Growth hormone endocrinology of Atlantic salmon (*Salmo salar*): pituitary gene expression, hormone storage, secretion and plasma levels during parr–smolt transformation

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Abstract

A number of studies on the Atlantic salmon (*Salmo salar*), have reported changes in plasma GH during parr–smolt transformation, but there is a lack of information about the endocrinology of the GH system during this process. In order to elucidate the mechanisms underlying these changes in plasma GH levels during the parr–smolt transformation of Atlantic salmon, GH mRNA expression in the pituitary was studied together with total pituitary GH content, *in vitro* GH secretion rate and plasma GH and IGF-I levels. Atlantic salmon were kept in outside tanks, under natural condition from early February until late June. Approximately three times a month fish were killed and pituitaries and blood were sampled for investigation. Further, pituitaries were moved to the laboratory for *in vitro* GH secretion studies. The results show that the GH system is first activated by an increase in GH secretion

rate, which leads to an increase in plasma GH levels and causes a drop in the total GH content of the pituitary. This drop in pituitary GH content is later reversed by an increased GH synthesis seen as an increase in GH mRNA expression. Maximal activation of the GH system is seen to occur in early May, when plasma IGF-I levels reach highest levels, after which a certain deactivation of the GH system takes place. The data show that plasma levels of GH are to a large extent regulated by the secretion rate from the pituitary, although changes in the GH clearance rate are also likely to take place and influence the plasma GH levels. The study further underlines the significant role that the GH–IGF-I axis plays in the parr–smolt transformation of the Atlantic salmon.

Journal of Endocrinology (2001) **170**, 227–234

Introduction

For growth hormone (GH), as for other endocrine factors, plasma levels are the result of secretion and clearance rates. Changes in circulating levels can be due to changes in either parameter. Thus, although increased plasma GH levels in salmonid fish are generally interpreted as the result of increased pituitary secretion, the extremely high GH levels during growth retardation have instead been explained by a down-regulation of hepatic GH receptors and lower clearance rate (Gray *et al.* 1990). Likewise, a change, e.g. a decrease, in pituitary GH content can be interpreted either as being the result of decreased synthesis rate or due to increased secretion rate. Thus, a measurement of a single GH-related parameter may not reveal if the GH system is activated or not. Data that help elucidate the causal relationships of GH dynamics, from gene expression, pituitary storage and secretion, and circulating levels, to peripheral physiological actions, are scarce. In

salmonids, plasma GH levels usually increase transiently following seawater (SW) entry (see Björnsson 1997). In coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*O. mykiss*), both the clearance rate and the calculated secretion rate of GH increased after transfer to SW (Sakamoto *et al.* 1990, 1991). Also, in amago salmon (*O. masou*), the response of GH mRNA to SW exposure appears to be related to the development of preparatory mechanisms for SW entry, indicating that GH mRNA increases before SW entry (Yada *et al.* 1992).

The parr–smolt transformation (smoltification) of anadromous salmonids is a very good model for studying the dynamics of the GH endocrine system. It is a well-defined life-history stage that involves profound physiological and biochemical changes with enhanced SW adaptability as one of the characteristic features (McCormick & Saunders 1987, Hoar 1988). Plasma GH levels increase significantly during smoltification and the hormone is considered to be one of the main regulators of

this developmental process, improving hypoosmoregulatory ability, as well as regulating growth and behaviour (Björnsson 1997). The smoltification process represents therefore a period where the GH system is highly activated.

The actions of GH may in part be mediated by insulin-like growth factor I (IGF-I) according to the dual effector hypothesis of action (Isaksson *et al.* 1987, Daughaday & Rotwein 1989), and/or have a role in a feed-back control of GH secretion as has recently been demonstrated in mice (Sjögren *et al.* 1999). Although IGF-I is expressed locally in many tissues, in mice it has recently been shown that the circulating IGF-I levels are primarily of hepatic origin (Sjögren *et al.* 1999, Yakar *et al.* 1999).

Data on fish reveal that the GH-IGF-I axis controlling growth has been highly conserved during vertebrate evolution (Bern *et al.* 1991, Siharath & Bern 1993, Duan 1997). In salmonids, the highest tissue density of GH receptors is in the liver (Sakamoto & Hirano 1991), and plasma IGF-I levels increase following GH treatment (Moriyama 1995). During salmon smoltification, liver IGF-I mRNA levels increase (Duan *et al.* 1995) and IGF-I along with GH can improve salinity tolerance (McCormick 1996), further indicating the synergy of these two hormones. For salmonids, as for other vertebrates, the circulating levels of IGF-I are thus likely to be a good indicator of peripheral GH action.

In order to elucidate some of the mechanisms underlying changes in circulating GH levels, the parr-smolt transformation of the Atlantic salmon was used as a model. A concomitant assessment of pituitary GH gene expression, and GH storage and secretion, was made to establish the chain of events leading to the observed changes in plasma GH. Further, plasma IGF-I levels were assessed as a measure of the peripheral action of GH, and to increase the understanding of the interplay between these two factors during this crucial developmental stage of the Atlantic salmon.

Materials and Methods

Fish and holding conditions

Juvenile Atlantic salmon, *Salmo salar*, (mean size 43 ± 0.7 g (S.E.M.) body weight (BW) and 16 ± 0.1 cm fork length) were obtained from and kept at a local hatchery, Fiskerman i Laxforsen AB, Anneberg, Sweden. At the start of the experimental period, on 4 January 1998, 250 salmon were transferred to each of two replicate 1 m^3 outdoor tanks under a natural photoperiod. The tanks were supplied by water from a nearby stream at ambient temperature, rising gradually from 2 to 10°C during the experimental period. The fish were fed commercial dry pellets, according to a feeding schedule used by the hatchery (EWOS (Livingston, UK), aquaculture feeding tables).

Experimental design

Sampling was conducted on 12 occasions from 4 February to 30 June; every second week during February and March, and then every tenth day. On each sampling date, from 1000 to 1600 h, 30 fish were sampled, 15 from each tank (some of the fish were sampled for purposes other than reported in this study). On each occasion, the fish were randomly netted (three times there were four fish and once three fish from each tank) and immediately killed by an overdose of anaesthetic (0.05% 2-phenoxyethanol/l; Sigma Chemical Co., St Louis, MO, USA). Then, the fish were weighed (wet weight) and measured (fork length), after which blood was drawn from the caudal vessels using 1 ml heparinised syringes. The blood was centrifuged at 3000 g for 5 min to obtain plasma, which was aliquoted, frozen immediately on dry ice and then stored at -80°C until analyses. The pituitaries were then dissected out. Five and eight pituitaries were frozen in liquid nitrogen and stored at -80°C for later analyses of GH mRNA and total GH content respectively. Another eight pituitaries were placed in ice-cold Hanks' balanced salt solution (HBSS) and transferred to the laboratory for measurement of GH secretion rate.

Plasma levels of GH and IGF-I

Plasma GH levels were measured by an RIA specific for salmon GH (Björnsson *et al.* 1994) and IGF-I levels were assessed using an RIA protocol by Moriyama *et al.* (1994).

In vitro perfusions

On each sampling date pituitaries were obtained from eight fish. Each pituitary was transferred to a perfusion chamber, made from a part of a 1 ml syringe barrel, containing 0.5 ml modified HBSS (containing 15 mM Hepes and 0.1% BSA (Sigma)) and placed between two layers of (approximately 0.05 g) microcarrier beads (Cytodex 1; Sigma) pre-swollen in HBSS. A temperature-controlled cooling mantle was placed around each perfusion chamber to keep the pituitaries at 10°C (Fig. 1).

The pituitaries were perfused with HBSS at a constant rate of 15 ml/h using a peristaltic pump. The reservoir containing HBSS solution was placed at the same height as the perfusion chambers to avoid pressure changes. In order to establish a reliable measure of secretion rate, the pituitaries were perfused for 3 h. The perfusate from the first hour, during which a base-line secretion rate was established (Ágústsson *et al.* 2000, Ágústsson & Björnsson 2000), was discarded. Five-minute fractions were collected during the last 2 h of the perfusion, frozen and stored at -80°C until analysis. For each pituitary, the secretion rate was calculated as the mean of all fractions (ng GH/pituitary per min), and then, a group mean was calculated for the eight individuals. No secretion rate measurements

Figure 1 *In vitro* perifusion chamber for measurement of whole pituitary GH secretion. The chamber is made from a part of a 1 ml syringe barrel containing 0.5 ml modified HBSS, with 15 mM Hepes and 0.1% BSA. The pituitary is placed between two layers of (approximately 0.05 g) microcarrier beads pre-swollen in HBSS. Each syringe barrel is then placed inside a cooling mantle with recirculating water to keep an optimum temperature of 10 °C.

could be carried out in late March and early May due to technical problems.

Pituitary GH content

Pituitaries from 8–20 fish from each sampling date were sonicated separately in 200 µl RIA buffer. After centrifugation, the supernatant was serially diluted to reach a valid GH detection range. GH levels in plasma (ng/ml), pituitary homogenate (recalculated as ng/pituitary per g BW) and pituitary perifusion fluid (ng/pituitary per min) were analysed by a specific salmon GH RIA (Björnsson *et al.* 1994).

GH gene expression

Pituitaries from four to six fish from each sampling date were used for quantification of GH mRNA. Total RNA was extracted from individual pituitaries using an RNA purification kit (Isogen; Wako Chemical, Osaka, Japan). All pituitaries were extracted at the same time to avoid procedure variability among the groups. Extracted RNA samples were dissolved in 10 µl ultra pure water and stored at –80 °C until Northern blot analysis. Oligonucleotide probes (40 mers) for salmon GH corresponding to nucleotides 60–99 of the GH I cDNA of rainbow trout, were labelled at the 3′-end with [³²P]dATP, using a labelling kit

(N4020; Amersham, Arlington Heights, IL, USA). Probe specificity was validated by Northern blot analysis and by competition tests used in *in situ* hybridisation studies (Yada *et al.* 1991, 1992). For Northern blot analysis, 1 µl for each of the total RNA samples (containing 5.6 µl RNA reconstituted in 0.5% SDS) was electrophoresed through a 1.4% agarose-formaldehyde gel and transferred on to a nylon membrane (Hybond-N+; Amersham) by capillary blotting (Sambrook *et al.* 1989). The RNA was covalently attached to the membrane by baking at 80 °C for 2 h and by UV cross-linking. Following prehybridisation, the membranes were hybridised with the probe at 55 °C for 18 h according to the method of Sambrook *et al.* (1989). The membranes were washed in 2 × SSC and 0.1% SDS at room temperature for 2 × 10 min. They were washed again with 1 × SSC containing 0.1% SDS at 65 °C for 2 × 20 min, 0.1 × SSC containing 0.1% SDS for 20 min at 65 °C, and then rinsed at room temperature. Internal standard (a pooled pituitary GH mRNA sample) was used in all gels to normalise the relative amount of the GH mRNA among blots. Serial dilutions of the RNA demonstrated linearity between the amount of RNA and the hybridisation signals obtained (data not shown). The membranes were exposed to imaging plate (Fuji imaging plate-Bas III; Fuji Film, Tokyo, Japan). Intensity of the hybridisation signals was assessed with an Auto Image Analyser (Bas 2000; Fuji Film) and GH mRNA was

represented in arbitrary units per pituitary. Molecular sizes were estimated relative to migration of RNA size marker (Toyobo, Osaka, Japan).

Calculations and statistics

Condition factor (CF) was calculated from BW (g) and fork length (cm) according to the equation $(BW/fork\ length^3) \times 100$.

The duplicates were compared using one-way ANOVA. As no significant differences existed, these were grouped together for further analysis. All data were log-transformed in order to obtain homoscedasity, and then analysed using one-way ANOVA followed by a Student–Newman–Keuls (SNK) *post-hoc* test. Data are presented as means $\pm$ S.E.M.

Results

Growth and morphological changes

During the study, the fish increased from 14.8 ± 0.2 to 18.3 ± 0.2 cm in length and from 36.7 ± 1.3 to 62.7 ± 2.5 g in weight, with most of the growth occurring in May and June (Table 1). No change in CF was observed until a significant decrease occurred in early May ($P<0.05$). This decrease was reversed in late May followed by another decrease towards the end of the experiment (Table 1).

The fish exhibited a gradual loss of parr marks and increasing silvering during the experiment (data not shown).

Pituitary GH mRNA expression

In the pituitaries, 1.7 kb transcripts for GH were detected. From being close to average in early February until mid-March, the GH mRNA expression decreased gradually during April to as low as 15% of average ($P<0.05$; Fig. 2a). From late April until mid-May, the mRNA expression increased from 15 to 132% ($P<0.05$; Fig. 2a), after which it decreased towards the average expression rate in June and July.

Pituitary GH content

No significant changes were observed in the total pituitary GH content during the first part of the study. From late March to early April, there was a significant increase in the pituitary GH content, from 0.35 ± 0.07 up to 6.31 ± 0.59 μ g/ml ($P<0.05$; Fig. 2b). In early May, there was a significant decrease compared with the preceding measurement ($P<0.05$), before the pituitary GH content increased again (Fig. 2b).

GH secretion in vitro

The pituitary GH secretion was low during the first part of the study until mid-April. From late April (2.47 ± 0.59 /ng

Table 1 BW, fork length and CF (means $\pm$ S.E.M.) of 1+ year aged Atlantic salmon during parr–smolt transformation under ambient temperature and photoperiod conditions

	4 February	27 February	12 March	26 March	5 April	15 April	24 April	5 May	13 May	25 May	10 June	30 June
Weight (g)	36.7 ± 1.25	33.1 ± 1.54	34.8 ± 1.42	35.2 ± 1.45	39.0 ± 1.35	42.2 ± 1.59	38.2 ± 1.61	42.9 ± 2.29	45.3 ± 2.49	48.1 ± 3.50	55.6 ± 2.71	62.7 ± 2.47
Length (cm)	14.8 ± 0.19	14.6 ± 0.24	14.8 ± 0.21	14.8 ± 0.21	15.3 ± 0.19	15.6 ± 0.20	15.3 ± 0.22	15.8 ± 0.30	16.5 ± 0.29	16.2 ± 0.44	17.4 ± 0.30	18.3 ± 0.24
CF	1.11 ± 0.01	1.05 ± 0.01	1.07 ± 0.02	1.07 ± 0.01	1.07 ± 0.01	1.09 ± 0.01	1.04 ± 0.01	1.05 ± 0.01	0.99 ± 0.02	1.08 ± 0.02	1.04 ± 0.03	1.01 ± 0.02

Figure 2 The GH-IGF-I endocrinology of 1+ year aged Atlantic salmon during parr-smolt transformation under ambient temperature and photoperiod conditions. (a) Pituitary GH mRNA levels ($n=4-6$). (b) Pituitary GH content ($n=8-20$). (c) *In vitro* GH secretion ($n=8$). (d) GH plasma levels ($n=30$). (e) IGF-I plasma levels ($n=30$). Data bars are means with vertical lines indicating S.E.M. Data were analysed using one-way ANOVA followed by a *post-hoc* SNK test. Within each histogram, bars not sharing a superscript letter differ significantly ($P<0.05$).

ml) the GH secretion starts to increase, reaching significantly elevated levels in mid-May (17.2 ± 2.54 ng/ml). After this, a significant decrease occurred in late May ($P < 0.05$; Fig. 2c).

Plasma GH levels

No changes were observed in plasma levels during winter and early spring. Then, two peaks in plasma GH levels were observed (Fig. 2d), the first in late April ($P < 0.05$), and a second in mid-May, being significantly higher than the previous one ($P < 0.05$; Fig. 2d). After these peaks, the plasma GH levels decreased before increasing again in June (Fig. 2d).

Plasma IGF-I levels

Two distinct peaks in plasma IGF-I levels were observed during the experiment. The first significant increase ($P < 0.05$; Fig. 2e) was observed in mid-March with a drop in late March. In early April, an increase in IGF-I levels was observed, becoming significant ($P < 0.05$; Fig. 2e) in early May. During May and until early June no significant changes were observed and the levels stayed high. In late June, the levels started to decrease and reached levels as seen in early April (Fig. 2e).

Discussion

During the parr-smolt transformation of the Atlantic salmon, a frequently observed feature is that the plasma GH profile has two or more distinct peaks (Prunet *et al.* 1989, Young *et al.* 1989a,b, Stefansson *et al.* 1991, McCormick *et al.* 1995, Björnsson *et al.* 2000). It has been speculated that this is due to interplay between GH secretion rate and the metabolic clearance rate of the hormone (Björnsson *et al.* 2000), but data explaining this have been lacking.

As the GH secretion rate is measured *in vitro*, the complex hypothalamic and peripheral feed-back control on GH secretion *in vivo* is removed. Thus, the obtained data may not fully represent the *in vivo* secretion rate, but rather the basal 'uncontrolled' secretion rate. Accepting that this is still an important component of the *in vivo* secretory activity, the present study indicates that the initial activation of the GH system during mid- to late April, when plasma GH levels start to increase, is caused by an increase in the GH secretion rate. This GH appears to come from pituitary stores, rather than new GH synthesis, as GH mRNA levels do not increase during this period. A likely reason why the GH content does not decline significantly is that the amount secreted is minor in relation to the total amount of GH stored.

From late April to mid-May, a second phase in the activation of the GH system is initiated, as the GH secretion rate continues to rise rapidly, in parallel with an

increased GH gene expression. During this period, there is first a decline and then a rise in pituitary GH content, indicating that initially the secretion rate is in excess of the GH synthesis rate, but from early May, there is enough *de novo* GH synthesis to replace the GH secreted as well as increase pituitary GH content. Although a measurement of secretion rate is not available for early May, the increased gene expression and decreased pituitary content suggest that secretion is increased from late April to mid-May. It is therefore somewhat surprising that plasma GH levels decline temporarily in early May, before increasing again. The result is a typical 'split-peak' plasma GH profile as often seen during salmon smoltification (Prunet *et al.* 1989, Young *et al.* 1989a,b, Stefansson *et al.* 1991, McCormick *et al.* 1995, Björnsson *et al.* 2000). It has been speculated that this is due to an increased metabolic clearance rate of GH as a result of increased GH receptor expression in peripheral tissues, representing a third stage in the activation of the GH system. Although no data exist on GH receptor densities or GH clearance rate during smoltification, the process is a pre-adaptation to life in SW, and SW transfer of rainbow trout has been shown to increase GH clearance rate (Sakamoto *et al.* 1990).

From mid- to late May, a deactivation of the GH system appears to start as GH secretion decreases significantly, with a concomitant decline in plasma GH levels. At the same time, there is a slight trend towards lower GH gene expression while the GH pituitary content does not change markedly.

Towards the end of the study, from late May to early July, there is a gradual increase in plasma GH levels without any significant changes in pituitary GH parameters. This may indicate a further deactivation of the GH system and thus decreased clearance rate of GH, due to a gradual down-regulation of GH receptors, as indicated by declining plasma IGF-I levels.

The above interpretation of the GH dynamics during the parr-smolt transformation and the peak in IGF-I plasma levels, suggest that the most intense GH stimulation of peripheral tissues occurs in early May. Physiological data from the fish of the presented study show that branchial Na^+ , K^+ -ATPase activity and intestinal fluid absorption are maximally stimulated in May (K Sundell, T Ágústsson & B Th Björnsson, unpublished observations), further supporting the above interpretations, and the accepted role of GH as stimulator of hypoosmoregulatory ability (see Björnsson 1997). In interpreting the physiological significance of changes in total plasma IGF-I levels, it should be kept in mind that it is largely (>99%) bound to specific IGF-I-binding proteins with the free IGF-I assumed to be the biologically active form (Shimizu *et al.* 1999).

It has been postulated from previous studies that there exists a 'light-pituitary' regulatory axis in salmonids (Komourdjian *et al.* 1976, Björnsson *et al.* 1989, 1995). These studies imply that increased photoperiod triggers an

elevation in plasma levels of GH and thereby the onset of the parr–smolt transformation. As a result, this would then result in an increase in pituitary GH secretion as well as increased plasma GH levels, production and storage, which is in agreement with the presented findings.

In summary, this study demonstrates that the activation of the GH system during smoltification is a complex process, involving increases in GH secretion rate as well as increased GH synthesis, resulting in increased plasma GH levels. At the same time, the data indicate that GH clearance rate may increase due to increased GH receptor binding. This GH-activation sequence culminates in May with strong activation of the GH–IGF-I axis, which is in agreement with a SW-adapting role of GH during the smoltification of the Atlantic salmon.

Acknowledgements

The authors thank Elisabeth Jönsson and Gunilla Eriksson for their assistance during sampling. This study was financed by grants from the Swedish Council for Agricultural and Forestry Research and the Wallenberg Foundation VIRTUE project to B Th B and K S, by the Royal Society of Arts and Sciences in Göteborg and C F Lundströms Stiftelse to K S, by grants from the Japan–Scandinavia Sasakawa Foundation, Helge Ax:son Johnson Stiftelse, Wilhelm och Martina Lundgrens vetenskapsfond, Kungl och Hvitfeldtska Överskottsfonden and Hierta Retzius Stipendiefond to T Á, and by grants from the Ministry of Education and Fisheries Agency, Japan to T S.

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Received in final form 12 March 2001

Accepted 26 March 2001