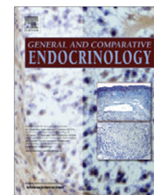
General and Comparative Endocrinology 182 (2013) 41–47

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Developmental transcription of genes putatively associated with growth in two sturgeon species of different growth rate

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ARTICLE INFO

Article history:
Received 30 July 2012
Revised 13 October 2012
Accepted 13 November 2012
Available online 7 December 2012

Keywords:
Growth hormone
Insulin-like growth factor-I
Ghrelin
Vascular endothelial growth factor
mRNA expression
Transcription
Sturgeon

ABSTRACT

In the present study, we surveyed developmental changes in the transcription of growth hormone (*gh*), insulin-like growth factor-I (*igf-I*), ghrelin (*ghrl*) and vascular endothelial growth factor (*vegf*) genes in the largest freshwater fish, European sturgeon (Beluga, *Huso huso*) and compared the same parameters to that of its phylogenically close moderate-sized species, Persian sturgeon (*Acipenser persicus*). The transcripts of *gh*, *igf-I*, *ghrl* and *vegf* were detected at all developmental time-points of Persian sturgeon and Beluga from embryos to juvenile fish. Changes in normalized *gh*, *igf-I*, *ghrl* and *vegf* transcription by using the geometric average of genes encoding ribosomal protein L6 (*RPL6*) and elongation factor (*EF1A*) over the time of development of Persian sturgeon and Beluga were statistically significant ($P < 0.05$). Our results showed that the mRNA expression levels of both *igf-I* and *ghrl* were low during early larval development and then increased significantly to the late larval time-points when larvae started exogenous feeding. In both Beluga and Persian sturgeon, after a low mRNA expression during the embryonic stage, the transcript levels of *vegf* displayed an increasing trend during yolk-sac fry, consistent with organogenesis. The *vegf* level remained constantly high in the time of exogenous feeding. The highest detection of *gh* transcripts coincided with the end of the embryonic stage (hatching time) in Persian sturgeon and 3 days-post-hatching (dph) in Beluga. In Persian sturgeon, the *gh* transcript started to decrease to the rest of the developmental time-points, whereas in Beluga *gh* transcript had a marked second increase from the time of exogenous feeding (20-dph). This Beluga specific increase in *gh* transcription may be associated with the marked growth rate and extraordinary size of this fish species.

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1. Introduction

Growth and development in fish, as in all vertebrates, are governed through the orderly release of hormones from the neuroendocrine system, which integrates environmental, physiological, and genetic information [46]. In fish, as in mammals, the endocrine control of growth works through the growth hormone (GH)–insulin-like growth factor (IGF) axis [40]. GH plays an essential role in the regulation of growth and development by promoting cell division, differentiation, and enlargement. The importance of the GH as a potential growth-promoting agent has long been recognized, and GH administration has been shown to accelerate growth rate in a number of animals, especially fish [1,9]. The early appearance of GH in larval fish also suggests its important role in growth. In fish, GH also influences osmoregulation and reproduction [27]. It has been known that GH stimulates growth directly by increasing DNA and protein synthesis and lipolysis in muscle and indirectly by inducing the production and release of a mitogen, IGF-I, which is produced both by the liver and by most peripheral tissues [7,46]. In vertebrates, IGF-I is the major regulator of growth and exerts its effects on cells through binding to the IGF-I receptor [43]. IGF-I acts on muscle, liver, adipose tissue, intestine, brain and most tissues to enhance growth [7]. In general, GH regulates IGF-I mRNA expression [7] and both GH and IGF-I are known to play a critical role during early larval development of fish [12].

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0016-6480/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygcen.2012.11.013

Ghrelin, a 28-amino acid peptide, is known to be an endocrine regulator of GH secretion and appetite [15,25]. As a hormone, ghrelin is secreted from the gastric endocrine cells, and stimulates GH release from the pituitary through growth hormone secretagogue receptor 1a (GHS-R1a) [25]. Such a stimulatory action has been observed in mammals [25,35,37,49,51], chicken [23], bullfrog [20], tilapia [22,46] and rainbow trout [21]. In a teleost fish, halibut, it has been demonstrated that ghrelin is expressed prior to exogenous feeding during larval development [33].

Vascular endothelial growth factor (VEGF), a selective mitogen for endothelial cells, is an important growth factor for vascular development and angiogenesis [28]. VEGF has emerged as the single most important regulator of blood vessel formation [18]. Various processes of early stage vascular development including vasculogenesis, large vessel formation (e.g., of the dorsal aorta), capillary sprouting, and the remodelling of the yolk sac vasculature are affected by VEGF [6]. It has been also found that VEGF is expressed throughout the zebrafish embryonic development [29] and it has been suggested that VEGF can not only stimulate endothelial cell differentiation but also hematopoiesis in zebrafish embryo by promoting the formation of terminally differentiated red blood cells [28].

The growth of fish is quite different from that of human and other warm-blooded animals. They do not stop their growth and reach a defined adult size at a certain age. Instead, most fish continue to grow until they die. This makes them a good model for understanding the hormonal regulation of growth [34]. Although fish grow until they die, they do not grow at the same rate. This makes it possible to investigate factors that may be related to growth regulation by using phylogenically close relatives differing in their growth. Such a species pair is European sturgeon (Beluga, *Huso huso*), which is the largest present-day sturgeon: a length of 5.5 m and a weight of 1000 kg has been reported for the species in Caspian Sea [26]. On the other hand, Persian sturgeon (*Acipenser persicus*) has a much more modest growth rate. It is well known that the growth intensity and length parameters in Beluga are significantly higher than any other sturgeon species during the larval and early juvenile development [47].

In this study we monitored the transcription of genes encoding growth-regulating hormones. While it was not possible to follow the developmental changes of the protein products because of the lack of available resources, transcription and transcriptional changes are a prerequisite for any effects at hormonal level. Because of the importance of the GH/IGF-I system in growth regulation of fish, especially during early larval period, and its association to ghrelin, we chose *gh*, *igf-I* and *ghrl* as genes in which developmental changes in transcription were studied. Although we did not measure the growth and muscle mass of the two species during development, we also looked at the transcription of the *vegf* gene, as the formation of vasculature in, e.g., the muscle, is associated with increased VEGF level.

2. Materials and methods

2.1. Animals and sampling protocol

Fertilized eggs of Persian sturgeon and Beluga were obtained from two artificially spawned sturgeon broodstocks located in North of Iran. Persian sturgeon samples were collected from Shahid Beheshti Artificial Sturgeon Propagation and Rearing Center located in Rasht and the samples of Beluga were obtained from Shahid Marjani Artificial Sturgeon Propagation and Rearing Center located in Aghalla, Iran. All the fish used in the present study were progeny of the artificial spawning of wild sturgeon broodstock. The Beluga and Persian sturgeon larvae hatched 7 and 6 days after fertilization, respectively, were reared in ferroconcrete and fiberglass tanks and fed with newly hatched Artemia five times a day. The Persian sturgeon and Beluga larvae started exogenous feeding at day 15 and 20 days, respectively. Sturgeon samples at ten developmental stages including 2 days before hatch (eyed eggs), newly hatched larvae (0), and larvae 1, 3, 6, 10, 15, 20, 25 and 50 days-post-hatching (dph) were collected. The time of feeding and sampling was synchronous in two species. Water temperature varied from 9 °C in March to 15 °C in May 2009. All individuals were killed by an overdose of tricaine methanesulfonate (MS-222), deep-frozen in liquid nitrogen as soon as they were collected and stored in a −80 °C freezer until RNA extraction.

2.2. Total RNA extraction and cDNA synthesis

The procedure of RNA extraction, control of RNA quality, measuring of RNA concentration and cDNA synthesis have earlier been described by Akbarzadeh et al. [2]. Briefly, whole larvae or eggs were placed in the recommended proportions of Tri Reagent and homogenized using a Qiagen Tissue Lyser. Total RNA was isolated from six individuals at each stage described above ($n = 6$) using a Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), treated with Invitrogen DNase I (Invitrogen, CA, USA) and cleaned up using the Nucleospin RNA II kit (Macherey–Nagel, Düren, Germany) in accordance with the manufacturers' instructions. The quality of RNA samples was evaluated by electrophoresis on a 1.5% agarose gel and their concentration was determined by a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) reading at 260/280 nm. Every sample was measured in duplicate and mean value was used. One microgram of total RNA was used to synthesize first-strand cDNAs using a DyNAmo™ cDNA Synthesis Kit (Finnzymes, Espoo, Finland) for RT-PCR, following the manufacturer's instructions and a mixture of oligo-dT as primer.

2.3. Quantitative real-time PCR (qPCR)

Quantitative real-time PCR was run on 7900 HT Fast Real-Time PCR System (Applied Biosystems) with Fermentas Maxima SYBR Green qPCR Master Mix (2×) (Fermentas) and all primers at 100 nM using standard protocol: initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. All reactions were run in triplicate. The mRNA expression levels of genes were recorded as Ct values that corresponded to the number of cycles at which the fluorescence signal can be detected above a threshold value. Baseline and threshold for Ct calculation were set manually using the SDS RQ manager v 1.2 (Applied Biosystems). Standard curves were constructed from dilution series of pooled cDNA (including seven dilutions from 1/10 to 1/2000), and the PCR efficiency was calculated using the equation $E\% = (10^{1/\text{slope}} - 1) \times 100$ [42].

The fold change in relative mRNA expression of *gh*, *igf-I*, *ghrl* and *vegf* was calculated by the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen [31]. The difference between Ct values of the reference genes and the target genes was calculated for each mRNA by taking the mean Ct of triplicate reactions and subtracting the mean Ct of triplicate reactions for the reference RNA measured on an aliquot from the same RT reaction ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$). All samples were then normalized to the ΔCt value of a calibrator sample to obtain a ΔΔCt value ($\Delta Ct_{\text{target}} - \Delta Ct_{\text{calibrator}}$). Among the developmental time-points, the sample with the lowest Ct value was chosen as the calibrator sample in order to evaluate the differential mRNA expression of target gene.

2.4. Primer design

The qPCR primers for *gh* and *igf-I* were designed based on the sequences from sturgeon available in the GenBank (Accession Nos.: HQ166628.1, AY941176.1, FJ428829.1, EU599640.2, GU325

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