

# Differential expression of two GH receptor mRNAs following temperature change in rainbow trout (*Oncorhynchus mykiss*)

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## Abstract

The recent genome duplication in salmonids has led to the presence of two GH receptors (GHRs). As temperature is a determining factor in fish growth, this study aims to determine whether the growth-promoting effect of temperature may be related to a change in GHR gene expression in embryo and juvenile rainbow trout. During embryonic development, using real-time PCR, we showed that high temperatures (12 vs 4 °C) increased the amounts of GHR1 transcript up to the hatching stage. By contrast, whatever the stage examined, the levels of GHR2 mRNA were unaffected by the incubation temperature. Nevertheless, incubating eggs with GH led to an enhanced embryo weight only after hatching, suggesting that GHR was not able to mediate the growth-promoting effect of GH before hatching. For juveniles, the GH-binding capacities of fish liver reared at 8, 12 or 16 °C revealed that high temperatures led to a lower GH binding. To better understand whether temperature regulates

GHR gene expressions independently of nutritional state, fish were reared at 8, 12 or 16 °C and either fed *ad libitum* or with the same ration (1.2% of body weight per day). In the muscle of fish fed *ad libitum*, a higher rearing temperature increased GHR1 ( $P < 0.001$ ) but not the GHR2 mRNA levels. When the fish were restricted, temperature no longer affected the levels of GHR1 and GHR2 transcript. In the liver of fish fed *ad libitum*, a higher rearing temperature increased both GHR1 and GHR2 mRNA levels ( $P < 0.001$ ), while in restricted fish, no difference was seen. In conclusion, the two GHR genes are differentially regulated following temperature change and this was related to the period of fish life (embryo or juvenile) and the tissue (liver or muscle). In juveniles, the GHR, by integrating the effect of temperature on plasma GH and nutritional state, could play a key role in the growth-promoting effect of temperature.

*Journal of Endocrinology* (2006) **190**, 29–37

## Introduction

The general organization of the somatotrophic axis is well conserved between higher vertebrates and fish, and includes growth hormone (GH), GH receptor (GHR), insulin-like growth factors (IGF-I and IGF-II), IGF receptor (IGFR) and IGF-binding protein (IGFBP) (Gabillard *et al.* 2005, Reinecke *et al.* 2005, Wood *et al.* 2005). On the other hand, fish in the family Salmonidae are among the few animal taxa believed to have a relatively recent (25–100 millions years ago) tetraploid ancestry (Allendorf & Thorgaard 1984). As a consequence, several genes that occur singly in mammals have two or more copies in salmonids. Indeed, in rainbow trout, two GH and IGFR-I genes have been described (Agellon *et al.* 1988, Greene & Chen 1999, Gabillard *et al.* 2003a), while there is no evidence of IGF-I or -II duplicates. Several works have reported a weak differential expression of both GH genes according to the physiological state (Salam *et al.* 1999, Mori *et al.* 2001, Gabillard *et al.* 2003a), while no obvious difference was

observed between the two IGFR-I genes (Gabillard *et al.* 2003b, 2003c). Recently, the GHR gene was found to be duplicated in salmon (Fukada *et al.* 2004), but the regulation of both GHR genes has not yet been studied.

Another particularity of fish is their sensitivity to environmental factors such as oxygen levels, water salinity, temperature, etc. (Brett 1979). Given that fish are poikilotherms, water temperature is a determining factor in fish biology and the most obvious effect of higher temperature is an increase of embryonic and postlarval growth (Vernier 1969, Brett & Groves 1979). Several studies have reported a close relationship between seasonal temperature and variations of GH or IGF-I levels (Marchant & Peter 1986, Mingarro *et al.* 2002, Taylor *et al.* 2003). Recently, our group and others have demonstrated the involvement of the GH/IGF system in the growth-promoting effect of high temperatures, during the embryonic and the postlarval period (Gabillard *et al.* 2003b, 2003c, 2003d, 2005, Beckman *et al.* 2004). There is abundant evidence that GH is the primary regulator of endocrine IGF-I production in juvenile

and adult fish (Moriyama 1995, Shablott *et al.* 1995, Duguay *et al.* 1996, Shepherd *et al.* 1997). Therefore, the regulation of the GHR is of particular interest in the study of growth regulation by the GH/IGF system. However, the involvement of the GHR in the growth-promoting effect of temperature is not known.

The aim of this study was to examine the transcriptional regulation of the two GHRs under different rearing temperatures and two periods in fish life (embryo and juvenile). Our results show that the high temperature decreased hepatic GH binding, probably as a result of higher GHR turnover. Moreover, we clearly demonstrated that GHR1 and GHR2 genes exhibited a distinct temperature-induced regulation depending on the period in fish life (embryo or juvenile) and the tissue (liver or muscle).

## Materials and Methods

### *Animals and experimental design*

**Trial 1** The experimental design was described in a previous work (Gabillard *et al.* 2003b). Briefly, after fertilization, eggs were incubated until hatching in a recycled water unit at three temperatures (4, 8 and 12 °C) in darkness. Under these conditions, more than 90% of embryos hatched.

**Trial 2** Six thousand eggs originating from a mix of 30 females were fertilized with milt from five males. Each treatment was in triplicate. Just after fertilization, eggs were incubated for 2 h with recombinant trout growth hormone, (rtGH, 15 ng/ml; Eurogentec, Liege, Belgium) in order to allow hormone penetration (Ruhle & Grieder 1989). After rinsing, the eggs were incubated at 10 °C in a recycled water unit.

**Trial 3** Fish ( $40 \pm 2.1$  g) were randomly distributed in six tanks of 1 m<sup>3</sup> ( $n=60$  per tank) in a recirculated water system under a controlled photoperiod (12 h darkness:12 h light) (SCRIBE, Rennes, France). During a 3-week acclimation period at 12 °C, fish were fed *ad libitum* (BioMar, Nersac, France). After that, the temperature was changed to 8 °C  $\pm$  0.5, 12 °C  $\pm$  0.5 or 16 °C  $\pm$  0.5, the six tanks being randomly assigned to 8, 12 or 16 °C (in duplicate). Fish were fed at 1.7, 2.2 or 2.7% of the body weight per day at 8, 12 and 16 °C respectively and maintained in these conditions for 5 weeks.

**Trial 4** The experiments were designed as described in a previous work (Gabillard *et al.* 2003d). Briefly, fish ( $8.4 \pm 0.4$  g) were randomly distributed in six tanks of 0.11 m<sup>3</sup> ( $n=78$  per tank) in a recirculated water system under a controlled photoperiod (12 h darkness:12 h light). During a 3-week acclimation period at 12 °C, fish were fed *ad libitum* ( $\Delta 26$ ; ProAqua, Palencia, Spain). After that, the temperature was changed to 8 °C  $\pm$  0.5, 12 °C  $\pm$  0.5 or 16 °C  $\pm$  0.5 (time 0). Fish were fed *ad libitum* and maintained in these conditions until they reached the weight of 50–60 g for comparison (6, 7

and 10 weeks at 16, 12 and 8 °C respectively). As expected, fish fed *ad libitum* (similar nutritional state) had a better growth rate at 16 °C than 8 °C (Gabillard *et al.* 2003d).

**Trial 5** The experiments were designed as described in a previous work (Gabillard *et al.* 2003d). Briefly, fish ( $9.6 \pm 0.4$  g) were randomly distributed in six tanks of 0.11 m<sup>3</sup> ( $n=80$  per tank) in a recirculated water system under a controlled photoperiod (12 h darkness:12 h light). During the acclimation period at 12 °C, fish were fed with a ration corresponding to 1.4% of body weight per day ( $\Delta 26$ , ProAqua). After that (time 0), the temperature in the tanks was changed as in experiment 1. In order to obtain a similar growth rate at each temperature, fish were fed at 1.2% of body weight per day. Fish were maintained in these conditions until they reached a mean weight of 50–60 g. As expected, fish fed with the same ration (different nutritional state) exhibited similar growth rates at the three temperatures (Gabillard *et al.* 2003d).

### *Sample collection*

**Trial 1** Embryos were collected at stages 22, 24, 25 and hatching. All embryo stages were identified using the developmental table of Vernier (1969) (stage 22, pigment on the choroid periphery and appearance of the cardinal vein; stage 24, six aortic arches and appearance of the caudal fin; stage 25, anal fin bud and the caudal artery and vein reach the tail extremity). The chorion and the yolk sac were removed from the embryos using forceps in order to eliminate most of the maternal mRNA. At hatching, the head was separated from the trunk. For RNA extraction, embryos (50 in triplicates) were directly frozen in liquid nitrogen and stored at –80 °C.

**Trial 2** For each treatment, 12–16 eggs were dissected in order to eliminate the vitellus and then the trunks (without heads) were weighed. Samples were stored at –20 °C until analysis.

**Trial 3** Livers were collected at week 1, 2, 4 and 5 and liver membranes were prepared directly according to Yao *et al.* (1991). After preparation, membranes were stored at –20 °C in Tris buffer (20 mM Tris–HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 0.1% NaN<sub>3</sub>) until the GH-binding assay.

**Trials 4 and 5** In the *ad libitum* feeding experiment (trial 4), samples were collected at weeks 0, 2 and 6 for all temperatures; week 7 for 8 and 12 °C and week 10 only for 8 °C. For the restricted feeding experiment (trial 5), fish were collected at 0, 2, 6 and 12 weeks at all temperatures. Liver and white muscle were collected and stored at –80 °C for total RNA extraction.

### *Sample analysis*

**GH-binding assay** The membrane preparations were obtained for the determination of the receptors number

according to the method developed by Yao *et al.* (1991). The rtGH was marked with  $^{125}\text{I}$  according to the Chloramide T method (60  $\mu\text{Ci}/\mu\text{g}$ ) and incubated at 12 °C (20 h) with the membrane preparation. The concentration of receptors in the hepatic preparation (expressed in femtomolar) was calculated from the saturation curve obtained with  $^{125}\text{I}$ -rtGH according to the method developed by Scatchard (1949). The affinity constant ( $K_d$ ) of the GHR, calculated according to the Scatchard method, was not affected by the rearing temperature.

**GHR cDNA cloning** We identified several expressed sequence tags (ESTs; BX082878, BX082877, CA356079, CA342500) with homology to the coho salmon (*Oncorhynchus kisuthi*) GHR sequence. These four ESTs came from two clones (tcac0005c.d.24 and 1RT91123-A-E12), homologous to the salmon GHR1 and GHR2. After complete sequencing of the clones, it appeared that one clone contained the full-length cDNA coding for GHR2, while the other contained only the 3'-untranslated region of GHR1. Based on the salmon GHR1 sequence, we designed primers (5'-ATACGGGTCAAACATCATGGCAA-3' and 5'-CTCTTTCTCCTTTTCCTTCTCATC-3') in order to clone the lacking fragment. cDNA was made with total liver RNA and the PCR was performed for 40 cycles, each cycle consisting of denaturation at 95 °C for 40 s, annealing at 60 °C for 1 min and extension at 72 °C for 2 min. PCR products were electrophoresed on 1.5% agarose gel and the expected amplified fragment was purified using Qiaquick columns (Qiagen no. 28704). The product was cloned into the pGEM-T easy vector according to the manufacturer's protocol (Promega). Sequencing was performed using an automatic sequencing system (ABI PRISM 310; Applied Biosystems, Foster City, CA, USA).

**Reverse transcription (RT)-PCR** Total RNA was extracted in triplicate from pools of embryos (50 embryos per pool) or from 100 mg tissue (muscle or liver) using TRIzol (Gibco BRL). Total RNA was quantified, based on absorbance at 260 nm, and the integrity checked for all RNA samples on 1% agarose gel, stained with ethidium bromide.

Quantitative real-time RT-PCR was performed as previously described (Gabillard *et al.* 2003a). Briefly, 1  $\mu\text{g}$  total RNA was used to perform the RT reaction (Applied Biosystems kit no. N808-0234). For each pool of embryos, RT was performed in duplicate.

Quantitative PCR analyses for GHR1 and GHR2 were carried out with one-tenth (10  $\mu\text{l}$ ) of the RT reaction using the SYBR Green PCR master mix (Applied Biosystems no. 4309155). Thermal cycling was initiated with incubation at 95 °C for 10 min for activation of AmpliTaq Gold DNA polymerase (Applied Biosystems). After this initial step, 35 cycles of PCR were performed (95 °C for 15 s; 60 °C for 30 s). The following primers were used: 5'-CGTCCTCATCCTTCCAGTTTTA-3' and 5'-GTTCTGTGAGGTTCTGGAAAAC-3' for GHR1 and

5'-TCTCCTCATCCTAACAGTTGTG-3' and 5'-GCTCTGTGAGGTTCTGGAAATGT-3' for GHR2. The absolute amount of the target mRNA was determined by comparison with a standard curve of the plasmid containing the cDNA. The PCR efficiency was identical with the standard and the RT reaction and ranged from 90 to 100%.

#### Statistical analysis

The temperature effect was analysed with a one-way ANOVA using the non-parametric Wilcoxon/Kruskal-Wallis rank test. If a significant temperature effect was found, the significance of the difference between the two means was determined by the non-parametric Mann-Whitney *U* test. All the data are presented as the mean  $\pm$  S.E.M. Pearson's linear regression was used to assess the relation between IGF-I mRNA and GHR mRNA.

## Results

#### cDNA cloning of the two GHRs

After cloning and sequencing, we obtained two distinct cDNA codings for GHR with an overall nucleotide identity of 88% between them. The GHR1 and GHR2 cDNAs have an open reading frame of 593 and 594 amino acids (Fig. 1) respectively and share an 85% amino acid identity. Our amino acid sequences of GHR1 and GHR2 have respectively four and two amino acids different from those recently reported (Very *et al.* 2005), but located outside the functional domains (box 1, box 2, FGEFS motifs or cysteine residues of the extracellular domain).

#### High temperatures increased GHR1 mRNA levels in embryo

In trial 1, in order to determine whether GHR genes were expressed during embryonic development, we measured the levels of GHR1 and GHR2 mRNA using real-time PCR. The GHR1 and GHR2 mRNAs were detected as early as stage 22 (Fig. 2) and at a similar level throughout the development. Water temperature significantly ( $P < 0.001$ ) increased (two-fold between 4 and 12 °C) the amount of GHR1 from stage 22 to hatching. At hatching, the increase of GHR1 mRNA levels was stronger in the trunk than in the head. On the other hand, the incubation temperature did not change GHR2 gene expression, whatever the stage or the compartment.

In trial 2, to determine whether GH can have an effect on embryonic growth, we incubated fertilized eggs with rtGH. Two weeks after treatment, rtGH was always detected in the vitellus ( $20.6 \pm 12.5$  ng/ml). At hatching, the weight of the trunk from GH-treated embryos was similar to the control group (Fig. 3). Nevertheless, 1 week after hatching, the GH-treated embryos exhibited a significantly ( $P < 0.05$ ) higher body weight ( $24.0 \pm 2.0$  vs  $19.7 \pm 1.9$  mg) and this effect of GH treatment was maintained at least 3 weeks after hatching.

GHR1	MATSHILFIC	LVLILPVLVSQ	EPPTSKQALF	QIRPQITGCV	SHDMNTRFCR	WNVGVFQNL	ELRDLRIFY	INDRNISPKE
rtGHR1	.....	.....	.....	.....	.....	.....	.....	.....
GHR2	.....	.L...T.V...	.....E...P	.....	.....	.....	.....T...PG...M...	.....NK.....
rtGHR2	.....	.L...T.V...	.....E...P	.....	.....	.....	.....T...PG...M...	.....NK.....
GHR1	WGECPRY-VD	RTNECFNFS	YTKVWITYSV	QLRPGDQDIL	YDEVIFTVED	IVEPDPPIAL	NWLLNVLGT	GSHFDIMLSW
rtGHR1	.....	.....	.....	.....S.....	.....	.....	.....	.....
GHR2	.S...N.MA	.ID.....	.....	.....S...N.	.....	.....	.....	.....V..
rtGHR2	.S...N.MA	.ID.....	.....	.....S...N.	.....	.....	.....	.....V..
GHR1	EPPHADVSM	GWMTLQYEVQ	YREVNSTLWR	TVDLEKGMQR	SLYGLRTNTD	NEVRVRCKTL	ASRN <u>FGEFS</u> D	SIFIHIPTKE
rtGHR1	.....	.....	.....	.....D.....	.....	.....	.....	.....
GHR2	.....	.....	.....	M.N...R.	.....	H.....	.....	P.V.....
rtGHR2	.....	.....	.....	M.N...R.	.....	H.....	.....	P.V.....
<u>Box 1</u>								
GHR1	SRLPVTLLV	FAALGLAVIL	MLLIYSQQQK	FMVILLPIP	<u>GPKIKGIDPE</u>	LFKKGNAEL	TSILGGIPDL	RPDLYSDDPW
rtGHR1	.....	.....	.....	.....	.....	.....	.....	.....
GHR2	.F.....	.....	I.V.....	L.M.....	.....	.....K.....	.....DH...	.....E..CE...
rtGHR2	.F.....	.....	I.V.....	L.M.....	.....	.....K.....	.....DH...	.....E..CE...
<u>Box 2</u>								
GHR1	VEFIELDMEE	PNDRLTVLDT	HCLMDHCASS	DCPPITIGFR	DDSGRASC	DPDLPDPEAP	FHSLLPNTSR	ALEPSCLAST
rtGHR1	.....	.....	.....	.....	.....	.....	.....	.....
GHR2	.....	.....E...Q...	RTP...L...G	.....	.....	.....E...P--S...P	S...G...	.....
rtGHR2	.....	.....E...Q...	RTP...L...G	.....	.....	.....E...P--S...P	S...G...	.....
GHR1	KASSPVQPT	TEDSPWASPG	REDLYTQVNE	VRLTGEVLLT	HEEQRNVEEN	SEKDEKE-KE	KEKKNRFQL	LNVNADVGGY
rtGHR1	.....	.....	.....	.....	.....D.....	.....	.....	.....
GHR2	E.....	.GMTL.V...	.....S...	..P.....M	P...K..KD	A.EKA..G...	.A.P.K.....	.....G...
rtGHR2	E.....	.GMTL.V...	.....S...	..P.....M	P...SK..KD	A.EKA..G...	.P.K.....	.....G...
GHR1	TSELDAGKMS	ARLPTGRASQ	PAPTESSSLV	QQQPFGEYQS	LYFEAEMMPI	PPASPVSPLL	PVSAYTMVEG	VDRQNSLLLK
rtGHR1	.....	.....	.....	.....	.....	.....	.....	.....
GHR2	.T.....	.K...G...	.....R..	.....	.....	.....S...	.L.V.....	.....
rtGHR2	.T.....	.K...G...	.....R..	.....	.....	.....S...	.L.V.....	.....
GHR1	PGPTPAPQPD	VTKLPLPTPT	PEGYLTPDLL	GNITP	.....	.....	.....	.....
rtGHR1	.....	.....	.....	.....	.....	.....	.....	.....
GHR2	.S.P....V	L.....	.....	..V..	.....	.....	.....	.....
rtGHR2	.S.P....V	L.....	.....	..V..	.....	.....	.....	.....

**Figure 1** Alignment of amino acid sequences of both GH receptors in the rainbow trout (GHR1 and GHR2) with the sequences published by Very *et al.* (2005) (rtGHR1: AAW56611 and rtGHR2: AAW27914). The dots represent conserved amino acids in the four sequences. FGEFS motifs (open square) and boxes (underlined with double line) are indicated.

### High temperatures increased GHR2 expression in the liver but not in muscle

In trial 3, the specific growth rate ( $100 \times \ln(W_2 - W_1) / (T_2 - T_1)$ );  $W_2$ , final weight and  $W_1$ , initial weight) was 1.2, 1.5 and 1.6% at 8, 12 and 16 °C respectively. The measurement of GH-binding capacities of liver in rainbow trout reared at 8, 12 or 16 °C revealed a significant decrease ( $P < 0.001$ ) of binding in relation to a higher water temperature (Fig. 4). This effect was seen as soon as 1 week after the temperature change ( $35.5 \pm 2.5$  vs  $19.5 \pm 1.0$  fmol/liver per  $\text{cm}^3$  at 8 and 16 °C respectively;  $P < 0.001$ ) and was maintained throughout the experiment.

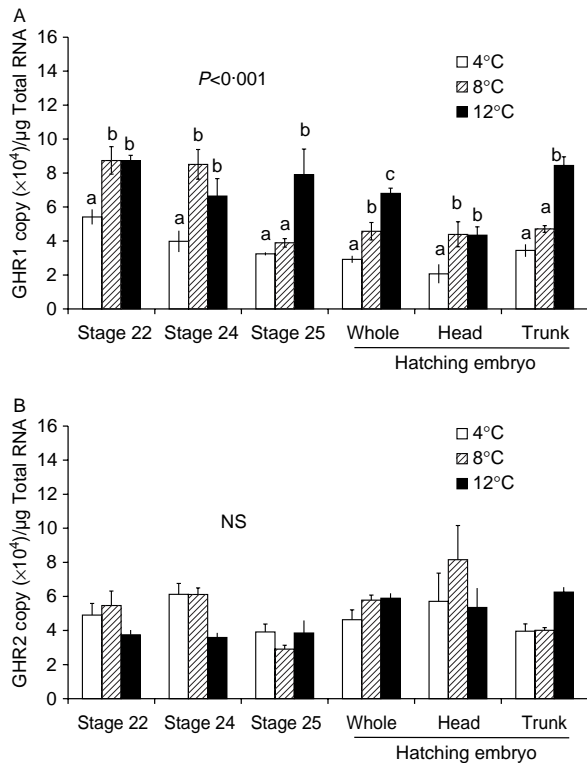
In trial 4 *ad libitum* feeding experiment, the analysis of GHR expression in the liver revealed a similar expression between both genes at time 0 (Fig. 5A and C). In the liver, high temperature led to an increase in GHR1 and GHR2 expression ( $P < 0.001$  and  $P < 0.05$  respectively). At week 6, GHR1 mRNA levels in fish reared at 16 °C were fourfold higher than at 8 °C ( $21.7 \pm 1.0$  vs  $5.6 \pm 1.5$  ( $\times 10^4$ ) copies/ $\mu\text{g}$  total RNA;  $P < 0.001$ ). At the same time, GHR2 mRNA levels were only twofold higher ( $26.1 \pm 3.8$  vs  $14.5 \pm 2.2$  ( $\times 10^4$ ) copies/ $\mu\text{g}$  total RNA;  $P < 0.01$ ) at 16 °C than 8 °C.

In muscle (Fig. 6A and C), the initial expressions of both GHR genes were similar and were fivefold lower than in the liver. The increase of temperature stimulated the muscle expression of the GHR1 gene ( $P < 0.001$ ), but not GHR2. At week 6, GHR1 mRNA levels in fish reared at 16 °C were fivefold higher than at 8 °C ( $6.3 \pm 1.3$  vs  $1.2 \pm 0.2$  ( $\times 10^4$ ) copies/ $\mu\text{g}$  total RNA;  $P < 0.001$ ).

In trial 5 (restricted feeding experiment), the analysis of GHR expression revealed a similar expression between both genes at time 0. In the liver (Fig. 5B and D) and muscle (Fig. 6B and D), no obvious effect of temperature was observed either on GHR1 or on GHR2 mRNA levels.

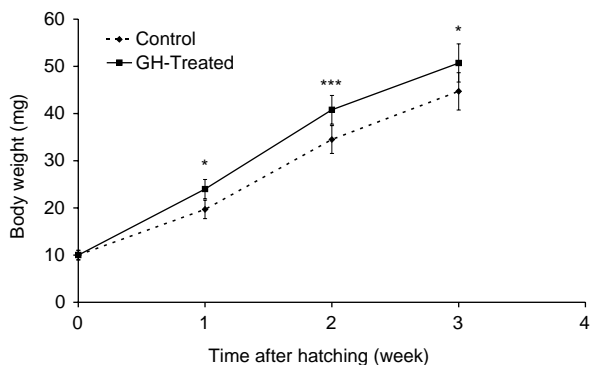
### Discussion

A salmonid-specific tetraploidization event is estimated to have occurred 25–100 million years ago (Allendorf & Thorgaard 1984). As a consequence, two GHR genes are present in rainbow trout. In this particular context, this study focussed for the first time on regulation of both GHRs and we observed that the two GHRs are differentially regulated by water temperature.

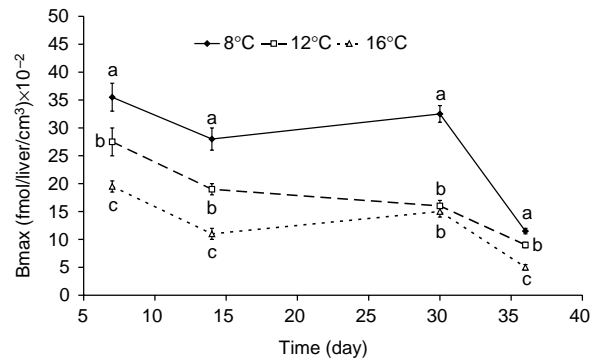


**Figure 2** Quantity of GHR1 (A) and GHR2 (B) mRNA in embryos incubated at 4, 8 or 12 °C. Results are expressed as molecules ( $\times 10^4$ ) per microgram of total RNA. Letters reveal the significant differences (non-parametric Mann–Whitney *U* test;  $P < 0.05$ ) between means within the same stage.

Our results showed the presence of both GHR mRNAs during embryonic development of rainbow trout. These data are in agreement with a mammalian study (Shoba *et al.* 1999) showing the presence of GHR mRNA during foetal development in mammals. Moreover, the real-time PCR study indicated a similar level of both GHR mRNAs in



**Figure 3** Body weight of trout larvae incubated for 2 h (treated) or not (control) with rtGH after fertilization. Each point corresponds to the mean of 12–16 larvae ( $\pm$  S.E.M.). Asterisks denote significant differences (\* $P < 0.05$ ; \*\*\* $P < 0.001$ ).

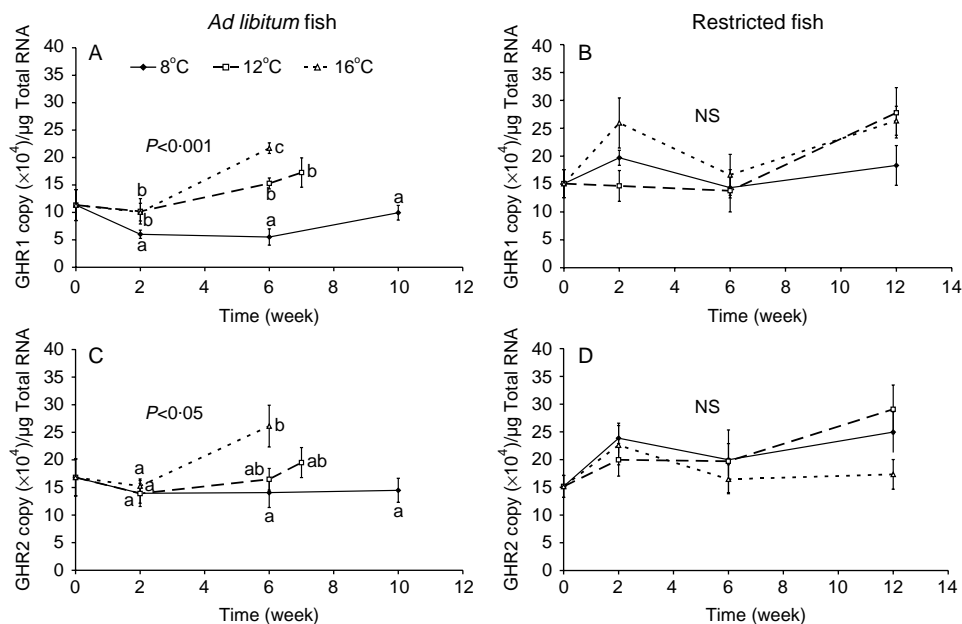


**Figure 4** GH-binding capacities of liver membrane of rainbow trout reared at 8, 12 and 16 °C. Fish were fed at 1.7, 2.2 or 2.7% of the body weight at 8, 12 and 16 °C respectively. Each mean ( $\pm$  S.E.M.) corresponds to the measurement of 8–10 fish. Within the same time, different letters indicate a significant difference between means ( $P < 0.05$ ).

embryos, suggesting that in standard conditions they are not differentially regulated. By contrast, when embryos were reared under different water temperatures, GHR1 mRNA abundance was higher at 12 °C than 4 °C while in contrast, no variation was observed for GHR2 mRNA levels. Thus, our results showed that water temperature differentially regulates GHR1 and GHR2 genes. By contrast, in a recent study (Gabillard *et al.* 2003b), we observed that water temperature has no effect on GH expression at transcript and protein level. Given that GHR1 mRNA abundance at high temperature is associated with an increase of the embryonic growth rate at 12 °C (Gabillard *et al.* 2003b), GHR1 could play a role in the growth-promoting effect of temperature.

In order to determine whether embryos were sensitive to GH, we incubated fertilized eggs with rtGH. The suitability of the method used by Ruhle and Grieder (1989) was confirmed by the detection of rtGH in the vitellus 2 weeks after hormone treatment. Embryo weight at hatching was similar in GH-incubated and control fish, whereas a growth-promoting effect of GH was observed from 1 week after hatching. So, under this experimental condition, GH cannot stimulate fish growth before the hatching stage, suggesting that the GHR would not be able to transduce the growth-promoting effect of GH during the embryonic period. Our data are reminiscent of the absence of any difference in weight in newborn GH-transgenic mice (Leroith *et al.* 2001). Moreover, when the GH/IGF-I system is fully functional, there is a good relationship between GHR and IGF-I mRNA levels (present study and Kajimura *et al.* 2004) but, in our experimental conditions, this was clearly not the case, since no effect of temperature on IGF-I expression was observed (Gabillard *et al.* 2003b). Together, these data strongly suggest that, during embryonic development, the GH/IGF-I system is not fully functional, as generally accepted in mammals (Leroith *et al.* 2001) and would not participate in the growth-promoting effect of temperature.

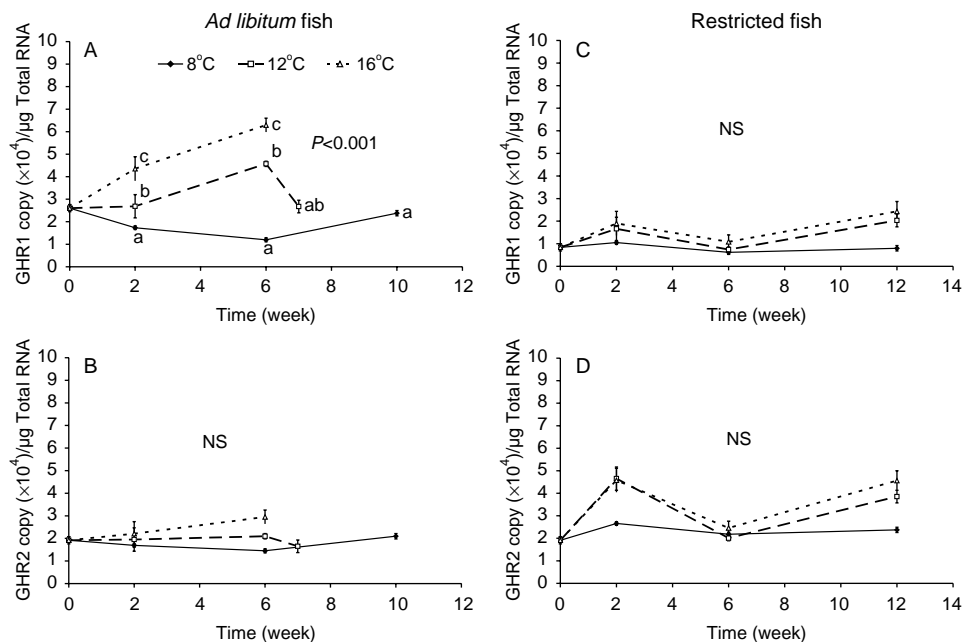
Although it was not possible to measure the GH-binding capacities in embryos, the experiment in juveniles (trial 3)



**Figure 5** Levels of GHR1 (A, B) and GHR2 (C, D) mRNA in liver of rainbow trout reared at 8, 12 and 16 °C. Fish were fed *ad libitum* (A, C) or with the same restricted ration (1.2% of body weight per day) (B, D). Each mean ( $\pm$  S.E.M.) corresponds to the measurement of five to six fish. *P* values correspond to the results of one-way (temperature) ANOVA (Kruskal–Wallis rank test). When there is a significant temperature effect, different letters indicate significant differences ( $P < 0.05$ ) between means. NS,  $P > 0.05$ .

showed an inverse relationship between water temperature and number of GHRs in the liver. Indeed, fish reared at 8 °C exhibited the highest GH-binding capacity of the liver throughout the experiment. It has been shown that high levels

(20–30 ng/ml) of GH decreased hepatic GH binding primarily by receptor occupancy, preventing reliable interpretation of GH-binding data (Sakamoto & Hirano 1991, Mori *et al.* 1992, Zhang & Marchant 1996). Given that in our experiment



**Figure 6** Levels of GHR1 (A, B) and GHR2 (C, D) mRNA in muscle of rainbow trout reared at 8, 12 and 16 °C. Further details as in Fig. 5.

(data not shown), as well as in a previous one (Gabillard *et al.* 2003d), high GH levels have been reported at high temperatures, we believe that the low GH-binding capacities in the liver at 16 °C could result from the high level of circulating GH rather than a down-regulation of the receptor.

In order to better understand the GHR regulation by the temperature, we studied the expression of GHR1 and GHR2 genes in liver and muscle. Because it has been reported that nutritional state regulates GHR expression (Pérez-Sánchez *et al.* 1995, Fukada *et al.* 2004), the difference of food intake caused by temperature (Brett 1979) should be taken into account as was previously done (Gabillard *et al.* 2003c, 2003d). In trials 4 and 5, fish were reared at 8, 12 or 16 °C and fed either to satiety or with the same ration. By comparing both trials, we were able to determine whether the effects were specific to temperature.

In both experiments (trials 4 and 5), liver GHR1 and GHR2 mRNA levels were similar before temperature change. Afterward, in fish fed *ad libitum*, a higher temperature increased GHR1 and GHR2 mRNA abundance, while no obvious effect was observed in restricted fish. Therefore, in the liver, temperature increased expression of both GHR genes only if fish were in an optimal nutritional state. In other words, food restriction could counteract the temperature effect on GHR gene expression. Indeed, it has been reported in mammals that the food restriction led to a GH resistance partly due to the impairment of the JAK/STAT signalling pathway (Beauloye *et al.* 2002). In addition, previous works reported a lower level of GHR mRNA in fasted salmon compared with controls (Fukada *et al.* 2004). Surprisingly, our GH-binding and GHR mRNA studies gave opposite results since high temperature decreased hepatic GH binding, while a higher abundance of GHR mRNA was measured at 16 °C. In addition to the influence of high endogenous GH on GH-binding measurements (Mori *et al.* 1992, Zhang & Marchant 1996), high temperature could also enhance the turnover of the GHR (Fauconneau & Arnal 1985), leading to an apparently lower number of GH-binding sites. Thus, despite this low level of GH-binding sites, high temperatures increased GHR gene expression. Previous works (Kajimura *et al.* 2004) reported that GHR mRNA abundance correlated well with that of IGF-I mRNA. Using our previous data on IGF-I expression in the liver (Gabillard *et al.* 2003c), we found a highly significant positive relationship between IGF-I mRNA and total GHR mRNA (GHR1 plus GHR2) in fish fed *ad libitum* ( $r=0.6$ ;  $P<0.0001$ ) as well as in restricted fish ( $r=0.76$ ;  $P<0.0001$ ). Furthermore, in trials 4 and 5, circulating IGF-I was well correlated with growth rate (Gabillard *et al.* 2003c). Together, these data indicate that the GHR is a key component in the temperature effect on growth rate by integrating the changes of plasma GH and nutritional state.

In muscle, our results showed that GHR1 mRNA abundance was increased by temperature in fish fed *ad libitum*, while no temperature effect was observed in restricted fish. By contrast, muscle expression of the GHR2 gene seems to be unaffected by temperature change and moderate food restriction. Therefore, our data showed a clear differential regulation of the two GHRs in muscle following the

temperature modification and this is reminiscent of what was observed in embryos (present study). By contrast to GHR1, the GHR2 gene is differentially regulated in the liver and muscle. Differential regulation of GHR expression between liver and muscle has been previously reported in mammals in response to fasting (Bornfeldt *et al.* 1989, Dauncey *et al.* 1994), but the mechanism is not yet known. In fish fed *ad libitum*, the temperature-induced increase of GHR mRNA levels that accompanied the high level of endogenous GH did not lead to a higher abundance of muscle IGF-I mRNA (Gabillard *et al.* 2003c), suggesting that GH was unable to stimulate IGF-I expression in muscle, as often reported in mammals (Florini *et al.* 1996, Leroith *et al.* 2001). Nevertheless, the high level of GH and the high expression of GHR in muscle may partly account for the enhanced growth rate, since it has been shown that GH can stimulate protein synthesis in trout muscle (Fauconneau *et al.* 1996).

The two highly homologous GHRs (85% amino acid identity) are the result of a recent genome duplication (Allendorf & Thorgaard 1984), which took place after the divergence of Salmonidae from other teleosts (25–100 million years ago) according to phylogenetic analysis (Very *et al.* 2005). This genetic redundancy is believed to have created new protein activities and/or to have modified their expression pattern (Shimeld 1999) as previously reported for the pro-opiomelanocortin genes (Salbert *et al.* 1992, Suzuki *et al.* 1997, Leder & Silverstein 2006). In the present study, we show that the two GHR genes also evolved separately, acquiring distinct regulations. While high temperatures constantly increase GHR1 expression in embryo, liver and muscle, GHR2 expression is stimulated by temperature only in the liver of juveniles. Thus, GHR genes are differentially regulated according to the period of life and the tissues. A differential regulation between GHR1 and GHR2 might be related to a structural difference in their promoter, which has not yet been characterized in any fish. On the other hand, the distinct regulation of GHR1 between liver and muscle would reflect a difference in the cellular context (transcription factors, signal transduction pathways, etc.).

In conclusion, the two GHR genes are differentially regulated following temperature change according to the period in fish life (embryo or juvenile) and the tissue (liver or muscle). Temperature stimulates GHR1 transcription during embryonic development, even if it is probably not implicated in the growth-promoting effect of temperature. By contrast, in juveniles, temperature increased gene expression of both GHR only in the liver, which probably participates in the increase of circulating IGF-I. Thus, the GHR, by integrating the effect of temperature on plasma GH and nutritional state, could play a key role in the growth-promoting effect of temperature.

## Acknowledgements

We would like to thank Dr P Y Rescan for critical reading of the manuscript and R H Britton for improving the English

of this manuscript. This work was supported by funding from the Institut National de la Recherche Agronomique. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 28 March 2006

Accepted 30 March 2006

Made available online as an Accepted Preprint

25 April 2006