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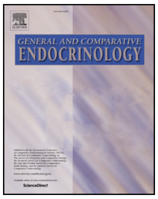

Effects of high and low temperature on expression of GnIH, GnIH receptor, GH and PRL genes in the male grass puffer during breeding season

Mohammad Lutfar Rahman[a,b], Md. Mahiuddin Zahangir[a,c], Takashi Kitahashi[a], Md. Shahjahan[b], Hironori Ando[a,*]

[a] Marine Biological Station, Sado Island Center for Ecological Sustainability, Niigata University, Sado, Niigata 952-2135, Japan
[b] Department of Fisheries Management, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
[c] Department of Fish Biology and Biotechnology, Chittagong Veterinary and Animal Sciences University, Chittagong 4225, Bangladesh

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ABSTRACT

Gonadotropin-inhibitory hormone (GnIH) is a multifunctional hypophysiotropic neurohormone and has a stimulatory role in the control of reproduction in the grass puffer. To clarify the neuroendocrine mechanisms underlying the effect of changes in water temperature on reproduction in fish, we previously revealed that, in parallel to gonadal regression, both low and high temperature significantly decreased the expressions of the genes encoding kisspeptin (*kiss2*), kisspeptin receptor (*kiss2r*), gonadotropin-releasing hormone 1 (*gnrh1*) in the brain and gonadotropin (GTH) subunits (*fshb* and *lhb*) in the pituitary of sexually mature male grass puffer. In this study, we examined the changes in expression of *gnih* and GnIH receptor gene (*gnihr*) in the brain and pituitary along with the genes for growth hormone (*gh*) and prolactin (*prl*) in the pituitary of male grass puffer exposed to low temperature (14 °C), normal temperature (21 °C, as initial control) and high temperature (28 °C) conditions for 7 days. The levels of *gnih* and *gnihr* mRNAs were significantly decreased in both low and high temperature conditions compared to normal temperature in the brain and pituitary. Similarly, the *gh* mRNA levels were significantly decreased in both low and high temperature conditions. The *prl* mRNAs showed no significant changes at high temperature, whereas drastically decreased at low temperature possibly by dysfunctional cold stress. Taken together, the present results suggest that, in addition to the inhibitory effect of temperature changes on the Kiss2/GnRH1/GTH system, the suppression of GnIH/GH system may also be involved in the termination of reproduction by high temperature at the end of breeding season.

1. Introduction

The regulation of reproduction is governed by a complex interaction between environmental signals and various neurohormones including gonadotropin-inhibitory hormone (GnIH) secreted from particular neurons in the brain (Khan and Kauffman, 2012; Simonneaux et al., 2013). GnIH, first identified in the quail brain as an inhibitor of luteinizing hormone (LH) secretion (Tsutsui et al., 2000), acts directly on pituitary gonadotropin (GTH) secretion (Tsutsui et al., 2010; Ogawa and Parhar, 2014; Di Yorio et al., 2019a). Subsequent studies have suggested that GnIH also acts in the brain of birds and mammals by antagonistic interaction with gonadotropin-releasing hormone (GnRH) (Bentley et al., 2006; Johnson et al., 2007; Clarke et al., 2008; Tsutsui et al., 2012). However, in teleosts, several evidences suggest controversial dual roles of GnIH. GnIH showed both stimulatory and inhibitory effects on GTH synthesis and release depending on species and gonadal stage (Amano et al., 2006; Zhang et al., 2010; Moussavi et al., 2012; Qi et al., 2013; Biran et al., 2014; Wang et al., 2015; Di Yorio et al., 2016; Paullada-Salmeron et al., 2016). In the grass puffer (*Takifugu alboplumbeus*), the expressions of the genes encoding follicle-stimulating hormone (FSH) β-subunit and LH β -subunit were stimulated by the goldfish LPXRFamide peptide-1 (gfLPXRFa-1) administration in vitro (Shahjahan et al., 2011) and in vivo (Ando et al., 2018).

Along with GTHs, synthesis and release of growth hormone (GH) and prolactin (PRL) are regulated by GnIH family peptides in various vertebrate species (Hinuma et al., 2000; Koda et al., 2002; Ukena et al., 2003; Johnson et al., 2007). In sockeye salmon, gfLPXRFa peptides increased the release of GH but PRL and somatolactin from the cultured pituitary cells (Amano et al., 2006). gfLPXRFa-3 showed inhibitory effects on basal and GnRH-induced GH release from the goldfish pituitary

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[*] Corresponding author at: Marine Biological Station, Sado Island Center for Ecological Sustainability, Niigata University, 87 Tassha, Sado, Niigata 952-2135, Japan.

E-mail address: hando311@cc.niigata-u.ac.jp (H. Ando).

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and these effects were seasonally-dependent (Moussavi et al., 2014). In the grass puffer, gfLPXRFa-1 was found to have stimulatory effects on the expressions of *gh* and *prl* as well as *fshb* and *lhb* (Shahjahan et al., 2016; Ando et al., 2018). It is therefore conceivable that GnIH may play as a multifunctional hypophysiotropic neurohormone in the brain of different fish species depending on their particular endocrine mechanism of reproduction and it has a stimulatory role in the control of reproduction in the grass puffer.

GH has been shown to be involved in the gonadal development and steroidogenesis in teleosts (Canosa et al., 2007; Reinecke, 2010). GH stimulated cell proliferation of spermatogonia in rainbow trout (Loir, 1999). GH stimulated steroid production in the testis and ovary of *Fundulus heteroclitis* (Singh et al., 1988). It also enhanced GTH-stimulated steroidogenesis in the goldfish ovary (Van der Kraak et al., 1990). Recently, it was demonstrated that GH was indispensable for normal folliculogenesis and spermatogenesis in zebrafish using a gene knockout model (Hu et al., 2019). Moreover, GH secreted from the gonad may regulate body growth in Mozambique tilapia (Bhatta et al., 2012). Therefore, the gonadotropic and somatotropic axes mutually interact through GH and their crosstalk is considered to be important to regulate the physiological transition from body growth to sexual maturation in fish. In the pituitary, both axes are modulated in part by steroid hormone feedback as has been shown that the expression of both GTH and GH genes is responsive to estradiol-17β (E2) in association with GnRH (Trudeau et al., 1992; Zou et al., 1997; Klausen et al., 2002; Ando et al., 2004; Onuma et al., 2005).

On the other hand, PRL is involved in various biological functions together with reproduction in teleosts (Whittington and Wilson, 2013). PRL has been shown to stimulate steroid production in guppy (Tan et al., 1988) and Nile tilapia (Rubin and Specker, 1992). The levels of PRL gene expression were higher in mature fish when compared to juvenile fish in blue gourami (Degani et al., 2010). Furthermore, PRL gene expression is also responsive to E2 and GnRH (Bhandari et al., 2003; Cavaco et al., 2003; Onuma et al., 2005). However, the physiological importance of PRL in the control of reproduction remains obscure.

Temperature has been considered as one of the most important environmental factors regulating the reproductive system in fish (Bromage et al., 2001; Wang et al., 2010; Pankhurst and Munday, 2011). Asymmetric fluctuation in water temperature can directly affects the different phases of fish reproductive cycle and both high and low water temperature has been shown to repress gonadal maturation in teleosts (Wang et al., 2010; Hermelink et al., 2011). In the Atlantic salmon, high water temperature disrupted the endocrine processes and consequently suppressed ovarian maturation, ovulation and successive fertility (King et al., 2007). GnRH-induced ovulation was inhibited with an increase in temperature in Chinese carps (Rottmann and Shireman, 1985). Furthermore, high temperature caused gonadal regression and associated decrease in plasma sex steroid concentration and reduced mRNA levels of the GTH subunit genes in the pejerrey (Soria et al., 2008) and red seabream (Okuzawa and Gen, 2013). On the other hand, a decrease in temperature can also hamper reproduction in fish. Low water temperature showed limited yolk deposition, delayed ovulation and lowered fecundity in *Prochilodus argenteus* (Arantes et al., 2011). Therefore, it is acknowledged generally that increase and decrease in water temperature suppress fish reproduction and lead to decrease in fish abundance and extinction in some cases. However, the endocrine and neuroendocrine mechanisms underlying the impairment of reproductive function by anomalous temperature remain unknown.

The grass puffer provides a unique and interesting animal model for studying the complex interaction between environmental signals and neuroendocrine systems regulating reproduction for its unique reproductive cycle, which is synchronized with seasonal, lunar and daily cycles (Ando et al., 2013, 2018). They breed seasonally from spring to early summer with the specialty of spawning that occurs only during spring tide every two weeks (Motohashi et al., 2010; Ando et al., 2013). On the day of spawning, spawning occurs 1.5–2 h before high tide and continues during the rising tidal phase (Motohashi et al., 2010; Ando et al., 2013). Therefore, spawning of the grass puffer is tightly connected with seasonal, lunar and tidal rhythms as well as daily rhythm where environmental factors such as light, tide, and water temperature play an important role in the control of reproduction (Ando et al., 2013, 2018).

Recently, we showed that both low and high temperature significantly decrease the expression levels of the genes encoding kisspeptin (*kiss2*), kisspeptin receptor (*kiss2r*) and *gnrh1* in the brain as well as *fshb* and *lhb* in the pituitary of the grass puffer (Shahjahan et al., 2017). It was suggested that the suppression of the *kiss2/kiss2r/gnrh1/fshb/lhb* expression pathway by the increase to 28 °C may lead to termination of reproduction at the end of breeding season in July in nature. Taking into account that the GnIH/GH/PRL system may also participate in the control of reproduction, we aimed to further investigate the effects of low and high temperature on the expression of those (*gnih*, *gh* and *prl*) and GnIH receptor (*gnihr*) genes using the same brain and pituitary samples to clarify their roles in reproduction. There is limited information on the effects of water temperature on GnIH, GH and PRL gene expression, except in fry and young European sea bass reared in high and low temperature, where the ontogeny of GnIH system was influenced with higher *gnih* and *gnihr* expressions under low temperature conditions during early developmental stage (Paullada-Salmeron et al., 2017).

2. Materials and methods

2.1. Fish

The male grass puffer with fully-matured testes were collected at spawning grounds in Sado, Niigata, Japan in June for the 1st experiment, and in Kawana, Shizuoka, Japan in July for the 2nd experiment. The fish were transferred to the Marine Biological Station, Niigata University, Japan, and reared in indoor tanks (500 L) with flow of seawater under natural photoperiod (LD 14:10) for two weeks. The water temperature during the acclimatization period was similar to that of sampling sites, which was 18 °C in the 1st experiment and 21 °C in the 2nd experiment. They were fed commercial pellets equivalent to 1% of body weight (BW) daily. BW and gonadosomatic index (GSI = gonad weight/BW × 100) of the fish are shown in Table 1.

2.2. Experimental design and sample collection

Two experiments were conducted as described in detail by Shahjahan et al. (2017). In brief, in the 1st experiment (preliminary) in June, the fish were exposed to low temperature (11 °C), normal temperature (18 °C) and high temperature (25 °C) for 3 and 7 days, which served as Day-3 and Day-7 groups, respectively (n = 5 for each group). To acclimatize the fish to the temperature change, temperature was gradually decreased or increased (Δ1°C per 12 h) from normal to the target temperature conditions. In addition, fish were sampled just before the temperature change and served as initial control (IC) group. In the 2nd experiment in July, the fish were exposed to low temperature (14 °C), normal temperature (21 °C) and high temperature (28 °C) for 7 days, and the fish of Day-7 and IC groups were sampled (n = 8).

The fish were anesthetized in 0.03% tricaine methane sulfonate (MS222, Sigma–Aldrich, Tokyo, Japan), and BW and gonad weight were measured to calculate GSI. The brains and pituitaries were removed and soaked in RNAlater (Ambion, Austin, TX) at 4 °C for 20 h. Then, the brains were trimmed to prepare diencephalon/midbrain samples that included the hypothalamus, and they were stored at −80 °C until extraction of total RNA. The experimental procedures followed the guidance approved by the Institutional Animal Care and Use Committee of Niigata University, Niigata, Japan.

Table 1
Body weight (BW) and gonadosomatic index (GSI) of the fish used in the present study.

		Initial control		Day-7	
1st experiment		18 °C	11 °C	18 °C	25 °C
	BW (g)	37.7 ± 5.8^{a}	37.3 ± 4.3^{a}	42.5 ± 6.8^{a}	37.3 ± 6.7^{a}
	GSI (%)	10.0 ± 1.4^{a}	10.0 ± 1.0^{a}	10.5 ± 0.5^{a}	9.6 ± 1.2^{a}
	n	5	5	5	5
2nd experiment		21 °C	14 °C	21 °C	28 °C
	BW (g)	66.9 ± 3.6^{a}	59.9 ± 3.1^{a}	68.0 ± 5.2^{a}	59.2 ± 4.8^{a}
	GSI (%)	11.9 ± 1.1^{a}	7.1 ± 0.4^{b}	12.4 ± 1.1^{a}	5.8 ± 0.8^{b}
	n	8	8	8	8

^{a,b}Values with different characters are significantly different among groups. Data are shown as the means ± SEM.

2.3. Real-time PCR assay of gnih, gnihr, gh and prl mRNAs

Real-time PCR assay was carried out as described previously (Shahjahan et al., 2011, 2016). Briefly, total RNA was extracted from the diencephalon/midbrain and pituitary samples and treated with DNase I (Takara, Ohtsu, Japan). Total RNA of 200 ng was used for the synthesis of first strand cDNA by reverse transcription reaction using Multiscribe Reverse Transcriptase (Applied Biosystems, USA) and an oligo d(T)$_{12\text{-}18}$ primer according to the manufacturer's instruction. PCR reaction mixture (10 µl) contained 2 µl of sample cDNA, 0.2 µM of forward and reverse primers (Table 2) and 5 µl of SYBR Premix Dimer Eraser (Takara, Ohtsu, Japan). Amplification was carried out at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 20 s. Specific amplification of each cDNA was verified by melting curve analysis and amplicon size check (Table 2) by gel electrophoresis of the product. The slope and correlation coefficient (r) of the standard curves and intra- and inter-assay CVs in each assay are shown in Supplementary Table 1.

2.4. Statistical analysis

The mRNA values are expressed as means ± standard error of the mean (SEM). Data were analyzed by ANOVA followed by Tukey's post hoc test to assess statistically significant differences among the different temperature conditions. Statistical significance was set at $P < 0.05$ or 0.1. Statistical analyses were performed using SPSS Version 14.0 for Windows (SPSS Inc., Chicago, IL).

3. Results

3.1. Changes in expression of gnih in the brain in the 1st experiment

In the 1st experiment, no distinct changes in GSI were observed in fish under different temperature conditions of Day-3 and Day-7 groups and they all showed similar GSI with IC group (Table 1). The amounts of *gnih* mRNA in the diencephalon/midbrain sample showed no noticeable changes in Day-3 and Day-7 groups, although there was a trend toward increased *gnih* mRNA in the high temperature (25 °C) conditions compared to the normal temperature (18 °C) conditions (Fig. 1).

Table 2
Primers used in real-time PCR.

Primers	Nucleotide sequences	Amplicon sizes
tnGnIH-qPCR-F1	5-TGATTCGTCTGTGCGAGGAC-3	114
tnGnIH-qPCR-R1	5-TCAGCAGCTGTGCATTGACC-3	
tnGnIH-R-qPCR-F1	5-AAGATGCTCATCCTGGTGGC-3	106
tnGnIH-R-qPCR-R1	5-AGATCCACCTGGTCACTGTCC-3	
tnGH-qPCR-F1	5-AGAGCACAACTCTTCAGCTAGC-3	123
tnGH-qPCR-R1	5-CCACCTTGTGCATGTCCTTC-3	
tnPRL-qPCR-F1	5-ACAGTGGAGGCAATGACTTGG-3	146
tnPRL-qPCR-R1	5-CTCTGGTACCATGTTTGCCATC-3	

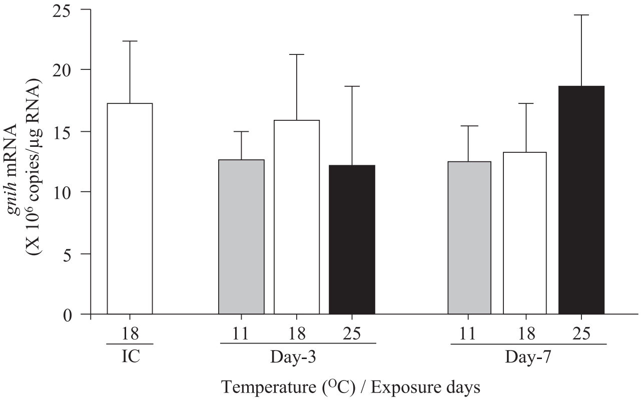

Fig. 1. Changes in the amounts of *gnih* mRNA in the brain of male grass puffer exposed to a low temperature (11 °C), normal temperature (18 °C), and high temperature (25 °C) for 3 and 7 days. Before starting to exposure to the target temperature conditions, fish was sampled to obtain baseline values which served as initial control (IC). Values represent the mean ± SEM (n = 5).

3.2. Changes in expression of gnih and gnihr in the brain and pituitary in the 2nd experiment

In the 2nd experiment, although the fish under the three temperature conditions were active in releasing milt, the GSI significantly decreased both in the low (14 °C) and high temperature (28 °C) when compared to the normal temperature (21 °C) of Day-7 group and IC group (Table 1). The amounts of *gnih* and *gnihr* mRNAs in the diencephalon/midbrain sample were significantly decreased in the low (14 °C) and high temperature (28 °C) when compared to the normal temperature (21 °C) of Day-7 group and IC group (Fig. 2A and 2B). Similarly, the amounts of *gnih* and *gnihr* mRNAs in the pituitary were decreased in the low and high temperature groups when compared to the group maintained at 21 °C and IC group, although the change in the high temperature group was not statistically significant (Fig. 3A and B).

3.3. Changes in expression of gh and prl in the pituitary in the 2nd experiment

Significantly lower amounts of *gh* mRNA were observed both in the low and high temperature groups compared to the normal temperature group and IC group (Fig. 4). Unlike *gh*, the amounts of *prl* mRNA significantly decreased only in the low temperature group but not in the high temperature group when compared to the normal temperature group and IC group (Fig. 5).

4. Discussion

Water temperature has been shown to have enormous effects on reproduction and different physiological activities in fish. In the present

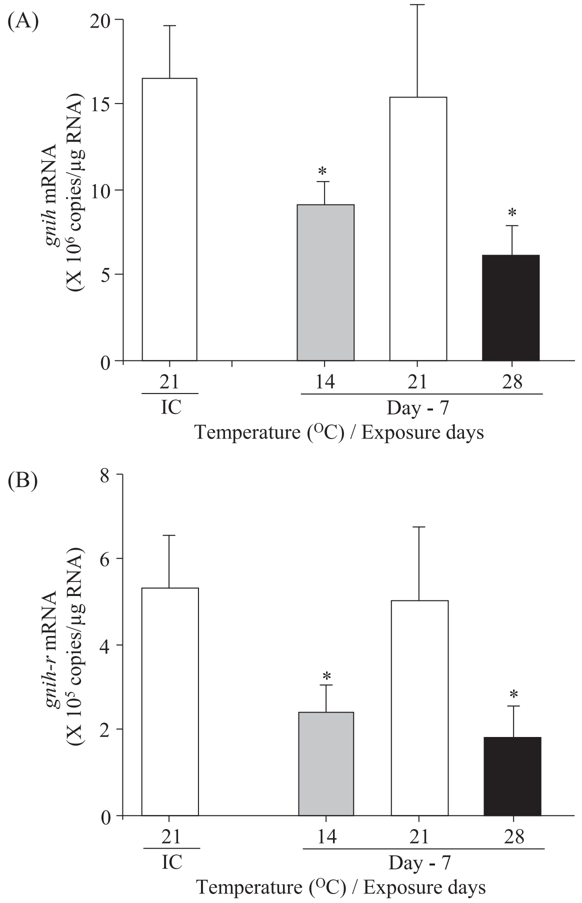

Fig. 2. Changes in the amounts of *gnih* (A) and *gnihr* (B) mRNA in the brain of male grass puffer exposed to a low temperature (14 °C), normal temperature (21 °C) and high temperature (28 °C) for 7 days. Before starting to exposure to the target temperature conditions, fish was sampled to obtain baseline values which served as initial control (IC). Values represent the mean ± SEM (n = 8). Asterisk indicates a significant difference in the low and high temperature groups compared to the normal temperature group (*, $P < 0.05$).

study, we investigated the effects of low and high temperature on the expressions of genes encoding GnIH and GnIH receptor in the brain and pituitary and genes encoding GH and PRL in the pituitary of sexually mature male grass puffer. The *gnih*, *gnihr* and *gh* mRNA levels were significantly decreased by the exposure to low and high temperature conditions along with gonadal regression. While significant suppression of *prl* expression was observed in the low temperature conditions, *prl* mRNA levels did not change in the high temperature conditions. The present and our previous results suggest that GnIH, in cooperation with GnRH and kisspeptin, has an important role in the control of gonadal maturation through stimulation of GH as well as FSH and LH gene expressions in the grass puffer.

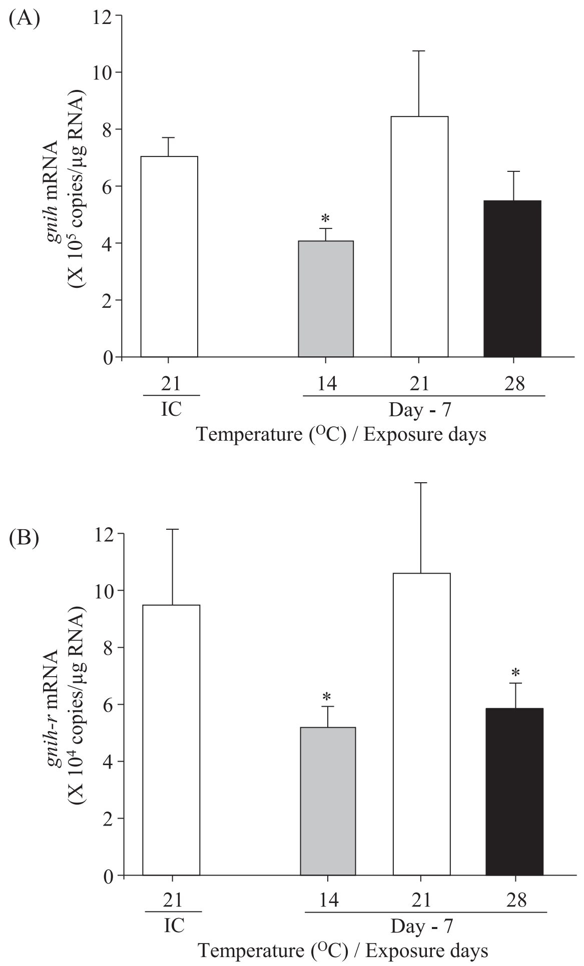

Fig. 3. Changes in the amounts of *gnih* (A) and *gnihr* (B) mRNA in the pituitary of male grass puffer exposed to a low temperature (14 °C), normal temperature (21 °C) and high temperature (28 °C) for 7 days. Before starting to exposure to the target temperature conditions, fish was sampled to obtain baseline values which served as initial control (IC). Values represent the mean ± SEM (n = 8). Asterisk indicates a significant difference in the low and high temperature groups compared to the normal temperature group (*, $P < 0.05$).

In the 1st experiment in June, the level of *gnih* mRNA did not show any significant changes under low (11 °C) and high (25 °C) temperature conditions at Day-3 and Day-7 but tended to increase in the high temperature conditions at Day-7 (Fig. 1). The GSI did not vary among different groups accordingly. In the 2nd experiment in July using the fish that were sexually more advanced (GSI of the IC group: 10.0% in the 1st experiment and 11.9% in the 2nd experiment), the *gnih* mRNA levels significantly decreased in both low (14 °C) and high (28 °C) temperature conditions at Day-7 concurrently with the decrease in the GSI (Fig. 2A). Since the breeding season of grass puffer starts in May at about 16 °C of water temperature and terminates at the end of July at about 28 °C, the low temperature condition both in 1st (11 °C) and 2nd (14 °C) experiment could keep fish under stress before starting breeding while the high temperature conditions at 25 °C in the 1st experiment may be acceptable for fish to progress sexual maturation and the *gnih* expression may remain augmented. However, high temperature at 28 °C in the 2nd experiment may be transmitted to the hypothalamus as an environmental cue that terminates reproduction and inhibit the expressions of *gnih* and associated genes in the GnIH/GH system.

GnIH has been shown to have stimulatory effects on the regulation of reproduction in the grass puffer: the administration of sexually matured fish with gfLPXRFa-1 stimulated the expressions of *fshb*, *lhb* and *gh* in vivo (Ando et al., 2018) and in vitro (Shahjahan et al. 2011, 2016). Our previous study on the effects of low and high temperature on the genes for the kisspeptin/GnRH/FSH/LH system clearly showed that the expressions of *kiss2*, *kiss2r*, *gnrh1*, *fshb* and *lhb*, but not *gnrh2* and *gnrh3*, decreased under the low (14 °C) and high (28 °C) temperature conditions (the 2nd experiment, Shahjahan et al., 2017). Therefore, these results suggest that GnIH may interact with GnRH1 and/or kisspeptin to stimulate FSH and LH secretion. In addition, our previous studies showing coordinated expressions of *gnih*, *gnihr*, *kiss2*, *kiss2r* and *gnrh1* in regard to seasonal, daily and circadian variations further support this notion (Shahjahan et al., 2010a,b, 2011; Ando et al., 2013, Ando et al., 2014, 2018). Neuroanatomical relationship of GnIH neurons and GnRH neurons has been examined in several fish species. In Nile tilapia, there was no close association of GnIH-immunoreactive (ir)

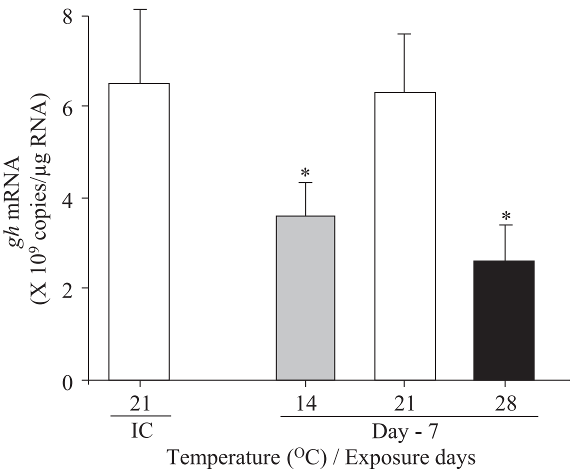

Fig. 4. Changes in the amounts of *gh* mRNA in the pituitary of male grass puffer exposed to a low temperature (14 °C), normal temperature (21 °C) and high temperature (28 °C) for 7 days. Before starting to exposure to the target temperature conditions, fish was sampled to obtain baseline values which served as initial control (IC). Values represent the mean ± SEM (n = 8). Asterisk indicates a significant difference in the low and high temperature groups compared to the normal temperature group (*, $P < 0.05$).

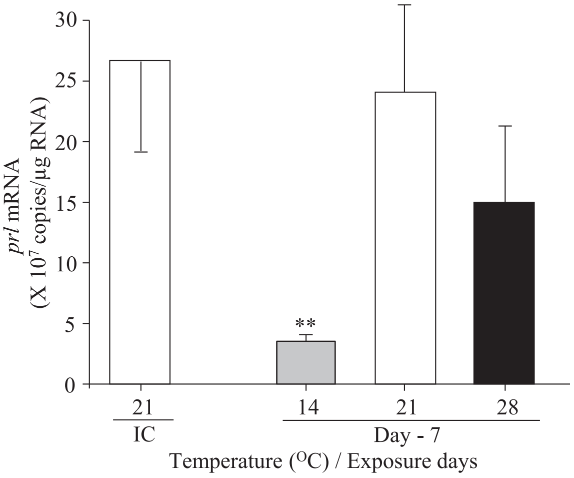

Fig. 5. Changes in the amounts of *prl* mRNA in the pituitary of male grass puffer exposed to a low temperature (14 °C), normal temperature (21 °C) and high temperature (28 °C) for 7 days. Before starting to exposure to the target temperature conditions, fish was sampled to obtain baseline values which served as initial control (IC). Values represent the mean ± SEM (n = 8). Asterisk indicates a significant difference in the low and high temperature groups compared to the normal temperature group (**, $P < 0.01$).

fibers and GnRH1 and GnRH3 neurons (Ogawa et al., 2016). In the South American cichlid fish, no apparent contacts between GnIH and GnRH1 neurons were observed; however, colocalization of GnIH and GnRH3 was found in the nucleus olfacto-retinalis (Di Yorio et al., 2019b). Moreover, in zebrafish, GnIH-ir fibers project to and contact with the hypophysiotropic GnRH3-ir neurons in the forebrain region (Spicer et al., 2017). Thus, it is possible that GnIH regulates the expression of GTH genes in the pituitary directly and indirectly through interaction with GnRH1 neurons in the grass puffer, although brain localization of GnIH soma and fibers and their interaction with GnRH neurons need to be determined. For the interaction with kisspeptin neurons, very limited information is available at present, except that there was no association of GnIH-ir fibers with kisspeptin (Kiss2) neurons in Nile tilapia, (Ogawa et al., 2016).

It has been reported that exposures to both high and low temperature suppress the expression of GnRH and kisspeptin genes. In the female blue gourami, the *gnrh3* mRNA levels were significantly decreased at low (23 °C) and high (31 °C) temperature compared to temperature at 27 °C (Levy et al., 2011). Negative effect was also observed by high water temperature on the expression of *gnrh1* in the pejerrey, whereas *gnrh2* and *gnrh3* were not (Elisio et al., 2012). Moreover, the associated decreases in the mRNA levels of *kiss2* and *gnrh3* was reported in the zebrafish exposed to low and high temperature (Shahjahan et al., 2013). Similar results of negative effects of high and low temperature on *kiss2*, *kiss2r* and *gnrh1* were found in the grass puffer (Shahjahan et al., 2017). On the other hand, the present study is the first report demonstrating the effects of temperature changes on *gnih* and *gnihr* expressions in mature fish, particularly in the fish where GnIH has a stimulatory role in the control of reproduction. It is therefore of considerable interest and importance to determine whether *gnih* and *gnihr* expression is suppressed or stimulated by temperature changes in the fish where GnIH negatively regulates reproduction.

In the present study, parallel to *gnih* and *gnihr*, the expression of *gh* was significantly suppressed under the low and high temperature conditions, while the *prl* mRNA levels was considerably decreased by the low temperature conditions but not by the high temperature conditions (Figs. 4 and 5). Our previous study showed significant decreases in *fshb* and *lhb* expressions under the low and high temperature conditions (the 2nd experiment, Shahjahan et al., 2017). Similar concomitant decreases in the *gh* mRNA levels with *fshb* and *lhb* mRNAs were observed in the female blue gourami exposed to low and high temperature (Levy et al., 2011). In addition, negative effects of high temperature on the *fshb* and *lhb* mRNA levels were reported in red seabream (Okuzawa and Gen, 2013). These results clearly indicate negative effect of anomalous temperature on the reproductive activity of teleost fishes through suppression of *gh* as well as *fshb* and *lhb* expressions. Taking into account that the *gh* expression is extensively activated during the breeding season possibly through the stimulation by GnIH (Shahjahan et al., 2016) and direct evidences showing stimulatory roles of GH in gonadal maturation and steroidogenesis (Singh et al., 1988; Van der Kraak et al., 1990; Hu et al., 2019), GH may have an important role in gonadal maturation during the breeding season in the grass puffer.

Although PRL has been shown to be involved in gonadal development and steroidogenesis and its levels are affected by GnRH and gonadal steroid (Whittington and Wilson, 2013), *prl* showed different expression patterns from *gh* and GTH subunit genes (Fig. 5). The *prl* mRNA levels did not decrease significantly under the high temperature conditions at 28 °C, suggesting that PRL may not be related to the termination of reproduction at the end of breeding season. Conversely, the *prl* mRNA levels decreased considerably under the low temperature conditions at 14 °C. This may be due to high plasma cortisol concentrations of the fish. In our previous study, the plasma levels of cortisol were significantly increased under the low temperature conditions but remained unchanged under the high temperature conditions, indicating that the fish could be under stress in the low temperature conditions but not in the high temperature conditions (Shahjahan et al., 2017). In teleosts, it is acknowledged generally that PRL and cortisol are important in osmoregulation, in adaptation to fresh water (FW) and seawater (SW) environments, respectively (McCormick, 2001; Seale et al., 2013). The relative importance of PRL in FW and cortisol in SW adaptation, respectively, agrees with the inhibition of PRL secretion by cortisol. Hyposmotically-induced PRL release was inhibited following exposure to cortisol in tilapia (Borski et al., 1991). Cortisol also directly suppressed the expressions of two PRL genes in the tilapia pituitary (Uchida et al., 2004). It is therefore conceivable that the suppression of *prl* expression seems to be due to high levels of plasma cortisol and the low temperature conditions may cause dysfunctional cold stress in this species.

In conclusion, we examined the differential effects of thermal regimes on the expressions of genes in the GnIH/GnIHR/GH/PRL system in the mature male grass puffer. Both low and high temperature suppressed the expressions of *gnih*, *gnihr* and *gh* along with the gonadal regression. *prl* mRNA levels did not decreased significantly under the high temperature conditions, whereas deceased considerably under the low temperature conditions possibly by cold stress. The present and previous studies on thermoregulation of neurohormone and pituitary hormone gene expression suggest that GnIH has a significant role in the control of gonadal maturation through stimulation of GH as well as FSH and LH gene expressions, and in addition to the Kiss2/GnRH1/GTH system, the suppression of GnIH/GH system may be important for the termination of reproduction by high temperature at the end of breeding season in the grass puffer.

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Appendix A. Supplementary data

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