The Embryonic to Larval Developmental Transition of Pikeperch (*Sander lucioperca*) – Analysis of a critical life phase

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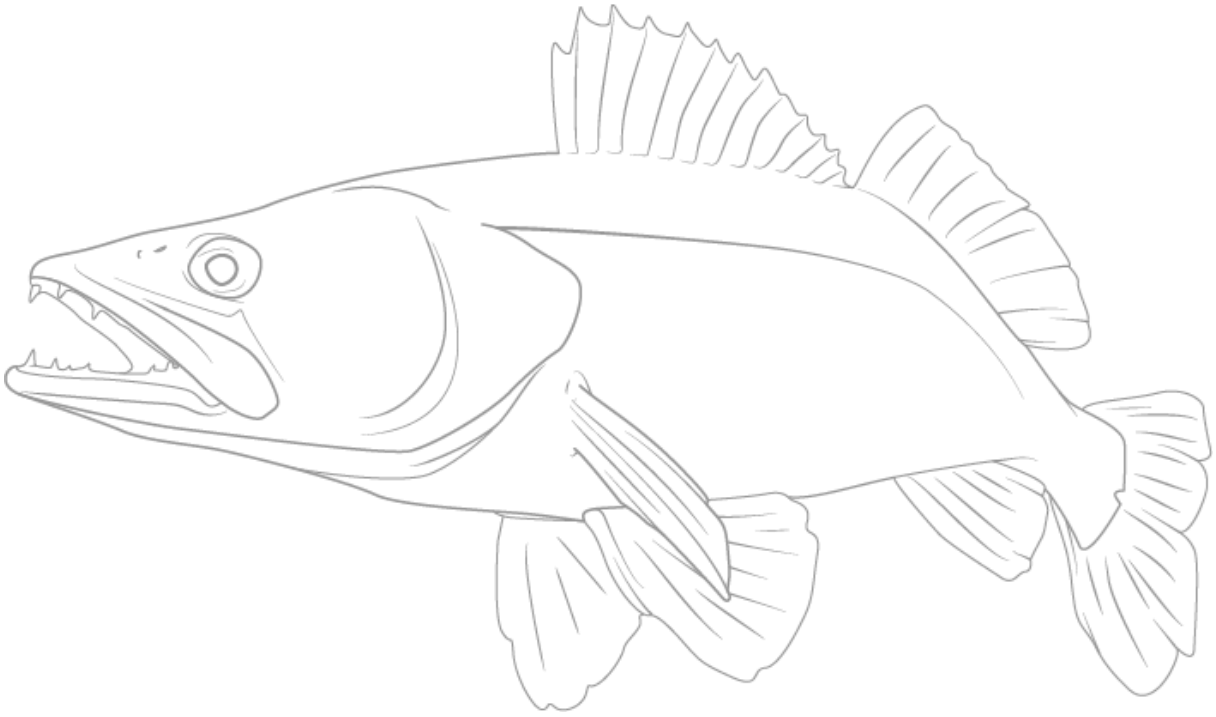


“We must plant the sea and herd its animals using the sea as farmers instead of hunters. That is what civilization is all about - farming replacing hunting.”

Jacques Yves Cousteau

Summary

The need for the diversification of utilised species has emerged in the present aquaculture production environment. Shifts in consumer interest, climate change-induced temperature increases, and major fish disease outbreaks have put a strain on this industry. In this context, the pikeperch (*Sander lucioperca*) has become a new target species for aquaculture in Central Europe. This new aquaculture focus species exhibits high numbers of offspring, fast growth, and high consumer acceptance. It can also effectively deal with higher temperatures and turbid water. However, the rate of successful rearing is still low, as various developmental transformations and environmental effects commonly lead to high mortality rates during the early ontogenetic stages. The aim of this doctoral project was thus to obtain insight into embryonic to larval developmental changes during pikeperch ontogeny. Specifically, the times of change that influence survival were of focus. Based on the available literature, particular attention was paid to general growth patterns and the connected developmental changes, the determination of myogenesis gene marker expression changes, and the support of animal welfare efforts for pikeperch rearing procedures. To achieve the aims of the study, a methodical setup consisting of morphometric and developmental observations was combined with transcriptome gene marker analysis for the different ontogenetic stages.

Three developmental phases were differentiated during the embryo-larval transition. Each of these possessed distinct growth patterns with different growth rates. The intermediate threshold phase showed internal organ development that focused on digestive, neuronal, and heart tissues. Three activity phases of myogenesis were determined: during early embryonic development, before hatching, and after hatching during the larval stages. Therefore, muscle development seemed to be regulated to balance energy expenditures. Additionally, two coinciding skeletogenic phases were found. Furthermore, a cell line from whole embryos was developed to support the replacement of animals in future experimental setups. A software system for video analyses was developed to support rearing procedures in aquaculture facilities. This prototype can be used to automate the counting of specimens and thus allows for faster responses to increasing mortalities. Based on the results of this thesis project, further insights into the early development of pikeperches were obtained. This will facilitate the design and adaptation of raising and husbandry protocols, which can help to further establish pikeperch as an aquaculture species and support its application in modern recirculatory systems.

Zusammenfassung

In der heutigen Aquakulturindustrie besteht die Notwendigkeit, das eingesetzte Artenspektrum zu erweitern. Veränderte Verbraucherinteressen, der durch den Klimawandel verursachte Temperaturanstieg und großflächige Krankheitsausbrüche in Aquakulturanlagen belasten die Branche. In diesem Kontext ist der Zander (*Sander lucioperca*) zu einer neuen Fokusart in der Aquakulturindustrie Mitteleuropas geworden. Der Zander verbindet eine hohe Anzahl von Nachkommen mit einem schnellen Wachstum und besitzt bereits eine hohe Akzeptanz beim Verbraucher. Allerdings ist die Erfolgsquote in der Aufzucht gering, da verschiedene Entwicklungsprozesse und Umwelteinflüsse zu einer allgemein hohen Sterblichkeitsrate, insbesondere während der frühen ontogenetischen Stadien, führen. Ziel dieses Promotionsprojektes ist es, einen besseren Einblick in die Entwicklung des Zanders während der Ontogenese von Embryo zur Larve zu gewinnen. Hierbei liegt der Schwerpunkt auf Zeitfenstern, während welcher das Überleben der Larve besonders gefährdet ist. Auf der Grundlage des Stands der bisherigen Forschungsliteratur wurde hierfür ein besonderes Augenmerk auf Wachstumsmustern und damit verbundenen entwicklungsbiologischen Veränderungen, den Expressionsveränderungen von Myogenese-Genmarkern und Möglichkeiten zur Unterstützung von Tierschutzmaßnahmen bei der Zanderaufzucht gelegt. Zu diesem Zweck wurden morphometrische und entwicklungsbiologische Beobachtungen mit Transkriptom-Genmarkeranalysen für die verschiedenen ontogenetischen Stadien kombiniert.

Während des Übergangs vom Embryo zur Larve konnten drei Entwicklungsphasen unterschieden werden. Jede dieser Phasen zeigte ein eigenes Wachstumsmuster und unterschiedliche Wachstumsraten. In der mittleren Phase, der Threshold-Phase gab es Anzeichen zur Entwicklung der inneren Organe, insbesondere bei Verdauungs-, Neuronal- und Herzgewebe. Des Weiteren wurde festgestellt, dass Myogenesegene besonders in drei Phasen verstärkt exprimiert wurden: Während der frühen Embryonalentwicklung, vor dem Schlüpfen sowie nach dem Schlüpfen im Larvenstadium. Die Muskelentwicklung scheint hierbei zum Ausgleich des Energiehaushalts zwischenzeitlich unterdrückt zu werden. Zusätzlich wurden zwei übereinstimmende Zeitfenster für die Skelettentwicklung festgestellt. Um zusätzlich Alternativen für Tierexperimenten zu finden, wurde eine Zelllinie aus embryonalen Zandern entwickelt, um den Einsatz von Tieren in zukünftigen Experimenten zu verringern. Außerdem wurde ein Softwaresystem zur Videoanalyse entwickelt, um Aufzuchtverfahren in Aquakulturanlagen zu unterstützen. Der hierbei entstandene Prototyp kann zum Zählen der kleinen Zanderlarven verwendet werden, was aufwendige manuelle Überprüfungen der fragilen Larven reduziert.

Mit den Ergebnissen aus diesem Dissertationsprojekt konnte ein tieferer Einblick in die frühe Entwicklung des Zanders gewonnen werden. Dies kann dazu beitragen Aufzucht- und Haltungsprotokolle weiterzuentwickeln, um den Zander als Aquakulturart weiter zu etablieren sowie seine Anwendung in modernen Rezirkulationssystemen zu unterstützen.

1. General introduction

The pikeperch (*Sander lucioperca*) is a highly valued food fish and is widely appreciated by consumers for its white flesh and few intermuscular bones (Kestemont, Dabrowski, & Summerfelt, 2015; Komolka *et al.*, 2020; Nelson, Grande, & Wilson, 2016). While its market accessibility and production rely mostly on wild catches, natural populations are already declining in some areas (Lehtonen, Hansson, & Winkler, 1996; Policar *et al.*, 2019; Steenfeldt *et al.*, 2015). Rearing and husbandry in aquaculture facilities is challenging and remains rare, as several bottlenecks, especially high mortalities during early ontogeny, inhibit economically successful rearing (Policar *et al.*, 2019; Steenfeldt *et al.*, 2015). In comparison to common aquaculture fish species like the salmonid rainbow trout (*Oncorhynchus mykiss*), research on the pikeperch remains sparse. Furthermore, the pikeperch was determined to be a possible candidate for successful species diversification processes in the European aquaculture sector (AG NASTAQ, 2020; EU Regulation 2013/1380). In these studies, it was found that the pikeperch could additionally support the development of advanced fish-farming systems, such as recirculation-setups (RAS) or aquaponics (Dalsgaard *et al.*, 2013; Overton, Toner, Policar, & Kucharczyk, 2015; Palm, Knaus, Appelbaum, Strauch, & Kotzen, 2019). With this in mind, this PhD project attempted to support pikeperch rearing by counteracting high mortality rates during embryonic and larval development.

1.1 The biology of the pikeperch

Pikeperch, *Sander lucioperca* (Linneaus 1758) is distributed in fresh and brackish waters of the Central to Eastern European regions (Froese & Pauly, 2022; Haponski & Stepien, 2013; Stepien & Haponski, 2015). It thrives well in warmer (≥ 25°C) and more turbid waters, such as eutrophicated lakes and rivers (Eschbach *et al.*, 2014; Lehtonen *et al.*, 1996; Winkler, Klinkhardt, & Buuk, 1989). The pikeperch is associated with the higher bony fishes (Teleostei), among which it has been placed in the order Perciformes and the family Percidae (Betancur-R *et al.*, 2013; Betancur-R *et al.*, 2017; Nelson *et al.*, 2016). Over time, several synonyms for the scientific names were developed, which include *Stizostedion lucioperca*, *Lucioperca sandra*, *Lucioperca linnei*, and *Centropomus sandat* (Eschmeyer, Fricke, & van der Laan, 2022). Presently, the pikeperch is associated with the genus *Sander*, formerly named *Stizostedion* (Bruner, 2011; Eschmeyer et al., 2022), which includes four other species: the Eurasian Volga-pikeperch (*Sander volgensis*, Gmelin, 1789), and sea-pikeperch (*Sander marinus*, Cuvier, 1828), the North American walleye (*Sander vitreus,* Mitchill, 1818) and the sauger (*Sander Canadensis,* Cuvier, 1828) (Haponski & Stepien, 2013; Nelson *et al.*, 2016).

In pikeperch, sexual maturity starts at the age of three to four years in males and four to five years in females, each of which has a body weight of around 300 to 700 grams (Tölg, Horváth,

& Tamás, 1981). Females are able to produce large quantities of eggs, with the ability to lay up to 200,000 eggs per kg of their bodyweight (Lappalainen, Dorner, & Wysujack, 2003; Lappalainen *et al.*, 2005). The eggs are placed in grooves or on plant materials, where they are fertilised by one male, which then guards the nest and fans fresh water to the eggs. After hatching, the embryos shift into a mixed feeding phase, during which they open their mouths and start to feed on their first prey while simultaneously depleting any remaining endogenous resources (Bastl, 1978; Study I). With the completed change to exogenous feeding, larval growth follows with the formation of the dorsal and anal fins, and their body proportions change until adult proportions are reached (Study I), which marks the beginning of juvenile development (Peňáz, 2001). Depending on the temperature, embryos of less than 5 mm in length are able to reach a size of 13 cm and a bodyweight of 50 g after a year, which demonstrates their fast growth compared to other fish species (Hilge & Steffens, 1996; Ložys, 2004; supported by personal communication with coorperating fisheries).

1.2 Previous research on pikeperch and the supporting literature

The research history on *Sander lucioperca* is sparse, especially in comparison to that of other aquaculture fish species, such as salmonids. This particular body of research has been influenced by historic-political circumstances and technological changes in the aquaculture sector. Consequently, even though some aspects have been addressed in previous research, a general need for more fundamental studies is still salient.

Earlier pikeperch research from 1945 onwards was dominated by Eastern European researchers. This is likely attributable to the distribution range of the European pikeperch. Exemplary of this is the Russian Ichthyologic Research conducted by Kryzhanovsky and Vasnetsov at Severtsov's Laboratory of Comparative Morphology in Moscow, where an ecomorphological approach to development and the theory of ontogenetic intervals in fishes were formulated (Kryzhanovsky, Disler, & Smirnova, 1953; Smirnov, Makeyeva, & Smirnov, 1995; Vasnetsov, 1948). Associated research aimed mainly to support fisheries and pond farming industries (Popova & Sytina, 1977; Steffens, Geldhauser, Gerstner, & Hilge, 1996; Tölg *et al.*, 1981), and the pikeperch commonly held the position of a secondary species, which more often than not was reared additionally to other fish species, like carp, in pond systems (Hilge & Steffens, 1996; Steffens *et al.*, 1996). Still, some general developmental descriptions of pikeperch have been provided (Bastl, 1978; Kryzhanovsky *et al.*, 1953). What remains problematic with this early Eastern European literature is the often sparse or completely absent translations into English, which hampers the recognition and accessibility of the works. In parallel, research in Western countries, especially in the United States and Canada, focused almost exclusively on the walleye (also called American pikeperch, or *Sander vitreus*) (Barton,

2011; Beyerle, 1975; Johnston & Mathias, 1993; Li & Mathias, 1982; McElman & Balon, 1979; Moodie, Loadman, Wiegand, & Mathias, 1989).

Since the 1990s, works have been published by researchers of various worldwide nationalities and also increasingly from nations outside of the original distribution range where the pikeperch was introduced, as in the case of research from France, Turkey, and China. However, countries that used to have a strong position in the pikeperch aquaculture remain dominant. With the increasing breadth of the research sector, larger international research collaborations associated with the industry have become more important. To specifically strengthen percid aquaculture (e.g., perch and pikeperch), the European Percid Fish Culture (EPFC) group of the European Aquaculture Society was founded as an association of researchers and fish farmers. As a result, the present research focus is more often placed on applicable research, such as gamete handling and selection (Khendek *et al.*, 2018; Nynca, Żarski, Bobe, & Ciereszko, 2020; Schäfer, 2016; Żarski *et al.*, 2020a; Żarski *et al.*, 2012; Żarski *et al.*, 2020b), out-of-season-spawning (Tielmann, 2017; Zakęś & Demska-Zakęś, 2009; Zakęś & Szczepkowski, 2004), domestication support and the design of breeding programmes (Eschbach *et al.*, 2014; Molnár, Benedek, Kovács, Zsolnai, & Lehoczky, 2020; Nguinkal *et al.*, 2019; Teletchea, 2016; Żarski *et al.*, 2020b), as well as the implementation of different aquaculture systems, like RAS or aquaponic facilities (Palm & Bischoff, 2016; Palm *et al.*, 2018; Rapp & Stüeken, 2019). In this vein, Steenfeldt *et al.* (2015) and Policar *et al.* (2019) provided a collective overview of the existing research on percids and their aquaculture status.

Nevertheless, as research on the pikeperch remains sparse, comparisons with closely related fish species, such as the walleye and Volga-pikeperch, provide helpful information for pikeperch researchers (Bokor *et al.*, 2007; Specziár, 2005; Specziár & Bíró, 2003). Furthermore, studies on the development of teleost model organisms, such as zebrafish (*Danio rerio*) or medaka (*Oryza latipes*) (Devoto *et al.*, 2006; Johnston, 2006; Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; Stickney, Barresi, & Devoto, 2000; Stoiber, Haslett, & Sänger, 1999) may generally support developmental studies on fishes and consequently also on pikeperch. However, research on these taxonomically divergent model organisms needs to be compared carefully, as developmental differences can occur. Even more fundamental mechanisms that emerge during development, such as those involved in myogenesis, may differ among fish taxa (Johnston, 1999). In addition, genome duplications, which occurred at least twice during teleost evolution (Glasauer & Neuhauss, 2014; Jaillon *et al.*, 2004; Sato & Nishida, 2010; Wittbrodt, Meyer, & Schartl, 1998), can result in difficulties for genetic analyses, such as the unambiguous detection of homologous genes between fish species.

1.3 Pikeperch aquaculture and the problem of rearing

Up until now, aquaculture setups have mainly been monocultures, focusing on a few of the economical top players (FAO, 2020; Naylor *et al.*, 2021). This low biodiversity has proven to be problematic, as illnesses and parasites, as well as climate change-induced temperature rises, create increasing risks for individual farms as well as entire industries, as disease outbreaks affect the whole stocks. These risk factors can lead to population breakdowns, which in the past have even resulted in large-scale economic crises. Some examples of this phenomenon are the salmon anaemia disease outbreaks in Chile between 2007 and 2009 (Asche, Hansen, Tveteras, & Tveteras, 2009; Mardones, Perez, Valdes-Donoso, & Carpenter, 2011), the occurrence of viral haemorrhagic septicaemia in the Great Lakes of the US between 2005 and 2008 (Bowser, 2009; Thompson *et al.*, 2011), and the regular outbreaks of pancreas disease in Norway and the United Kingdom (Aunsmo, Valle, Sandberg, Midtlyng, & Bruheim, 2010; Jensen, Kristoffersen, Myr, & Brun, 2012). As a result, efforts are being made to include more species in the aquaculture sector to diversify the species range and reduce the risks for the industry in general. As newer aquaculture infrastructures, especially those in Europe and North America, become increasingly technologically advanced, an appropriate number of well-trained staff members are required. Additionally, there have been increasing animal welfare efforts in aquaculture settings, which are leading to adaptations in feeding and handling, maximum stock densities, and environment enrichment (Ashley, 2007; Brown, Davidson, & Laland, 2003; Evans, 2009). Such practices are combined with stress-level monitoring of the kept fishes (Fanouraki *et al.*, 2008; Guardiola, Cuesta, & Esteban, 2016; Swirplies *et al.*, 2019; Wendelaar Bonga, 1997). However, these new infrastructure adaptations make these systems costly and mark the need for more expensive fish species to offset costs. In this context, high-priced pikeperch have received additional support from this sector (Overton *et al.*, 2015; Policar *et al.*, 2019; Rapp & Stüeken, 2019).

As the pikeperch is still a new species of focus in aquaculture, adequate husbandry procedures need to be developed and optimised. However, a major bottleneck for successful pikeperch aquaculture is the raising during the early ontogenetic stages. An over 130-year-old citation from Nevin (1887), referring to the American pikeperch, describes the long struggles with rearing the *Sander* species: *“If fifty per cent of the eggs can be hatched it can be considered very fair success. My opinion is that the general average is lower, […]”*

These rates are still present in pikeperch rearing with natural spawning on nests (30–50%; Zienert & Heidrich, 2005). While this rate of survival up to and after hatching in some cases can be higher with adapted raise procedures (80%; Schmidt & Kühn, 2021), it still lags behind the survival rates of established species, such as rainbow trout (94%; Shah, Balkhi, & Asimi, 2009). This demonstrates the difficulties that arise during the rearing of the pikeperch. During

this process, the mortality rate increases, as further developmental processes like hatching, the change to exogenous feeding, and cannibalism in the larval stage occur during pikeperch ontogeny (Policar *et al.*, 2019). As these losses add up over time, only around five to ten percent of all pikeperch currently develop into a saleable fattening fish (Landesforschungsanstalt MV, Dr. Ralf Borchert - personal communication).

To understand these critical life stages, a study focusing on developmental burdens and the analysis of environmental requirements during ontogeny is important (Irie & Kuratani, 2014; Riedl, 1978; Schank & Wimsatt, 1986; Wimsatt, 1986). Consequently, working on pikeperch requires a combination of applied and fundamental research, which can help to successfully establish the pikeperch in aquaculture. To improve the understanding of pikeperch development and to overcome high mortalities, previous studies have focused on the development of specific organ systems in pikeperch, such as the skeleton and the digestive tract (Löffler, Ott, Ahnelt, & Keckeis, 2008; Ostaszewska, 2005; Ott, Löffler, Ahnelt, & Keckeis, 2012), or on general morphology (Bastl, 1978; Güralp *et al.*, 2017). Based on this fundament, three **open research topics** were chosen for analysis within this doctoral thesis. With this objective in mind, observations of naturally raised specimens were preferred to isolate potential parameter adaptations for aquaculture settings.

First, the general growth patterns and early development needed to be analysed to gain insight into the superordinate changes during early ontogeny, and to find the critical life phase.

Second, the myogenesis process required further analysis, as it had not yet been studied in detail in pikeperch. Accordingly, adult muscle properties also needed to be compared.

Third, fundamental insights for the implementation of animal welfare in research and aquaculture need to be determined.

2. Research aims and project fundamentals

Based on the open research topics obtained from the review of previous studies and literature, the following points were developed as the **aims of this dissertation**:

1. Analyse the early ontogeny of pikeperch to identify critical life stages, developmental thresholds, and phases of change.
2. Gain insights into muscle development during early ontogeny.
3. Find ways of introducing animal welfare efforts into research and aquaculture procedures on pikeperch. To accomplish this, the description of stress levels during ontogeny, the development of alternative testing models, and the support of appropriate rearing procedures should be targeted.

The doctoral project was based on the working group “Fish Growth Physiology” at the Research Institute for Farm Animal Biology (FBN) in Dummerstorf. To provide a starting point for the following work on aquaculture fish development, the doctoral project had two **further objectives**:

- The study was to have a strong background in applied science to support aquaculture procedures in the broader context. Consequently, possible implications for aquaculture rearing also needed to be considered.
- The work was to be carried out in cooperation with other FBN research units and industry collaboration partners.

In addition to these objectives, several previously known factors played a role in conducting the research. In particular, the availability of specimens was identified as a major limiting factor. Presently, the production of pikeperch larvae is generally limited. However, the fishery partner at the nearby Lake Hohensprenz was able to supply the required quantity of specimens. The current rearing setup is a flow-through rearing system that is closely connected to the nearby lake. The use of lake water, including water parameters, temperatures, and local zooplankton feed, as well as the availability of adults from the wild lake population, allowed us to gain insights into the near-natural rearing process of pikeperch (Study I, Study III). Nonetheless, some aquaculture practices had to be applied to the specimens. These included keeping the parental animals in cages within the lake, desticking the eggshells with a light formalin solution to prevent clustering, rearing the fertilised eggs in Zuger jars, and keeping hatchlings in net cages. During all the procedures, water from the lake was used. These practices allowed us to obtain a sufficient number of specimens once a year during the spawning season. Therefore, three samplings with around three to five nests were able to be taken in the scheduled time

(Fig. 3). Since most of the methodological procedures were designed for other species, mostly model organisms, the protocols had to be adapted to this non-model species. Consequently, a longer initial phase of methodological establishment had to be included, which involved protocol design, adaptation, and testing.

3. Growth patterns and development during the embryonic to larval transition

The development and growth of organisms are influenced by internal and external factors. In particular, temperature can influence development (Hayes, 1949), as described by the well-known van't Hoff rule (also known as the Q10 rule; Běhrádek, 1930; van't Hoff, 1884). As a near-natural setup with the according temperature regime used, it was established that fluctuating temperatures during rearing would consequently have an impact on pikeperch development. To counteract this, the specimens' ages were provided in degree-days (dd), which was calculated on the basis of the age (given in days) multiplied by the mean daily temperature, thus allowing for the comparison of fish development under different temperature regimes (Bonhomme, 2000). In general, teleost development is not uniform. This is related to the large teleost biodiversity and is reflected in a number of terms that are only valid for specific developmental stages of specific taxa, resulting in a not-yet-resolved nomenclature chaos (Balon, 1975; Peňáz, 2001; Urho, 2002). Peňáz (2001) summarised previous systems for teleost development and formulated a scheme for ontogenetic stages suitable for a broad range of teleost taxa. Therefore, to further improve the comparability of pikeperch developmental stages, ontogenetic stage definitions were included following Peňáz (2001). These definitions, which separate embryonic development from the following larval stages by the start of exogenous food ingestion, rather than hatching, are followed in the studies conducted here.

3.1 Growth patterns

Previous studies have pointed out a high mortality rate between the late embryonic phase and the larval stages. Consequently, this problematic developmental phase was observed in more detail. However, this phase contains different developmental events, such as hatching and the change to exogenous nutrition (Bastl, 1978; Peňáz, 2001; see also Studies I, II, and III), which may lead to different critical windows during this developmental period. Critical windows describe times when higher mortality rates occur, as external factors have a stronger influence on the development of specific organ systems and associated developmental/morphologic complexes (Burggren & Mueller, 2015; Li & Mathias, 1987). Therefore, as a first step, a morphometric approach (see Study I) was chosen to determine growth changes and differentiate the effects of the occurring developmental events, and to discover which body parts, and consequently associated organs, might be affected.

Overall growth did not follow a uniform linear line of behaviour during the observed time, but instead contained biphasic growth with different growth rates during embryonic and larval development, as well as an intermediate plateau phase (Study I). This observed growth pattern

was further confirmed by the gene expression of insulin growth factors (IGFs; Studies II and III), which demonstrated increased expressions at times of increased growth during the larval stages and even a small reduction in expression levels during the plateau phase (Study III, Fig. 3). This intermediate gene expression reduction was also found for the growth hormone receptor (GHR; Study II).

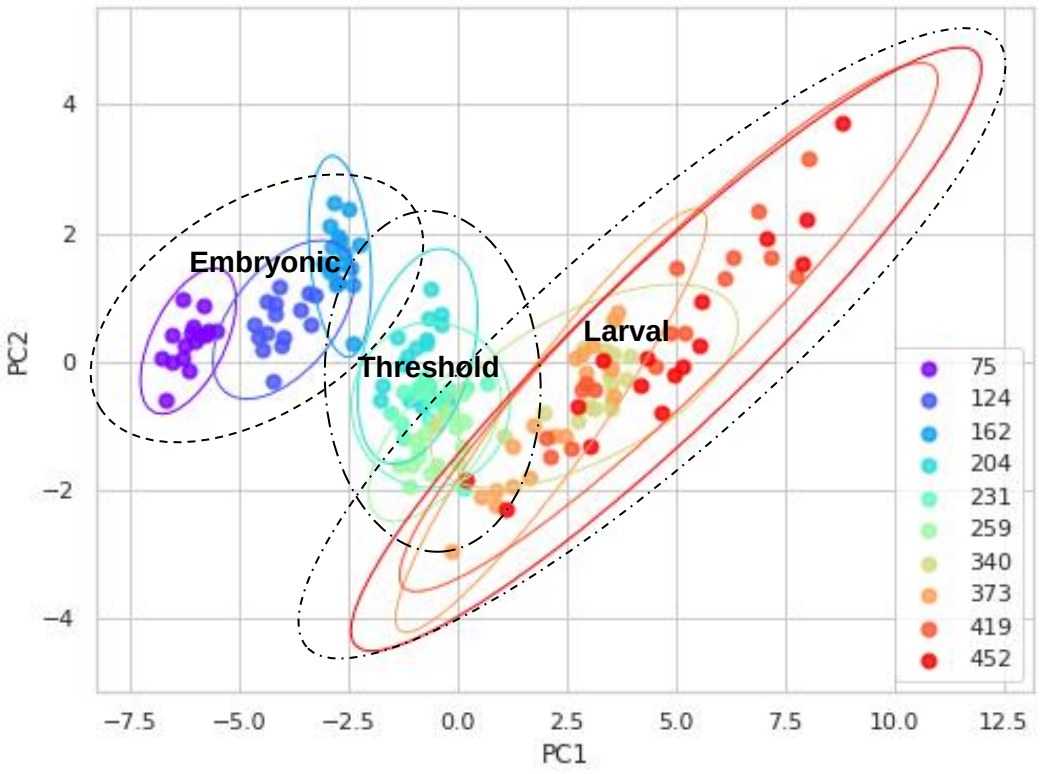


Fig. 1: Results of the Principal Component Analysis for the different age stages from 75 to 542 dd. Age stages are colour-coded and the resulting three developmental phases are circled (see also Study I).

Single parameters were found to be especially influential on the overall body form changes, as demonstrated by the principal component analysis (PCA; for an explanation see Study I, Materials and Methods, and Fig. 4). The first principal component was influenced by a majority of the length-correlated parameters. The second principal component was influenced by fewer parameters, such as pectoral fin length, interorbital diameter, body width at the caudal peduncle, body width at the anus, and head angle.

The PCA demonstrated uniform development in size and growth rate during the embryonic stages. This changed with the beginning of the threshold phase (Fig. 1; see also Study I, Fig.

4c). As a result, the influence of the interorbital distance and head angle were concluded to represent developmental processes in the head and gill region with connected neuronal, respiratory system (gill arches), and heart development (Güralp *et al.*, 2017; Chapter 3.2, Studies I, II, and III). The pectoral fin length and the body width at the anus and at the caudal peduncle play roles in locomotor abilities. The growth of these parameters was determined to allow first movements and enable first feeding events, which afterwards increased further, as shown by the growing muscle tissue during the larval stages (Study I, Study III). This timing pattern has been interpreted as a consequence of the energy limitations known for larval fishes (Osse & van den Boogaart, 1995; Osse, van den Boogaart, van Snik, & van der Sluys, 1997; Wieser, 1995) and the size-relative viscosity of the surrounding water (China & Holzman, 2014; Fuiman & Webb, 1988; Webb, 1988; Webb & Weihs, 1986). In addition, an intermediate shift from a longer post- to a longer preanal length was observed during the threshold phase (Study I, Fig. 6a–b). This suggests that this particular phenomenon is related to the simultaneous switch to exogenous nutrition, as the remaining endogenous resources were depleted and the associated digestive tissue transformation had taken place (Bastl, 1978; Ostaszewska, 2005; Studies I and III)

Altogether, the growth patterns and growth-related gene expression patterns reveal the considerable changes that occur from embryonic to larval pikeperch development. The shifts in the PCA highlight the period from about 200 to about 300 dd as a critical phase for such development. Similarly, more rapid morphometric changes have been associated with developmental modifications in the *Salvelinus* species, where a higher shape change also occurs during the free-embryo period (Fischer-Rousseau, Cloutier, & Zelditch, 2009). This is consistent with general developmental studies on teleosts that identify early development around the hatch as a critical life phase leading to high mortalities (Balon, 1984; Fischer-Rousseau *et al.*, 2009; Gisbert, 1999; Li & Mathias, 1982, 1987; Osse *et al.*, 1997; van Snik, van den Boogaart, & Osse, 1997). Although morphometric data can contribute to various applied aquaculture procedures (Klingenberg, 2013; Poulet, Berrebi, Crivelli, Lek, & Argillier, 2004; also see Chapter 5.2), this deductive approach from the resulting secondary phenotype (with the primary being organ development, and the secondary being growth changes) can only indicate changes and consequently requires further validation by examining the actual process of organ development.

3.2 General developmental changes in pikeperch early ontogeny

To gain insight into the critical developmental changes during embryonic to larval development and especially during the threshold phase, the development of the near-naturally reared specimens (see also the developmental descriptions included in Studies I and III) was observed in detail. Additionally, descriptions of pikeperch ontogeny in the literature (Bastl,

1978; Güralp *et al.*, 2017; Kryzhanovsky *et al.*, 1953), or of walleye (McElman & Balon, 1979) or other fish model organisms (Kimmel *et al.*, 1995) were taken as comparisons As the specimens were mostly transparent, primarily normal and oblique light microscopy were used. Additionally, whole-mount alcian blue tissue counterstaining was applied to enhance the visibility of the cartilages and other organs by staining their mucin polysaccharides (Steedman, 1950; Fig. 2a). Further combination with trypsin-based tissue digestion and alizarin red for calcified structure marking and cleared and double-stained whole-mount staining after Taylor and van Dyke (1985) was utilised to check the skeletal development of the selected age stages. In addition, the specimens' internal organs were analysed using microdissections to gain insight into the different ontogenetic stages and their organ development. In the end, the development was further compared with the expression of developmental marker genes (Studies II, and III) and gene markers for metabolism and energy expenditure (Study II), as well as the decrease in yolk and oil globule volume (Study I). The results show that pikeperch development passes through profound changes during the transition from embryo to larva. Late embryotic development emphasises general organogenesis, which forms fundamental organs, while larval growth focuses on the differentiation of organ tissues (Studies I, II, and III). Overall, there was a slow depletion of endogenous resources during embryonic development and a strong reduction during the threshold phase, which is situated in the early larval development (Studies I and II).

3.2.1 Embryonic development

Following the definition of Peňáz (2001), embryonic development starts with the activation of the egg. Here, the earliest observed specimens were in the phases of gastrulation (26 dd, Study III) and somite formation (53 dd, Study III, Fig. 3a). In the following stages (75 dd, Study I; 80 dd, Study III, Fig. 3b), the basic body shape was already present. The head was bent ventrally and connected to the oil globule surface, but the tail was separated from the yolk (Study I and III). The eye anlagen formed, and their pigmentation started, and an nasal placode was present anterior to the eye (Fig. 2b). The pericardium contained an already beating heart (Fig. 2b), and the dorsal aorta was visible. This coincided with elevated levels of the marker gene MYH6, encoding myosin heavy chain 6 (MYH6), which was found to have a maximal expression (Study III, Fig. 4a). MYH6 is predominantly expressed in cardiomyocytes (Schiaffino, 2018), thus reflecting regional muscle development in the heart, but it may also indicate the development of the heart itself (Study III).

From 124 dd on (Study I, Study III), pectoral fin buds formed as cell accumulations on the dorsolateral body. During their progressing development, they shifted ventrally, while the head started to bend dorsally (Fig. 2b). The skeletal development of the specimens generally followed the descriptions in previous literature (Löffler *et al.*, 2008; Ott *et al.*, 2012). With the

uplifting of the head, cartilaginous skeletal precursors formed in the skull, which consisted of trabecular bars and the auditory capsule that began to cover the ventral otic region. At the same time, Meckel's, hyosymplectic, and palatoquadrate cartilage formed, which produced elements of the jaw in the following stage (Fig. 2c-d). This first formation of skeletal elements was accompanied by increased expression of collagen gene markers (COL1A1 and COL1A2; Study III), which can be attributed to the increasing collagen formation in the skeletal precursor cartilages (Gelse, Pöschl, & Aigner, 2003).

At 204 dd (Study I), the mouth was open. The digestive tract was still straight and uniform, but the liver had started to form. A still-large yolk sac and oil globule were present. In the caudal fin, no skeletal elements had yet developed, but at the end of the embryonic phase, a distinct caudal fin fold formed out of the straight undifferentiated fin fold margin. Skin pigmentation was minimal and focused on the ventral surface of the yolk. Most embryos hatched between 180 and 220 dd. However, when the specimens were disturbed, a prehatch of single specimens was observed with increasing numbers from 75 dd onwards.

3.2.2 Larval development

The larval development started with the first feeding, observed at 231 dd (Study I) and 248 dd (Study III), and was examined in focus until flexion of the posterior notochord end (Stage L3 of Peňáz, 2001). This notochord flexion can also be used to separate early from late larval development (Balon, 1975; Bensam, 1989). After the first feeding, the structural development focused on the jaw structures and the differentiation of the digestive tract. With the first external feeding, the digestive tract continued to grow and formed a distinct narrowing between the mid- and hindgut. Proximal to the liver, the gallbladder was observable for the first time. At around 259 dd (Study I), the posterior section of the oesophagus formed the swim bladder as a small dorsal protrusion. In the skull, further jaw and gill elements differentiated and increased in size. The gill slit opened, and the opercular elements formed. A further MYH6 expression increase occurred at this time (Study III, Fig. 4a), which may be related to the circulatory tissue increase associated with the onset of gill development (Grimes & Kirby, 2009). Also, further gene markers connected to skeletal development showed increased expression (musculoskeletal embryonic nuclear protein 1 - MUSTN1 and COL1A; Study III, 280 dd). Additionally, the expression of Sry-box transcription factor 9c was elevated at that time (SOX9, Study II 252 dd), which is known to be connected to the development and maturation of gonads (Klüver, Kondo, Herpin, Mitani, & Schartl, 2005; Yokoi *et al.*, 2002).

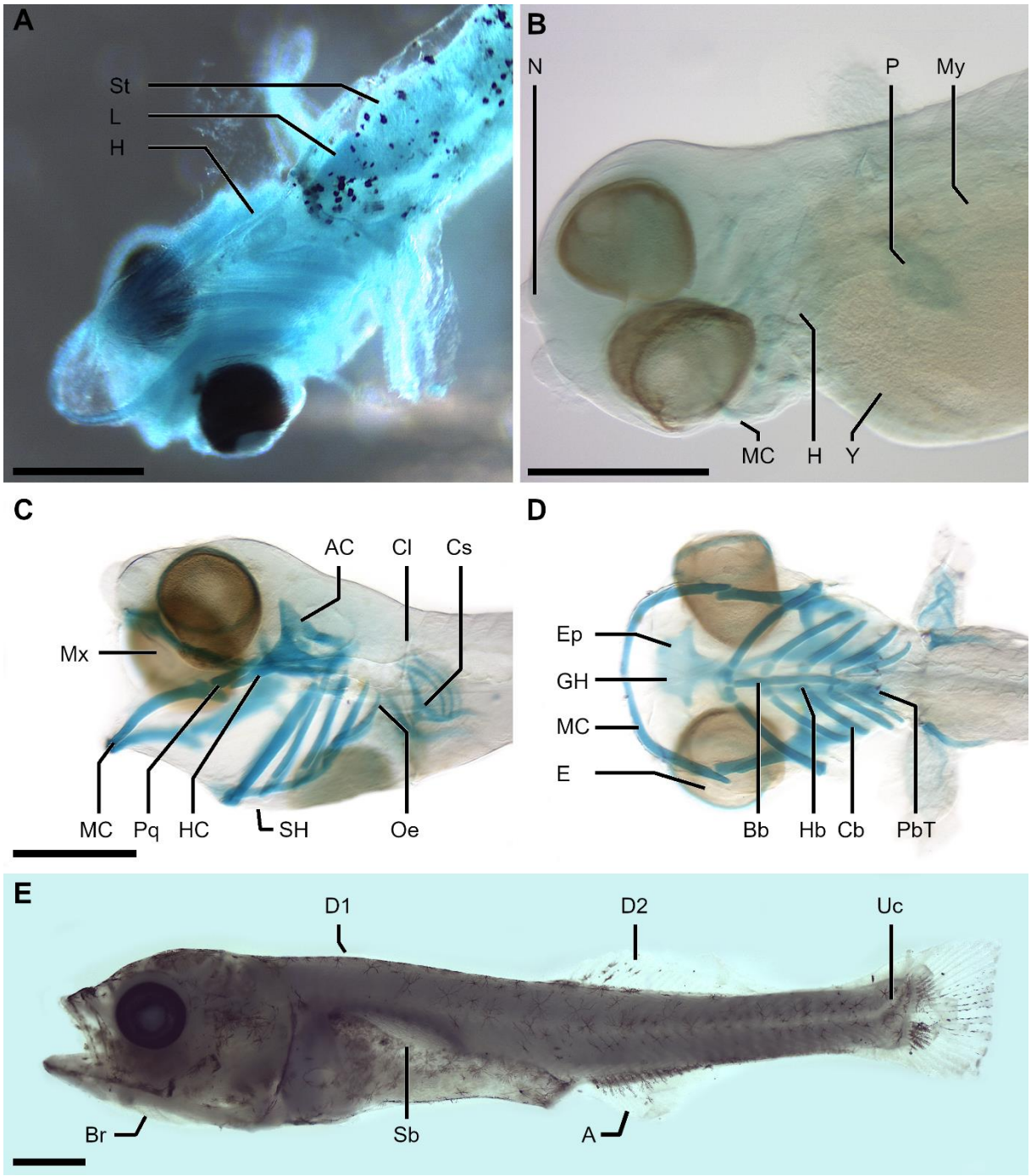


Fig. 2: Development of the pikeperch and observation methods utilised. A: Alcian blue tissue staining for contrast enhancement. Ventrolateral view on the head and organs of a larva at 297 dd (19 dph). B: Cleared and Stained embryo in dorsolateral view at 199 dd (14 dph) before the fulfilled ventral shift of the pectoral fins. C+D: Lateral (C) and ventral (D) view of the head of a cleared and stained larval specimen at 280 dd (19 dph). The cartilaginous structures of the skull and gill arch skeleton are visible. Ossifications are sparse and remain mostly transparent. E: Lateral view of a larval specimen after the dorsal shift of the notochord end at 665 dd (38 dpf). Scale = 0.5 mm (A-D); 1 mm (E). A anal

fin, Bb basibranchial copula cartilage, Br branchiostegal rays, Cb ceratobranchial, Cl cleithrum, D dorsal fin, E eye, GH geniohyoid muscle, H heart, HC hyosymplectic cartilage, L liver, MC Meckel's cartilage, My myomers, Mx maxillary, N nasal placode, Oe oesophagus, P pectoral fin, Pq palatoquadrate cartilage, PbT pharyngobranchial toothplate, Sb swim bladder, SH Sternohyoid muscle, St stomach U ural centrum, Y yolk (see also developmental depictions in Study I Fig. 1, Study III Fig. 1, Study IV Fig. 6, Study II Fig. 2a-b)

With progressing early larval development from 325 dd (Study III) to 340 dd (Study I), the feeding mode became solely exogenous as the yolk and oil reserves were depleted (at 340 dd, only a single specimen was observed with oil droplet remnants in Study I (see also Study I Fig. 5e + f). This was also found for the burbot (*Lota lota)*, where it could be attributed to a more efficient uptake of the fats in the yolk, but also to the possible buoyancy support given by the oil during swim bladder inflation (Palińska-Żarska *et al.*, 2014). For the pikeperch, gene markers reflected this utilisation. The transcription factor EB (TFEB; Study II) is activated by starvation responses and results in the catabolism of lipids (Settembre *et al.*, 2013). In this case, TFEB had its highest expression levels at the end of the yolk absorption. At the same time, the energy metabolism marker peroxisome proliferative activated receptor gamma coactivator 1 alpha (PGC1A; Study III), which had significantly reduced expression. As PGC1A is related to mitochondrial energy metabolism (Cheng, Ku, & Lin, 2018; Liang & Ward, 2006), it was concluded that this low expression highlights a general need to save energy in pikeperches at that developmental time. Altogether, the results demonstrated a high rate of resource usage, which was concluded to be connected to the developmental changes and resulting energy allocation pressures during the early larval phase. This energetically challenging phase and the connected developmental processes, as well as the growth arrest time with the threshold phase (Chapter 3.1; Study I, Fig. 1), further support this developmental period as a critical life phase.

Also timed with the completed food change, the muscles of the jaw and gill arches grew and thus, the adductor mandibulae and adductor hyoideus muscles, as well as the ventral jaw opening muscles sternohyoideus and a smaller geniohyoideus became visible (Fig. 2c-d). This development of jaw muscles was also reflected by the elevated expression of engrailed 2 during the larval stages that passed the food change (EN2, Study III). EN2 is specifically expressed in myoblasts and later muscles that are related to the jaw (Degenhardt & Sassoon, 2001; Yokoyama, Yoshimura, Suzuki, Higashiyama, & Wada, 2021). At 340 dd (Study I), first teeth were visible on the dental and premaxillar. First gill filaments formed, starting on Ceratobranchial 3. Additionally, connected musculature and gill arteries and dorsal artery

branches became visible. The swim bladder became rounded and increasingly pigmented but remained connected to the oesophagus via the ductus pneumaticus. At 452 dd (Study I), first branchiostegal rays had formed. On the right dorsal side of the stomach, the pancreas forms. The differentiated stomach region (see also Ostaszewska, 2005) starts to twist to the left. In addition, locomotive structures increased, supporting the start of prey hunting behaviour. This included the increase of the pectoral fin during early larval development. Additionally, the mesenchyme in the caudal fin formed the first elements, starting at 340 dd (Study I) with the hypural 1, followed by the parhypural, hypural 2, and mesenchymal ray anlagen (419 dd, Study I). Next to this, the muscular tissue in the trunk increased in size. At 452 dd (Study I), the notochord flexion started and first dorsal and anal fin mesenchymal rays appeared in the fin margin. With this notochord flexion, the early larval development ended.

3.3 Influence of a near-natural environment

In this project, near-naturally reared (Study I, Study III) and RAS-reared specimens (Study II) were observed. While temperature regimes are more uniform in industrial settings, natural environments are connected to stronger temperature fluctuations (see Fig. 3). General development and growth patterns are influenced by temperature and other water parameters. Consequently, differences in rearing durations can occur.

Since the pikeperch is considered a preferred warm-water fish (Hilge & Steffens, 1996; Willemsen, 1978), one might also assume higher temperatures to be more suitable for rearing. However, this might not be the case throughout the entire development process (Frisk, Skov, & Steffensen, 2012). Especially during the early ontogeny, temperature is known to have a substantial influence on fish development (Brett, 1970; Fowler, Hamilton, & Currie, 2009; Jonsson & Jonsson, 2019; Lee, Yoon, Park, Lee, & Lim, 2021; Rombough, 1997; Vandeputte *et al.*, 2020). In the observed specimens, growth patterns differed between the embryonic and larval phases, which could be attributed to the temperature regime. However, Ott *et al.* (2012) also described lower temperatures as more beneficial for embryonic than for larval growth (see Fig. 4, compare also Study I, Chapter 4.2, “Study design and limitations”). Although not described further, a plateau-like phase at about 10 dpf was depicted by Ott *et al.* (2012) for both tested temperatures of 15.5 and 18.0°C. This supports the idea that the observed threshold phase is not an artefact of a near-natural raise with its temperature fluctuations, but instead must be a consequence of developmental processes.

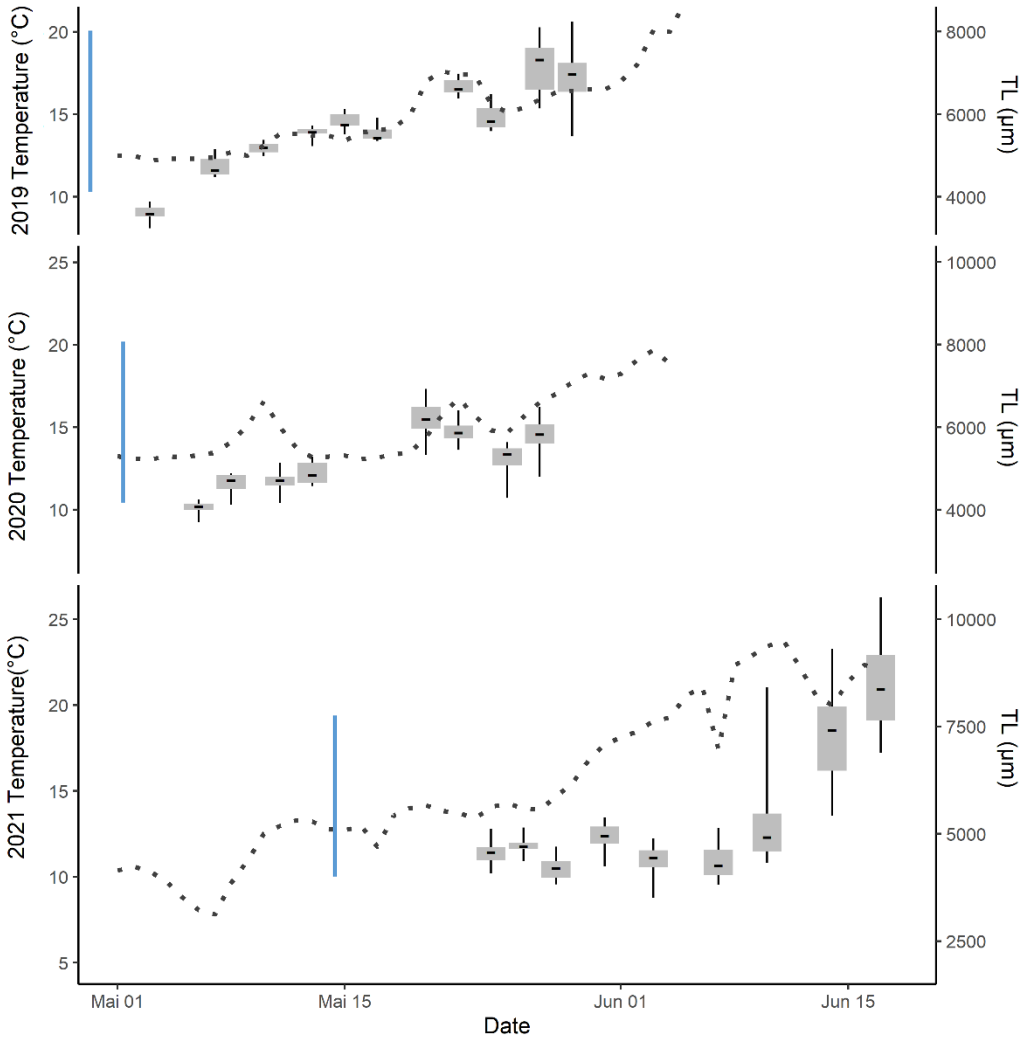


Fig. 3: Pikeperch size distributions and lake water temperatures during the three samplings conducted during the thesis project. Temperatures are depicted as dotted line. Size in form of the total length (TL, see Study I) is shown as box plots for each sampling group (Min-Max range, quartiles and mean are shown). Fertilisations for the analysed nests, marked as blue vertical line, were on the 28.04.2019, 03.05.2020, and the 14.05.2021.

With the stronger effects of temperature on the early life stages in fish in general, these critical life phases can especially benefit from an adapted temperature protocol. In pikeperch aquaculture, growth and survival might be enhanced by fine-tuning the temperature regimes during these stages. The observations taken from natural growth patterns can further support breeding approaches for aquaculture-reared specimens and afford insights into the growth occurring under “natural” parameter regimes to which the species have evolutionarily adapted.

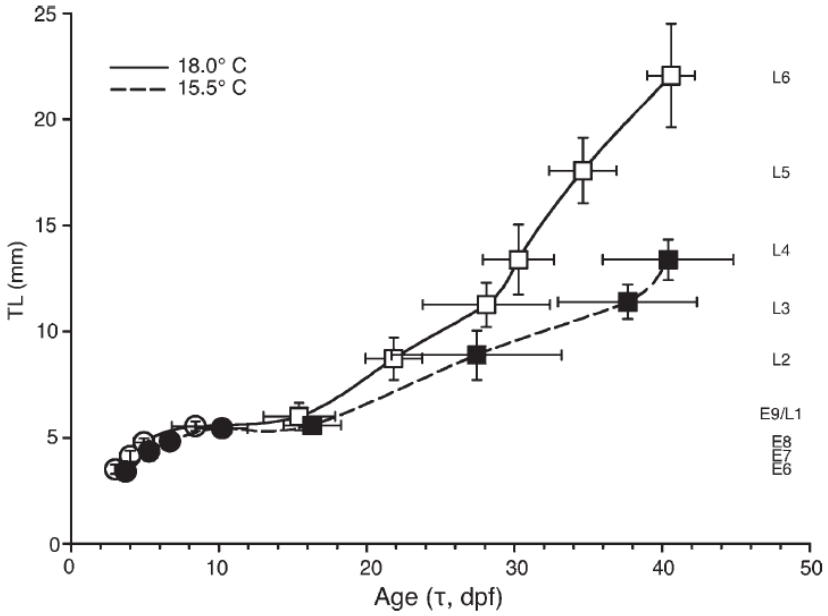


Fig. 4: Total length (TL) change during the early ontogeny of pikeperch under 15.5 and 18°C rearing temperature. Mean age is given in days post fertilisation (dpf) and developmental stages after Peňáz (2001) are provided. Figure taken from Ott *et al.* (2012).

4. Early development of the musculoskeletal system

Muscles play a vital role in a vertebrate's life, and this is consequently also the case for pikeperch. Muscle cells are essential not only for movement, but also for respiration, the function of digestive organs, and blood flow (Brunet *et al.*, 2016; Grimes & Kirby, 2009; Hale, 1965; Hanel, Karjalainen, & Wieser, 1996; Schiaffino, 2018; Totland *et al.*, 1987). Already during the early development of fish, locomotion and other movements are crucial for predator avoidance, as well food intake and hunting prey. However, due to the small size of the pikeperch in the early stages of development, water is relatively more viscous, which is described by the small Reynolds numbers. This results in different swimming performances (China & Holzman, 2014; Fuiman & Webb, 1988; Muller, Stamhuis, & Videler, 2000; Voesenek, Muijres, & van Leeuwen, 2018). Additional energy expenditures can become critical for smaller and younger age stages, as energy is limited during that time (Xu *et al.*, 2017). Consequently, myogenetic processes need to be balanced and adapted with development.

Generally, myogenic processes in vertebrates are controlled by a typical set of gene markers (Berkes & Tapscott, 2005; Buckingham *et al.*, 2003; Johnston, 2006; Rossi & Messina, 2014; Rudnicki & Jaenisch, 1995; Shahjahan, 2015; Steinbacher, Haslett, Saenger, & Stoiber, 2006; Stoiber *et al.*, 1999; Watabe, 1999). Additionally, the development and morphogenesis of the muscles are also influenced by the surrounding connective tissue system via which the muscles attach to the skeletal system (Kardon, 2011; Sefton & Kardon, 2019). Consequently, the general gene markers, as well as the connective tissue and skeletal markers, were observed here to provide a holistic view of the expression patterns during pikeperch ontogeny.

4.1 Myogenesis

Three phases of myogenetic activity were detected during the embryonic to larval transition in the pikeperch. Their respective times align with different developmental processes, and they possess distinct qualities. Overall, the expressions of typical markers from the myogenetic cascade in vertebrates were found to be supported in pikeperch (see Study III; Akolkar, Asaduzzaman, Kinoshita, Asakawa, & Watabe, 2016; Berkes & Tapscott, 2005; Johnston, 1999; Johnston & Bower, 2009; Kobiyama *et al.*, 1998; Rudnicki & Jaenisch, 1995; Watabe, 1999; Watabe, 2001). The variations in the expression activity of marker genes have provided insight into the varying qualities of the different phases. Overall, the analysis had to focus on the striated muscles, as they support the locomotor system and allow food intake and gill respiration. In contrast, smooth muscles possess different genetic regulations and varying physiological properties (Brunet *et al.*, 2016; Hoggatt *et al.*, 2013; Nishida *et al.*, 2002; Wang, Wang, Pipes, & Olson, 2003).

During the first phase, coinciding with somitogenesis, the activation of progenitor cells could be detected, as MYOD and MYF5 were highly expressed. Only the suggested influence of MRF4 on somitogenesis in pikeperches differs from the known regulator patterns of other fish model organisms, as its expression elevation untypically coincided with somite formation (see Study III). However, differences in MRF4 expression between the teleost taxa seem to occur, as a somite-specific expression of MRF4 was also found for the olive flounder (*Paralichthys olivaceus*) (Tan, Xu, Zhang, & Zhang, 2019).

A second myogenetic phase occurred in the pikeperch before hatching. During this phase, modestly enhanced expression of all myogenetic markers occurred. This likely reflects the formation of the first muscle tissue before hatching. This is necessary for hatching itself and enables first locomotion activities (Budick & O'Malley, 2000; Fuiman & Webb, 1988). For pikeperch, this effect on first larval locomotion capabilities is supported by the simultaneous completed development and ventral shift of the pectoral fins (see Chapter 3.2). As a possible consequence of this up-to-now only fundamental muscle presence, the early pikeperch hatchlings appear to be mostly resting on the bottom, with only sparse movements. However, the muscle tissue needs to be sufficiently developed for them to reach the surface and allow for inflation of the swim bladder by gulping up air (Kitajima, Watanabe, Tsukashima, & Fujita, 1994; Palińska-Żarska *et al.*, 2014).

Muscle growth continued with a third myogenetic phase after the threshold phase, during which markers for myotube and myofibre formation were especially highly expressed. This reflected the increase of postanal muscle tissue observed in the larval pikeperch (Study I) and was accompanied by the formation and growth of muscles in other body parts (e.g., the viscerocranial muscles; see Chapter 3.2). Additionally, the break in myogenesis during the threshold phase seemed to be significantly inhibited by myostatin (MSTN), as the highest levels of expression were present at that time. Thereafter, the expression decreased to a lower level, which may be necessary for the overall regulation of muscle growth and repair (Koganti, Yao, & Cleveland, 2020; Rodriguez *et al.*, 2014). This suppression by MSTN during the threshold phase can additionally shift energy expenditure to other necessary developmental processes at that time, such as internal organ development (as described in Chapter 3.2).

4.2 Skeletogenesis

Based on the markers for bone formation, ossification, and collagen, two skeletogenic phases were determined. These coincided with the second and third observed phases of myogenesis around the threshold phase, which was supported by microscopic observations of the embryonic and larval specimens (Study I; Study III, see Chapter 3.2) and the previous literature (Löffler *et al.*, 2008; Ott *et al.*, 2012). In particular, MUSTN1 can be interpreted as a suitable marker that reflects bone formation and endochondral ossification (Gersch & Hadjiargyrou,

2009; Liu, 2007). In this case, its peak coincided with an increase in skeletal element formation and ossification processes during the larval stages following the threshold phase (see Study III). In conjunction, the collagenous marker COL1A (Gelse *et al.*, 2003; Ytteborg, Torgersen, Baeverfjord, & Takle, 2010) can afford insight into the formation of the cartilages needed for the enchondral mode of bone formation. COL1A highlighted additional skeletogenic processes prior to hatching. However, the highest expression levels, especially prominent for the COL1A1 variant, were detected during post hatch skeletal development (Study III).

For the analysed bone morphogenetic protein 4 gene (BMP4), the observed increased expression after hatching coincided with the second skeletogenic phase, demonstrating its potential as a marker (Study II, Study III), as described by previous studies (Bonilla-Claudio *et al.*, 2012; Kan, Hu, Gomes, & Kessler, 2004; Ma, Zhang, Qin, Fu, & Jiang, 2016; Rosen & Thies, 1992). Nonetheless, the participation of BMPs in a wide variety of developmental processes is well-known (Bei & Maas, 1998; Hwang, Tsou, Lin, & Liu, 1997; Jungwirth & Winkler, 1984; Stickney, Imai, Draper, Moens, & Talbot, 2007; Su & Dong, 2018). This general developmental influence was here demonstrated by the high expression levels observed during the first stage at 26 dd, as this stage aligns with fundamental processes such as gastrulation and beginning segmentation (see Chapter 3.2). Consequently, BMP4 should be used only with caution as a marker for skeletogenesis, especially if embryonic developmental stages are analysed, as expression from other tissues can result in enhanced expression levels.

4.3 Muscoskeletal development and effects on adult life

During the early formation of skeletal elements and muscular tissues, malformations can occur that may have detrimental effects on adult life. Consequently, the early development and effects of environmental factors are of interest to aquaculture-related research to ensure and improve animal welfare, as well as later product quality (Fjelldal *et al.*, 2021; Johnston, 1999; Kitajima *et al.*, 1994). Since the muscoskeletal elements form and, in particular differentiate, after the switch to exogenous feeding, late larval and juvenile development should be of interest to future researchers. Specifically, skeletal malformations in the jaw or the vertebral column, such as lordosis or scoliosis, can occur during pikeperch larval development. In fact, in pikeperch larvae and juveniles, these well-known skeletal deformations can be observed regularly (Fig. 4). During these developmental phases, skeletal changes, such as the formation of axial fins and vertebral ossification processes, occur (Löffler *et al.*, 2008; Ott *et al.*, 2012; own observations). Furthermore, another coinciding increase in BMP7 was observed during the juvenile stage at 175 dph (Study II).

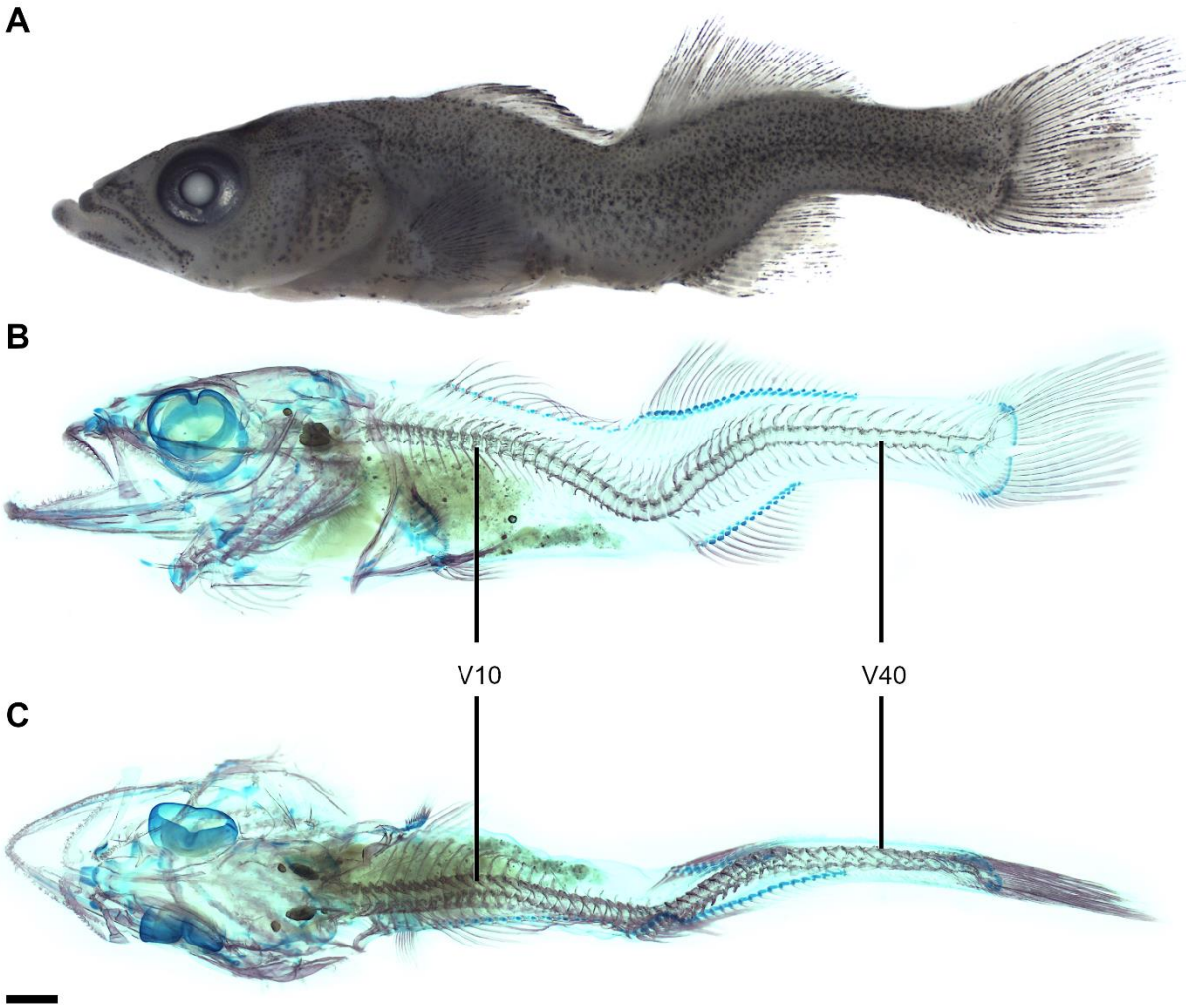


Fig. 5: Depiction of malformed vertebral column development in pikeperch larvae of 55 days post hatch (dph). A: Lateral view before staining. B+C: Lateral (B) and dorsal (C) view of the cleared and stained specimen (after Taylor and van Dyke 1985). The visible vertebral column shows a dorsoventral (Lordosis) and lateral bend (Scoliosis). Vertebrae 10 and 40 are marked (V10, V40). Scale = 1 mm.

Overall, various processes are known to possibly result in these artefacts during the skeletal development of fishes (Boglione *et al.*, 2013; El Kertaoui *et al.*, 2019; Kestemont, Xueliang, Hamza, Maboudou, & Toko, 2007). These processes also include increased muscular activity due to higher swimming activity (Basaran, Ozbilgin, & Ozbilgin, 2007; Kihara, Ogata, Kawano, Kubota, & Yamaguchi, 2002; Kranenbarg, Waarsing, Muller, Weinans, & van Leeuwen, 2005; Printzi *et al.*, 2021), which again highlights the interconnection of the muscoskeletal tissues during development. Notably, genetic markers, such as BMPs and SOX9, were also found to differ between normal and deformed morphologies (Ma *et al.*, 2016; Ytteborg *et al.*, 2010).

Thus, these genes hold promise as suitable subjects for research into these age stages in pikeperch, for which the here-described gene primers and expression levels (Studies II and III) may be of great help.

5. Animal welfare efforts in pikeperch rearing and research

In aquaculture as well as science, the animals' well-being and the reduction of stressful influences is a major goal. To achieve this aim, procedures are increasingly exchanged or adapted to more appropriate conditions that support animals' well-being. As a part of animal welfare efforts, the 3R-principles with the goal of reducing, replacing, and refining animal experiments have been formulated by Russell and Burch (1959). These have served as the basis for the correct handling of animals in science and the design of animal experimental setups up to the present time (De Angelis, Ricceri, & Vitale, 2019; Díaz *et al.*, 2020).

Current procedures to determine fishes' state of health commonly rely on external controls, such as the inspection of skin and fins, the occurrence of parasites, and feeding and swimming behaviours (Ashley, 2007; Folkedal *et al.*, 2012; González, Marín, Mancilla, Cañon-Jones, & Vargas-Chacoff, 2020; Kulczykowska, 2019; Mameri, Branco, Ferreira, & Santos, 2020; Wendelaar Bonga, 1997). Additionally, stress levels can be determined via cortisol or genetic analyses, blood samples (Wendelaar Bonga, 1997; Wu *et al.*, 2015), skin mucus samples (Guardiola *et al.*, 2016; Kulczykowska, 2019), and even water samples (Fanouraki *et al.*, 2008; Sadoul & Geffroy, 2019). Improved knowledge of stress markers is a practical tool for analysing animal health and well-being (Study II), as stress can also negatively affect fertility and offspring (Mileva, Gilmour, & Balshine, 2011), as well as the product quality of the fillet (Anders, Eide, Lerfall, Roth, & Breen, 2020). To determine optimal abiotic parameters and the reaction to certain hormonal treatments, growth factors, or antibiotics, alternative test methods, such as in vitro models, can be used in scientific tests, thus reducing the number of sacrificed animals or even replacing them fully (Bergmann *et al.*, 2020; Grunow, Franz, & Tönißen, 2021; Noguera *et al.*, 2017). Due to this large spectrum of applications, the observation of markers and the development of alternative test methods are crucial tasks for research on aquaculture models and their developmental stages.

In the studies conducted, the determination of stress gene markers that can be used to check for the animals well-being can be considered a refinement, as better husbandry environments can be created based on these findings. A reduction, as in the exploitation of fewer specimens for experimental setups, can be achieved by finding methodical adaptations that use fewer specimens, for example, for isolating RNA (e.g., comparing the numbers of specimens needed for RNA isolation between Study II and Study III). In addition, replacement can include the formation of a cell culture model for experimental setups.

5.1 Refinement: Markers to detect stressful environments

Cortisol was found to be suitable for determining stress levels in fish. In pikeperch, some gene markers were found for the detection of specific stressors, such as heat stress-related physiological reactions (Swirplies *et al.*, 2019; Study II). As these markers are also affected by the changing physiology during individual development, the levels of stress markers change during ontogeny (Study II). To set a baseline for stress marker expression, these stress markers were analysed at different developmental stages in pikeperch.

In the related study (Study II), gene markers for heat stress response (HSF – heat shock transcription factor) and hypoxia stress markers (HIF1A – hypoxia inducible factor 1 subunit alpha) were focused on to determine stress gene expression levels during the early development of pikeperch. Both temperature and oxygen levels are major factors in aquatic organisms. Consequently, they also influence fish physiology and are important for aquaculture husbandry. While HSF1 followed a steady course of expression, the expression of HIF1A was increased at two times during the observed embryonic to larval development. This may be connected to developmental changes, as both elevated expression levels coincided with physiological changes in which higher oxygen depletion was detected in the developing pikeperch (Anderson & Podrabsky, 2014; also see description in Study II). For future applications, these gene markers can be examined to check the physiological reactions associated with climate change and the connected increase in temperature and reduced oxygen levels in water (Little, Loughland, & Seebacher, 2020; Rajesh & Rehana, 2022). These expression markers can already be analysed using transcriptomic methods, and, as a result, adaptations can be made in aquaculture husbandry that reduce stress for the animals.

5.2 Refinement: Morphometry and automatised measuring in aquaculture rearing

The obtained growth patterns (see Chapter 3.1) provide a basis for the future automation of early-age monitoring processes (Study IV). Presently, monitoring systems are already used in larger fish farms, but they are generally adapted to larger developmental stages of economical top players, such as salmon (Pinkiewicz, Purser, & Williams, 2011; Risholm *et al.*, 2022). As technologies develop and become less expensive, these approaches are becoming more available for other species and smaller aquaculture facilities. For instance, a model system has been developed for the observation of small pikeperch larval specimens under laboratory conditions (Study IV). Due to their size of only around 5 mm and their minimal pigmentation, this is a particularly challenging task compared to observing the larger larvae of other fish species, such as those of salmonids, which are already double the size after the hatch (Cyr, Downing, Lalonde, Baines, & Pace, 1992; Houde, 1994; Kestemont *et al.*, 2003). Based on video sequences and frame-to-frame comparisons, counting the small pikeperch larval

specimens is possible with this system (Study IV). As this software aims to obtain specimen sizes in future versions, this comparable data on individual parameters, such as total length and head length, could contribute greatly to facilitating a basic comparison of the animals within a setup. If size variances increase too much, an alarm can be given out, which would then be followed by a size-sorting procedure. This could prevent losses due to cannibalism, as large size differences in a group are the most common reason for cannibalistic activities in pikeperch larvae (Kestemont *et al.*, 2003; Szczepkowski, Zakęś, Szczepkowska, & Piotrowska, 2011). Furthermore, a simultaneous understanding of the developmental stages can highlight growth periods when higher maintenance is needed in aquaculture settings. As already seen with automatised counting, the implementation of these techniques could improve larval survival rates and thus has the potential to support the economic success of fish farms and animal welfare efforts in the near future.

5.3 Replacement: Cell culture alternative testing model

For scientific research, the development of alternate research models, such as in vitro models, is of critical importance. Cell culture models can be used to test cellular physiological reactions to external influences (Study II; Dang, van der Ven, & Kienhuis, 2017) or to analyse factors that influence tissue development (Duran, Dal-Pai-Silva, & Garcia de la Serrana, 2020; Salmerón, 2018), while requiring less animals to be sacrificed. For aquaculture-related research, the influences of temperature changes or water oxygen levels can also be tested in cell culture models. However, comparable baseline levels for marker expressions and comparisons with the in-vivo state are necessary to perform such tests (Study II). Next to this fundamental research, cell cultures are also used productively in applied research, such as toxicology and fish virology. In the field of ecotoxicology, acute toxicity testing is performed by checking for mortality rates, a procedure that is also legally required in the European Union (Dang *et al.*, 2017; see also 2010/63/EU; Grunow *et al.*, 2021; OECD, 2019). In the future, these standardised models could not only limit the need to sacrifice animals for such tests, but also avoid the influence of natural influences, such as diurnal rhythms, in these organisms (Thoré, Brendonck, & Pinceel, 2021).

As differences between the reactions of cells from different fish species were found in previous studies (Grunow *et al.*, 2021) the choice of the cell culture model significantly influences the gained results. Cell cultures for testing should include models that are taxonomically close or even species-specific and consist of well-characterised cell lines (Goswami, Yashwanth, Trudeau, & Lakra, 2022). To decrease the exploitation of animal specimens in pikeperch research, the cell line SLUlar1 based on whole embryos was established (Study II). Even though differences in some marker expression levels were found, the application is a promising alternative to the use of animal specimens. The established SLUlar1 cell line can also enable

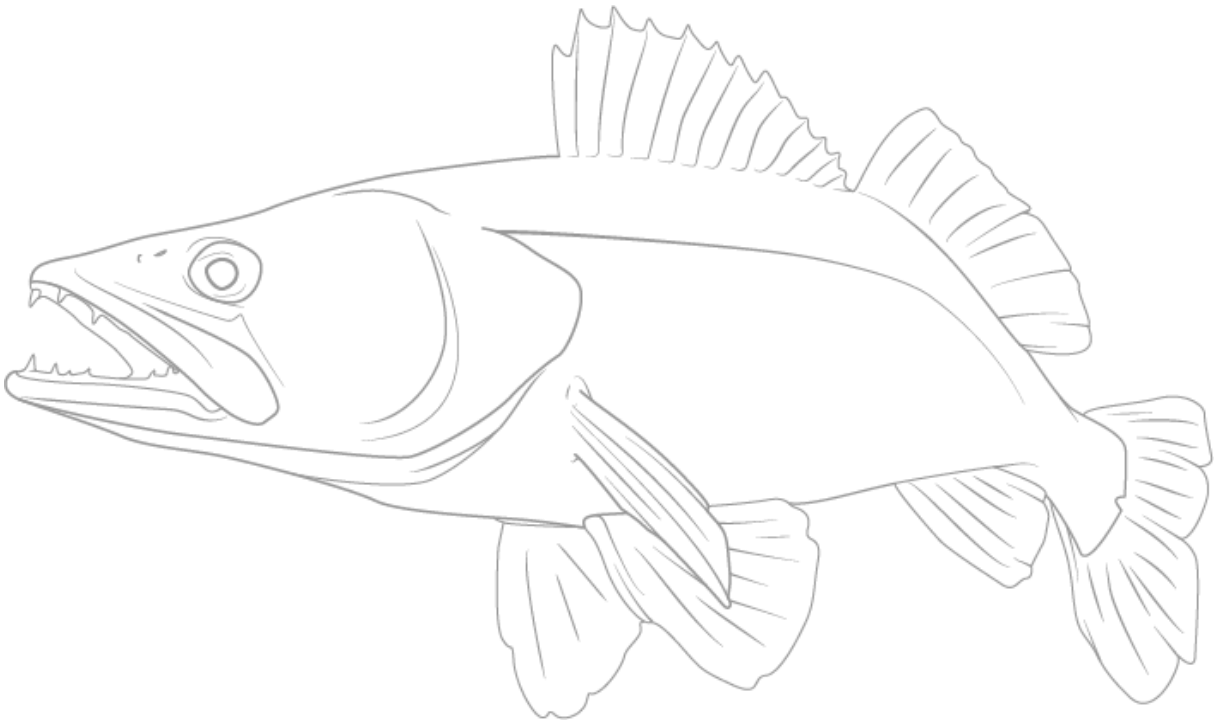
the testing of developmental processes in the future. However, in SLUlar1, the expression of stress markers and the cellular actin filament structure indicated that the cultivation settings for the cell line needed to be further adjusted (see Study II).

6. General conclusions

The observed ontogenetic stages of the pikeperch passed through a series of developmental processes that influence its survival and consequently must be considered for successful rearing. These processes constitute ontogenetic phases that are not influenced by hatching, but by subsequent processes. From the results, the following conclusions can be drawn:

1. Three developmental phases were identified based on changes in the growth rate. An intermediate “threshold phase” occurred during the early larval development, which started shortly after the hatch and ended around the time of the fulfilled switch to exogenous feeding.
2. Embryonic and larval growth patterns differed, with embryonic growth being slower and more uniform, and larval growth being faster and diverse between the same age specimens. The intermediate threshold phase showed a stop in body-length-related growth.
3. During the threshold phase, indications for the development of digestive tissue, neuronal tissue, and heart tissue were found. These findings were in accordance with previous studies on pikeperch.
4. Three myogenetic phases were detected, in which different myogenetic activities associated with different phases of the myogenic cascade were present. They occurred [first] during somitogenesis, [second] before hatch, and [third] after the completed switch to exogenous feeding.
5. The second and third myogenetic phases occurred before and after the threshold phase, respectively. During the threshold phase, MSTN proved to be an inhibitor of myogenetic activity.
6. Two skeletal growth phases around the threshold phase were found. They coincided with the second and third phases of myogenetic activity.
7. The highest rate of endogenous resource utilisation occurred during the threshold phase, the timing of which was alongside the differentiation of organ tissue. The shift to organ differentiation and the simultaneous restriction of body size increase and muscle growth highlight a developmental shift associated with changes in energy expenditures.
8. For the reduction of animal experiments, the cell line model SLUlar1 was established, and its expression levels and cell morphology were analysed. Further adjustments to handling protocols are required for widespread application.

7. Manuscript collection



Study I: “Observations of growth changes during the embryonic–larval transition of pikeperch (*Sander lucioperca*) under near-natural conditions”

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Background and Aim: In this study, growth patterns during early pikeperch ontogeny were analysed. Specimens from a close-to-natural environment were chosen to observe naturally occurring growth patterns during developmental phases that are also problematic in aquaculture rearing. From the results, connections to ontogenetic processes could be derived.

Materials and Methods: Near-natural rearing was achieved by using parental animals from a wild pikeperch population. The specimens were raised under a temperature regime, and water parameters were obtained from a nearby lake via a flow-through system. Using a light trap, zooplankton was obtained as feed. On 162 specimens from 10 age stages between 75 dd and 452 dd (n ≥ 16 per stage), 18 morphometric parameters were analysed. Additionally, yolk and oil globule volumes were calculated. Overall growth tendencies were calculated based on all anatomic parameters by applying principal component analysis (PCA).

Results and Discussion: Three phases of growth activity were established as the result of changes in the principal component analysis. After an embryonic phase until 204 dd, a threshold phase occurred in which length growth paused. Afterwards, during the larval stages from 340 dd onwards, the fastest length growth was observed. Based on the single parameters, it was shown that the depletion rate of endogenous energy resources was highest during the growth intermission in the threshold phase. Here, parameters attributable to internal development were growing predominantly. Additionally, a shift between a longer preanal and postanal length occurred during the threshold phase.

Conclusion: Pikeperch larvae undergo a transformation during the embryonic to larval transition, which leads to a change in growth patterns, with a near stop in length-correlated growth (threshold phase). This allows us to distinguish between an embryonic phase, the specific threshold phase as part of early larval development, and the remaining larval phase.

Supplemental in the appendix

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REGULAR PAPER

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Observations of growth changes during the embryonic-larval-transition of pikeperch (*Sander lucioperca*) under near-natural conditions

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Abstract

Sander lucioperca is an organism of growing importance for the aquaculture industry. Nonetheless, the rearing of *S. lucioperca* larvae is proving to be a difficult task as it is facing a high mortality rate during hatching and the change to exogenous feeding. To gain insight into growth patterns during this period, the authors analysed pikeperch embryos and larvae from 9 days before hatching to 17 days after hatch. Hereby they were able to describe a natural development by using close to natural conditions based on using a direct flow-through supply of lake fresh water on specimens from a local wild population. The results show that between the early embryonic stages a steady growth was visible. Nonetheless, in between hatching and the start of exogenous feeding, a phase of growth stagnation took place. In the following larval stages, an increased growth with large size variations between individual specimens appeared. Both factors are conspicuous as they can indicate a starting point for cannibalism. With this analysis, the authors can provide a fundament to support the upcoming research on *S. lucioperca* and aid to optimize size-sorting procedures for a higher survival of pikeperch stock in aquaculture.

KEYWORDS
larval fishes, morphometry, ontogeny, PCA, Percidae

1 | INTRODUCTION

Early development in fishes is known to proceed in phases that are separated by "thresholds" (Balon, 1979; Balon, 1986; Kryzhanovsky *et al*., 1953; Smirnov *et al*., 1995). The beginning of the exogenous feeding marks such a transition in the ontogeny of fishes and defines the change from embryonic to larval development (Balon, 1979; Kováč *et al*., 1999; Peňáz, 2001; Urho, 2002). In percids high mortality rates occur during this phase (Kestemont *et al*., 2015). For pikeperch *Sander lucioperca* (Linnaeus 1758), the loss rate can rise to 52% of the fry before the start of the larval development (Schmidt & Kühn, 2013). Mortality is largely attributed to cannibalism and defects in organ development (Demska-Zakęś *et al*., 2003; Kestemont *et al*., 2015; Ostaszewska, 2005; Steenfeldt *et al*., 2011). In addition, developmental and physiological changes during embryonic to larval ontogeny (Peňáz, 2001) are connected to a differing energy metabolism and are known to result in variations between phases of growth and differentiation (Osse *et al*., 1997; Osse & Van den Boogaart, 1995; Wieser, 1995).

The high mortality is especially problematic as *S. lucioperca* is of growing importance for the aquaculture industry (Lappalainen *et al*., 2003; Lehtonen *et al*., 1996; Policar *et al*., 2019). Specimens are

needed to restock regional decreasing populations (Mustamäki *et al*., 2014) and support freshwater fisheries as well as recreational fishing (Bninska & Wolos, 2001). Furthermore, as a piscivorous predator, *S. lucioperca* is used as a biomanipulation tool to improve the water quality (Benndorf, 1990).

All this led to the fact that the development of *S. lucioperca* was studied in the past. With the exception of a general developmental description (Bastl, 1978) and an embryogenetic study (Güralp *et al*., 2017), the major focus was placed on separate organ systems that are attributed to larval survival (Demska-Zakęś *et al*., 2003; Kowalska *et al*., 2006; Löffler *et al*., 2008; Ostaszewska, 2005; Ott *et al*., 2012). Ostaszewska (2005) and Kowalska *et al*. (2006) described the differentiation of the digestive system that showed extensive developmental changes starting with the beginning of the exogenous feeding. Moreover, Demska-Zakęś *et al*. (2003) characterized the formation of the swimbladder (SB) and related malformations that reduce larval survival. Löffler *et al*. (2008) as well as Ott *et al*. (2012) analysed the development of the skeletal structures and additionally mentioned size changes that they attributed to the start of external feeding and the starting gill respiration.

To the best of the authors' knowledge, no study focuses on general growth tendencies, thus showing possible growth and differentiation phases. In general, except the basic parameters such as the standard length (SL), no morphometric study of the developmental stages is present for *S. lucioperca*. For this reason, the aim of this study is to morphometrically analyse the ontogeny of *S. lucioperca* during the embryonic-larval-transition as it is a phase of extensive anatomical changes and high mortality. As temperatures affect growth (Réalis-Doyelle *et al*., 2018), the specimens are raised under natural temperature and water conditions, to observe a close to natural state, thus providing a comparison for developmental studies using steady temperature rearing, as well as aquaculture pond rearing. Furthermore, the authors provide information on the development and include measurements attributable to locomotion, endogenous nutrition and muscle tissue increase to reflect physiological changes.

2 | MATERIALS AND METHODS

Embryos and larvae of *S. lucioperca* (Figure 1) were obtained from a fishery at the Lake Hohensprenz in Mecklenburg-Western Pomerania, Germany. Parental animals, originating from the lake's wild population and fed with smaller fishes from the lake, were kept in net cages in a group of eight females and three males until spawning occurred naturally on coco mats. Eggs from a single nest of one female were taken and placed in a Zuger jar, resulting in a sibling to half sibling relation of all studied specimen. Shortly before hatching, the eggs were transferred to hatching trays. Directly after hatching, they were transferred to tanks of 2 m length × 80 cm width and 80 cm depth and were fed *ad libitum* with a zooplankton mixture (Cladocera: *Bosmina* and *Daphnia*; copepod nauplii) obtained from the lake using light traps. During the rearing of the fish in the zugar glass and the hatching trays, the water of the lake (flow-through system) was used over the whole time so that natural conditions existed. The water temperature was measured daily (HACH HQ30D digital multimeter kit, accuracy ±0.3°C) and used to calculate the age [degree-days (dd)] for every developmental stage. The temperature

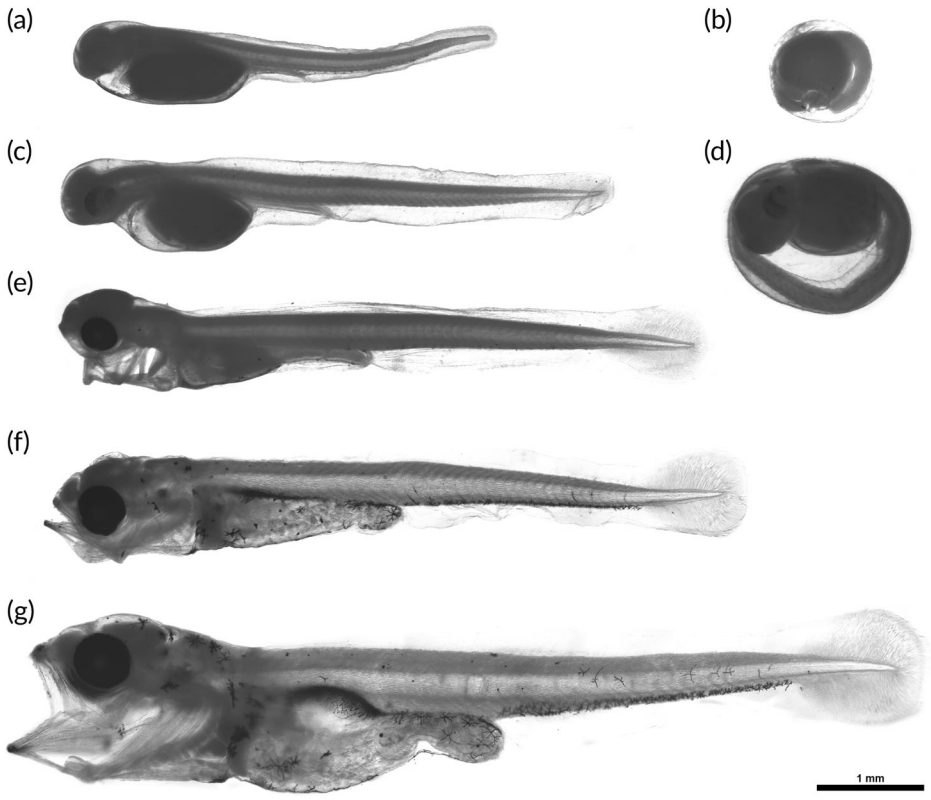

FIGURE 1 Selected developmental stages of *Sander lucioperca*. Age is presented in degree-days (dd) and days post fertilization (dpf). (a + b) Specimens of embryonic stage 1 at 75 dd/5 dpf outside (a) and inside of the egg (b). (c and d) Specimens of embryonic stage 3 at 162 dd/12 dpf outside (c) and inside of the egg (d). (e) Larval stage 5 at 231 dd/17 dpf, 3 days after hatching. (f) Larval stage 7 at 340 dd/24 dpf, 10 days after hatching. (g) Larval stage 9 at 419 dd/29 dpf, 15 days after hatching

increased during the 1 month collection (May till June) with a rate of 0.4 ± 0.9°C day^{-1} and ranged between 12.5 and 23°C with a mean of 15.1 ± 2.8°C. Oxygen concentrations were measured simultaneously with the same equipment ranged around 9.2 ± 1.1 mg l^{-1} (accuracy ±0.01 mg l^{-1}). The pH was measured once per week and had a mean of 7.4 ± 0.2 (accuracy ±0.5). Using a spotlight over one tank side, the light regime was set to a 12:12 day–night setting with *c.* 400 lx below and lower light intensities in the majority of the tank, following recommendations of Steenfeldt (2015). Specimens were euthanized using a Tricain solution overdose (0.25 g l^{-1}, Serva, E10521) and stored in a 70% ethanol solution. For each stage, the development was observed using a stereomicroscope (Leica SD9) in combination with micro-dissection. If necessary, Alcian blue (Roth, 3082.1) was added overnight to enhance visibility of cartilages and organ structures. Based on their development, the age stages were attributed to the embryonic and larval phases following the description of Peňáz (2001) who used the start of exogenous feeding to define the beginning of larval development. For *S. lucioperca*, main hatching took place at 190 dd and the exogenous feeding began after 3 days. All animals were treated according to the animal welfare law Directive 2010/63/EU and § Act (§ 4(3) TierSchG.

2.1 | Measurements

Altogether 162 specimens spread over 10 age stages were used for measurements (n = 16 per stage except stage 419 dd where n = 18; Table 1). For each specimen, lateral and dorsal photographs were taken using a stereomicroscope (Leica SD9) in combination with a Moticam 5.0 (Motic). Eighteen morphometric parameters (Figure 2, Supporting Information Table S1) were analysed using the Software Motic Images Plus (Version 3.0).

In addition, the volume of the yolk and oil globule was calculated to analyse the consumption of nutrients before and during the start of exogenous feeding (Avila & Juario, 1987; Cetta & Capuzzo, 1982; Hempel & Blaxter, 1963). The postanal body volume was calculated to measure the increase in the tail muscle tissue growth as muscle mass can be attributed to the maximum metabolic rate (Wieser, 1995), and their growth is attributed to changes in locomotive pattern during the ontogeny of zebrafishes (Fuiman & Webb, 1988).

Formula 1: The oil globule volume (V_{OG}) was calculated using the formula for spheres where d is the oil globule diameter (OGD):

$$V_{OG} = \frac{4}{3}\pi \times (d/2)^3$$

Formula 2: The yolk volume (V_Y) was determined using the formula for prolate spheroids, where L and H are the length and height of the yolk, respectively:

$$V_Y = \frac{\pi}{6} \times L \times H^2$$

Formula 3: Postanal body volume (V_{PAB}) was obtained with the formula for an elliptic truncated cone where L is the postanal length

TABLE 1 Overview of the examined developmental stages of *Sander lucioperca*

Stage no.	Life phase	dd	dpf/dph	*n*	Developmental characteristics
1	Embryonic	75	5/–	16	Embryo inside egg, head bend towards yolk, eye lenses forming, retina slightly pigmented
2		124	9/–	16	Tail straightens, first integument pigmentation, pectoral fin buds form, first single specimens may hatch
3		162	12/–	16	Head straightens, mouth pit forms, gill arches visible, pectoral fin buds shift ventral, pectoral fin fold forms
4		204	15/1	16	All embryos hatched (main hatch around 190 dd), mouth and opercular cover open, lateral pectoral fins now moveable, caudal fin lobe separates
5	Larval (threshold)	231	17/3	16	First external feeding, hindgut and midgut separated
6		259	19/5	16	Stomach separated from the midgut, swimbladder (SB) forms
7	Larval	340	24/10	16	Teeth on the premaxilla and dentary, SB with short ductus, mesenchymal concentration in caudal fin, first gill filaments
8		373	26/12	16	Only exogenous feeding, stomach turns, intestinal integuments fold, SB inflates
9		419	29/15	18	Increased dentition, increased intestinal integument folding, first caudal fin elements (first hypural and parhypural)
10		452	31/17	16	Beginning notochord flexion, further caudal fin elements and first fin rays, dorsal and ventral fin mesenchymal condensations

Note. For each stage, the age in degree-days (dd), days post fertilization (dpf) and days post-hatch (dph) are mentioned. In addition, notes on the developmental characteristics are given. Stage numbers represent the collected age groups. Life phases are applied after Peňáz (2001) with an additionally marked threshold phase.

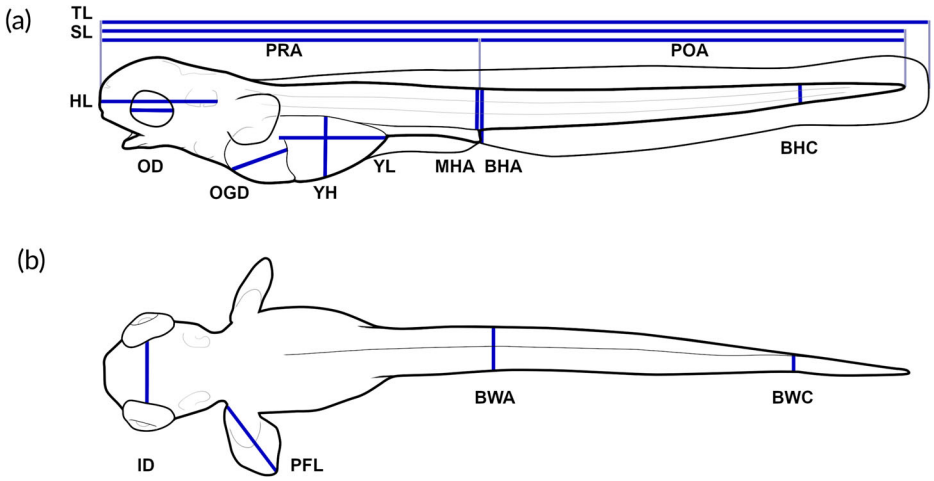

FIGURE 2 Position of the measurements taken on the developmental stages of *Sander lucioperca*. (a) Measurements taken from the lateral side; BHA, body height at the anus; BHC, body height at the caudal peduncle; HL, head length; MHA, myomer height at the anus; OD, orbita diameter; OGD, oil globule diameter; POA, postanal length; PRA, preanal length; SL, standard length; TL, total length; YH, yolk height; YL, yolk length. (b) Measurements taken from the dorsal side. BWA, body width at the anus; BWC, body width at the caudal peduncle; ID, interorbital distance; PFL, pectoral fin length

(POA), A is the body width at the anus (BWA), *B* is the myomer height at the anus (MHA) and *C* is the height at the caudal peduncle:

$$V_{PAB} = \frac{\pi \times (A/2) \times L}{3 \times (B/2)} \times \left((B/2)^2 + (B/2) \times (C/2) + (C/2)^2 \right)$$

2.2 | Data analysis

To detect the superordinate trait variations during the developmental change, a principal component analysis (PCA) was conducted. After Klingenberg (1996), a PCA was used to summarize multivariate data by a reduced number of principal components (PC). The first PC geometrically represents the longest axis through the data point cloud, therefore including the highest variation, and subsequent PCs for the remaining maxima orthogonal to the proceeding axes. For this, data were normalized by setting the mean of each feature to 0 and the standard deviation to 1. The PCA was executed with the Python package scikit-learn (Pedregosa *et al*., 2011) that was applied to the 17 measured parameters (excluding the only noted SB inflation). Plots were generated with the Python package Matplotlib (Hunter, 2007) and Seaborn (Waskom *et al*., 2020). To determine the number of PCs that are relevant for the explanation of the variance, their eigenvalues were compared to a Parallel Analysis (Horn, 1965) of uncorrelated random variables of the same size as the data.

Then, the separate morphometric parameters were further analysed based on developmental phases gained from the PCA results. As the time between the obtained stages differed, parameters were compared to their change over the continuous dd. For this, multiple linear regression analysis was applied. Hereby, the developmental phases determined by the PCA were treated as separate linear growth phases. The calculation of the multiple linear regressions and the plotting of the graphs were done using R (Version 4.0.3) with the libraries psych (Version 2.0.9) and ggplot2 (Version 3.3.2) in R-Studio (Version 1.3.1093). The development of the parameters is described by their overall mean during the developmental phases as well as by the slope of their regression graphs (*m*). A complete overview of resulting data is provided in the supporting information (Supporting Information Table S2). As the general data analysis determined a strong correlation with the body length, the morphometric parameters with the exception of the total length (TL) and SL were additionally analysed in relation to the SL with the methods described earlier.

3 | RESULTS

3.1 | Correlation analysis and PCA

To get an overview of the relation between the individual factors determined in this study, correlation analyses were carried out (Figure 3). In *S. lucioperca* ontogeny, numerous features [such as TL, preanal length (PRA), POA, MHA, head length (HL), body height at the anus (BHA) and body height at the caudal peduncle (BHC)] were highly positively correlated with SL having a Pearson correlation coefficient greater than 0.9. Exceptions were the head angle (HAN), interorbital distance (ID), pectoral fin length (PFL) and body width at the caudal peduncle (BWC), which did not show such strong linear dependency with a correlation coefficient ranging between 0.46 and 0.89. Compared to the length features, a highly negative correlation between −0.70 and −0.82 was measured for the features belonging to the embryonic nutrition [yolk height (YH), yolk length (YL), and OGD].

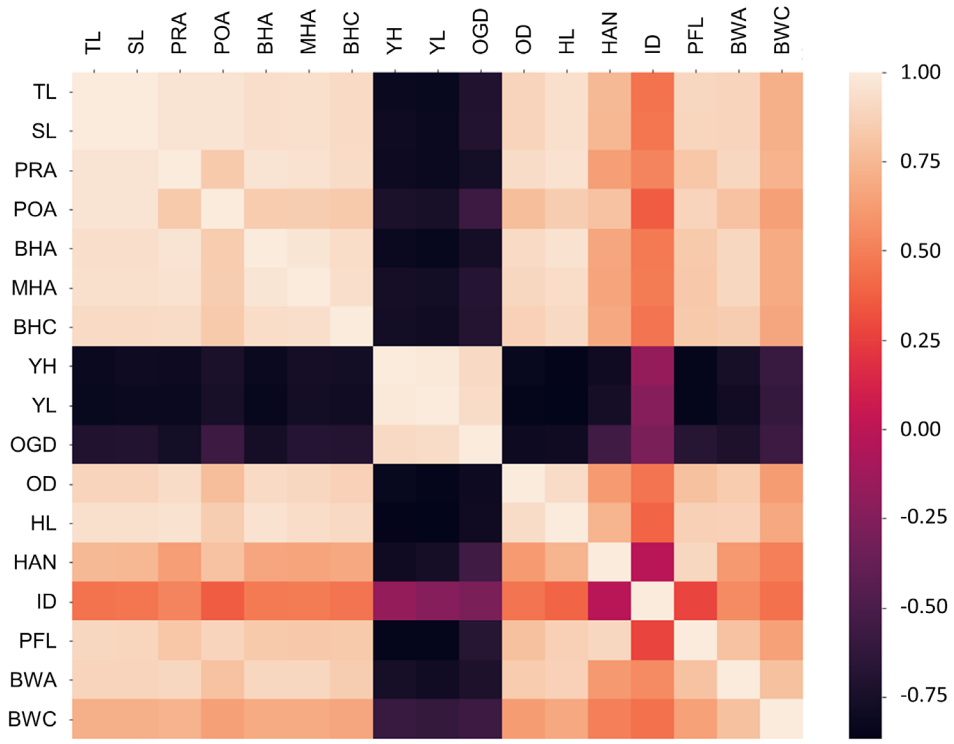

FIGURE 3 Correlation matrix of the obtained body parameters. The values for the Pearson correlation coefficient are colour-coded. Abbreviations follow Figure 2

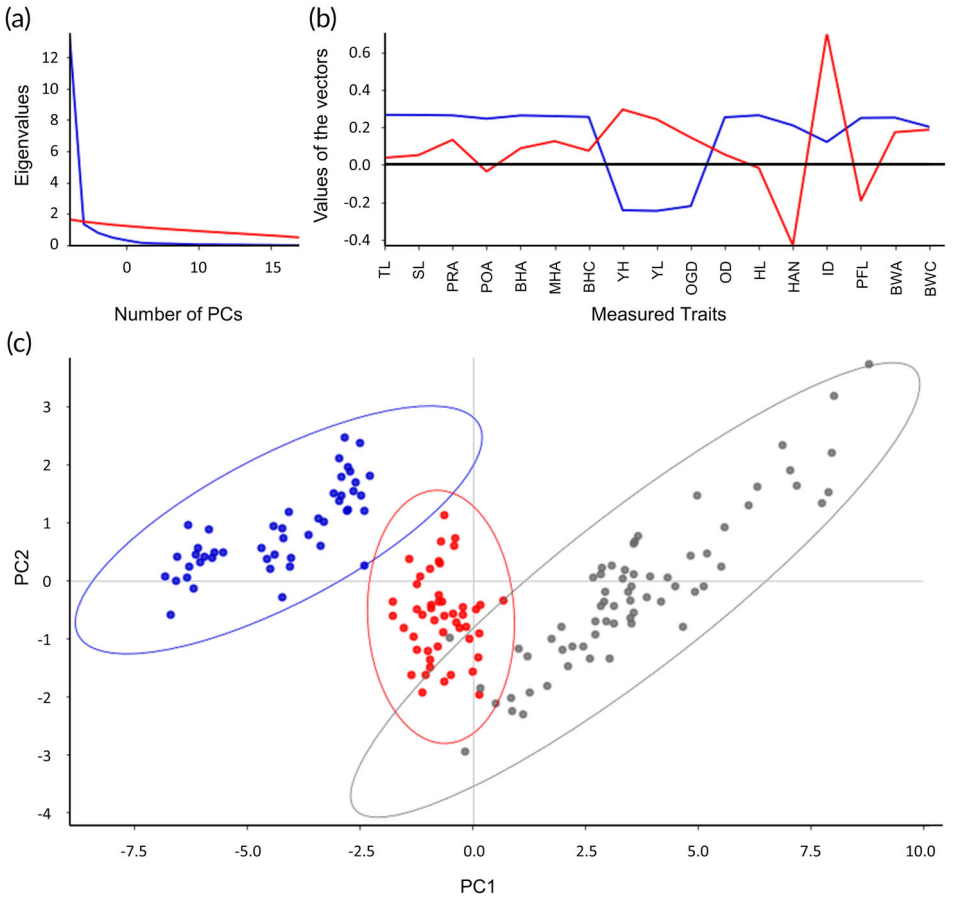

FIGURE 4 Results of the principal component analysis (PCA). (a) The eigenvalues of the PCA over the number of components for the *Sander lucioperca* data (data, blue). Sufficient contribution of the principal components was determined by a Parallel Analysis, which is based on the mean of 1000 artificial samples of uncorrelated random variables of same size (red, random) ——, data; ——, random. (b) Entries of the values for the eigenvectors for the first two principal components (PC). Contribution of the parameters to each PC is shown by the value of the eigenvector——, PC1; ——, PC2. (c) Plot of the first two PCs. Results are shown for each developmental phase (colour-coded). The ellipses are the covariance confidences with a standard deviation (σ = 3) ●, embryonal; ●, threshold; ●, larval

As during the ontogeny of *S. lucioperca* a correlation between growth and most of the analysed traits was present, the aim was to determine the features, which are independent of size and contribute to the measured morphological variation. To achieve this aim, a PCA was performed with normalized parameter values. Based on the Parallel Analysis, a significant impact was found for the first principle component (PC) and with some limitations for the second PC, because the second PC had approximately the same level as the artificial samples of the Parallel Analysis (Figure 4a). Then, two PCs were used. The corresponding eigenvalue of PC1 with 13.53 was much larger than the ones of all other PCs (PC2 eigenvalue 1.35) and therefore showed most of the variance. Together, both PC1 (79.1%) and PC2 (7.9%) accounted for 87% of the total variance in the data.

The measured parameters were associated with the PCs based on their values for the eigenvectors (Figure 4b). The first PC was composed of nearly all normalized measured features equally. Generally, the correlated features were added to the same PC. Their equal distribution with a value between 0.25 and 0.3 resembled the high correlation of the length scales. The same applied to descriptive parameters of endogenous resources that had a similar negative value. ID and HAN were the only parameters that differed from this scheme decidedly. The second principal axis mainly consisted of contributions from ID, and further from HAN, YH, PFL and BWC.

All data points were mapped to the first two components of the common PCA (Figure 4c). The first PC exhibited a steady growth with minor standard deviations between 75 and 162 dd old animals, visible by clusters for each of these stages. Following, a plateau with means close to zero and low standard deviations occurred. In the oldest examined stages, between 340 dd and 452 dd, PC1 increased further while high standard deviations were present.

In the second PC, an increase in the earliest stages, similar to PC1, was present. Later between 204 dd and 259 dd, PC2 decreased. Until this, a nearly constant standard deviation was present. In the oldest larval stages after 340 dd, another increase with high standard deviations took place.

Based on the observed changes in the PCA, three developmental phases could be distinguished: An early phase ending between 162 dd and 204 dd named embryonic phase, an intermediate phase labelled as threshold phase and a late phase that starts between 259 dd and 340 dd named larval phase. Then, the single parameters were analysed based on these three developmental phases.

3.2 | Analysis of the separate parameters

3.2.1 | General parameters

For the analysis of the parameter growth over time, all parameters were described by their means during the phases obtained from the PCA and the behaviour of the regression graphs. The TL (Supporting Information Table S2) and SL (Figure 5a) behaved overall similarly. During the embryonic phase, an increase with m = 18.34 (TL) and m = 17.47 (SL) was present. The embryonic means were 4493 ± 692 μm (TL) and 4392 ± 659 μm (SL) with connected low standard deviations. In the threshold phase, nearly no growth was visible with m = 1.74 (TL) and 1.02 (SL). The means were 5644 ± 222 μm (TL) and 5458 ± 206 μm with remaining low standard deviations. In the larval phase, a further growth [m = 7.85 (TL) and m = 7.72 (SL)] with higher standard deviations was present. During the larval stages, means of 6647 ± 728 μm (TL) and 6402 ± 711 μm were measured.

The highly correlated (Pearson coefficient >0.95) PRA and POA (Supporting Information Table S2) behaved correspondingly to the SL. The PRA had a higher mean but a lower rise during the embryonic phase (2251 ± 198 μm, m = 5.07) than the POA (2141 ± 474 μm, m = 12.41). During the threshold phase, the POA had a higher mean than the PRA, while the growth rates stagnated (PRA: 2585 ± 120 μm, m = −0.07; POA: 2873 ± 117 μm, m = 1.09). During the larval phase, the PRA had a larger mean with a steeper rise than the POA (PRA: 3270 ± 442 μm, m = 5.42; POA: 3133 ± 296 μm, m = 2.30).

3.2.2 | Parameters of the anterior body

The HL (Supporting Information Table S2) showed a growth through all developmental phases, which was reduced only during the threshold phase. It grew with m = 2.32 during the embryonic, m = 1.01 during the threshold and further with m = 2.25 during the larval phase. The means increased from 620 ± 102 μm (embryonic), over 888 ± 71 μm (threshold), to 1243 ± 204 μm (larval). Hereby, the standard deviations were the highest during the latest stages of the larval phase, reaching 1376 ± 224 μm at 452 dd.

Similar to the HL, the orbita diameter (OD; Supporting Information Table S2) showed an increase during stages, which was reduced during the threshold phase. Within all observed stages, the OD showed minimal changes in a range of only 396 μm. It grew from 288 ± 37 μm in the embryonic phase (m = 0.73) to 326 ± 24 μm during the threshold phase (m = 0.52) and reached 435 ± 55 μm in the larval phase (m = 0.70).

During all the observed parameters, the ID (Figure 5b) remained at a similar size. Still, a growth was observable during the embryonic and larval phases, whereas it decreased in size during the threshold phase. The associated mean was 430 ± 51 μm in the embryonic (m = 1.06), dropped to 390 ± 35 μm during the threshold (m = −0.79) and grew to 450 ± 70 μm in the larval phase (m = 0.28).

The PFL (Figure 5c) increased strongest directly after its formation during the embryonic phase (m = 3.25), whereas its growth was reduced later (threshold phase m = 1.00; larval phase m = 0.75). The PFL had a mean of 143 ± 137 μm during the embryonic phase, 585 ± 64 μm during the threshold phase and only slightly increased to 742 ± 103 during the larval phase.

During the development of the head and gills, the HAN (Supporting Information Table 2) increased from 131 ± 6° at the earliest stage at 75 dd until it achieved a straightened position. During the embryonic phase, it had a mean of 138 ± 8 (m = 0.16). Its strongest growth occurred during the threshold phase (m = 0.30), where its

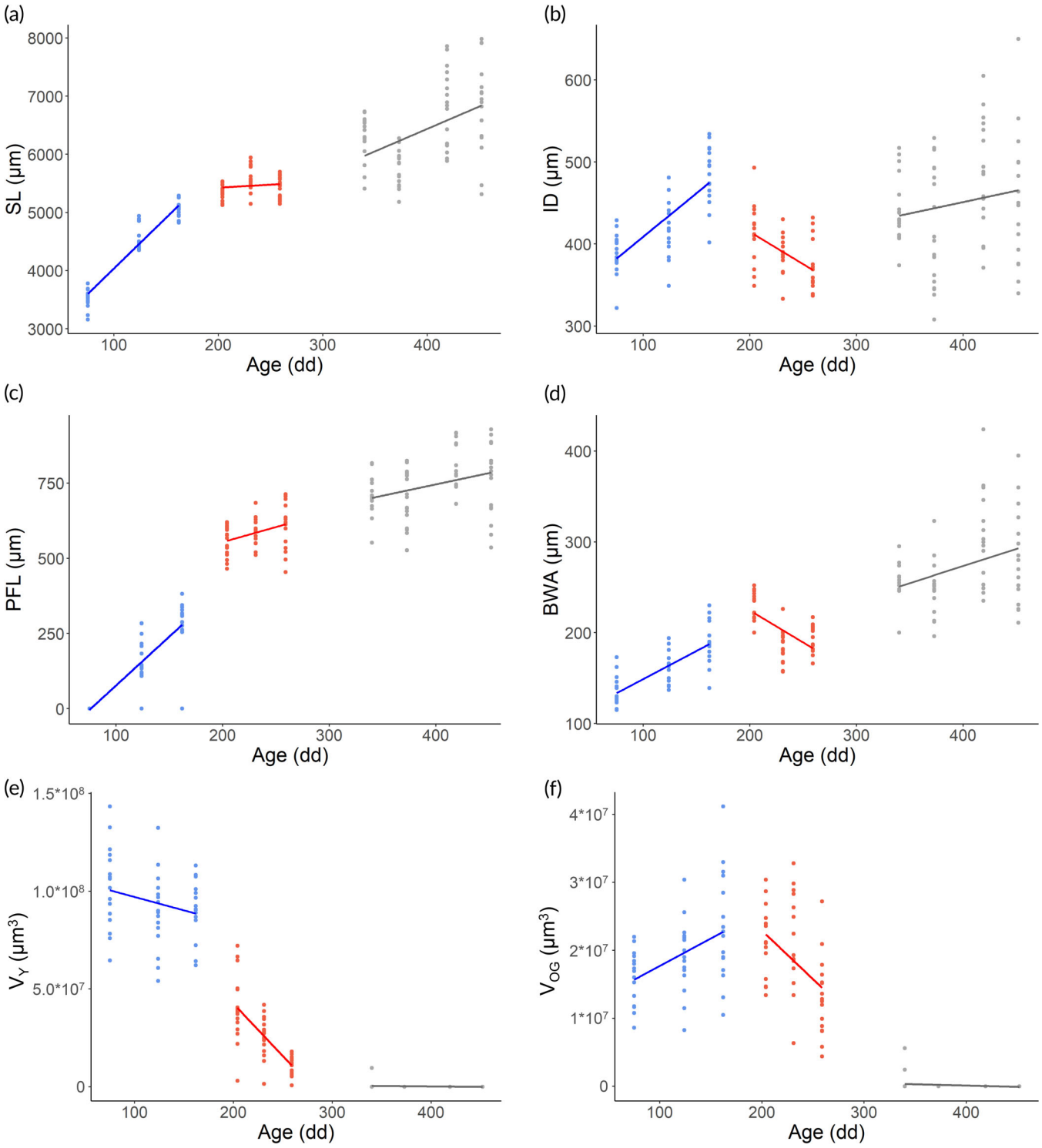

FIGURE 5 The changes in selected body parameters of *Sander lucioperca* with increasing age in degree-days (dd). The separate developmental phases are colour-coded. Blue, embryonic phase; red, threshold phase; grey, larval phase. Linear regression lines are given for the associated phases. (a) SL, standard length. (b) ID, interorbital distance. (c) PFL, pectoral fin length. (d) BWA, body width at the anus. (e) V_Y, volume of the yolk. (f) V_{OG}, volume of the oil globule

mean reached 183 ± 8°. During this, the HAN shortly overstretched to a maximum of 191 ± 3° at 259 dd. In the larval stages no change (m = −0.02) was observable, whereas the mean levelled off at 184 ± 4°.

3.2.3 | Parameters of the postanal body

The parameters measured in the anal area and at the caudal peduncle showed correlated changes over time, resulting in similar growth

tendencies. Although a growth was present in the embryonic and larval phases, a stagnation and even reduction were observed during the threshold phase.

At the anus, the BHA (Supporting Information Table S2) increased from 301 ± 60 μm (m = 1.54) during the embryonic phase, to a plateau of 440 ± 33 μm (m = −0.32) in the threshold phase and increased further during the larval phase to 646 ± 140 with m = 1.52. Thereby, the MHA (Supporting Information Table S2) equalled 69.4 ± 5.1% of the BHA until the end of the threshold phase and changed to 62.0 ± 5.9% during the larval phase. The BWA (Figure 5d) showed a mean of 162 ± 29 μm during the embryonic phase and an ascending slope with m = 0.62. In the threshold phase, a mean of 202 ± 26 μm was present, which was combined with a decrease in the regression graph of m = −0.70, being the strongest decrease rate in the postanal body region. Later, during the larval phase, a further growth of the BWA with a mean of 272 ± 46 μm and m = 0.38 occurred.

Although the growth tendencies were similar at the caudal peduncle, the rates of growth lead to a change in the transverse section. During the embryonic phase, the caudal peduncle was round (BHC: 101 ± 23 μm, BWC: 95 ± 16 μm, both Supporting Information Table S2) with increased rates of m = 0.51 (BHC) and m = 0.23 (BWC). It became dorsolaterally widened from the threshold phase on (BHC: 152 ± 14 μm, BWC: 109 ± 15 μm), whereas the regression slopes decreased with m = −0.16 (BHC) and m = 0.02. In the larval stages, this ratio was kept with a mean BHC of 201 ± 42 μm and a mean BWC of 134 ± 28 μm, and a further BHC increase of m = 0.45 while the BWC only had a slightly increasing slope of m = 0.06.

Resulting out of the above parameters, the postanal body volume (V_{PAB}; Supporting Information Table S2) showed a slight growth during the embryonic phase ($m = 46 \times 10^4$) with a mean volume of $0.35 \pm 0.18 \times 10^8$ μm^3 and decreased in size during the threshold stages (mean $0.81 \pm 0.14 \times 10^8$ μm^3; $m = -32 \times 10^4$). During the larval phase, a strong increase with $m = 84 \times 10^4$ and a mean of $1.65 \pm 0.78 \times 10^8$ μm^3 was present.

3.2.4 | Parameters of the endogenous resources

In pikeperch, the endogenous resources comprise the yolk and oil globule. Both decreased until the larval phase, but showed different

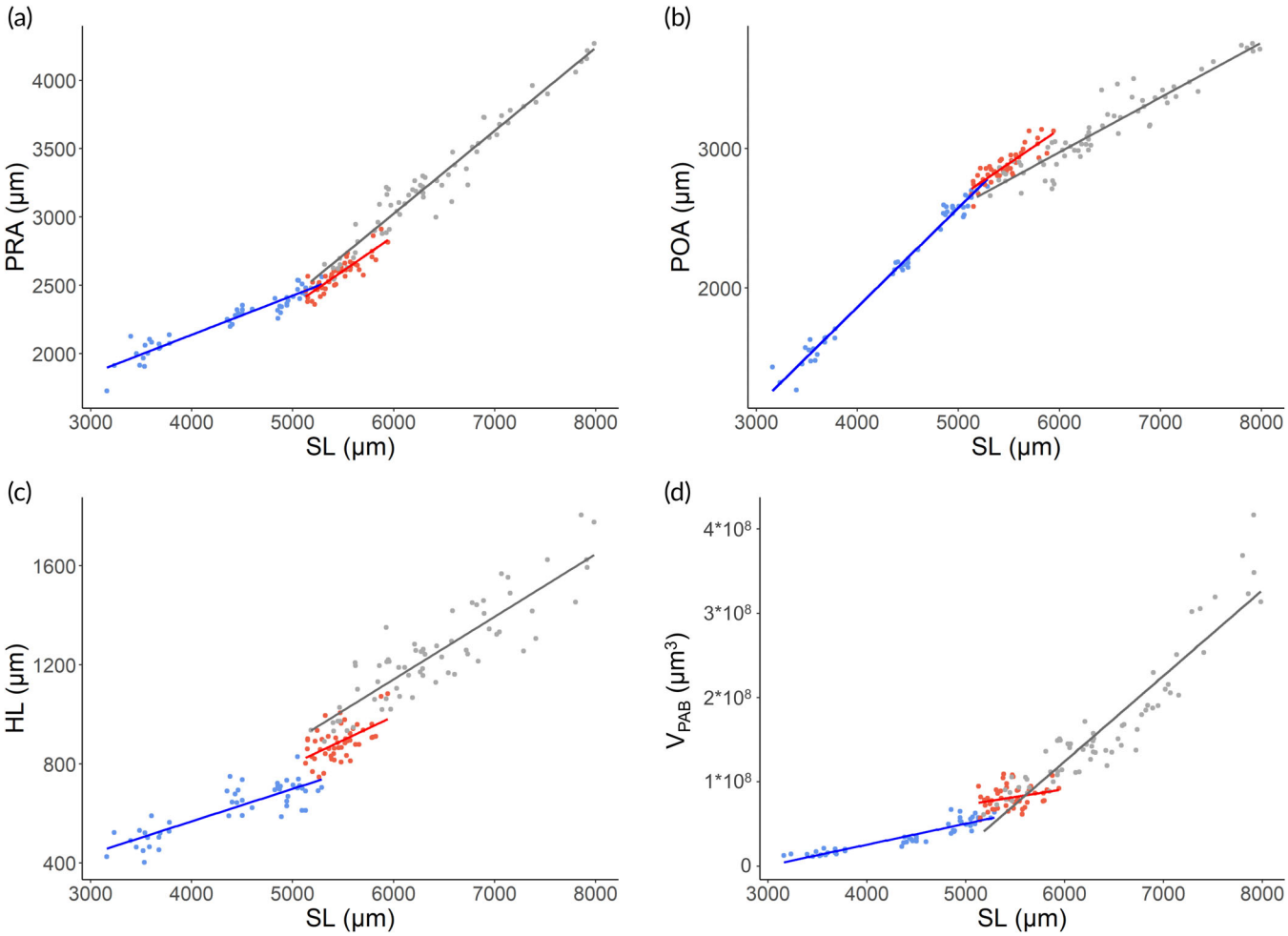

FIGURE 6 The changes in selected body parameters of *Sander lucioperca* compared to the standard length (SL) of the specimens. The separate developmental phases are colour-coded. Blue, embryonic phase; red, threshold phase; grey, larval phase. Linear regression lines are given for the associated phases. (a) PRA, preanal length. (b) POA, postanal length. (c) HL, head length. (d) V_{PAB}, volume of the postanal body

changing behaviour. At 373 dd shortly after the start of the larval phase, both parameters were fully depleted.

The YH (Supporting Information Table S2) and YL (Supporting Information Table S2) showed similar reductions, which resulted in a ratio of *c*. 1 to 2. During the embryonic phase, a steady YH was present (m = 0.03) with a mean of 448 ± 37 μm. It decreased by almost half during the threshold phase (268 ± 77 μm, m = −2.35). Compared to the YH, the YL showed already a decrease during the embryonic phase (m = −1.37, mean 889 ± 84 μm) and decreased even stronger during the threshold phase (m = −3.32, mean 578 ± 142 μm). In general, the yolk was completely depleted latest at 343 dd. The calculated yolk volume (V_Y; Figure 5e) had a decrease of $m = -137 \times 10^3$ during the embryonic phase and a decrease of $m = -536 \times 10^3$ during the threshold phase.

Compared to the V_Y, the changes in the volume of the oil globule (V_{OG}, Figure 5f) were different. The analyses showed that the OGD (Supporting Information Table S2) even increased in the embryonic phase (m = 0.53, mean 398 ± 46 μm). The consumption of the oil globule was only recorded from the threshold phase onwards (m = −1.18, mean 390 ± 55 μm). The associated V_{OG} increased in the embryonic phase with $m = 81 \times 10^3$ and decreased in the threshold phase with $m = -143 \times 10^3$. The oil globule was usually depleted at 343 dd.

3.2.5 | Parameters in relation to the SL

As growth trends in relation to SL were mostly similar in the comparisons of the parameters, only some peculiarities are addressed below.

The general length proportions included a shift to a prolonged POA during the threshold phase (Figure 6a,b). Although the POA had its strongest linear relation to the SL (m = 0.71) during the embryonic phase, the PRA had its highest increase in the regression slope during the larval phase (m = 0.60). During the threshold phase, both PRA and POA exhibited increases close to m = 0.50. Altogether, these growth differences lead to the POA being longer than the PRA during the threshold phase with a size-specific break point at a PRA of 2500 μm and a POA of 3000 μm.

In the anterior body region, the HL (Figure 6c) showed a changing relation to the SL even though it had a strong correlation with the SL (Figure 3). The linear relation between the HL and SL was weaker during the embryonic phase (m = 0.13) and nearly doubled during the larval phase (m = 0.25).

The ratio of V_{PAB} to SL (Figure 6d) was a weak linear relationship ($m = 25 \times 10^3$) during the embryonic phase, which was even reduced to $m = 19 \times 10^3$ during threshold phase. In the larval phase, nonetheless, the linear relationship was very strong with $m = 102 \times 10^3$. These different linear relationships of the developmental stages were caused by the postanal body parameters (MHA, BHC, BWA and BWC) included in the equation. Although these parameters showed an overall positive linear relation, it was the weakest during the threshold phase with the BWA even becoming slightly negatively related (m = −0.01).

4 | DISCUSSION

Observing the overall data, the PCA revealed changing growth patterns during the ontogeny of *S. lucioperca*. Because of this, a differentiation of three distinct developmental phases, an embryonic phase, an intermediate threshold phase from 204 to 259 dd shortly after hatching and a following larval phase, were distinguishable. During the embryonic and larval phase, a mainly length-correlated growth occurred, but it stagnated during the threshold phase.

4.1 | Changes during the developmental phases

During the embryonic phase, a steady linear growth with low standard deviations between the individuals within one nest occurred in all analysed parameters. Because of the inclusion in the egg shell, less energy is spent on interactions with the environment and instead on growth itself during the early ontogeny (Rombough, 2011). In addition, a less differing metabolism between embryos of the same age occurs (Wieser, 1995). Both factors consequently result in a more uniform growth close to the metabolic maximum.

The following threshold phase started directly after hatching. A strongly reduced growth was present in the measured parameters. In addition, the standard deviation was the lowest in most of the measurements. This stagnation of growth displays an ontogenetic shift for *S. lucioperca*, which can be seen as the result of high energetic costs at that time, as the depletion rate of endogenous resources simultaneously reached its maximum. High energetic costs during this transition are common in teleosts and can be seen in weight loss (Osse *et al*., 1997). For the rabbitfish *Siganus guttatus* (Bloch 1787), this was additionally noticed in a decrease in the age groups mean body size (Avila & Juario, 1987). For *S. lucioperca*, the high energetic costs were attributed to simultaneous internal changes (Ostaszewska, 2005). During the threshold phase, the digestive tract undergoes major changes starting with the beginning of the exogenous feeding. It could be observed that in all analysed specimens, a straight digestive tract was present right after hatch and a separation of the mid- and hindgut as well as the torsion of the stomach took place during the threshold phase. Changes in the digestive tract were also found in sea bass *Dicentrarchus labrax* (Linnaeus 1758) (García Hernández *et al*., 2001), turbot *Scophthalmus maximus* (Linnaeus 1758) (Segner *et al*., 1994) and Eurasian perch *Perca fluviatilis* (Linnaeus 1758) (Kestemont *et al*., 1996). For pikeperch, the authors additionally found a coinciding short-term shift towards a longer POA during the threshold phase. This shift was followed by a stronger preanal growth after the threshold phase during the larval stages, which has been named common for percids (Urho, 2002).

With the start of the larval phase, endogenous resources were fully depleted and the larvae completely transitioned to exogenous feeding. In all animals, a high growth rate and increasing standard deviations between animals of the same age were measured. Demska-Zakęś *et al*. (2003) concluded a missing inflation of SBs to result in growth differences in *S. lucioperca*. In the here-observed

specimens, the SB inflation occurred in only around 65% of all larval stage specimens from 340 dd on. From 419 dd, specimens with an inflated SB had a significantly longer SL ($P \leq 0.05$) compared to animals without inflated SB, which supports this hypothesis.

It is known that pikeperch is subject to very high cannibalistic pressure during its larval development (Colchen *et al*., 2019; Steenfeldt *et al*., 2011; Szczepkowski *et al*., 2011). The here-observed high size variances of all parameters within larval age groups support these investigations. For example, a large head is important for survival, as it is helpful for feeding prey and avoiding predation by individuals of the same size. Its growth can therefore be regarded as evolutionary driven during these crucial developmental stages. Coinciding, the authors observed the strongest linear HL to SL relation during the larval phase, which supports this assumption. Next to this, a growth change of morphometric parameters fundamental to locomotion was present in the larval phase. Although the pectoral fin decreased its growth, a stronger growth in the postanal muscle tissue volume was present, thus allowing fast spurts. Faster locomotion contributes to prey capture success, thus resulting in increased growth (Fuiman & Webb, 1988; Hunter, 1981; Webb & Weihs, 1986). As a conclusion, the size differences may increase over time, as larger and different prey becomes obtainable (Graeb *et al*., 2005; Hunter, 1977). As *S. lucioperca* has a broad spawning season (mainly April–May) that can span temperatures from 4 to 24°C (Lappalainen *et al*., 2003) and a high amount of offspring (138–643 eggs per gram body weight) (Kosior & Wandzel, 2001), the increasing growth variance consequently leads to cannibalism in a natural habitat (Claessen *et al*., 2000; Nellen, 1986; Osse *et al*., 1997).

To prevent the high rate of cannibalism in aquaculture, size sorting of specimen is used. Although the onset of cannibalistic activity in *S. lucioperca* has already been observed between 14 and 22 dph (Colchen *et al*., 2019; Kestemont *et al*., 2007), sorting was only tested in older larvae from 48 dph. Nonetheless, even this late sorting has led to higher larval survival (Szczepkowski *et al*., 2011). As the authors of this study were able to find a strong increase in variance to occur after the completion of exogenous feeding at 10 dph already, an earlier starting of sorting procedures needs to be implemented to increase the survival of *S. lucioperca* larvae. Herby, the high correlation rate of most of the analysed morphological traits to the fish length leads to the conclusion that the starting point of exogenous feeding can be determined using the SL or TL. The results of the PCA support this statement, as the first PC had a generally size-based input that by itself was able to explain 79.1% of the occurring variation.

In summary, within this study it could be shown that the morphometric growth pattern of pikeperches shows strong changes in the phase of transition from embryo to larvae. It includes a threshold phase of external growth stagnation and low variation in the morphological traits. In later larval stages, the increased variance in body size parameters can be seen as a driving force for intraspecific competition. Given the high increase in variance already in early larval development, an adaption of sorting procedures in aquaculture is necessary. This study displayed that the size-sorting of the larvae does not have to begin 2 months post-hatching but already around 10 dph periodically to stop the high loss of animals in aquaculture caused by cannibalism.

4.2 | Study design and restrictions

For this study, the authors have chosen eggs from a semi-natural environment. The reason for this is that unlike for other species, pikeperch eggs from a conventional aquaculture farm cannot be obtained in sufficient quantity and quality so far. Obtaining eggs from a natural environment as well proves to be difficult, as the pikeperch spawns at depths of up to 3 m in turbid eutrophic waters with the nest being protected by the male (Lappalainen *et al*., 2003). The construction of this design therefore represents an opportunity to analyse the development of this species from a natural environment, which could be used, *e.g*., as a comparison with aquaculture rearing. Nonetheless, such a study design also has disadvantages. Even if no extreme temperatures occurred in the year of the study, the uniqueness of the combination of water temperature and water quality parameters makes it difficult to repeat the situation in subsequent seasons. Moreover, within a season, the differences between specimens from different nests may be large because of the very long spawning period (March to May). The supply of plankton species and therefore protein composition as a food source varies with the water temperature. In addition, the larger animals naturally eat the smaller larvae, so that specimens of late spawners have a very low chance of survival. In total, this study can only provide an insight into the development of the population at one time point. Nevertheless, various aspects of the presented data coincide with other studies. For example, the growth stagnation the authors observed in this semi-natural environment was also mentioned for pikeperch larvae under controlled conditions (Löffler *et al*., 2008; Ott *et al*., 2012). The aim should be to further understand the development of the pikeperch in more detail, like the impact of food particle size, protein composition and temperature in general, to ensure a successful breeding in aquaculture.

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AUTHOR CONTRIBUTIONS

B.G. devised the project and procured the funding for the research. G.P.F. carried out the project, collected samples, conducted measurements and analysed the data. L.L. contributed the correlation and principal component analysis. All authors contributed to data interpretation and writing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Study II: “Insights into early ontogenesis: Characterization of stress and development key genes of pikeperch (*Sander lucioperca*) in vivo and in vitro”

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Background and Aim: Embryonic and larval age stages were analysed based on their respective RNA expression. Genes connected to general developmental processes, energy, lipid metabolism, and stress were observed. Additionally, the results were compared to a newly developed cell model based on whole-larvae pikeperch.

Materials and Methods: For the genetic analysis, pikeperches from the strain “Sachsen” raised in the RAS testing facility were examined with a nanofluidic qPCR approach. For the total RNA analysis, six age stages between 0 and 175 days post hatch (dph) were analysed based on pools of 20 specimens or liver tissue from the oldest two stages. The cell model was established based on 12 pikeperch embryos with an age of 125 dd. Gene expression and cell morphology were observed to analyse the status of the cells.

Results and Discussion: In vivo, the observed stress markers showed different expression patterns. The general stress marker HSF1 had continuous and steady expression, and HIF1A contrasted with biphasic expression during these stages. This was attributable to their connection with either environmental stressors (HSF1) or hypoxia stressors (HIF1A). Developmental gene expression connected to bone formation (BMP4 and BMP7) and heart development (MYH6) coincided with the general developmental processes of pikeperch at these age stages. The first cell line from pikeperch was established. The cells became bigger and more homogenous during cultivation. However, their morphology and gene expression indicated enhanced stress levels. Stress gene markers were compared with a cell line from the walleye (*Sander vitreus*), which confirmed the finding of enhanced stress levels in the pikeperch cells. We attributed this to the more general sensitivity of the pikeperch cell line.

Conclusion: The gene expression of developmental, stress, and metabolism gene markers was described for pikeperch reared under aquaculture conditions. The cell model was established and can support animal welfare efforts in accordance with the 3R-principles.

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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

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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 Zak ś 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 Cl 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).

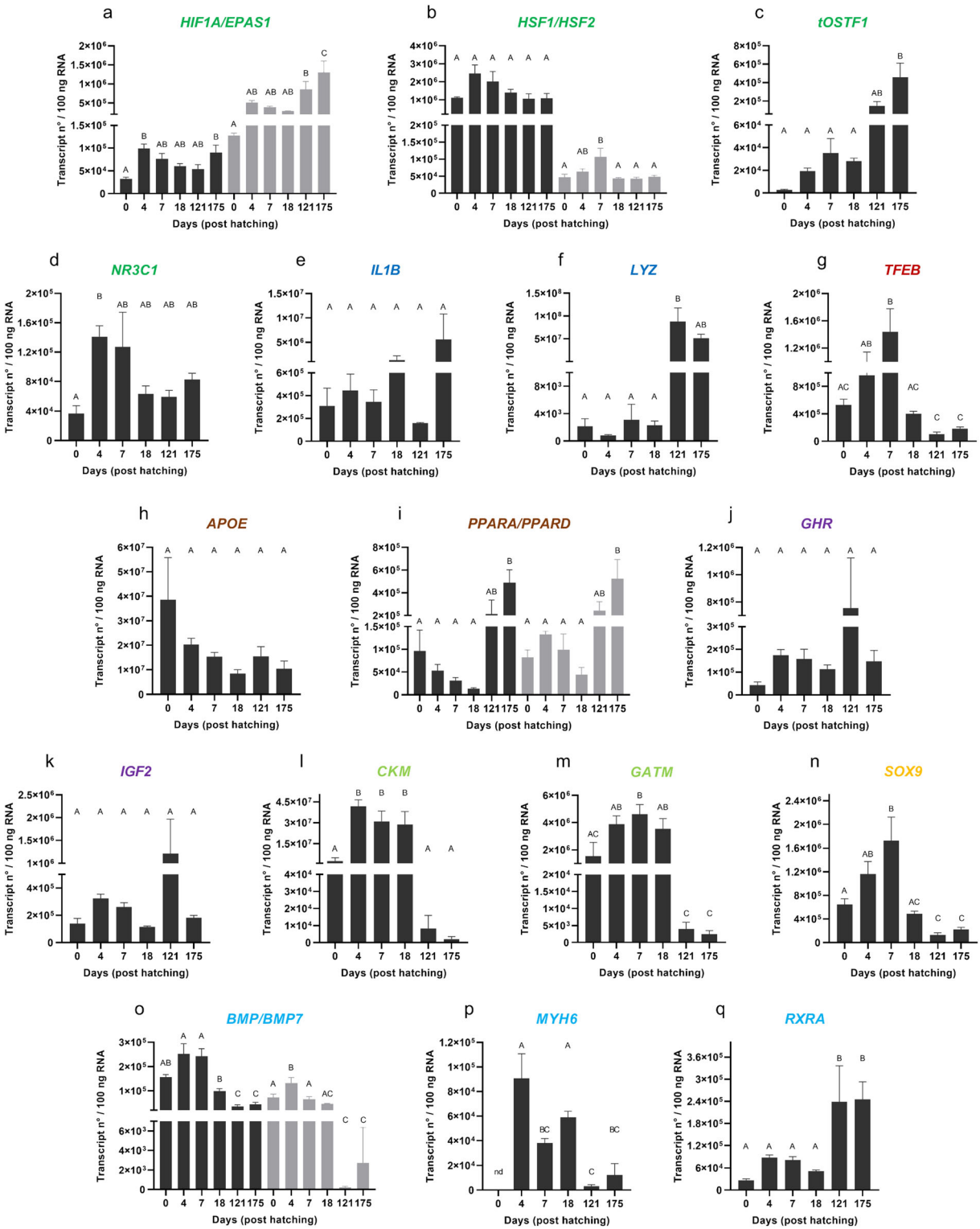
The genes *GHR*, *IGF2*, and *LYZ* were most strongly expressed at 121 dph, whereby the expression of *LYZ* was highly significantly ($p = 0.003$) increased from early (0–18 dph) to late developmental stages (121 dph). *EPAS1*, *IL1B*, *tOSTF1*, *RXRA*, *PPARA*, and *PPARD* revealed the highest transcript 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)
0 4 7 18 121 175

◀ **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

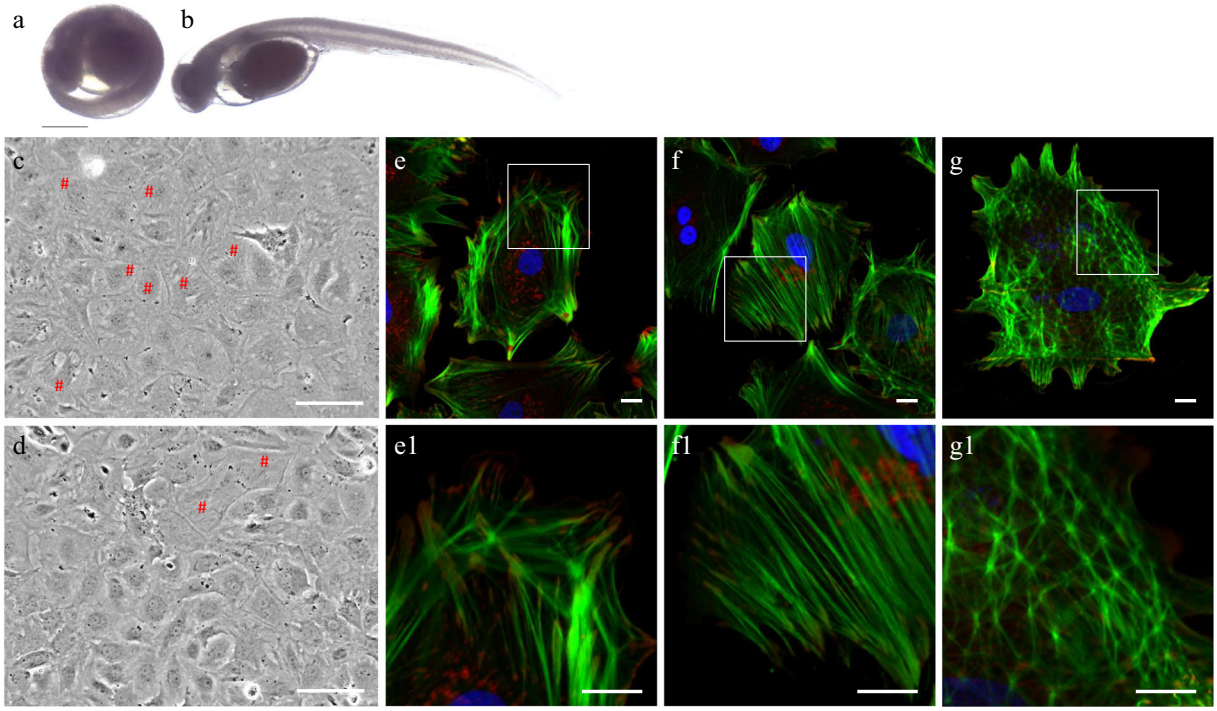


Fig. 2 Source and morphology of SLUlar1 cells from *Sander lucioperca*. Larvae at an age of 125 degree days (DD; nine days after fertilization) in (a) the egg and (b) isolated from the eggshell, phase contrast microscopy of isolated SLUlar1 cells from (c) passage five and (d) passage six. High concentrations 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

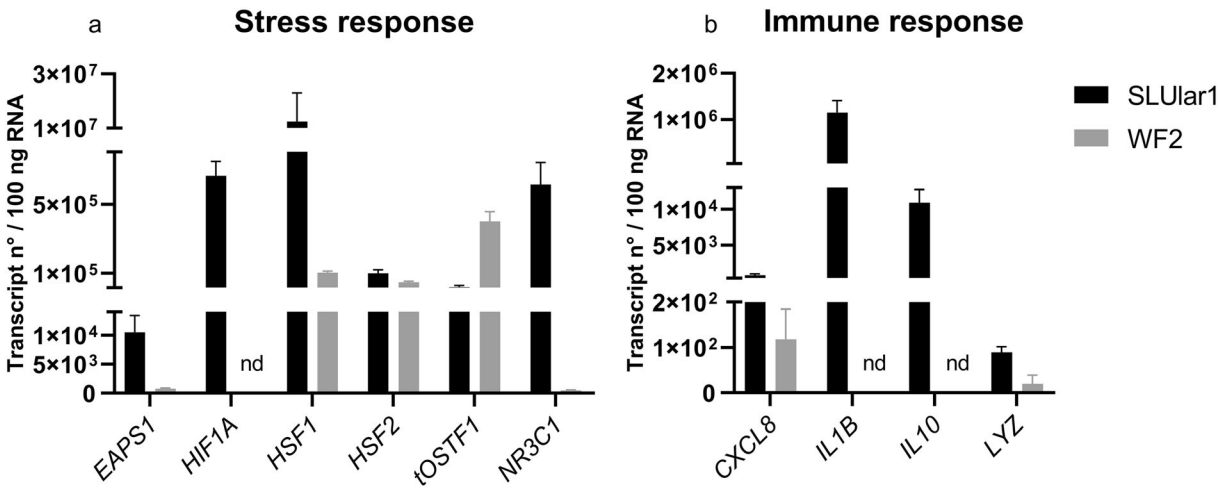


Fig. 3 Basal transcript levels of stress and immune marker in unchallenged cell models. Transcription patterns of genes important for stress (a) and immune response (b) in SLUlar1 (black columns) and WF2 cells (grey columns). Columns represent normalized means of three individual samples (+ SEM), calculated 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*	*LYZ*	*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.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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Study III: “The expression of myogenic gene markers during the embryo-larval-transition in pikeperch (*Sander lucioperca*)”

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Background and Aim: In this study, the developmental adaptation of myogenesis patterns was analysed. Bone and cartilage gene markers were also included, as muscle development is also influenced by the muscoskeletal system overall.

Materials and Methods: Eight age stages of pikeperches were obtained from a near-natural environment. The development around the embryonic to larval change was of focus. For RNA extraction, 15 specimen pools were utilised. Applying a nanofluidic qPCR method, the relative RNA expression of genes from the myogenic cascade, local muscle formation regulating genes, and skeletal and general developmental gene markers were analysed. The interconnection between the analysed markers was depicted using the IPA tool.

Results and Discussion: Three different phases of myogenic activity were found. A first occurs during early development coinciding with somitogenesis, a second occurs during embryonic stages, and a third occurs during larval development. Between the embryonic and larval stages, the muscle growth-inhibiting gene MSTN was expressed more dominantly. Gene expression for local muscle formation and skeletal gene marker expression coincided with related developmental processes.

Conclusion: The detected myogenic phases during pikeperch ontogeny occurred alongside previously described growth phases. Myogenic expression patterns differed between the phases, indicating the formation of fundamental muscle tissue before hatching and further muscle growth after hatching during the larval stages. Two phases of elevated skeletal gene expression were found, which concurred with the myogenic expression activities around hatching.

Supplemental in the appendix

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ORIGINAL ARTICLE

The expression of myogenic gene markers during the embryo-larval-transition in Pikeperch (*Sander lucioperca*)

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Abstract

Pikeperch (*Sander lucioperca*) has become a species of interest in aquaculture. It is a popular and economically valuable food fish and can produce high numbers of offspring. However, during early development, there are transition phases when high mortality rates concur with growth changes, vital organ transformations and a limited energy budget. Up to now, no study focused on the developmental adaption of muscle tissue in pikeperch, regardless of muscle tissue influencing essential traits such as locomotion and thus the competence to hunt prey and avoid predators. In the present study, therefore, the developmental myogenesis of pikeperch was analysed using specimens from early embryonic to larval development. Myogenic and developmental genes were utilized to gain insights into transcriptomic regulation during these stages by applying a nanofluidic qPCR approach. Result, three phases of myogenic gene expression, during somitogenesis, during the late embryonic development and during the larval development were detected. Increased myostatin expression showed an interim arrest of muscle formation between embryonic and larval myogenesis. Expression patterns of satellite cell gene markers indicated an accumulation of stem cells before myogenesis interruption. The here gained data will help to broaden the knowledge on percid myogenesis and can support pikeperch rearing in aquaculture.

KEYWORDS
Fluidigm array, gene expression, larval fish, ontogeny, Percidae, regulatory networks

1 | INTRODUCTION

Pikeperch is a new focus species in freshwater aquaculture. It has large economical potential as it already has a broad consumer base as well as the biological capability to produce large numbers of offspring (Lappalainen et al., 2003; Steenfeldt et al., 2015). Still, it is rare in aquaculture, as high mortality rates occur during its rearing and husbandry (Policar et al., 2019). This is especially severe during the early development between embryonic and larval ontogeny. During this period, the loss of around half or more of the reared specimens can occur, which highly affects the economic success of pikeperch aquaculture (Ostaszewska et al., 2005; Szkudlarek & Zakęś, 2007).

Several developmental changes occur during this time including late embryonic organ formation, hatching, the transition from endogenous to exogenous feeding and several organ changes connected to these (Bastl, 1978; Demska-Zakęś et al., 2003; Kimmel et al., 1995;

Ostaszewska, 2005). At the same time, the energy budget of fish larvae is generally restricted, which requires an adjustment of energy distribution (Rombough, 2011; Wieser, 1995). In pikeperch larvae, the present energy resource is especially sparse, leading to the death of larvae after overstepping a point of no return 1–2 days after the depletion of endogenous resources (Xu et al., 2017).

During the early development of fish and other vertebrates three phases of myogenic activity are present (Rowlerson & Veggetti, 2001; Steinbacher et al., 2007). In these early stages after hatching, muscle development is crucial for survival. In zebrafish (*Danio rerio*), variable swimming modes are already present in larval stages where they are applied as well for general locomotion as for capturing prey items (Budick & O'Malley, 2000). However, since the water resistance is different for smaller than for larger organisms, concurrent with smaller Reynolds numbers, the water becomes more viscous for smaller fish or developmental stages, and the energy expenditure is thus higher (Muller et al., 2000). This viscosity can lead to additional difficulties during development also for the young and small pikeperch larvae, causing locomotion to become crucial for survival. Thus, problems of, for example, hydrodynamic starvation during initial feeding events (China & Holzman, 2014) as well as difficulties with breaking through the water surface during swim bladder inflation (Blecha et al., 2019; Summerfelt, 2013) can occur occasionally during early development of pikeperch larvae. Up to now, no study was undertaken to analyse muscle development and its regulation during that time in pikeperch. To fill this gap, we conducted a gene expression analysis of near-wild reared pikeperch specimens from embryonic to larval development. We chose specimens from a wild population, which were reared under natural as possible conditions. While this involves unsteady environmental conditions (like water temperature), it also allows observing specimens free or otherwise present cultivation or breeding effects (Berg et al., 1990; Mas-Muñoz et al., 2013; Molnár et al., 2020), which possibly are present in aquaculture specimens.

Generally, muscle development relies on a cascade from myogenic progenitor cells derived from the dermomyotomal somite tissues, through myoblasts and myotubes, to the final myofibres (Chal & Pourquié, 2017; Cossu et al., 1995; Kato & Gurdon, 1993; Watabe, 1999). For vertebrates, different master genes are commonly associated with these steps. After the initial formation, further growth of muscle occurs via an increase in the number of cells (hyperplasia) or an enlargement of the cells (hypertrophia) in myofibres (Churova et al., 2017). Due to hyperplasia even in the adult stages, fish exhibit lifelong muscle growth, which differs from the other vertebrate groups (Rowe & Goldspink, 1969; Watabe, 1999).

In this study, the expression of selected master genes was analysed for the early developmental stages of pikeperch. Genes with a contribution to myogenesis in other teleosts were chosen (Watabe, 1999; Watabe, 2001). As this study focuses primarily on early ontogeny, we included genes that influence general development as well as genes that regulate local muscle formation (Degenhardt & Sassoon, 2001; Kimmel et al., 2001). As muscle localization during development is controlled by the musculoskeletal system (Sefton & Kardon, 2019), additional gene markers for collagenous (Gelse et al., 2003) and skeletal development (Gersch & Hadjiargyrou, 2009; Hadjiargyrou, 2018) were included. The goal of this study was to provide a basic expression profile of developmental genes to gain insight into early ontogeny, and in particular, the myogenesis of pikeperch.

2 | MATERIALS AND METHODS

2.1 | Specimen rearing and collection

Pikeperch specimens were gained from a wild population of parental animals from the Hohen Sprenz Lake in Mecklenburg Western-Pomerania, Germany. During spawning season, a group of eight females and three males were kept in a net cage of 4 m × 4 m × 2 m and fed with fish from the lake. Eggs were spawned on coco-mats and fertilized naturally. Afterwards, fertilized eggs were placed in Zuger-jars. Shortly before hatch, the specimens were transferred to a net cage of 1 m × 0.8 m × 0.8 m in size situated in a larger tank with 2500 L volume. A flow-through system provided natural water conditions including temperatures conforming to the lake during the whole period. The temperature was measured in the lake as well as in the flow-through system using temperature loggers (iButton MF1921G, Maxim Integrated) to control for similar temperature levels between the measuring points and to determine the developmental age in degree-days (dd). A spotlight lamp provided illumination with a 12:12 h day/night setting. Altogether, eight age stages were taken with embryonic stages at 1 dpf (26 dd), 3 dpf (53 dd), 5 dpf (80 dd), 8 dpf (127 dd) and 10 dpf (154 dd), and larval stages at 17 dpf (248 dd), 19 dpf (280 dd) and 22 dpf (325 dd). Embryonic and larval development were separated based on the start of external food ingestion (Peňáz, 2001). For each stage, three pools of 15 specimens were euthanized using MS222 (Serva) in a 0.25 g/L concentration. They were transferred to RNA-later (Thermo Fisher) and stored at −80°C until RNA extraction. To record developmental changes between the sampled stages, specimens were examined under the stereomicroscope (Leica SD9) and photographed using the Moticam 5.0 with associated Software (Motic, Motic Images Plus Version 3.0.). This study followed international, national and institutional guidelines for animal treatment and sacrifice and complied with Directive 2010/63/EU and the German Animal Welfare Act [§ 4(3) TierSchG].

2.2 | Selection of genes and primer design

Altogether, 19 genes were selected for analysis (Table 1). The focus was placed on genes regulating myogenesis with the transformation of myogenic progenitor cells to myoblasts (myogenic factor 5, myogenic differentiation 1, paired box 3, paired box 7a and msh homeobox 1), the formation of myotubes (myogenin, myocyte enhance factor 2a, insulin-like growth factor 1, insulin-like growth factor 2 and transforming growth factor beta 1) and muscle fibre

TABLE 1 Overview of selected genes and primers for Fluidigm qPCR analysis

Gene symbol	For/Rev sequence (5′- 3′)	Accession no./source	Product length [bp]
Target genes			
BMP4	CCGTAAACGCAACCGCAACTG AGACCGCCTAGTAGACTTGAGT	Schäfer et al. (2021)	151
COL1A1	TCCGCTGGCAACCTCAAGAAG TGATGGGCAGGCGAGATGTTTT	JN112557.1	181
COL1A2	AAGAGGCCAACCCGGAAACATT AGACCAGTGGGACCAGTGGAT	XM_028596714.1	99
EN2	CTCAAACCCAGCCCAGTCTCA CGTCCGTGGTCGCTTGTCCT	XM_028568870.1	142
IGF1	TGTGTGGAGAGAGAGGCTTTTAT AGCGAGCAGCCTTGCTAGTCT	XM_031313442.1	158
IGF2	GAGGCTTCTATTTCAGGTAGGC ACGGGTATGACCTGCAGAGAG	XM_031282902.1	162
MSX1	CGAGAGCCCCGACAGTAATGA GGGGTCCGGGGCTTTCTGTT	XM_028576570.1	132
MUSTN1	TATTGCCCCAAAGCGCTACAAG TTGATGGGCGACATCTTGGTAG	Swirplies et al. (2019)	153
MEF2A	AGAGTGCGGGAAGAAATCGTGT ACGCTCAGCTCATAGGCCTTTT	XM_028584207.1	182
MYOD1	CTCCGACGGCATGACGGATTT GAGATCCGCTCCACAATGCTG	XM_028584397.1	165
MYF5	GTGGAAAACTACTACGGCCTAC TCGTTCCTCGCATATGAATAACC	HM190249.1	152
MRF4	TGATGGGCTTATGTGGGAGTCT CTCTCCGACGGTCCATGGTAA	HM190251.1	177
MYOG	TTCTACGAGGGAGGGGACAG GAACTGGGCTCACTTGACGA	XM_031323467	512
MYH6	CTTGCTGGAGAAGTCACGGT CGAGAGCGCCAAGAGAGTAG	HM050076	485
MSTN	ACTGGGGCATCGAGATCAACG TTGGGGCCCTCTGAGATCTTAA	XM_028591409.1	121
PAX3	ACCCACGCTGGCTCAGAACTA CTCCACGATTTTATGTCGGATGT	XM_031283761.1	145
PAX7A	ACTGCGAGTTTCTCACGGTT ATGGAGCTCACAGATGAAGCC	XM_031302059.1	252
PGC1A	CCGTTTGAGAACAAAACCATTGAA TGACCGATGCTTTGAAAGGATTC	XM_028564706.1	124
TGFB1	GCGTGAGGAGCTTGTACATCG TGATGCTGGTACAGGGCCAAAA	XM_031285154	157
Reference genes			
EEF1A1	ATGGACAGACCCGTGAGCATG TTCTTGATGTAGGTGCTCACTTC	Swirplies et al. (2019)	151
RPL32	GGCGTAAACCCAGAGGTATTGA ACCTCGAGCTCCTTGACATTGT	Swirplies et al. (2019)	157
RPS5	GCAGGATTACATTGCTGTGAAAG TCATCAGCTTCTTGCCATTGTTG	Swirplies et al. (2019)	161

development (myogenic regulatory factor 4 and myostatin). Further, genes regulating regional specific myogenesis (engrailed 2 and myosin heavy chain 6), structural tissues formation (collagen type 1 alpha 1, collagen type 1 alpha 2, bone morphogenetic protein 4 and musculoskeletal embryonic nuclear protein 1) and energy metabolism (peroxisome proliferative activated receptor gamma coactivator 1 alpha) were chosen. Following the MIQE Guidelines (Bustin et al., 2009), three reference genes (elongation factor 1 alpha, ribosomal protein L32 and ribosomal protein S5) were chosen and validated to allow normalizing the expression data (Pfaffl, 2001; Swirplies et al., 2019).

For primer design, sequences were obtained from the NCBI GenBank database. If no pikeperch gene sequences (RefSeq NCBI: GCA_008315115) were available, sequences from other perciform species (*Perca flavaescens* and *Epinephelus coioides*) were chosen and their homology was checked using BLAST algorithms against the genome of *Sander lucioperca* (RefSeq NCBI: GCA_008315115; PMID: 31540274/DOI: 10.3390/genes10090708). The oligonucleotide primer sequences (Table 1) were designed using the software PSQ-Assay Design (Version 1.0.6, Biotage) and synthesized by SIGMA–Aldrich (Merck). Primers were evaluated by performing a standard PCR, gel electrophoresis, as well as qPCR on a LightCycler96 instrument (Roche) to ensure the formation of single products and to prevent primer dimer-formation.

2.3 | Extraction of RNA and RT-qPCR

For RNA extraction, pools of 15 specimens each were mechanically homogenized using Precellys Evolution (VWR) followed by Trizol-Chloroform precipitation. Total RNA was further purified using the RNeasy Micro Kit (Qiagen) combined with an RNase-free DNase Set (Qiagen) according to the manufacturer's instructions. The concentration of RNA was determined using the 260/280 nm absorbance ratio obtained with the NanoDrop ND-1000 spectrophotometer (Peqlab). Until further measurements, the RNA was stored at −80°C.

Transcriptional analysis was conducted using a quantitative real-time PCR based on a microfluid circuit system (Biomark, FLUIDIGM) using EvaGreen fluorescence dyes (Bio-Rad). The protocol was described in detail by Rebl et al. (2019). The design of Fluidigm 48.48 Dynamic Array IFC allows the parallel quantification of 48 transcripts in 48 samples each. Three biological replicates were assessed in a form of two technical replicates each. The raw cycle thresholds (CT-values) were retrieved using the real-time PCR analysis software (Fluidigm, Version 3.0.2).

2.4 | Data analysis and graph design

The resulting CT-values were used as a base for a relative quantification established by Pfaffl (2001) assuming a constant efficiency (E = 2.0) for all sequences. The values obtained from all examined stages were converted to fold changes (FC) to the expression of the first age stage. Consequently, the gene-expression data during the first stage was set at one. Based on the obtained relative ratios of the age stages, three biological replicates and their respective geometric mean data were analysed using R (Version 4.0.3). Statistically significant differences between the expression of all examined stages were determined by conducting an ANOVA followed by a Tukey-test ($p = 0.05$) with the library stats (Version 4.0.3). Graphs were designed by applying the ggplot2 library (Version 3.3.2). Correlation between gene expressions of embryonic and larval stages was checked using corrplot library (Version 0.84; provided as supplemental only, Tables S1 and S2). The depiction of the expression networks in comparison to the first age stage (26 dd) was generated using Ingenuity Pathway Analysis (IPA) Software (Qiagen, Release March 2021).

3 | RESULTS

3.1 | Development of the analysed stages

The earliest obtained embryonic stage, taken at 1 day post fertilization (dpf) with an age of 26 dd, progressed during the latest stages of gastrulation up to early segmentation. The following embryonic stages at 3 dpf (53 dd) had an increasing number of somites (Figure 1a), the eye anlagen became visible, the heart started to beat and the tail detached from the yolk (Figure 1b). At 8 and 10 dpf (127 and 154 dd) some first prehatchlings occurred. At this phase, the pectoral fin bud formed and subsequently started to shift laterventrally. The head, which was previously bent to the yolk, began to straighten up. The eyes were fully pigmented and the mandibular was positioned ventral to the eyes. The first larval stage at the 17 dpf stage (248 dd) had an open mouth and ingested the first food particles (Figure 1c). Additionally, the pectoral fins were well developed and arrived in their final body position (Figure 1c). In the 19 dpf stage (280 dd), the yolk was depleted in most specimens, but in a few specimens oil droplet remnants remained present. Simultaneously, the stomach was now distinguishable from the lower intestines. At 22 dpf (325 dd), all specimens had switched to solely exogenous nutrition (Figure 1d). The formation of skeletal elements progressed further (Figure 1d,e).

3.2 | Activation of myogenic progenitor cells and stem cell regulation

The expression of the genes associated with muscle stem cells and satellite cells was not uniform across the different age groups (Figure 2). However, myogenic differentiation 1 (MYOD1, Figure 2a) and myogenic factor 5 (MYF5, Figure 2b) followed similar expression patterns. A maximum expression was present at 53 dd. The following lower expression levels were interrupted by a slightly increased expression from 127 to 154 dd and again at 280 dd. The paired box

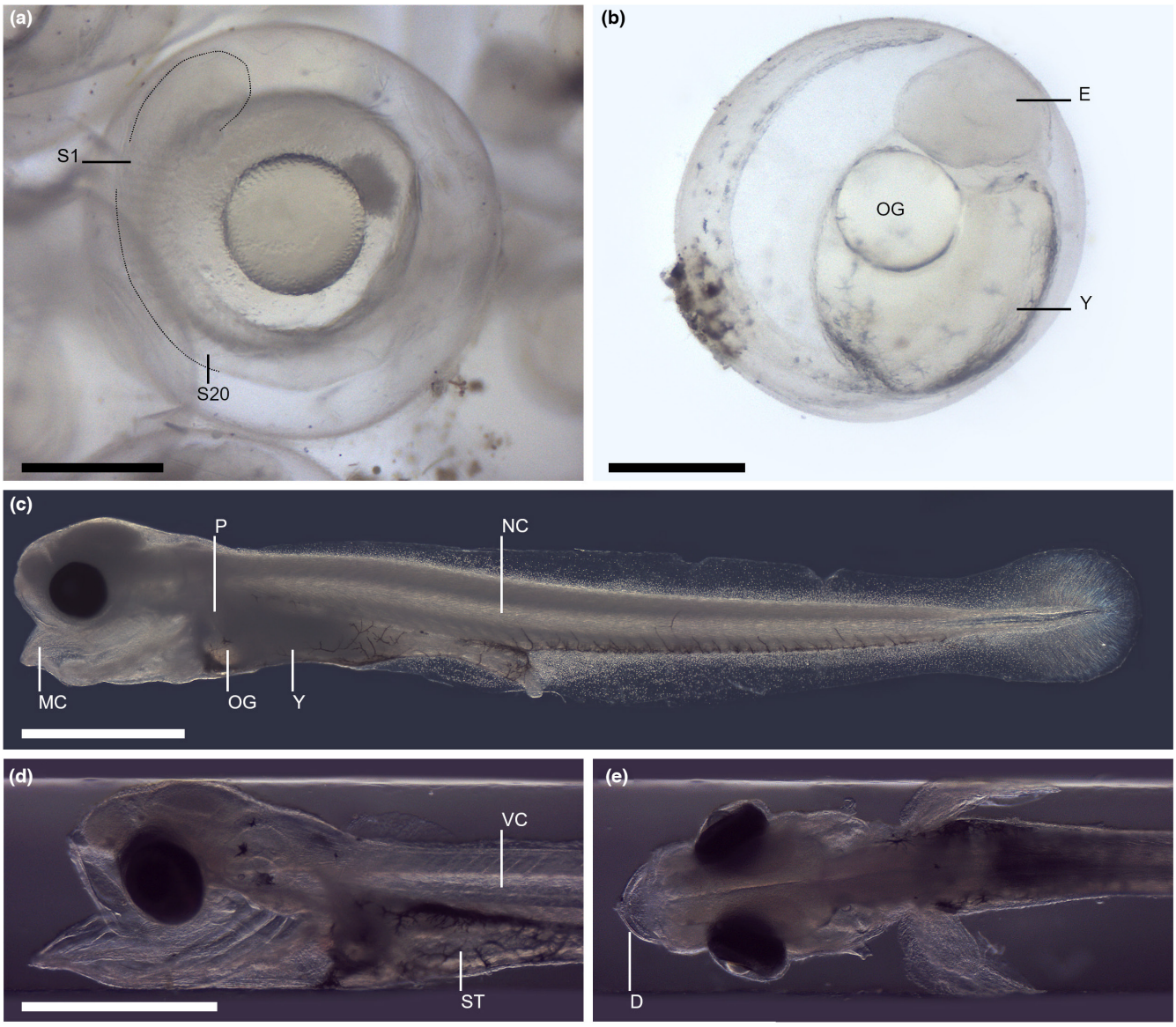

FIGURE 1 Light microscopy images of selected developmental stages of pikeperch. (a) 3 dpf/53 dd stage, the embryonic stage during somitogenesis with 20 visible somites, scale = 0.5 mm. (b) 5 dpf/80 dd stage, embryonic specimen showing early eye anlagen and a tail separated from the yolk, scale = 0.5 mm. (c) 17 dpf/248 dd stage, larval specimen during the onset of exogenous feeding. Scale = 1 mm. (d, e) 22 dpf/325 dd stage, lateral (d) and dorsal (e) anterior body of a larval specimen after the completed transition to exogenous feeding, oblique light applied to emphasize internal morphology. Scale = 1 mm. D, dental ossification; E, eye anlage; MC, meckels cartilage; NC, notochord; OG, oil globule; P, pectoral fin; S, somite; ST, stomach; VC, vertebral centrum ossification; Y, yolk.

genes (PAX3, PAX7A, Figure 2c,d) had both lowered expression at 248 dd and their maximum at the latest stage at 325 dd. The expression of msh homeobox 1 (MSX1, Figure 2e) was at its highest level during the first examined age at 28 dd. It decreased significantly afterwards, reaching a steady low expression level from stage 80 dd on, which was solely interrupted by a significantly increased expression at 280 dd. Peroxisome proliferative activated receptor gamma coactivator 1 alpha (PGC1A, Figure 2f) had a low-level expression during the earlier stages until 127 dd and as well at 248 dd. At 154 dd, and from 280 dd onward, the expression was significantly higher with around 2.3–2.6 FC.

3.3 | Proliferation of myoblasts, myotube and muscle fibre formation

The expression of myogenin (MYOG, Figure 3a) fluctuated modestly between an FC of 1.0 and 3.5 but had no significant expression changes overall. The myocyte enhancer factor 2A (MEF2A, Figure 3b) expression remained steady until 80 dd. Two significant increases between 80 dd and 154 dd lead to an FC of 5.0, which remained steady afterwards, except for a single intermediate drop at 248 dd. The expression of the insulin-like growth factor genes (IGF1, IGF2, Figure 3c,d) increased during the progressive ontogeny,

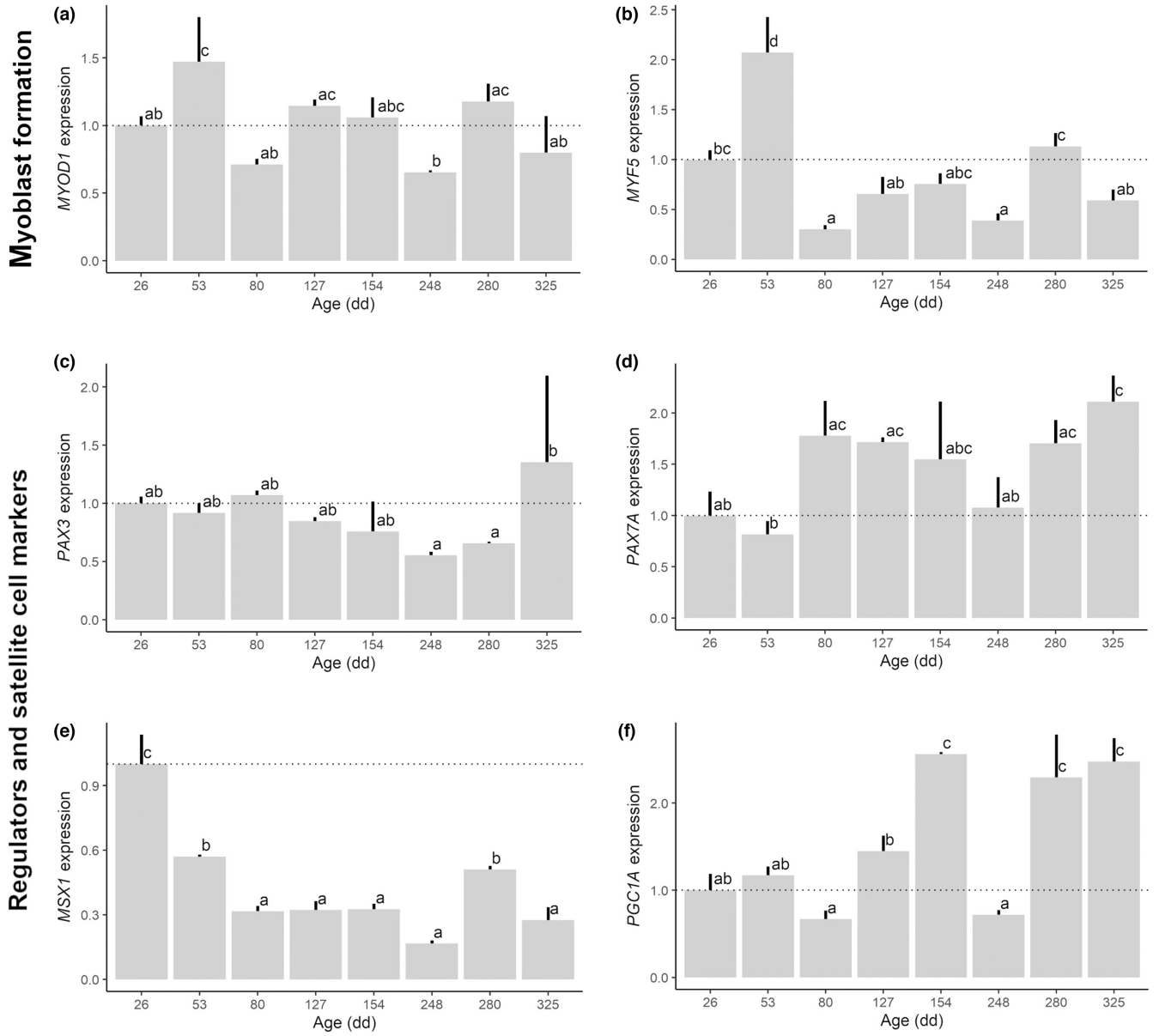

FIGURE 2 Relative expression patterns of genes regulating muscle stem cells and the formation of myogenic progenitor cells in pikeperch. (a) MYOD1. (b) MYF5. (c) PAX3. (d) PAX7A. (e) MSX1. (f) PGC1A. The dotted line indicates an FC of 1, significant differences are shown by groupwise letters given for $P \leq 0.05$.

leading to a maximum at the last examined stage. Compared with IGF2, IGF1 were more uniformly expressed during the observed stages. Transforming growth factor-beta 1 (TGFB1, Figure 3e) had an increased expression over time leading to a maximum of 4.5 FC in the latest studied stage. In between, a slight decrease occurred at 248 dd.

The expression pattern of myogenic regulatory factor 4 (MRF4, Figure 3f) was raised significantly three times during ontogeny. The first time at 53 dd had the overall highest FC of 2.4. Further, similar increases were present from 127 dd to 154 dd, and at 280 dd. Myostatin (MSTN, Figure 3g) expression did not change significantly during the first 4 examined age stages. Afterwards, expression increased to more than 10-fold expression at 248 dd, followed by a significant increase at 280 dd to a maximum of 32.4 FC. The latest stage decreased significantly again, reaching expression levels comparable to the ones measured at 248 dd.

3.4 | Regional muscle markers and structural tissue regulating genes

The expression of the cardiac-specific myosin heavy chain 6 (MYH6, Figure 4a) was elevated mainly during the earlier stages. Hereby, a maximum occurred at 80 dd. Following this maximum, the levels decreased significantly only showing an intermediately higher expression again at 248 dd. The expression of the

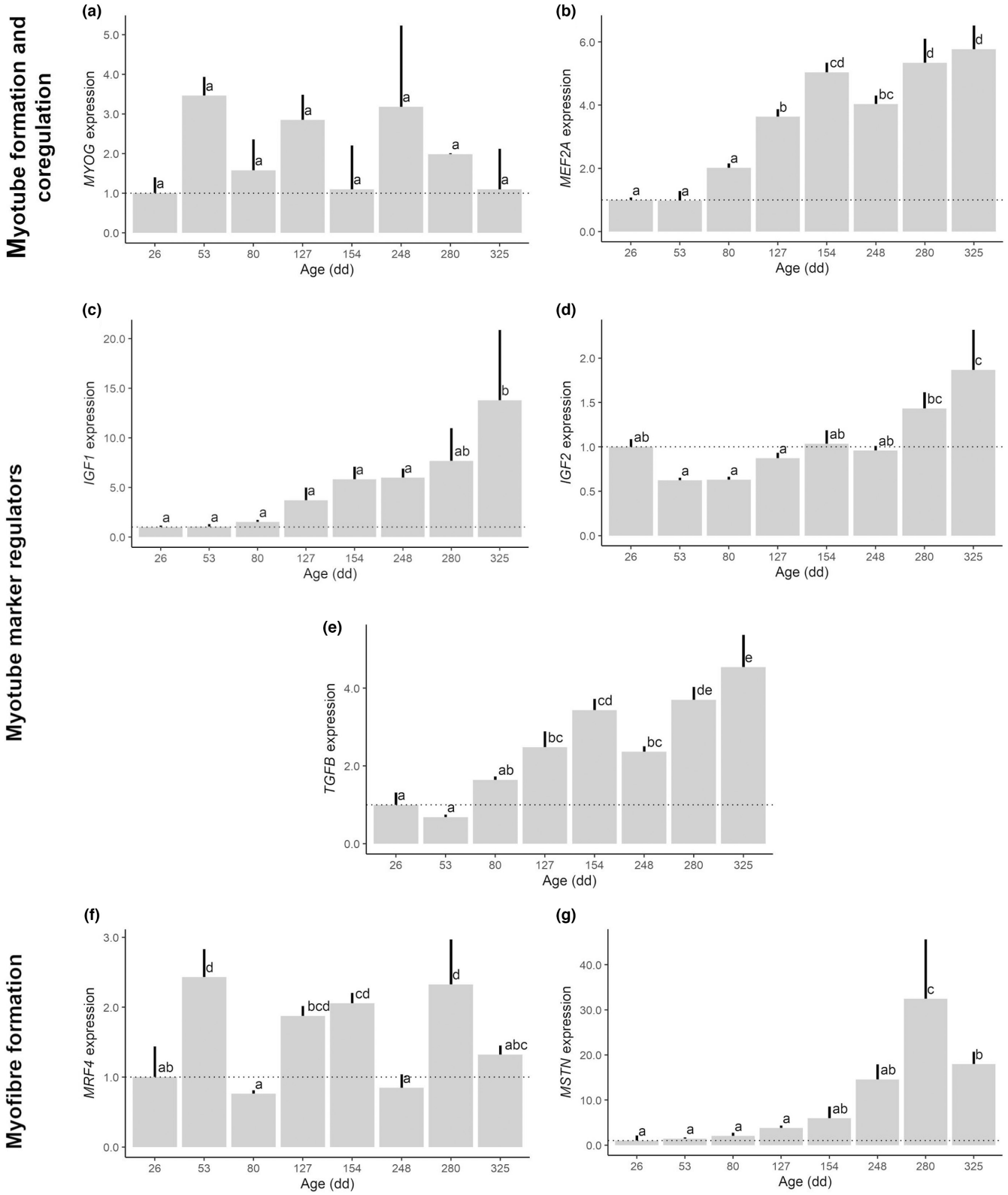

FIGURE 3 Relative expression patterns of genes associated with the proliferation of myoblasts and the formation of myotubes and myofibres in pikeperch. (a) MYOG. (b) MEF2A. (c) IGF1. (d) IGF2. (e) TGFB1. (f) MRF4. (g) MSTN. The dotted line indicates an FC of 1, significant differences are shown by groupwise letters given for $p \leq 0.05$.

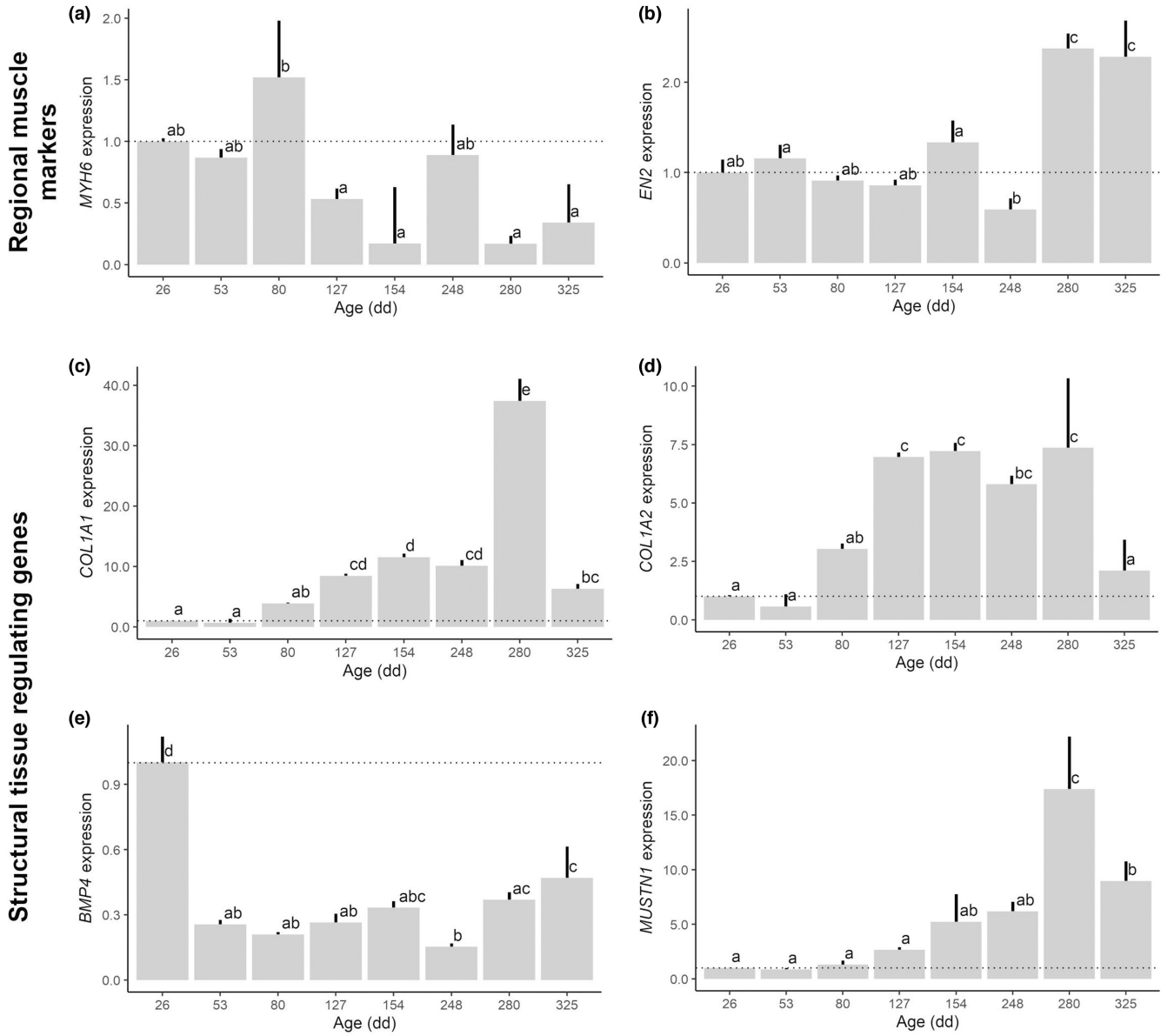

FIGURE 4 Relative expression patterns of genes controlling regional muscle development and structural tissue growth in pikeperch. (a) MYH6. (b) EN2. (c, d) COL1A1 and COL1A2. (e) BMP4. (f) MUSTN1. The dotted line indicates an FC of 1, significant differences are shown by groupwise letters given for $p \leq 0.05$.

mandibular arch-muscle specific engrailed 2 (EN2, Figure 4b) remained constant until 154 dd. A subsequent significant decrease in expression at 248 dd was followed by a significant increase at 280 dd to a steady level during the last two stages observed.

The two analysed collagen 1 alpha variants (COL1A1, COL1A2; Figure 4c,d) had overall similar expression patterns. Apart from the expression levels at 280 dd, which represent the maximum for both gene variants, the expressions correspond to a bell-shaped pattern, with a steady increase until stage 154 dd and a steadily decreasing expression in the following stages. For COL1A1, the change in expression level was more pronounced reaching 11.5 FC at the vertex and 37.4 FC at 280 dd.

The expression pattern of the gene coding for bone morphogenetic protein 4 (BMP4, Figure 4e) had its peak in the first stage. Afterwards, lower expression occurred with a mean FC of 0.3, a minimum of 0.2 FC at 248 dd, and a subsequent renewed increase until the latest stage. The musculoskeletal embryonic nuclear protein 1 (MUSTN1, Figure 4f) was expressed sparsely until 127 dd. From 154 dd, increasing expression levels occurred with a prominent maximum of 17.4 FC at 280 dd.

3.5 | Gene network

We used the IPA tool to illustrate linkages of analysed myogenic genes (Figure 5). Additional genes or regulators, next to the here

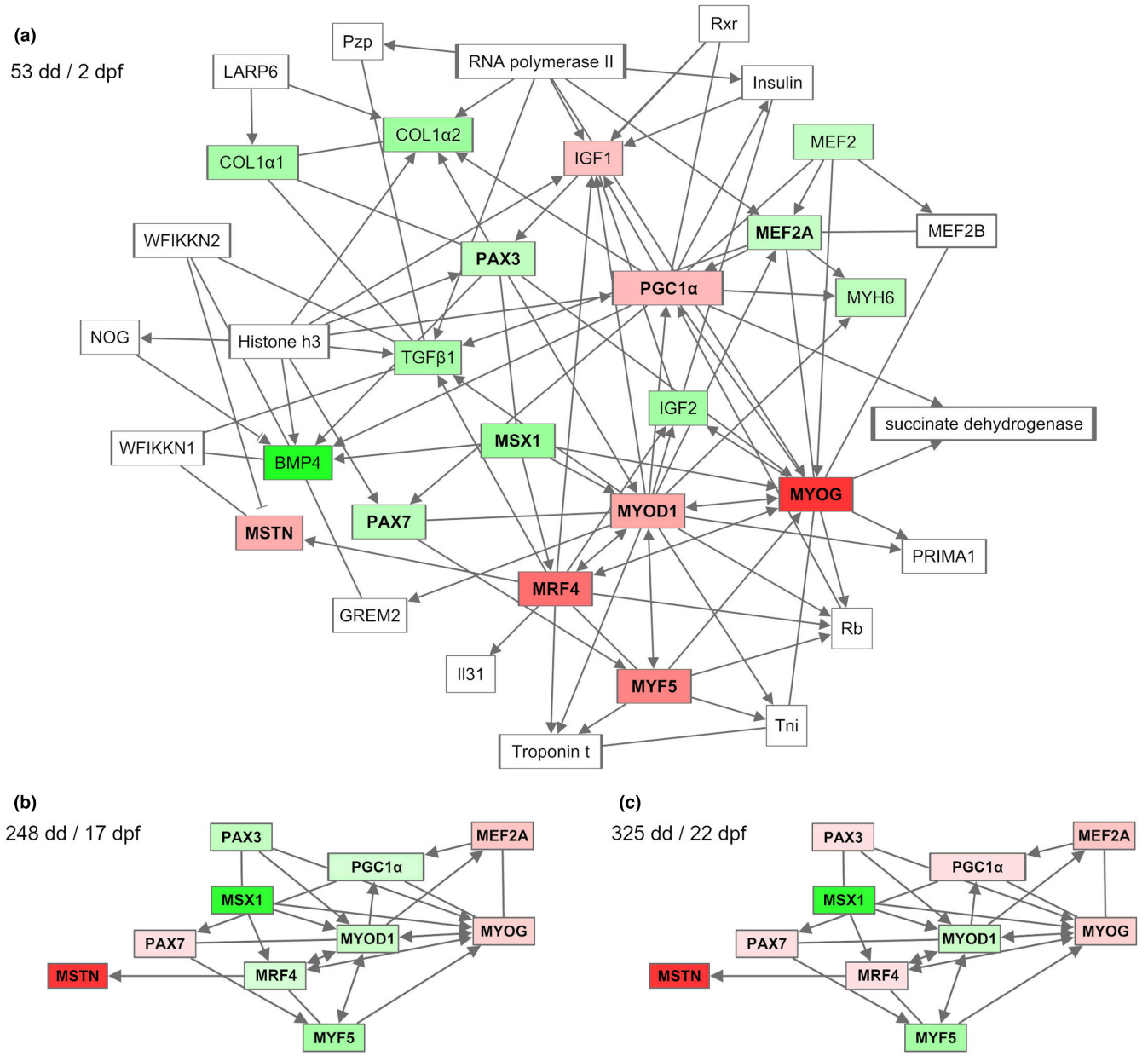

FIGURE 5 Network of myogenesis-associated genes displayed with the IPA-tool. Expression levels for pikeperch are included and given in relation to the first stage at 26 dd/1 dpf. Age is given in degree-days (dd) and days post fertilization (dpf). Interactions are depicted as arrows. Green labels show downregulation and red labels upregulations. (a) Myogenesis associated network for the 53 dd stage. (b, c) Section of the myogenesis associated network for 248 dd (b) and 325 dd (c). Full networks for 248 dd and 325 dd are added as supplemental (Figures S1–S3).

analysed ones, were given as output based on connections from the underlying IPA database (Release March 2021). Hereby, differences between the software-included genes were present in between the stages, especially for genes whose products have a plasma membrane localization (Figures S1–S3). At the same time, similarly connected genes were found between all stages, mainly in relation to gene expression and, in addition, to membrane transport and proteases (Figures S1–S3). This provides further genes that may be relevant for the here investigated regulatory and developmental patterns (Figure 5a; Figures S1–S3).

4 | DISCUSSION

4.1 | Activation of progenitor cells and myoblast formation

The main actors of the muscle progenitor activation and the resulting formation of myoblasts are MYOD and MYF5 (Berkes & Tapscott, 2005; Rudnicki et al., 1993; Rudnicki & Jaenisch, 1995; Tapscott, 2005; Ustanina et al., 2007; Watabe, 1999; Zhu et al., 2016). These genes are commonly regulated positively by PAX3 and PAX7

and negatively by MSX1 (Bendall et al., 1999; Buckingham, 2007; Buckingham & Relaix, 2007; Koganti et al., 2020; Song et al., 1992). In pikeperch, MYOD1 and MYF5 are positively correlated in embryonic and larval stages. Their elevated expressions coincide with somitogenesis and the phases of muscle growth before and after hatching. This supports the common coregulation and joint control of myogenic progenitor cells by MYOD1 and MYF5 in pikeperch. However, the IPA network shows that they are differentially regulated, as also inferred from the correlation of MSX1 and PAX7A expression with MYF5 but not with MYOD1. One reason for these differences could be an earlier onset in MYF5 expression compared with MYOD expression, as shown for common carp (*Cyprinus carpio*) (Kobiyama et al., 1998).

Here, MSX1 was found to negatively regulate myogenesis in pikeperch. Phases of elevated MSX1 expression coincided with times of low myogenic activity. The expression of the PAX genes differed in pikeperch, indicating varying roles during development. PAX3 had a constant expression level throughout embryonic development, whereas PAX7A expression showed significant differences between the stages. Since we analysed the whole specimen in this study, a clear statement on the influence on muscle development is not possible here.

4.2 | Satellite cell regulation

We found possible indications for satellite cell formation before hatch and during the later larval stages in pikeperch. As a marker for these quiescent muscle stem cells (Devoto et al., 2006; Jiao et al., 2015; Koganti et al., 2020; Nord et al., 2021; Relaix, Montarras, et al., 2005; Relaix, Rocancourt, et al., 2005), PAX7A showed elevated expression levels during these times. The additionally increased expression during the late embryonic development is of interest, as it may indicate the initial formation of satellite cells before the energy-demanding early larval stages and the subsequent proliferation of muscle tissue. Spatial expression data are required to fully clarify this finding. Control of energy metabolism that includes PGC1A represents a further mechanism regulating muscle growth and regeneration (Williamson et al., 2009). However, the functions of PGC1A gene variants were found to differ, at least partially, in fish (LeMoine et al., 2008). Thus, its connection to satellite cell formation remains ambiguous. Consequently, PGC1A itself remains useful as the regulator of the energy metabolism (Liang & Ward, 2006; Peng et al., 2017), by highlighting phases of elevated energy expenditure. For this reason, the significantly reduced expression at 248 dd is particularly interesting.

4.3 | Myotube and muscle fibre formation and regulation

MYOG is the key regulator in the formation of myotubes (Devoto et al., 2006; Hasty et al., 1993; Nabeshima et al., 1993). In pikeperch, it was expressed in a fluctuating wave-like pattern, which we attribute to three phases of myogenesis during pikeperch ontogeny.

The formation of muscle fibres out of single myotubes is promoted by MEF2A and MRF4 (Hinits et al., 2009; Hinits & Hughes, 2007; Moretti et al., 2016). The common reciprocal coregulation of MEF2A with MYOG (Watabe, 1999; Watabe, 2001) was not clearly supported by the obtained data. Still, the overall expression of MEF2A followed the increase in muscle tissues, confirming its role in muscle fibre formation. MRF4 expression resembled that of MYOG with three elevated phases, during somite formation, before hatch and during larval stages. The elevated expression at 53 dd implies some influence on somite formation, which would contradict previous work on *D. rerio* (Hinits et al., 2009). However, an initiating effect of MRF4 on somite myogenesis is supported by studies on mice (Kassar-Duchossoy et al., 2004).

While both IGFs seem necessary for overall muscle growth in pikeperch, their incorporation into myogenesis differs. IGF1 and IGF2 control the increase in muscle fibre mass (Barton-Davis et al., 1999; Fuentes et al., 2013; Fuentes et al., 2014; Johnston, 2006; Wood et al., 2005). For pikeperch, IGF1 and IGF2 expression correlated with one another during the embryonic and even more during the larval phase. Still, during the larval stages, only IGF1 had a positive correlation with MYOD1 and a negative correlation with MYOG. This pattern could be attributed to different developmental tasks (Fuentes et al., 2013; Wood et al., 2005), and differential association with overall growth (Fuentes et al., 2013; Mingarro et al., 2002), which becomes more dominant during larval stages.

The role of TGFB1 in pikeperch myogenesis remains ambiguous, but a connection to fibre type differentiation might is justifiable. In previous studies, TGFB1 inhibited myogenesis in chickens (Li & Velleman, 2009), but in rodents, a connection to the differentiation of fast muscle fibres was found (McLennan, 1993; McLennan et al., 2000; Noirez et al., 2006). Linked to fibre type differentiation, TGFB1 supports the formation of extracellular matrix proteins like collagen (Herpin et al., 2004; Schmidt et al., 2016; Vial et al., 2008). In pikeperch, TGFB1 expression coincided with the late embryonic and larval phase of myogenesis, possibly negating an inhibiting effect on myogenic activity. The strong correlations of TGFB1 with both analysed COL1A variants during embryonic stages support a connection to collagen formation. Nevertheless, as TGFB1 influences various developmental processes (Grafe et al., 2018; Massagué, 2012; Massagué & Xi, 2012; Zinski et al., 2018) no clear statement can be given here and further research remains necessary. In contrast to TGFB1, MSTN is clearly linked to myogenesis as a negative key regulator (Gabillard et al., 2013; Østbye et al., 2007; Rodgers et al., 2001; Rodgers & Garikipati, 2008). This can be concluded for pikeperch as well, as the MSTN expression maximum was situated in a growth gap, between the late embryonic and larval myogenesis phase. Hereby, a general increase in expression with age could be attributed to growth regulation and homeostasis, as also shown in tilapia and white bass (Rodgers & Garikipati, 2008).

4.4 | Regional muscle markers

In this study, two regional muscle markers MYH6 and EN2 were analysed, both of which can be attributed to local muscle formation

in pikeperch. MYH6 is specifically expressed in the heart muscle cells (Schiaffino, 2018; Singleman & Holtzman, 2012). In the gill arches, EN2 regulates the formation of muscles associated with the first visceral arch that forms the jaw elements (Degenhardt & Sassoon, 2001; Yokoyama et al., 2021). In our study, the expression maxima of MYH6 coincides with the observed formation of the heart and takes place shortly before the first heartbeats. The further increased expression occurs after hatching and coincides with the switch to gill respiration, which is accompanied by another increase in myocardial tissue and a combined increase in the circulatory system (Grimes & Kirby, 2009). EN2s expression maxima coincided with the point of full exogenous feeding, which was coupled with increased jaw development. Additionally, a first minor elevated expression was detected in the specimens at 154 dd shortly before hatching. This increase could additionally be influenced by the formation of slow muscle tissue, which is known to also express EN2 at higher levels (Du et al., 1997; Hammond et al., 2009; Hatta et al., 1991). Consistent with studies in zebrafish, this slow muscle tissue is known to form during embryogenesis, where they are required for initial post-hatching movements (te Kronnié, 2000).

4.5 | Markers for skeletal development

In our specimens, skeletal growth generally matched with the pikeperch skeletal development described by Löffler et al. (2008) and Ott et al. (2012). In the embryonic stages before hatching, fundamental cartilages with single ossifications were present. During larval stages, further skeletal elements formed and ossification considerably increased. The expression of the studied skeletal markers coincided with times of skeletal development and demonstrated differences between embryonic and larval skeletogenesis. Since muscles and bones develop partially codependently together as elements of the musculoskeletal system (Sefton & Kardon, 2019), these two skeletal growth phases could be linked to muscle development.

For the COL1A variants, the bell-shaped expression pattern in pikeperch could be linked to embryonic cartilage formation (Gelse et al., 2003; Min et al., 2021; Watson et al., 2017). An additional peak at 280 dd matched with increased skeletal development during this time. The transformation of intestinal tissues during the change to exogenous feeding shortly after hatching could further influence this peak value (Ostaszewska, 2005), as it also involves the formation of collagenous matrices (Nakase et al., 2006). Together with COL1A, the skeletal marker MUSTN1 (Hadjiargyrou, 2018; Lombardo et al., 2004) showed significant expression maxima during the increasing skeletal growth and ossification at 280 dd.

In contrast, BMP4 cannot be associated only with skeletal development. While it has been found to influence intermuscular ossification (Su & Dong, 2018) and bone repair (Rosen & Thies, 1992), it was also linked to other developmental processes (Hwang et al., 1997), including tooth development (Bei & Maas, 1998), inhibition of muscle pioneer cells (Du et al., 1997) and dorsoventral patterning (Stickney et al., 2007). The highest expression of BMP4 occurred during the earliest studied stage, while the expression in pikeperch otherwise correlated with age. This maximum could highlight a foremost attribution to general developmental processes in pikeperch.

5 | CONCLUSIONS

In this study on pikeperch myogenesis during early development, the three phases of myogenic activity were found to be chronologically adapted to the change from endogenous to exogenous feeding shortly after hatch. After a first myogenic phase during somitogenesis, a second occurs in embryos shortly before hatch (127–154 dd) and a third starts at around 280 dd during larval development. Additionally, skeletal gene marker expression was found to coincide with the second and third phases of myogenic gene expression.

The increased expression of some muscle gene markers in pikeperch indicates that the muscle tissue fundamental for initial locomotion is already formed before the embryos hatch. Between embryonic and larval development, an interruption in muscle development occurs, as evidenced by decreased expression of myogenesis marker genes. Additionally, two different phases of skeletal growth were supported by our expression analysis. A first phase is present before hatching, in which precursor cartilages are formed, and a second phase after hatching, in which ossification processes are enhanced.

In this fundamental study, gene expression was analysed using the whole specimen. To clarify the exact spatial expression and analyse the resulting phenotypic changes during myogenesis, follow-up studies are needed. Nevertheless, this study provides the first insight into the regulation of myogenic genes in near-natural raised pikeperch, which is important for comparisons with aquaculture-reared pikeperch.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are contained in the article or are available as supplementary material.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Study IV: “Konzeption eines Prototyps zur Videoüberwachung von Zanderlarven”

[“Conceptual design of a prototype for the video monitoring of pikeperch larvae”]

Mitteilungen der Landesforschungsanstalt für Landwirtschaft und Fischerei 63, 134-142, 2021 (non-peer reviewed)

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Background and Aim: To support rearing procedures in the aquaculture industry, a prototype system was developed to monitor early larval pikeperches. For this purpose, a video system combined with software for automatised counting, tracking, and measuring was designed on a laboratory scale. First, pikeperch were analysed using this setup during a two-week trial observation, and the number of larvae was determined by applying the software algorithms.

Materials and Methods: For the trial phase, a small aquarium inside a climate chamber was equipped with three GoPro cameras. Shortly before their hatch, two test series of pikeperch specimens were obtained from a hatchery at Lake Hohensprenz and transferred to the aquarium. The specimens were fed microworms. To reduce stress on the specimens, the aquarium was supplied with lake water, and the temperature was set to the before-measured temperatures of the lake. A programme for processing the video frames, as well as a neuronal net for detecting specimens in the frames, was designed using Jupyter Notebook with the OpenCV Python library and Tensorflow.

Results and Discussion: After the first test series was utilised to establish the methods and train the neuronal net, the second series of specimens was analysed. Over the course of 12 days, 10-minute video sequences were taken for each day at the same time. Altogether, over 50,000 frames were analysed using the software. Video frame resolution proved to be sufficient in the lower tested settings. Time lapse has been shown to require a minimum of at least 15 frames per second to allow the algorithm to function properly.

Conclusion: The study provided a basic setup for counting less than five millimetres of small pikeperch larvae. Further improvement of the neuronal net and more light sensitive cameras and were found to possibly be beneficial for following setups. Based on existing software parameters, the measurement of specimens can be included in future versions.

Konzeption eines Prototyps zur Videoüberwachung von Zanderlarven

Digitalisation of pikeperch raise

George P. Franz[1,2], Lars Lewerentz[1,3], Prof. Dr. Ralf Schneider[3], Dr. Bianka Grunow[1]

Abstract: *Pikeperch (Sander lucioperca) has become a new target species for the aquaculture industry. Nonetheless, rearing remains complicated as high mortality rates occur during the early ontogenetic stages. To facilitate larvae observation and thus reduce mortality, the development of a video observation system is presented here. The goal is to combine counting, tracking and measuring of pikeperch larvae using image analysis software and further developed video analysis algorithms. Based on the data gained hereby, the determination of appropriate times for size sorting will be improved, which will reduce cannibalism between specimens. A first series of pikeperch larvae were analyzed during a two weeks observation. For these, the number of larvae were extracted automatically using the video analysis algorithms.*

Einleitung

Um sowohl die ökonomische als auch die ökologische Situation der Aquakultur zu verbessern, ist ein Ziel, die in der Industrie genutzte Artenbreite weiter zu diversifizieren. Insbesondere der Zander hat sich dabei als idealer Kandidat hervorgetan, da dieser bereits eine hohe Marktakzeptanz erzielt und sein Fleisch im Vergleich zu anderen Fischarten besondere Eigenschaften aufweist (KOMOLKA et al., 2020).

Um zudem die Nachfrage an Satzfischen zu erfüllen, sind Verbesserungen in der Aufzucht der Tiere erforderlich. Insbesondere die frühe Entwicklung vom Schlupf bis zu den Larvenstadien ist hierbei problematisch, da diese durch eine hohe Sterblichkeitsrate gekennzeichnet ist (POLICAR et al., 2019; SCHMIDT und KÜHN, 2013). Neben dem energetisch kostenintensiven Schlupf und der Umstellung auf externe Nahrungsquellen führt besonders der Kannibalismus zwischen den Jungtieren zu hohen Verlusten (COLCHEN et al., 2019; LEHTONEN et al., 1996; OSTASZEWSKA, 2005). Um diesem Verlust durch Kannibalismus entgegenzuwirken, werden die Tiere regelmäßig anhand ihrer Größe sortiert, um eine höhere Überlebensrate zu gewährleisten und damit eine höhere Anzahl an adulten Tieren für die Nahrungsproduktion zu erhalten (SZCZEPKOWSKI et al., 2011). Derzeit ist hierfür eine regelmäßige manuelle Überwachung der Tiere notwendig. Da Zander jedoch eine große Zahl an Nachkommen (200.000 Eier pro kg Weibchen) produzieren können (LAPPALAINEN et al., 2003), ist die Überwachung nicht nur aufwendig, sondern wegen der geringen Ei- und Larvengröße schwierig zu realisieren. Bisher verwendete Systeme zur Fischüberwachung sind bei diesen Tieren in diesem Entwicklungszeitraum somit nicht anwendbar.

Das hier vorgestellte, durch den EMFF geförderte Projekt (MV-II.1-LM-011) hatte zum Ziel, die Überwachung von Zanderlarven zu automatisieren. Als nicht-invasive Methode für Tiere von geringer Größe wurde hierfür das Verfahren der Videoanalyse genutzt. Insgesamt sollen mit dem neu entwickelten System sowohl die Erkennung und Auszählung von Larven als auch eine Größenmessung möglich sein. Im Verlaufe der Aufzucht könnten die steigende Größenvarianz bei den Tieren eines Geleges detektiert sowie die Reduktion in der Anzahl der Larven frühzeitig erkannt und so der Kannibalismus in der Aufzucht verringert werden. Mithilfe dieses Systems können folglich die Aufzucht der Zander erleichtert und somit die Zahl der Tiere für den späteren Besatz erhöht werden.

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Test der Auswertemethode unter Laborbedingungen

Für die Entwicklung der notwendigen Software zur Zanderlarvenerkennung wurde ein Prototyp im kleineren Maßstab hergestellt. Ziel war es, mit einfachem reproduzierbaren Aufbau in kontrollierten Testbedingungen die Auswertemethode zu erproben und dabei flexibel anpassen zu können. Die Umsetzung in Großanlagen wäre mit einem höheren Zeit- und technischen Aufwand verbunden.

Für die Erstellung eines Testaufbaus dienten die ermittelten Bedingungen der Larven-Aquakultur als Orientierung und Grundlage. So wurde zur Gewährleistung konstanter abiotischer Faktoren (z. B. Temperatur, Licht) ein Aquarium in einem Klimaschrank für die Aufzucht der Larven verwendet. Als Kamerasystem wurden GoPro Kameras genutzt, da diese Bilder mit hoher Auflösung (Videos mit 30 Bildern pro Sekunde) produzieren können. Die Nutzung von drei Kameras ermöglichte hierbei eine 3D-Raumbetrachtung der Larven, wodurch die Erkennung und Unterscheidung der Einzeltiere sichergestellt werden sollte (Abb. 1). Für die Aufnahmen wurde eine Einmessung der Kameras durchgeführt, die mithilfe von Schachbrett-Hintergründen eventuelle Linsenfehler korrigierte. Anhand eines Startsignals konnten die einzelnen Videoaufnahmen folgend für die Auswertung synchronisiert werden

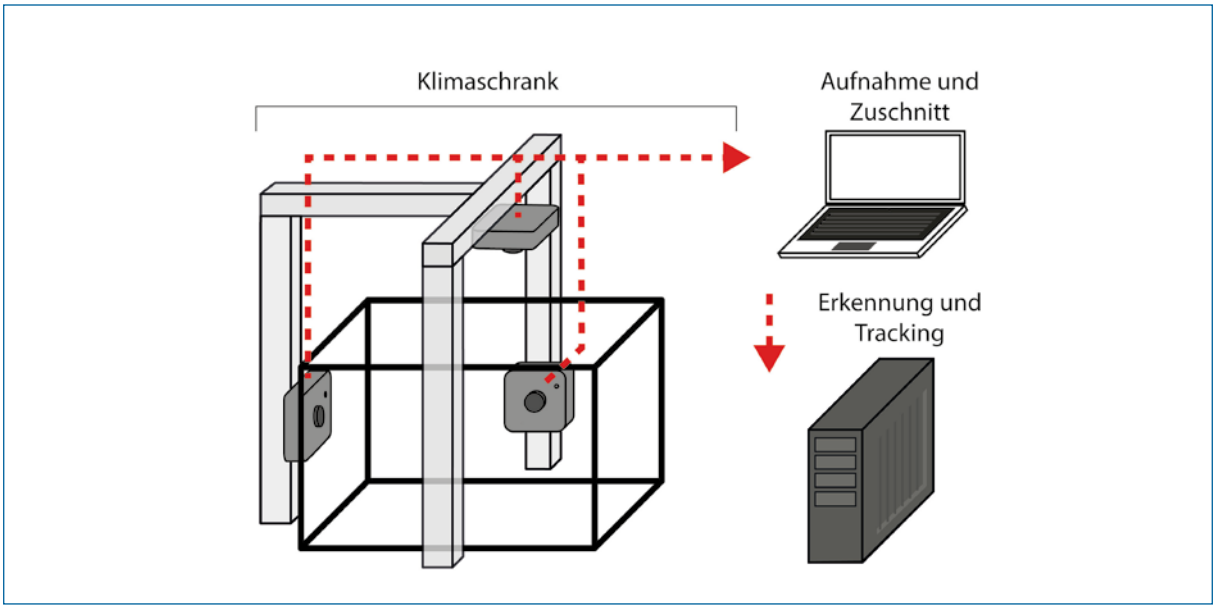

Abb. 1: Schema des Aufbaus und der Abfolge der Datenauswertung für die Messreihen. Der Aufbau ist in seinen Ausmaßen an die Größe des Klimaschranks angepasst. Die folgenden Auswertungsschritte umfassen einen Grobzuschnitt und die weitere Analyse mit Hilfe der erstellten Algorithmen.

Probentiere

Die Fischerei Werner Loch, Hohen Sprenz, stellte die Tiere für mehrere Versuchsreihen zur Verfügung. Hierfür wurden adulte Zander aus dem Hohensprenzer See gefangen, welche dann in Netzkäfigen vor Ort gehalten wurden. Die Tiere laichten unter seminatürlichen Bedingungen auf Kokosmatten ab. Folgend wurden die einzelnen Nester eingesammelt, die Eier entklebt und in Zugergläser transferiert (Abb. 2a), in denen sie von Seewasser umspült wurden. In der Fischerei Werner Loch werden die Tiere kurz vor dem Schlupf in Aufzuchtrinnen transferiert (Abb. 2b). Für das Digitalisierungsprojekt wurden die Zanderlarven kurz vor dem Schlupf in den Aquarium-Prototyp überführt. Um Stress zu minimieren, wurde dauerhaft Wasser aus dem Hohensprenzer See für den regelmäßig erfolgenden Teilwasserwechsel genutzt. Die Wassertemperatur im Prototyp wurde basierend auf der ursprünglichen Temperatur im See auf konstante 13,5 °C eingestellt. Um den Kannibalismus im System zu verringern, wurden die Tiere neben dem im Seewasser ausreichend befindlichen Zooplankton zusätzlich mit Mikrowürmern (*Panagrellus* spec.) gefüttert.

Abb. 2: Aufzuchtanlage in der Fischerei W. Loch, Hohen Sprenz. a: Die befruchteten Eier werden in Zugergläsern mit Seewasser durchspült. b: Kurz vor dem Schlupf werden die Eier in die Aufzuchtrinnen verlagert.

Datenvorbereitung und Test der Datenauswertung mit neuronalen Netzen

Der Laichabfolge der Zander entsprechend wurden Tiere aus zwei Nestern untersucht. Die erste Messreihe mit Tieren aus dem ersten Nest diente vorwiegend der Verbesserung des Aufbaus und Tests der Erkennungsalgorithmen. Die Tiere aus dem zweiten Nest wurden zur Softwareerprobung verwendet. Die täglichen Zeitrafferaufnahmen mit einer Dauer von je 10 min wurden analysiert, sodass eine erstmalige Überwachung der Tiere stattfinden konnte.

Um die Datenmenge für die Softwareentwicklung und das Softwaretraining zu reduzieren und damit die Datenauswertung zu erleichtern, wurden die Rohvideos manuell auf zeitliche und räumliche Bereiche zugeschnitten. Hierbei konnten der Start, vor dem Synchronisieren der Videos, und der Endvorgang herausgenommen sowie Einzelframes auf Fokusbereiche reduziert werden (Abb. 3 a, b). Der Fokusbereich wurde hinsichtlich geringer Bildstörungen wie Reflexionen und Spiegelungen sowie einzeln erkennbarer Larven ausgewählt. Aufgrund der kurzen Projektdauer und der Larvenanzahl im Fokusbereich wurden die ersten Videoanalysen in 2D als ausreichend betrachtet. Die Erweiterung der Auswertung auf 3D ist derzeit in Bearbeitung.

Zur Analyse der einzelnen Videobilder (Frames) wurde eine Open-Source Python-Bibliothek (OpenCV) genutzt, welche sich bereits als Standard im Bereich der Computer-Vision etabliert hat (BRADSKI, 2000). Durch Differenzbildung zum vorherigen Bild wurde von jedem ursprünglichen Frame der Hintergrund entfernt, wodurch die sich bewegenden Einzeltiere herausgefiltert wurden (Abb. 3 c). Als Ergebnis entstand eine Maske, welche den Vordergrund in Graustufen darstellt. Weitere Erosions- und Dilatationsfilter entfernten Bildrauschen. Weiter wurde das Bild auf ein binäres Schwarzweißbild reduziert (Abb. 3 d). Durch eine folgende Routine wurden die Konturen bestimmt, welche mit Quadraten umzogen wurden. Einzelne Larven erzeugten bei schneller Bewegung mehrere Konturen, sodass ein einzelner Fisch mehrere Quadrate erzeugen konnte. Um diese Mehrfacherkennung zu verhindern, wurden Quadrate ab einer bestimmten Überlappungsmenge zusammengefasst (Abb. 3e).

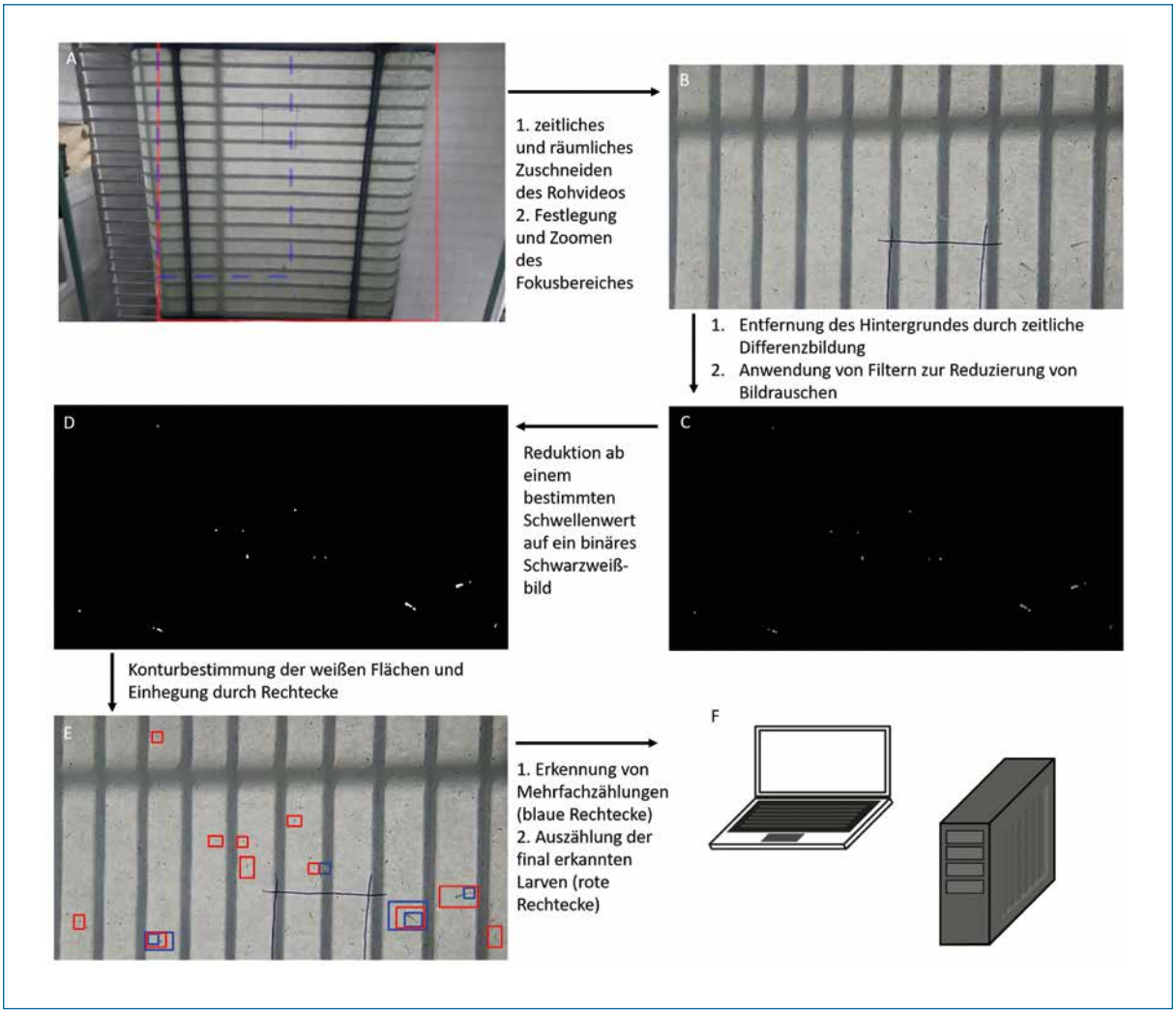

Abb. 3: Schematische Darstellung der Verarbeitungsschritte. A: Überwachungsbereich und räumliches Beschneiden des Rohvideos. Der rote Rahmen stellt den ersten Zuschnitt dar. Der blaue Rahmen markiert den Fokusbereich, welcher für das Training des Neuronalen Netzes verwendet wurde. B: Zoom des räumlichen Bereiches; C: Graustufenbild als Resultat der ersten Verarbeitungsschritte; D: Binärbild erstellt aus dem Graustufenbild; E & F: Die Erkennung der Larven geschieht durch das automatisierte Markieren der Konturen mithilfe von Rechtecken. Die blauen Rahmen sind das Ergebnis der Konturbestimmung. Da schnelle Fische in mehrere Konturen zerfallen können (blaue und rote Quadrate), werden diese bei hinreichender Überlappung zusammengefasst, sodass daraus die finale Ausgabe im roten Rahmen generiert und die Larvenanzahl automatisiert ermittelt wird.

Zur Verbesserung der Erkennung konnten mit den ausgewerteten Bildern ein Neuronales Netz und ein zur Abstraktion befähigter Erkennungsalgorithmus, welcher auf Tensorflow (ABADI et al., 2015) basiert, trainiert werden. Neuronale Netze kommen aus der Forschung zur Künstlichen Intelligenz und basieren auf einem von biologischen Prozessen inspirierten Konzept im Bereich des maschinellen Lernens. Sie werden z. B. bei der automatischen Erkennung von Verkehrszeichen in Autopilotsystemen eingesetzt.

Anhand der vorher vorgestellten Auswertung wurden die Larven in den einzelnen Videobildern automatisiert erkannt und damit ein Testbilddatensatz mit 565 Bildern mit jeweils mehreren Fischlarven erstellt, wobei die Einzelbilder per Hand für die Larvenerkennung überprüft und gegebenenfalls nachkorrigiert wurden. Durch das Training des Neuronalen Netzes konnte die Auswertequalität weiter verbessert werden.

Ergebnisse der Videoanalysen und Wachstum der Larven

Mithilfe der mit der ersten Messreihe etablierten Methoden und dem trainierten Neuronalen Netz wurden die weiteren Videos der zweiten Zanderlarven-Messreihe in 2D ausgewertet. Über 12 Tage betrug dies insgesamt über 50.000 Frames. Zusätzlich wurde ein Vergleich zwischen standardmäßigen Full-HD-Aufnahmen (1920 x 1080) und 4K-Testsaufnahmen (3840 x 2160 px) erstellt. Eine höhere Auflösung führte jedoch zu keiner Verbesserung, sodass Full-HD–Aufnahmen als ausreichend angesehen wurden. Die Zeitraffer sollten nicht stärker als 0.5x (15 Bilder pro Sekunde) gewählt werden, da sonst wegen der zu geringen Folgebildveränderung keine ausreichende Verfolgung der Larven möglich ist.

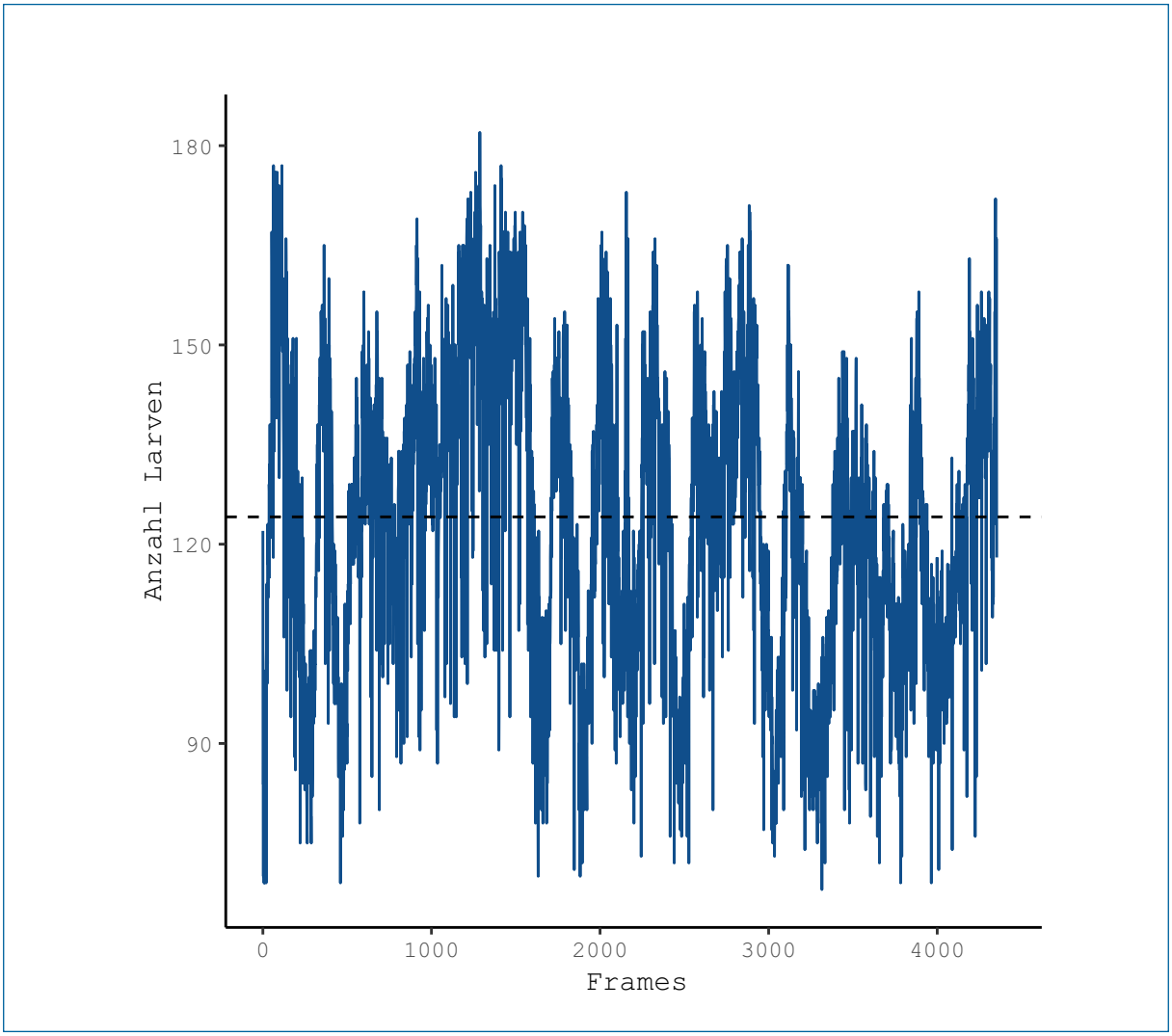

Abb. 4: Exemplarische Darstellung der automatisiert ermittelten Anzahl der gelabelten Tiere im Fokusbereich eines Videos. Über die Zeit sind abwechselnd Hoch- und Tiefpunkte zu erkennen, die u. a. durch das Schwimmen über das Gitter verursacht wurden. Der Durchschnittswert über die Gesamtdauer des Videos ist als gestrichelte Linie dargestellt.

Innerhalb der Videos sind periodische Schwankungen bei den einzelnen Frames in der Anzahl an gelabelten Tieren sichtbar (Abb. 4). Diese Schwankungen entstanden durch die Bewegung der Tiere in und aus dem Beobachtungsbereich und vor dem regelmäßigen Gitter, auf dem sich das Aquarium befand. Zusätzlich sind auch weiterhin Mehrfacherkennungen möglich, welche durch besonders hohe Geschwindigkeiten der Tiere oder durch Spiegelungen erzeugt wurden. Innerhalb dieses Projektes wurde daher für die ermittelte Larvenanzahl jeweils der Mittelwert der Tage gewählt.

Über den gesamten Messzeitraum konnte so eine sich verringernde Anzahl an Larven beobachtet werden, bis nur noch wenige Tiere im Becken vorhanden waren (Abb. 5). Trotz all dieser Auswertungsprobleme sind die gewonnenen Zahlen repräsentativ für die Anzahl der aktiven und mobilen Larven und können als Marker für die Populationsentwicklung genutzt werden.

In den hierbei ermittelten Daten sind zwei Reduktionsphasen erkennbar (Abb. 5). Die erste Phase fand simultan vom Einzug in die Testanlage und zur Umstellung von der endogenen (bis 260 dd) zur exogenen Nahrungsquelle statt. Infolge des Umzugs von der Fischerei Hohen Sprenz in die Versuchsanlage sind nach dem ersten Tag Verluste zu verzeichnen gewesen. Die Nahrungsumstellung, die kurz darauf erfolgte, ist ein energetisch aufwendiger Prozess, der diverse Veränderungen in der Anatomie des Darmtrakts beinhaltet und durch eine hohe Sterblichkeit gekennzeichnet ist (OSTASZEWSKA, 2005). Nach einer kurzen Plateauphase wurde eine zweite Reduktionsphase bei der Larvenanzahl sichtbar (ab 310 Tagesgraden). Diese verlief simultan zu einem Anstieg der Größenvarianz zwischen den gleichaltrigen Stadien. Die Larvengröße wurde in einem separaten Versuch mikroskopisch ermittelt, um einen Vergleich mit der automatisierten Überwachung und dem Wachstum der Tiere zu ermöglichen. Bei je 10 Tieren aus 10 verschiedenen Altersgruppen wurden die Totallänge sowie die Distanz zwischen Kopfspitze und Schwanzflossenende vermessen. Die Messung ermöglichte einen Einblick in die Wachstumsverhältnisse in der für den Zander kritischen Entwicklungsphase (Abb. 5). Hierfür wurde das Alter in Tagesgraden angegeben, um eine bessere Vergleichbarkeit zwischen den beiden Projekten zu erzielen. Die Ergebnisse zeigen eine generelle Größenzunahme mit dem Alter von etwa 1 mm pro 100 Tagesgraden und eine auffällige Zunahme der Größenvarianz innerhalb späterer Altersgruppen (Abb. 5, 6). Diese deutlichere Größenvarianz begann ab etwa 300 Tagesgraden, dem Zeitpunkt kurz vor dem Vollenden der Nahrungsumstellung. Diese Zunahme der Größenvarianz markiert einen weiteren kritischen Zeitraum der larvalen Entwicklung, da diese Kannibalismus fördert (RAPP und STÜEKEN, 2019). Um detailliertere Kenntnisse über nötige Größensortierungen zu erhalten, finden bereits weitere Arbeiten zur Morphometrie der frühen Zanderentwicklungsstadien am FBN – Arbeitsgruppe „Wachstumsphysiologie der Fische“ statt.

Für eine zukünftig automatisierte Vermessung der Zanderlarven könnte anhand der Rechteck-Umrandungen der Larven eine Größenauswertung erfolgen. Dabei wäre die Größe der Umrandungen repräsentativ für die Größe der Einzeltiere.

Fazit und Ausblick

Videoüberwachung und -analyse

Durch die Arbeiten in diesem Projekt konnten das Grundprinzip für eine automatisierte Videoanalyse von Zanderlarven entwickelt und deren Auszählung im kleinen Maßstab erfolgreich angewendet werden. Mit der zusätzlich angestrebten Größenermittlung der Tiere und der Erweiterung der Datenanalyse auf 3D wäre eine automatisierte Untersuchung der Tiere in der Aquakultur möglich. Auch wenn die Mortalitätsraten im hier benutzten experimentellen Prototyp-Aufbau nicht mit denen aus üblichen Haltungen vergleichbar sind, konnten die beobachteten Reduktionen der Tieranzahl bereits auf die aus der Aquakultur bekannten problematischen Entwicklungsphasen des Zanders zurückgeführt werden.

Insgesamt besitzt die Anwendung der automatisierten Videoanalyse von Fischlarven ein großes Potential. Allein durch weiteres Training des Neuronalen Netzes könnte die Erkennung von bewegungslosen ruhenden Fischen ebenfalls ermöglicht und die von schnell bewegenden Fischen verbessert werden. Dies würde einen besseren Einblick in das Verhalten der Tiere bieten und so Rückschlüsse auf deren Gesundheit zulassen. Die Verwendung

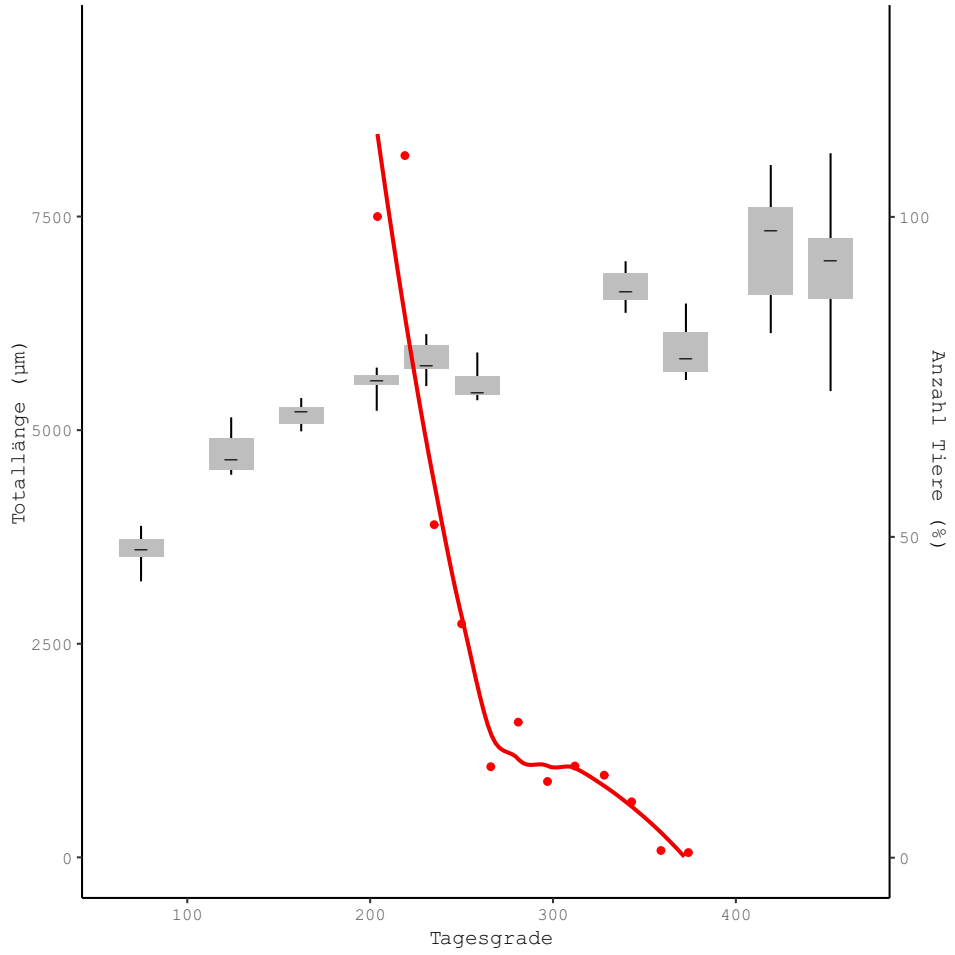

*Abb. 5: Verlauf der Anzahl der überwachten Tiere und Darstellung der Entwicklung anhand der Totallänge. Die durchschnittliche Anzahl der Tiere für jeden Tag (rote Punkte, linke Achse) wurde genutzt, um die Veränderung der Anzahl der Tiere zu interpolieren (rote Linie, linke Achse). Die Boxplots stellen die Totallänge in den bestimmten Altersstadien dar (rechte Achse). Das Alter ist über Tagesgrade (Durchschnittstemperatur * Anzahl Tage) dargestellt .*

Abb. 6: Zwei larvale Zander aus demselben Nest im Alter von 450 Tagesgraden (31 Tage nach Befruchtung). Die Umstellung auf externe Nahrung ist bei beiden Tieren vollzogen. Die erkennbaren Größenunterschiede können Kannibalismus fördern.

des Systems wäre zudem auch auf andere Arten übertragbar. Hier wären insbesondere andere Perciden, wie der Europäische Flussbarsch (*Perca fluviatilis*), im Fokus, da diese zum einen ebenfalls zunehmend interessant für die Aquakultur werden (BUHRKE et al., 2019) und zum zweiten dessen Larven eine große Ähnlichkeit zu denen des Zanders besitzen (URHO, 1996). Für eine Nutzung im Aquakulturbereich wird die Anwendung anderer Kamerasysteme interessant, bei denen die Option zu Unterwasseraufnahmen sowie der Einsatz von Filterobjektiven mit größerem Tiefenschärfebereich möglich wird. Dies wird durch die Entwicklung bei digitalen Kameras zu besserer Lichtstärke und Auflösung unterstützt. Mit diesen Überwachungssystemen könnte zukünftig der Einsatz in weniger gut belichteten oder anders gefärbten Aufzuchtbecken ermöglicht und damit die hohe Mortalitätsrate während der Aufzucht durch automatisierte Früherkennung reduziert werden.

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Contribution of Authors

The parts of this cumulative thesis were collaborations by the following authors and their named participations:

Study I – "Observations of growth changes during the embryonic –larval transition of pikeperch (Sander lucioperca) under near-natural conditions"

Journal of Fish Biology 99, 425-436, 2021

- Franz, G.P. 60%
- Lewerentz, L. 10%
- Grunow, B. 30%

Research design: BG; GPF; Funding acquisition: BG; Sample collection and handling: GPF; Data acquisition: GPF; Data analysis: GPF; LL; Illustration: GPF; LL; Supervision: BG; All authors contributed to data interpretation and writing.

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

Fish Physiology and Biology 47(2), 1-18, 2021

- Schäfer, N. 40%
- Kaya, Y. 20%
- Rebl, H. 3%
- Stüeken, M. 1%
- Rebl, A. 1%
- Nguinkal, J.A. 1%
- Franz, G.P. 1%
- Brunner, R.M. 1%
- Goldammer, T. 2%
- Grunow, B. 15%
- Verleih, M.1 15%

Research design: MV, BG, AR; Funding acquisition: TG; Fish production: MS; Experimental procedures: RMB; Fish sampling: NS, JAN, MS, MV; Cell culture: YK, GPF, BG, NS; Immunofluorescence: HR; RTqPCR assays: NS, MV, AR; Data acquisition and analysis: NS, HR, AR, JAN, YK, GPF, BG, MV; Supervision: MV, AR BG, TG; Paper writing: NS; Paper reviewing and editing: NS, HR, AR, MS, TG, RMB, BG, MV; All authors read and approved the final manuscript.

Study III – "The Expression of Myogenic Gene Markers during the Embryo-Larval-Transition in Pikeperch (Sander lucioperca)"

Aquaculture Research (accepted 09.06.22)

- Franz, G.P. 50%
- Tönißen, K. 10%
- Rebl, A. 10%
- Lutze, P. 5%
- Grunow, B. 25%

Research design: BG; AR; Funding acquisition: BG; Sample collection and handling: GPF; Experimental procedures: GPF; Primer design and testing: GPF; KT, PL; FLUIDIGM-qPCR assays: AR, GPF; Data acquisition and analysis: GPF; Illustration: GPF; Supervision: BG; AR; Paper writing: GPF; Paper reviewing and editing: GPF, KT, AR, BG; All authors read and approved the final manuscript.

Study IV – "Konzeption eines Prototyps zur Videoüberwachung von Zanderlarven"

Mitteilungen der Landesforschungsanstalt für Landwirtschaft und Fischerei 63, 134-142, 2021 (non-peer reviewed)

- Franz, G.P. 30%
- Lewerentz, L. 30%
- Schneider, R. 10%
- Grunow, B. 30%

Research design: BG; RS; **Funding acquisition:** BG; **Sample collection:** GPF; **Experimental procedures:** LL, GPF, BG; **Software design:** LL, RS; **Data acquisition and analysis:** LL, GPF; **Illustration**: GPF, LL; **Supervision:** RS, BG; **Paper writing:** GPF, BG; **Paper reviewing and editing** GPF, LL, RS, BG; All authors read and approved the final manuscript.

Signature - Supervisor

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Further publications during the doctorate project

Komolka, K., Bochert, R., **Franz, G. P.**, Kaya, Y., Pfuhl, R., & Grunow, B. (2020). Determination and comparison of physical meat quality parameters of Percidae and Salmonidae in aquaculture. *Foods*, **9** (4), 388.

Grunow, B., **Franz, G. P.**, & Tönißen, K. (2021). In vitro fish models for the analysis of exotoxins and temperature increase in the context of global warming. *Toxics*, **9** (11), 286.

Tönißen, K., Pfuhl, R., **Franz, G. P.**, Dannenberger, D., Bochert, R., & Grunow, B. (2022). Impact of spawning season on fillet quality of wild pikeperch (*Sander lucioperca*). *European Food Research and Technology*, **248** (5), 1277-1285.

Franz, G. P., Warth, P., Grunow, B., & Konstantinidis, P. (2022). Osteology of the white barracudina, *Arctozenus risso* (Bonaparte)(Aulopiformes: Paralepididae). *Ichthyology & Herpetology,* **110** (1), 115-130. [Publication of former Master thesis]

Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Unterschrift des*der Promovend*in:

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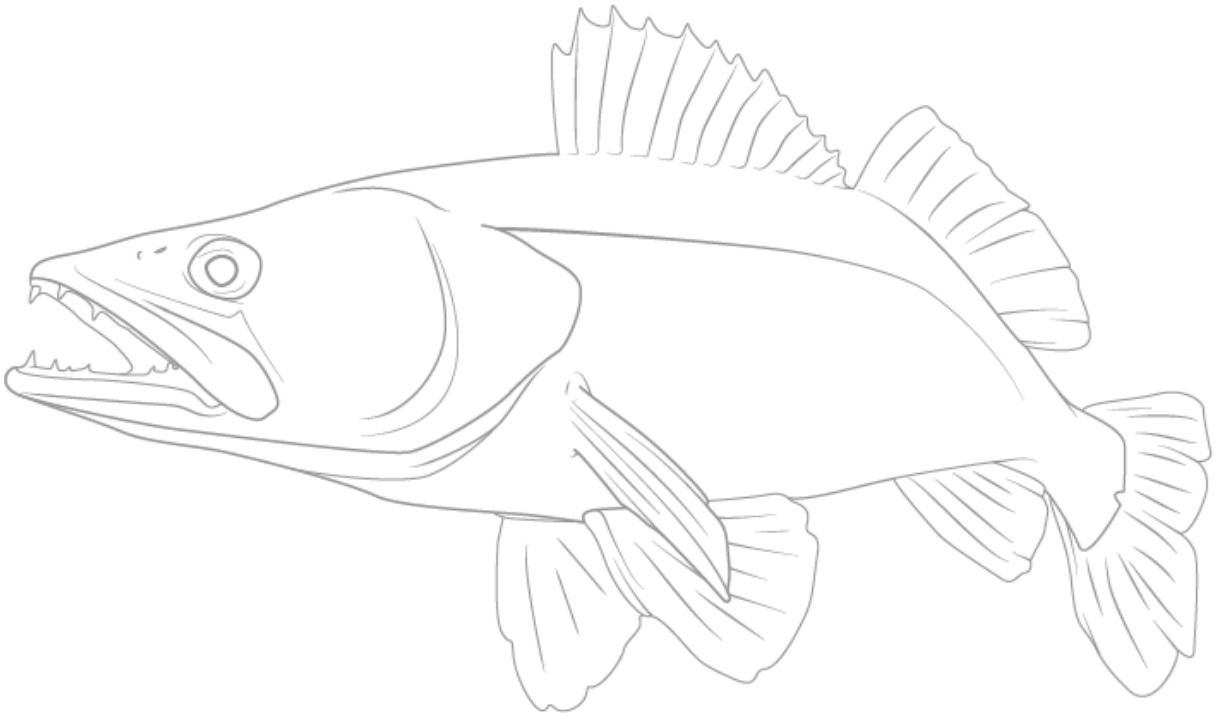
Appendix

Study I – Supplemental



SUPPLEMENTAL

Supplemental 1 - Table. Abbreviations and descriptions of the measured parameters. Values in µm and for volumes in μm^3.

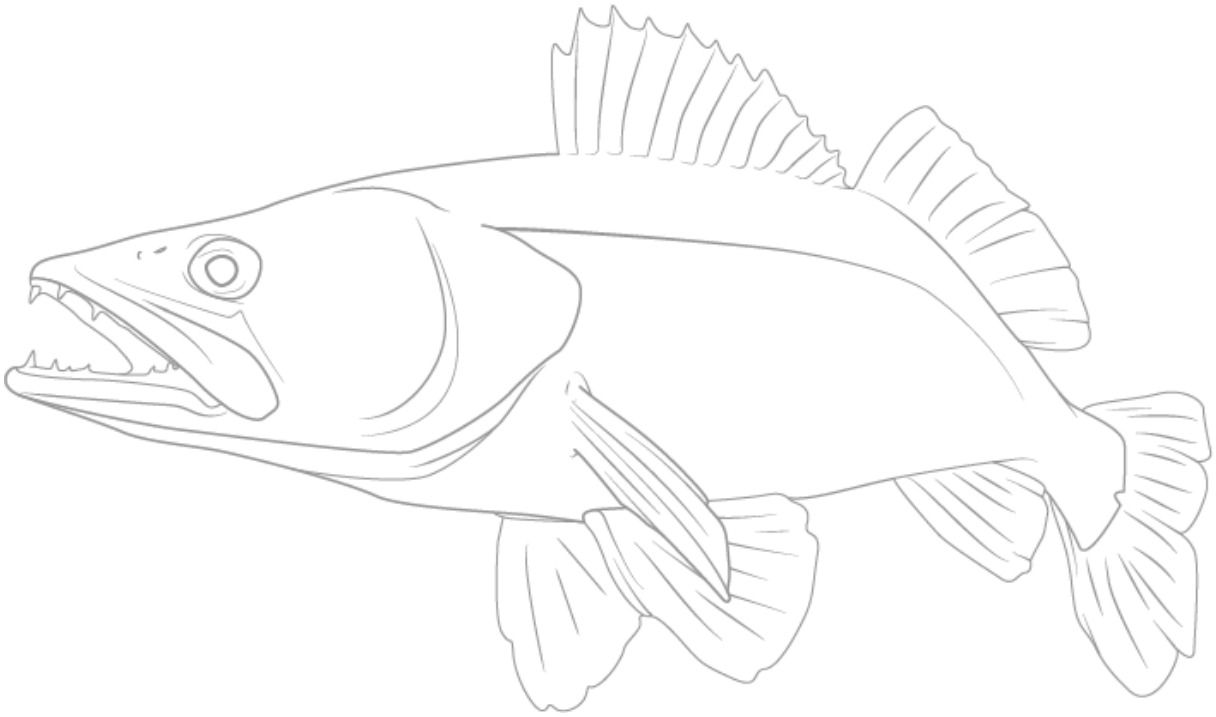
Abbreviation	Name	Description
BHA	body height at the anus	maximal distance dorsal to ventral, myomer height including intestines
BHC	body height at the caudal peduncle	maximal distance dorsal to ventral
BWA	body width at the anus	maximal width of the dorsal body at the anus
BWC	body width at the caudal peduncle	maximal width of the dorsal body at the caudal peduncle
HAN	head angle	angle between rostral tip, otic capsule midpoint and middle of the chorda at the position of the pectoral fin
HL	head length	rostral tip to the posterior end of the otic capsule
ID	interorbital distance	distance on the frontals between the eyes
MHA	myomer height at the anus	maximal distance dorsal to ventral
OD	orbita diameter	anterior to posterior eye diameter
OGD	oil globule diameter	maximal diameter of the oil globule
PFL	pectoral fin length	distance from the anterior attachment to the fin margin
POA	postanal length	middle of the notochord at the anus to the posterior notochord end
PRA	preanal length	rostral tip to middle of the chorda at the anus
SB	swim bladder inflation	laterally visible inflation noted
SL	standard length	rostral tip to notochord end
TL	total length	rostral tip to caudal fin margin
YH	yolk height	maximal distance dorsal to ventral
YL	yolk length	maximal distance anterior to posterior
$\mathbf{V_{OG}}$	volume of the oil globule	calculated, see Formula 1
$\mathbf{V_{Y}}$	volume of the yolk	calculated, see Formula 2
$\mathbf{V_{PAB}}$	Volume of the postanal body	calculated, see Formula 3

Supplemental 2 - Table. Result overview of the multilinear regression. The results are separated based on the developmental phases obtained from the PCA. The function for the associated regression graphs are given as increase (m) and Y-axis-intersection (n).

Parameter				EMBRYONIC			Regression Graph	
	n	mean	SD	median	min	max	Reg n	Reg m
BHA	48	301	60	317	175	402	115.37	1.54
BHC	48	101	23	97	50	146	39.94	0.51
BWA	48	162	29	160.5	115	230	86.94	0.62
BWC	48	95	16	93.5	55	129	67.39	0.23
HAN	48	138	8	139	121	159	119.20	0.16
HL	48	620	102	644.5	403	829	340.85	2.32
ID	48	430	51	420.5	322	534	302.51	1.06
MHA	48	208	36	214	137	269	99.14	0.91
OD	48	288	37	295.5	191	345	199.42	0.73
OGD	48	398	46	398.5	304	519	335.02	0.53
PFL	48	143	137	135.5	0	382	-248.17	3.25
POA	48	2141	474	2184.5	1269	2753	648.18	12.41
PRA	48	2251	198	2296.5	1728	2561	1640.94	5.07
SL	48	4392	659	4501.5	3160	5290	2289.11	17.47
TL	48	4493	692	4608.5	3230	5375	2286.42	18.34
V_{OG}	48	$1.93*10^7$	$6.70*10^6$	$1.86*10^7$	$8.27*10^6$	$4.12*10^7$	$9.62*10^6$	$8.09*10^4$
V_{PAB}	48	$3.53*10^7$	$1.77*10^7$	$3.32*10^7$	$1.15*10^7$	$6.73*10^7$	$-2.01*10^7$	$4.60*10^5$
V_Y	48	$9.43*10^7$	$1.93*10^7$	$9.40*10^7$	$5.41*10^7$	$1.43*10^8$	$1.11*10^8$	$-1.37*10^5$
YH	48	448	37	450	367	554	444.50	0.03
YL	48	889	84	886.5	716	1064	1053.99	-1.37
				THRESHOLD			Regression Graph	
	n	mean	SD	median	min	max	Reg n	Reg m
BHA	48	440	33	440	379	508	514.14	-0.32
BHC	48	152	14	152	119	177	188.91	-0.16
BWA	48	202	26	200	157	252	365.33	-0.70
BWC	48	109	15	110	76	145	113.38	-0.02
HAN	48	183	8	184	164	197	113.74	0.30
HL	48	888	71	889	747	1084	654.37	1.01
ID	48	390	35	390	333	493	572.11	-0.79
MHA	48	303	21	300.5	261	357	370.42	-0.29
OD	48	326	24	326	269	372	206.35	0.52
OGD	48	390	55	395	246	481	662.35	-1.18
PFL	48	585	64	592.5	454	713	354.05	1.00
POA	48	2873	117	2853	2584	3138	2620.33	1.09
PRA	48	2585	120	2591	2360	2911	2600.58	-0.07
SL	48	5458	206	5450.5	5129	5942	5220.91	1.02

TL	48	5644	222	5642	5227	6124	5240.50	1.74
V_{OG}	48	$1.84*10^7$	$7.07*10^6$	$1.82*10^7$	$4.38*10^6$	$3.28*10^7$	$5.15*10^7$	$-1.43*10^5$
V_{PAB}	48	$8.13*10^7$	$1.37*10^7$	$7.99*10^7$	$5.47*10^7$	$1.09*10^8$	$1.56*10^8$	$-3.25*10^5$
V_Y	48	$2.57*10^7$	$1.70*10^7$	$2.43*10^7$	$6.61*10^5$	$7.21*10^7$	$1.50*10^8$	$-5.36*10^5$
YH	48	268	77	267.5	63	438	811.57	-2.35
YL	48	578	142	600.5	229	989	1345.89	-3.32
				LARVAL			**Regression Graph**	
	n	**mean**	**SD**	**median**	**min**	**max**	**Reg n**	**Reg m**
BHA	66	646	140	619.5	374	951	45.23	1.52
BHC	66	201	42	199	125	297	24.14	0.45
BWA	66	272	46	259.5	196	424	123.05	0.38
BWC	66	134	28	131	94	221	108.19	0.06
HAN	66	184	4	184.5	176	194	190.85	-0.02
HL	66	1243	204	1218	890	1806	353.76	2.25
ID	66	450	70	444	308	650	340.12	0.28
MHA	66	399	91	379.5	253	649	-55.78	1.15
OD	66	435	55	431	331	587	157.21	0.70
OGD	66	7	41	0	0	267	93.77	-0.22
PFL	66	742	103	750.5	526	929	444.39	0.75
POA	66	3133	296	3058.5	2661	3753	2223.46	2.30
PRA	66	3270	442	3210	2481	4271	1127.33	5.42
SL	66	6402	711	6288.5	5182	7985	3350.79	7.72
TL	66	6647	728	6523.5	5380	8241	3540.82	7.86
V_{OG}	66	$1.22*10^5$	$7.48*10^5$	0.00	0.00	$5.61*10^6$	$1.61*10^6$	$-3.76*10^3$
V_{PAB}	66	$1.65*10^8$	$7.76*10^7$	$1.45*10^8$	$6.12*10^7$	$4.17*10^8$	$-1.68*10^8$	$8.42*10^5$
V_Y	66	$1.45*10^5$	$1.18*10^6$	0.00	0.00	$9.57*10^6$	$1.91*10^6$	$-4.47*10^3$
YH	66	3	24	0	0	197	39.39	-0.09
YL	66	7	58	0	0	471	94.17	-0.22

Study III – Supplemental



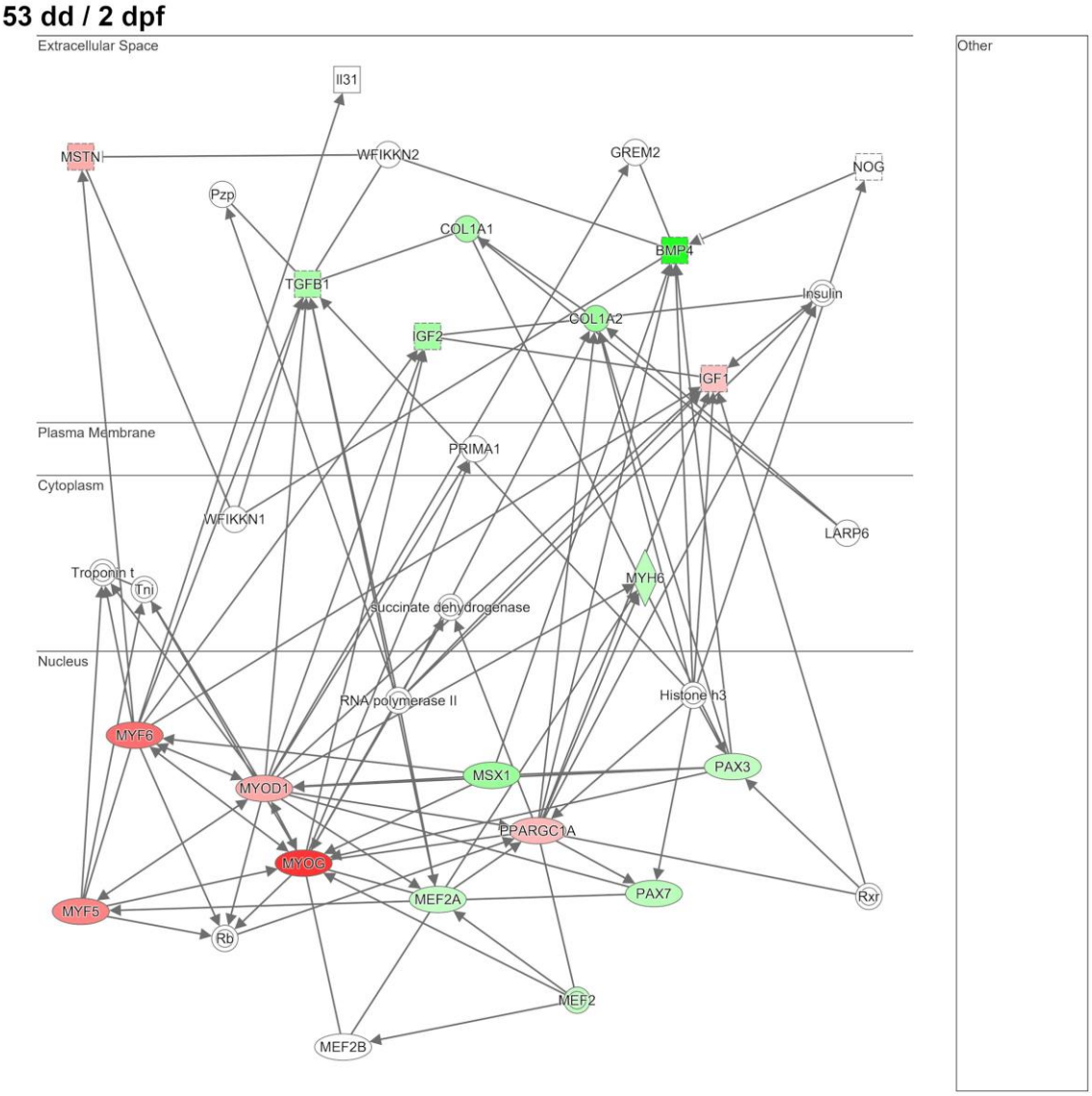


Figure S1 IPA Network for 53 dd pikeperch.

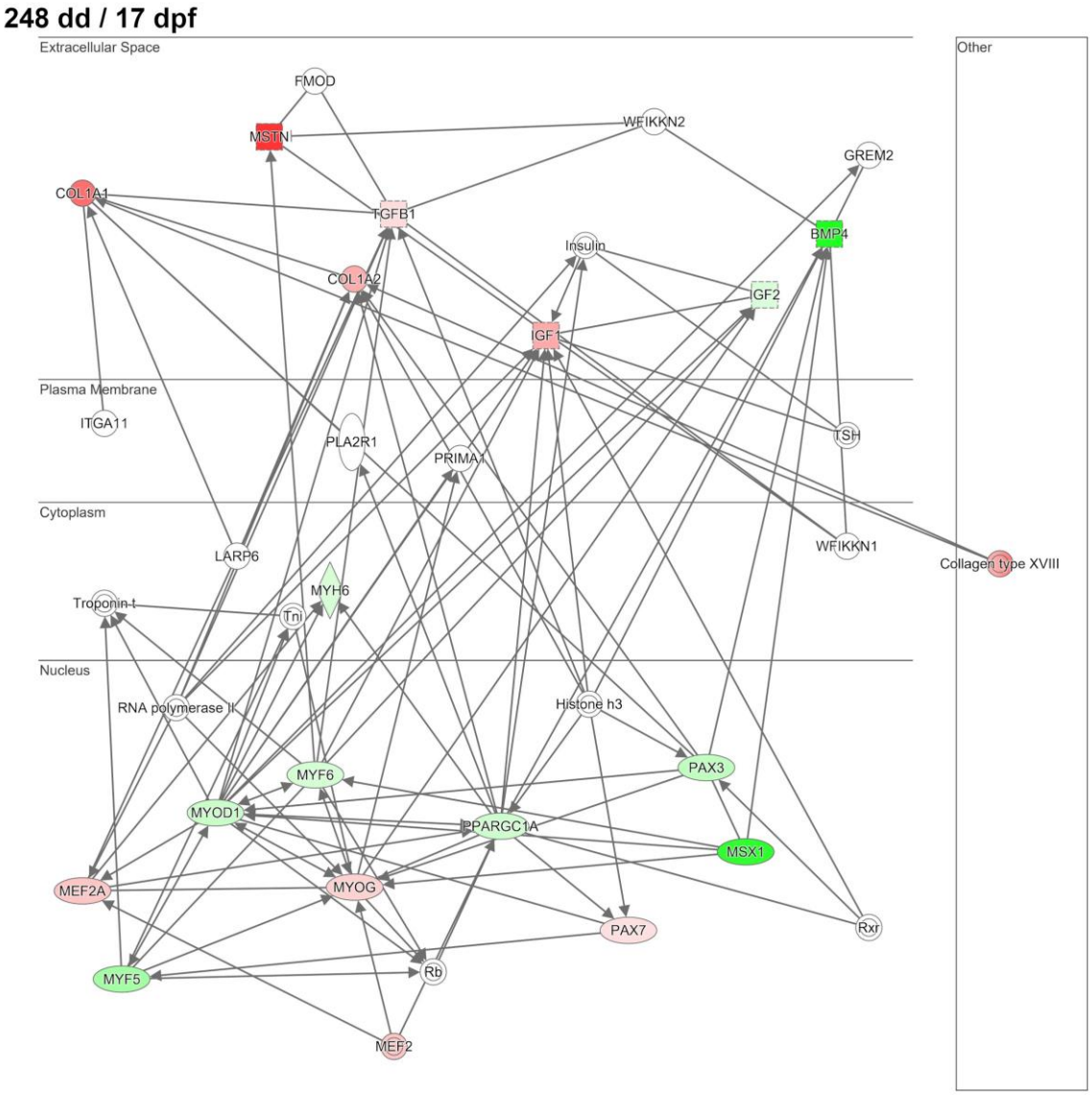


Figure S2 IPA Network for 248 dd pikeperch.

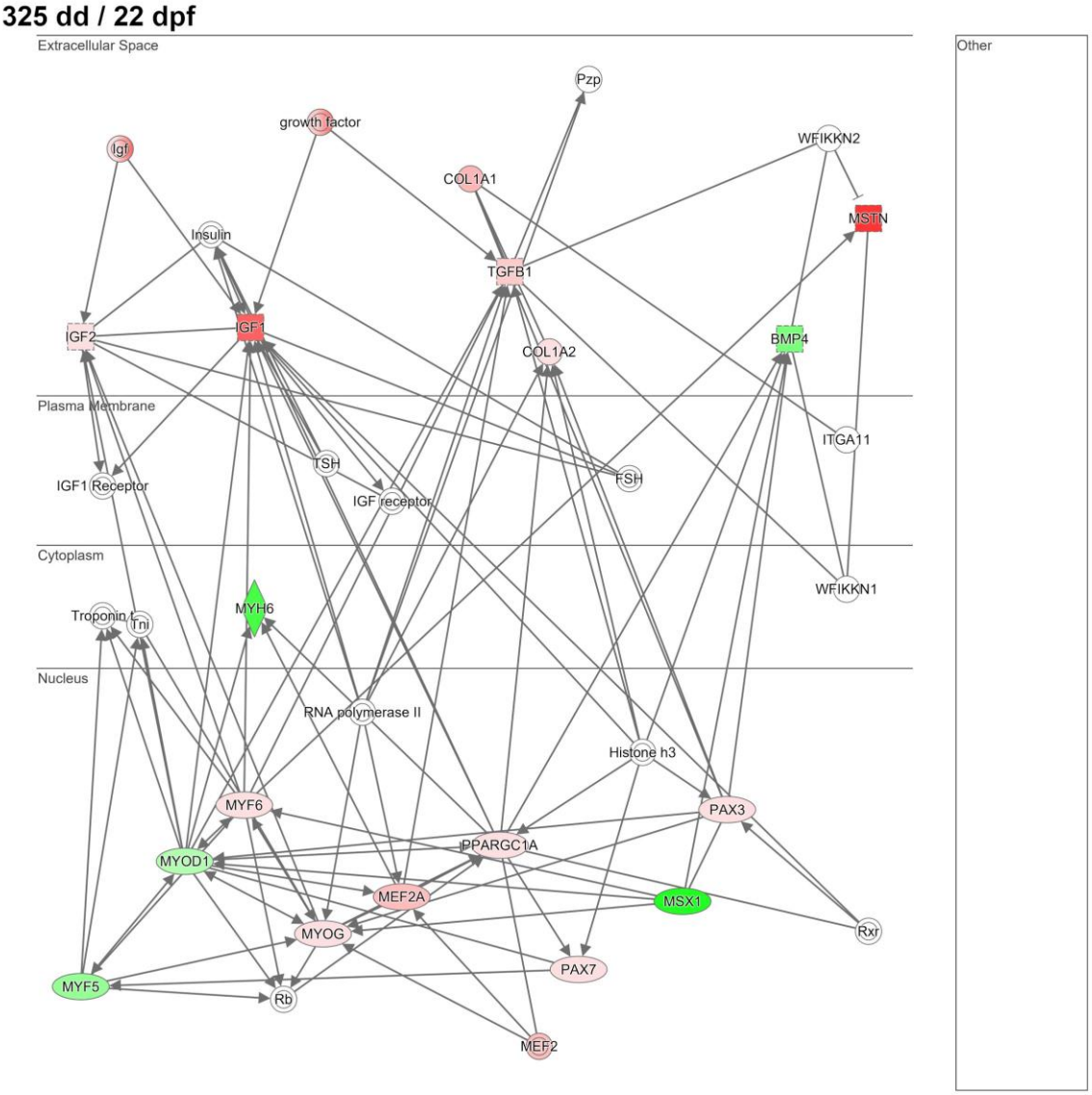


Figure S3 IPA Network for 325 dd pikeperch.

Table S1. Correlation matrix for embryonic gene expression FC in *Sander lucioperca*. Grey numbers mark P >0.05. Relation to age is provided by degree-days (dd).

	dd	***BMP4***	***COL1A1***	***COL1A2***	***EN2***	***IGF1***	***IGF2***	***MEF2A***	***MRF4***	***MSTN***	***MSX1***	***MUSTN1***	***MYF5***	***MYH6***	***MYOD***	***MYOG***	***PAX3***	***PAX7A***	***PGC1A***	***TGFB1***
dd	1.00	-0.57	0.97	0.96	0.24	0.89	0.31	0.97	0.33	0.8	-0.79	0.79	-0.42	-0.54	-0.08	0.03	-0.53	0.6	0.76	0.93
Pval	-	0.03	<0.01	<0.01	0.39	<0.01	0.26	<0.01	0.23	<0.01	<0.01	<0.01	0.12	0.04	0.79	0.92	0.04	0.02	<0.01	<0.01
BMP4	-0.57	1.00	-0.4	-0.41	-0.05	-0.28	0.54	-0.39	-0.37	-0.28	0.9	-0.24	0.05	0.05	-0.12	-0.49	0.19	-0.4	-0.16	-0.32
Pval	0.03	-	0.14	0.13	0.87	0.31	0.04	0.16	0.18	0.32	<0.01	0.4	0.85	0.86	0.66	0.07	0.5	0.14	0.58	0.24
COL1A1	0.97	-0.4	1.00	0.97	0.25	0.92	0.49	0.99	0.23	0.81	-0.67	0.83	-0.5	-0.58	-0.12	-0.13	-0.54	0.59	0.8	0.98
Pval	<0.01	0.14	-	<0.01	0.37	<0.01	0.06	<0.01	0.4	<0.01	0.01	<0.01	0.06	0.02	0.66	0.65	0.04	0.02	<0.01	<0.01
COL1A2	0.96	-0.41	0.97	1.00	0.09	0.89	0.43	0.96	0.2	0.79	-0.69	0.73	-0.54	-0.53	-0.14	-0.03	-0.5	0.65	0.7	0.94
Pval	<0.01	0.13	<0.01	-	0.74	<0.01	0.11	<0.01	0.47	<0.01	<0.01	<0.01	0.04	0.04	0.62	0.92	0.06	0.01	<0.01	<0.01
EN2	0.24	-0.05	0.25	0.09	1.00	0.42	0.32	0.3	0.55	0.29	-0.05	0.32	0.39	-0.26	0.35	0.05	-0.03	-0.02	0.62	0.28
Pval	0.39	0.87	0.37	0.74	-	0.12	0.24	0.28	0.03	0.29	0.86	0.25	0.15	0.35	0.2	0.87	0.92	0.94	0.01	0.3
IGF1	0.89	-0.28	0.92	0.89	0.42	1.00	0.54	0.95	0.37	0.84	-0.53	0.7	-0.3	-0.55	0.00	-0.03	-0.42	0.56	0.87	0.94
Pval	<0.01	0.31	<0.01	<0.01	0.12	-	0.04	<0.01	0.17	<0.01	0.04	<0.01	0.28	0.03	1.00	0.92	0.12	0.03	<0.01	<0.01
IGF2	0.31	0.54	0.49	0.43	0.32	0.54	1.00	0.48	-0.02	0.33	0.27	0.47	-0.26	-0.49	-0.03	-0.5	-0.3	0.1	0.58	0.53
Pval	0.26	0.04	0.06	0.11	0.24	0.04	-	0.07	0.95	0.23	0.33	0.08	0.35	0.07	0.91	0.06	0.29	0.74	0.02	0.04
MEF2A	0.97	-0.39	0.99	0.96	0.3	0.95	0.48	1.00	0.26	0.86	-0.65	0.81	-0.46	-0.57	-0.12	-0.1	-0.51	0.59	0.83	0.97
Pval	<0.01	0.16	<0.01	<0.01	0.28	<0.01	0.07	-	0.35	<0.01	0.01	<0.01	0.08	0.03	0.67	0.72	0.05	0.02	<0.01	<0.01
MRF4	0.33	-0.37	0.23	0.2	0.55	0.37	-0.02	0.26	1.00	0.34	-0.25	0.25	0.68	-0.57	0.72	0.59	-0.41	-0.2	0.56	0.16
Pval	0.23	0.18	0.4	0.47	0.03	0.17	0.95	0.35	-	0.22	0.37	0.36	0.01	0.03	<0.01	0.02	0.13	0.47	0.03	0.57
MSTN	0.8	-0.28	0.81	0.79	0.29	0.84	0.33	0.86	0.34	1.00	-0.52	0.73	-0.31	-0.55	-0.15	-0.11	-0.48	0.42	0.79	0.81
Pval	<0.01	0.32	<0.01	<0.01	0.29	<0.01	0.23	<0.01	0.22	-	0.05	<0.01	0.26	0.03	0.59	0.7	0.07	0.12	<0.01	<0.01
MSX1	-0.79	0.9	-0.67	-0.69	-0.05	-0.53	0.27	-0.65	-0.25	-0.52	1.00	-0.46	0.36	0.14	0.13	-0.28	0.26	-0.63	-0.32	-0.59
Pval	<0.01	<0.01	0.01	<0.01	0.86	0.04	0.33	0.01	0.37	0.05	-	0.09	0.19	0.62	0.66	0.31	0.35	0.01	0.25	0.02
MUSTN1	0.79	-0.24	0.83	0.73	0.32	0.7	0.47	0.81	0.25	0.73	-0.46	1.00	-0.31	-0.7	-0.07	-0.33	-0.79	0.17	0.82	0.79
Pval	<0.01	0.4	<0.01	<0.01	0.25	<0.01	0.08	<0.01	0.36	<0.01	0.09	-	0.26	<0.01	0.81	0.23	<0.01	0.54	<0.01	<0.01
MYF5	-0.42	0.05	-0.5	-0.54	0.39	-0.3	-0.26	-0.46	0.68	-0.31	0.36	-0.31	1.00	-0.16	0.78	0.54	-0.02	-0.69	-0.01	-0.54
Pval	0.12	0.85	0.06	0.04	0.15	0.28	0.35	0.08	0.01	0.26	0.19	0.26	-	0.58	<0.01	0.04	0.94	<0.01	0.98	0.04
MYH6	-0.54	0.05	-0.58	-0.53	-0.26	-0.55	-0.49	-0.57	-0.57	-0.55	0.14	-0.7	-0.16	1.00	-0.42	0.09	0.82	0.24	-0.75	-0.5
Pval	0.04	0.86	0.02	0.04	0.35	0.03	0.07	0.03	0.03	0.03	0.62	<0.01	0.58	-	0.12	0.76	<0.01	0.38	<0.01	0.06
MYOD	-0.08	-0.12	-0.12	-0.14	0.35	0.00	-0.03	-0.12	0.72	-0.15	0.13	-0.07	0.78	-0.42	1.00	0.59	-0.3	-0.43	0.21	-0.18
Pval	0.79	0.66	0.66	0.62	0.2	1.00	0.91	0.67	<0.01	0.59	0.66	0.81	<0.01	0.12	-	0.02	0.28	0.11	0.46	0.52
MYOG	0.03	-0.49	-0.13	-0.03	0.05	-0.03	-0.5	-0.1	0.59	-0.11	-0.28	-0.33	0.54	0.09	0.59	1.00	0.08	0.08	-0.1	-0.22
Pval	0.92	0.07	0.65	0.92	0.87	0.92	0.06	0.72	0.02	0.7	0.31	0.23	0.04	0.76	0.02	-	0.78	0.79	0.73	0.43
PAX3	-0.53	0.19	-0.54	-0.5	-0.03	-0.42	-0.3	-0.51	-0.41	-0.48	0.26	-0.79	-0.02	0.82	-0.3	0.08	1.00	0.24	-0.61	-0.44
Pval	0.04	0.5	0.04	0.06	0.92	0.12	0.29	0.05	0.13	0.07	0.35	<0.01	0.94	<0.01	0.28	0.78	-	0.38	0.02	0.1
PAX7A	0.6	-0.4	0.59	0.65	-0.02	0.56	0.1	0.59	-0.2	0.42	-0.63	0.17	-0.69	0.24	-0.43	0.08	0.24	1.00	0.18	0.64
Pval	0.02	0.14	0.02	0.01	0.94	0.03	0.74	0.02	0.47	0.12	0.01	0.54	<0.01	0.38	0.11	0.79	0.38	-	0.52	0.01
PGC1A	0.76	-0.16	0.8	0.7	0.62	0.87	0.58	0.83	0.56	0.79	-0.32	0.82	-0.01	-0.75	0.21	-0.1	-0.61	0.18	1.00	0.8
Pval	<0.01	0.58	<0.01	<0.01	0.01	<0.01	0.02	<0.01	0.03	<0.01	0.25	<0.01	0.98	<0.01	0.46	0.73	0.02	0.52	-	<0.01
TGFB1	0.93	-0.32	0.98	0.94	0.28	0.94	0.53	0.97	0.16	0.81	-0.59	0.79	-0.54	-0.5	-0.18	-0.22	-0.44	0.64	0.8	1.00
Pval	<0.01	0.24	<0.01	<0.01	0.3	<0.01	0.04	<0.01	0.57	<0.01	0.02	<0.01	0.04	0.06	0.52	0.43	0.1	0.01	<0.01	-

Table S1. Correlation matrix for larval gene expression FC in *Sander lucioperca*. Grey numbers mark P >0.05. Relation to age is provided by degree-days (dd).

	dd	***BMP4***	***COL1A1***	***COL1A2***	***EN2***	***IGF1***	***IGF2***	***MEF2A***	***MRF4***	***MSTN***	***MSX1***	***MUSTN1***	***MYF5***	***MYH6***	***MYOD***	***MYOG***	***PAX3***	***PAX7A***	***PGC1A***	***TGFB1***
dd	1.00	0.9	-0.11	-0.85	0.77	0.81	0.98	0.71	0.41	0.32	0.44	0.34	0.4	-0.68	0.63	-0.63	0.97	0.92	0.8	0.94
Pval	-	0.01	0.82	0.02	0.04	0.03	<0.01	0.07	0.36	0.49	0.32	0.45	0.37	0.09	0.13	0.13	<0.01	<0.01	0.03	<0.01
BMP4	0.9	1.00	0.3	-0.6	0.95	0.8	0.91	0.75	0.71	0.67	0.77	0.58	0.73	-0.85	0.82	-0.64	0.78	0.85	0.95	0.95
Pval	0.01	-	0.52	0.15	<0.01	0.03	<0.01	0.05	0.07	0.1	0.04	0.17	0.06	0.01	0.02	0.12	0.04	0.02	<0.01	<0.01
COL1A1	-0.11	0.3	1.00	0.57	0.5	0.27	-0.1	0.4	0.83	0.65	0.83	0.8	0.86	-0.49	0.67	-0.11	-0.32	-0.07	0.49	0.13
Pval	0.82	0.52	-	0.18	0.25	0.56	0.83	0.37	0.02	0.11	0.02	0.03	0.01	0.27	0.1	0.81	0.49	0.88	0.27	0.78
COL1A2	-0.85	-0.6	0.57	1.00	-0.4	-0.5	-0.86	-0.28	0.11	-0.03	0.04	0.15	0.11	0.38	-0.15	0.56	-0.9	-0.72	-0.41	-0.7
Pval	0.02	0.15	0.18	-	0.37	0.25	0.01	0.55	0.81	0.95	0.93	0.75	0.82	0.4	0.75	0.19	0.01	0.07	0.36	0.08
EN2	0.77	0.95	0.5	-0.4	1.00	0.85	0.78	0.78	0.81	0.69	0.87	0.75	0.83	-0.92	0.89	-0.63	0.6	0.69	0.99	0.91
Pval	0.04	<0.01	0.25	0.37	-	0.02	0.04	0.04	0.03	0.08	0.01	0.05	0.02	<0.01	0.01	0.13	0.16	0.09	<0.01	<0.01
IGF1	0.81	0.8	0.27	-0.5	0.85	1.00	0.75	0.86	0.59	0.25	0.61	0.76	0.59	-0.87	0.85	-0.77	0.66	0.61	0.9	0.88
Pval	0.03	0.03	0.56	0.25	0.02	-	0.05	0.01	0.16	0.59	0.14	0.05	0.16	0.01	0.02	0.04	0.11	0.15	0.01	0.01
IGF2	0.98	0.91	-0.1	-0.86	0.78	0.75	1.00	0.61	0.39	0.39	0.46	0.28	0.4	-0.68	0.58	-0.65	0.95	0.9	0.79	0.95
Pval	<0.01	<0.01	0.83	0.01	0.04	0.05	-	0.14	0.39	0.38	0.3	0.54	0.37	0.09	0.17	0.12	<0.01	0.01	0.03	<0.01
MEF2A	0.71	0.75	0.4	-0.28	0.78	0.86	0.61	1.00	0.77	0.29	0.68	0.8	0.72	-0.69	0.9	-0.39	0.6	0.69	0.86	0.76
Pval	0.07	0.05	0.37	0.55	0.04	0.01	0.14	-	0.04	0.53	0.1	0.03	0.07	0.08	0.01	0.38	0.15	0.08	0.01	0.05
MRF4	0.41	0.71	0.83	0.11	0.81	0.59	0.39	0.77	1.00	0.75	0.96	0.87	0.99	-0.71	0.92	-0.27	0.23	0.48	0.82	0.56
Pval	0.36	0.07	0.02	0.81	0.03	0.16	0.39	0.04	-	0.05	<0.01	0.01	<0.01	0.08	<0.01	0.56	0.61	0.28	0.02	0.19
MSTN	0.32	0.67	0.65	-0.03	0.69	0.25	0.39	0.29	0.75	1.00	0.84	0.46	0.8	-0.62	0.59	-0.3	0.18	0.39	0.61	0.44
Pval	0.49	0.1	0.11	0.95	0.08	0.59	0.38	0.53	0.05	-	0.02	0.29	0.03	0.13	0.16	0.51	0.71	0.39	0.15	0.32
MSX1	0.44	0.77	0.83	0.04	0.87	0.61	0.46	0.68	0.96	0.84	1.00	0.85	0.99	-0.79	0.9	-0.42	0.24	0.45	0.85	0.62
Pval	0.32	0.04	0.02	0.93	0.01	0.14	0.3	0.1	<0.01	0.02	-	0.02	<0.01	0.03	0.01	0.35	0.6	0.31	0.01	0.13
MUSTN1	0.34	0.58	0.8	0.15	0.75	0.76	0.28	0.8	0.87	0.46	0.85	1.00	0.87	-0.78	0.93	-0.48	0.12	0.25	0.79	0.52
Pval	0.45	0.17	0.03	0.75	0.05	0.05	0.54	0.03	0.01	0.29	0.02	-	0.01	0.04	<0.01	0.28	0.79	0.59	0.03	0.23
MYF5	0.4	0.73	0.86	0.11	0.83	0.59	0.4	0.72	0.99	0.8	0.99	0.87	1.00	-0.74	0.91	-0.33	0.21	0.45	0.83	0.58
Pval	0.37	0.06	0.01	0.82	0.02	0.16	0.37	0.07	<0.01	0.03	<0.01	0.01	-	0.06	<0.01	0.47	0.65	0.31	0.02	0.18
MYH6	-0.68	-0.85	-0.49	0.38	-0.92	-0.87	-0.68	-0.69	-0.71	-0.62	-0.79	-0.78	-0.74	1.00	-0.86	0.83	-0.48	-0.49	-0.91	-0.8
Pval	0.09	0.01	0.27	0.4	<0.01	0.01	0.09	0.08	0.08	0.13	0.03	0.04	0.06	-	0.01	0.02	0.27	0.27	<0.01	0.03
MYOD	0.63	0.82	0.67	-0.15	0.89	0.85	0.58	0.9	0.92	0.59	0.9	0.93	0.91	-0.86	1.00	-0.56	0.45	0.57	0.94	0.75
Pval	0.13	0.02	0.1	0.75	0.01	0.02	0.17	0.01	<0.01	0.16	0.01	<0.01	<0.01	0.01	-	0.19	0.31	0.18	<0.01	0.05
MYOG	-0.63	-0.64	-0.11	0.56	-0.63	-0.77	-0.65	-0.39	-0.27	-0.3	-0.42	-0.48	-0.33	0.83	-0.56	1.00	-0.49	-0.31	-0.64	-0.66
Pval	0.13	0.12	0.81	0.19	0.13	0.04	0.12	0.38	0.56	0.51	0.35	0.28	0.47	0.02	0.19	-	0.27	0.5	0.12	0.11
PAX3	0.97	0.78	-0.32	-0.9	0.6	0.66	0.95	0.6	0.23	0.18	0.24	0.12	0.21	-0.48	0.45	-0.49	1.00	0.93	0.64	0.84
Pval	<0.01	0.04	0.49	0.01	0.16	0.11	<0.01	0.15	0.61	0.71	0.6	0.79	0.65	0.27	0.31	0.27	-	<0.01	0.12	0.02
PAX7A	0.92	0.85	-0.07	-0.72	0.69	0.61	0.9	0.69	0.48	0.39	0.45	0.25	0.45	-0.49	0.57	-0.31	0.93	1.00	0.72	0.84
Pval	<0.01	0.02	0.88	0.07	0.09	0.15	0.01	0.08	0.28	0.39	0.31	0.59	0.31	0.27	0.18	0.5	<0.01	-	0.07	0.02
PGC1A	0.8	0.95	0.49	-0.41	0.99	0.9	0.79	0.86	0.82	0.61	0.85	0.79	0.83	-0.91	0.94	-0.64	0.64	0.72	1.00	0.92
Pval	0.03	<0.01	0.27	0.36	<0.01	0.01	0.03	0.01	0.02	0.15	0.01	0.03	0.02	<0.01	<0.01	0.12	0.12	0.07	-	<0.01
TGFB1	0.94	0.95	0.13	-0.7	0.91	0.88	0.95	0.76	0.56	0.44	0.62	0.52	0.58	-0.8	0.75	-0.66	0.84	0.84	0.92	1.00
Pval	<0.01	<0.01	0.78	0.08	<0.01	0.01	<0.01	0.05	0.19	0.32	0.13	0.23	0.18	0.03	0.05	0.11	0.02	0.02	<0.01	-