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Characterization of *igf1* and *igf2* genes during maraena whitefish (*Coregonus maraena*) ontogeny and the effect of temperature on embryogenesis and *igf* expression

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ABSTRACT

The insulin-like growth factors IGF-1 and IGF-2 play important roles in the growth, development, and metabolism of teleost fish. We isolated cDNA sequences of *igf1*, and *igf2* genes from maraena whitefish. We quantified the mRNA and protein expressions of IGFs in different tissues of marketable juvenile maraena whitefish. Moreover, we analyzed the gene expression profiles during maraena whitefish development from unfertilized egg to fingerling and examined the effect of incubation temperature on *igf1*, and *igf2* gene expression during embryonic and early larval development.

Transcripts encoding IGF-1 or IGF-2 were detected in all tested tissues, with the greatest abundance in the liver. We measured higher *igf2* than *igf1* copy numbers in all tissues and at all developmental stages examined, even at advanced juvenile stages. Using the Western blot technique, we demonstrated that several isoforms of IGF-1 are expressed in the liver and gills but not in muscle tissue, indicating tissue-specific protein expression of IGF-1. We observed an accelerated embryonic development with increasing temperature, resulting in shortened hatching periods. Out of the three tested temperatures, we observed the highest hatching rate, larval hatching size, and larval growth at 6 °C. At 9 °C, hatching rate, larval hatching size and larval growth were reduced compared to the values we observed at 4 °C and 6 °C, since incubation temperature might have exceeded the optimum. To our knowledge, our data show for the first time that both *igf1* and *igf2* expression were upregulated due to elevated incubation temperature within embryonic development of fish. Further, we found significantly higher *igf* expression for the best-developing larvae (6 °C group) at specific life stages of maraena whitefish.

1. Introduction

The insulin-like growth factors IGF-1 and IGF-2 play important roles in growth, development and metabolism in all vertebrates [1–4]. Both insulin-like growth factors are single-chain polypeptide hormones with structural homology to pro-insulin, and both are highly conserved between species [5–7]. IGFs mediate their biological actions through binding to membrane bound IGF-1 receptor (IGF-1R) [8]. Additionally, IGF binding proteins (IGFBPs) both positively and negatively influence IGF effects on cells and modulate their tissue-specific delivery [8]. From a total of six IGF binding proteins known in mammals, four proteins—IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-5—have so far been identified in fish [8–12].

IGF-1 is a mediator of growth hormone (GH) and relevant for postnatal growth, as has been reported for humans, mice, and several fish species [8,13–16]. Furthermore, the GH/IGF-1 axis influences reproduction and osmoregulation and the immune systems of teleost fish [9,17–21]. Within embryological development, IGF-1 also has a major role but mediates its function in a GH-independent manner, as has been shown for transgenic mice [15,16]. IGF-2 in general acts more independently from GH and seems to be crucial for early embryonic growth, as has been demonstrated in mouse embryos [13,22–24].

IGF-1 and IGF-2 are mainly produced in the livers of all vertebrates but are ubiquitously expressed, as they stimulate cell proliferation and DNA synthesis through their mitogenic action [3,8,25,26]. Because of these diverse interactions, IGFs, especially IGF-1, are often discussed for

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their potential as biomarkers, e.g. as a growth indicator for aquaculture [4,27–30].

However, not only is a fast fish growth crucial for successful aquaculture, but the initial steps of egg incubation, hatching, and the onset of feeding are elementary for a successful fish production cycle. In the present study, we analyzed IGF-1 and IGF-2 during pre- and post-natal development and in marketable juvenile maraena whitefish.

In fish in general, and particularly in salmonids, IGF-1 and IGF-2 expression has already been detected in various organs and at all life stages from unfertilized egg to adult [3,23,31–33]. For maraena whitefish (*Coregonus maraena*, Bloch), which is a new salmonid species in aquaculture compared to well domesticated trout, data about IGF-1 and IGF-2 are still missing. Therefore, in the present study, we sequenced the open reading frames (ORFs) of *igf1* and *igf2* genes in maraena whitefish. To get insight into growth regulation mechanisms, we quantified IGF expression levels in different tissues by qPCR and Western blot, during ontogenetic development of maraena whitefish from unfertilized egg to fingerling. Moreover, we analyzed the effect of different incubation temperatures on *igf1* and *igf2* transcript levels during embryonic development and early life stages post-hatching of maraena whitefish larvae, since the mechanisms of temperature-dependent fish development, particularly around hatching, are still poorly understood.

2. Materials and methods

2.1. Rearing and sampling of maraena whitefish

Ethics statement: Animal care and tissue collection processes followed the guidelines of the German Law of Animal Protection and the Animal Care Committee of the Leibniz Institute for Farm Animal Biology (FBN, Dummerstorf, Germany).

Unfertilized and fertilized maraena whitefish eggs, larvae, fry, fingerlings and juvenile fish were collected at the Institute of Fishery in Born, Germany. All samples were flash-frozen in liquid nitrogen immediately and stored at −80 °C until further use.

For every experiment described in the following, water quality was regularly monitored regarding temperature, oxygen supply, pH and nutrient concentration (NO_{2-}, NO_3^-, NH_3).

For the qPCR-based tissue comparison approach (see Section 3.2), 10 different tissues were dissected from four juvenile fish that were reared in a recirculation system until the age of 344 dph, a total weight of $\bar{x}$ = 300.9 ± 88.4 g, and a total length of $\bar{x}$ = 30.6 ± 2.4 cm. The recirculation system was supplied with UV-purified, tempered, brackish water (19.7 ± 1.1 °C) and fish were fed a commercial pelleted diet (Biomar, Inicio Plus, 3 mm) at a daily rate of 0.8% of their biomass.

For assessment of ontogeny (see Section 3.2) eggs and milt were stripped from wild-caught maraena whitefish and artificially fertilized. Then, eggs were transferred to hatching jars (Zuger) supplied with freshwater constantly at 4 °C ($\bar{x}$ = 4.0 ± 0.3 °C) until hatching. After hatching, alevins were reared in temperature-controlled freshwater tanks at 18 °C ($\bar{x}$ = 18.0 ± 0.5 °C). Ad libitum feeding with *Artemia salina* set in around five days post-hatch (dph) and complete yolk sack resorption. 59 dph fingerlings were transferred to flow-through tanks (0.33 m^3) with natural environmental conditions regarding water temperature and quality. Sampling started immediately before egg fertilization and continued up to 60 days post-hatch.

For the analysis of incubation temperature effects on growth and development (see Section 3.4), fertilized eggs were first incubated in hatching jars (Zuger) with freshwater constantly at 4 °C till 22 days post-fertilization (dpf). Then, the spawn was split into three groups, each with a 200 mL volume (calculated average of eggs: $\bar{x}$ = 8514) and reared until hatching in McDonald jars at the following temperatures: 4 °C ($\bar{x}$ = 4.5 ± 0.3), 6 °C ($\bar{x}$ = 6.0 ± 0.3) and 9 °C ($\bar{x}$ = 9.4 ± 0.9). After hatching, larvae were transferred in groups according to their previous incubation temperature to a freshwater recirculation system at 15 °C ($\bar{x}$ = 15.2 ± 0.9) equipped with a biofilter, a drum filter, and UV disinfection. The ad libitum feeding of alevins with *Artemia salina* set in after complete yolk sack resorption around 5 dph. For qPCR analysis sampling started two weeks after allocation of the spawn to the three different temperature groups and continued until four weeks post hatch for each group. Additionally, after hatching five individuals per group were sampled weekly for length determination.

2.2. RNA extraction and cDNA production

For the ontogenetic and temperature-dependent measurements, total RNA was extracted from 16 eggs pooled into four samples for each time point and from four whole larvae, fry, and fingerlings at each time point, respectively. For tissue-specific measurements, RNA was extracted from the brains, hearts, eyes, gonads, gills, livers, spleens, muscles, kidneys, and skin of four juvenile maraena whitefish. For RNA isolation, flash-frozen samples were homogenized individually (for larvae and fry) or pooled (four eggs per time point) in 1 mL TRIzol® Reagent (Invitrogen, Karlsruhe, Germany). The maximum length of fish possible to homogenize as a whole was 30.0 mm. RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase treatment (15 min, at 20–30 °C) to digest and remove genomic DNA. RNA integrity was verified by agarose gel electrophoresis. Quality and quantity were evaluated by a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). 1.5 µg total RNA of every sample were reverse-transcribed using Superscript II™ (Invitrogen, Karlsruhe, Germany), Oligo-d$(T)_{24}$-primers, and random hexamers. The generated cDNA was purified with the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and then diluted in 100 µL nuclease-free water.

2.3. Isolation of igf1 and igf2 sequences

Since maraena whitefish *igf1* and *igf2* gene sequences were unknown so far, gene specific primers were derived from conserved sequence regions of the evolutionary related salmonid species Atlantic salmon (*Salmo salar*; NM_001123623.1; NM_001123647.1) and rainbow trout (*Oncorhynchus mykiss*; NM_001124696.1; NM_001124697.1). Clustal Omega [34,35] was used to align the respective orthologous *igf1* and *igf2* sequences obtained from NCBI GenBank. With the help of Primaclade software [36], one pair of consensus primers, flanking the complete coding region, was designed for each gene. Gene specific primers and cDNA obtained from whole larvae RNA were used to amplify a 677-bp fragment for *igf1* and a 742-bp fragment for *igf2* by PCR. All primers used in this study are listed in Table 1. The PCR was carried out using HotStarTaq Plus DNA polymerase (Qiagen) according to a touchdown-PCR protocol. The PCR program comprised 43 cycles in total, including a 5 min pre-incubation at 95 °C, a denaturing step at 94 °C for 30 s, an annealing step at temperatures decreasing from 68 °C to 59 °C during the first 10 cycles (temperature decline of 1 °C per cycle) for 30 s, and an elongation step at 72 °C for 90 s, followed by 32 cycles with 30 s at 94 °C, 30 s at 60 °C, 90 s at 72 °C, and final elongation at 72 °C for 6 min. The obtained fragments were purified with the High Pure PCR Product Purification Kit (Roche) and then cloned into pGEM-T Easy vectors (Promega, LaJolla, CA, USA). Subsequently, sequencing was carried out independently at least three times with vector-specific primers (SP6 and T7), using the ABI Big Dye® Terminator v3.1 Cycle Sequencing Kit and the ABIPrism DNA sequencer (Applied Biosystems, Darmstadt, Germany).

2.4. Transcript quantification

Tissue-specific mRNA expression of *igf1* and *igf2* was examined by semi-quantitative PCR and qPCR. For this purpose, 1.5 µg of the isolated RNA (per fish and tissue) were used for cDNA synthesis with Superscript II™ (Invitrogen). All primers applied for semi-quantitative