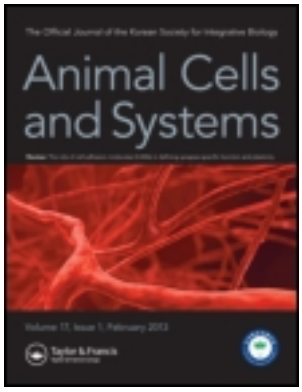

Animal Cells and Systems
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tacs20

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Published online: 17 Apr 2014.

To cite this article: Hyun Suk Shin, Jin Ah Song, Ji Yong Choi, Na Na Kim, Young Jae Choi, Si Nae Sung, Mi Seon Park, Byung Hwa Min & Cheol Young Choi (2014) Effects of various photoperiods on Kisspeptin and reproductive hormones in the goldfish, Carassius auratus, Animal Cells and Systems, 18:2, 109-118, DOI: 10.1080/19768354.2014.902863

To link to this article: http://dx.doi.org/10.1080/19768354.2014.902863

Animal Cells and Systems, 2014
Vol. 18, No. 2, 109–118, http://dx.doi.org/10.1080/19768354.2014.902863

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(*Received 22 September 2013; received in revised form 4 March 2014; accepted 5 March 2014*)

The study aimed to test differences in the hormones of the hypothalamus–pituitary–gonad axis, Kisspeptin (Kiss) and sex steroids, and the sexual maturation of goldfish (*Carassius auratus*) according to various photoperiods: 10 h light (L):14 h dark (D) (short day), 12L:12D (control), and 14L:10D (long day). To investigate the sexual maturation under various photoperiods, quantitative real-time PCR (QPCR) assays for salmon gonadotropin-releasing hormone, chicken gonadotropin-releasing hormone, gonadotropin hormones (GTHs), Kisspeptin 1 (Kiss1), and its receptor, G protein-coupled receptor 54 (GPR54), were developed. mRNA expression was monitored in cultured pituitary cells (*in vitro*) of various photoperiod groups. Furthermore, we injected or treated the animals with Kiss and found that Kiss treatment increases the mRNA expression levels of GTHs. We measured the plasma follicle-stimulating hormone (FSH), luteinizing hormone (LH), and 17α-hydroxypregnenolone levels. The gonadotropin-releasing hormones (GnRHs), GTHs, Kiss1/GPR54 mRNA expression, plasma FSH, LH, and 17α-hydroxypregnenolone levels in the 14L:10D group were significantly higher than in the other groups. These results suggest that light length regulates sex maturation by GnRH and Kiss1 in the brain of the goldfish.

Keywords: gonadotropin-releasing hormone; kisspeptin; photoperiod; sex maturation; sex steroid hormone

Introduction

Sexual development and maturation in teleosts are regulated by various sex hormones in the hypothalamus–pituitary–gonad (HPG) axis, including gonadotropin-releasing hormone (GnRH), gonadotropin hormone (GTH), and steroid hormones, and elsewhere by neuroendocrine materials and gonadal hormones (Baroiller et al. 1999).

To date, the role of GnRH in the control of pituitary hormone secretion, in the direct GnRH actions on oocyte meiosis, and in the steroidogenesis (Habibi et al. 1988; Pati & Habibi 2002) has been well documented. Fifteen GnRH isoforms have been isolated from vertebrates and protochordates (Fernald & White 1999; Adams et al. 2002). Using the goldfish (*Carassius auratus*) as experimental fish, the presence of two distinct forms of GnRH, namely, salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II), in the brain of a single fish has been characterized (Yu et al. 1988). Generally, in certain teleosts, such as striped bass (*Morone saxatilis*), gilthead seabream (*Sparus aurata*), and Nile tilapia (*Oreochromis niloticus*), sGnRH is produced as a third form of GnRH in neuronal groups localized in the ventral forebrain along the terminal nerve (Senthilkumaran et al. 1999). cGnRH-II neurons are localized in the midbrain tegmentum, project their axons widely throughout the central nervous system, and modulate sexual and feeding behaviors (White et al. 1998).

The role of GnRH, the hormone that regulates sex maturation in the hypothalamus, is well characterized. In contrast, Kisspeptin (Kiss) is a neuropeptide that has recently been detected in the hypothalamus (Lee et al. 1996) and regulates sexual differentiation and spawn time in fish through the activation of the HPG axis (Funes et al. 2003; Seminara et al. 2003). Additionally, Kiss acts as a sex maturation-regulating factor by activating certain nerves in the brain through the regulation of GnRH expression (Chang et al. 2012).

There are two types of Kiss isoforms in teleosts and amphibians (Lee et al. 2009; Um et al. 2010). Kisspeptin 1 (Kiss1) is one of the important neuroendocrine factors that regulate the sexual maturation of teleosts (Roa et al. 2011), and it plays a role in the initiation of sexual maturation in the GnRH neurons of the hypothalamus (Colledge 2009). Additionally, histological observations have shown that Kiss1 and its receptor, G protein-coupled receptor 54 (GPR54), are located with the GnRH neurons in the hypothalamus. Interactions between GnRH have also been reported (Irwig et al. 2004; Messager et al. 2005). According to these various research results, the Kiss1-GPR54 signaling system is one of the circuits that regulate GnRH secretion in the hypothalamus.

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†These authors contributed equally to this work.

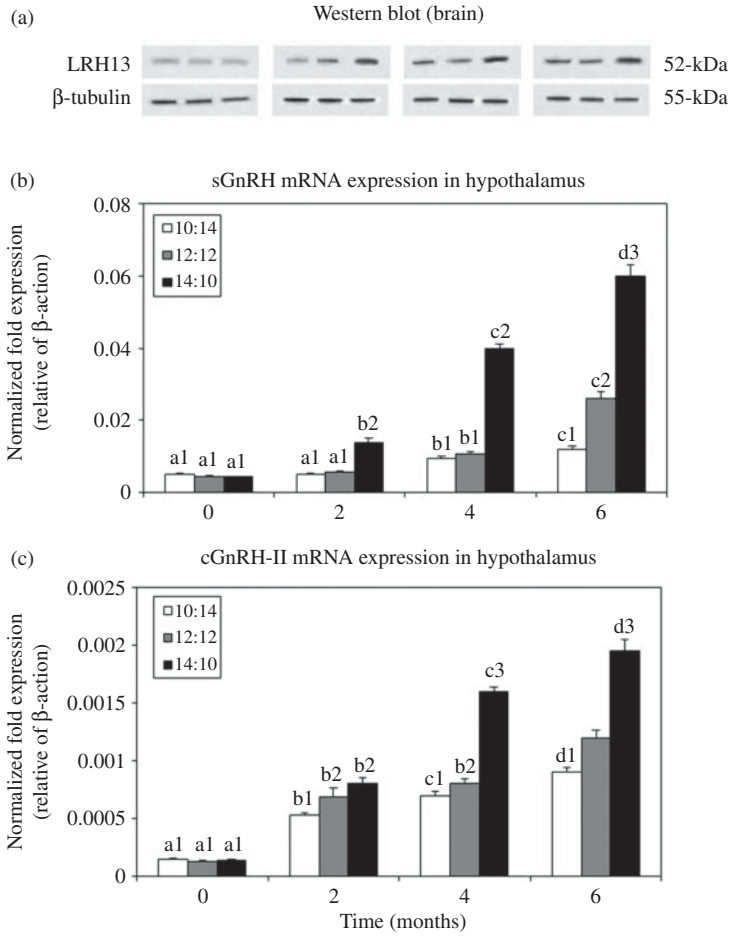

Figure 1. Changes in the expression levels of the LRH13 protein (identified using a monoclonal mouse antiserum that recognizes most vertebrate GnRH forms; 52-kDa; Park & Wakabayashi 1986) in the brain (a) and sGnRH (b) and cGnRH-II (c) mRNA levels in the hypothalamus of goldfish maintained under different photoperiods – 10L:14D (short day), 12L:12D (control), and 14L:10D (long day) – using a white fluorescent bulb. Total hypothalamus RNA (2.0 μg) was reverse-transcribed and amplified. The results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. Values marked with different characters are significantly different at different times (months) in fish exposed to the same photoperiod ($P < 0.05$). The numbers indicate significant differences between different photoperiods within the same month ($P < 0.05$). All values are means ± SD ($n = 10$).

The synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the pituitary are stimulated by GnRH and Kiss1 protein synthesis and secretion in the hypothalamus, and these hormones play an important role in regulating reproduction in teleosts (Andrews et al. 1988). There are two types of GTH in fish, FSH and LH (Van Der Kraak et al. 1998). FSH and LH induce gonad development and gonad steroid hormone synthesis (Andrews et al. 1988). During the induction process, the precursor of gonad steroid hormone, 17α-hydroxypregnenolone, is secreted, and cholesterol is changed to 17α-hydroxypregnenolone by LH, which affects the theca follicular cells and granulosa cells during the final stage of maturation. 17α-hydroxypregnenolone also plays an important role in the synthesis of sex hormones (estradiol and testosterone [T]), which interact with FSH (Dickey and Swanson 2000; Yamato et al. 2010).

Various factors influence sex maturation, and external factors such as photoperiod, temperature, pH, or feeding behavior affect the endocrinological side (Baroiller et al. 1999). Among these factors, photoperiod influences the regulation of diurnal endocrine rhythm (Duston & Saunders 1990) and growth in fish (Gross et al. 1995) and sex maturation (Biswas & Takeuchi 2002). In particular, photoperiod regulates sex maturation (Rodrlguez et al. 2000) by strongly affecting neuroendocrine control and the HPG axis (Koumoundouros et al. 2002).

Therefore, we investigated the changes in GnRH, GTHs, and Kiss1/GPR54 mRNA expression under different photoperiods using *in vivo* and *in vitro* (pituitary cell culture) methods to examine the influence of light exposure time on goldfish sex maturation. Additionally, we observed the changes in GTHs mRNA expression in pituitary cultures treated with Kiss to investigate the influence of Kiss on the

Table 1. Primers used for QPCR amplification.

Genes (accession no.)	Primer	DNA sequences
cGnRH-II (U40567)	Forward	5′-TTC AGA GGT TTC AGA AGA AAT CAA-3′
	Reverse	5′-GCG TCC AGC AGT ATT GTC-3′
sGnRH (AB017272)	Forward	5′-CCA ACA GAC GAG GAA GAG-3′
	Reverse	5′-CGA TTC AGG ACG CAA ACT-3′
Kisspeptin (FJ236327)	Forward	5′-TGA ACC TAC TTA CCA TAA TTT TGA TG-3′
	Reverse	5′-CCTGAG ACC CTG GAG TGA -3′
GPR54 (FJ465139)	Forward	5′-AGT GGT CAT TGT TGT TCT CTT-3′
	Reverse	5′-AGG AGT TGG CAT AGG ACA T-3′
GTHα (D86552)	Forward	5′- TAT CGG TGG TGC TGG TTA -3′
	Reverse	5′- GCT GTC CTC AAA GTC GTT A -3′
FSHβ (D88023)	Forward	5′-CCT GGA AAG TGA GGA ATG-3′
	Reverse	5′GTT CTG GTA AGA CAG CAT CA-3′
LHβ (D88024)	Forward	5′-TGT CCT ATT CTC TGT AAT TGT CC-3′
	Reverse	5′-GTC TCA TTA ACT GGC TCA CA -3′
β-actin (AB039726)	Forward	5′- TTC CAG CCA TCC TTC CTA T-3′
	Reverse	5′- TAC CTC CAG ACA GCA CAG -3′

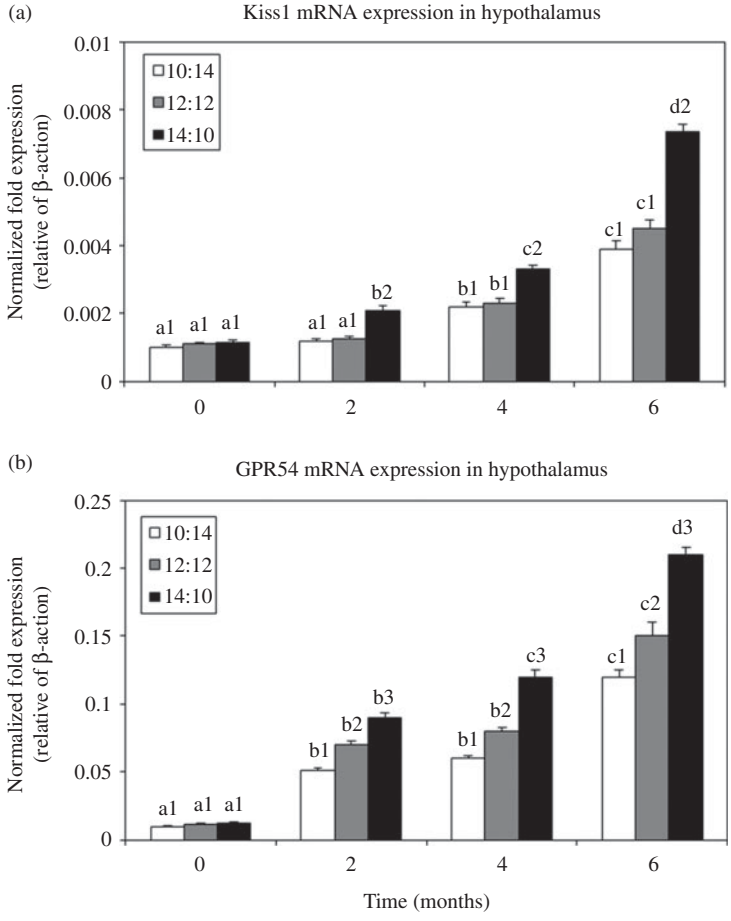

Figure 2. Changes in the mRNA expression levels of Kiss1 (a) and its receptor, GPR54 (b) in the hypothalamus in goldfish maintained under different photoperiod conditions – 10L:14D (short day), 12L:12D (control), and 14L:10D (long day) – using a white fluorescent bulb, as measured by QPCR. Total hypothalamus RNA (2.0 μg) was reverse-transcribed and amplified. The results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. Values marked with different characters are significantly different at different times (months) in fish exposed to the same photoperiod ($P < 0.05$). The numbers indicate significant differences between different photoperiods within the same month ($P < 0.05$). All values are means ± SD ($n = 10$).

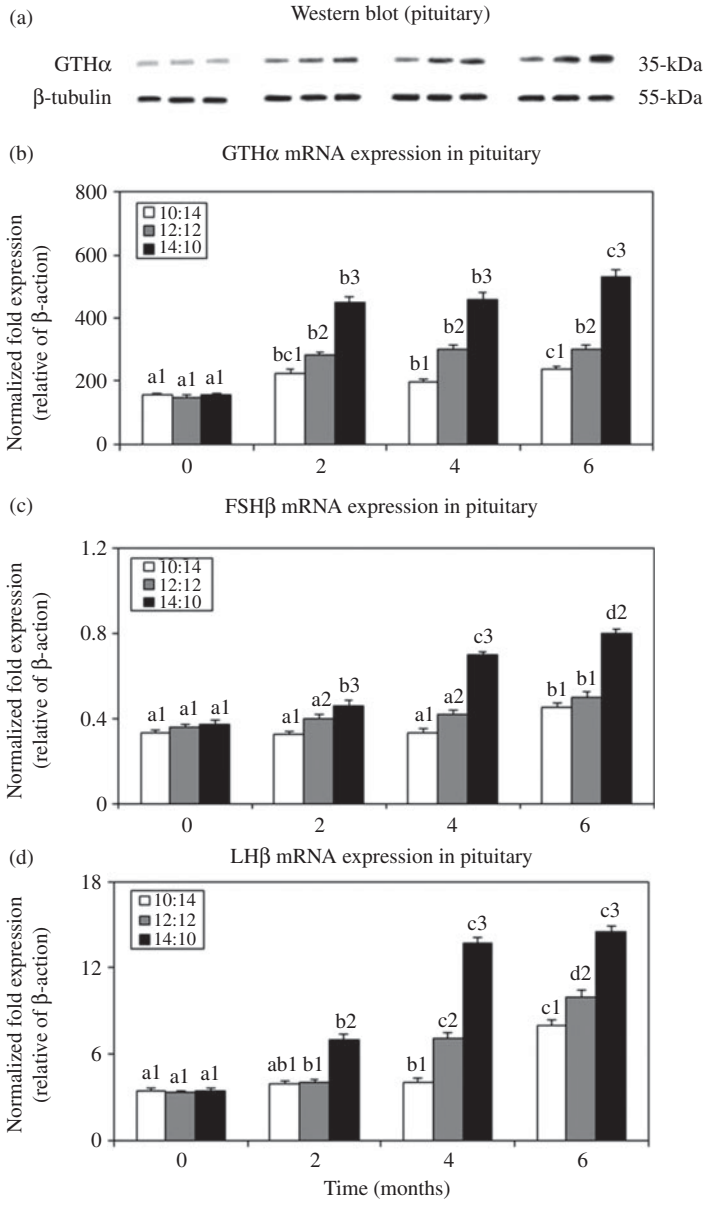

Figure 3. Changes in the expression levels of the GTHα protein (detected using anti-goldfish GTHα; a polyclonal rabbit antibody; 35-kDa; Kobayashi et al. 2006) in the pituitary (a) and GTHα (b), FSHβ (c), and LHβ (d) mRNA in the pituitary of goldfish maintained under different photoperiod conditions – 10L:14D (short day), 12L:12D (control), and 14L:10D (long day) – using a white fluorescent bulb, as measured by QPCR. Total pituitary RNA (2.0 µg) was reverse-transcribed and amplified. The results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. Values marked with different characters are significantly different at different times (months) in fish exposed to the same photoperiod ($P < 0.05$). The numbers indicate significant differences between different photoperiods within the same month ($P < 0.05$). All values are means ± SD ($n = 10$).

expression of gonadal hormones secreted by the HPG axis. Furthermore, we examined the difference of goldfish sex maturation by photoperiod regulation by measuring plasma FSH, LH, and the 17α-hydroxypregnenolone levels.

Experimental fish and conditions

The immature goldfish (length 5.2 ± 1.2 cm, weight 8.1 ± 3.2 g) were purchased from Choryang aquarium (Busan, Korea). The goldfish were transferred into 300-L circulation filter tanks in the laboratory and reared for 6 months. The fish were randomly divided into three tanks, and each tank was exposed to one of three photoperiods (10 h light (L):14 h dark (D), 12L:12D, 14L:10D). The start of the light period for the 12L:12D and 14L:10D groups was 07:00 and for the 10L:14D group was 09:00. The light source was a white fluorescent bulb, and the lights were set 50 cm above the water surface. The water temperature

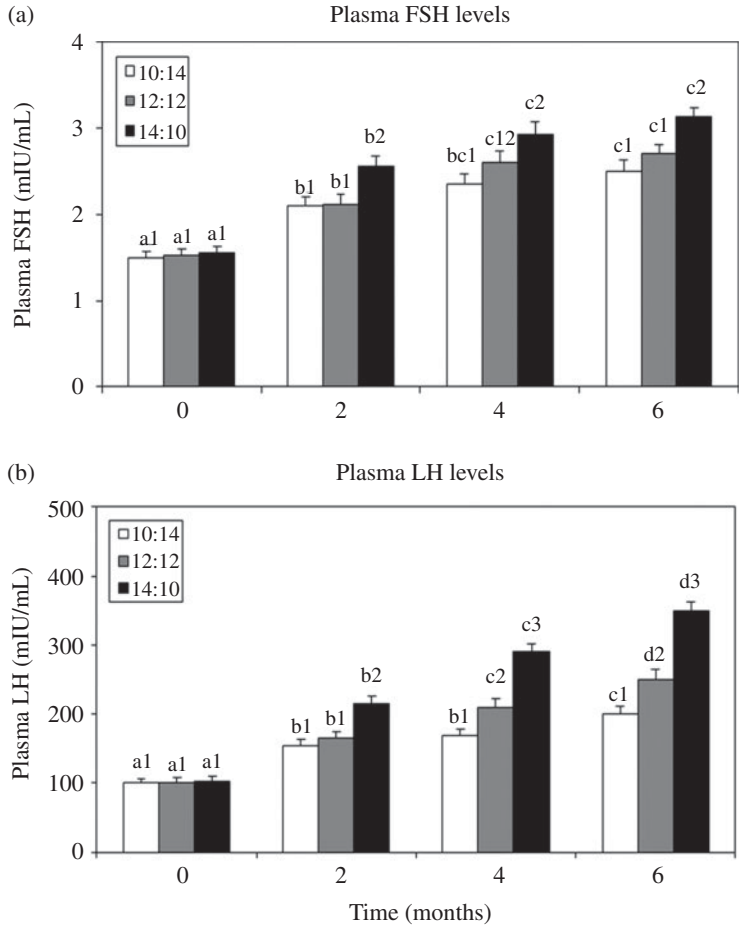

Figure 4. Changes in the levels of plasma FSH (a) and LH (b) in goldfish under different photoperiod conditions – 10L:14D (short day), 12L:12D (control), and 14L:10D (long day) – using a white fluorescent bulb, as measured by a plate reader. Values with different characters are significantly difference at different times (months) in fish exposed to the same photoperiod ($P < 0.05$). The numbers indicate significant differences between different photoperiods within the same months ($P < 0.05$). All values are means ± SD ($n = 10$).

was maintained at 22 ± 1°C. The goldfish were provided with commercial feed twice daily (09:00 h and 17:00 h) at a 4–5% wet body weight per day.

The experiments were performed under these conditions for 6 months at intervals of two months. After 2, 4, and 6 months, female goldfish were analyzed in this study. Prior to each experiment, the fish were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222, Sigma, USA), and their body masses and total lengths were recorded.

Results

Changes in sGnRH, cGnRH-II mRNA, and GnRH protein expression levels

GnRH protein levels using protein extracted from goldfish brain were increased in all three photoperiod groups as rearing time increased. In particular, the levels in 14L:10D were high compared with the other photoperiods (Figure 1a). Additionally, the expression of sGnRH and cGnRH-II mRNA levels using cDNA extracted from goldfish hypothalamus was significantly higher in 14L:10D group compared with the other photoperiods by quantitative real-time PCR (QPCR) (Figure 1b, c).

Changes of Kiss1 and GPR54 mRNA expression levels

To compare the expression levels of Kiss1 and GPR54 mRNA in the three photoperiod groups, we performed QPCR (Table 1) using cDNA extracted from goldfish hypothalamus in each experimental group. As a result, Kiss1 and GPR54 mRNA expression levels in 14L:10D were significantly higher compared with the other groups (Figure 2). Additionally, we observed increases in the Kiss1 and GPR54 mRNA expression levels in the three photoperiod groups as rearing time increased.

Changes of GTHs mRNA and GTHα protein expression levels

The GTHα protein levels measured in protein extracted from the goldfish pituitary increased with rearing time in

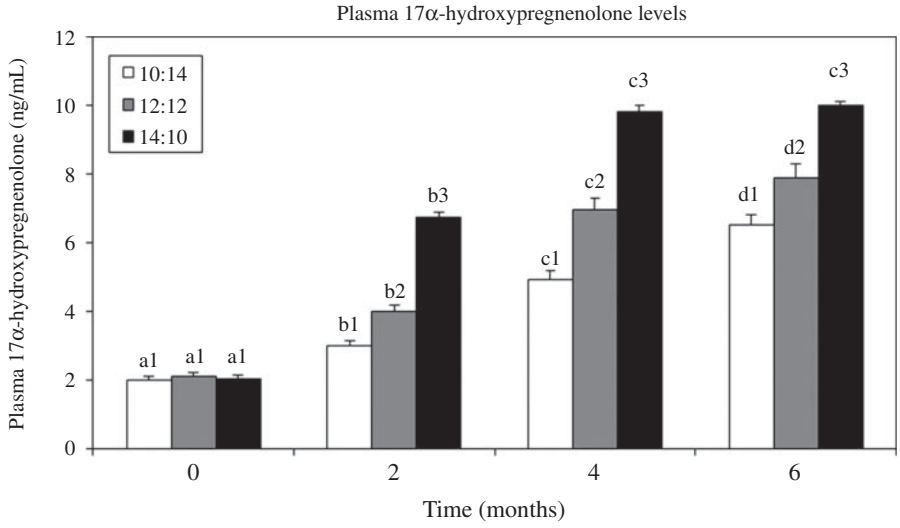

Figure 5. Changes in the levels of plasma 17α-hydroxypregnenolone in goldfish under different photoperiod conditions – 10L:14D (short day), 12L:12D (control), and 14L:10D (long day) – using a white fluorescent bulb, as measured by a plate reader. Values marked with different characters are significantly different at different times (months) in fish exposed to the same photoperiod ($P < 0.05$). The numbers indicate significant differences between different photoperiods within the same month ($P < 0.05$). All values are means ± SD ($n = 10$).

all three photoperiod groups. In particular, the levels in 14L:10D were high compared with those in the other photoperiods (Figure 3a). Additionally, we performed QPCR using cDNA extracted from goldfish pituitary in each experimental group to compare the expression levels of GTHs (GTHα, LHβ, and FSHβ) mRNA. The levels of GTHα, LHβ, and FSHβ mRNA expressions were significantly higher in the 14L:10D group, which was illuminated longer than the other photoperiod groups, compared with the other photoperiods (Figure 3b, c, and d).

Plasma FSH, LH, and 17α-hydroxypregnenolone levels

Plasma FSH, LH, and 17α-hydroxypregnenolone levels were increased in all three photoperiod groups as rearing time increased. In particular, the levels in 14L:10D were high compared with those in the other photoperiod groups. The FSH levels increased from 1.55 ± 0.08 mIU/mL to 3.13 ± 0.10 mIU/mL, and the LH levels increased from 103.03 ± 6.18 mIU/mL to 350.14 ± 13.11 mIU/mL (Figure 4). Plasma 17α-hydroxypregnenolone levels were significantly increased from 2.05 ± 0.10 ng/mL to 10.10 ± 0.13 ng/mL after 6 months (Figure 5).

Changes of GTHs mRNA expression levels by treated Kiss (in vitro)

We investigated the changes in the GTHs (GTHα, LHβ, and FSHβ) mRNA expression levels by pituitary culturing treated with Kiss (0, 100, and 1000 nM) for 5 days under the three photoperiod conditions: 10L:14D, 12L:12D, and 14L:10D. As a result, GTH mRNA expression levels were significantly higher in the 14L:10D group treated with 100 nM Kiss. However, the GTH mRNA expression levels were significantly decreased in the group treated with 1000 nM Kiss. In particular, the GTH mRNA expression levels in 14L:10D were significantly lower than those of the other groups (Figure 6).

Body weight

We have compared the body weight to investigate the sex maturation levels of goldfish according to rearing period in each photoperiod experimental group. The body weight at the initial stage was about 9.0 ± 0.5 g, but rearing time increased, the weights were significantly increased in all experimental groups. The highest value of weight of 25.07 ± 1.30 g was observed in 14L:10D compared with the other groups (Figure 7).

Discussion

To investigate the effects of photoperiod on the sexual maturation of goldfish, we reared the goldfish under three photoperiod conditions (10L:14D, 12L:12D, and 14L:10D) for 6 months and examined various parameters every two months. We examined the mRNA levels of GnRH, GTHs, the neuropeptide Kiss1 and its receptor GPR54, and the changes in GTH mRNA levels in an *in vitro* pituitary culture treated with Kiss. Furthermore, we investigated the changes in plasma FSH, LH, and 17α-hydroxypregnenolone levels.

First, we observed that the GnRH protein and the mRNA expression levels of two types of GnRH, sGnRH, and cGnRH-II were significantly higher in the 14L:10D group (Figure 1). The present results are in agreement

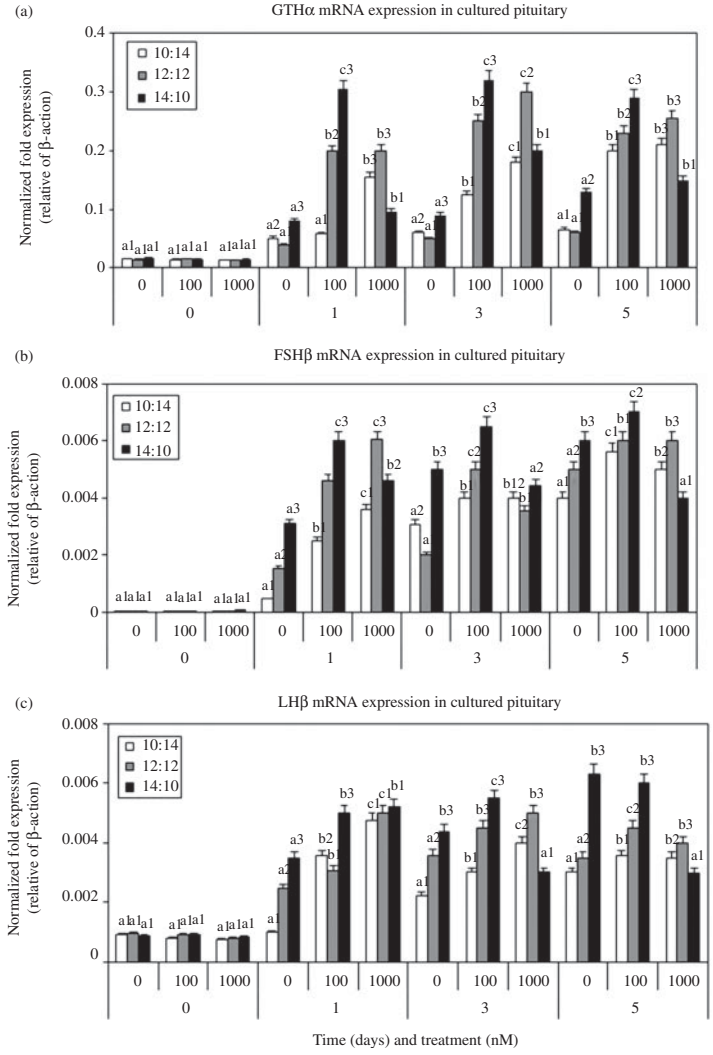

Figure 6. Changes in the expression levels of GTHα (a), FSHβ (b), and LHβ (c) mRNA in the cultured pituitary (kiss treatment) of goldfish under different photoperiod conditions – 10L:14D (short day), 12L:12D (control), and 14L:10D (long day) – using a white fluorescent bulb, as measured by quantitative real-time PCR. Total cultured pituitary RNA (2.0 μg) was reverse-transcribed and amplified. The results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. Values with different characters are significantly different at different Kisspeptin treatment concentrations (nM) in fish exposed to the same days ($P < 0.05$). The numbers indicate significant differences between different photoperiods within the same days ($P < 0.05$). All values are means ± SD ($n = 5$).

with a previous report by Carrillo et al. (2010), which demonstrated growth and sexual maturation of European sea bass (*Dicentrarchus labrax*) illuminated with light for long period (18L:6D) were significantly higher than natural photoperiod.

Furthermore, the changes in the neuropeptide Kiss1, which affects the mRNA expression levels of GnRH and its receptor GPR54 in the hypothalamus, were significantly higher in 14L:10D, and the results were similar to the changes in the GnRH mRNA expression (Figure 2). These results demonstrate that a longer exposure to light positively affects sexual maturation, and the results are in agreement with those of a study that showed high levels of Kiss1 mRNA expression in Atlantic cod (*Gadus morhua*) illuminated by long light exposure (Cowan et al. 2012). Additionally, the sexual maturation and Kiss1 expression levels in a mammal, the Syrian hamster, were significantly increased by a long-day photoperiod (Revel et al. 2006).

According to a report from Irwig et al. (2004), histological observations show that Kiss1 is localized within the GnRH neurons in the hypothalamus. Colledge (2009) also reported that the interaction between GnRH and Kiss1 is a function of the GnRH regulator, which regulates the gonad steroid hormones; these hormones interact with Kiss1, and their signals are conveyed to the brain through a feedback mechanism. According to these results, GTHα protein and GTHα, FSHβ, LHβ mRNA expression levels were significantly higher in 14L:10D, the group that

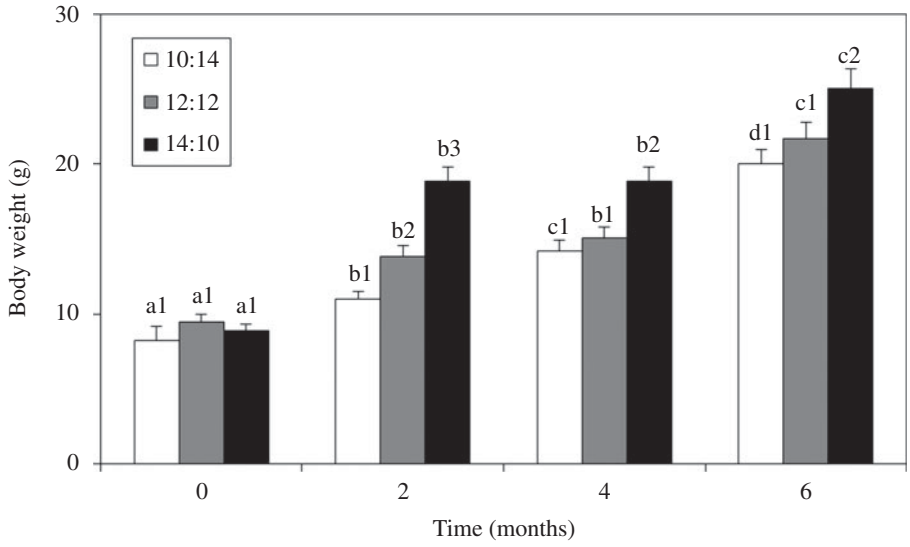

Figure 7. Changes in the body weight in goldfish maintained under different photoperiod conditions – 10L:14D (short day), 12L:12D (control), and 14L:10D (long day) – using a white fluorescent bulb. Values marked with different characters are significantly different at different times (months) in fish exposed to the same photoperiod ($P < 0.05$). The numbers indicate significant differences between different photoperiods within the same month ($P < 0.05$). All values are means ± SD ($n = 10$).

showed the highest expression levels of Kiss1 and GPR54 (Figure 3). Additionally, plasma FSH and LH levels were significantly increased in the 14L:10D group (Figure 4).

Meanwhile, Kiss1 is reported to be expressed in the hypothalamus of various fish (Kanda et al. 2008; Kitahashi et al. 2009). Kiss functions as a GnRH regulator in the hypothalamus (Colledge 2009), and it plays a positive and negative feedback role by causing sex hormones to be secreted (Colledge 2008). Therefore, we hypothesized that the significant high expression levels of Kiss1 and GPR54 mRNA in the hypothalamus and GTH mRNA in the pituitary suggested that the increased Kiss1 in 14L:10D enhanced GTH secretion in the pituitary, and then the secretion of reproductive hormones are significantly increased in 14L:10D compared with the other photoperiod conditions. To examine this hypothesis, we examined the expression of GTH mRNA in the pituitary culture (*in vitro*) treated by Kiss and observed significantly high levels (Figure 6). This result is consistent with the hypothesis that Kiss enhances GTH secretion in the pituitary and previous reports that show increasing LH mRNA expression levels from HPG axis stimulation of Kiss when the pituitaries of mature Wistar rats (Gutiérrez-Pascual et al. 2007) and goldfish (Bo et al. 2010) are treated with Kiss. Additionally, previous pharmacological research has demonstrated that Kiss is able to stimulate FSH secretion in the rat (Messager et al. 2005).

However, we observed that the GTH mRNA expression levels were weakly decreased in the 14L:10D group treated with a high concentration (1000 nM) of Kiss. This result is in agreement with Rance (2009), who reported a decrease in GTH expression through a decrease in GnRH expression according to the action of negative feedback from an increased concentration of Kiss in the HPG axis. In particular, we hypothesized that the observed decrease in GTHs in only 14L:10D means that 14L:10D is sufficient to stimulate the secretion of the reproductive hormones required for sexual maturation without external Kiss treatment based on high expression levels of Kiss1, GnRH, and GTH mRNA. Therefore, negative feedback action occurred in 14L:10D due to the treatment with Kiss.

Additionally, the levels of the precursor of gonad steroid hormone secreted in the final stage of maturation, 17α-hydroxypregnenolone, were significantly higher in 14L:10D (Figure 5). Plasma steroids are converted to 17α-hydroxypregnenolone during steroidogenesis by stimulation of LH and FSH (Hu et al. 2001); therefore, we hypothesized that the levels of this hormone have a close relationship with sexual maturation because its expression level patterns are similar to those of FSH and LH.

These results are in agreement with previous studies showing that sexual maturation under continuous light conditions for a few months is enhanced through a significant increase in sex hormones and the number of oocytes and developing oocytes in Atlantic salmon (*Salmo salar*) (Taranger et al. 1999), Atlantic cod (Hansen et al. 1995), and rainbow trout (Duston & Bromage 1986). Also, several previous studies have shown that long photoperiods stimulate reproduction in the goldfish and golden shiner (e.g. De Vlaming 1975; Stacey et al. 1979; Delahunty et al. 1980). In addition, Bon et al. (1999) reported that a photoperiod involving a longer day length simulates initial reproduction development. Thus, this study results supported the theory that there is a relationship between light and sexual maturation (Stacey et al. 1979; Duston & Bromage 1986; Hansen et al. 1995; Revel et al. 2006).

In conclusion, we hypothesized that Kiss1 is an important factor in the mechanism regulating FSH, LH, and GnRH. Furthermore, based on these results, GnRHs, GTHs, Kiss1/GPR54 mRNA expression, plasma FSH, LH, and 17α-hydroxypregnenolone levels were significantly higher in 14L:10D, the longer day length photoperiod. As the results, we have examined the possibility of female sex maturation by various photoperiods. The maturation stage for this experimental period was for the initial maturation stage, not the final sex maturation stage in this study.

Furthermore, we have plans to investigate the further research about final sex maturation by various photoperiods. These results (1) suggest that a longer day length photoperiod affects the stimulation of the sexual maturation of goldfish and (2) provide the basic information needed for the investigation of the mechanism through which Kiss mediates sexual maturation. Moreover, as one of the genes that regulate maturation in an epistatic fashion, Kiss is one of the important factors in the regulation of sexual maturation.

Acknowledgments

This study was supported by a grant from the National Fisheries Research & Development Institute [NFRDI; RP-2013-AQ-222], and by the Information Technology Research Center (ITRC) support program supervised by the National IT Industry Promotion Agency (NIPA) [2013-H0301-13-2009].

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