La mer 49 : 111-117, 2011
Société franco-japonaise d'océanographie, Tokyo

Daily expression patterns of growth-related genes in growth hormone transgenic coho salmon, *Oncorhynchus kisutch*

Toshiki NAKANO[1)*], Yui SHOJI[1)], Hitoshi SHIRAKAWA[2)], Yoshihito SUDA[3)], Toshiyasu YAMAGUCHI[1)], Minoru SATO[1)], Robert H. DEVLIN[4)]

Abstract: Growth in fish is regulated by the growth hormone (GH)/ growth hormone receptor (GHR)/ insulin-like growth factor (IGF)-I axis. This GH-IGF-I axis is influenced by several factors. With regard to hormonal effect, it is known that salmon transgenic for GH show increased growth. However, very little is known about the expression patterns of growth-related genes in GH transgenic fish. The present study examined the daily expression patterns of mRNAs for the growth-related genes after feeding in GH transgenic coho salmon (*Oncorhynchus kisutch*). GH mRNA was detected in both liver and muscle as well as in the pituitary from transgenic fish. GHR mRNA levels in the liver and muscle were higher in transgenic fish than in non-transgenic wild type fish but lower in the pituitary. The expression level of hepatic IGF-I mRNA was greater in transgenic fish. The daily GH, GHR and IGF-I mRNA expression patterns reached their peak at roughly 4–8 h after feeding, however, GHR and IGF-I mRNA expressions in the liver was observed to be irregular in non-transgenic fish. These results suggest that the daily expression patterns for growth-related genes are particularly pronounced in GH transgenic fish. Furthermore, our findings would be useful for consideration of the time of feeding and the optimum time of day for tissue sampling for analysis of growth-related genes in fish.

Keywords: *Growth-related gene; Growth hormone, Daily expression pattern; Transgenic coho salmon*

1) Marine Biochemistry Laboratory, Graduate School of Agricultural Science, Tohoku University, Aoba-ku, Sendai 981–8555, Japan
2) Nutrition Laboratory, Graduate School of Agricultural Science, Tohoku University, Aoba-ku, Sendai 981–8555, Japan
3) Animal Breeding and Genetics Laboratory, School of Food, Agricultural and Environmental Sciences, Miyagi University, Taihaku-ku, Sendai 982–0215, Japan
4) West Vancouver Laboratory, Center for Aquaculture and Environment Research (CAER- DFO/ UBC), Fisheries and Oceans Canada, 4160 Marine Drive, West Vancouver, BC, V7V 1N6, Canada
* Corresponding author:
Fax: +81–22–717–8739;
E-mail: nakanot@bios.tohoku.ac.jp

1. Introduction

The aquaculture is known to be very important to world food production. The enhancement of growth in cultured fish, therefore, has been desired and researched. In fish, growth is regulated to a major extent by liver-derived insulin-like growth factor (IGF) -I in response to pituitary-secreted growth hormone (GH) binding to GH receptors (GHR) of liver. The GH-IGF-I axis is believed to play an important role in the regulation of growth. In fish, secretion of GH is known to be under hypothalamic regulation by means of many modulators The actions of IGFs are controlled by GH and IGF binding protein and a specific receptor on the surface of target cells (DONALDSON *et al.*, 1979; DUAN, 1998; MORIYAMA *et al.*, 2000; DEANE and

Woo, 2009; Reinecke, 2010).

Growth in fish is genetically regulated and is also influenced by cellular, endocrinological and environmental factors. The responses of endocrinal tissue are affected by the integration of external stimuli (Duan, 1998; Moriyama *et al.*, 2000; Nakano *et al.*, 2008; Deane and Woo, 2009; Reinecke, 2010). The environmental factors such as nutrition (food availability), temperature and photoperiod are major factors regarding growth and development, and seem to condition biological rhythms in fish. In the natural environment, fish exhibit feeding flexibility according to various conditions. On the other hand, in aquaculture, feeding in fish is controlled artificially and the feeding rhythms might be modified (Ayson and Takemura, 2006; Montoya *et al.*, 2010).

In many fish, growth can be significantly stimulated by treatment with exogenous GH (Donaldson *et al.*, 1979). More recently, GH transgenes have been transferred to fish with strong stimulation of growth (Devlin *et al.*, 1994). The levels of both GH and IGF-I in plasma are known to be high in GH transgenic fish. Growth abnormalities, and altered muscle and pituitary structures, high feeding motivation have been observed in GH transgenic salmon relative to normal wild type fish (Devlin *et al.*, 1995, 2004; Mori and Devlin, 1999; Rise *et al.*, 2006; Hallerman *et al.*, 2007). Accordingly, expression of transgenes might affect the many aspects of fitness such as metabolism, behavior and viability in the fish. However, little is known about the daily expression patterns of genes concerning GH-IGF-I axis in transgenic fish. We hypothesized that the expressions of growth-related genes are held high levels all day long and show unique characters in GH transgenic fish.

In the present study, we examined the changes in the mRNA expression patterns of GH, GHR and IGF-I genes after feeding in GH transgenic coho salmon (*Oncorhynchus kisutch*).

2. Materials and Methods

2.1. Fish, rearing conditions and sampling

Transgenic coho salmon (strain M77) containing the GH gene construct OnMTGH1 (Devlin *et al.*, 1994, 2004) and non-transgenic wild type coho salmon from the Chehalis River, BC, Canada were reared in the CAER Aquarium Facility. The two types of fish (transgenic and non-transgenic) were fed by hand to apparent satiation twice a day with commercially available diets (Skretting Canada, Canada) and matched their size according to the method reported previously (Rise *et al.*, 2006; Raven *et al.* 2008). Six individuals of each of transgenic fish and older non-transgenic fish of similar size were placed into each of five tanks supplied with running 10–11 ℃ well water and acclimatized for two days under natural photoperiod. Tissues from transgenic fish (mean ± SD, 20.34 ± 3.32 g) and non-transgenic fish (19.27 ± 2.15 g) were sampled at 0, 2, 4, 8 and 24 h after feeding (at 9:00 AM). Fish were sampled from separate experimental tanks at each time period, thereby avoiding repeated sampling from the same tank. Fish were anaesthetized with 100 mg/L tricane methane sulphonate buffered with $NaHCO_3$ and rapidly team-sampled for blood and tissues. Plasma was stored frozen at −80 ℃, and all tissues were immediately immersed in RNAlater (Ambion-ABI, USA) and then stored at −80 ℃ until analysis. All the protocols of fish treatment were approved by the DFO Pacific Region Animal Care Committee.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from tissue using TRIzol (Invitrogen, USA) and complementary DNA (cDNA) was synthesized using the ReverTra Ace reagent kit (Toyobo, Japan) were carried out following the manufacturer's protocol and Raven *et al.* (2008).

2.3. Determination of GH, GHR, IGF-I and β-actin mRNA levels

The levels of GH, GHR and IGF-I mRNA expressions in the tissues were determined by real-time quantitative PCR (qPCR) with an equipment of Applied Biosystems Prism 7300 Sequence Detection System (USA) using β-actin as an internal standard according to Raven *et al.* (2008). Gene specific primers and TaqMan probes were used as the qPCR assays (Table 1). Values for GH, GHR and IGF-I were normalized with those of β-actin. Levels of β-actin were confirmed not to change with

Table 1. Sequences of primers and probes used in qPCR analysis.

Gene	Forward Primer (5'–3')	Reverse Primer (5'–3')	TaqMan Probe (5'–3')
GH	CAAGATATTCCTGCTGGACTT	GGGTACTCCCAGGATTCAATCA	CAGTCCTGAAGCTGC
GHR	CACTGTGGAAGACATCGTGGAA	CAAAGTGGCTCCCGGTTAGA	AACTGGACCCTGCTGAA
IGF-I	GGCATTTATGTGATGTCTTCAAGAGT	CCTGTTGCCGCCGAAGT	TCTCACTGCTGCTGTGC
β-actin	ACGGCCGAGAGGGAAATC	CAAAGTCCAGCGCCACGTA	CACAGCTTCTCCTTGATGT

respect to treatment.

2.4. Measurements of glucose levels in plasma

Plasma glucose was measured using an enzymatic assay method available in a kit (Wako Pure Chemical Industries, Ltd., Japan).

2.5. Statistical analysis

All samples were run in duplicate and results are reported as mean ± SD. All data were subjected to one-way analysis of variance (ANOVA). Multiple comparisons between groups were made by Tukey-Kramer method and results were determined statistically significant at $P < 0.05$.

3. Results

3.1. GH, GHR and IGF-I mRNA levels

GH mRNA was detected in non-pituitary tissues such as liver and muscle from transgenic fish as well as in the pituitary. GH mRNA level in pituitary was higher than in other tissues. The daily GH mRNA expression pattern in pituitary and other tissues was observed to peak at roughly 4–8 h after feeding (Fig. 1). This tendency regarding expression pattern of GH mRNA was almost same with liver and muscle in transgenic fish (data not shown).

As shown in Fig. 2, GHR mRNA levels in liver and muscle were found to be higher in transgenic fish than in non-transgenic fish. In contrast, pituitary GHR mRNA levels were low in transgenic fish, compared with non-transgenic fish (data not shown). The daily expression pattern of GHR mRNA revealed relatively high level in transgenic fish, in both pituitary and muscle 8 h after feeding, and in the liver 4 h after feeding. GHR mRNA levels were not significantly different throughout after feeding in non-transgenic fish.

The expression levels of IGF-I mRNA in the liver were found to be greater in transgenic fish, with a peak at 4 h after feeding. On the other hand, the expression pattern of IGF-I mRNA in non-transgenic fish was irregular and there was no significant difference in levels

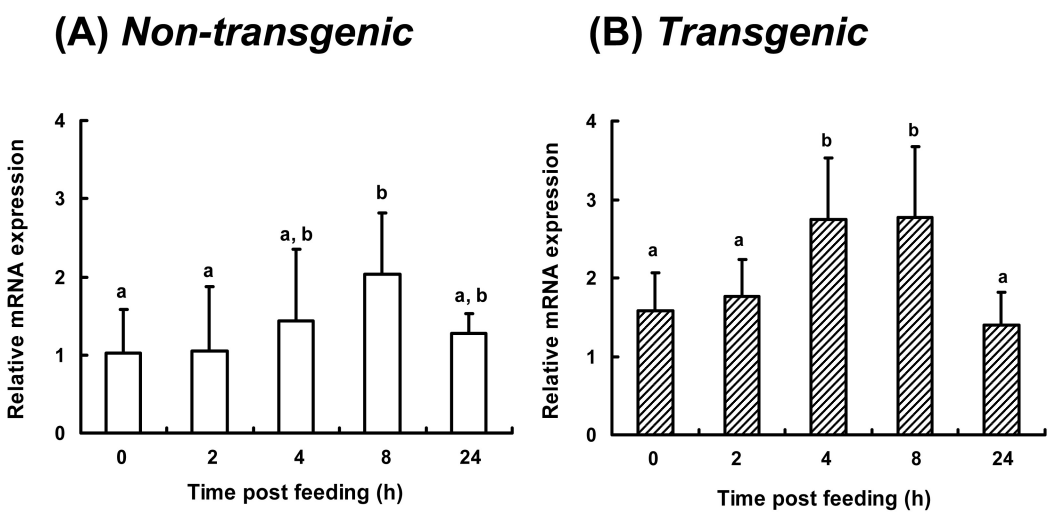

Fig. 1. GH mRNA levels in the pituitary of non-transgenic (A) and GH transgenic (B) coho salmon. Values with different letter superscripts are significantly different ($P < 0.05$). Bars are means ± SD, $n=4$.

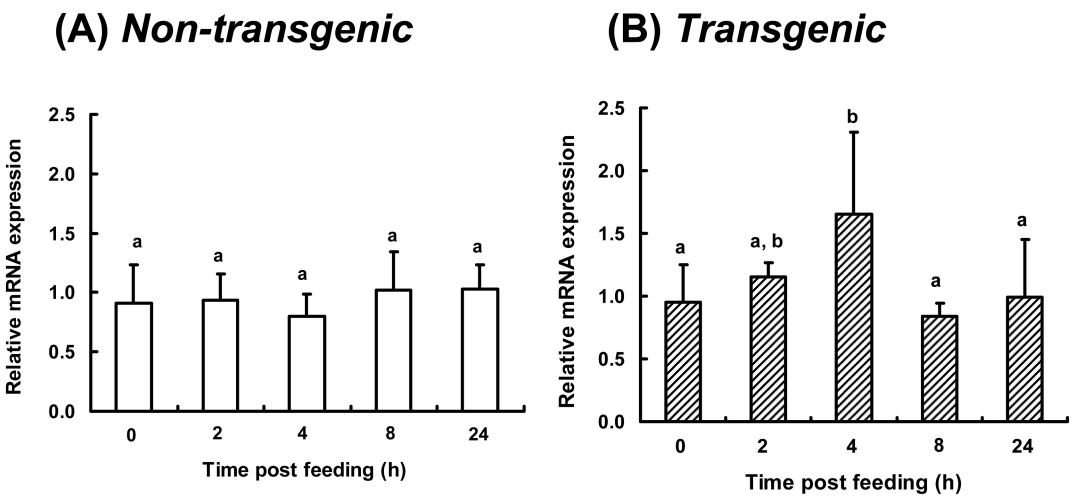

Fig. 2. GHR mRNA levels in the liver of non-transgenic (A) and GH transgenic (B) coho salmon. Values with different letter superscripts are significantly different ($P < 0.05$). Bars are means ± SD, $n=4$.

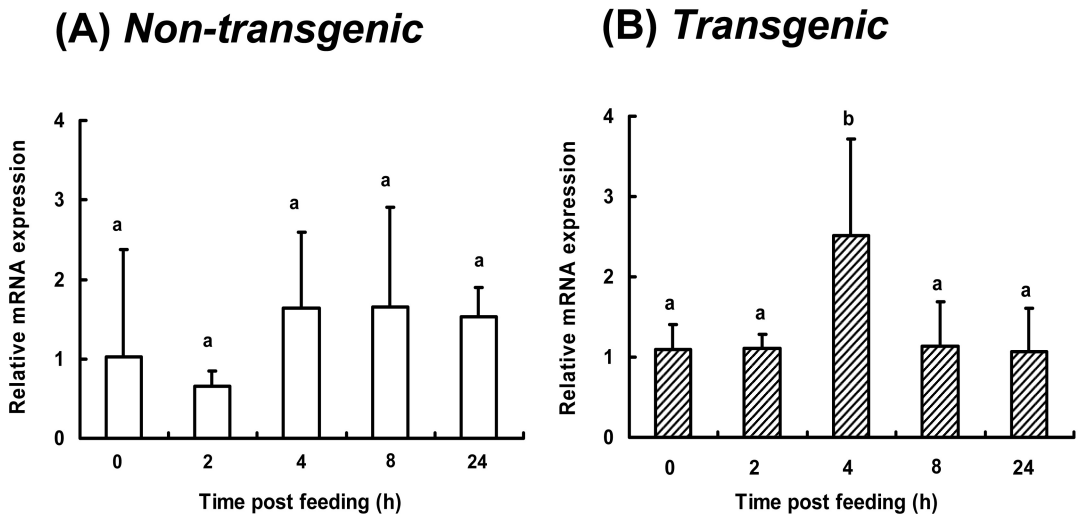

Fig. 3. IGF-I mRNA levels in the liver of non-transgenic (A) and GH transgenic (B) coho salmon. Values with different letter superscripts are significantly different ($P < 0.05$). Bars are means ± SD, $n=4$.

at anytime during the 24 h sampling period after feeding (Fig. 3).

3.2. Glucose levels in plasma

Plasma glucose levels in transgenic fish were higher than those in non-transgenic fish, although in both groups they increased gradually after feeding (data not shown).

4. Discussion

In the present study, the results indicate for the first time that the rhythms in the expression patterns for growth-related genes exist according to the time of feeding in GH transgenic fish and seem to be pronounced in transgenic fish compared to non-transgenic fish.

As shown in Fig. 1, significant differences

were observed in pituitary GH mRNA levels during the 24 h period after feeding in both transgenic and non-transgenic fish. In liver and muscle from transgenic fish, there were no significant differences in GH mRNA expressions during the post-feeding 24 h period, although the highest GH mRNA levels occurred 4–8 h after feeding. This expression patterns suggest the presence of a diurnal pattern of GH expression in coho salmon, which therefore differs from results obtained for several fish species (GOMEZ *et al.*, 1996; AYSON *et al.*, 2007). In rabbitfish, GH mRNA levels during day time were lower compared with the levels during night time (AYSON *et al.*, 2007), and plasma GH levels in fasting fish increased (THISSEN *et al.*, 1999).

GHR mRNA expression in transgenic fish is regular and increases gradually after feeding compared to non-transgenic fish (Fig. 2). Both mRNA and protein levels of GHR are affected by several factors, such as exogenous cortisol, stress and fasting, in fish and mammals (MORIYAMA *et al.*, 2000; FOX *et al.*, 2006; SMALL *et al.*, 2006; NAKANO *et al.*, 2008; KAMEDA *et al.*, 2008; DEANE and WOO, 2009). In the present study, a clear increase in GHR mRNA levels after feeding was observed in all tissues analyzed from transgenic fish. Consequently, food appears to be one of the important factors influencing the expressions of GH and GHR mRNAs in GH transgenic fish.

The mRNA expression of IGF-I in the liver of transgenic fish reached its peak at 4 h after feeding (Fig. 3). IGF-I gene expression in the liver of fish has also been reported to be influenced by environmental factors (LEUNG *et al.*, 2008; NAKANO *et al.*, 2008). Liver expression of IGF-I is regulated by GH and GHR located on the surface of hepatocytes (MORIYAMA *et al.*, 2000). Hence, the patterns of IGF-I mRNA liver expression would synchronize with the mRNA expressions of both GH and GHR in coho salmon. This phenomenon seems to be obvious in transgenic coho salmon (Figs. 1–3), and higher expression levels of growth-related genes in GH transgenic fish than in non-transgenic fish is consistent with RAVEN *et al* (2008).

At present, the reason for the irregularity of GHR and IGF-I gene expressions in the liver of non-transgenic fish is not clear. The daily expression patterns of growth-related genes alter under several conditions in fish (AYSON, *et al.*, 2006; AYSON, *et al.*, 2007).

The higher plasma glucose levels in GH transgenic fish than in non-transgenic fish observed in the present study have been reported (EBERT *et al.*, 1988; JHINGAN *et al.*, 2003). High levels of GH in GH transgenic animals should promote lipolytic and gluconeogenic metabolism, so enhancing the utilization of glycogen stores (JHINGAN *et al.*, 2003). Higher glycolysis in muscle is observed in growth-enhanced transgenic fish owing to a higher energy requirement (KRASNOV *et al.*, 1999; HILL *et al.*, 2000). Indeed, the food (energy) intakes, food conversion efficiency, carbohydrate degradation, utilization of lipids and proteins of GH transgenic fish are greater than those of non-transgenic fish (HUANG *et al.*, 2004; RAVEN *et al.*, 2006; LEGGATT *et al.*, 2009). Accordingly, GH transgenic fish are characterized by enhanced metabolism and energy availability due to their high levels of circulating GH.

In conclusion, the daily expression patterns for growth-related genes in GH transgenic coho salmon were found to be rhythmic. Most organisms display daily rhythms in biological and physiological functions, which affect growth, development and ability to adapt to environmental conditions (AYSON and TAKEMURA, 2006; MONTOYA *et al.*, 2010). Our results reveal that feeding time should also be important as a major synchronizer of growth-related gene expression rhythms in GH transgenic fish.

Studies of the beneficial effect of nutritional conditions on growth-related gene expression are now in progress to enhance the performance of GH transgenic fish. Also under consideration are experiments with coho salmon to determine the effects of environmental factors such as stress and photoperiod on physiological rhythms, since there is much yet to learn about the relationship between physiological and environmental factors, growth-related gene expression and growth in GH transgenic fish.

Acknowledgments

The authors are grateful to C. Biagi, K. Eom, B. Goh, T. Hollo, M. Nomura, P.A. Raven, G. Rigter, M. Rowshandeli and D. Sakhrani at CAER for the support on the lab work. T.N. acknowledge Dr. E.M. Donaldson, Scientist Emeritus, at CAER for his encouragement. We thank Drs. I. Gleadall, N. Ito, M. Osada and K. Takahashi at Tohoku Univ. for their help. This research was partly supported by Grant KAKENHI (#20580219) from MEXT Japan to T.N.

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Received: December 20, 2010
Accepted: March 15, 2011