Comparative Biochemistry and Physiology, Part A 179 (2015) 17–24

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Kisspeptin regulates the somatic growth-related factors of the cinnamon clownfish *Amphiprion melanopus*

Na Na Kim [a], Young-Ung Choi [b], Heung-Sik Park [b], Cheol Young Choi [a,*]

[a] Division of Marine Environment & BioScience, Korea Maritime and Ocean University, Busan 606-791, Republic of Korea
[b] Korea Institute of Ocean Science & Technology, Ansan P.O. Box 29, 426-744, Republic of Korea

ARTICLE INFO

Article history:
Received 29 May 2014
Received in revised form 31 July 2014
Accepted 19 August 2014
Available online 15 September 2014

Keywords:
Cinnamon clownfish
Kisspeptin
Growth hormone
Insulin-like growth factor I
Somatolactin
Melatonin

ABSTRACT

This study aimed to test the effects of kisspeptin (Kiss) on somatic growth in the cinnamon clownfish *Amphiprion melanopus*. We investigated the effects of Kiss treatment on the growth by measuring the mRNA expressions of the growth hormone (GH), insulin-like growth hormone factor (IGF-I), somatolactin (SL), and melatonin receptor (MT). The expression levels of GH and SL of the pituitary gland and IGF-I of the liver increased after Kiss treatment (*in vivo* and *in vitro*). In addition, the MT mRNA expression increased in the pituitary gland and brain after Kiss treatment (*in vivo* and *in vitro*). These results support the hypothesis that Kiss directly regulates the somatic growth-related factors, such as GH, SL, and MT, and IGF-I in the cinnamon clownfish. Further, injection of Kiss resulted in significantly higher levels of plasma melatonin than that in the control. We, therefore, conclude that Kiss plays a role in modulating growth and artificially induced rapid growth in cinnamon clownfish.

1. Introduction

Kisspeptin (Kiss), a member of the RF-amide peptide family, is a neuropeptide located in the preoptic area (POA) of the hypothalamus. The RF-amide family is known to regulate various central nervous system functions, including the energy balance, behavior, growth, and reproduction *in vivo* (Osugi et al., 2006; Bechtold and Luckman, 2007). In addition, the RF-amide family has been implicated in the regulation of almost all of the major pituitary hormones, including luteinizing hormone (LH), follicle stimulating hormone (FSH), growth hormone (GH), somatolactin (SL), adrenocorticotrophic hormone (ACTH), and arginine vasopressin (AVP), although some of these are likely to be regulated directly/indirectly *via* other hypophysiotrophic regulatory hormones in the autocrine/paracrine action. Thus, their sphere of influence on endocrine functions is likely to include maternal physiology, reproduction, growth, and stress responses (Anderson, 2011).

Recently, Kiss has been shown to stimulate the secretions of gonadotropin-releasing hormone (GnRH) and gonadotropins (GTH) and regulate maturation and reproduction (Roseweir and Millar, 2009; Roa et al., 2011; Kim et al., 2014); GTH secretion was shown to increase in the pituitary GTH cells after Kiss treatment (Gutierrez-Pascual et al., 2007). Meanwhile, Kiss, a hormone in the hypothalamus, directly affects the somatotroph, lactotrophs, gonadotrophs, and neurointermediate lobe (NIL) of pituitary cells (Ramaswamy et al., 2009; Yang et al., 2010).

GH is the hormone that is directly related to the growth of the organism, as is GTH. GH is synthesized in the somatotroph and gonadotrophs of pituitary gland, is required for the normal growth of vertebrates, and controls somatic growth through the complex modulation of various metabolic processes (Canosa et al., 2007). In addition, GH is closely related to energy metabolism and stimulates the synthesis of insulin-like growth factor I (IGF-I), which selectively induces the growth of various tissues and accelerates cellular protein synthesis (Holly and Wass, 1989; Wood et al., 2005).

SL is a hormone that belongs to the GH/PRL family and is regulated during various important physiological activities such as development, growth, body-color regulation, lipid metabolism, cortisol secretion, and reproduction *in vivo* (Fukada et al., 2004, 2005). In zebrafish *Danio rerio*, SL is known to play an important role in the growth and developmental stage (Wan and Chan, 2010). Unlike GH and PRL with expression restricted to pituitary cells in the pars distalis, SL is only expressed in the NIL of the fish pituitary gland (Ono et al., 1990). That being said, study of the relationship between Kiss, a hypothalamic peptide, and the GH/PRL family, including the SL and GH, is lacking.

Melatonin is similar to other hormones that promote the growth of organisms; it is secreted from the pineal gland, hypothalamus, and pituitary gland and regulates the biological rhythm (Zeman et al., 1993) and GH synthesis by adjusting the central nervous pathway (John et al., 1990). Melatonin, an environmental promoter affecting

* Corresponding author. Tel.: +82 51 410 4756; fax: +82 51 404 4750.
E-mail address: choic@kmou.ac.kr (C.Y. Choi).

http://dx.doi.org/10.1016/j.cbpa.2014.08.020
1095-6433/

the endocrine system, is known to be involved in physiological processes, such as the growth and development of vertebrates—including fish (Vlaming, 1980; Bromage et al., 2001). Recent studies reported that melatonin synthesis modulates the secretion of the Kiss hypothalamus peptide (Ansel et al., 2010; Maitra et al., 2013). Also, Kiss levels are driven by changes in the pattern of melatonin secretion during photoperiodic change (Revel et al., 2007; Simonneaux et al., 2009). The effects of melatonin are mediated by melatonin receptors (MTs), which belong to the G-protein-coupled receptor superfamily (Reppert et al., 1996; Iigo et al., 2003). MTs are distributed in the central nervous system and peripheral tissues of vertebrate species and mediate the various physiological functions of melatonin in these tissues (Dubocovich, 1995; Reppert et al., 1996).

More recently, studies on the maturation and reproduction of Kiss have been reported (Chang et al., 2012; Trombley and Schmitz, 2013; Kim et al., 2014), but Kiss has not been studied closely; with studies limited in determining its physiological functions, such as regulation of growth and metabolism in the fish.

Therefore, in this study, we used the cinnamon clownfish *Amphiprion melanopus*, whose gender and social status (sexual maturity side) are determined by the size of the individual (growth side). Cinnamon clownfish typically live as part of a mated adult pair or an adult pair and an immature individual, and social ranking in the group controls the sexes of the fish (Godwin and Thomas, 1993). In general, the female is the larger, dominant individual. If a dominant female dies or is absent, the male partner experiences a sex change to become a female, and an immature individual experiences a sex change to become a male (Godwin and Thomas, 1993).

In this study, we studied the relationship between growth regulation and growth-related genes using Kiss. We experimented with the production of a pair of male and female groups and investigated the effects of weekly injections of Kiss into large female cinnamon clownfish over a period of 6 weeks. By sampling the female in 2-weeks intervals, we confirmed the mRNA expression of GH, IGF-I, SL, and MT and plasma levels of GH, IGF-I, and melatonin. In addition, we investigated the direct effects of Kiss on the regulation of growth in cinnamon clownfish, as assessed by studying cultures of pituitary and liver cells, and examined the maturity of female gonads using histological analyses.

2. Materials and methods

2.1. Experimental fish

We used cinnamon clownfish *A. melanopus* [average length, 7.4 ± 0.5 cm, body mass, 14.6 ± 0.5 g, gonadosomatic index (GSI; gonad mass/body mass × 100) = 0.15 ± 0.03]. Fish were purchased from the Center of Ornamental Reef & Aquarium (CCORA, Jeju, Korea). The water temperature and photoperiod were 27 ± 1 °C and a 12 L:12D period (lights on from 07:00 to 19:00 h), respectively. The fish were fed a commercial feed twice daily (at 09:00 and 17:00 h) at a rate of 4–5% of wet body weight per day. pH was 7.8–7.9, and ammonia was not detected in the water. The fish were reared under the above conditions for 6 weeks.

2.2. Kiss treatment and sampling (in vivo)

To investigate the effects of Kiss, the fish were reared in 100-L circulating filter tanks in the laboratory and were anesthetized using tricaine methane sulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) prior to injection. Kiss (metastin 45–54 amide; #445888, Calbiochem Merck KGaA, Darmstadt, Germany; 80% similarity with fish species) was dissolved in 0.9% physiological saline, and each fish was injected with Kiss [0.1 and 0.5 μg/g body mass (BM)] at a volume of 10 μL/g BM. A control group of fish was injected with an equal volume of 0.9% NaCl (10 μL/g BM) once per week at 14:00 h. The control samples were removed prior to injection. The pituitary gland, brain, and liver samples were collected from the fish at 14:00 h of 2, 4, and 6 weeks after the injection. During the experimental period, the water temperature and photoperiod were maintained at 27 ± 1 °C and 12 L:12D, respectively.

All fish were anesthetized with tricaine methane sulfonate (Sigma) and decapitated prior to tissue collection. The pituitary gland, brain, and liver samples from the fish were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until the total RNA was extracted for analysis. The plasma samples were separated by centrifugation (4 °C, 10,000 *g*, 5 min) and stored at −80 °C.

2.3. Culture of liver and pituitary cells and Kiss treatment (in vitro)

2.3.1. Pituitary cell culture

After the fish were anesthetized, the pituitary was dissected and placed in ice-cold medium (pH 7.5) composed of 25 mM HEPES, 4 mM $NaHCO_3$, 0.3% BSA, 0.1% collagenase, 0.25 mg/mL fungizone, and RPMI medium containing antibiotics (100 U/L penicillin and 100 mg/L streptomycin; Penicillin–Streptomycin, Gibco, Carlsbad, CA, USA). The pituitary gland was cut by using a scalpel, into 1–3-mm^3 pieces, weighed, and placed in a 24-well culture plate (SPL Life Science, Gyeonggi, Korea) containing 1 mL of medium and incubated at 20 ± 1 °C in an incubator for 1 day. Kiss was dissolved in an equal volume of 0.9% NaCl at the appropriate doses (0.1 and 1 μg/mL) and added to the culture medium. The pituitary cells cultured for 0, 6, 12, 24, and 48 h in an incubator at 28 °C, 100% humidity, and 5% CO_2 in air. Following the incubation period, each sample was centrifuged (20 °C, 10,000 *g*, 15 s), and the supernatant was removed and stored in individual microcentrifuge tubes at −80 °C.

2.3.2. Liver cell culture

The livers were removed from five cinnamon clownfish, cut by using a scalpel, into 1–3-mm^3 pieces, weighed, and placed in a 24-well culture plate. Under a sterile hood, the liver pieces were washed several times with culture medium solution (M199, Invitrogen, Carlsbad, CA, USA). The liver pieces were added in equal amounts (approximately 50 mg) to each well of a 24-well plate, and a total of 2 mL of fresh culture media was added. The liver pieces were allowed to acclimatize at room temperature with access to ambient air for 2 h, after which the indicted concentrations of Kiss (0.1 and 1 μg/mL) were added, with an equal volume of distilled water (dH_2O) being added to control group. The liver pieces were cultured for 0, 6, 12, 24, and 48 h in an incubator at 28 °C, 100% humidity, and 5% CO_2 in air. Following the incubation period, each sample was centrifuged (20 °C, 10,000 *g*, 15 s), and the supernatant was removed and stored in individual microcentrifuge tubes at −80 °C.

2.4. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative mRNA expressions of GH, IGF-I, SL, and MT by using the total RNA extracted from the cinnamon clownfish tissues. The primers for QPCR are shown in Table 1.

Table 1
Primers used for amplification of QPCR.

Genes (GenBank accession no.)	Primer	DNA sequences
GH (ADJ57589)	Forward	5′-CTT AGC AAC AGA ACG GTC G-3′
	Reverse	5′-CCA GGT AGC CAG CCA TAC-3′
IGF-I (JX494724)	Forward	5′-TGT GTC TGG GAG CCC AGC T-3′
	Reverse	5′-CAG AGG TGG TAA ATG AGT CCA-3′
SL (KJ009391)	Forward	5′-GCG TCA CCA AAG CCT TAC-3′
	Reverse	5′-CCA GCA TCA GCA CAG AGT-3′
MT (HM107821)	Forward	5′-TCA CCT CCA TCT TCC ACA A-3′
	Reverse	5′-TGT AGC AGT AGC GGT TGA-3′
β-actin (JF273495)	Forward	5′-GGA CCT GTA TGC CAA CAC TG-3′
	Reverse	5′-TGA TCT CCT TCT GCA TCC TG-3′

These primers were designed for each gene using the Beacon Designer software (Bio-Rad, Hercules, CA, USA). Primer alignments were performed with the BLAST database to ensure the specificity of primers. QPCR amplification was conducted similarly to previous studies using a BIO-RAD CFX96™ Real-Time System (Bio-Rad). QPCR was performed in the following manner: 1 cycle of denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 20 s, and annealing at 55 °C for 20 s. Each reaction was run in triplicate to confirm consistency. The experiments were duplicated with β-actin as an internal control. The efficiencies of the reactions were determined by performing the QPCR. All data were expressed as changes with respect to the corresponding β-actin-calculated cycle threshold (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated as: $\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$.

2.5. Western blot analysis

The total protein isolated from the pituitary during sexual maturation was extracted using protein extraction buffer (5.6 mM Tris, 0.55 mM EDTA, 0.55 mM EGTA, 0.1% SDS, 0.15 mg/mL PMSF, and 0.15 mg/mL leupeptin), sonicated, and quantified using the Bradford method (Bio-Rad). The total protein (30 μg per lane) was loaded onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel, and a protein ladder (Bio-Rad) was used for reference. Thereafter, the membranes were blocked with 5% milk in TBS (pH 7.4) for 45 min and then washed in TBS. The membranes were incubated with MT antibody (dilution, 1:8000; NBP1-28912; Novus Biologicals, Littleton, USA), followed by a horseradish peroxidase conjugated anti-goat IgG secondary antibody (1:5000; Bio-Rad) for 60 min. The internal control was a β-tubulin antibody (dilution, 1:5000; ab6046, Abcam, UK), followed by a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:5000; Bio-Rad) for 60 min. The bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA) with a 30 s exposure and a Molecular Imager® ChemiDoc™ XRS + System (Bio-Rad). The membrane images were scanned with a high-resolution scanner, and the band density was estimated using a computer program (Image Lab™ Software, version 3.0, Bio-Rad). The ratios of the internal control (β-tubulin) to the MT for each concentration were calculated and plotted against the concentration of the internal control.

2.6. Plasma parameter analysis

The levels of plasma GH and IGF-I were analyzed using the immunoassay technique with the ELISA kit (GH, E12121Fh and IGF-I, EL012870FI; Cusabio Biotech, Hubei, China). An anti-antibody that was specific to the antibody of the hormones (GH and IGF-I) was precoated onto a microplate with a biotin-conjugated polyclonal antibody preparation specific for each hormones, as follows: Add 100 μL of sample per well. Following the last wash, any remaining Wash Buffer was aspirated or decanted off, and 50 μL of substrate solution was added to each well. The substrate solutions were then incubated for 15 min at 37 °C in the dark, during which time they changed from colorless or light blue to darker shades of blue. Following incubation, 50 μL of stop solution was added to each well, resulting in a color change from blue to yellow. In addition, then, the optical density of each well was determined at once, using a plate reader set to 450 nm.

The melatonin concentration in the plasma was determined using the enzyme-linked immunosorbent assay (ELISA) kit (RE54041; IBL, Hamburg, Germany). 50 μL of each sample was added to different wells of an ELISA plate that was precoated with capture antibody. The samples were incubated with the melatonin-biotin and antiserum solutions for 15 h at 4 °C. The wells were then washed with the assay buffer and the plate was incubated with the enzyme-labeled solution for 2 h at room temperature with constant shaking. After the plate was washed a second time, it was incubated with the *p*-nitrophenyl phosphate solution for 30 min before 50 μL of the stop solution was added. Absorbance was read at 405 nm.

2.7. Histological analysis

The gonadal tissues of each Kiss injection group (control, 2, 4, and 6 weeks) were fixed in Bouin's solution to analyze the gonads during the growth period. The samples were dehydrated in increasing ethanol concentrations, clarified in xylene, and embedded in paraffin. Sections (5-μm thick) were selected and stained with hematoxylin–eosin for observation under a light microscope (DM 100; Leica, Wetzlar, Germany). The images were captured using a digital camera (DFC 290; Leica).

2.8. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). A one-way analysis of variance, followed by Tukey's post-hoc test, was used to test for significant differences in the data ($p < 0.05$). The values are expressed as the means ± standard error (SE).

3. Results

3.1. Changes in the total length of the cinnamon clownfish

The total lengths of fish injected with Kiss were significantly greater than those of the control fish (Table 2), and the greatest effect was seen by the end of the experiment (6 weeks), at which point the Kiss injection group (0.1 and 0.5 μg/g) exhibited the greatest total length (9.4 ± 0.4 and 9.6 ± 0.6 cm, respectively, for the two doses).

3.2. Time-course and dose-related effects of Kiss on GH (in vivo)

The effects of Kiss injection on the expression of GH mRNA in the pituitary are shown in Fig. 1. Treatment with 0.1 and 0.5 μg/g BM of Kiss significantly increased the mRNA levels of the GH in the pituitary (Fig. 1A). In particular, the level of GH mRNA significantly increased and reached levels that were approximately 5.6-fold higher than that in the control at 6 weeks. In addition, following the Kiss injection, the plasma GH levels significantly increased and reached levels that were approximately 2.7-fold higher than that in the control at 6 weeks.

3.3. Time-course and dose-related effects of Kiss on GH (in vitro)

The mRNA expression levels of GH in the cultured pituitary cells of the cinnamon clownfish following a Kiss injection are shown in Fig. 2. Treatments with Kiss significantly increased GH mRNA level, especially at 48 h after treatment with Kiss.

3.4. Time-course and dose-related effects of Kiss on IGF-I (in vivo)

The effects of Kiss injection on the expression of IGF-I mRNA in the liver are shown in Fig. 3. Treatment with 0.1 and 0.5 μg/g BM of Kiss significantly increased the mRNA levels of the IGF-I in the liver (Fig. 3A).

Table 2
Changes in the total length of cinnamon clownfish.

weeks	Control	Sham	Kiss	
			0.1 μg/g	0.5 μg/g
0	7.3 ± 0.4^{a1}	7.3 ± 0.4^{a1}	7.5 ± 0.5^{a1}	7.4 ± 0.5 ^{a1}
2	7.5 ± 0.6^{a1}	7.4 ± 0.5^{a1}	8.4 ± 0.4^{a12}	8.2 ± 0.5^{a12}
4	7.8 ± 0.5^{a1}	7.8 ± 0.4^{a1}	8.7 ± 0.4^{b2}	8.9 ± 0.3^{b2}
6	7.7 ± 0.4^{a1}	7.8 ± 0.5^{a1}	9.4 ± 0.4^{b2}	9.6 ± 0.6^{b2}

Values with lower-case letters indicate significant differences between the treated Kiss concentrations at the same time after the Kiss treatment. The numbers indicate significant differences between initiation and 6 weeks of rearing ($P < 0.05$).

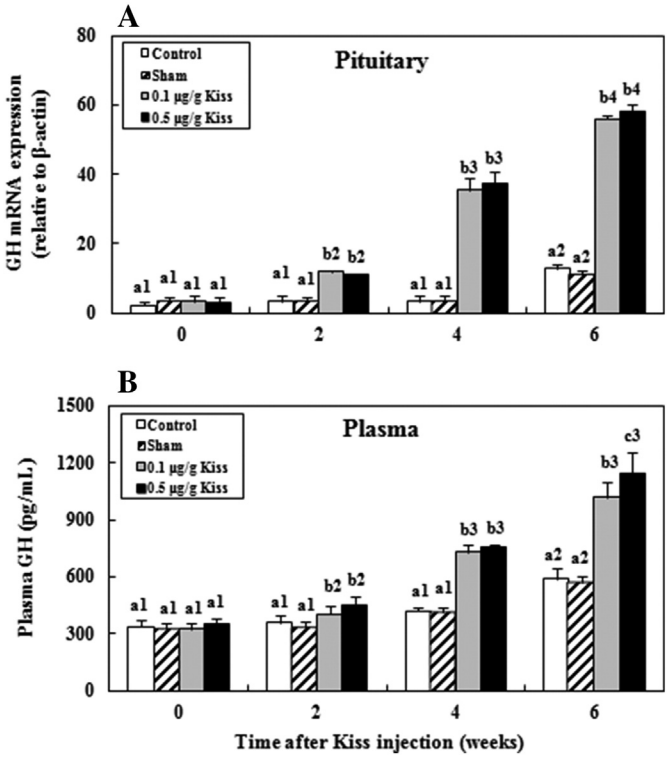

Fig. 1. The expression and activity of GH in the pituitary of cinnamon clownfish at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 0.5 μg/g) injection. (A) GH mRNA levels was relative to the β-actin mRNA levels in the pituitary of cinnamon clownfish. (B) The plasma GH activity of cinnamon clownfish at 2, 4, and 6 weeks after the Kiss injections were also analyzed using a plate reader. The numbers indicate significant differences between different time points within the same Kiss concentration injection, and the lower-case letters indicate significant differences between different Kiss concentrations injected at the same time ($P < 0.05$). All values are means ± SE ($n = 5$).

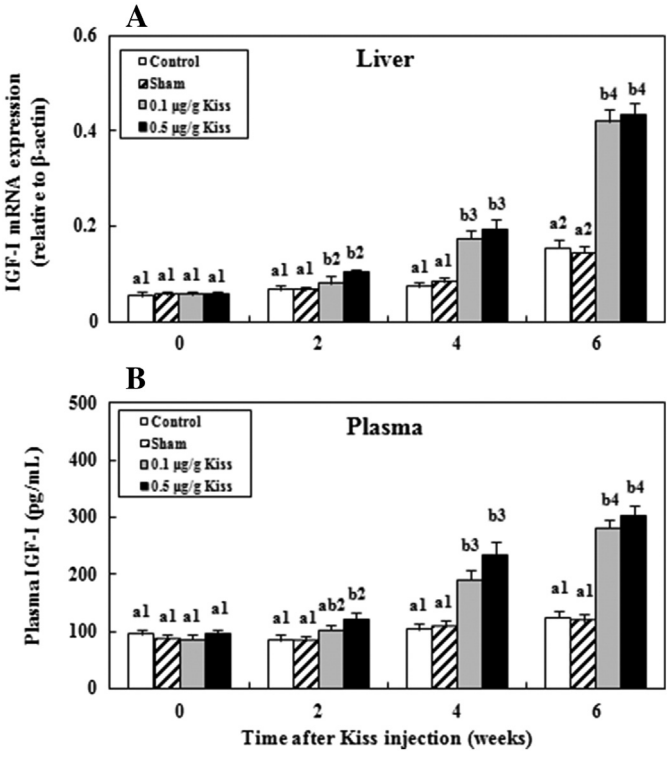

Fig. 3. The expression and activity of IGF-I in the liver of cinnamon clownfish at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 0.5 μg/g) injection. (A) IGF-I mRNA levels was relative to the β-actin mRNA levels in the liver of cinnamon clownfish. (B) The plasma IGF-I activity of cinnamon clownfish at 2, 4, and 6 weeks after the Kiss injections were also analyzed using a plate reader. The numbers indicate significant differences between different time points within the same Kiss concentration injection, and the lower-case letters indicate significant differences between different Kiss concentrations injected at the same time ($P < 0.05$). All values are means ± SE ($n = 5$).

In particular, the level of IGF-I mRNA was significantly increased and reached levels that were approximately 2.6-fold higher than that in the control at 6 weeks. Further, following the Kiss injection, the plasma IGF-I level was 94.6 ± 10.2 pg/mL at the experiment start. Most notably, the levels of IGF-I levels increased to 302.7 ± 18.5 pg/mL at 6 weeks (0.5 μg/g of Kiss, Fig. 3B).

3.5. Time-course and dose-related effects of Kiss on IGF-I (in vitro)

The mRNA expression levels of IGF-I in the cultured liver cells of cinnamon clownfish following a Kiss injection are shown in Fig. 4. Treatments with Kiss significantly increased the IGF mRNA level; approximately 6.2-fold higher than the control at 48 h.

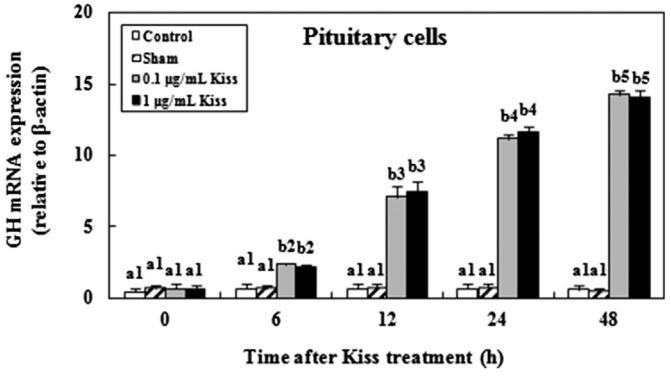

Fig. 2. The expression of GH mRNA in the cultured pituitary cells of cinnamon clownfish at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 1 μg/mL) treatment. GH mRNA levels was relative to the β-actin mRNA levels in the cultured pituitary of cinnamon clownfish. The numbers indicate significant differences between different time points within the same Kiss concentration injection, and the lower-case letters indicate significant differences between different Kiss concentrations injected at the same time ($P < 0.05$). All values are means ± SE ($n = 5$).

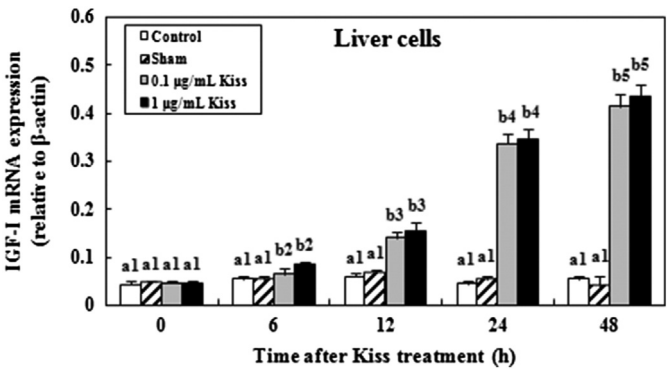

Fig. 4. The expression of IGF-I mRNA in the cultured liver cells of cinnamon clownfish at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 1 μg/mL) treatment. IGF-I mRNA levels were relative to the β-actin mRNA levels in the cultured liver of cinnamon clownfish. The numbers indicate significant differences between different time points within the same Kiss concentration injection, and the lower-case letters indicate significant differences between different Kiss concentrations injected at the same time ($P < 0.05$). All values are means ± SE ($n = 5$).

3.6. Time-course and dose-related effects of Kiss on SL (in vivo and in vitro)

The mRNA expression levels of SL in the pituitary cells following a Kiss injection are shown in Fig. 5. Treatment with 0.1 and 0.5 μg/g BM of Kiss significantly increased SL mRNA levels that were approximately 2.8-fold (*in vivo*; at 6 weeks in Fig. 5A) and 4.9-fold (*in vitro*; at 48 h in Fig. 5B) higher than that in the control, respectively.

3.7. Time-course and dose-related effects of Kiss on MT and melatonin

The effects of Kiss injection on the mRNA expressions of MT and melatonin in the pituitary and brain are shown in Figs. 6 and 7. Western blot analysis revealed a protein with antioxidant enzyme-specific immunoreactivity and with a mass that corresponded to the predicted mass for cinnamon clownfish MT (45 kDa). The expression pattern of the immunoreactive proteins resembled that of MT transcript levels in the pituitary and brain cinnamon clownfish MT. In addition, treatment with 0.1 and 0.5 μg/g BM of Kiss significantly increased the mRNA levels of the MT in the pituitary (Fig. 6A, B) and brain (Fig. 7). In particular, the level of MT mRNA was significantly increased and reached levels that were approximately 2.4-fold (at 6 weeks in Fig. 6A; *in vivo*), 2.3-fold (at 6 weeks in Fig. 7; *in vivo*) and 4.4-fold (at 48 h in Fig. 6C; *in vitro*) higher than that in the control. In addition, we measured the circulating level of melatonin in cinnamon clownfish following injection with Kiss (Fig. 6B). The plasma melatonin level was 9.3 ± 2.3 pg/mL at the experiment start period. Most notably, the levels of melatonin increased to 45.1 ± 3.5 pg/mL at 6 weeks after treatment of Kiss.

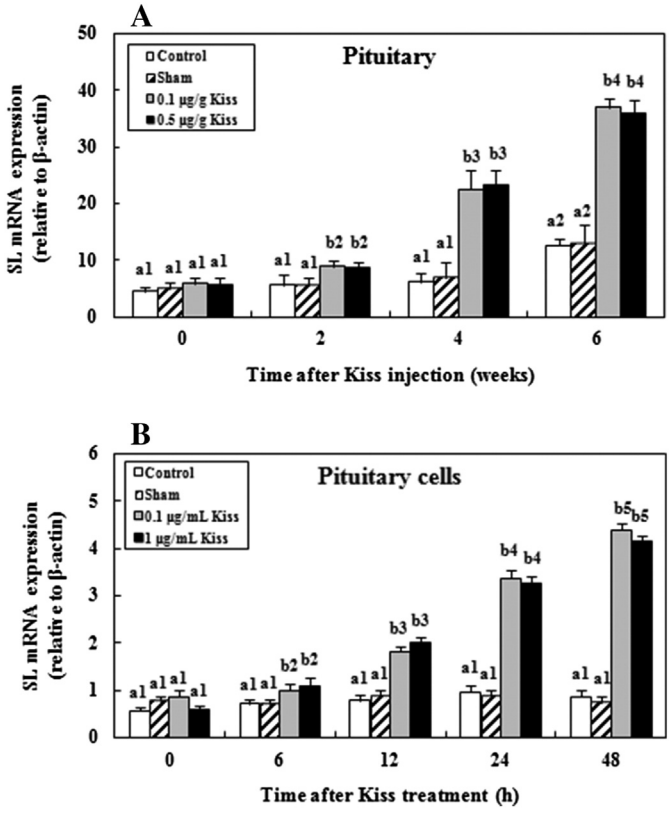

Fig. 5. The expression of SL mRNA in the pituitary glands of cinnamon clownfish at 2, 4, and 6 weeks after Kisspeptin (Kiss; *in vivo*, 0.1 and 0.5 μg/g; *in vitro*, 0.1 and 1 μg/mL) treatment. (A) SL (*in vivo*) and (B) SL (*in vitro*) mRNA levels was relative to the β-actin mRNA levels in the pituitary of cinnamon clownfish. The numbers indicate significant differences between different time points within the same Kiss concentration injection, and the lower-case letters indicate significant differences between different Kiss concentrations injected at the same time ($P < 0.05$). All values are means ± SE ($n = 5$).

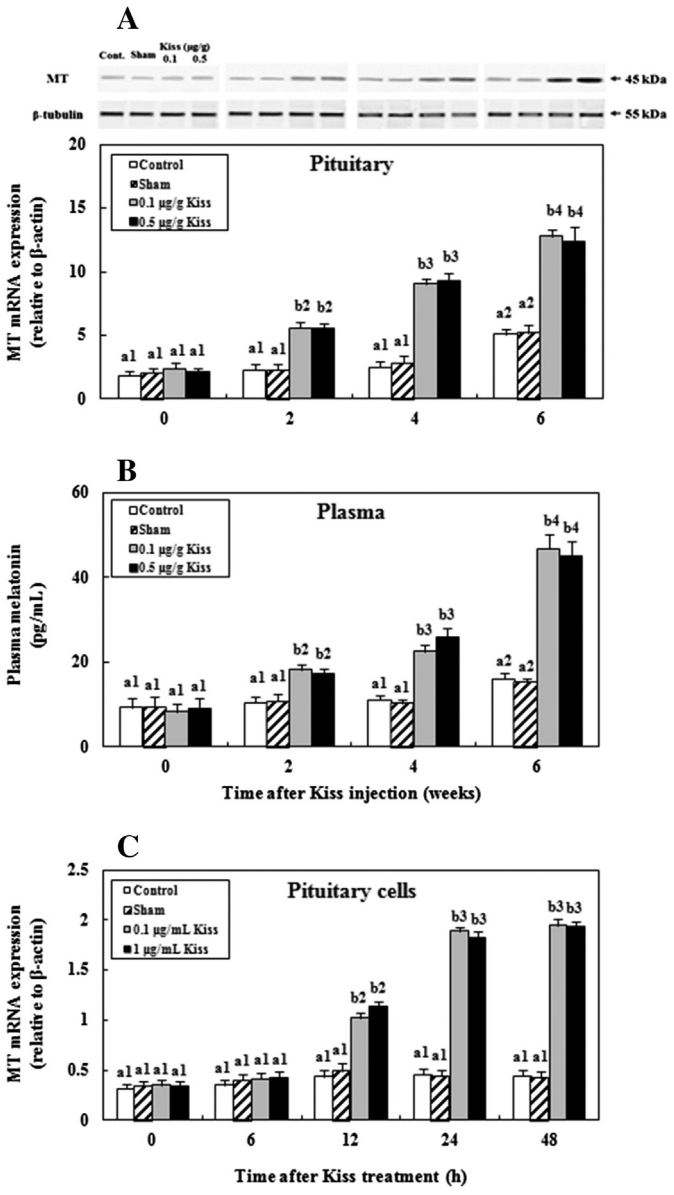

Fig. 6. The expression of MT mRNA and protein in the pituitary glands of cinnamon clownfish at 2, 4, and 6 weeks after Kisspeptin (Kiss; *in vivo*, 0.1 and 0.5 μg/g; *in vitro*, 0.1 and 1 μg/mL) treatment. Western blotting using MT (45 kDa) was used to examine protein expression in the pituitary. The β-tubulin (55 kDa) was used as the internal control. (A) MT (*in vivo*) and (C) MT (*in vitro*) mRNA levels were relative to the β-actin mRNA levels in the pituitary of cinnamon clownfish. (B) The levels of plasma melatonin levels were analyzed using a plate reader. The numbers indicate significant differences between different time points within the same Kiss concentration injection, and the lower-case letters indicate significant differences between different Kiss concentrations injected at the same time ($P < 0.05$). All values are means ± SE ($n = 5$).

3.8. Histological observations

The gonads of cinnamon clownfish mainly consisted of testicular tissue with primary oocytes (Fig. 8A). The oocytes had developed and were largest in size (Fig. 8C, from the Kiss injection to 4 weeks), the diameter of the oocytes increased, the testicular tissue regressed, and finally, the fish became female (Fig. 8D, from the Kiss injection to 6 weeks).

4. Discussion

We investigated the effects of 6-weekly injections of Kiss (0.1 and 0.5 μg/g; weekly) on the regulation mechanism of growth in cinnamon

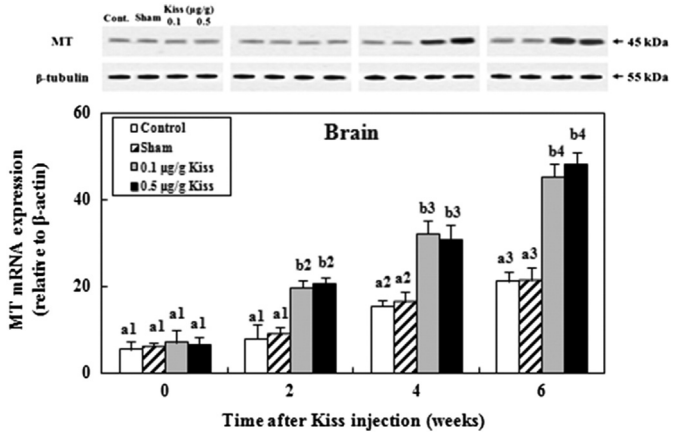

Fig. 7. The expression of MT mRNA and protein in the brain of cinnamon clownfish at 2, 4, and 6 weeks after Kisspeptin (Kiss 0.1 and 0.5 μg/g) treatment. Western blotting using MT (45 kDa) was used to examine protein expression in the brain. The β-tubulin (55 kDa) was used as the internal control. The numbers indicate significant differences between different time points within the same Kiss concentration injection, and the lower-case letters indicate significant differences between different Kiss concentrations injected at the same time ($P < 0.05$). All values are means ± SE ($n = 5$).

clownfish, as assessed by studying the effects of Kiss on the mRNA expressions of GH, IGF-I, and SL, expression of MT mRNA/protein, and plasma concentrations of GH, IGF-I, and melatonin.

Cinnamon clownfish reared for 6 weeks with weekly Kiss injections exhibited a significantly more rapid growth rate (Table 2), in addition to an increase in mRNA expression of GH and IGF-I and levels of GH and IGF-I activity (Figs. 1 and 3). Our result is similar to that of a previous study that showed that the expression of GH mRNA, distribution of GH cells in the pituitary (Yang et al., 2010), and levels of GH in the pituitary cells (Chang et al., 2012) of the goldfish *Carassius auratus*, were significantly elevated by Kiss injection. Thus, we consider that Kiss is controlled *via* neuroendocrine GH, and the synthesis of GH stimulates the pituitary, showing a positive effect on the growth of fish.

A previous study showed that GH function is regulated by IGF-I in the liver and that GH, in turn, facilitates the synthesis of IGF-I (Moriyama et al., 2000). Furthermore, IGF-I is an autocrine/paracrine growth factor that is produced in both the liver and skeletal muscle (Yamaguchi et al., 2006). The growth of skeletal muscle is controlled by myostatin negative feedback (Biga et al., 2004).

In this study, the expression of IGF-I mRNA increased upon Kiss injection (Fig. 3), we suggested that Kiss stimulated the GH, whose effect increased IGF-I stimulates and thus myostatin activity, and thereby affects the growth and feeding ability after treatment with Kiss. Yang et al. (2010) reported that pituitary hormones stimulated by Kiss affected various physiological functions, such as reproduction, digestive function, and energy metabolism *in vivo*. This result suggested that the somatic growth of cinnamon clownfish increases, as well as the secretion of GH and IGF-I, growth-related hormones, by Kiss treatment.

Furthermore, in this study, mRNA expressions of GH and IGF-I are increased in cultured experiments of pituitary and liver cells by Kiss treatment (Figs. 2 and 4), we have determined that Kiss directly affects pituitary and liver cells.

In this study, we observed the changes of SL mRNA expression and pituitary hormone, by Kiss treatment. Our results showed that the levels of SL mRNA increased significantly in all experimental groups by Kiss treatment (treatment concentration and time after treatment; Fig. 5A). In addition, expression level of SL mRNA was increased in the cultured pituitary cells (*in vitro*) to examine the direct effects of Kiss on the stimulator NIL (SL cells) in the pituitary gland (Fig. 5B).

In other words, the present study confirmed that Kiss increased the secretion of both GH and SL through the stimulation of pituitary cells (Figs. 1 and 5). Fukamachi and Meyer (2007) have reported that the interaction with GH and SL regulated important physiological functions, such as growth metabolism, in lungfish *Protopterus dolloi*, because of

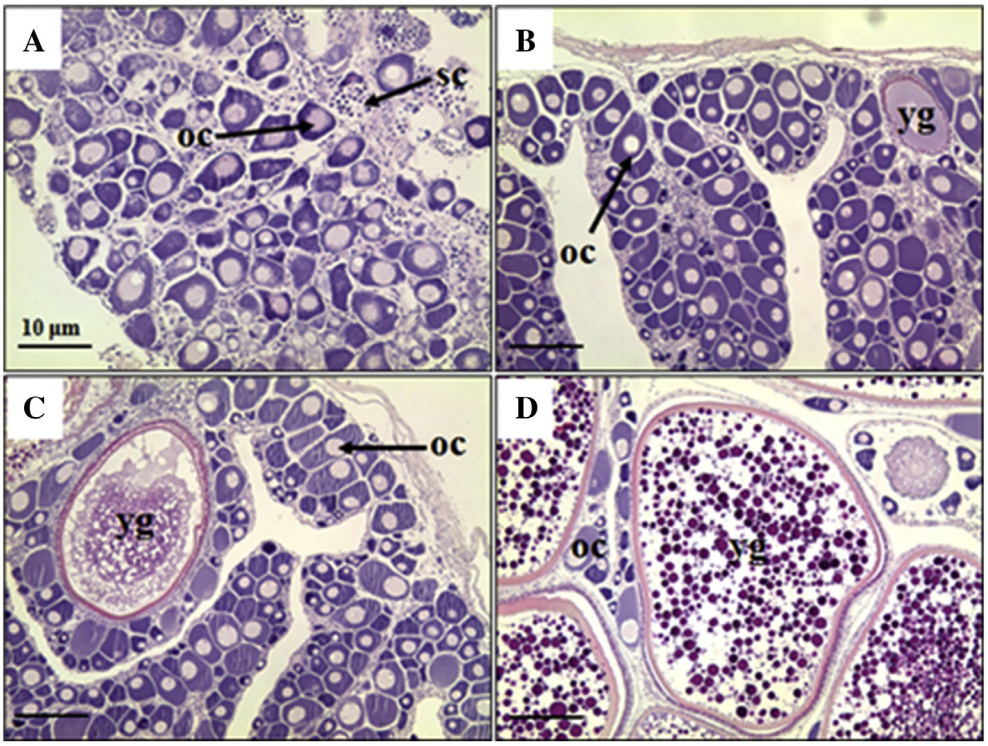

Fig. 8. Photomicrographs of cross sections of cinnamon clownfish after treatment with Kisspeptin (Kiss, 0.5 μg/g). Females: (A) controls (un-injected control), (B) 2 weeks after treatment with Kiss, (C) 4 weeks after treatment with Kiss, and (D) 6 weeks after treatment with Kiss. sc: spermatocytes, st: spermatids, oc: oocytes, yg: yolk granules. Scale bar = 10 μm. Reprinted from Kim et al. (2014), with permission from Comparative Biochemistry and Physiology, Part-B.

high homology to each of the receptors. Thus, we have suggested that Kiss directly affected the growth of fish by acting on the pituitary.

Furthermore, in the present study, we have confirmed the relevance of melatonin, which serves to promote and regulate the growth of cinnamon clownfish by Kiss processing. In the brain and pituitary, we have observed that not only the expression level of MT mRNA but also the melatonin concentration increases in accordance with the treatment concentration and time after treatment (Figs. 6, 7). In addition, the levels of plasma melatonin increased by Kiss treatment (Fig. 6B), as did the expression of MT in the cultured pituitary (*in vitro*) as a direct effect of Kiss treatment (Fig. 6C). Melatonin is implicated in numerous physiological processes, including circadian rhythms, stress, and reproduction, many of which are mediated by the hypothalamus and pituitary (Wu et al., 2006). MTs, which modulate the action of melatonin, are present in the arcuate nucleus (ARC) of the hypothalamus, coexisting with Kiss neurons. Li et al. (2011) reported that as the expression level of Kiss increased, the expression level of MT mRNA also increased. Ansel et al. (2010) reported that Kiss is a melatonin-driven photoperiodic gene and is regulated by melatonin. Also, in the present study, we found abundant expression of the MT around the brain-pituitary axis. In a similar study, Hong et al. (2014) reported that expression of the MT around the brain and pituitary gland, which agreed with previous reports concerning the detection of different MT genes. Additionally, in pike *Esox lucius* pituitary glands, ^{125}IMel binding was found to be predominant in the anteroventral part of the hypophysis, suggesting a direct action of melatonin on adenopituitary cells and melatonin increased pituitary cAMP content, which indicated that the response to melatonin depends on reproductive and growth status, or on the type of MT expressed in the pituitary glands, at the time the experiments were performed (Gaildrat and Falcon, 2000). Also, Porter et al. (1998) and Gaildrat and Falcon (2000) reported high levels of melatonin in POA of 2-[^{125}I]-iodomelatonin binding sites (melatonin binding site) of Atlantic salmon brains and pikes pituitary, further demonstrating the relationship between growth and melatonin. Therefore, from the results of this study, we have confirmed the correlation between melatonin and Kiss in fish.

After 6 weeks of Kiss treatment, mature oocyte cells were observed in many Kiss-treated gonad tissues, confirming that sexual maturation was accelerated (Fig. 8D). The sex of cinnamon clownfish is determined depending on the size the female. We have confirmed that rapid growth-induced fish matured into females by Kiss treatment.

In summary, (1) Kiss, which is located in the POA cells of the hypothalamus, stimulated the activity of anterior pituitary cells, and played an important role in the expression growth-related genes, such as GH/IGF-I and SL. (2) Melatonin, which is one of the growth regulators of the organism, and its receptor, MT, was also directly/indirectly controlled by Kiss. (3) Kiss induced somatic growth and large cinnamon clownfish became female. Thus, we have confirmed that artificially induced rapid growth in cinnamon clownfish, causing the individuals to mature into females at the same time.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (2014R1A2A2A01002747), and by Korea Institute of Ocean Science and Technology (PE99234).

References

Anderson, G.M., 2011. Pituitary actions RF-amide peptides: a critique of the evidence. Open Neuroendocrinol. J. 4, 51–63.
Ansel, L., Bolborea, M., Bentsen, A.H., Klosen, P., Mikkelsen, J.D., Simonneaux, V., 2010. Differential regulation of kiss1 expression by melatonin and gonadal hormones in male and female Syrian hamsters. J. Biol. Rhythm. 25, 81–91.
Bechtold, D.A., Luckman, S.M., 2007. The role of RF-amide peptides in feeding. J. Endocrinol. 192, 3–15.
Biga, P.R., Cain, K.D., Hardy, R.W., Schelling, G.T., Overturf, K., Roberts, S.B., Goetz, F.W., Ott, T.L., 2004. Growth hormone differentially regulates muscle myostatin-1 and -2 and increases circulating cortisol in rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 138, 32–41.
Bromage, N., Porter, M., Randall, C., 2001. The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture 197, 63–98.
Canosa, L.F., Chang, J.P., Peter, R.E., 2007. Neuroendocrine control of growth hormone in fish. Gen. Comp. Endocrinol. 151, 1–26.
Chang, J.P., Mar, A., Wlasichuk, M., Wong, A.O.L., 2012. Kisspeptin-1 directly stimulates LH and GH secretion from goldfish pituitary cells in a Ca^{2+}-dependent manner. Gen. Comp. Endocrinol. 179, 38–46.
Dubocovich, M.L., 1995. Melatonin receptors: are there multiple subtypes? Trends Pharmacol. Sci. 16, 50–56.
Fukada, H., Ozaki, Y., Pierce, A.L., Adachi, S., Yamauchi, K., Hara, A., Swanson, P., Dickhoff, W.W., 2004. Salmon growth hormone receptor: molecular cloning, ligand specificity, and response to fasting. Gen. Comp. Endocrinol. 139, 61–71.
Fukada, H., Ozaki, Y., Pierce, A.L., Adachi, S., Yamauchi, K., Hara, A., Swanson, P., Dickhoff, W.W., 2005. Identification of the salmon somatolactin receptor, a new member of the cytokine receptor family. Endocrinology 146, 2354–2361.
Fukamachi, S., Meyer, A., 2007. Evolution of receptors for growth hormone and somatolactin in fish and land vertebrates: lessons from the lungfish and sturgeon orthologues. J. Mol. Evol. 65, 359–372.
Gaildrat, P., Falcon, J., 2000. Melatonin receptors in the pituitary of a teleost fish: mRNA expression, 2-[^{125}I]-iodomelatonin binding and cyclic AMP response. Neuroendocrinology 72, 57–66.
Godwin, J.R., Thomas, P., 1993. Sex change and steroid profiles in the protandrous anemonefish *Amphiprion melanopus* (Pomacentridae, Teleostei). Gen. Comp. Endocrinol. 91, 144–157.
Gutierrez-Pascual, E., Martinez-Fuentes, A.J., Pinilla, L., Tena-Sempere, M., Malagon, M.M., Castano, J.P., 2007. Direct pituitary effects of kisspeptin: activation of gonadotrophs and somatotrophs and stimulation of luteinizing hormone and growth hormone secretion. J. Neuroendocrinol. 19, 521–530.
Holly, J.M.P., Wass, J.A.H., 1989. Insulin-like growth factors, autocrine, paracrine or endocrine? New perspectives of the somatomedin hypothesis in the light of recent developments. J. Endocrinol. 122, 611–618.
Hong, L.Y., Hong, W.S., Zhu, W.B., Shi, Q., You, X.X., Chen, S.X., 2014. Cloning and expression of melatonin receptors in the mudskipper *Boleophthalmus pectinirostris*: their role in synchronizing its semilunar spawning rhythm. Gen. Comp. Endocrinol. 195, 138–150.
Iigo, M., Furukawa, K., Tabata, M., Aida, K., 2003. Circadian variations of melatonin binding sites in the goldfish brain. Neurosci. Lett. 347, 49–52.
John, T.M., Viswanathan, M., George, J.C., Scanes, C.G., 1990. Influence of chronic melatonin implantation on circulating levels of catecholamines, growth hormone, thyroid hormones, glucose, and free fatty acids in the pigeon. Gen. Comp. Endocrinol. 79, 226–232.
Kim, N.N., Shin, H.S., Choi, Y.J., Choi, C.Y., 2014. Kisspeptin regulates the hypothalamus–pituitary-gonad axis gene expression during sexual maturation in the cinnamon clownfish, *Amphiprion melanopus*. Comp. Biochem. Physiol. B 168, 19–32.
Li, Q., Rao, A., Pereira, A., Clarke, I.J., Smith, J.T., 2011. Kisspeptin cells in the ovine arcuate nucleus express prolactin receptor but not melatonin receptor. J. Neuroendocrinol. 23, 871–882.
Maitra, S.K., Chattoraj, A., Mukherjee, S., Moniruzzaman, M., 2013. Melatonin: a potent candidate in the regulation of fish oocyte growth and maturation. Gen. Comp. Endocrinol. 181, 215–222.
Moriyama, S., Anyson, F.G., Kawauchi, H., 2000. Growth regulation by insulin-like factor-I in fish. Biosci. Biotechnol. Biochem. 64, 1553–1562.
Ono, M., Takayama, Y., Rand-Weaver, M., Sakata, S., Yasunaga, T., Noso, T., Kawauchi, H., 1990. cDNA cloning of somatolactin, a pituitary protein related to growth hormone and prolactin. Proc. Natl. Acad. Sci. U. S. A. 87, 4330–4334.
Osugi, T., Ukena, K., Sower, S.A., Kawauchi, H., Tsutsui, K., 2006. Evolutionary origin and divergence of PQRF-amide peptides and LPXRF-amide peptides in the RF-amide peptide family. Insights from novel lamprey RF-amide peptides. FEBS J. 273, 1731–1743.
Porter, M.J.R., Randall, C.F., Bromage, N.R., Thorpe, J.E., 1998. The role of melatonin and the pineal gland on development and smoltification of Atlantic salmon (*Salmo salar*) parr. Aquaculture 168, 139–155.
Ramaswamy, S., Gibbs, R.B., Plant, T.M., 2009. Studies of the localisation of kisspeptin within the pituitary of the rhesus monkey (*Macaca mulatta*) and the effect of kisspeptin on the release of non-gonadotropic pituitary hormones. J. Neuroendocrinol. 21, 795–804.
Reppert, S.M., Weaver, D.R., Godson, C., 1996. Melatonin receptors step into the light: cloning and classification of subtypes. Trends Pharmacol. Sci. 17, 100–102.
Revel, F.G., Ansel, L., Klosen, P., Saboureau, M., Pevet, P., Mikkelsen, J.D., Simonneaux, V., 2007. Kisspeptin: a key link to seasonal breeding. Rev. Endocr. Metab. Disord. 8, 57–65.
Roa, J., Navarro, T.M., Tena-Sempere, M., 2011. Kisspeptins in reproductive biology: consensus knowledge and recent developments. Biol. Reprod. 85, 650–660.
Roseweir, A.K., Millar, R.P., 2009. The role of kisspeptin in the control of gonadotrophin secretion. Hum. Reprod. Update 15, 203–212.
Simonneaux, V., Ansel, L., Revel, F.G., Klosen, P., Pevet, P., Mikkelsen, J.D., 2009. Kisspeptin and the seasonal control of reproduction in hamsters. Peptides 30, 146–153.
Trombley, S., Schmitz, N., 2013. Leptin in fish: possible role in sexual maturation in male Atlantic salmon. Fish Physiol. Biochem. 39, 103–106.
Vlaming, V.D., 1980. Effects of pinealectomy and melatonin treatment on growth in the goldfish, *Carassius auratus*. Gen. Comp. Endocrinol. 40, 245–250.
Wan, G., Chan, K.M., 2010. A study of somatolactin actions by ectopic expression in transgenic zebrafish larvae. J. Mol. Endocrinol. 45, 301–315.
Wood, A.W., Duan, C.M., Bern, H.A., 2005. Insulin-like growth factor signaling in fish. Int. Rev. Cytol. Surv. Cell Biol. 243, 215–285.

Wu, Y.H., Zhou, J.N., Balesar, R., Unmehopa, U., Bao, A., Jockers, R., Van Heerikhuize, J., Swaab, D.F., 2006. Distribution of MT1 melatonin receptor immunoreactivity in the human hypothalamus and pituitary gland: colocalization of MT1 with vasopressin, oxytocin, and corticotropin-releasing hormone. J. Comp. Neurol. 499, 897–910.

Yamaguchi, A., Fujikawa, T., Tateoka, M., Soya, H., Sakuma, K., Sugiura, T., Morita, I., Ikeda, Y., Hirai, T., 2006. The expression of IGF-I and myostatin mRNAs in skeletal muscle of hypophysectomized and underfed rats during postnatal growth. Acta Physiol. 186, 291–300.

Yang, B., Jiang, Q., Chan, T., Ko, W.K.W., Wong, A.O.L., 2010. Goldfish kisspeptin: molecular cloning, tissue distribution of transcript expression, and stimulatory effects on prolactin, growth hormone and luteinizing hormone secretion and gene expression via direct actions at the pituitary level. Gen. Comp. Endocrinol. 165, 60–71.

Zeman, M., Výboh, P., Juráni, M., Lamošová, D., Koštál, B., Bilčík, B., Blažíček, P., Jurániová, E., 1993. Effects of exogenous melatonin on some endocrine, behavioral and metabolic parameters in Japanese quail *Coturnix coturnix japonica*. Comp. Biochem. Physiol. A 105, 323–328.