in-vivo studies of the current dissertation.

Gene symbol	Gene product	Function	Study
Reference			
ACTB	Beta-actin	Cytoskeletal composition	III
EEF1A1	Eukaryotic elongation factor 1 alpha 1	Translation	I, II, III
GADPH	Glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate metabolism	III
MTRNR2	Mitochondrially encoded 16S RNA	Cell survival	III
RNA18S	18S ribosomal RNA	Protein synthesis	III
RPL32	Ribosomal protein L32	Protein synthesis	I, II, III
RPS5	Ribosomal protein S5	Protein synthesis	I, II, III
RPS9	Ribosomal protein S9	Protein synthesis	III
Stress response			
CYSTM1	Cysteine rich transmembrane module-containing protein 1	Stress and Immune responses	III
DNAJA1	DnaJ heat shock protein family (Hsp40) member A1	Regulation of ATP hydrolysis activity, recruiting of substrate	III
DNAJA3	DnaJ heat shock protein family (Hsp40) member A3	Protein folding, regulation of proliferative and apoptotic processes	III
DNAJB11	DnaJ heat shock protein family (Hsp40) member B11	Protein folding	III
EPAS1	Endothelial PAS domain protein 1	Developmental processes, response to hypoxia	I, II
HIF1A	Hypoxia inducible factor 1 subunit alpha	Response to hypoxia, development: angiogenesis, glucose metabolism, cellular proliferation, apoptosis	I, II

Table 1: **Candidate genes** (continued)

Gene symbol	Gene product	Function	Study
Stress response			
HMOX1	Heme oxygenase 1	Haeme catabolism, response to stress	II, III
HMX1	H6 homeobox 1	Morphogenesis, response to stress	III
HSF1	Heat shock transcription factor 1	Response to environmental stress, protein and lipid metabolism, contribution to immune processes, protein refolding	I, II
HSF2	Heat shock transcription factor 2	Signal transduction during heat stress, development	I, II
HSP90AA1	Heat-shock protein 90 alpha family class A member 1	Protein folding, response to stress	II, III
HSP90AB1	Heat shock protein 90 alpha family class B member 1	Protein folding, signal transduction	III
HSPA1A	Heat-shock protein family A (Hsp70) member 1A	Protein folding, response to stress	III
HSPA4	Heat-shock protein family A (Hsp70) member 4	Protein folding, response to stress	III
HSPA6	Heat-shock protein family A (Hsp70) member 6	Protein folding, response to stress	III
HSPA8a1 (HSC70)	Heat-shock protein family A (Hsp70) member 8a1	Protein folding, response to stress	III
HSPA8a2 (HSC70)	Heat-shock protein family A (Hsp70) member 8a2	Protein folding, response to stress	III
HSPA8b (HSC70)	Heat-shock protein family A (Hsp70) member 8b	Protein folding, response to stress	III
HSPB1	Heat-shock protein family B (small) member 1	Protein folding, response to stress	III
HSPB6	Heat-shock protein family B (small) member 6	Protein folding, vasodilation, platelet function, insulin resistance	III
HSPB8	Heat-shock protein family B (small) member 8	Cell proliferation, apoptosis and carcinogenesis	III
HSPD1	Heat-shock protein 60	Protein folding, signalling in innate immunity	III
SERPINH1	Serpin family H member 1	Collagen biosynthesis, response to heat stress	III
tOSTF1	*Teleost-specific* Osmotic stress transcription factor 1	Signal transduction after osmotic stress	I
NR3C1	Nuclear receptor subfamily 3 group c member 1	General stress response, signal transduction	I, II, III
OSER1	Oxidative stress responsive serine-rich protein 1	Response to oxidative stress, signal transduction	III
OSGIN2	Oxidative stress-induced growth inhibitor family member 2	Growth regulation during stress, response to oxidative stress, apoptosis	III
C3	Complement C3	Acute-phase protein, complement system activation	II, III

Table 1: **Candidate genes** (continued)

Gene symbol	Gene product	Function	Study
Immune response			
STK39	Serin/threonine kinas 39	Response to cellular stress	III
CSF2	Colony stimulating factor 2	Regulation of myeloid cell mobilisation, acute-phase protein	II
CXCL8	Interleukin 8	Pro-inflammatory cytokine	I, II, III
FGL2	Fibrinogen-like protein 2	Acute-phase protein, regulation of immune response	III
FKBP4	FK506 binding protein 4	Protein folding, regulation of immune responses	III
FTH1	Ferritin heavy chain 1	Iron storage, regulation of immune responses	II, III
HP	Haptoglobin	Plasma haemoglobin-binding, acute-phase protein	II, III
IL1B	Interleukin 1 beta	Pro-inflammatory cytokine	I, II
LYZ	Lysozyme	Antimicrobial agent	I
MHC II alpha	Major histocompatibility complex II alpha	Antigen presentation to CD4+ T lymphocytes	II
MPO	Myeloperoxidase	Lysosomal enzyme, response to inflammation	II
RAG1	Recombination activating 1	Immunoglobin V-D-J recombination	II
SAA	Serum amyloid A	Acute-phase response, tissue protection	II, III
TCRG	T-cell receptor gamma chain	Antigen recognition (T cell activation)	III
TNF	Tumor necrosis factor	Proinflammatory cytokine	II, III
Cell homeostasis			
TFEB	Transcription factor EB	Lipid metabolism, bone resorption, immune response	I
UCP2	Uncoupling protein 3	ATP synthesis, protection form oxidative stress	III
Metabolism			
APOE	Apolipoprotein E	Lipid metabolism	I
CS	Citrate synthase	Oxidative metabolism	III
CKM	Creatine kinase, M	Energy metabolism (creatine system)	I
ENO1	Enolase 1	Glycolysis	III
GATM	Glycine amidinotransferase	Energy metabolism (creatine system)	I

Table 1: **Candidate genes** (continued)

Gene symbol	Gene product	Function	Study
Metabolism			
PGM3	Phosphoglucomutase 3	Glycolysis	III
PPARA	Peroxisome proliferator activated receptor alpha	Lipid metabolism	I
PPARD	Peroxisome proliferator activated receptor delta	Lipid metabolism	I
Growth			
GHR	Growth hormone receptor	Growth regulation, Signal transduction	I
IGF1	Insulin like growth factor 1	Growth regulation, anabolism	III
IGF2	Insulin like growth factor 2	Growth regulation	I
MSTN1	Muscoskeletal embryonic nuclear protein 1	Growth regulation	III
Gonadal maturation			
SOX9	SRY-box transcription factor 9 c	Sex determination, gonad development & maturation; chondro- & skeletogenesis	I
Organogenesis			
BMP4	Bone morphogenetic protein 4	Chondro- & skeletogenesis, morphogenesis; organogenesis	I
BMP7	Bone morphogenetic protein 7	Chondro- & skeletogenesis, morphogenesis	I
MYH6	Myosin heavy chain	Major component of motor protein myosin (heart muscle)	I
RXRA	Retinoid x receptor alpha	Organogenesis, morphogenesis, lipid metabolism	I

Table **2: Selected blood parameters of Study II**

Individual glucose and lactate levels of juvenile pikeperch during hypoxic challenge.

Treatment	**Day of the experiment**	**Glucose (mmol/l)** Detection range: 1.1–33.3 mmol/l	**Lactate (mmol/l)** Detection range: 0.7-26 mmol/l
Control	1	5.88	3.30
Control	1	8.21	2.80
Control	1	10.10	3.90
Control	1	1.78	LOW
Control	1	3.05	3.10
Hypoxic challenge	1	4.88	2.60
Hypoxic challenge	1	6.33	3.90
Hypoxic challenge	1	6.88	2.40
Hypoxic challenge	1	4.27	2.90
Hypoxic challenge	1	6.05	3.60
Control	7	6.38	1.90
Control	7	7.22	1.50
Control	7	3.39	LOW
Control	7	3.05	LOW
Control	7	3.77	LOW
Hypoxic challenge	7	4.66	LOW
Hypoxic challenge	7	6.94	LOW
Hypoxic challenge	7	3.89	2.00
Hypoxic challenge	7	7.94	1.00
Hypoxic challenge	7	6.94	0.80
Control	14	6.38	0.90
Control	14	5.83	0.90
Control	14	1.78	n.a.
Control	14	1.89	LOW
Control	14	2.72	0.80
Hypoxic challenge	14	8.27	LOW
Hypoxic challenge	14	2.22	2.10
Hypoxic challenge	14	6.33	1.50
Hypoxic challenge	14	6.05	LOW
Hypoxic challenge	14	6.66	1.00

Table **2 Blood parameters** (continued)

Treatment	**Day of the experiment**	**Glucose (mmol/l)** Detection range: 1.1–33.3 mmol/l	**Lactate (mmol/l)** Detection range: 0.7-26 mmol/l
Control	21	3.39	LOW
Control	21	3.83	1.20
Control	21	7.10	0.80
Control	21	6.27	LOW
Control	21	3.11	1.00
Hypoxic challenge	21	3.39	LOW
Hypoxic challenge	21	2.33	LOW
Hypoxic challenge	21	3.39	LOW
Hypoxic challenge	21	3.39	LOW
Hypoxic challenge	21	3.11	LOW
Control	28	10.71	3.20
Control	28	2.89	1.00
Control	28	10.71	1.10
Control	28	2.89	2.20
Control	28	2.83	1.00
Hypoxic challenge	28	2.89	0.80
Hypoxic challenge	28	2.94	1.60
Hypoxic challenge	28	8.49	1.40
Hypoxic challenge	28	2.50	2.00
Hypoxic challenge	28	4.27	0.90

n.a. = not applicable

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6.3. Declaration of authorship

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6.4.Curriculum Vitae

The CV was deleted for data protection reasons.

Publications, Conferences and Projects

Publications

Goldammer T, Verleih M, Brunner RM, Rebl A, Nguinkal JA, de los Ríos-Pérez L, Schäfer N, Stüeken M, Swirplies F, Wittenburg D (2021). **Zandergenomdaten – Basis für das Smart Farming in Aquakultur.** *Der Zander – Beiträge zur Produktion in Aquakultur in Mitteilungen der Landesforschungsanstalt für Landwirtschaft und Fischerei,* 63: 125-133. ISSN 1618-7938

Schäfer N, Matoušek J, Rebl A, Stejskal V, Brunner RM, Goldammer T, Verleih M, Korytář T (2021). **Effects of Chronic Hypoxia on the Immune Status of Pikeperch (*Sander lucioperca* Linnaeus, 1758).** Biology, 10(7), 649. https://doi.org/10.3390/biology10070649

Schäfer N, Kaya Y, Rebl H, Stüeken M, Rebl A, Nguinkal JA, Franz GP, Brunner RM, Goldammer T, Grunow B, Verleih M (2021). **Insights into Early Ontogenesis: Characterization of Stress and Developmental Key Genes of Pikeperch (*Sander lucioperca*) in vivo and in vitro.** *Fish Physiology and Biochemistry*, 47, 515-532. https://doi.org/10.1007/s10695-021-00929-6

De los Ríos-Pérez L, Nguinkal JA, Verleih M, Rebl A, Brunner RM, Klosa J, Schäfer N, Stüeken M, Goldammer T, Wittenburg D (2020). **An ultra-high density SNP-based linkage map for enhancing the pikeperch (*Sander lucioperca*) genome assembly to chromosome-scale.** *Scientific Reports*, 10, 22335. https://doi.org/10.1038/s41598-020-79358-z

Nguinkal JA, Brunner RM, Verleih M, Rebl A, de Los Ríos-Pérez L, Schäfer N, Hadlich F, Stüeken M, Wittenburg D, Goldammer T (2019). **The First Highly Contiguous Genome Assembly of Pikeperch (*Sander lucioperca*), an Emerging Aquaculture Species in Europe.** *Genes*, 10(9):708. https://doi:10.3390/genes10090708

Schäfer N, Verleih M, Korytář T, Rebl A, Matoušek J, Chabera J, Stejskal V, Brunner RM, Goldammer T (2019). **Influence of hypoxia stress on the immune response of pikeperch (*Sander lucioperca* L., 1758).** *Fish & Shellfish Immunology*, 91: 427. https://doi:10.1016/j.fsi.2019.04.170

Swirplies F, Wuertz S, Baßmann B, Orban A, Schäfer N, Brunner RM, Hadlich F, Goldammer T, Rebl A (2019). **Identification of molecular stress indicators in pikeperch *Sander lucioperca* correlating with rising water temperatures.** *Aquaculture*, 501, 260-271. https://doi.org/10.1016/j.aquaculture.2018.11.043

Conferences

38th International Society for Animal Genetics (ISAG) 2021, Virtual Conference:

Pikeperch genome data – basis for the smart farming in aquaculture. Tom Goldammer, Marieke Verleih, Ronald M. Brunner, Alexander Rebl, Julien A. Nguinkal, Lidia de los Ríos-Pérez, Nadine Schäfer, Marcus Stüeken, Fabian Swirplies, Dörte Wittenburg. *Abstract for poster presentation* #P230 & *Abstract for oral presentation* #W237

37th International Society for Animal Genetics (ISAG) 2019, Lleida Spain:

Analysis of adaptive plasticity of pikeperch (*Sander lucioperca* L., 1758) after temperature change based on gene expression data. Tom Goldammer, Fabian Swirplies, Sven Wuertz, Björn Baßmann, Axel Orban, Nadine Schäfer, Ronald M. Brunner, Frieder Hadlich, Alexander Rebl, Marieke Verleih. *Abstract for oral presentation in the category Genetics and Genomics of Aquaculture Species* #OP76

Aquaculture Europe (EAS) 2019, Berlin Germany:

Study of genomic variation with whole genome sequencing in pikeperch Sander lucioperca. Lidia de los Ríos-Pérez, Ronald M. Brunner, Marieke Verleih, Alexander Rebl, Julien A. Nguinkal, Nadine Schäfer, Tom Goldammer, Marcus Stüeken, Dörte Wittenburg. *Abstract for poster presentation in the category Genomic Research, Tools and Applications* Board #317

Aquaculture Europe (EAS) 2019, Berlin Germany:

The draft genome of pikeperch *Sander lucioperca*. Julien A. Nguinkal, Lidia de los Ríos-Pérez, Nadine Schäfer, Frieder Hadlich, Marcus Stüeken, Olaf Wolkenhauer, Ronald M. Brunner, Alexander Rebl, Marieke Verleih, Dörte Wittenburg, Tom Goldammer. *Abstract for oral presentation in the category Genomic Research, Tools and Applications*

Aquaculture Europe (EAS) 2019, Berlin Germany:

Genomic tools for the aquaculture of pikeperch *Sander lucioperca* L., 1758. Tom Goldammer, Julien Nguinkal, Lidia de los Ríos-Pérez, Nadine Schäfer, Frieder Hadlich, Marcus Stüeken, Olaf Wolkenhauer, Ronald M. Brunner, Alexander Rebl, Marieke Verleih, Dörte Wittenburg. *Abstract for poster presentation in the category Genomic Research, Tools and Applications* Board #290

Aquaculture Europe (EAS) 2019, Berlin Germany:

Quantification of stress and developmental key genes during early ontogenesis of pikeperch *Sander lucioperca.* Marieke Verleih, Nadine Schäfer, Fabian Swirplies, Sven Wuertz, Sebastian Kanne, Ronald M. Brunner, Julien A. Nguinkal, Lidia de los Ríos-Pérez, Marcus Stüeken, Dörte Wittenburg, Alexander Rebl, Tom Goldammer. *Abstract for poster presentation in the category Fish Welfare* Board #263

Aquaculture Europe (EAS) 2019, Berlin Germany:

Impact of hypoxia stress on the immune status of farmed pikeperch (*Sander lucioperca* L., 1758). Nadine Schäfer, Marieke Verleih, Tomáš Korytář, Alexander Rebl, Jan Matoušek, Jan Chabera, Vlastimil Stejskal, Marcus Stüeken, Ronald M. Brunner, Julien A. Nguinkal, Lidia de los Ríos-Pérez, Tom Goldammer. *Abstract for poster presentation in the category Fish Welfare* Board #259

3rd International Society of Fish and Shellfish Immunology (ISFSI) 2019, Las Palmas de Gran Canaria Canary Islands:

Influence of hypoxia stress on the immune response of pikeperch (*Sander lucioperca* L., 1758). Nadine Schäfer**,** Marieke Verleih, Tomáš Korytář, Alexander Rebl, Jan Matoušek, Jan Chabera, Vlastimil Stejskal, Ronald M. Brunner, Tom Goldammer. *Abstract for oral presentation in the category Fish Immunology* #0-016

Fish Immunology Workshop 2018, Wageningen The Netherlands:

The significance of stress-inducible transcription factors for early stress immune responses of *Sander spp*. Nadine Schäfer, Marieke Verleih, Fabian Swirplies, Tomáš Korytář, Alexander Rebl, Tom Goldammer. *Abstract for poster presentation*

Projects

TNA-project #AE080004 “Influence of Hypoxia on the Immune Response of Pike-perch” within the infrastructure support programme “AQUAECXEL^{2020} – Aquaculture infrastructures for excellence in European fish research” (re. n. 652831) from the EU programme Horizon 2020.