DISSERTATION FOR THE DOCTORAL DEGREE

Studies on the role and regulation of kisspeptin and gonadotropin-inhibitory hormone system in the reproduction of grass and tiger puffers: Implication of melatonin in semilunar spawning rhythm

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Chapter 1

General introduction

1.1. Hypothalamic–pituitary–gonadal (HPG) axis in fish

1.1.1. HPG axis in the control of reproduction

Reproduction, the fundamental biological process necessary for the existence and continuation of a species is governed by the hypothalamus-pituitary-gonadal (HPG) axis. The HPG axis consists of a three-tier organization: the hypothalamus in the brain, the pituitary, and the gonads. The HPG axis is the key neuroendocrine axis regulating the reproduction in vertebrates. This axis receives complex external and internal signals at the brain and pituitary levels and integrates and translates these incitements into physiological and behavioral outputs to achieve successful reproduction. The HPG axis is generally conserved in animal classes from agnathans to humans and controls the reproduction by regulating the gonadal maturation and also sexual behavior.

In the hypothalamus, the reproductive neuroendocrine system involves the release of three peptidic neurohormones, namely gonadotropin-releasing hormone (GnRH), kisspeptin, and gonadotropin-inhibitory hormone (GnIH). These neurohormones are involved in the control of the synthesis and release of two gonadotropins (GTHs), namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the pituitary. FSH and LH act on the gonad to stimulate the gonadal maturation and the production of sex steroid hormones. The sex steroids, in turn, feedback to the brain and pituitary to complete the HPG axis and regulate the reproductive cycle in a periodic manner. Besides, multiple environmental factors, such as temperature, photoperiod, tides and moonlight, and many peptidic and non-peptidic

neuromediators are involved in the control of reproduction through direct and indirect interactions with the HPG axis. Although the overall organization of the HPG axis is shared among the vertebrate classes, the structure, function and regulation of the hormones in the HPG axis vary depending on the animal species, sex and stage of gonadal development.

1.1.2. Gonadotropin-releasing hormones (GnRHs)

Among the hypothalamic neurohormones, GnRH is the principal regulator of the GTH secretion in many vertebrate species (Ando and Urano, 2005; Zohar et al., 2010). It was first isolated from pigs and sheep as a LH-releasing factor (Amoss et al., 1971; Matsuo et al., 1971) and later named GnRH due to its ability to stimulate the secretion of both LH and FSH. The GnRH precursor consists of a signal peptide, a GnRH decapeptide and a GnRH-associated peptide (GAP). To date, 15 different forms of GnRH have been identified in vertebrates, and all vertebrate species possess two or three GnRH forms, namely GnRH1, GnRH2, and GnRH3. In teleosts that possess three GnRH forms, GnRH1 has a hypophysiotropic role through stimulating the secretion of the pituitary FSH and LH, and GnRH1 neurons are mainly located in the preoptic area (POA). The second group is referred as GnRH2, formally known as chicken GnRH-II is involved in appetite-related reproductive function and localized in the midbrain tegmentum (Matsuda et al., 2008; Nishiguchi et al., 2012; Marvel et al., 2019). GnRH3 have neuromodulatory action related to sexual behavior and GnRH3 neurons are localized in the terminal nerve ganglion-POA region (Okuyama et al., 2014; Li et al., 2017).

1.1.3. Kisspeptin and kisspeptin receptor

Kisspeptin is also a potential key player in the neuroendocrine control of reproduction in vertebrates. Kisspeptin was initially identified as a metastasis suppressor gene for melanoma cells (Lee et al., 1996) and was later found to be the ligand for the G–protein coupled receptor

(GPR) 54, now named Kiss1r. Mature kisspeptin peptides in mammals are cleaved into endogenous fragments such as Kp54, Kp16, Kp14, Kp13 and Kp10 (Beltramo et. al., 2014) and the C-terminus decapeptide Kp10 is the minimum active site and allows them to bind to Kiss1r (Muir et al., 2001; Ohtaki et al., 2001). In mammals, kisspeptin has been shown to play a central role in the control of reproduction through stimulating the GnRH secretion. In fish, there are two paralogous genes for kisspeptin, namely *kiss1* and *kiss2* (Felip et al., 2009; Kitahashi et al., 2009). In some fish species, however, there is only one gene, *kiss2* (Shahjahan et al., 2010b; Ogawa and Parhar, 2013). Kisspeptin has been shown to play an important role in fish reproduction like in mammals, although the role of two kisspeptin isoforms, Kiss1 and Kiss2 is different, and some authors described as stimulatory and inhibitory or no effect on reproduction depending on the species (Kanda et al., 2013; Nakajo et al., 2017; Ohga et al., 2018; Ando et al., 2018).

The piscine kisspeptin system was first reported in tilapia showing an expression of kisspeptin receptor with GnRH neurons (Parhar et al., 2004). Four subtypes of kisspeptin receptors (Kiss1r, Kiss2r, Kiss3r and Kiss4r) have been identified in non-mammalian vertebrates and there are two subtypes (Kiss1r and Kiss2r) in fish so far (Pasquier et al., 2014). The *kiss1r*- and *kiss2r*-expressing cells are localized in the POA and hypothalamus and their co-localization and close proximity with GnRH neurons suggests that kisspeptin may be involved in the regulation of reproduction by regulating the GnRH secretion (Oka, 2009). Besides, widely expressed *kiss2r* mRNA has been reported in the fish brain, which includes the olfactory bulb, telencephalon, POA, midbrain, hypothalamic nuclei, cerebellum, and the spinal cord (Grone et al., 2010; Servili et al., 2011; Ogawa et al., 2012).

1.1.4. Gonadotropin-inhibitory hormone (GnIH) and GnIH receptor

GnIH was first identified as a novel hypothalamic neuropeptide in birds as an inhibiting

factor of LH release (Tsutsui et al., 2000). The GnIH precursor is cleaved into two to four mature peptides which possess a characteristic putative C-terminal LPXRFamide (LPXRFa, X = L or Q) depending on the species (Ubuka et al., 2016; Tsutsui et al., 2018). Most of the fish GnIH precursors encode three LPXRFa or LPXRFa-like peptides. In mammals and birds, GnIH inhibits GTH secretion directly and indirectly by antagonistic interaction with GnRH (Tsutsui et al., 2012; Ubuka et al., 2016). However, in fish, GnIH has both stimulatory and inhibitory effects on the secretion of GTH depending on fish species and gonadal stage (Moussavi et al., 2012, 2014; Biran et al., 2014; Wang et al., 2015; Di Yorio et al., 2016; Paullada-Salmeron et al., 2016).

The action of GnIH is mediated by GnIH receptor (GnIH-R, GPR147). The cDNA of GnIH-R was first identified in birds (Ikemoto and Park, 2005; Yin et al., 2005) and their cognate receptors have been also identified in zebrafish (Zhang et al., 2010), grass puffer (Shahjahan et al., 2011), goldfish (Qi et al., 2013) and tilapia (Biran et al., 2014). The presence of *gnih-r* transcripts has been determined in the brain and pituitary of some fish species (Zhang et al., 2010; Shahjahan et al., 2011). In the brain of tilapia, GnIH-R-immunoreactive (ir) neurons are widely distributed in the olfactory bulb, ventral/dorsal telencephalon, POA, hypothalamus, optic tectum, semicircular torus, and caudal midbrain tegmentum. GnIH-R-ir cells are also extended to the pituitary level (Ogawa et al., 2016).

1.1.5. Role of pituitary hormones in reproduction

Hormonal inputs from the hypothalamus act on the pituitary through the hypophyseal-portal system promoting the synthesis and release of GTHs. FSH and LH are principal glycoprotein hormones regulating the gonadal maturation in vertebrates (Yaron et al., 2003). These two hormones are heterodimers which consist of a common glycoprotein α (GPα) subunit and a hormone-specific β subunit (FSHβ and LHβ) (Pierce and Parsons, 1981). FSH is mostly

involved in promoting early gonadal development and gametogenesis (spermatogenesis and oogenesis), whereas LH plays an important role in the late stage of reproduction, stimulating the final gamete maturation and release (spermiation and ovulation) and the production of sex steroid hormones (Ogiwara et al., 2013; Chauvigne et al., 2014).

1.2. Periodic control of reproduction in fish

1.2.1. Environmental factors in regulating the HPG axis

Synchronous reproduction is crucial for most vertebrate species to achieve reproductive success and the linkage between environmental periodicity to reproduction is recognized as an important adaptive feature in the life cycles of most vertebrate species. Many organisms adopt a combined action of photic (e.g., daylight and moonlight) and non-photic (e.g., temperature, tidal changes and nutrition) environmental signals to synchronize their sexual maturation and spawning to specific places, particular time of the day, season and moon phases. The environmental cues are integrated by the HPG axis and regulate the synthesis and release of hypothalamic neurohormones, pituitary hormones and gonadal sex steroids. The complex mechanism of photic and non-photic signal transduction and the periodic control of the HPG axis is largely remained obscure.

Among the environmental cues that control the reproduction in vertebrates, light is one of the most important environmental factors regulating the HPG axis. Photoperiod is the most reliable cue involved in the seasonal control of reproduction in vertebrate species, especially in seasonal breeders. The photoperiodic control of reproduction has long been manifested by the endogenous clock which helps the animals to anticipate the changes in the environment and prepares the animals intrinsically for the precisely timed migration, hibernation, molt and reproduction. The circadian clock which entrains the daily rhythm and the circannual clock

which regulates the seasonal response is intrinsically self-sustained clock mechanisms that anticipate the changes in the environment (Gwinner, 2003). It is becoming increasingly obvious that the daily and photoperiodic control of reproduction involves the cyclic changes in the activity of the reproductive neuroendocrine system in fish. For example, in the grass puffer (*Takifugu alboplumbeus*), a semilunar spawner, the expressions of the genes for kisspeptin, GnIH and their receptors, and GnRH2 demonstrate diurnal and circadian oscillations in the spawning season (Shahjahan et al., 2011; Ando et al., 2014). These facts suggest that the expressions of these genes are regulated by the circadian clock and also by melatonin, an indoleamine hormone produced from the pineal gland, that transmits the photoperiodic information to entire body including the brain (Ando et al., 2018). However, the molecular and cellular mechanisms of the photoperiodic control of reproduction are still largely unknown.

Marine and coastal organisms have evolved lunar and semilunar endogenous rhythms, especially in the context of the reproductive cycles and this has been well established in multiple animal groups from marine invertebrates to vertebrates (Numata and Helm, 2014). Monthly and semi-monthly oscillations in the environmental stimuli, such as moonlight and mechanical spring/neap tide cycles, cause variation in the animal physiology, pigmentation, behavior and subsequent hormonal changes which are translated into the observable biological rhythm (Raible et al., 2017).

Field and laboratory investigations on the diverse animal groups have proven that lunar and semilunar rhythms are endogenous and under clock control (Enright, 1972; Quilter and Lewis, 1989; Zantke et al., 2013). Several studies have tried to identify signal molecules that are linked to the lunar/tidal cycle information and reproduction. For example, in the golden rabbitfish, which spawns around the first quarter moon, the plasma levels of melatonin at midnight are higher on the day of the new moon than the full moon (Takemura et al., 2004a). This lunar phase-dependent variation in the plasma melatonin concentrations at night has been considered

to be critical for the occurrence of the lunar-synchronized spawning in golden rabbitfish (Takemura et al., 2004b). In the grass puffer (*Takifugu alboplumbeus*), melatonin receptor gene expression (*mel1b*) demonstrates a unique ultradian oscillation (Ikegami et al., 2015) which may be involved in the tidal/lunar synchronized spawning in grass puffer. However, it remains obscure how melatonin regulates the GnRH/kisspeptin/GnIH system in the lunar and semilunar spawners.

Water temperature is also important environmental factor regulating the HPG axis in fish. Environmental temperature directly affects the molecular, biochemical and physiological processes, especially in the ectotherm animals (Strussmann et al., 2010). Asymmetric fluctuations in water temperature inhibit gonadal development, maturation, spawning, and successive fertility in fish (Soria et al., 2008; Wang et al., 2010; Shahjahan et al., 2017; Rahman et al., 2019). Several studies reported that the expression of GnRH gene is suppressed by anomalous increase and decrease in water temperature (Levy et al., 2011; Okuzawa and Gen, 2013). In grass puffer, both high and low temperature conditions suppress the expression of the HPG axis genes and affect the reproductive function (Shahjahan et al., 2017; Rahman et al., 2019). However, the underlying molecular mechanisms of how temperature regulates the HPG axis have remained unknown. In recent years, anthropogenic climate change along with temperature rise and the impact on the reproductive processes needs especial investigation.

1.2.2. Melatonin: A key regulator in light-dependent reproduction

It has been shown that melatonin plays an important role in the photoperiodic regulation of reproduction. The secretion of melatonin is under control of light and time, being high in the nighttime and low during the daytime (Reiter, 1993). Thus, melatonin, “the nocturnal hormone” can transmit the information of day length and its seasonal changes to the central and peripheral organs (Falcon et al., 2007). In birds and mammals, melatonin has been shown to be involved

in the regulation of GnIH expression and thus participates in the neuroendocrine control of seasonal reproduction (Ubuka et al., 2005; Revel et al., 2008). Daily and circadian variations in the melatonin receptor gene expression have been reported in golden rabbitfish (Park et al., 2006, 2007) and grass puffer (Ikegami et al., 2009, 2015). Besides, plasma melatonin level and melatonin receptor gene expression were found to higher in the new moon than the full moon in golden rabbitfish (Takemura et al., 2004a). It is therefore conceivable that melatonin signals may oscillate with the tidal and/or lunar cycle and interplay with the tidal and/or lunar-related reproductive rhythmicity in fish (Ikegami et al., 2015).

1.3. Grass puffer as a model animal for study on the periodic control of reproduction

1.3.1. Semilunar-synchronized reproduction of the grass puffer

Grass puffer is a common intertidal puffer in Japan and shows unique reproductive physiology which is synchronized with the seasonal, lunar and daily cycles. During the spawning season from spring to early summer, the fish aggregate at certain seashore places for spawning 2.5−3 hours before high tide at dusk only during the new and full moon period (Motohashi et al., 2010; Ando et al., 2013). Spawning takes place in a particular seashore site in a group of 10−60 individuals among which one is female. Spawning starts 1.5–2 hours before high tide and continues for about 1 hour during the rising tidal phase. Therefore, the spawning of grass puffer is tightly connected to the seasonal, lunar, daily and tidal rhythms where the environmental factors, such as water temperature, light (daylight and moonlight) and tidal cycle may interplay in the control of reproduction. Since we are aware of the spawning time and place, spawning fish can be easily caught by hand net from the coastal area. Altogether with the interesting facts, the grass puffer provides an excellent model for studying the molecular and neuroendocrine framework for the periodic control of reproduction.

1.3.2. Reproductive physiology of the grass puffer

Grass puffer displays semilunar spawning at the new and full moon night. In the brain of grass puffer, three forms of GnRHs display differential expression patterns throughout the spawning season. The expression levels of *gnrh1* and *gnrh3* are significantly elevated during spawning season while *ghrh2* does not show such variations (Shahjahan et al., 2010a). The expression levels of GTH subunits (GPα, FSHβ and LHβ) are also increased significantly in parallel with *gnrh1* and *gnrh3* (Shahjahan et al., 2010a). The genes encoding kisspeptin, GnIH and their receptors also elevate during the spawning season (Shahjahan et al., 2010b, 2011). Besides, during the spawning season, these genes demonstrate diurnal, circadian and lunar oscillations (Shahjahan et al., 2011, Ando et al., 2014, Rahman, 2020). In addition, four subtypes of melatonin receptor genes display ultradian oscillations, indicating the possibility of a tidal cycle-related biological clock in the grass puffer (Ikegami et al., 2015).

On the other hand, to examine the role of GnIH in the control of reproduction, a heterologous GnIH peptide (goldfish LPXRFa-1) was administered to the pituitary in vivo and in vitro. In both studies, goldfish LPXRFa-1 stimulated the expression of not only *fshb* and *lhb* expressions but also *gh* and *prl* expressions, indicating that GnIH may have a stimulatory role in the reproduction and is a multifunctional hypophysiotropic neurohormone in the grass puffer (Shahjahan et al., 2011, 2016; Ando et al., 2018).

1.4. Tiger puffer, an alternative model fish for the periodic control of reproduction

Tiger puffer (*Takifugu rubripes*) belongs to the family Tetraodontidae and closely related to the grass puffer. Both puffers are migratory and spawn in coastal area in spring. Tiger puffer exhibits long migration: juveniles remain in the spawning area from spring to summer, then moved to the wider nursery grounds in the oceans covering the Japan sea, the Yellow sea, and

the East China Sea and then return to the spawning area in Japan (Sato et al., 1999; Nakajima and Nitta, 2005; Matsumura, 2006). At the spawning ground, the mature fish aggregate and spawn in a group like grass puffer.

Tiger puffer is one of the most commercial and delicate fish species in Japan having great aquaculture importance (Matsubara, 1955). The wild populations of tiger puffer are gradually decreasing since ’90s, therefore, about 6 million hatchery-reared fry have been released every year to restore wild populations since 2006 (Katamachi, 2015). Although artificial induction of ovulation by hormonal treatment has been successively applied to the cultured tiger puffer, studies on the reproductive neuroendocrine system in wild population is scarce because of their long migration.

In addition, tiger puffer is one of model fish for genomic studies since the genomic resource of tiger puffer has been established in early 2000s (Aparicio et al., 2002). Later, it was revealed that *Takifugu* species share extremely high similarity in their genome sequences (Yamanoue et al., 2009). Indeed, the grass and tiger puffers share 92−98% similarity in the nucleotide sequence of the precursor genes for GnRH, kisspeptin, GnIH, vasotocin and isotocin, even in their non-coding regions (Motohashi et al., 2010; Shahjahan et al., 2011). As the hatchery-reared fry, fingerlings, and adult fish are available, we can use the tiger puffer for studies of physiology and molecular biology in close proximation and also in laboratory conditions. Findings on the reproductive physiology may be similar to the grass puffer especially in terms of periodic control of reproduction.

1.5. The purpose of the present study

Based on the finding of the previous studies on the neuroendocrine regulation of the semilunar-synchronized spawning in the grass puffer, it is still unknown whether kisspeptin and GnIH have stimulatory roles or not and how they functionally interact with each other in the

periodic control of reproduction of the grass puffer. Moreover, the possible implication of melatonin in the regulation of the kisspeptin/GnIH system is still obscure. On the other hand, there is no information regarding the reproductive neuroendocrine system in the wild tiger puffer. Here in this study, effects of kisspeptin and GnIH administration on the HPG axis gene expression were examined in vivo in the grass puffer. To further clarify the role of the kisspeptin/GnIH system, changes in its activity was examined in a wild tiger puffer population at different reproductive stages. Moreover, a comprehensive study on the regulation of the kisspeptin/GnIH system by melatonin was also analyzed in the grass puffer. Therefore, the following points were considered in the present study to clarify the neuroendocrine mechanisms of semilunar spawning in the grass and tiger puffers:

1. To understand the effect of kisspeptin administration on the expressions of the HPG axis genes in the grass puffer;
2. To understand the effect of native GnIH administration on the expressions of the HPG axis genes in the grass puffer;
3. To determine changes in the expressions of the HPG axis genes in the tiger puffer at different reproductive stages;
4. To clarify the regulation of the kisspeptin/GnIH system by melatonin in the grass puffer.

Chapter 2

Effect of kisspeptin administration on the expression of the HPG axis genes in the grass puffer at different reproductive stages

2.1. Introduction

The reproduction in vertebrates is regulated by the complex interaction among multiple environmental factors and the reproductive neuroendocrine system, which is composed of kisspeptin, gonadotropin-inhibitory hormone (GnIH), gonadotropin-releasing hormone (GnRH) in the hypothalamus and two pituitary gonadotropins (GTHs), namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Khan and Kauffman, 2012; Simonneaux et al., 2013). Kisspeptin, a member of the RFamide peptide family, is encoded by the KISS1/Kiss1 gene. *KISS1* was originally identified as a metastasis suppressor gene (Lee et al., 1996) and its product was found to be the ligand for an orphan G-protein coupled receptor, GPR54, later named Kiss1r. Since mutations in *GPR54* were found to be responsible for idiopathic hypogonadotropic hypogonadism (de Roux et al., 2003; Seminara et al., 2003), kisspeptin has received considerable attention as a potential key player in the neuroendocrine regulation of reproduction. It is now well established that kisspeptin regulates reproductive events including puberty and ovulation through stimulating GnRH secretion in mammals (Oakley et al., 2009).

Unlike most mammals that possess a single kisspeptin gene (*Kiss1*), most teleost possesses two paralogous genes for kisspeptin (*kiss1* and *kiss2*) and kisspeptin receptor (*kiss1r* and *kiss2r*) (Kanda et al., 2008; Kitahashi et al., 2009; Saha et al., 2016) and this increases the complexity of the kisspeptin system in teleosts. It has been shown that the role of two kisspeptin forms in

the regulation of reproduction largely varies among fish species. For example, the administration of Kiss1 increased the plasma LH levels in goldfish (Li et al., 2009) and stimulated spermiation in chub mackerel (Selvaraj et al., 2013). In striped bass, Kiss1 showed stimulatory but Kiss2 exhibited inhibitory effects on the expression of *fshb* and *lhb* (Zmora et al., 2014). In contrast, Kiss2 stimulated the secretion of FSH and LH in several fish species such as sea bass, zebrafish, and chub mackerel (Felip et al., 2009; Kitahashi et al., 2009; Ohga et al., 2014; Espigares et al., 2015; Park et al., 2016). In addition, the actions of two kisspeptin forms are dependent on the stage of gonadal development. For example, in mature fish of *Morone* species, Kiss2 was more potent than Kiss1 in upregulating plasma LH levels and *gnrh1* and *kiss1r* expressions. However, during the recrudescence period in the same species, Kiss1 was more potent than Kiss2 in inducing LH release, and Kiss2 downregulated the expression of *gnrh1* and *kiss1r* (Zmora et al., 2012).

In addition, it has recently been shown that the kisspeptin system is dispensable for reproduction in several fish species such as zebrafish (Tang et al., 2015) and medaka (Nakajo et al., 2017) using gene knockout models. Moreover, GnRH neurons do not co-express kisspeptin receptors in European sea bass (Escobar et al., 2013) and medaka (Kanda et al., 2013; Nakajo et al., 2017). Therefore, the roles of kisspeptin in the control of reproduction are currently controversial in fish, and it seems that the functional significance of the kisspeptin system in reproduction varies depending on fish species and also gonadal development.

The grass puffer, *Takifugu alboplumbeus*, shows unique reproductive physiology that is synchronized with the seasonal, lunar, and daily cycles. During the spawning season from spring to early summer, spawning occurs on seashore only for several days around the new and full moon days every two weeks (Motahashi et al., 2010; Ando et al., 2013). Mature fish usually aggregate for spawning at certain seashore locations 2–3.5 hours before high tide in the evening, and spawning occurs for 1.5–2 hours during the rising tidal phase. Therefore, the timing of

spawning is tightly connected with seasonal, lunar, and tidal cycles as well as daily rhythm. Since we are aware of the time and place of the spawning, we can obtain spawning fish easily by dip net at the spawning bed. Thus, the grass puffer provides a unique animal model for studying the neuroendocrine mechanisms underlying the seasonal, lunar, and circadian control of reproduction.

Grass puffer has only a single pair of genes for kisspeptin (*kiss2*) and kisspeptin receptor (*kiss2r*) and previous studies on their expression patterns with respect to seasonal, daily and circadian changes have indicated the possible importance of the kisspeptin system in the semilunar synchronized spawning. The expression levels of both *kiss2* and *kiss2r* show distinct changes during reproductive cycle with a significant increase from the early stage of gametogenesis to the post-spawning stage (Shahjahan et al., 2010a; Ando et al., 2013). The seasonal variations of *kiss2*/*kiss2r* expressions are certainly important for the spawning in early summer and are recently found be regulated by water temperature: high temperature conditions in summer (over 28°C) suppress the *kiss2*/*kiss2r* expressions, leading to the termination of spawning period (Shahjahan et al., 2017). Furthermore, *kiss2* and *kiss2r* exhibit diurnal and seasonal variations in expression during the spawning period (Ando et al., 2014). These results suggest that the kisspeptin system is most probably important in the stimulation, maintenance, and cyclicity of reproductive function in the grass puffer.

In the present study, the effects of Kiss2 administration on the expressions of the genes for various hormones and receptors that are comprised in the hypothalamus-pituitary-gonadal (HPG) axis, (i.e. *kiss2*, *kiss2r*, *gnih*, *gnih-r*, *gnrh1*, *gnrh2*, *gnrh3*, *gpa*, *fshb* and *lhb*) were examined to clarify the functional significance of Kiss2 in the grass puffer. For the possible roles of Kiss2 at multiple stages during gonadal development, fish samples at three different reproductive stages, namely immature, mature, and regressed stages, were used in the present study.

2.2. Materials and methods

2.2.1. Fish

Male fish with fully matured testes were collected from the spawning ground in Kawana, Shizuoka in June. Male fish with regressed testes were collected from the spawning ground in Minamiise, Mie, Japan at the end of July. The mature and regressed fish were transferred to the Marine Biological Station, Niigata University, Japan, and reared in indoor tanks (500 L) with the flow of seawater under natural photoperiod (LD 14:10) for two weeks. The fish were fed daily with commercial pellets equivalent to 1% body weight (BW) until the experiment was conducted. Since immature grass puffer is unavailable from wild source, juvenile fish were artificially reared at the Fisheries Laboratory, University of Tokyo, Hamamatsu, Shizuoka, Japan, and they were transferred to the Marine Biological Station, Niigata University and reared in indoor conditions for one year.

2.2.2. Kiss2 administration

Grass puffer Kiss2 (gpKiss2, SKFNLPFGLRFamide) was synthesized and dissolved in saline solution (0.9% NaCl) and stored at -80ºC until use. The fish were anesthetized in 0.008% tricaine methanesulfonate (MS222, Sigma-Aldrich, Tokyo, Japan) for 30 sec. and were immobilized with its ventral side upward. The immature and mature fish were intraperitoneally (ip) injected with gpKiss2 (0, 0.1, and 1.0 μg/g BW, n = 8−9) using a fine needle (25G, Terumo Corporation, Tokyo, Japan). For the regressed fish, a preliminary experiment was conducted to examine the effect of gpKiss2 administration (0, 0.01, 0.1 μg/g BW, n = 4) because there had been few reports on the effect of kisspeptin on animals at recrudescence stage. Because there was a trend toward increased *kiss2* and *gnih* expressions at the 0.01 μg/g BW in the preliminary experiment, the regressed fish were ip injected with gpKiss2 at 0 and 0.01 μg/g BW (n = 7). In

all experiments, fish were injected with gpKiss2 at 7:00 AM (Zeitgeber time (ZT) 2:00) and left in indoor tanks (100 L, n = 7−9 per tank) for 12 hours.

2.2.3. Sample collection

The fish were anesthetized in 0.03% MS222 and total length and BW were recorded. Brains and pituitaries were removed after decapitation and soaked in RNAlater (Ambion, Austin, TX) and kept at 4ºC overnight. Gonads were removed and weighed for the calculation of gonadosomatic index (GSI = gonad weight/BW × 100). In the next day, brains were trimmed to prepare the forebrain sample that contained the telencephalon and diencephalon and they were stored at -80ºC until RNA extraction. All the experimental procedures were carried out following the approved guidance by the Institutional Animal Care and Use Committee of the Niigata University, Niigata, Japan. Total length, BW, and GSI of the fish are shown in Table 2.1.

2.2.4. Real-time PCR assay

Real-time PCR assays for *kiss2*, *kiss2r, gnih*, *gnih-r*, *gnrh1*, *gnrh2*, *gnrh3*, *gpa*, *fshb* and *lhb* were carried out as described previously by Shahjahan et al., 2010a. Briefly, total RNA was extracted from the brain samples and pituitary using guanidium thiocyanate-phenol-chloroform method (Chirgwin et. al., 1979). Total RNA was quantified by NanoDrop One Spectrophotometer (ThermoFisher Scientific, Japan) and verified the quality and integrity of total RNA by gel electrophoresis. Total RNAs (200-500 ng) were used for synthesis of first strand of cDNAs using MultiScribe Reverse Transcriptase (Applied Biosystem, USA) and an oligo $d(T)_{12\text{-}18}$ primer as per manufacturer's instructions. The profile for reverse transcription reaction was 25ºC for 10 min, 48ºC for 30 min and 95ºC for 5 min. Real-time PCR was carried out with a Thermal Cycler Dice Real Time System III (TP 970, TaKaRa Bio, Japan). The

absolute amount of mRNA was determined using sense reference RNA, which was synthesized in vitro by a MAXIscript kit (Ambion) according to the manufacturer's instruction and were serially diluted to 1 x 10^3 − 1 x 10^8 copies/µl. The standard sense RNAs were reverse transcribed and used as standard cDNAs to establish a standard curve. PCR reaction mixture (10 µl) contained 1 µl of standard sample cDNA, 0.4 µl of forward and reverse primers (Table 2.2) and 5 µl of TB Green Premix DimerEraser (TaKaRa, Ohtsu, Japan). Amplification was carried out at 95ºC for 30 sec, followed by 40 cycle at 95ºC for 5 sec, 60ºC for 30 sec and 72ºC for 30 sec. Specific amplification of each cDNA was verified by melting curve analysis and gel electrophoresis of the product.

2.2.5. Statistical analysis

The relative mRNA values with respect to control (0 µg/g BW) are expressed as mean ± standard error of the mean (SEM). To assess the statistically significant difference among different doses of gpKiss2 in the immature and mature fish, data were analyzed by ANOVA followed by Tukey's HSD post hoc test. Statistical significance was set at $p < 0.05$ unless described anywhere in the text. Student t-test were performed to compare significant difference between gpKiss2 injected and control groups in the regressed fish. All statistical analyses were performed using SPSS Version 23.0 for windows (SPSS Inc., Chicago, IL).

2.3. Results

2.3.1. Effect of gpKiss2 on kiss2, kiss2r, gnih, gnih-r and three gnrh expressions in the brain of immature and mature fish

The administration of gpKiss2 did not alter the expression levels of *kiss2* in the immature and mature fish (Fig. 2.1A). However, the expression of *kiss2r* was significantly stimulated in

the gpKiss2 injected fish at both immature and mature stages when compared to the control (Fig. 2.1B). The fold changes in *kiss2r* expression in response to gpKiss2 seemed to be higher in the immature fish than the mature fish. gpKiss2 did not show any noticeable effect on the expression of *gnih* and *gnih-r* in the brain of both immature and mature fish (Figs. 2.2A and 2.2B).

gpKiss2 significantly elevated the expression of *gnrh1* in the brain of immature and mature fish and the fold changes in *gnrh1* expression in response to gpKiss2 seemed to be higher in the mature fish than the immature fish (Fig. 2.3A). In the case of *gnrh2* and *gnrh3*, gpKiss2 did not show any effect at both immature and mature stages at any doses (Figs. 2.3B and 2.3C).

2.3.2. Effect of gpKiss2 on kiss2r and GTH subunit gene expressions in the pituitary of immature and mature fish

In the pituitary, the mRNA levels of *kiss2r* were significantly increased by the gpKiss2 administration in both immature and mature fish and gpKiss2 showed higher potency to stimulate the *kiss2r* expression in the immature fish compared to the mature fish (Fig. 2.4). Similarly, gpKiss2 significantly stimulated the expression of *fshb* and *lhb* in the immature and mature fish (Figs. 2.5B and 2.5C), whereas no noticeable changes were observed for *gpa* (Fig. 2.5A).

2.3.3. Effect of gpKiss2 on kiss2, kiss2r, gnih, gnih-r and three gnrh expressions in the brain of regressed fish

In the brain of regressed fish, gpKiss2 did not show any change in the *kiss2* expression but significantly stimulated the expression of *kiss2r* (Fig. 2.6A). The expression levels of *gnih* and *gnih-r* tended to increase by the gpKisss2 administration, although these changes were not statistically significant (Fig. 2.6B). There were no significant changes in the expression levels

of three *gnrhs* (Fig. 2.6C).

2.3.4. Effect of gpKiss2 on kiss2r and GTH subunit gene expressions in the pituitary of regressed fish

In the pituitary of regressed fish, gpKiss2 significantly decreased the *kiss2r* expression (Fig. 2.7A). There was a trend toward increased *gpa* and *fshb* expression by the gpKiss2 administration (Figs. 2.7B and 2.7C) and no noticeable changes were observed in the *lhb* expression (Fig. 2.7D).

2.4. Discussion

The effect of gpKiss2 administration on the expression of the genes for the HPG axis were examined at three gonadal stages to clarify the functional importance of the kisspeptin system in the grass puffer. The present results showed that gpKiss2 significantly stimulated the expression of *kiss2r* and *gnrh1* in the brain and *kiss2r*, *fshb* and *lhb* in the pituitary of the immature and mature fish, demonstrating a stimulatory role of gpKiss2 on the reproduction by activating the pituitary GTH secretion through stimulating the GnRH1 synthesis. In the regressed fish, however, gpKiss2 was not effective in stimulating the GnRH1 and GTH gene expression, suggesting that the stimulatory role of gpKiss2 in reproduction varies depending on the gonadal stage. Moreover, gpKiss2 did not alter the expression of *gnih* and *gnih-r* as well as its own gene, *kiss2*.

In mammals, kisspeptin has a strong stimulatory effect on the GTH secretion from the pituitary and this is mainly mediated through the stimulatory action on GnRH secretion. Kiss1r is colocalized with GnRH neurons in the hypothalamus and the direct interaction between kisspeptin and GnRH is primarily important in the control of ovulatory cycle in mammals. In

the present study, in vivo effect of gpKiss2 was evaluated on the expression of three GnRH genes, namely *gnrh1*, *gnrh2* and *gnrh3* at different reproductive stages. In fish, the localization and function of three GnRH neuronal groups are diversified. GnRH1 neurons are mainly located in the preoptic area (POA) and have a hypophysiotropic role through stimulating the GTH secretion. GnRH2 neurons are localized in the midbrain tegmentum and are involved in appetite-related reproductive function (Matsuda et al., 2008; Nishiguchi et al., 2012; Marvel et al., 2019). GnRH3 neurons are localized in the terminal nerve ganglion-POA region and have neuromodulatory action related to sexual behavior (Okuyama et al., 2014; Li et al., 2017). The mRNA levels of *gnrh1* as well as *kiss2r* in the brain were significantly elevated in the immature and mature fish 12 hours after gpKiss2 injection. Immunohistochemical localization of Kiss2 and Kiss2r in the grass puffer showed that both Kiss2- and Kiss2r-immunoreactive (ir) cells are localized in the magnocellular preoptic nucleus pars magnocellularis (PMm) in the POA (Rahman, 2020), which is one of the major hypothalamic nuclei that consist of hypophysiotropic neurons including GnRH1 neurons (Munoz-Cueto et al., 2020). Although the colocalization of GnRH1 neurons with Kiss2r needs to be determined, the present results suggest that gpKiss2 directly stimulates the secretion of GnRH1 and then activates FSH and LH secretion in the grass puffer. The present results are consistent with the previous studies on the expression patterns of *kiss2*, *kiss2r* and three *gnrhs* with respect to seasonal, daily and circadian variations (Shahjahan et al., 2010b; Ando et al., 2013, 2014, 2018) and also response to temperature change (Shahjahan et al., 2017).

In the regressed fish, there was no significant changes in the expression of gpKiss2 injected and control groups and this suggests that kisspeptin action is dependent on the reproductive stages. The expression of *kiss2r* was increased in the brain of regressed fish by the gpKiss2 injection, whereas it decreased in the pituitary in accordance with no significant increases in *lhb* expression. Kiss2 showed stimulatory and inhibitory effects on the *kiss2r* expression

depending on the gonadal stage in hybrid bass (Zomra et al., 2012). In yellowtail kingfish, no changes in the *kiss2r* mRNA level were observed in the hypothalamus after Kiss1 administration in the breeding season, while Kiss1 administration significantly augment the expression of *kiss2r* in the pituitary in the non-breeding season (Nocillado et al., 2013). Therefore, the regulation of kisspeptin receptor gene expression by Kiss may be different between the hypothalamus and pituitary and also dependent on reproductive stage.

In this study, augmented expression of *fshb*, *lhb* as well as *kiss2r* in the pituitary of the immature and mature fish suggests that gpKiss2 could have both direct and indirect action on the regulation of pituitary. In the previous studies in grass puffer, the *kiss2r* expression in both the hypothalamus and pituitary showed seasonal variations with an increase during the spawning season (Shahjahan et al., 2010a). The stimulation of GTH secretion from the pituitary by kisspeptin administration has been shown in many fish species such as zebrafish, goldfish, cinnamon clownfish, European seabass, and Nile tilapia (Kitahashi et al., 2009; Li et al., 2009; Felip et al., 2009; Kim et al., 2014; Park et al., 2016), although it has been unclear whether kisspeptin has direct and/or indirect actions on the pituitary. The present and previous results indicate that gpKiss2 is certainly effective to stimulate the GTH secretion via direct local action and indirect neuroendocrine action through GnRH1 neurons.

The effect of gpKiss2 on the expression of *gnih* and *gnih-r* was also examined in the present study. gpKiss2 did not show any significant changes in the expression of *gnih* and *gnih-r* at any reproductive stages. In the sole, Kiss2 significantly upregulated the expression of *gnih* and downregulated the expression of *gnih-r* in vitro (Wang et al., 2017). In the previous studies in the grass puffer, *gnih* and *gnih-r* showed seasonal, daily and circadian variations like *kiss2* and *kiss2r* (Shahjahan et al., 2011; Ando et al., 2013, 2018) and GnIH administration experiments using a heterologous peptide (goldfish LPXRFamide) on the pituitary of the grass puffer showed that GnIH is a multifunctional hypophysiotropic neurohormone, simulating the

expression of the genes for GTH subunits as well as growth hormone and prolactin (Shahjahan et al., 2011, 2016; Ando et al., 2018). Moreover, immunohistochemical localization of GnIH and GnIH-R in the grass puffer showed that both GnIH- and GnIH-R-ir cells are localized the PMm, suggesting the colocalization with Kiss2, Kiss2r and GnRH1 neurons (Rahman, 2020). These facts strongly support the notion that GnIH has a stimulatory role in the control of reproduction with interaction with Kiss2 and GnRH1. Therefore, it is possible that gpKiss2 may have some effect on the *gnih* and *gnih-r* expressions, and this warrants further investigation.

In conclusion, gpKiss2 significantly stimulated the expression of *kiss2r* and *gnrh1* in the brain and *kiss2r*, *fshb* and *lhb* in the pituitary at immature and mature stages, suggesting that kisspeptin functions as a stimulatory neurohormone in reproductive functions via direct local action and indirect neuroendocrine action through stimulating the GnRH1 secretion in the grass puffer.

Table 2.1. Total length, body weight and gonadosomatic index (GSI) of fish samples. Values are presented as mean ± SEM.

Gonadal condition	No. of fish	Total length (cm)	Body weight (g)	GSI (%)
Immature	26	7.9 ± 0.1	8.7 ± 0.3	0.6 ± 0.1
Mature	24	14.9 ± 0.3	63.7 ± 0.3	16.3 ± 1.6
Regressed	13	13.4 ± 0.5	43.5 ± 5.1	1.1 ± 0.1

Table 2.2. Primers used in the real-time PCR assays in this study

Primers	**Nucleotide sequences**
GnRH1-qPCR-F1	5′-CGGGAGTCTGATGTCACAGCTC-3′
GnRH1-qPCR-R1	5′-AACACTGACGACGACCGTGTCC-3′
GnRH2-qPCR-F1	5′-CAGGAGCTCACCTGTCCAAC-3′
GnRH2-qPCR-R1	5′-CTGCATTCTCCTGCTTCACAG-3′
GnRH3-qPCR-F1	5′-AAGCAAACAGGGTGATGGTG-3′
GnRH3-qPCR-R1	5′-CTGATGGTTGCCTCCAACTC-3′
GnIH-qPCR-F1	5′-TGATTCGTCTGTGCGAGGAC-3′
GnIH-qPCR-R1	5′-TCAGCAGCTGTGCATTGACC-3′
GnIH-R-qPCR-F1	5′-AAGATGCTCATCCTGGTGGC-3′
GnIH-R-qPCR-R1	5′-AGATCCACCTGGTCACTGTCC-3′
Kiss2-qPCR-F1	5′-GACCTTCAGGGACAACGAGGAC-3′
Kiss2-qPCR-R1	5′-ATGAAGCGCTTGCCAAAGC-3′
Kiss2r-qPCR-F1	5′-TCCCGTTTCTGTTCAAGCACAAG-3′
Kiss2r-qPCR-R1	5′-ATTGTTGTTGCGCTCCTCTGC-3′
GPα-qPCR-F1	5′-AAGGTGAGGAACCACACCGAG-3′
GPα-qPCR-R1	5′-AGCTCAAGGCCAGGATGAAC-3′
FSHβ-qPCR-F1	5′-ACACATTGAGGGCTGTCCAGTGG-3′
FSHβ-qPCR-R1	5′-TCCCCATTGAAGCGACTGCAG-3′
LHβ-qPCR-F1	5′-CACTTGGTGCAAACAAGCATC-3′
LHβ-qPCR-R1	5′-CAACTTAGAGCCACGGGGTAG-3′

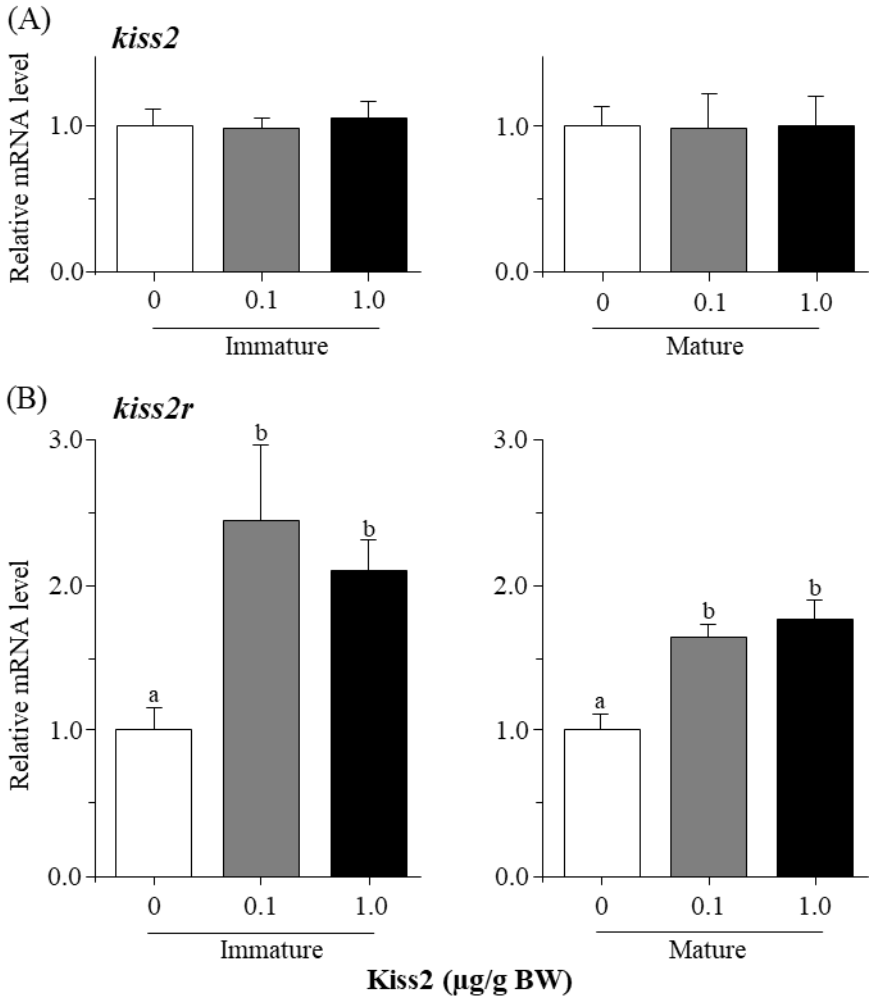

Fig. 2.1. Changes in the relative mRNA levels of *kiss2* (A) and *kiss2r* (B) in the brain of the grass puffer at immature and mature stages. Values are presented as mean ± SEM (n = 6−8). Values accompanied by different letters are statistically significantly different ($p < 0.05$).

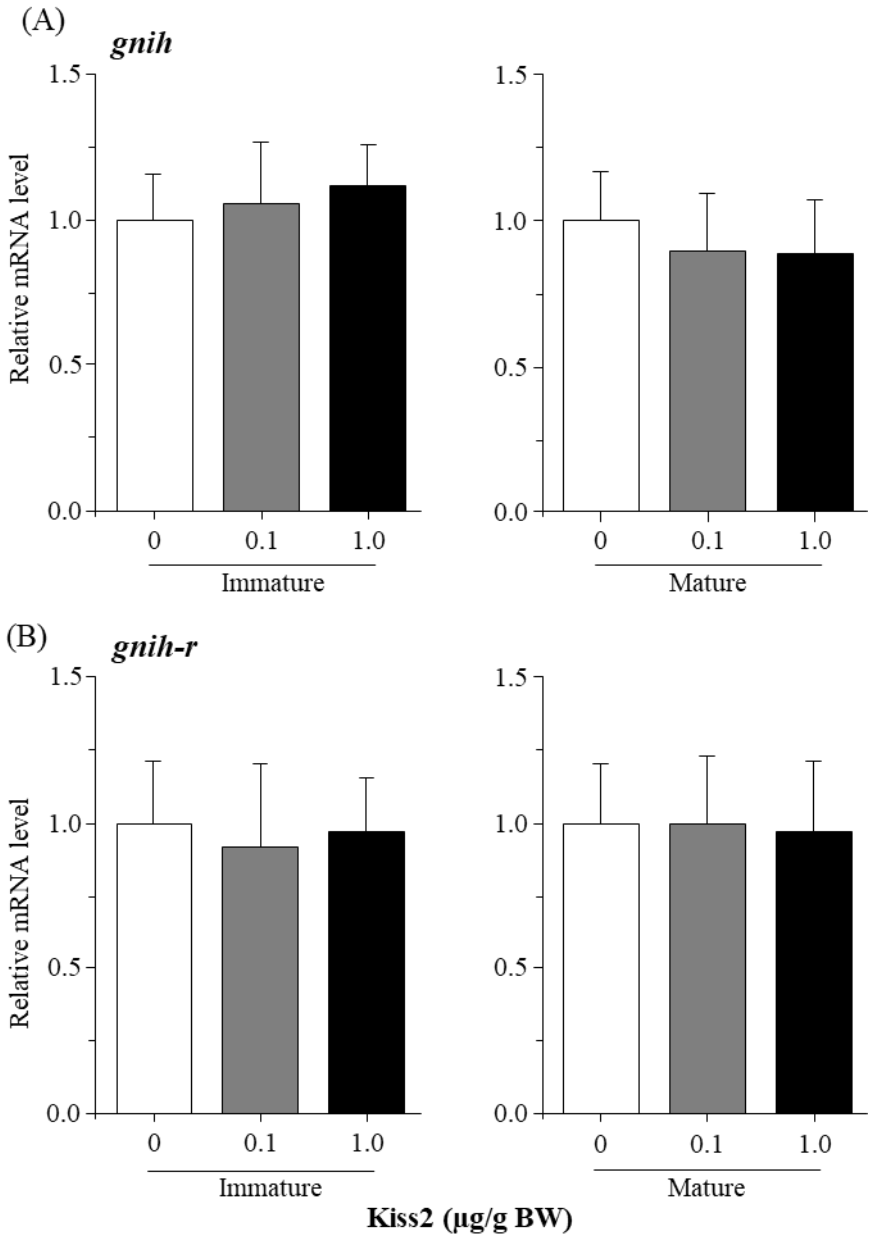

Fig. 2.2. Changes in the relative mRNA levels of *gnih* (A) and *gnih-r* (B) in the brain of the grass puffer at immature and mature stages. Values are presented as mean ± SEM (n = 6−8).

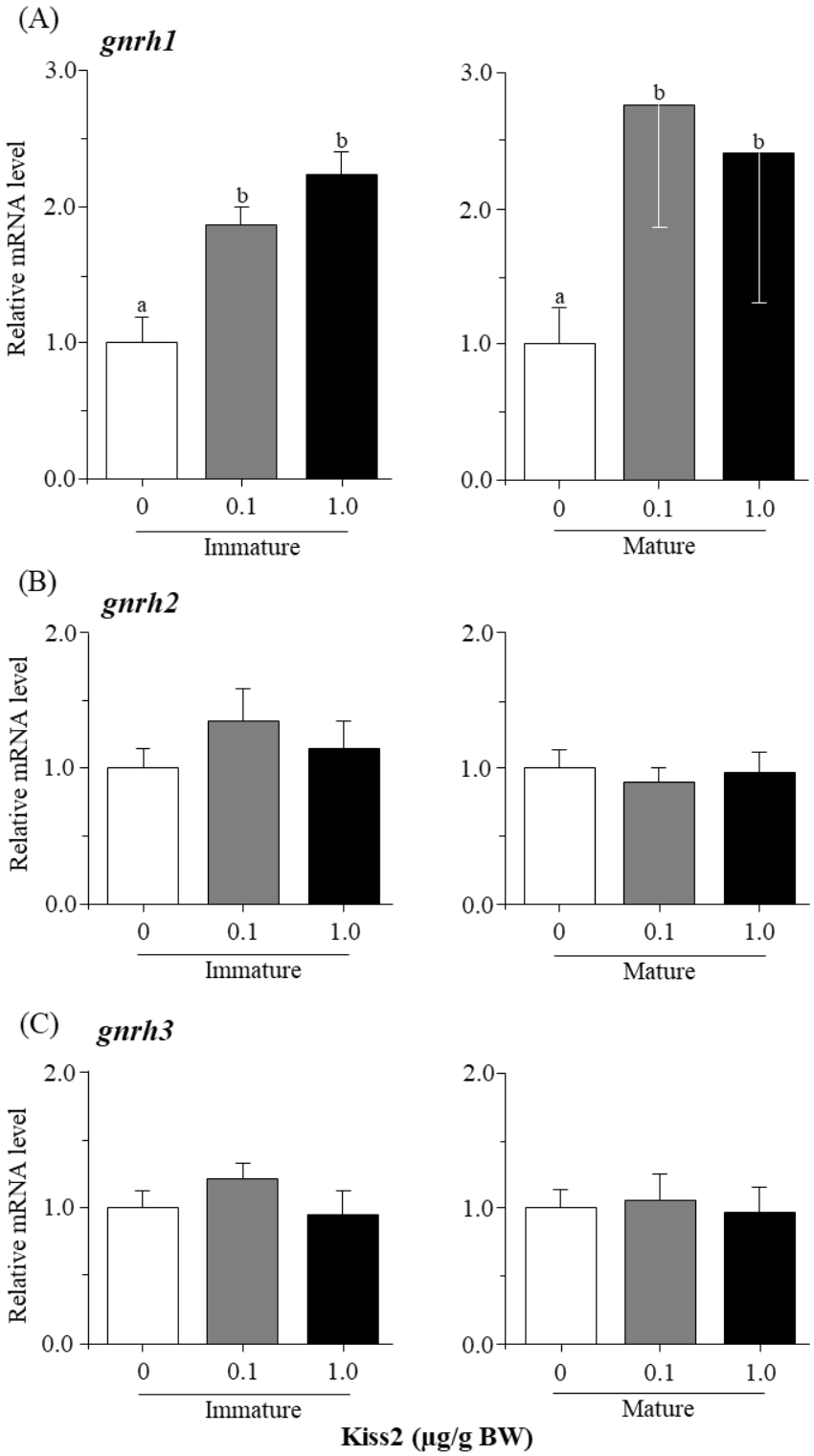

Fig. 2.3. Changes in the relative mRNA levels of *gnrh1* (A), *gnrh2* (B) and *gnrh3* (C) in the brain of the grass puffer at immature and mature stages. Values are presented as mean ± SEM (n = 6−8). Values accompanied by different letters are statistically significantly different ($p < 0.05$).

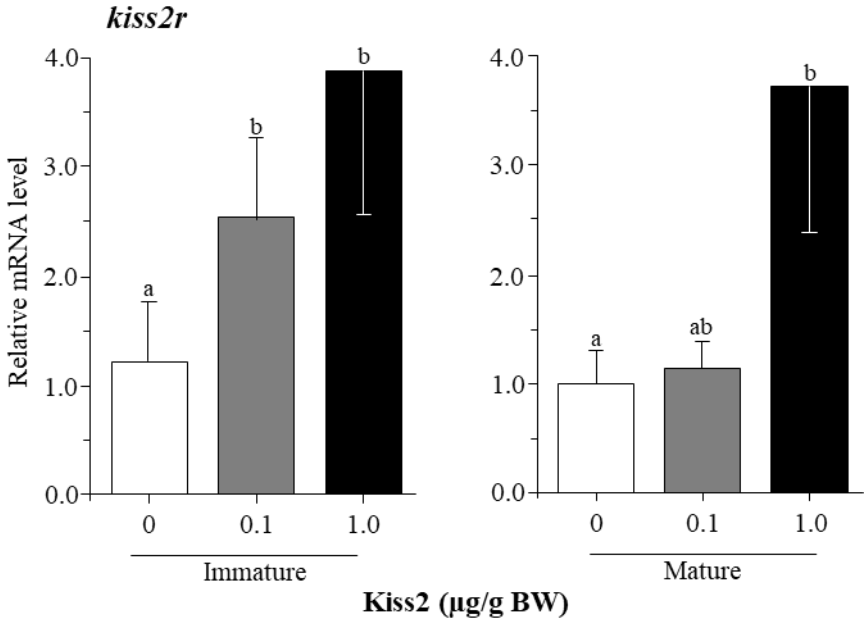

Fig. 2.4. Changes in the relative mRNA levels of *kiss2r* in the pituitary of the grass puffer at immature and mature stages. Values are presented as mean ± SEM (n = 6−8). Values accompanied by different letters are statistically significantly different ($p < 0.05$).

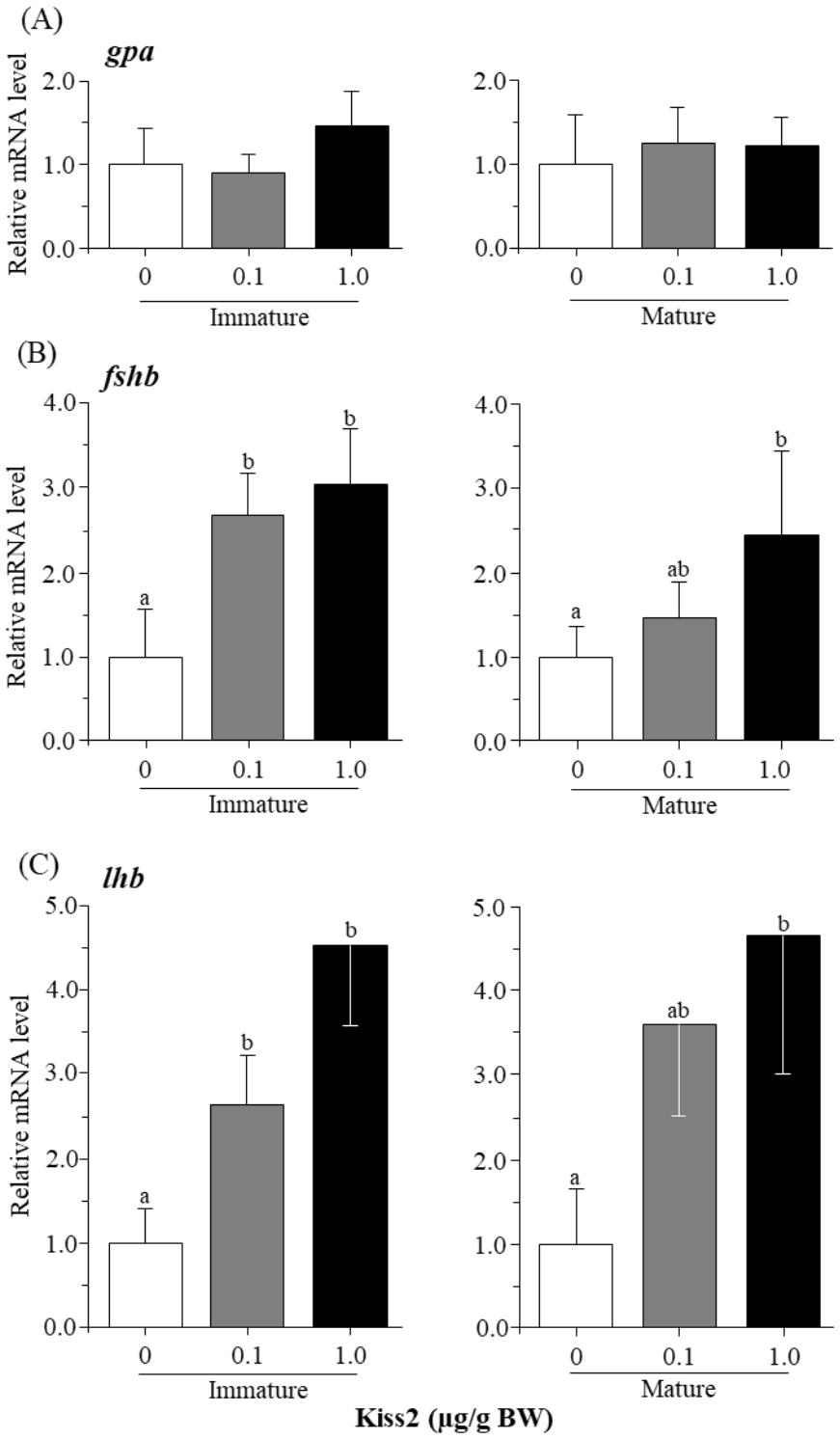

Fig. 2.5. Changes in the relative mRNA levels of *gpa* (A), *fshb* (B) and *lhb* (C) in the pituitary of the grass puffer at immature and mature stages. Values are presented as mean ± SEM (n = 6−8). Values accompanied by different letters are statistically significantly different ($p < 0.05$).

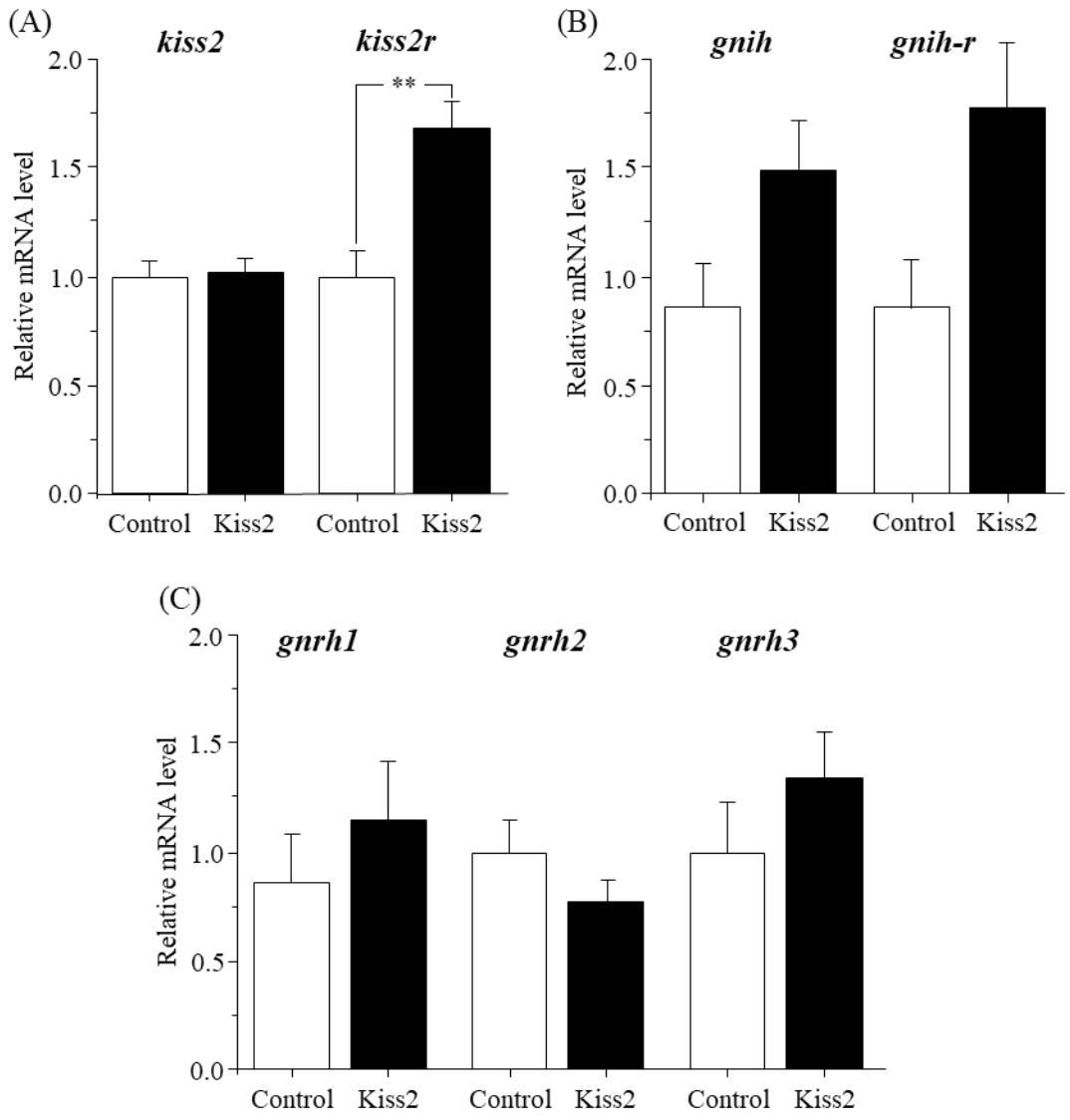

Fig. 2.6. Changes in the relative mRNA levels of *kiss2*, *kiss2r* (A), *gnih*, *gnih-r* (B) and three *gnrh* (C) in the brain of the grass puffer at regressed stage. Values are presented as mean ± SEM (n = 6−8). Asterisks denotes a significant difference between the control and gpKiss2 injected fish (**, $p < 0.01$).

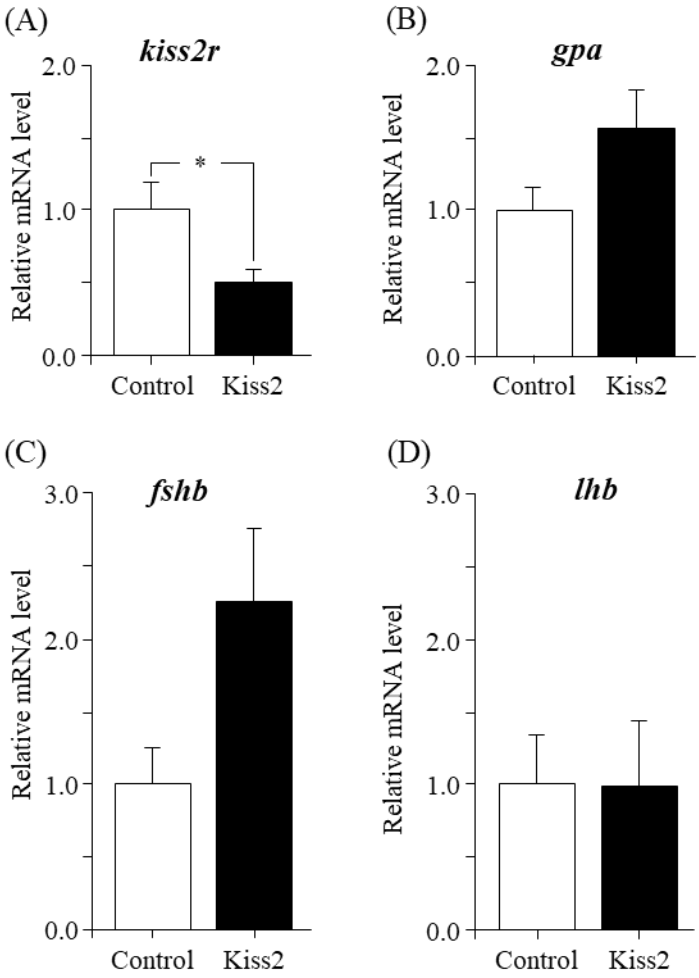

Fig. 2.7. Changes in the relative mRNA levels of *kiss2r* (A), *gpa* (B), *fshb* (C) and *lhb* (D) in the pituitary of the grass puffer at regressed stage. Values are presented as mean ± SEM (n = 6−8). Asterisks denotes a significant difference between the control and gpKiss2 injected fish (*, $p < 0.05$).

Chapter 3

Effect of GnIH administration on the expression of the HPG axis genes in the grass puffer at different reproductive stages

3.1. Introduction

Reproduction in vertebrates is regulated by the hypothalamus-pituitary and gonadal (HPG) axis which translate external and internal incitements into endocrine signals and reproductive outputs. This axis integrates a complex mechanism at the brain and pituitary level which includes a number of neurohormones like gonadotropin-inhibitory hormone (GnIH), kisspeptin and gonadotropin-releasing hormone (GnRH). These hormones are released from the hypothalamus and are involved in the control of pituitary gonadotropins (GTHs). GTHs (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)) in turn, modulates the gametogenesis and sexual maturation through the arbitration of gonadal steroids and growth factors (Zohar et al., 2010). GnIH, members of the RFamide peptide family were incipiently discovered from quail as a novel hypothalamic neuropeptide as an LH release inhibiting factor (Tsutsui et al., 2000). Subsequently, GnIH homologs have been identified in a variety of vertebrate species and its involvement was reported in diverse physiological functions depending on the species, sex and gonadal conditions (Tsutsui and Ukuba, 2020).

The GnIH precursor is possibly cleaved into two to four mature peptides which possess a characteristic putative C-terminal LPXRFamide (X = L or Q) peptides depending on the species (Ubuka et al., 2016; Tsutsui et al., 2018). For example, quail GnIH precursor comprised of three LPXRFamide peptides and also two GnIH-related peptide (RP)-1 and GnIH-RP-2 (Satake et al., 2001). Human LPXRFamide precursor includes one C-terminal LPLRFamide peptide and one LPQRFamide peptide and followed by the name human RFamide related peptide

(RFRP)-1 and -3, respectively. Likewise, in amphibians, four mature peptides (fGRP, fGRPRP-1, fGRPRP-2, and fGRPRP-3) are formed (Koda et al., 2002; Ukena et al., 2003). On the other hand, most of the teleost GnIH precursor encode three LPXRFamide or LPXRFamide-like peptides, which align to human RFRP-1/quail GnIH-RP-1, human RFRP-2/quail GnIH, and human RFRP-3 respectively. The grass puffer LPXRFa precursor contains two putative RFamide peptide (LPXRFa-1 and LPXRFa-2) and one possible RYamide peptide. In teleosts, evidence suggests that functional significance of the multiple GnIH peptide from a species varied depending on the peptide and conditions of the fish (Tsutsui and Ubuka et al., 2020). For example, in *Cichlasoma dimerus*, LPQRFa-1 decreased the expression of *lh* and *fshb*, and at the same time, LPQRFa-2 at a higher dose stimulated the expression of *fshb* (Di Yorio et al., 2016). In flatfish, intramuscular injection of GnIH-3 significantly reduced the expression of *gnrh3* and *lh*, meanwhile, GnIH-2 did not affect the HPG axis (Aliaga-Guerrero et al., 2018).

Accumulating evidence suggests that GnIH has important functions in regulating reproduction in mammals and birds through its inhibitory action on the GnRH and GTHs from the brain and pituitary respectively. However, in teleosts, stimulatory and inhibitory effects on GnRH neurons as well as on the secretion of GTHs have been reported depending on the species, gonadal stages and types of peptide (Shahjahan et al., 2011, 2016; Moussavi et al., 2014; Biran et al., 2014; Di Yorio et al., 2016; Wang et al., 2019; Zhai et al., 2020). Additionally, involvement of GnIH in the periodic control of reproduction has been demonstrated in many vertebrate species. It has also been shown that GnIH serves as a key component in the HPG axis driving photoperiodic control of reproduction through its interaction with melatonin (Ubuka et al., 2005; Revel et al., 2008). Photic condition-dependent *gnih* expression has been reported in zebrafish (Yumnamcha et al., 2017) and European sea bass (Paullada-Salmeron et al., 2017). It is therefore most probable that GnIH/GnIH-R system is correlated with seasonal

reproduction, although the functional significance of GnIH/GnIH-R in association with the periodic control of reproduction remain obscure in teleosts.

The grass puffer (*Takufugu alboplumbeus)*, a semilunar spawner, spawning occurs on the beach in the new moon and full moon period from spring to early summer and is synchronized with seasonal, diurnal, and lunar cycles (Yamahira, 2004; Motohashi et al., 2010; Ando et al., 2013, 2018). Spawning fish aggregate at certain seashores 2−3.5 hours before spawning and spawning occurs 1.5−2 hours before high tide and continues for 1 hour during the rising tidal phase before evening in a group of 10−60 individuals, among which one is female (Motohashi et al., 2010). Thus, grass puffer provides a unique model animal for studying the neuroendocrine mechanism underlying seasonal, lunar and circadian control of reproduction.

Previously, it was demonstrated that grass puffer GnIH and its receptor gene expression displayed diurnal and circadian oscillations during spawning season (Shahjahan et al., 2011). Moreover, studies found elevated expression of *gnih* and *gnih-r* during spawning season in compared to the pre-spawning and recrudescence period (Shahjahan et al., 2011). Earlier, to understand the role of GnIH in the grass puffer, a heterologous peptide, goldfish LPXRFamide (gfLpxrfa) was tested in vivo and in vitro on the expression of pituitary GTHs and found to have stimulatory effect in this species (Shahjahan et al., 2011, 2016; Ando et al., 2018). Previous findings indicated that GnIH/GnIH-R system is functionally important in the periodic control of semilunar spawning in the grass puffer. For further clarification of the GnIH/GnIH-R system in the semilunar spawning in the grass puffer, two native peptides (gpGnIH-1 and gpGnIH-2) was tested at different reproductive stages in this study.

3.2. Materials and Methods

3.2.1. Fish

Mature male grass puffer were collected from the spawning ground in Kawana, Shizuoka Japan in June. Male fish with regressed testes were collected from the spawning ground in Minamiise, Mie, Japan at the end of July. The collected fishes were transferred to the Sado Island Center for Ecological Sustainability, Niigata University, Japan and reared in indoor tanks (500L capacity) with running seawater under natural photoperiod (LD 14:10) prior experiment. The water temperature during the acclimation period was similar to that in the spawning sites. The fishes were fed with commercial pellets equivalent to 1% body weight (BW) prior to the in vivo experiment.

3.2.2. Experimental design and sample collection

Grass puffer GnIH (gpGnIH-1, PHHQHVNMPMRFamide and gpGnIH-2, DGVQGGDHVPNLNPNMPQRFamide) were synthesized and dissolved in saline solution (0.9% NaCl) and stored at -80ºC until use. The fish were anesthetized in 0.008% tricaine methanesulfonate (MS222, Sigma-Aldrich, Tokyo, Japan) for 30 sec. and injected GnIH intraperitoneally (ip) using a fine needle (25G, Terumo Corporation, Tokyo, Japan). The gpGnIH-1 was administered at different doses (0, 0.01, 0.1, and 1.0 μg/g BW, n = 6−7) and sampling was conducted after 12 and 24 hours of injection. A preliminary experiment was conducted to examine the effect of gpGnIH-2 administration (0, 0.01, 0.1 μg/g BW, n = 4) for a period of 12 hours, because there had been few reports on the effect of GnIH on animals at recrudescence stage and had an increasing trend in the expression of *gnih* and *kiss2* at the 0.01 μg/g BW. So, gpGnIH-2 was injected at 0.01 μg/g BW in regressed fish and sampling was done after 12 hours of administration. Each fish was anesthetized and immobilized with its ventral side upward and the solution was injected slowly and released into the treatment tank. The fish were anesthetized in 0.03% MS222 before sampling, and body weight (BW) and total length (TL) were recorded. Brains and pituitaries were removed after decapitation and soaked

in RNAlater (Ambion, Austin, TX) and kept at 4°C overnight. Gonads were removed and weighed for the calculation of gonadosomatic index (GSI, gonad weight/body weight × 100). In the next day, brains were trimmed to prepare the forebrain sample that contained the telencephalon and diencephalon and they were stored at -80ºC until RNA extraction. All the experimental procedures were carried out following the approved guidance by the Institutional Animal Care and Use Committee of the Niigata University, Niigata, Japan. TL, BW, and GSI of the fish used in this study are shown in Table 3.1.

3.2.3. Real-time PCR assay

Real-time PCR assays for *gnih*, *gnih-r*, *kiss2*, *kiss2r, gnrh1*, *gnrh2*, *gnrh3*, *gpa*, *fshb* and *lhb* were carried out as described previously by Shahjahan et al., 2010a. Briefly, total RNA was extracted from the brain samples and pituitary using guanidium thiocyanate-phenol-chloroform method (Chirgwin et. al., 1979). Total RNA was quantified by NanoDrop One Spectrophotometer (ThermoFisher Scientific, Japan) and verified the quality and integrity of total RNA by gel electrophoresis. Total RNAs (200-500 ng) were used for synthesis of first strand of cDNAs using MultiScribe Reverse Transcriptase (Applied Biosystem, USA) and an oligo $d(T)_{12-18}$ primer as per manufacturer's instructions. The profile for reverse transcription reaction was 25ºC for 10 min, 48ºC for 30 min and 95ºC for 5 min. Real-time PCR was carried out with a Thermal Cycler Dice Real Time System III (TP 970, TaKaRa Bio, Japan). The absolute amount of mRNA was determined using sense reference RNA, which was synthesized in vitro by a MAXIscript kit (Ambion) according to the manufacturer's instruction and were serially diluted to $1 \times 10^3 - 1 \times 10^8$ copies/µl. The standard sense RNAs were reverse transcribed and used as standard cDNAs to establish a standard curve. PCR reaction mixture (10 µl) contained 1 µl of standard sample cDNA, 0.4 µl of forward and reverse primers (Table 2.2) and 5 µl of TB Green Premix DimerEraser (TaKaRa, Ohtsu, Japan). Amplification was

carried out at 95ºC for 30 sec, followed by 40 cycle at 95ºC for 5 sec, 60ºC for 30 sec and 72ºC for 30 sec. Specific amplification of each cDNA was verified by melting curve analysis and gel electrophoresis of the product.

3.2.4. Statistical analysis

Datasets for the measured absolute amount of mRNA are expressed as mean ± standard error of the mean (SEM). To assess the statistically significant difference among the different doses of gpGnIH-1 peptide, mRNA values were subjected to one-way analysis of variance (ANOVA) followed by the comparison of means by Tukey's HSD post hoc test. Significant differences were indicated by p-values < 0.05 unless described anywhere in the text. Student t-test was performed to compare the significant difference between gpGnIH-2 injected and control groups in the regressed fishes. All statistical analyses were performed using Microsoft Excel (Microsoft Inc., USA) and SPSS Version 23.0 for windows (SPSS Inc., Chicago, IL).

3.3. Results

3.3.1. Effect of gpGnIH-1 on the expression of gnih and gnih-r in the brain of mature fish

Administration of gpGnIH-1 stimulates the expression of *gnih* and *gnih-r* at 0.10 µg/g BW doses after 12 hours in the brain of mature grass puffer (Figs. 3.1A and 3.1B). Injection at low (0.01 µg/g BW) and high (1.00 µg/g BW) doses did not alter the GnIH and GnIH-R mRNA levels compared to control.

A significant increase in the expression of *gnih* was observed in the brain of mature fish when administered with a low dose (0.01 µg/g BW) of gpGnIH-1 after 24 hours (Fig. 3.1C). In the same way, the expression of *gnih-r* was also higher in fish at the same dose of gpGnIH-1 (Fig. 3.1D). However, higher doses (0.10 and 1.00 µg/g BW) of gpGnIH-1 did not alter the

GnIH and GnIH-R mRNA level in the brain of mature grass puffer after 24 hours of gpGnIH-1 injection.

3.3.2. Effect of gpGnIH-1 on the expression of kiss2 and kiss2r in the brain of mature fish

In vivo effect of gpGnIH-1 was analyzed for the expressions of *kiss2* and *kiss2r* from the mature grass puffer. Increased mRNA levels of Kiss2 and Kiss2r was observed after 12 hours of gpGnIH-1 injection at 0.10 µg/BW doses (Figs. 3.2A and 3.2B). No significant changes were observed in the Kiss2 and Kiss2r mRNA levels at low (0.01 µg/BW) or high (1.00 µg/BW) doses in compared to control after 12 hours (Figs. 3.2A and 3.2B).

Administration of gpGnIH-1 at lower (0.01 µg/BW) doses after 24 hours significantly increased the expression of *kiss2* and *kiss2r* (Figs. 3.2C and 3.2D). However, higher doses of gpGnIH-1 (0.10 and 1.00 µg/BW) did not show any noticeable changes in the Kiss2 and Kiss2r mRNA level when compared to control in the brain of mature grass puffer.

3.3.3. Effect of gpGnIH-1 on the expression of three gnrh genes in the brain of mature fish

Significantly higher expression of *gnrh3* was observed after 12 hours of gpGnIH-1 administration at 0.10 µg/BW doses (Fig. 3.4C). No significant changes were observed in the expression of *gnrh1* and *gnrh2* after 12 hours of gpGnIH-1 administration when compared to control (Figs. 3.4A and 3.4B).

The administration of gpGnIH-1 with the low dose (0.01 µg/BW) stimulated the expression of *gnrh1*, *gnrh2* and *gnrh3* after 24 hours injection of the peptide (Figs. 3.4D−F). However, at higher doses, expressions of *gnrhs* did not show any noticeable changes except slight elevation in the expression of *gnrh3* at 1.00 µg/BW doses in compared to control.

3.3.4. Effect of gpGnIH-1 on the expression of gnih-r, gpa, fshb and lhb in the pituitary of

mature fish

No significant changes in the expression of *gnih-r* were observed after 12 hours of gpGnIH-1 injected fish compared to the control (Fig. 3.4A). However, expression of *gnih-r* was decreased in the pituitary at higher doses (0.10 and 1.00 µg/g BW) after 24 hours of gpGnIH-1 administration (Fig. 3.4B).

Significantly higher expression of *lhb* was observed at 0.01 µg/g BW after 12 hours of gpGnIH-1 injection (Fig. 3.5C). In the case of *gpa*, 12 hours post-injection of gpGnIH-1 did not alter the mRNA levels at any doses (Fig. 3.5A) while, *fshb* mRNA level was slightly elevated only at 0.01 µg/g BW doses compared to control (Fig. 3.5B). Increased expression of *gpa*, *fshb* and *lhb* was noticed at 0.01 µg/g BW doses, while, higher doses (0.10 and 1.00 µg/g BW) of gpGnIH-1 did not alter the mRNA level after 24 hours (Fig. 3.5D−F).

3.3.5. Effect of gpGnIH-2 on the expression of kiss2, kiss2r, gnih, gnih-r and three gnrh in the brain of regressed fish

In vivo functional analysis of gpGnIH-2 peptide (single dose, 0.01 µg/g BW, 12 hours) was also assessed in the brain of regressed grass puffer. Expression of *gnih*, *gnih-r, kiss2*, *kiss2r*, *gnrh1*, *gnrh2* and *gnrh3* did not show any noticeable changes in the brain after injection of gpGnIH-2 peptide compared to control (Figs. 3.6−3.7).

3.3.6. Effect of gpGnIH-2 on the expression of gnih-r, gpa, fshb and lhb in the pituitary of regressed fish

In the pituitary, expression of *gnih-r* significantly decreased in the gpGnIH-2 injected fish compared to the control (Fig. 3.8A). However, gpGnIH-2 significantly elevated the expression of *gpa* (Fig. 3.9B). A slight elevation in the expression of *fshb* (Fig. 3.8C) and no changes in the expression of *lhb* was noticed in the gpGnIH-2 injected fish compared to the control (Fig.

3.8D).

3.4. Discussion

The effect of homologous GnIH peptide on the expression of the genes for the HPG axis were examined in mature and regressed fish to clarify the functional significance of the GnIH in the semilunar spawning in the grass puffer. The result of the present study revealed that gpGnIH-1 has a stimulatory effect on the expression of *gnih*, *gnih-r, kiss2*, *kiss2r*, *gnrh2* and *gnrh3* in the brain and *gpa*, *fshb*, and *lhb* in the pituitary of mature grass puffer. The results suggest that gpGnIH-1 has a stimulatory role on the reproduction by activating pituitary GTHs through GnRH and kisspeptin synthesis. In contrast, gpGnIH-2 did not alter the expression of HPG axis genes at the regressed stage suggesting that the stimulatory role of gpGnIH-2 in the reproduction varies depending on the gonadal stage.

The role of GnIH in the avian and mammalian reproductive axis have been clearly established the inhibitory action on GnRH and gonadotropin secretion (Clarke et al., 2008; Ubuka et al., 2014; Tsutsui and Ubuka, 2020). However, in teleost, the role of GnIH on GnRH and GTHs is controversial depending on the species, sex and gonadal conditions. In this study, intraperitoneal administration of gpGnIH-1 significantly elevated the expression of *gnih-r*, *kiss2*, *kiss2r*, *gnrh2* and *gnrh3* in the brain and *fshb* and *lhb* in the pituitary of mature grass puffer which further confirms the findings of the previous studies (Shahjahan et al., 2011; Ando et al., 2018). Immunohistochemical localization of GnIH and GnIH-R in the grass puffer showed that both GnIH- and GnIH-R-ir cells are localized the magnocellular preoptic nucleus pars magnocellularis (PMm) in the pre-optic area (POA) (Rahman, 2020) which is also the major hypothalamic area consist of GnRH and kisspeptin neurons (Munoz-Cueto et al., 2020). Although the colocalization of GnIH neurons with kisspeptin and GnRH needs to be

determined, the present results suggest that gpGnIH-1 directly stimulates the secretion of kisspeptin and GnRH in the brain and then activates FSH and LH secretion in the grass puffer. The present results are consistent with the previous studies on the expression patterns of *kiss2*, *kiss2r* and three *gnrhs* with respect to seasonal, daily and circadian variations (Shahjahan et al., 2010b; Ando et al., 2013, 2014, 2018) and also response to temperature change (Shahjahan et al., 2017; Rahman et al., 2019).

In this study, intraperitoneal injection of gpGnIH-1 in mature fish stimulates the expression of *gnih* indicating the possible autocrine regulation in mature fishes (Fig. 3.1). These findings are in agreement with the European seabass where administration of GnIH-2 significantly elevated the expression of *gnih* and *gnih-r* in the brain and both GnIH-1 and GnIH-2 stimulated the testicles in spermiated male (Paullada-Salmeron et al., 2016). Autocrine regulation of GnIH/GnIH-R system was also reported in immature, male and female protandrous cinnamon clownfish when injected with GnIH-3 peptides (Choi et al., 2016). This result of the present study indicates the autocrine regulation of gpGnIH-1 through positive feedback in the GnIH system of grass puffer.

In this study, in the pituitary of mature fish, expressions of *gnih-r* were consistent or decreased after 12 and 24 hours of gpGnIH-1 administration at different doses (Fig. 3.4). However, in vivo and in vitro studies using gfLPXRFa demonstrates the direct stimulatory effect of GnIH on the expression of GTH genes in the pituitary of grass puffer (Shahjahan et al., 2011, Ando et al., 2018). Previous and present results indicate the two possible ways of GnIH action in grass puffer. First, the action of GnIH on GTH release is mediated via the GnRH and/or kisspeptin neurons in the grass puffer, secondly, direct innervations of the GnIH to the pituitary and stimulates the release of GTHs. In tilapia, LPXRFa-ir fibers were found innervated to FSH and LH cells (Ogawa et al., 2016) and this result was supported by the study performed in sea bass (Paullada-Salmerón et al., 2016) where GnIH-2-ir fibers were closely

associated with FSH and LH soma in the proximal pars distalis. Localization and projections of GnIH neurons and co-localization study between GnIH-R and GTH cells in the pituitary will further elucidate the mechanism of GnIH action in this species.

In the regressed fish, gpGnIH-2 did not alter the expressions of hypothalamic genes in the grass puffer (Fig. 3.6−3.7). In the pituitary, expression of *gnih-r* was significantly decreased (Fig. 3.8A) while increased the expression of *gpa* and *fshb* (Fig. 3.8B and 3.8C) suggesting that the regulation of GnIH-R gene expression by gpGnIH-2 may be different between the hypothalamus and pituitary and also dependent on the reproductive stage. Multiple factors such as types of peptides, dose and route of administration, elapsed time after treatment of peptides is also involved in the diversified physiological regulation of the HPG axis in fishes (Munoz-Cueto et al., 2017). No changes were observed in the Kiss1 and Kiss2 mRNA level while injected with three GnIH peptides in grouper (Wang et al., 2015) and flatfish GnIH-2 and GnIH-3 did not alter the expression of *kiss2v1* mRNA level (Aliaga-Guerrero et al., 2017). Moreover, inhibitory or no effect of GnIH peptide has also been reported in some other species on the expression of GnRH genes depending on the peptides (Paullada-Salmeron et al., 2016; Spicer et al., 2017; Zhai et al., 2020). In tilapia, no association was found on GnIH-ir fibers with kisspeptin (Kiss2) neurons (Ogawa et al., 2016). Besides, immunohistochemical studies showed that GnIH-ir axon terminals is in probable contact with GnRH neurons and controlling the secretion of gonadotropin by decreasing the activity of GnRH (Ubuka et al., 2016). Therefore, the findings of the present study suggest that multiple GnIH peptides produced from the same precursor may have differential effects on the regulation of the HPG axis depending on the types of peptide and reproductive stages.

In conclusion, the present findings depict that gpGnIH-1 in mature fishes stimulates the expression of the genes for GnIH, GnIH-R, Kiss2, Kiss2R, GnRH-2 and GnRH-3 in the brain and releases the GTHs from the pituitary, while gpGnIH-2 only stimulates the expressions of

gpa and *fshb* in the pituitary in regressed fishes. Taken together with previous and present results suggest that GnIH might be involved in semilunar synchronized spawning in the grass puffer and may regulate gonadotropin synthesis and/or release through direct interaction with adenohypophysial hormone cells (FSH or LH or both) or indirectly with hypothalamic hypophysiotropic GnRH, Kisspeptin neurons.

Table 3.1. Total length, body weight and gonadosomatic index (GSI) of fish samples. Values are presented as mean ± SEM.

Peptide	**Time course (h)**	**Maturational stages**	**No of fish**	**Total length (cm)**	**Body weight (g)**	**GSI (%)**
gpGnIH-1	12	Mature	24	15.1 ± 0.4	57.6 ± 3.6	10.6 ± 1.1
gpGnIH-1	24	Mature	27	15.3 ± 0.3	57.9 ± 3.3	6.2 ± 0.9
gpGnIH-2	12	Regressed	13	13.3 ± 0.4	42.6 ± 3.5	1.1 ± 0.1

Table 3.2. Primers used in the real-time PCR assays in this study

Primers	**Nucleotide sequences**
GnRH1-qPCR-F1	5′-CGGGAGTCTGATGTCACAGCTC-3′
GnRH1-qPCR-R1	5′-AACACTGACGACGACCGTGTCC-3′
GnRH2-qPCR-F1	5′-CAGGAGCTCACCTGTCCAAC-3′
GnRH2-qPCR-R1	5′-CTGCATTCTCCTGCTTCACAG-3′
GnRH3-qPCR-F1	5′-AAGCAAACAGGGTGATGGTG-3′
GnRH3-qPCR-R1	5′-CTGATGGTTGCCTCCAACTC-3′
GnIH-qPCR-F1	5′-TGATTCGTCTGTGCGAGGAC-3′
GnIH-qPCR-R1	5′-TCAGCAGCTGTGCATTGACC-3′
GnIH-R-qPCR-F1	5′-AAGATGCTCATCCTGGTGGC-3′
GnIH-R-qPCR-R1	5′-AGATCCACCTGGTCACTGTCC-3′
Kiss2-qPCR-F1	5′-GACCTTCAGGGACAACGAGGAC-3′
Kiss2-qPCR-R1	5′-ATGAAGCGCTTGCCAAAGC-3′
Kiss2r-qPCR-F1	5′-TCCCGTTTCTGTTCAAGCACAAG-3′
Kiss2r-qPCR-R1	5′-ATTGTTGTTGCGCTCCTCTGC-3′
GPα-qPCR-F1	5′-AAGGTGAGGAACCACACCGAG-3′
GPα-qPCR-R1	5′-AGCTCAAGGCCAGGATGAAC-3′
FSHβ-qPCR-F1	5′-ACACATTGAGGGCTGTCCAGTGG-3′
FSHβ-qPCR-R1	5′-TCCCCATTGAAGCGACTGCAG-3′
LHβ-qPCR-F1	5′-CACTTGGTGCAAACAAGCATC-3′
LHβ-qPCR-R1	5′-CAACTTAGAGCCACGGGGTAG-3′

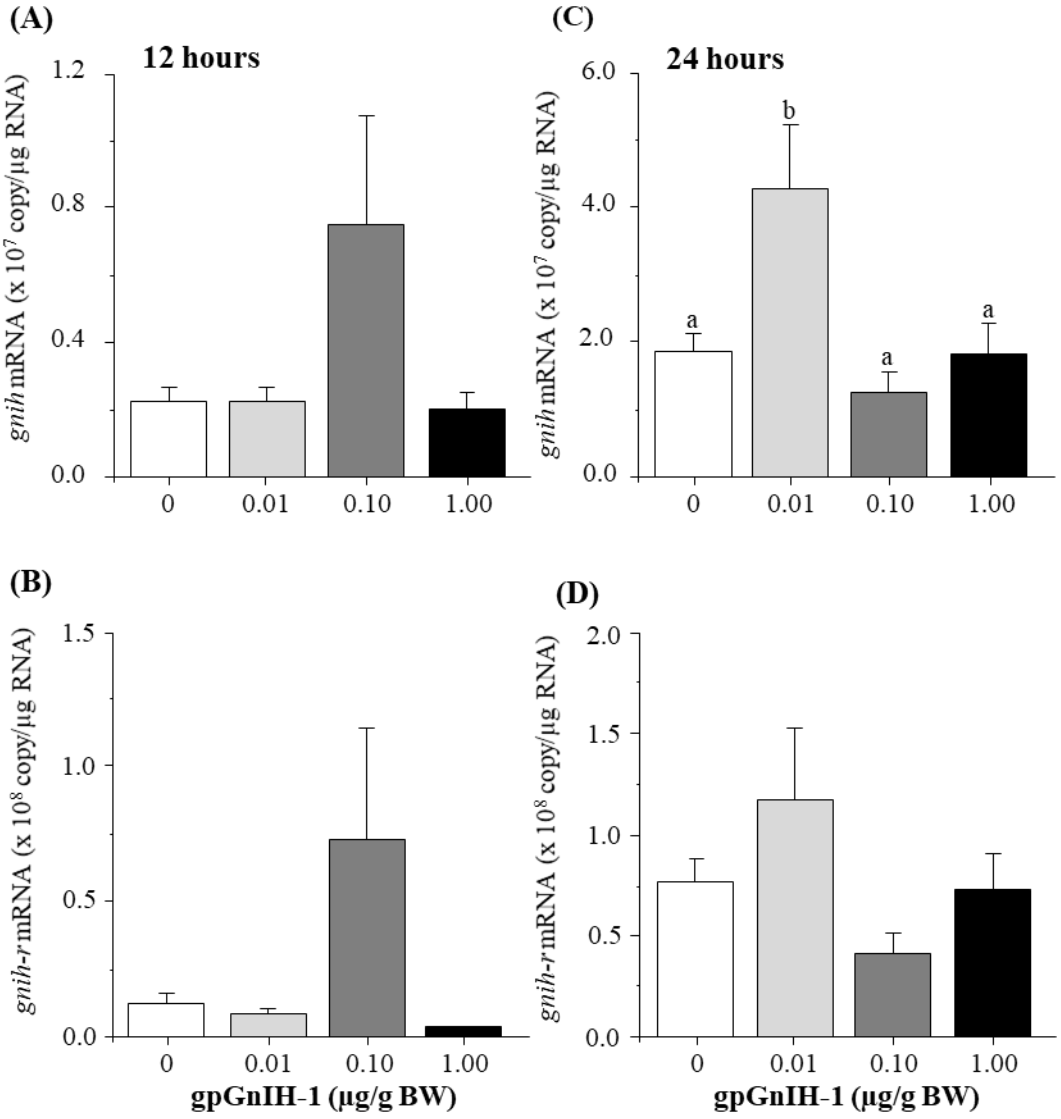

Fig. 3.1. Effect of gpGnIH-1 administration on the expressions of *gnih* and *gnih-r* after 12 hours (A and B) and 24 hours (C and D) in the brain of mature grass puffer. Values are presented as mean ± SEM (n = 5−7). Different subscript of alphabets indicates significant differences between doses ($p < 0.05$).

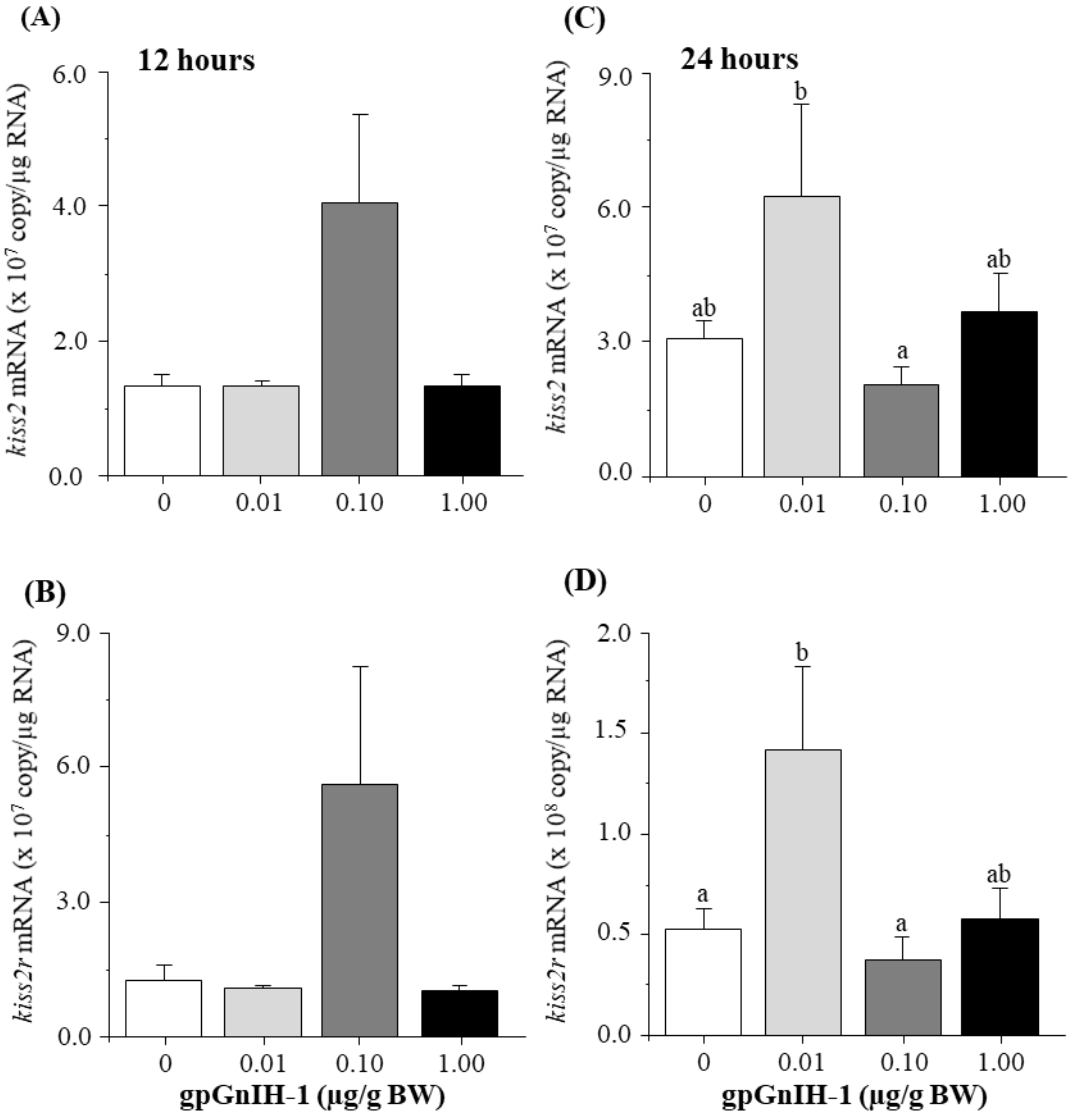

Fig. 3.2. Effect of gpGnIH-1 administration on the expressions of *kiss2* and *kiss2r* after 12 hour (A and B) and 24 hours (C and D) in the brain of mature grass puffer. Values are presented as mean ± SEM (n = 5−7). Different subscript of alphabets indicates significant differences between doses ($p < 0.05$).

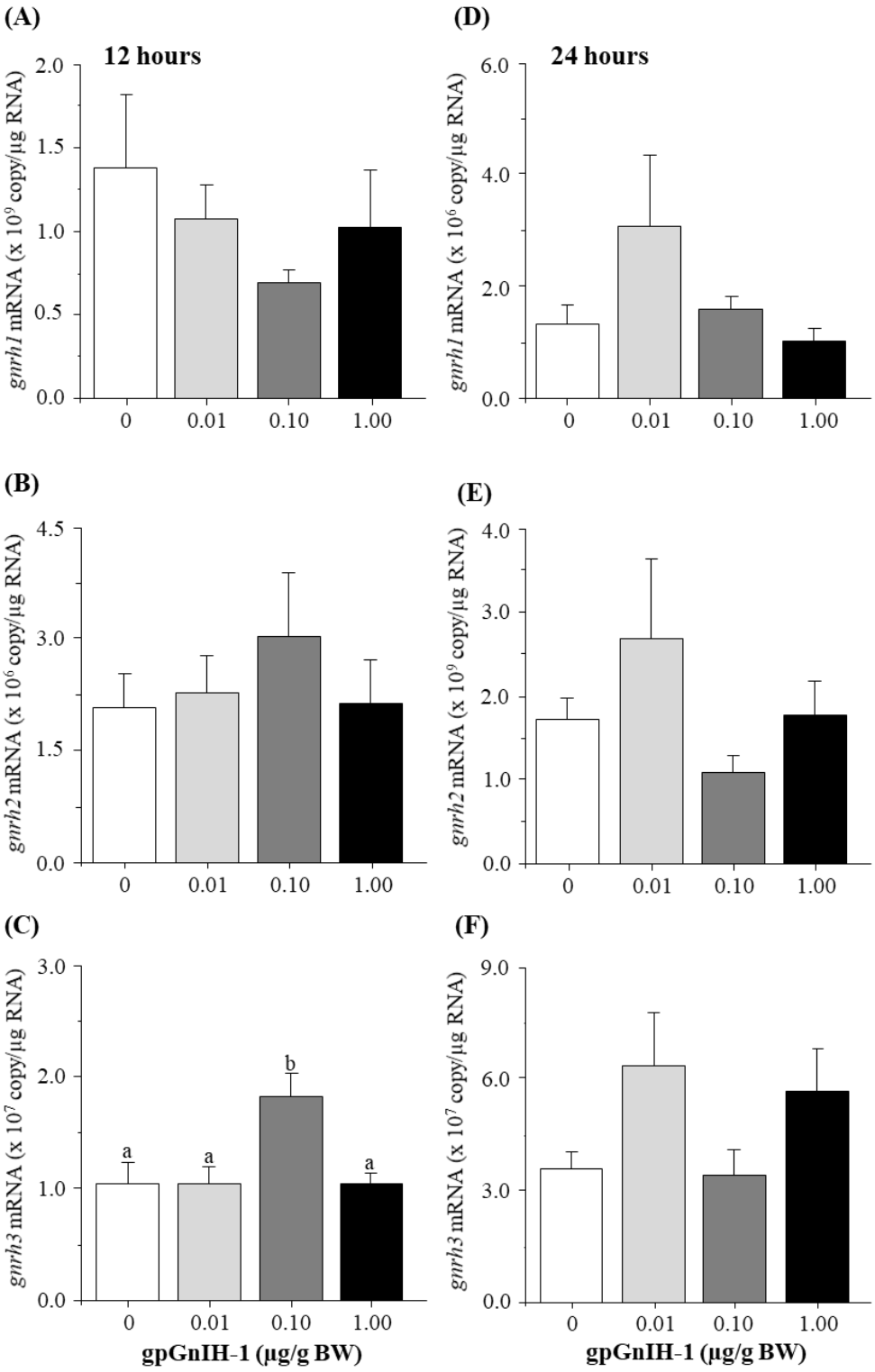

Fig. 3.3. Effect of gpGnIH-1 administration on the expressions of *gnrh1, gnrh2* and *gnrh3* after 12 hours (A−C) and 24 hours (D−F) in the brain of mature grass puffer. Values are presented as mean ± SEM (n = 5−7). Different subscript of alphabets indicates significant differences between doses ($p < 0.05$).

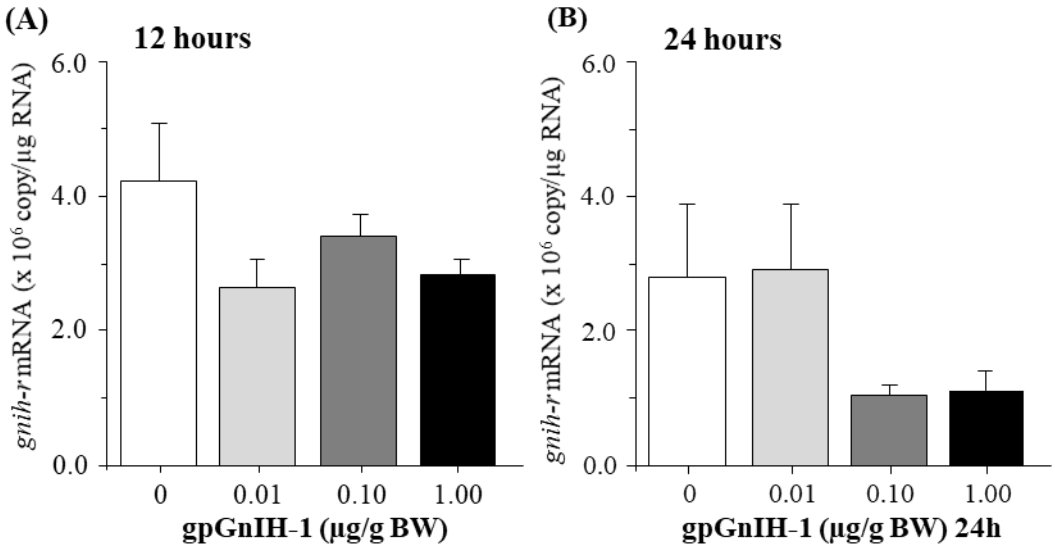

Fig. 3.4. Effect of gpGnIH-1 administration on the expressions of *gnih-r* after 12 hours (A) and 24 hours (B) in the pituitary of mature grass puffer. Values are presented as mean ± SEM (n = 5−7).

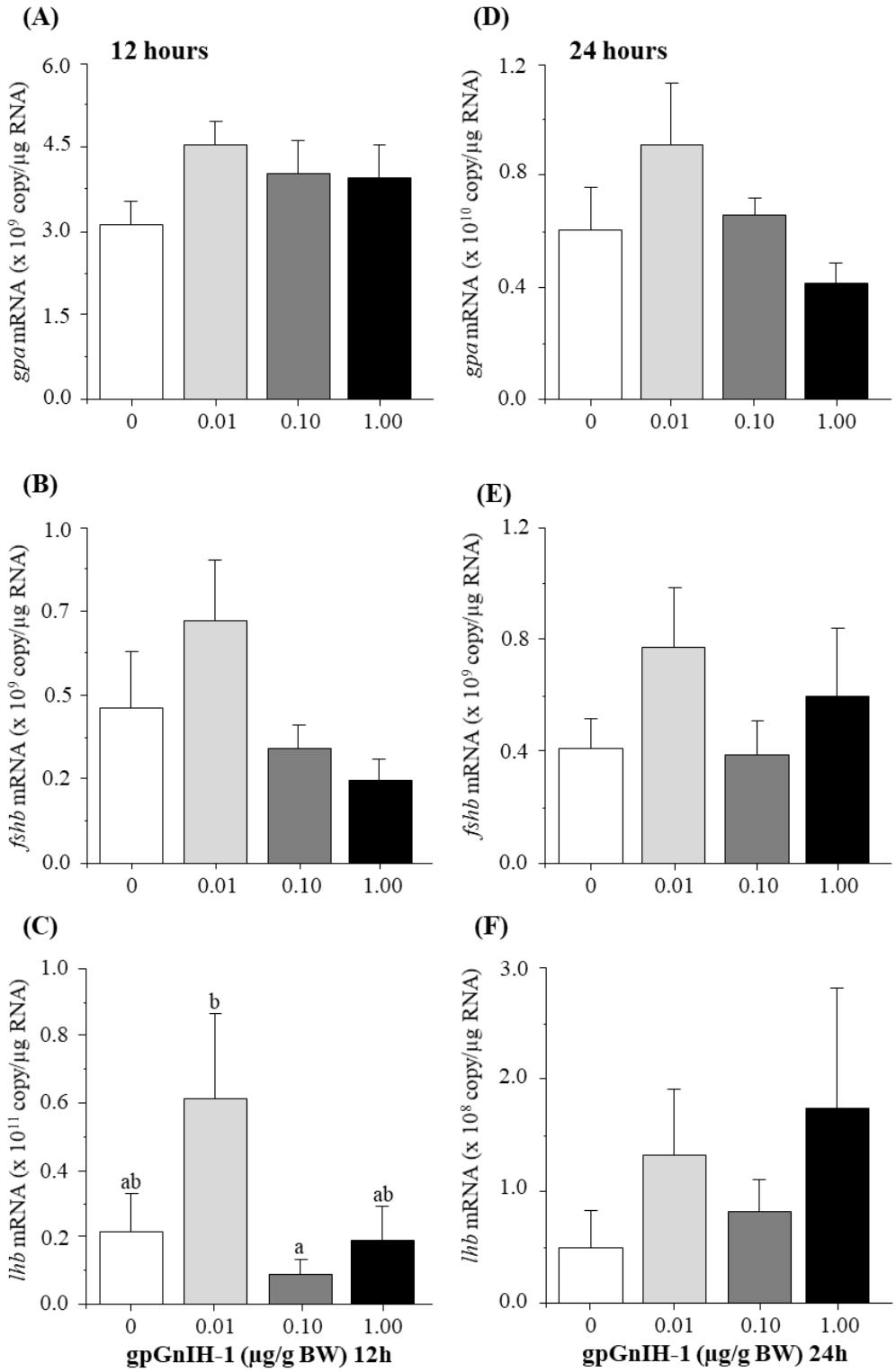

Fig. 3.5. Effect of gpGnIH-1 administration on the expressions of *gpa* (A and D), *fshb* (B and E), and *lhb* (C and F) after 12 hours (A−C) and 24 hours (D−F) in the pituitary of mature grass puffer. Values are presented as mean ± SEM (n = 5−7). Different subscript of alphabets indicates significant differences between doses ($p < 0.05$).

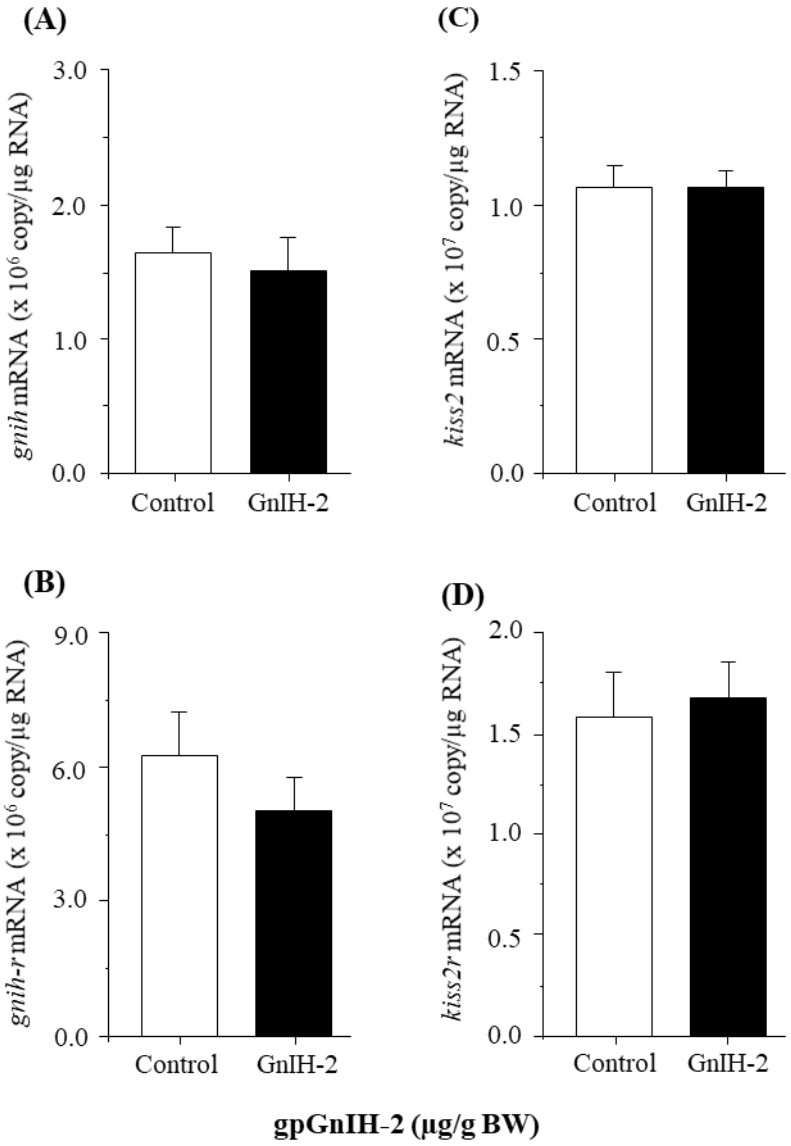

Fig. 3.6. Effect of gpGnIH-2 administration on the expressions of *gnih* (A), *gnih-r* (B), *kiss2* (C), and *kiss2r* (D) after 12 hours in the brain of grass puffer at regressed stage. Values are presented as mean ± SEM (n = 5−7).

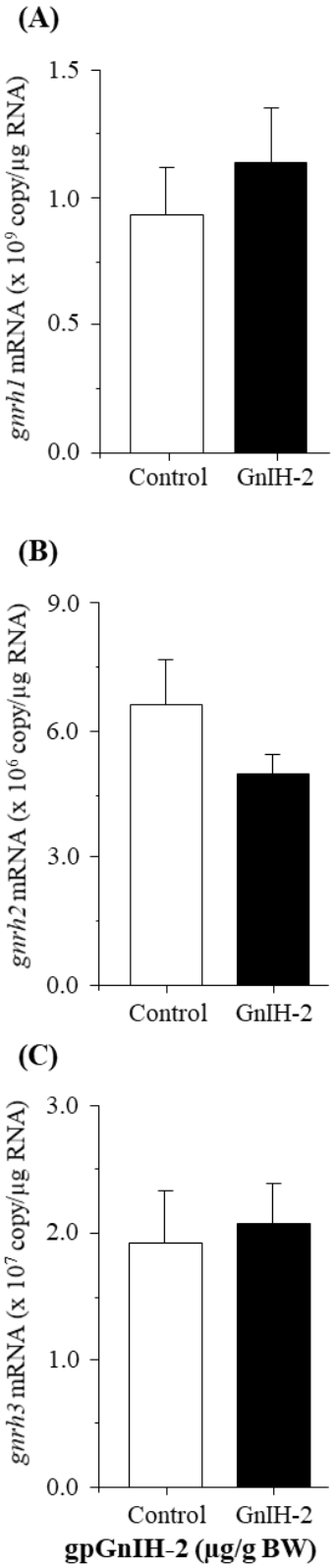

Fig. 3.7. Effect of gpGnIH-2 administration on the expressions of *gnrh1* (A), g*nrh2* (B) and *gnrh3* (C) after 12 hours in the brain of grass puffer at regressed stage. Values are presented as mean ± SEM (n = 5−7).

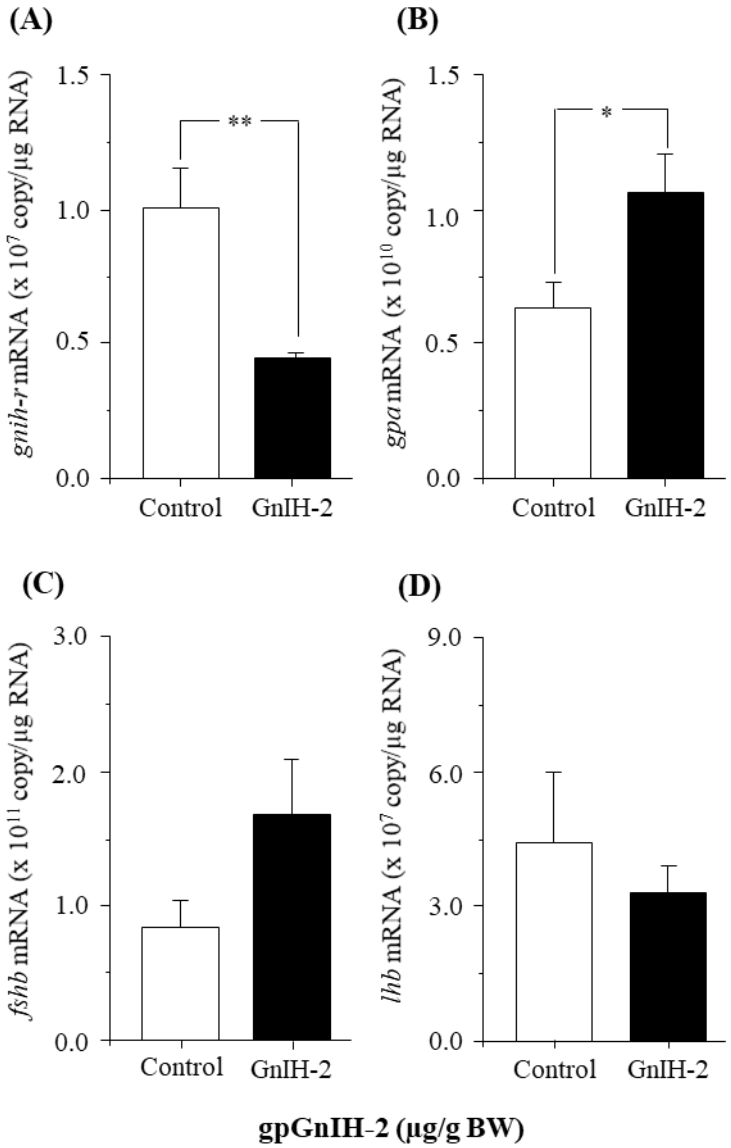

Fig. 3.8. Effect of gpGnIH-2 administration on the expressions of *gnih-r* (A), *gpa* (B), *fshb* (C), and *lhb* (D) after 12 hours in the pituitary of grass puffer at regressed stage. Values are presented as mean ± SEM (n = 5−7). Asterisks denotes a significant difference between the control and gpGnIH-2 injected fish (*, $p < 0.05$ and **, $p < 0.01$).

Chapter 4

Changes in expression of the genes for the HPG axis in the tiger puffer at different reproductive stages

4.1. Introduction

In vertebrates, reproduction is regulated by the hypothalamo-pituitary-gonadal (HPG) axis and the hypothalamus releases gonadotropin-releasing hormone (GnRH), kisspeptin and gonadotropin-inhibitory hormone (GnIH) which are involved in the control of the secretion of two pituitary gonadotropins (GTHs), namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH acts on the gonad to stimulate gonadal maturation and secretion of sex steroids. The sex steroids, in turn, feed back to the brain and the pituitary to complete the HPG axis and regulates the reproductive cycle in a periodical manner (Levavi-Sivan et al., 2010; Zohar et al., 2010). FSH and LH play an essential role in stimulating gonadal development, maturation and production of sex steroid hormones in most vertebrate species. However, the roles of GnRH, GnIH and kisspeptin in the control of reproduction vary depending on animal class, species and also reproductive status.

Among the three hypothalamic neurohormones, GnRH is the principal regulator for the secretion of GTHs and have role in the regulation of reproductive behavior in many vertebrate species (Ando and Urano, 2005; Zohar et al., 2010; Munoz-Cueto et al., 2020). To date, 15 different forms of GnRHs have been identified in vertebrates, and two or three GnRH forms (GnRH1, GnRH2 and GnRH3) are present in all vertebrate species. In teleosts that possess three GnRH forms, GnRH1 neurons located in the preoptic area (POA) has a hypophysiotropic role and GnRH1 stimulates the secretion of the pituitary GTHs. GnRH2 neurons are located in

the midbrain tegmentum and secrete cGnRH-II, which is involved in appetite-related reproductive function (Matsuda et al., 2008; Nishiguchi et al., 2012; Marvel et al., 2019). GnRH3 neurons localized in the terminal nerve ganglion-POA region, secreting sGnRH having neuromodulatory action related to sexual behavior (Okuyama et al., 2014; Li et al., 2017).

The expression patterns of three GnRH genes are differentially regulated during reproductive cycle. For example, in the brain of grass puffer (*Takifugu alboplumbeus*), the expression levels of *gnrh1* and *gnrh3* were significantly augmented during the spawning season, while *gnrh2* did not show any noticeable changes in the entire reproductive cycle (Shahjahan et al., 2010a; Ando et al., 2013). However, in red seabream, the expression levels of *gnrh1* and *gnrh2* mRNAs gradually increased during gonadal development and peaked at spawning season, while *gnrh3* remained constant (Okuzawa et al., 2003). In barfin flounder, *gnhr1* is temporally expressed during spawning season, whereas no noticeable changes in *gnrh2* and *gnrh3* were reported (Amano et al., 2004). In chum salmon, which possess two GnRH forms (midbrain GnRH2 and POA GnRH3), the amounts of both mRNAs increased during upstream migration from the coast to the natal hatchery for spawning (Onuma et al., 2010). These results indicate the dominant relevance of the preoptic GnRH to reproduction. However, the expression patterns of multiple GnRH isoforms are species-specific.

GnIH is a RFamide peptide and regulates the secretion of GTHs, growth hormone (GH) and prolactin (PRL) from the pituitary (Tsutsui et al., 2012; Ogawa and Parhar, 2014). In birds and mammals, GnIH inhibits GTH secretion directly and indirectly by antagonistic interaction with GnRH (Tsutsui, 2009; Tsutsui et al., 2012; Ubuka et al., 2016). However, in teleosts, GnIH has both stimulatory and inhibitory effects on the secretion of GTH and also GH depending on the species and gonadal stages (Moussavi et al., 2012, 2014; Biran et al., 2014; Wang et al., 2015; Di Yorio et al., 2016; Paullada-Salmeron et al., 2016). In the grass puffer, significantly higher expressions of GnIH and its receptor (GnIH-R, G-protein-coupled receptor (GPR) 147) genes

were observed during spawning season compared to the early gametogenesis, pre-spawning and post-spawning stages (Shahjahan et al., 2011; Ando et al., 2013). In addition, GnIH administration experiments using goldfish GnIH peptide stimulates the expression of *fshb*, *lhb*, *gh* and *prl* in the mature fish (Shahjahan et al., 2011, 2016; Ando et al., 2018).

Kisspeptin is also a potential key player in the neuroendocrine regulation of reproduction. In mammals, kisspeptin regulates the secretion of GnRH (Oakley et al., 2009). In teleosts, however, the role of kisspeptin in the control of reproduction is controversial, some studies find stimulatory while other author reported inhibitory or no effect depending on species (Kanda et al., 2013; Nakajo et al., 2017; Ando et al., 2018; Ohga et al., 2018). Two kisspeptin genes (*kiss1* and *kiss2*) have been identified from several fish species (Kitahashi et al., 2009; Zmora et al. 2012; Saha et al. 2016) and two kisspeptin receptor (Kissr, GPR54) genes (*kiss1r* and *kiss2r*) with different brain localization suggest multiple functional roles of kisspeptin in teleosts (Ogawa and Parhar, 2018). In the grass puffer, which has only a single pair of *kiss2* and *kiss2r*, the expression levels of both genes were significantly increased in the pre-spawning, spawning and post-spawning season compared to the early gametogenesis stages (Shahjahan et al., 2010b). In the female chub mackerel, both *kiss1* and *kiss2* expression were increased during the final oocyte maturation and ovulation stages in addition to a temporal increase at the onset of puberty (Selvaraj et al., 2012; Ohga et al., 2018). In the male European sea bass, the expression levels of *kiss1*, *kiss2* and *kissr* were significantly higher in mid and late recrudescence stages before spawning when compared to fully spermiating and post-spawning stages (Migaud et al., 2012). Moreover, it has been reported that substantial increase in *kissr* expression occur before onset of puberty or during early puberty (Mohammed et al., 2007; Nocillado et al., 2007; Filby et al., 2008; Mechaly et al., 2010). Therefore, kisspeptin system is considered to be activated at the onset of puberty and during the final sexual maturation in teleosts.

GnIH serves as a key component in the HPG axis driving photoperiodic control of

reproduction through its interaction with melatonin (Ubuka et al., 2005; Revel et al., 2008). Daily changes in the expression levels of *gnih* and *gnih-r* was reported in the grass puffer (Shahjahan et al., 2011) and photic condition-dependent *gnih* expression has been reported in zebrafish (Yumnamcha et al., 2017) and European sea bass (Paullada-Salmeron et al., 2017). It is therefore most probable that GnIH/GnIH-R system is involved in the photic control of reproduction, although the temporal expression patterns in association with seasonal reproduction remain obscure in teleosts.

Tiger puffer (*Takifugu rubripes*) is closely related to the grass puffer (*Takifugu alboplumbeus*) and is one of the most commercially important fish species in Japan, distributed in the north-western side of the Pacific Ocean (Yamanoue et al., 2009). The wild populations of tiger puffer are gradually decreasing since 90's in Japan, and artificial propagation is conducted widely both for aquaculture and stock enhancement programs (Katamachi and Ishida, 2013). Although artificial induction of sexual maturation and ovulation by hormonal treatment has been successively applied to the cultured tiger puffer, there is a paucity of information concerning the reproductive neuroendocrine system in wild populations because they show a long-distance migration for spawning (Sato et al., 1999; Matsumura, 2006). In this study, to clarify the role of reproductive neuroendocrine system, which is composed of GnRH, GnIH, kisspeptin and the pituitary GTH in *Takifugu* species, the expression profile of these genes were examined in immature, mature and post-ovulatory fish of the wild tiger puffer populations.

4.2. Materials and methods

4.2.1. Fish and sample collection

Wild tiger puffer of both sexes was obtained from the spawning ground of Nanao Bay, Ishikawa, Japan during the breeding season from May to July in 2017–2019. Fish were caught

using settled net and/or hook and line around the north middle of Noto island. Collected fish (immature and mature) were then transferred to the Marine Biological Station, Sado Island Center for Ecological Sustainability, Niigata University, Japan. The fish were kept in indoor tanks (200L) with running seawater in natural photoperiods (LD 14:10) for three days before sampling. The temperature was 20°C which was similar to that in the spawning area.

For post-ovulatory fish samples, GnRH analogue (GnRHa) treatment was applied to induce ovulation (Chuda et al., 1997). Mature females were transferred to the Noto Center for Fisheries Science and Technology, Kanazawa University, and kept in indoor tanks (3000L) with running seawater in natural photoperiods for 7 days. A GnRH agonist, [D-Ala^6, Pro^9-N ethylamide]-luteinizing hormone-releasing hormone (AgGnRHa), was synthesized by a custom peptide synthesis service (Anygen, Jeollanam-do, Korea). Single implantation of AgGnRHa (400 μg/kg BW) was applied for 5 days to induce ovulation. After ovulation, the fish were used for sampling.

The fish were anesthetized in 0.01%–0.03% tricaine methane sulfonate (MS222, Sigma-Aldrich, Tokyo, Japan) or by carbon dioxide tablet (Patent No. 6202570) before sampling, and standard length (SL) and body weight (BW) were recorded. Brains and pituitaries were removed after decapitation and soaked in RNAlater (Ambion, Austin, TX) and kept at 4°C for overnight. Gonads were removed and weighted for the calculation of gonadosomatic index (GSI, gonad weight/body weight × 100). In the next day, brains were trimmed to prepare the olfactory bulb (OB), telencephalon (T) and diencephalon/midbrain tegmentum (D/M) samples and they were stored at -80°C until RNA extraction. All the sampling procedure were carried out following the approved guidance by the Institutional Animal Care and Use Committee of the Niigata University, Niigata, Japan. SL, BW, and GSI of the fish used in this study are shown in Table 4.1.

4.2.2. Real-time PCR assay

Total RNA was extracted from the brain and pituitary samples using guanidium thiocyanate-phenol-chloroform method (Chirgwin et. al., 1979). To avoid DNA contamination, the RNA samples were treated with DNase I (TaKaRa Bio, Japan). Total RNA was quantified by NanoDrop One Spectrophotometer (ThermoFisher Scientific, Japan) and verified the quality and integrity of total RNA by gel electrophoresis. Total RNAs (200−500ng) were used for synthesis of first strand of cDNAs using MultiScribe Reverse Transcriptase (Applied Biosystem, USA) and an oligo $d(T)_{12-18}$ primer as per manufacturer's instructions. The profile for reverse transcription reaction was 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Real-time PCR was carried out with a Thermal Cycler Dice Real Time System III (TP 970, TaKaRa Bio, Japan). To determine the absolute amount of mRNA, sense RNA was synthesized in vitro by a MAXIscript kit (Ambion) according to the manufacturer's instruction, and were serially diluted to $1 \times 10^3 - 1 \times 10^8$ copies/µl. The standard sense RNAs were reverse transcribed and used as standard cDNAs to establish a standard curve. PCR reaction mixture (10 µl) contained 1 µl of standard sample cDNA, 0.4 µl each of forward and reverse primers (Table 4.2) and 5 µl of TB Green Premix DimerEraser (TaKaRa, Ohtsu, Japan). Amplification was carried out at 95°C for 30 sec, followed by 40 cycle at 95°C for 5 sec, 60°C for 30 sec and 72°C for 30 sec. Specific amplification of each cDNA was verified by melting curve analysis and gel electrophoresis of the product.

4.2.3. Statistical analysis

The mRNA values are expressed as mean ± standard error of the mean (SEM). Student t-test were performed to compare significant difference between immature and mature male groups. To assess the statistically significant difference among different groups of females, data were analyzed by ANOVA followed by Tukey's HSD post hoc test. Statistical significance was

set at $p < 0.05$ unless described anywhere in the text. All statistical analyses were performed using SPSS Version 23.0 for windows (SPSS Inc., Chicago, IL).

4.3. Results

4.3.1. Changes in the expression level of kiss2 and kiss2r at different reproductive stages in the brain

Tiger puffer of both sexes at different reproductive stages (immature, mature and post-ovulatory; Table 4.1) were obtained from the Nanao Bay during the spawning season. The mature females had pre-ovulatory mature oocytes. The post-ovulatory females were obtained by artificial ovulation because of the limited number of ovulated females available in the wild. The absolute amounts of mRNA for the brain neuropeptides and their receptors were examined in the OB, T and D/M. In our preliminary experiment, in which the mRNA levels of all genes were determined in the three regions, the mRNA levels of the genes for GnIH and kisspeptin, were higher in the T and D/M compared to the OB (data not shown). In the case of *gnrh1* and *gnrh3* were comparable among the three regions, while *gnrh2* mRNA was exclusively high in the D/M. Therefore, *kiss2*, *kiss2r*, *gnih,* and *gnih-r* mRNAs were quantified in the T and D/M samples, *gnrh1* and *gnrh3* mRNAs were quantified in the OB, T and D/M samples, while *gnrh2* mRNA was measured in the D/M sample.

Expression profile of *kiss2* and *kiss2r* were characterized in the T and D/M samples, in which similar expression levels were obtained for both genes (Figs. 4.1 and 4.2). In the males, there was no noticeable difference between the immature and mature fish for both genes except significant decrease in *kiss2r* in mature fishes in T (Figs. 4.1A and 4.1B). In the females, the expression levels of both genes were similar between the immature and mature fish, whereas the levels were significantly lower in the post-ovulatory females for both genes (Figs. 4.2A and

4.2B).

4.3.2. Changes in the expression level of gnih and gnih-r at different reproductive stages in the brain

Expression profile of *gnih* and *gnih-r* were characterized in the T and D/M samples, in which similar expression levels were obtained for both genes (Figs. 4.3 and 4.4). In the males, no significant difference was observed between the immature and mature fish for both genes (Figs. 4.3A and 4.3B). In contrast, in the females, the expression levels of both genes in the T were significantly higher in the mature fish compared to the immature fish, and the levels were decreased in the post-ovulatory fish (Figs. 4.4A and 4.4B). In the D/M, however, both genes did not show significant changes except for lower levels of *gnih* mRNA in the post-ovulatory females.

4.3.3. Changes in the expression level of three gnrhs at different reproductive stages in the brain

The amount of *gnrh1* mRNA was relatively higher in the OB than T and D/M in both sexes, especially in the mature fish (Figs. 4.5A and 4.6A). Expression of *gnrh3* showed similar levels in these three regions in mature fish (Figs. 4.5C and 4.6C). As a whole, the amounts of *gnrh3* mRNA were much higher than those of *gnrh1* and *gnrh2* in both sexes (Figs. 4.5 and 4.6).

In the males, significantly higher expression levels were found in the mature fish compared to the immature fish in the OB for *gnrh1* and *gnrh3*, in the T for *gnrh3* and in the D/M for *gnrh2* (Figs. 4.5A, 4.5B and 4.5C). In contrast, *gnrh1* in the T were significantly lower in the mature fish compared to the immature fish (Fig. 4.5A). No significant difference was observed in the D/M for *gnrh1* and *gnrh3* between the immature and mature fish (Figs. 4.5A and 4.5C). In the females, the expression levels of *gnrh1* and *gnrh3* were significantly higher in the mature fish

compared to the immature fish in all brain regions, and these values were decreased in the post-ovulatory females to similar levels of the immature fish except for *gnrh3* in the OB (Figs. 4.6A and 4.6C). In contrast, the expression levels of *gnrh2* were significantly higher in the post-ovulatory females compared to the immature and mature females (Fig. 4.6B).

4.3.4. Changes in the expression level of gpa, fshb, and lhb at different reproductive stages in the pituitary

Expression levels of the three GTH subunit genes (*gpα*, *fshb* and *lhb*) were quantified in the pituitary. In the males, the amounts of all three mRNAs were significantly increased in the mature fish compared to the immature fish (Figs. 4.7A−C). Especially, *fshb* and *lhb* expressions were substantially activated in the mature males. Likewise, in the females, *fshb* and *lhb* showed significant increases in the mature fish, although no change was observed for *gpa*. In the post-ovulatory females, the expression levels of all three genes were extremely lower compared to the mature fish.

4.4. Discussion

In the present study, the expression patterns of the HPG axis genes were examined at different reproductive stages in the tiger puffer captured in the wild. The genes encoding GnIH, GnIH-R, GnRHs, and GTH subunits showed extensive elevation in expression in the mature fish compared to the immature fish, especially in the females. Moreover, these augmented expressions were drastically decreased in the post-ovulatory females, in which the ovulation was artificially induced. In contrast, the expression of the genes for kisspeptin and kisspeptin receptor did not show significant changes in the males but significantly decreased in the post-ovulatory females. The present results demonstrate the expression dynamics of the HPG axis

genes associated with the reproductive conditions and the possible involvement of GnRH/GnIH/GTH system in the regulation of sexual maturation and ovulation in the wild tiger puffer.

In this study, we did not find any noticeable changes in the amount of *kiss2* and *kiss2r* mRNAs between the immature and mature fishes in the males, but their levels were significantly lower in the post-ovulatory females compared to the immature and mature fishes (Figs. 4.1 and 4.2). In the grass puffer, the expression levels of *kiss2* and *kiss2r* were higher in the mature fish during the pre-spawning and spawning season compared to the preparative fish in both sexes, though the fold-change differences were low (< 2) (Shahjahan et al., 2010b). Considering that the prepubertal activation of kisspeptin and kisspeptin receptor gene expression in many fishes (Mohammed et al., 2007; Nocillado et al., 2007; Filby et al., 2008; Mechaly et al., 2010; Miguad et al., 2012; Selvaraj et al., 2012; Ohga et al., 2018) and their dominant expressions in the hypothalamus during the gonadal maturation in the grass puffer (Ando et al., 2013), *kiss2* and *kiss2r* expressions are likely to be activated at puberty and keep augmented during gonadal maturation followed by decrease at post-breeding stage.

The regulatory role of GnIH on GTH release has been established in many fishes and both stimulatory and inhibitory actions were reported depending on species and gonadal condition. In this study, we found significant elevation of *gnih* and *gnih-r* mRNAs in the T of the mature females and extremely low levels in the post-ovulatory females, suggesting that GnIH may have the stimulatory role in the regulation of the HPG axis (Fig. 4.4). In contrast, no such changes were observed in the males. In the grass puffer, *gnih* and *gnih-r* expressions were highly activated in the mature fish of both sexes; however, these mRNA levels in the spawning fish of both sexes were as low as preparative fish (Shahjahan et al., 2011). The spawning fish in the previous study were caught at the spawning ground when they were performing group spawning on seashore (Motohashi et al., 2010; Ando et al., 2013) which suggest that GnIH has an

important role in the final stage of sexual maturation prior to spawning and its expression rapidly ceases once spawning commences. The mature males in the present study were highly active in spermiogenesis and were considered to experience spawning in the wild before being caught, while the mature females were pre-ovulatory fish that were ready for spawning. Therefore, the present results support the notion of the possible role of GnIH in the final stage of sexual maturation. In the grass puffer, the administration of goldfish GnIH (gfLPXRFa-1) stimulates the expression of *fshb* and *lhb* in vivo and in vitro (Shahjahan et al., 2011, 2016; Ando et al., 2018). Taken together, it is most probable that GnIH acts as a key player in regulation of gonadal maturation through direct stimulation of FSH and LH release in Tetraodontidae fishes.

In teleosts that have three GnRH forms, functional regulation of these three forms is different and their roles in regulating the HPG axis are diverse. Tetraodontidae family possesses three forms of GnRHs and their expression patterns were found quite different during sexual maturation (Shahjahan et al., 2010a). In the present study, the levels of *gnrh1* mRNA were extensively higher in the mature fish compared to the immature fish in the OB of the males and in all brain regions of the females (Figs. 4.5A and 4.6A). In particular, in the females the levels in the mature fish were observed to be approximately 300- and 20-fold greater compared to the immature fish in the OB and T/DM regions, respectively (Fig. 4.6A). Furthermore, the levels in the post-ovulatory females were as low as the immature fish. Similar drastic changes in *gnrh1* expression was reported in the grass puffer (Shahjahan et al., 2010a). GnRH1 neurons are localized in the ventral forebrain region including the OB/terminal nerve (TN), ventral telencephalon and POA and directly project their axons to the pituitary to regulate GTH secretion in many fishes (Kah et al., 2007; Munoz-Cueto, et al. 2020). In the present study, the significantly higher levels of *fshb* and *lhb* mRNAs were observed in the pituitary of the mature fish in both sexes, and the levels of *gnrh1* mRNA in the post-ovulatory females also coincides

with the mRNA levels of *fshb* and *lhb* of the same group in the pituitary (Figs. 4.7B and 4.7C). Therefore, the present results suggest the potentially important role of GnRH1 to stimulate GTH secretion and sexual maturation in the tiger puffer. However, in the males, there was lower levels of *gnrh1* mRNA in the T of the mature fish and similar levels in the D/M between the immature and mature fish (Fig. 4.5A). The lower expression levels in the T might be due to negative feedback response by gonadal sex steroids (Ando et al., 2004). However, the *gnrh1* mRNA levels in the OB were higher in the mature males, suggesting that the response to sex steroids might be different in brain regions due to localization of steroid receptors. This needs to be examined in further study.

In this study, we found increased mRNA levels of *gnrh2* in the mature males and the post-spawning females (Figs. 4.5B and 4.6B). Similar increased expression of *gnrh2* was detected in the fully matured males and post-spawning females in the grass puffer, although the fold-change differences were smaller than the present results (Shahajahan et al., 2010a). GnRH2 neurons in the midbrain tegmentum project throughout the brain and GnRH2 has been shown to have neuromodulatory roles related to anorexigenic action and also reproduction (Matsuda et al., 2008; Nishiguchi et al., 2012; Marvel et al., 2019). In addition, GnRH2 has a hypophysiotropic role in some fishes, in which GnRH2 has been detected in the pituitary. In the striped seabass, positive correlation was observed in the pituitary GnRH2 and gonadal development (Holland et al., 2001), and the administration with GnRH2 induced LH release in goldfish (Chang et al., 1990). The knockout of *gnrh2* in zebrafish decreased *lhb* expression and the treatment with GnRH2 increased the *lhb* expression (Marvel et al., 2019), consistently with the projection of GnRH2 fibers to the pituitary (Xia et al., 2014). The physiological significance of the increased *ghrh2* expression in the mature tiger puffer is unknown at present. Considering the low GTH subunit gene expression in the pituitary of the post-ovulatory females, this might not be related to a hypophysiotropic role but related to some behavioral function, e.g. fasting

during spawning (Elskus et al., 2005), though further studies will be needed to examine the role of GnRH2 in sexual maturation and spawning in the tiger puffer.

The amount of *gnrh3* mRNA was substantially increased in the mature fish of both sexes in all brain regions (Figs. 4.5C and 4.6C) and the relative mRNA levels in the mature fish were also high compared to *gnrh1* and *gnrh2* (Figs. 4.5 and 4.6). Although further study will be needed, the present results suggest that GnRH3 could be important not only for its possible involvement in sexual maturation, but also its multiple roles in reproductive and non-reproductive functions, which may or may not be directly associated with sexual maturation. Increased expression of *gnrh3* has been reported in adult fish in salmonids (Ando et al., 2001; Onuma et al., 2005, 2010) and blue gourami (Levy at al., 2009). GnRH3 neurons in fish species with three GnRH isoforms are localized in the OB/TN, ventral telencephalon and POA and they project throughout the brain, having neuromodulatory actions related to reproductive behavior (Munoz-Cueto et al., 2020). The significant increase in *gnrh3* expression in the present study may correspond to homing migration and spawning behavior of the tiger puffer. In particular, GnRH3 neurons in the OB, where the post-ovulatory females also showed high expression levels, might be involved in integration of olfactory information which is important in homing migration (Ueda, 2011; Ueda et al., 2016). Tiger puffer exhibits homing migration to specific spawning grounds including Nanao Bay in the present study (Sato et al., 1999; Matsumura, 2006). In Pacific salmon, which home to natal river after long-distance migration, *gnrh3* expression was highly activated in the OB during homing migration (Onuma et al., 2005; 2010). Moreover, GnRH has been shown to have a positive role in the salmon migration: the administration with GnRH analog shortened the duration of homing migration in sockeye salmon (Sato et al., 1997; Kitahashi et al., 1998). It is therefore possible that GnRH3 may have a positive role in olfactory memory retrieval during homing migration in the tiger puffer. The physiological significance of the activation of *gnrh3* expression in the OB, T and D/M need to

be further investigated.

To delineate the possible roles of reproductive neuropeptides and pituitary hormones in the regulation of sexual maturation in the tiger puffer, the relative expression levels of these hormone and receptor genes are summarized in Fig. 4.8. In the brain, the expression of these genes was extensively activated in the mature females that were at the pre-ovulatory stage before spawning in comparison to the immature females and the levels drastically decreased after ovulation (Fig. 4.8A). On the other hand, in the males, three GnRH genes were significantly activated in the mature fish that were highly active in spermatogenesis and spermiation, though the expression levels of the genes for Kiss2/Kiss2r and GnIH/GnIH-R showed few changes between the immature and mature fish. Considering the prepubertal activation of *kiss2* and *kissr* and temporal activation of *gnih* and *gnihr* during the pre-spawning stage, these neuropeptide/receptor genes are differentially regulated during sexual maturation and they all are likely to be associated with reproduction. In particular, GnRH/GnIH are most probably important in the regulation of the final stage of gonadal maturation and spawning in both sexes. In the pituitary, *lhb* expression was substantially activated in the mature fish compared to the immature fish, supporting the important role of LH in the final stage of gonadal maturation and spawning in both sexes (Fig. 4.8B). Importantly, these expression patters are quite similar to that in the grass puffer (Ando et al., 2013; Shahjahan et al., 2016) which suggest that the reproductive neuroendocrine system is similar between tiger and grass puffers.

In conclusion, the present results showed differential expression patterns of the HPG axis genes at different reproductive stages in the tiger puffer. The mRNA levels of the genes encoding GnIH, GnIH-R, three GnRHs, and GTH subunits showed significant elevation in the mature fish, especially in the females, indicating their active involvement in the sexual maturation and spawning. On the other hand, the expression of the genes for Kiss2 and Kiss2r did not show significant changes in the males but significantly decreased in the post-ovulatory

females. The present results suggest the possible involvement of the GnIH/GnRH/GTH system in the regulation of sexual maturation and spawning in the wild tiger puffer. Furthermore, the reproductive neuroendocrine system may be similar between tiger and grass puffers.

Table 4.1. Standard length, body weight and gonadosomatic index (GSI) of fish samples. Values are presented as mean ± SEM.

Year	Sex	Maturational stages	No. of fish	Standard length (cm)	Body weight (kg)	GSI (%)
2017	Male	Immature	9	23.3 ± 1.2	0.35 ± 0.04	0.14 ± 0.02
	Female	Immature	7	21.1 ± 0.8	0.29 ± 0.03	0.24 ± 0.02
2018	Male	Mature	9	36.5 ± 1.0	1.68 ± 0.13	11.70 ± 1.96
	Female	Immature	1	33.8	1.55	0.58
		Mature	1	47.5	3.90	16.9
2019	Female	Mature	6	43.5 ± 1.0	2.94 ± 0.38	20.07 ± 2.07
		Post-ovulatory	8	43.4 ± 0.7	2.00 ± 0.09	2.40 ± 0.84

Table 4.2. Primers used in the real-time PCR assays in this study

Primers	**Nucleotide sequences**
Kiss2-qPCR-F1	5′-GACCTTCAGGGACAACGAGGAC-3′
Kiss2-qPCR-R1	5′-ATGAAGCGCTTGCCAAAGC-3′
Kiss2r-qPCR-F1	5′-TCCCGTTTCTGTTCAAGCACAAG-3′
Kiss2r-qPCR-R1	5′-ATTGTTGTTGCGCTCCTCTGC-3′
GnIH-qPCR-F1	5′-TGATTCGTCTGTGCGAGGAC-3′
GnIH-qPCR-R1	5′-TCAGCAGCTGTGCATTGACC-3′
GnIH-R-qPCR-F1	5′-AAGATGCTCATCCTGGTGGC-3′
GnIH-R-qPCR-R1	5′-AGATCCACCTGGTCACTGTCC-3′
GnRH1-qPCR-F1	5′-CGGGAGTCTGATGTCACAGCTC-3′
GnRH1-qPCR-R1	5′-AACACTGACGACGACCGTGTCC-3′
GnRH2-qPCR-F1	5′-CAGGAGCTCACCTGTCCAAC-3′
GnRH2-qPCR-R1	5′-CTGCATTCTCCTGCTTCACAG-3′
GnRH3-qPCR-F1	5′-AAGCAAACAGGGTGATGGTG-3′
GnRH3-qPCR-R1	5′-CTGATGGTTGCCTCCAACTC-3′
GPα-qPCR-F1	5′-AAGGTGAGGAACCACACCGAG-3′
GPα-qPCR-R1	5′-AGCTCAAGGCCAGGATGAAC-3′
FSHβ-qPCR-F1	5′-ACACATTGAGGGCTGTCCAGTGG-3′
FSHβ-qPCR-R1	5′-TCCCCATTGAAGCGACTGCAG-3′
LHβ-qPCR-F1	5′-CACTTGGTGCAAACAAGCATC-3′
LHβ-qPCR-R1	5′-CAACTTAGAGCCACGGGGTAG-3′

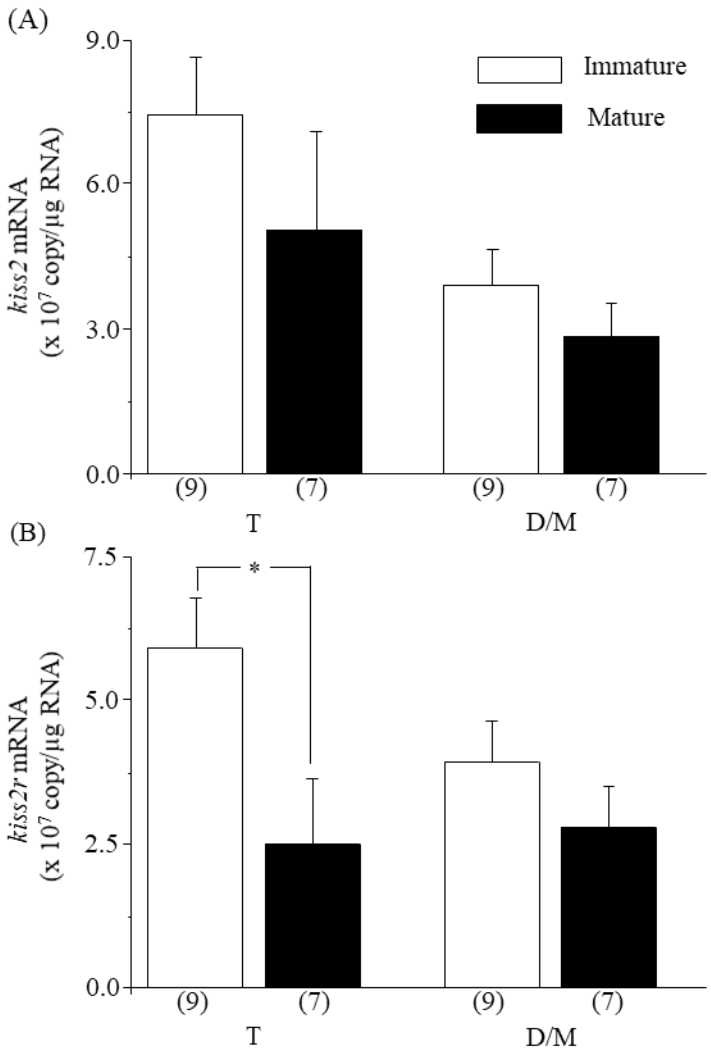

Fig. 4.1. Changes in the amounts of *kiss2* (A) and *kiss2r* (B) mRNAs in different brain regions of tiger puffer at different reproductive stages in the males. Values are presented as mean ± SEM. Asterisks denotes a significant difference between the immature and mature fish (*, $p < 0.05$). T, telencephalon; D/M, diencephalon/midbrain. Number in the parentheses represents the number of fish analyzed in each group.

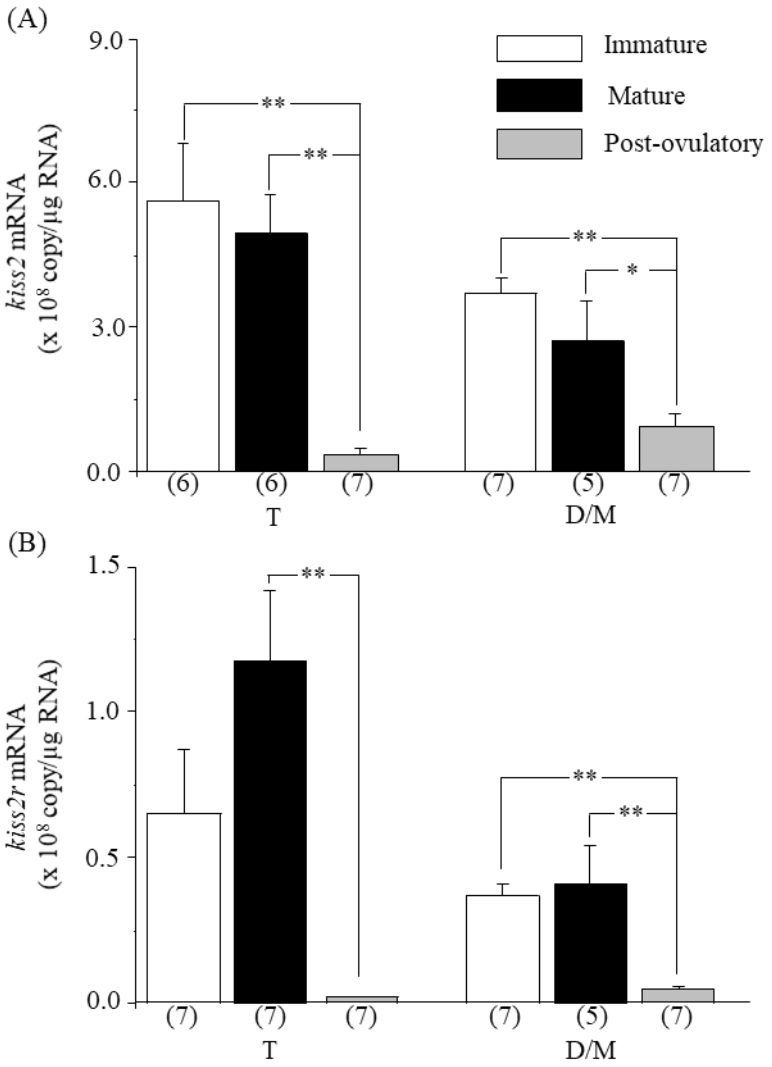

Fig. 4.2. Changes in the amounts of *kiss2* (A) and *kiss2r* (B) mRNAs in different brain regions of tiger puffer at different reproductive stages in the females. Values are presented as mean ± SEM. Asterisks denotes a significant difference between the immature, mature and post-ovulatory fish (*, $p < 0.05$ and **, $p < 0.01$). T, telencephalon; D/M, diencephalon/midbrain. Number in the parentheses represents the number of fish analyzed in each group.

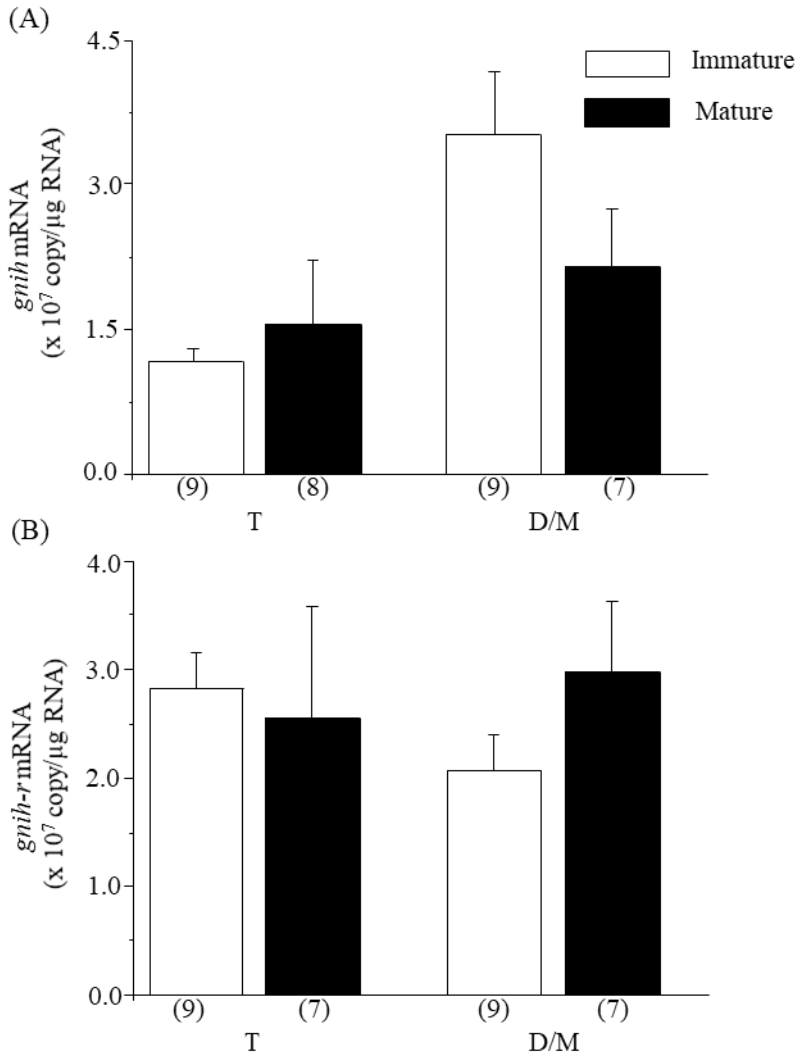

Fig. 4.3. Changes in the amounts of *gnih* (A) and *gnih-r* (B) mRNAs in different brain regions of tiger puffer at different reproductive stages in the males. Values are presented as mean ± SEM. T, telencephalon; D/M, diencephalon/midbrain. Number in the parentheses represents the number of fish analyzed in each group.

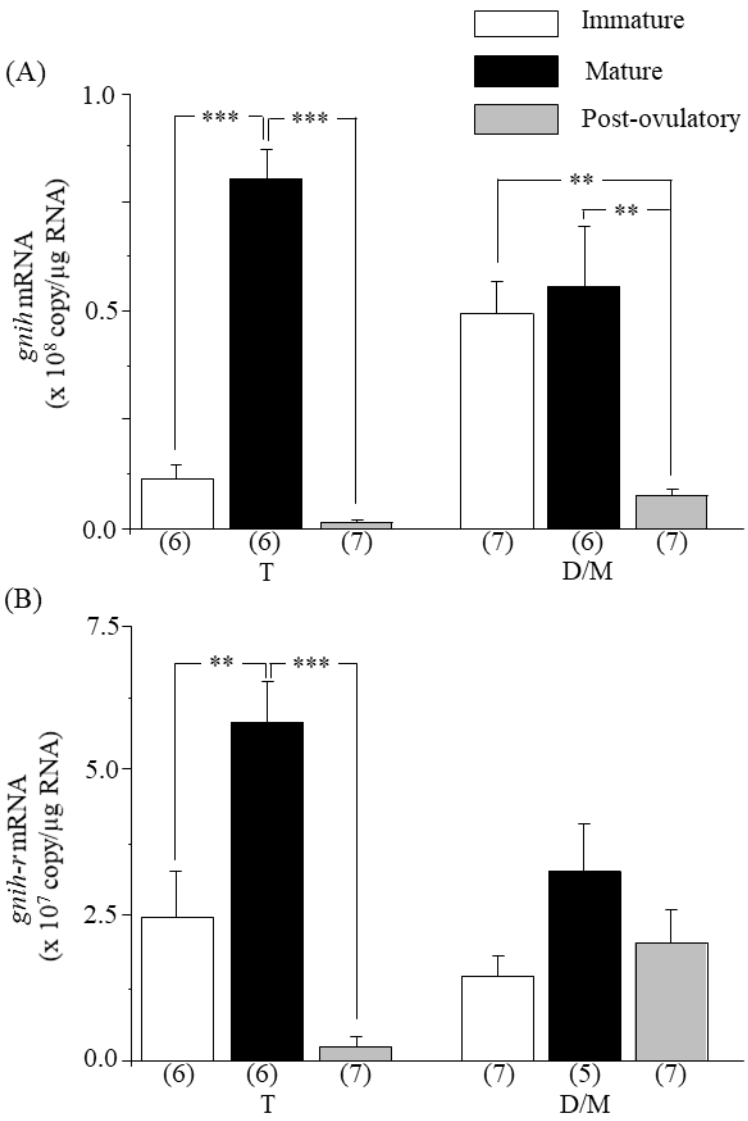

Fig. 4.4. Changes in the amounts of *gnih* (A) and *gnih-r* (B) mRNAs in different brain regions of tiger puffer at different reproductive stages in the females. Values are presented as mean ± SEM. Asterisks denotes a significant difference between the immature, mature and post-ovulatory fish (**, $p < 0.01$ and ***, $p < 0.001$). T, telencephalon; D/M, diencephalon/midbrain. Number in the parentheses represents the number of fish analyzed in each group.

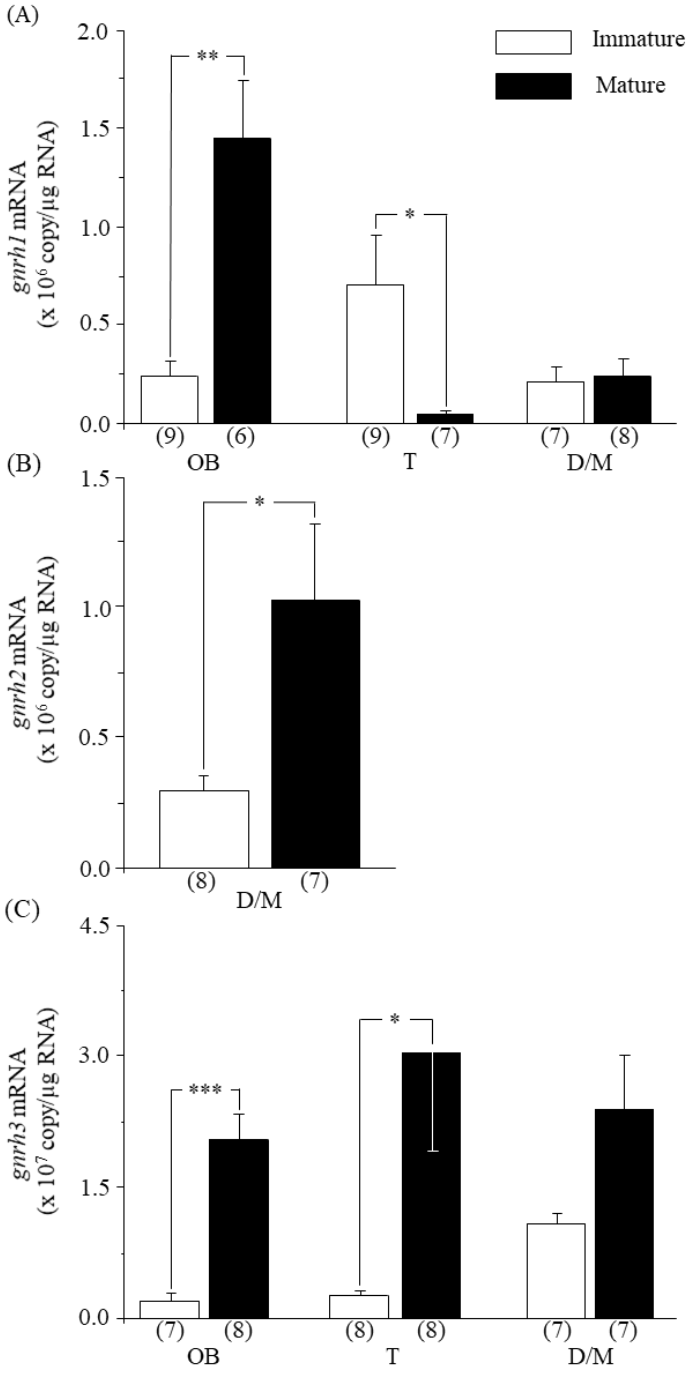

Fig. 4.5. Changes in the amounts of *gnrh1* (A), *gnrh2* (B) and *gnrh3* (C) mRNAs in different brain regions of tiger puffer at different reproductive stages in the males. Values are presented as mean ± SEM. Asterisks denotes a significant difference between the immature and mature fish (*, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$). OB, olfactory bulb; T, telencephalon; D/M, diencephalon/midbrain. Number in the parentheses represents the number of fish analyzed in each group.

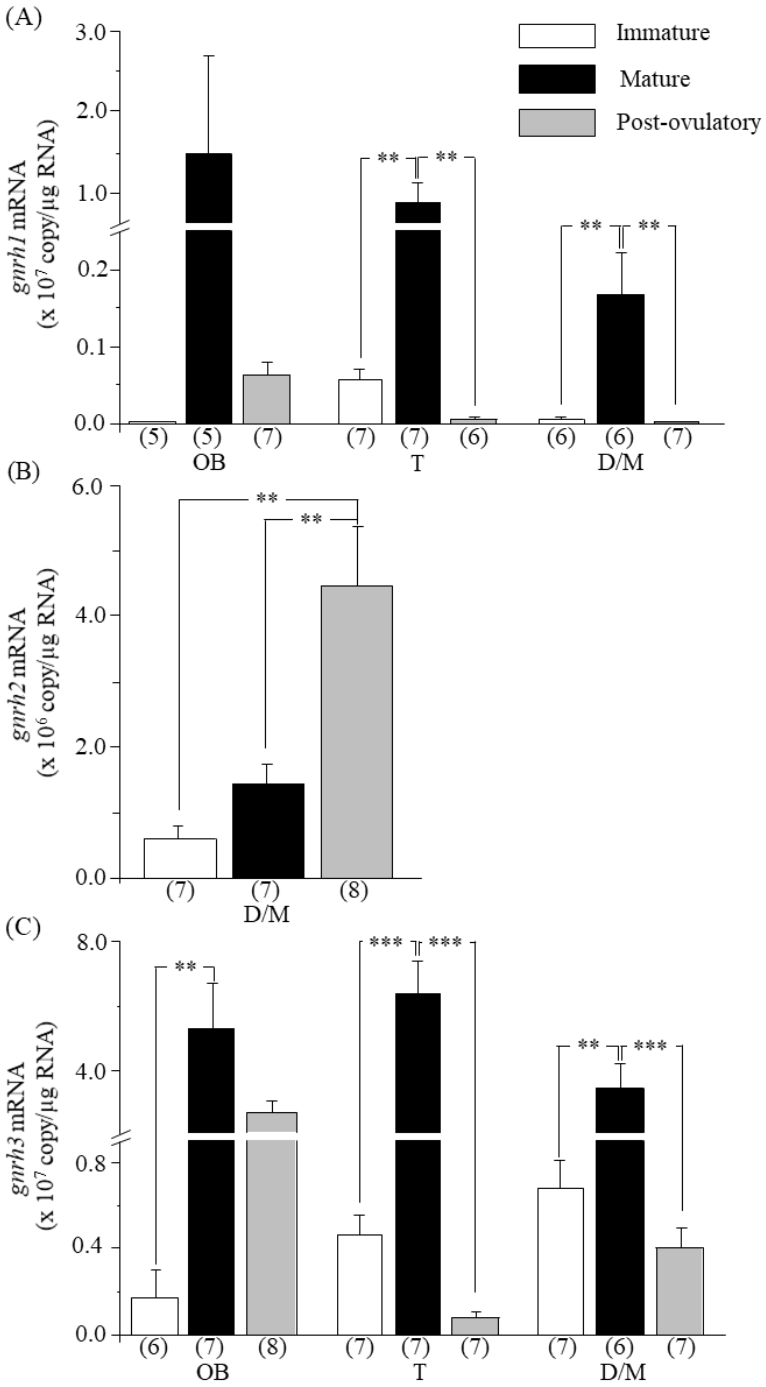

Fig. 4.6. Changes in the amounts of *gnrh1* (A), *gnrh2* (B) and *gnrh3* (C) mRNAs in different brain regions of tiger puffer at different reproductive stages in the females. Values are presented as mean ± SEM. Asterisks denotes a significant difference between the immature, mature and post-ovulatory fish (**, $p < 0.01$ and ***, $p < 0.001$). OB, olfactory bulb; T, telencephalon; D/M, diencephalon/midbrain. Number in the parentheses represents the number of fish analyzed in each group.

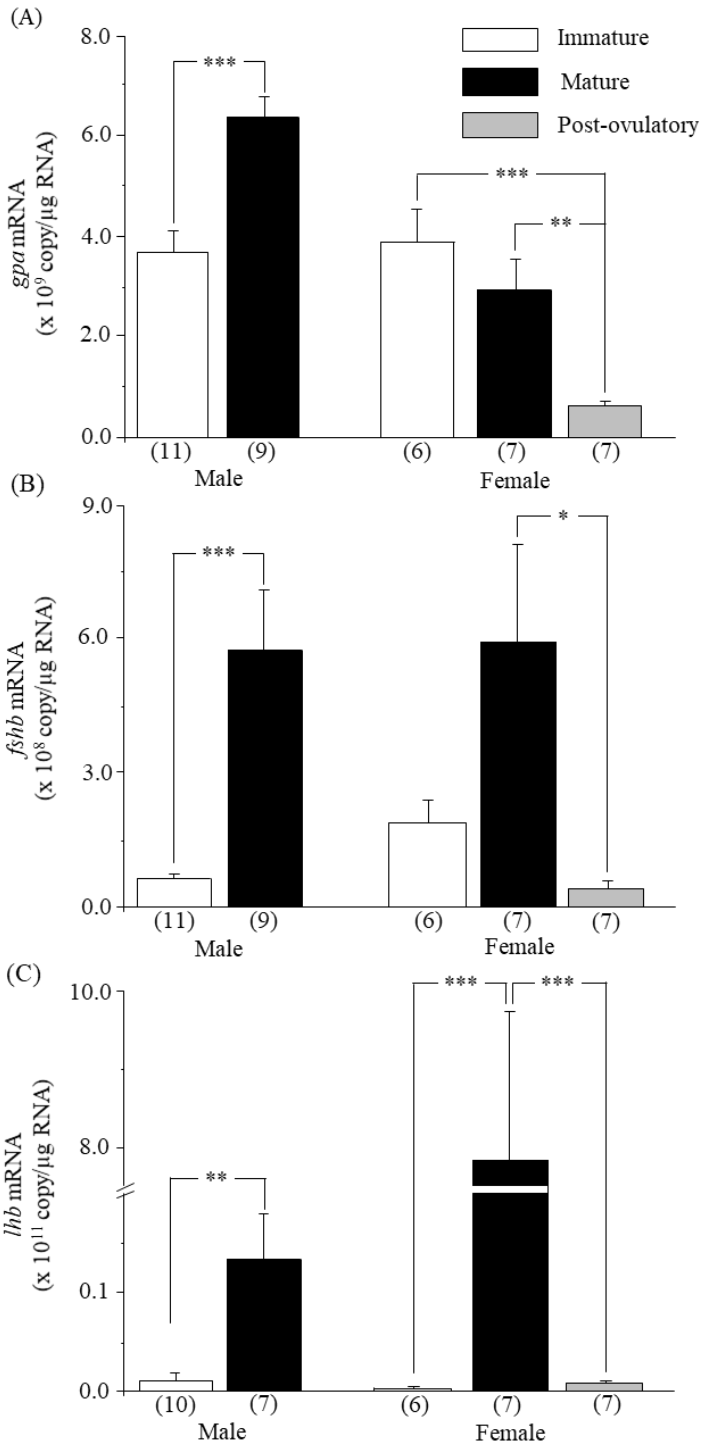

Fig. 4.7. Changes in the amounts of *gpa* (A), *fshb* (B) and *lhb* (C) mRNAs in the pituitary of tiger puffer at different reproductive stages. Values are presented as mean ± SEM. Asterisks denotes a significant difference between the immature, mature and post-ovulatory fish (*, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$). Number in the parentheses represents the number of fish analyzed in each group.

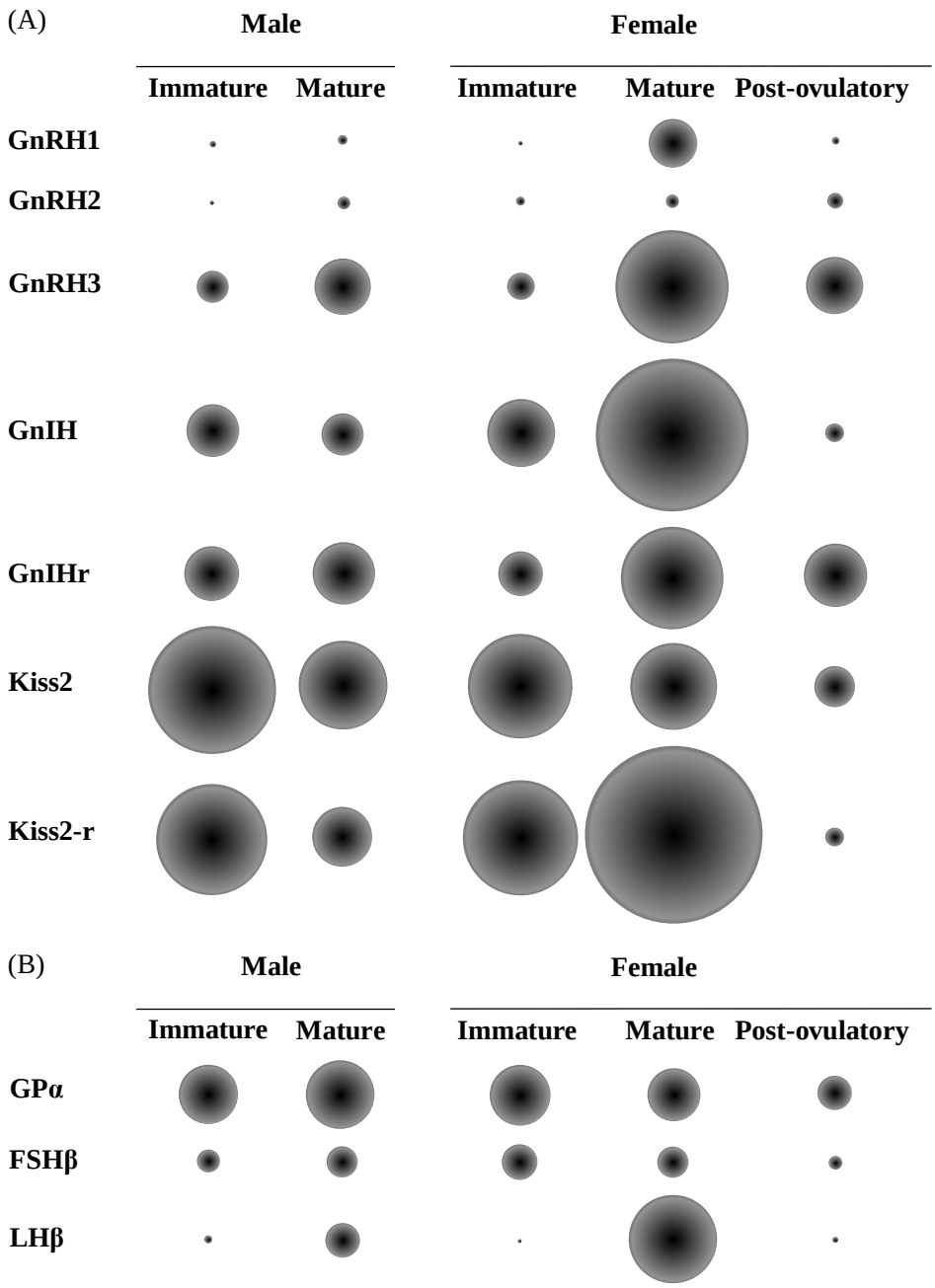

Fig. 4.8. Expression profile of the HPG axis genes in the brain (A) and pituitary (B) at different reproductive stages in the males and females in tiger puffer. The absolute amounts of mRNAs in the whole brain and pituitary are represented by the circle size, respectively.

Chapter 5

Regulation of the kisspeptin/GnIH system by melatonin in the grass puffer

Subchapter 5.1

Effects of melatonin administration on the expression of the HPG axis genes in the grass puffer

5.1.1. Introduction

Melatonin is a multifunctional biomolecule exclusively produced in the pineal gland in a circadian manner during night. Melatonin plays a major role in circadian and seasonal rhythms in various physiological and behavioral functions such as reproduction, food intake, sleep and locomotion in animals (Falcon et al., 2010). The role of melatonin in reproduction is species-specific (Goldman, 2001). The administration of melatonin induces or inhibits reproductive functions in mammals depending on reproductive strategy of the species (Carter and Goldman, 1983; Prendergast, 2005). For example, following melatonin increase, reproductive function is induced in short-day breeding animals such as sheep (Goodman and Inskeep, 2006), whereas inhibited in long-day breeding animals such as hamsters (Kauffman et al., 2007; Ansel et al., 2010). The actions of melatonin are mediated via melatonin receptors that belong to the G protein-coupled receptor superfamily (Reppert et al., 1996). The presence of melatonin receptors in distinct nuclei of the brain, pituitary and gonads suggests that melatonin has multiple sites of action in the modulation of reproduction (Woo et al., 2001; Barrett et al., 2003; Dubocovich et al., 2003; Falcon et al., 2010).

The reproduction in vertebrates is centrally regulated by kisspeptin and gonadotropin-

releasing hormone (GnRH) neurons through stimulation of the secretion of pituitary gonadotropins (GTHs), namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Parhar et al., 2004; Chang et al., 2012; Tena-Sempere et al., 2012; Espigares et al., 2015). Another RFamide neuropeptide, gonadotropin-inhibitory hormone (GnIH) is also involved in the regulation of reproduction through inhibiting or releasing GTHs depending on species and gonadal conditions (Tsutsui, 2009; Shahjahan et al., 2011; Shahjahan and Ando, 2011; Shahjahan et al., 2016). It has been shown that kisspeptin has important roles in transmitting multiple environmental signals, such as photoperiod, energy status and stress, to GnRH neurons in cooperation with GnIH neurons (Tsutsui, 2009; Parhar et al., 2012).

In mammals, it has been considered that melatonin regulates kisspeptin and GnRH neurons in a photoperiod-dependent manner and mediates seasonal control of reproduction (Simonneaux et al., 2009). In hamsters, long-day breeder, a short photoperiod induces gonadal regression being the HPG axis reactivated after kisspeptin administration (Greives et al., 2007; Mason et al., 2007; Ansel et al., 2011). However, the molecular mechanisms of melatonin aciton on kisspeptin and GnRH neurons remain to be determined.

In fish, melatonin has been shown to play an important role in the control of gonadal maturation (Amano et al., 2000; Singh et al., 2012). In male tilapia, melatonin treatment decreased the spawning frequency and the number of eggs in females, while decreased sperm count and spermatozoa activity index in the males (Kim et al., 2018b). However, very few studies have explored the neuroendocrine mechanism through which melatonin acts to influence the activity of the HPG axis. In the zebrafish, melatonin treatment was shown to increase the expression of *kiss1* and *kiss2* in association with an increase in *gnrh3* and *lhb* expression, suggesting that melatonin may have a stimulatory role in reproduction in this species (Carnevali et al., 2011). On the other hand, in the European eel, the melatonin administration decreased the expression of *fshb* and *lhb* and plasma levels of sex steroid

hormones, thus inhibiting the reproductive function (Sebert et al., 2008). Therefore, melatonin may play either a stimulatory or inhibitory role in reproduction also in fish and the regulatory mechanism of melatonin action on the reproductive neuroendocrine system is still unclear in fish.

The involvement of melatonin in the regulation of GnIH has been reported in several vertebrates. Melatonin was found to act directly on GnIH neurons through its receptor to induce GnIH expression in Japanese quail (Ubuka et al., 2005). The expression of GnIH was regulated by changing photoperiod in the sheep (Dardente et al., 2008; Smith et al., 2008), and melatonin regulated GnIH gene expression in the Syrian hamster (Revel et al., 2008). However, there is no report in the regulation of GnIH by melatonin in fish so far and this warrants investigation.

In the present study, the grass puffer (*Takifugu alboplumbeus*) was used as an experiment model in order to clarify the role of melatonin in the neuroendocrine control of reproduction, because this fish shows unique reproductive physiology that is synchronized with seasonal, lunar, and daily cycles. Spawning occurs only during spring tide every two weeks from spring to early summer (Motohashi et al., 2010; Ando et al., 2013). The fish aggregate at a certain seashore location for spawning 2.5–3 h before high tide. Before spawning, several tens of males actively pursue one female. Then, spawning starts 1.5–2 h before high tide and continues for 1 h during the rising tidal phase (Motohashi et al., 2010). Therefore, the timing of spawning is tightly connected with lunar and tidal rhythms as well as daily rhythms. Previous studies demonstrated in the grass puffer that the expression levels of the *kiss2* and *kiss2r* significantly increase during the spawning period in concert with specific expression patterns of three GnRH genes (*gnrh1*, *gnrh2* and *gnrh3*) (Shahjahan et al., 2010a,b; Ando et al., 2013). In addition, the expression levels of *gnih* and *gnih-r* are augmented during the spawning period (Shahjahan et al., 2011). Interestingly, *kiss2*, *kiss2r*, *gnih*, and *gnih-r* genes showed daily and circadian fluctuations in expression in the hypothalamus in association with the expression of melatonin

receptor gene (Ikegami et al., 2009; Shahjahan et al., 2011; Ando et al., 2014), suggesting the role of melatonin in controlling the reproduction of this fish species. Therefore, we investigated the effects of melatonin administration on the expression of *kiss2*, *kiss2r*, *gnih*, *gnih-r*, three *gnrhs*, and *mel1b* (melatonin receptor) in the brain and three GTH subunit genes (*gpa*, *fshb* and *lhb*) in the pituitary were examined, with the aim of investigating possible roles of melatonin in the control of the periodic reproductive activity in the grass puffer.

5.1.2. Materials and methods

5.1.2.1. Fish

Sexually mature male grass puffer was collected at spawning grounds in Kawana, Shizuoka, Japan in July. They were transferred to the Sado Island Center for Ecological Sustainability, Niigata University, Japan. The fish were transferred into indoor tanks (500 L) and acclimatized with the flow of seawater under natural photoperiod (LD 14:10) for two weeks. They were fed commercial pellets equivalent to 1% of body weight daily. Body weight (BW) and gonadosomatic index (GSI = gonad weight/BW × 100) of the fish (n = 32) were 48.5 ± 2.2 g and 5.9 ± 0.7%, respectively.

5.1.2.2. Melatonin administration and sample collection

Melatonin (*N*-acetyl-5-methoxytryptamine) was purchased commercially (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and dissolved in ethanol (4 mg/ml, stock solution) and diluted with saline solution (0.9% NaCl). The fish were anesthetized in 0.008% tricaine methanesulfonate (MS222, Sigma-Aldrich, Tokyo, Japan) for 30 sec. and were immobilized with its ventral side upward. The fish were intraperitoneally injected with different doses of melatonin (0, 0.11, 0.33 and 1.0 μg/g BW, n = 8) using a fine needle (25G, Terumo Corporation,

Tokyo, Japan) at 6:00AM (Zeitgeber time (ZT) 1:00) and left in indoor tanks (100 L, n = 8 per tank) for 6 hours. The fish were anesthetized in 0.03% tricaine methanesulfonate (MS222, Sigma-Aldrich, Tokyo, Japan) before sampling. Total length and BW was measured. Blood samples were collected from the celiac artery, kept on ice, and were later centrifuged at 10000 rpm for 10 min to obtain plasma samples, which were stored at -50°C until assayed. Gonads were removed after decapitation and weighted for calculation of the 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 the forebrain sample containing the telencephalon and diencephalon, and stored at -80°C until extraction of total RNA. The experimental procedures followed the guidance approved by the Animal Care and Use Committees of Niigata University, Niigata, Japan.

5.1.2.3. Real-time PCR assay

Real-time PCR assays for *kiss2*, *kiss2r*, *gnih*, *gnih-r*, *gnrh1*, *gnrh2*, *gnrh3*, *mel1b*, *gpa*, *fshb* and *lhb* were carried out as described previously (Ikegami et al., 2009; Shahjahan et al., 2010a, b; Shahjahan et al., 2011). Briefly, total RNA was extracted from the brain samples and pituitary, and 200−500 ng of total RNA was used for the synthesis of the first-strand cDNA by reverse transcription reaction using Multiscribe Reverse Transcriptase (Applied Biosystems, USA) according to the manufacturer's instruction. Real-time PCR was carried out with a Thermal Cycler Dice Real Time System III (TP 970, TaKaRa Bio, Japan). The absolute amount of mRNA was determined using sense reference RNA, which was synthesized in vitro by a MAXIscript kit (Ambion) according to the manufacturer's instruction and were serially diluted to 1 x 10^3−1 x 10^8 copies/µl. The standard sense RNAs were reverse transcribed and used as standard cDNAs to establish a standard curve. PCR reaction mixture (10µl) contained 1µl of sample cDNA, 0.4µl of forward and reverse primers (Table 5.1.1) and 5µl of TB Green Premix

DimerEraser (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 30 s, and 72°C for 30 s. Specific amplification of each subtype cDNA was verified by melting curve analysis and gel electrophoresis of the product.

5.1.2.4. Measurement of plasma melatonin

The plasma melatonin concentration was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as described by Iwashita et al. (2021). Briefly, 100 µl of the plasma sample was extracted with 400 µl of acetone and the acetone phase was evaporated to dryness at 65°C under a stream of nitrogen gas. After dissolving residues into 50 µl of Milli-Q water, the mixtures were passed through filters with 0.22 µm pores (Centrifugal Filter Units Ultrafree-MC-GV; Merck Millipore, Guyancourt, France). The samples (10 µl) were injected into a high performance liquid chromatography system (AC30AD; Shimadzu Corporation, Kyoto, Japan) equipped with a C18 2.0 x 150 mm, 3 µm Kinetex column (Tosoh, Japan). The mobile phase utilized 10 µmol/l ammonium acetate in 0.05% (v/v) acetic acid with varying concentrations of MeOH. The linear gradient was run over 20 min. from 5% to 50% MeOH maintained thereafter in 100% MeOH for 10 min. The flow rate was 0.3 ml/min and the autosampler and column oven were maintained at 4°C and 25°C, respectively. Melatonin was detected using a triple quadrupole mass spectrometer (LCMS-8050; Shimadzu) and were quantified using multiple reaction monitoring with the transition of parent ions to product ions. The transition of melatonin was m/z 233.0−130.0.

5.1.2.5. 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 doses of melatonin. Statistical significance was set at $p < 0.05$.

Statistical analyses were performed using SPSS Version 23.0 for Windows (SPSS Inc., Chicago, IL).

5.1.3. Results

5.1.3.1. Changes in the plasma melatonin levels after injection

Melatonin injection experiment was conducted to examine the effect of exogeneous melatonin treatment on the kisspeptin/GnIH system. Melatonin was ip injected at ZT1:00 when the plasma melatonin levels were considered to be the basal levels as shown in Fig. 5.2.1 of Subchapter 5.2. The plasma melatonin levels after injection were measured to confirm that the levels in the experiment groups were high as physiologically relevant levels. The plasma melatonin levels were increased in the experimental groups at 0.11 μg/g BW (70.8 ± 7.1 pg/ml, n = 8) and at 0.33 μg/g BW (81.4 ± 14.3 pg/ml, n = 8) compared to the control (11.3 ± 1.3 pg/ml, n = 8), although these changes were not statistically significant (Fig. 5.1.1). The melatonin levels were significantly elevated in the experimental group at 1.00 μg/g BW (440.6 ± 86.0 pg/ml, n = 7).

5.1.3.2. Effect of melatonin on the expression of kiss2 and kiss2r in the brain

The mRNA levels for *kiss2* and *kiss2r* did not show any significant changes after melatonin administration (Fig. 5.1.2). However, a slight increase in the expression of *kiss2* was noticed at higher doses (0.33 and 1.00 μg/BW) (Fig. 5.1.2A). Likewise, there was a trend toward increased *kiss2r* expression at 0.11 and 0.33 μg/BW doses of melatonin administration (Fig. 5.1.2B).

5.1.3.3. Effect of melatonin on the expression of gnih and gnih-r in the brain

Melatonin treatment significantly increased the expression of *gnih* and *gnih-r* by the melatonin administration at 0.33 μg/BW dose (Fig. 5.1.3). However, no such increment was observed at high and low doses of melatonin administration compared to the control.

5.1.3.4. Effect of melatonin on the expression of three gnrhs in the brain

The expression levels of *gnrh1* and *gnrh3* were increased by the melatonin administration at 0.33 μg/BW dose, although the change in the *gnrh1* expression was not statistically significant (Figs. 5.1.4A and 5.4.1C), while expression of *gnrh2* did not show any noticeable changes at any doses (Fig. 5.1.4B).

5.1.3.5. Effect of melatonin administration on the expression of mel1b in the brain

The expression levels of *mel1b* were significantly increased by the melatonin administration at 0.33 μg/BW dose and showed a trend toward increased expression at 0.11 and 1.00 μg/BW doses compared to the control (Fig. 5.1.5).

5.1.3.6. Effect of melatonin on the expression of gpa, fshb and lhb in the pituitary

The expression levels of *gpa* did not show any changes at different doses compared to the control (Fig. 5.1.6A). A significant increase in the expression of *fshb* was observed at 0.33 μg/BW doses of melatonin treatment (Fig. 5.1.6B). Similarly, the mRNA level of *lhb* tended to increase extensively at 0.33 μg/BW doses (Fig. 5.1.6C), although this change was not statistically significant.

5.1.4. Discussion

To clarify the role of melatonin in the neuroendocrine control of reproduction in the grass

puffer, the effects of melatonin on the HPG axis gene expression was examined in addition to the effect on melatonin receptor gene (*mel1b*) expression. The melatonin treatment increased the expression of many genes in the HPG axis including *kiss2*, *kiss2r*, *gnih*, *gnih-r*, *gnrh1*, *gnrh3*, and *mel1b* in the brain and *fshb* and *lhb* in the pituitary at the dose of 0.33 μg/BW. These results suggest that melatonin has a stimulatory role in the HPG axis and plays an important role in the control of the periodic reproductive activity in the grass puffer.

The effect of melatonin was examined by ip injection at three different doses (0.11, 0.33 and 1.00 μg/BW) and the fish were sampled 6 hours after injection. Plasma melatonin levels of both 0.11 and 0.33 μg/BW groups were 70–80 pg/ml, being a submaximal level of plasma melatonin that shows daily changes up to 100−200 pg/ml during nighttime (See Fig. 5.2.1). Though the levels did not differ between 0.11 and 0.33 μg/BW groups, the levels of melatonin metabolites such as 6-Hydroxymelatonin (6HMEL) and N-Acetyl-5-methoxylkynuramine (AMK) in the plasma samples, that were also determined in the LC-MS/MS analysis, were significantly higher in the 0.33 μg/BW group compared to the 0.11 μg/BW group (data not shown). Thus, it is considered that the significant increases in the expression of many HPG axis genes in the 0.33 μg/BW group, but 0.11 μg/BW group may be caused by the temporal melatonin increase during the 6 hours of treatment in the 0.33 μg/BW group. In the 1.00 μg/BW group, the levels rose up to about 400 pg/ml, which may be beyond the physiologically relevant levels, and thus no stimulation was observed in the expression of the HPG axis genes.

In mammals, the plasma melatonin levels are correlated with a decrease in *kiss1* expression in Syrian hamster (Kauffman et al., 2007; Ansel et al., 2010) but with an increase in *kiss1* expression in sheep (Goodman and Inskeep, 2006), indicating the possible role of melatonin in the control of reproduction through kisspeptin neurons in a photoperiod dependent manner (Kauffman et al., 2007). In fish, melatonin has been shown to influence reproductive function concomitantly with changes in kisspeptin expression. Long-term melatonin treatment (10 days)

stimulated the follicular maturation with increased expression of *kiss2* in zebrafish (Carnevali et al., 2011). In the male sea bass, however, long-term melatonin administration (30 days) inhibited spermatogenesis, while the mRNA levels of kisspeptin and GnRH genes were increased (Alvarado et al., 2015). Therefore, although melatonin most probably plays as a signal mechanism for reproduction through regulating the kisspeptin expression, the effect of melatonin on reproduction through kisspeptin neurons may be different depending on fish species. In the grass puffer, in which the *kiss2* expression is highly corelated to the periodic reproductive function (Shahjahan et al., 2010b, 2017; Ando et al., 2013, 2014, 2018; Chapter 2 in this thesis), melatonin stimulates Kiss2 expression, which may in turn stimulate hypothalamic GnRH1 neurons and pituitary GTH secretion.

The expression of *gnih* and *gnih-r* was also significantly stimulated by the melatonin administration in the present study. The action of melatonin on GnIH has been studied in several vertebrate species. Melatonin induced the expression of the GnIH gene directly through melatonin receptor 1c in quail (Ubuka et al., 2005; Chowdhury et al., 2010). Similarly, melatonin stimulated the expression of the gene encoding LPXRFamide precursor, GnIH ortholog in the urodele and anuran brains (Chowdhury et al., 2008; Chowdhury et al., 2011). In tilapia, higher plasma melatonin level and increased expression of *gnih* in nighttime and decrease in melatonin level and *gnih* expression in daytime was observed, and ip injection of melatonin significantly increased the *gnih* expression (Kim et al., 2018a). In the grass puffer, *gnih* and *gnih-r* expressions show diurnal and circadian oscillations with high expression levels under constant dark conditions (Shahjahan et al., 2011). It is thus conceivable that these periodic expressions of *gnih* and *gnih-r* are regulated by melatonin, probably directly through melatonin receptor, of which expression was also stimulated in the present study (*mel1b*).

GnRH is a primarily important neurohormone in the hypothalamus with its direct action of stimulating the secretion of GTH from the pituitary. In the present study, *gnrh1* and *gnrh3*

expressions were stimulated by the melatonin treatment with concomitant increases in *fshb* and *lhb* expressions. Considering the hypophysiotropic GnRH1 neurons in the POA and neuromodulatory GnRH3 neurons in the terminal nerve ganglion-POA region (Munoz-Cueto et al., 2020), melatonin may have important roles not only in stimulating gonadal maturation via GnRH1/GTH system but also in stimulating sexual behavior via GnRH3 neurons. It has been reported that melatonin does not appear to have a direct influence on GnRH neurons (reviewed by Kauffman et al., 2007). It is also notable that the distribution of melatonin receptor-expressing cells does not match that of *gnrh1* and *gnrh3* neurons in the sea bass (Herrera-Perez et al., 2010). Therefore, the increased expression of *gnrh1* and *gnrh3* may be mediated through Kiss2 and/or GnIH neurons. Immunohistochemical localization of Kiss2- and GnIH in the grass puffer showed that both Kiss2- and GnIH-immunoreactive cells are localized the magnocellular preoptic nucleus pars magnocellularis (PMm) in the POA (Rahman, 2020), which is one of the major hypothalamic nuclei that consist of hypophysiotropic neurons including GnRH1 neurons (Munoz-Cueto et al., 2020), suggesting the colocalization of GnRH, Kiss2 and GnIH neurons. It should also be of considerable interest and importance to clarify the colocalization of melatonin receptor with these neurons, and this warrants further investigation.

In conclusion, the present results indicate that melatonin stimulates the expression of *kiss2*, *kiss2r*, *gnih*, *gnih-r*, *gnrh1*, *gnrh3*, and *mel1b* in the brain and *fshb* and *lhb* in the pituitary of the grass puffer. These results suggest that melatonin plays a pivotal role in the regulation of the periodic reproductive function in the grass puffer, of which spawning is tightly dependent on seasonal, lunar, and daily cycles.

Table 5.1.1. Primers used in the real-time PCR assays in this study

Primers	**Nucleotide sequences**
Kiss2-qPCR-F1	5′-GACCTTCAGGGACAACGAGGAC-3′
Kiss2-qPCR-R1	5′-ATGAAGCGCTTGCCAAAGC-3′
Kiss2r-qPCR-F1	5′-TCCCGTTTCTGTTCAAGCACAAG-3′
Kiss2r-qPCR-R1	5′-ATTGTTGTTGCGCTCCTCTGC-3′
GnIH-qPCR-F1	5′-TGATTCGTCTGTGCGAGGAC-3′
GnIH-qPCR-R1	5′-TCAGCAGCTGTGCATTGACC-3′
GnIH-R-qPCR-F1	5′-AAGATGCTCATCCTGGTGGC-3′
GnIH-R-qPCR-R1	5′-AGATCCACCTGGTCACTGTCC-3′
GnRH1-qPCR-F1	5′-CGGGAGTCTGATGTCACAGCTC-3′
GnRH1-qPCR-R1	5′-AACACTGACGACGACCGTGTCC-3′
GnRH2-qPCR-F1	5′-CAGGAGCTCACCTGTCCAAC-3′
GnRH2-qPCR-R1	5′-CTGCATTCTCCTGCTTCACAG-3′
GnRH3-qPCR-F1	5′-AAGCAAACAGGGTGATGGTG-3′
GnRH3-qPCR-R1	5′-CTGATGGTTGCCTCCAACTC-3′
Mel1b-qPCR-F1	5′-CCATAGATCCGTCCCACGTA-3′
Mel1b-qPCR-R1	5′-TGTTGAGCAGGCCATAGATG-3′
GPα-qPCR-F1	5′-AAGGTGAGGAACCACACCGAG-3′
GPα-qPCR-R1	5′-AGCTCAAGGCCAGGATGAAC-3′
FSHβ-qPCR-F1	5′-ACACATTGAGGGCTGTCCAGTGG-3′
FSHβ-qPCR-R1	5′-TCCCCATTGAAGCGACTGCAG-3′
LHβ-qPCR-F1	5′-CACTTGGTGCAAACAAGCATC-3′
LHβ-qPCR-R1	5′-CAACTTAGAGCCACGGGGTAG-3′

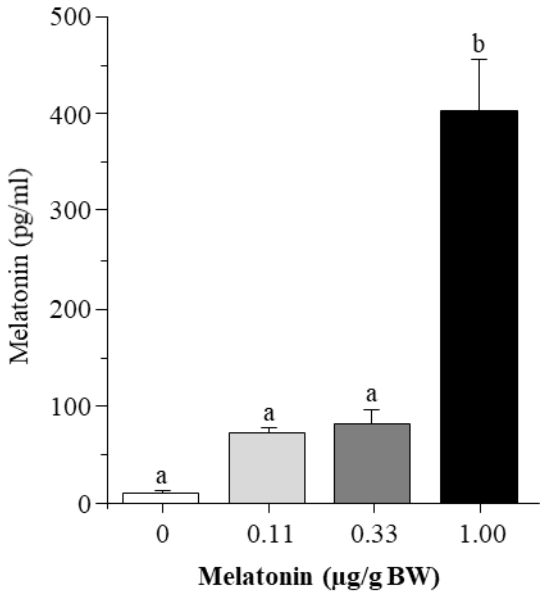

Fig. 5.1.1. Effect of melatonin administration on the plasma melatonin levels 6 hours after treatment in the male grass puffer. Values are presented as mean ± SEM (n = 8). Different subscript of alphabets indicates significant differences between doses of melatonin administration ($p < 0.05$).

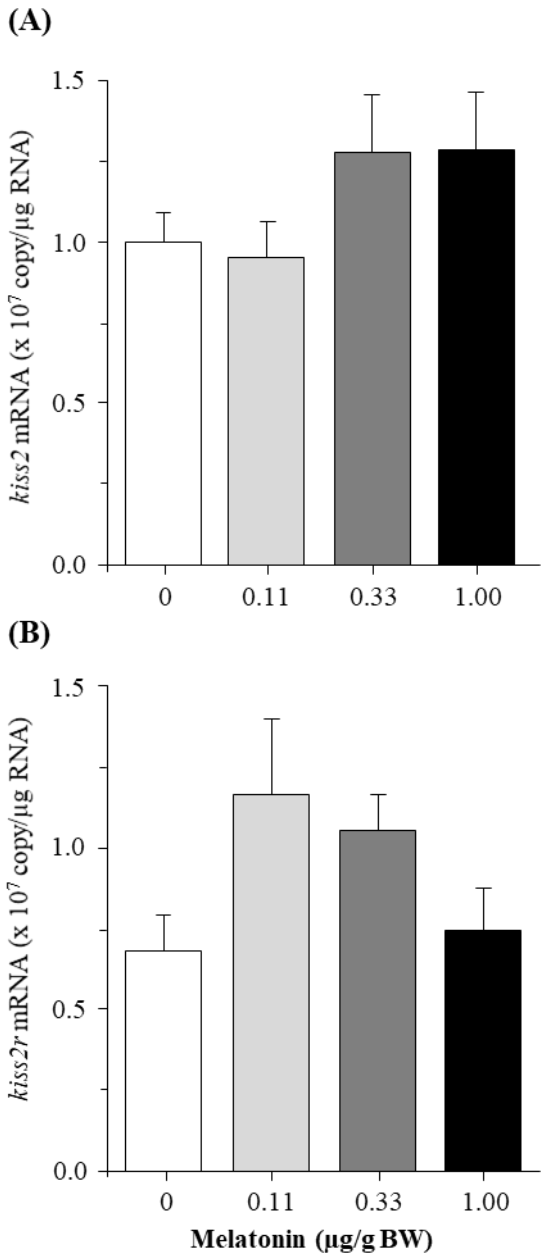

Fig. 5.1.2. Effect of melatonin administration on the expressions of *kiss2* (A) and *kiss2r* (B) in the brain of male grass puffer 6 hour after treatment. Values are presented as mean ± SEM (n = 8).

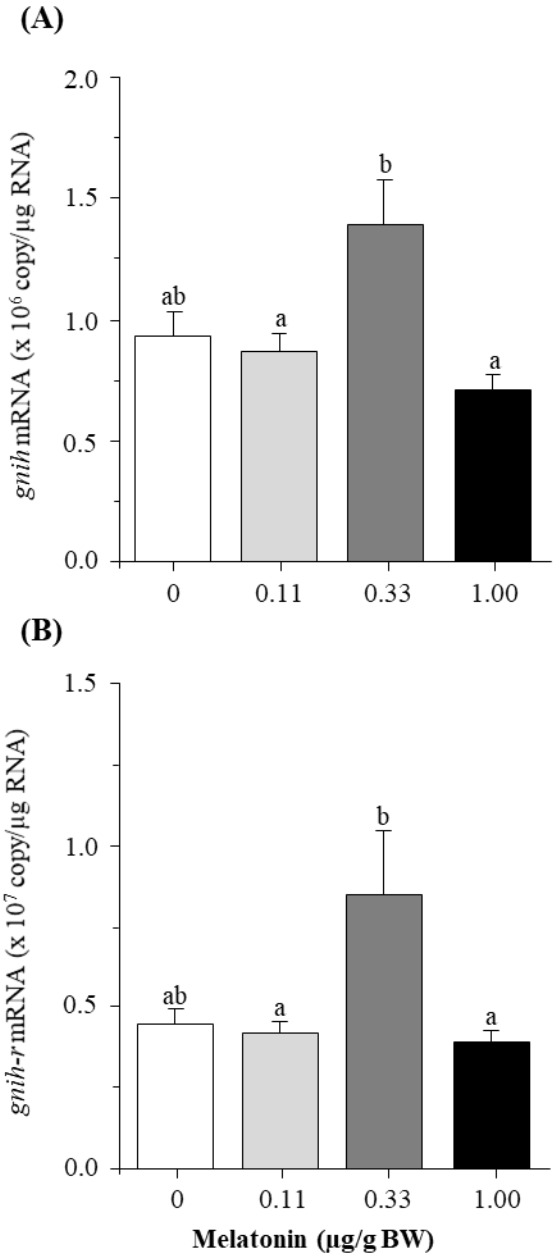

Fig. 5.1.3. Effect of melatonin administration on the expressions of (A) *gnih* and *gnih-r* (B) in the brain of male grass puffer 6 hour after treatment. Values are presented as mean ± SEM (n = 8). Different subscript of alphabets indicates significant differences between doses of melatonin administration ($p < 0.05$).

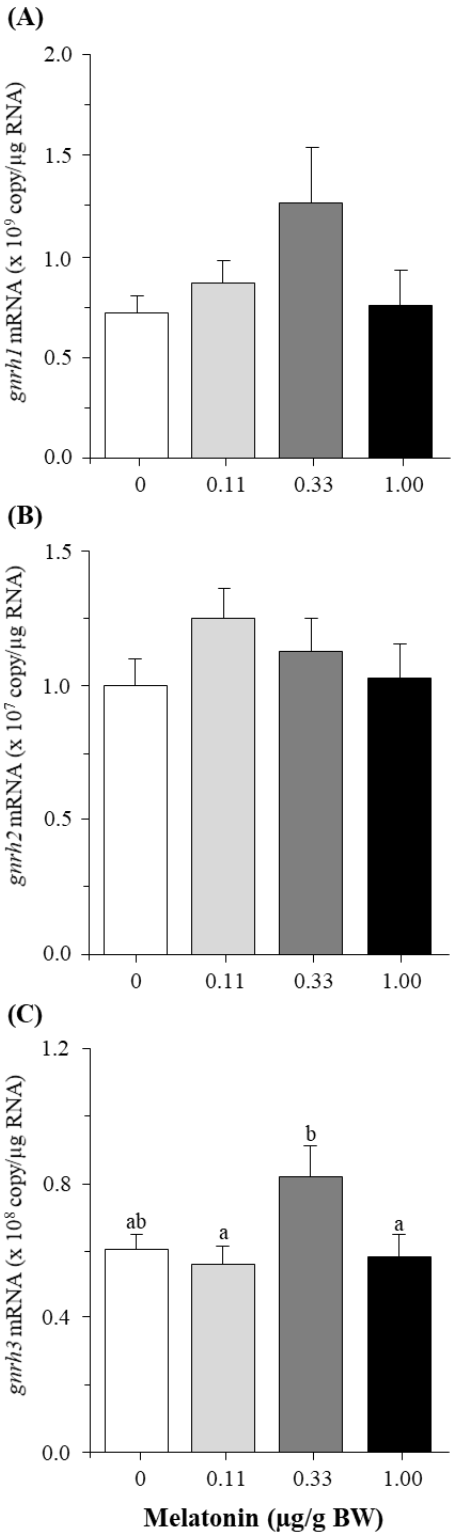

Fig. 5.1.4. Effect of melatonin administration on the expressions of *gnrh1* (A), *gnrh2* (B) and *gnrh3* (C) in the brain of male grass puffer 6 hour after treatment. Values are presented as mean ± SEM (n = 8). Different subscript of alphabets indicates significant differences between doses of melatonin administration ($p < 0.05$).

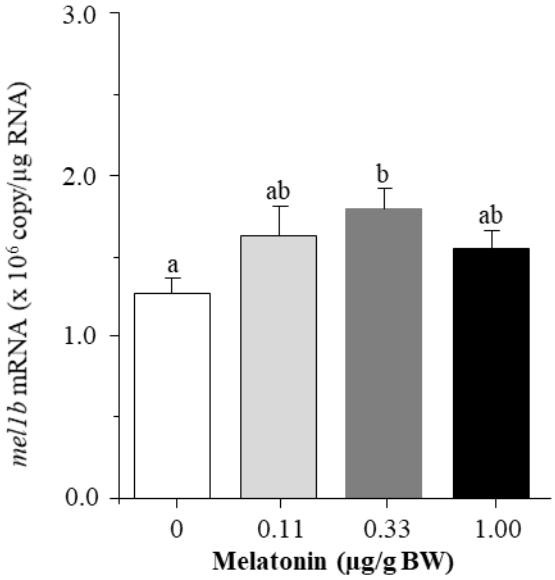

Fig. 5.1.5. Effect of melatonin administration on the expression of *mel1b* in the brain of male grass puffer 6 hour after treatment. Values are presented as mean ± SEM (n = 8). Different subscript of alphabets indicates significant differences between doses of melatonin administration ($p < 0.05$).

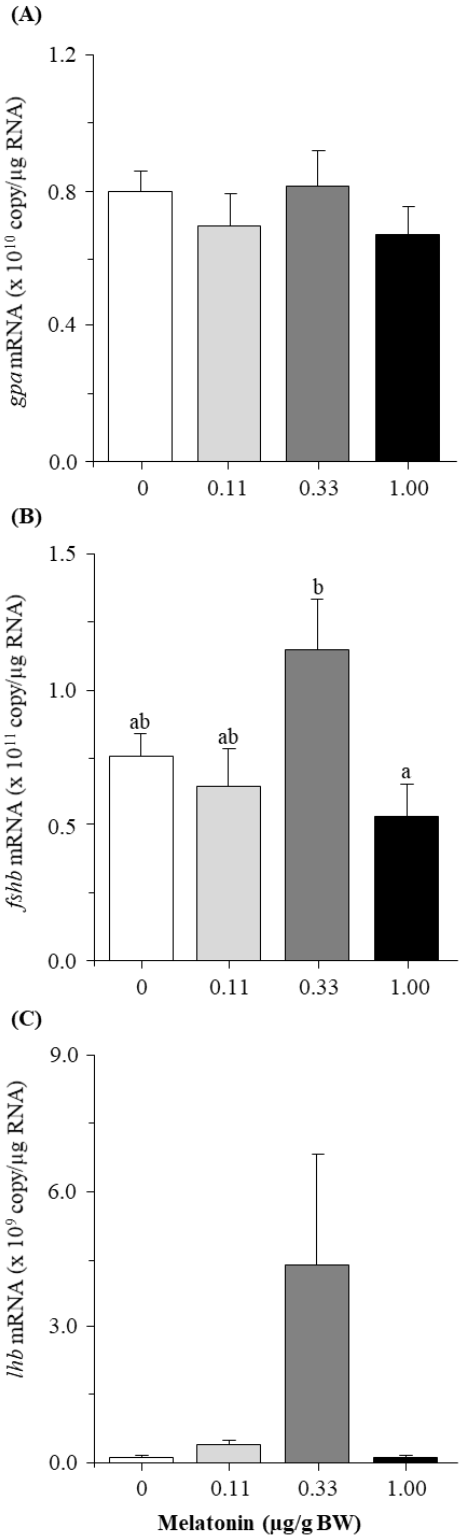

Fig. 5.1.6. Effect of melatonin administration on the expressions of *gpa* (A), *fshb* (B) and *lhb* (C) in the pituitary of male grass puffer 6 hour after treatment. Values are presented as mean ± SEM (n = 8). Different subscript of alphabets indicates significant differences between doses of melatonin administration ($p < 0.05$).

Chapter 5

Regulation of the kisspeptin/GnIH system by melatonin in the grass puffer

Subchapter 5.2

Moonlight and plasma melatonin levels in the grass puffer during spawning season

5.2.1. Introduction

The initiation of the reproductive activity in the seasonal breeders is the perception of changes in the photoperiodic circumstances by the photosensory organs. Photoperiod is one of the key periodical cues that function as an entrainer in the seasonal reproduction. In the long-day (LD) breeder, at the end of winter solstice, a steady increase in the photoperiod stimulates the initiation of gonadal development, while a decreasing photoperiod plays the same role in the short-day (SD) breeders. Following the perception of changes in the environmental factors by the sensory system, the signal is transduced in the hypothalamus-pituitary-gonadal (HPG) axis, the key neuroendocrine system controlling reproduction in vertebrates (Migaud et al., 2010; Pankhurst and Munday, 2011). Most animal species breed under suitable environmental conditions to ensure the survival of their offspring in a particular breeding season by sensing the various environmental cues (Dufour et al., 2010; Falcon et al., 2010). It has been demonstrated that in teleosts, the pineal gland in the brain and the retina in the eye is the key center to perceive photoperiodic information. These two organs possess structurally similar photoreceptor cells and relayed the perceived information to the HPG axis through secreting melatonin, an internal chemical messenger of environmental signals (Falcon et al., 1992; Okimoto and Stetson, 1999; Sanchez-Vazquez et al., 2000).

Melatonin, an indoleamine, is secreted into the circulatory system and involved in a variety of physiological and behavioral processes in the central and peripheral tissues at 24 hours interval (Klein, 2007). Secretion of melatonin follows a rhythmic fashion with high levels at nighttime and low levels during the daytime (Falcon et al., 2010). So, melatonin has important biological activity in the regulation of daily rhythm. Furthermore, the oscillations in the melatonin secretion in response to photoperiodic changes also affect the reproductive neuroendocrine system in fish (Falcon et al., 2007, 2010; Maitra et al., 2013).

Lunar-related phenomena play important role in the synchronization of growth, reproduction, and migratory events in many marine organisms (Letherland et al., 1992; Takemura et al., 2004a). A lunar reproductive cycle is persistent in many animals including fish, corals and insects that are repeated monthly during the spawning season. Moon-derived cues are a potent entrainer in physiological and behavioral synchrony but the mechanisms of driving perceived signals into the lunar rhythmicity varies among species. For example, rabbitfishes and white-spotted spinefoot release their gamete during the full-moon period (Hoque et al., 1999; Park et al., 2006), while gold-lined spinefoot and streamlined spinefoot spawn during the first quarter moon and last quarter moon, respectively (Rahman et al., 2000; Park et al., 2006). The exposure of gold-lined spinefoot to constant new moon or full moon conditions caused a disruption in the spawning activity during the predicted moon phase (Takemura et al., 2004b). Moreover, exposing fish to moonlight lowered the plasma melatonin levels and in vitro culture of the pineal gland with moonlight suppressed the synthesis of melatonin (Takemura et al., 2006). These findings indicate that fish with lunar synchronization are capable of perceiving the changes in the moonlight and melatonin may transduce these signals to the HPG axis. However, the molecular and neuroendocrine mechanisms of the moonlight signal-dependent reproduction remain unknown.

The grass puffer (*Takifugu alboplumbeus*) shows unique reproductive physiology that is

synchronized with seasonal, lunar and daily cycles. Spawning occurs only during spring tide on the day of new moon and full moon every two weeks from spring to early summer (Motohashi et al., 2010; Ando et al., 2013). The fish aggregate at a certain seashore location for spawning 2.5–3 h before high tide. Before spawning, several tens of males actively pursue one female. Then, spawning starts 1.5–2 h before high tide and continues for 1 h during the rising tidal phase (Motohashi et al., 2010). Therefore, the timing of spawning is tightly connected with lunar and tidal rhythms as well as daily rhythms. Previous studies in the grass puffer demonstrate that melatonin secretion from the pineal gland displayed diurnal and circadian variations in vitro (Ikegami et al., 2015). Besides, four subtypes of melatonin receptor genes displayed ultradian oscillations in about 15 hours of cycle in the pineal gland of grass puffer (Ikegami et al., 2015). These results suggest that the melatonin may play an important role in the transmission of moonlight signals to the HPG axis. As described in Subchapter 5.1, melatonin was found to stimulate the expression of the HPG axis genes in the mature grass puffer. Therefore, it is hypothesized that this species can sense the moonlight and transduce the moonlight signals into the HPG axis to regulate the reproductive functions in a semilunar-synchronized way. However, to date, there is no information regarding the plasma melatonin levels in the grass puffer in terms of daily and lunar age-dependent variations. Therefore, in this study, changes in the plasma melatonin levels were examined during the breeding season with respect to daily and lunar age-dependent variations. Additionally, fish were exposed to artificial dim light conditions to examine the impact of moonlight on the synthesis of melatonin.

5.2.2. Materials and Methods

5.2.2.1. Fish

Mature male grass puffer was collected from spawning grounds in Tomioka, Kumamoto,

Japan and Kawana, Shizuoka, Japan during spawning season from May to July in different years (Table 5.2.1). In addition, for a preliminary experiment, immature fish were collected from a spawning ground in Minamiise, Mie, Japan. Collected fish were transferred to the Marine Biological Station, Sado Island Center for Ecological Sustainability, Niigata University, Japan. Fish were acclimatized in 500 L indoor tanks with a flow of running seawater under natural photoperiod (LD 14:10) for two weeks. The water temperature during the acclimation period was similar to the spawning ground. Fish were fed commercial pellets equivalent to 1% of body weight (BW) daily prior to the experiment. Total length, BW and gonadosomatic index (GSI = gonad weight/BW × 100) of the fish are shown in Table 5.2.1. The experimental procedures followed the guidelines approved by the Animal Care and Use Committees of Niigata University, Niigata, Japan.

5.2.2.2. Experimental design and sample collection

Daily variation of the plasma melatonin level was examined using fish collected from Tomioka in June 2010 (n = 64). The fish was anesthetized in 0.03% tricaine methanesulfonate (MS222, Sigma-Aldrich, Tokyo, Japan) and blood sample was collected from the celiac artery at a 3-hour interval for one day at Zeitgeber time (ZT) 3, ZT6, ZT9, ZT12, ZT15, ZT18, ZT21, and ZT24 (n = 8 in each time point). Plasma sample was obtained by centrifugation at 10,000 rpm at 4°C for 10 min. and were stored at -50°C until assayed.

Lunar age-dependent variation of the plasma melatonin level was examined in 2017 and 2018 using fish collected from Kawana (n = 48 in each year). The fish were anesthetized in 0.03% MS222 and blood sample was collected at ZT18 (11:00PM, middle of dark period) at a five-day interval on lunar age 0, 5, 10, 15, 20, and 25 from July 18 to August 12 in 2017 and from June 5 to June 29 in 2018 (n = 8 for each lunar age). Plasma sample was obtained as described above.

Plasma melatonin levels in the fish exposed to artificial dim light were examined in 2018, 2019 and 2020. For preliminary investigation in 2018, immature fish collected from Minamiise were used in August (n = 6). Mature male fish collected from Tomioka were used in June 2019 (n = 18) and those from Kawana were used in July 2020 (n = 21). Prior to exposure to dim light conditions, fish were transferred and acclimatized in three aquariums (100 L, n = 6−7 for each aquarium in 2019 and 2020) with circulating seawater at 20°C under natural photoperiod conditions (LD 14:10) for three days. On day-4, light was turned off at ZT14 and three different lighting conditions were set using fluorescent light placed at the top of the aquarium and by covering the aquarium with black sheets: one aquarium was left under darkness for control (D), second aquarium was left under dim light (Dim, 0.02–0.05 lux at the bottom and 0.70–2.00 lux at the surface) and third aquarium was left under bright light (L, 12–51 lux at the bottom and 85–215 lux at the surface). The light intensity at the bottom and surface of these aquariums was measured by an illuminometer (LX-1102, Lutron Electronic Enterprise Co. Ltd., Taiwan). At ZT20, after six hours of light exposure, the fish were anesthetized in 0.03% MS222 and blood sample was collected from the celiac artery, kept on ice and plasma sample was obtained as described above.

5.2.2.3. Measurement of plasma melatonin

The plasma melatonin concentration was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as described by Iwashita et al. (2021). Briefly, 100 µl of the plasma sample was extracted with 400 µl of acetone and the acetone phase was evaporated to dryness at 65°C under a stream of nitrogen gas. After dissolving residues into 50 µl of Milli-Q water, the mixtures were passed through filters with 0.22 µm pores (Centrifugal Filter Units Ultrafree-MC-GV; Merck Millipore, Guyancourt, France). The samples (10 µl) were injected into a high performance liquid chromatography system (AC30AD; Shimadzu Corporation,

Kyoto, Japan) equipped with a C18 2.0 x 150 mm, 3 µm Kinetex column (Tosoh, Japan). The mobile phase utilized 10 µmol/l ammonium acetate in 0.05% (v/v) acetic acid with varying concentrations of MeOH. The linear gradient was run over 20 min. from 5% to 50% MeOH maintained thereafter in 100% MeOH for 10 min. The flow rate was 0.3 ml/min and the autosampler and column oven were maintained at 4°C and 25°C, respectively. Melatonin was detected using a triple quadrupole mass spectrometer (LCMS-8050; Shimadzu) and were quantified using multiple reaction monitoring with the transition of parent ions to product ions. The transition of melatonin was m/z 233.0-130.0.

5.2.2.4. Statistical analysis

The plasma melatonin levels are expressed as means ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test to assess the statistically significant differences in melatonin levels at different time points and lighting conditions. Student t-test were performed to compare significant difference between the dim light (Dim) and control (D) groups in the preliminary experiment in 2018. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using SPSS Version 23.0 for Windows (SPSS Inc., Chicago, IL).

5.2.3. Results

5.2.3.1. Daily variation in the plasma melatonin levels

Daily rhythmicity was observed under the natural photoperiodic cycle having significantly higher levels during nighttime compared to the daytime (Fig. 5.2.1). Plasma melatonin levels were low between ZT0 to ZT6 with basal levels of 30–50 pg/ml. The levels gradually increased from ZT9 and reached significantly higher levels from ZT12 to ZT21 when compared to the

basal levels with a peak value of 196 pg/ml at ZT15 (Fig. 5.2.1).

5.2.3.2. Lunar age-dependent variation in the plasma melatonin levels

Plasma samples were taken at ZT18, middle of dark period, every five days in a lunar month on the day of new moon, after waxing crescent moon, before waxing gibbous moon, full moon, after waning gibbous moon, and before waning crescent moon phase in two different years (Figs. 5.2.2A and 5.2.2.B). The plasma melatonin levels at midnight did not vary significantly in association with the lunar age in both years.

5.2.3.3. Effect of dim light exposure on the plasma melatonin levels

Plasma melatonin levels were examined in the fish left under dim light conditions to further investigate the impact of moonlight intensity on the semilunar spawning in the grass puffer. In 2018, the preliminary experiment using four fish for the Dim group and two fish for the control (D) group showed that the melatonin levels in Dim were significantly lower than that in D (Fig. 5.2.3A). In 2019, the plasma melatonin levels were significantly higher in Dim compared to D and L (under bright light conditions) (Fig. 5.2.3B). In 2020, however, the levels in Dim were almost the same as D and the levels were significantly decreased in L (Fig. 5.2.3C).

5.2.4. Discussion

The present study examined periodic changes in the plasma melatonin levels in the grass puffer in terms of daily and lunar-age dependent variations. Furthermore, the changes in the plasma melatonin levels after dim light exposure were examined to investigate the possible involvement of moonlight intensity to influence the melatonin secretion and thus to mediate lunar information to the HPG axis. The plasma melatonin levels clearly showed a daily change

with high levels during the nighttime. However, the nighttime levels did not change in association with lunar age, suggesting that relationship between lunar periodicity and melatonin production proposed in rabbitfish is missing in this species. Furthermore, the effects of the dim light exposure at nighttime on the plasma melatonin levels were variable among the three experiments conducted in different years. These results suggest that moonlight may not be a cue that is transmitted to the HPG axis through melatonin to serve as an entrainer in the semilunar-synchronized spawning rhythm.

The plasma melatonin levels were significantly higher during the nighttime compared to the daytime (Fig. 5.2.1). The previous study using in vitro culture of the grass puffer pineal gland also demonstrated a drastic increase in the melatonin secretion after the light was turned off and ceased immediately after the light turned on (Ikegami et al., 2015). This light- and time-dependent melatonin secretion has been reported in many vertebrate species including fish (Kezuka et al., 1988; Porter et al., 1999; Bayarri et al., 2002; Takemura et al., 2004b; Vera et al., 2007) and it has been well established that environmental light/dark cycle and endogenous circadian clock are involved in the daily variation in the plasma melatonin (Falcon, 1999). Therefore, the plasma melatonin levels are certainly important in the performance of daily changes in physiological and behavioral activities also in the grass puffer.

It has been considered in rabbitfish, a lunar spawner, changes in moonlight intensity are expressed as monthly changes in the plasma melatonin levels that signal the difference among the moon phases, for the plasma melatonin levels vary according to the moon age with high levels during new moon and low levels during full moon (Takemura et al., 2004b; Rahman et al., 2004). In the present study, experiments conducted in two different years, the plasma melatonin levels over a lunar month at midnight (ZT 18) in the grass puffer did not show any changes (Fig. 5.2.2). Moreover, exposing the fish to the artificial dim light during nighttime caused different responses in the melatonin secretion in three years. Although the variations in

the wild stocks and gonadal conditions may affect the response and further study will be needed to clarify the relation between moonlight and melatonin, the present results suggest that moonlight does not have any impact on the plasma melatonin levels and thus do not function as an important entrainer in the semilunar spawning in this species. This may consist with the fact that grass puffer repeats spawning on both new moon and full moon nights and this is different from the rabbitfish.

The molecular mechanism of circalunar periodicity has been poorly understood because of limited model species exhibiting lunar periodicity under laboratory conditions. However, in the pineal gland of grass puffer, four melatonin receptor genes show unique ultradian oscillations in the expression with a period of 14.0−15.4 hours, suggesting that melatonin may be involved in tidal cycle-related synchronization (Ikegami et al., 2015). Considering that melatonin stimulates the expression of the HPG axis genes (Subchapter 5.1), melatonin signal may be involved in the coordination of the tidal related rhythmicity and the semilunar spawning. One possible explanation of melatonin action relies on beating reaction of circatidal (ca. 12.4 hours of cycle) and circadian (ca. 24 hours of cycle) clocks to produce the semilunar rhythm: two oscillators only coincide once per semilunar month (ca. 14.7 days of cycle) (Bünning and Müller, 1961). Further studies will be required to clarify the role of melatonin signal in the control of semilunar spawning rhythm.

In conclusions, the physiological significance of plasma melatonin in the daily variation is evident in the grass puffer. However, the plasma melatonin levels do not correlate to lunar age and moonlight intensity. The present results suggest that moonlight do not play a role as a lunar synchronizer in the grass puffer, which spawns on the day of new moon and full moon in a semilunar rhythm.

Table 5.2.1. Total length, body weight and gonadosomatic index (GSI) of fish samples. Values are presented as mean ± SEM.

Experiment	**Collection site**	**Year**	**Total length (cm)**	**Body weight (g)**	**GSI (%)**
Diurnal variation	Tomioka	2010	11.3 ± 0.1	45.2 ± 0.7	12.9 ± 0.5
Lunar age-dependent variation	Kawana	2017	15.4 ± 0.2	57.0 ± 1.7	4.6 ± 0.8
	Kawana	2018	15.1 ± 0.1	66.2 ± 1.7	15.8 ± 0.6
Dim light exposure	Minamiise	2018	12.8 ± 2.7	38.6 ± 7.8	0.001 ± 0.0001
	Tomioka	2019	11.6 ± 0.4	30.4 ± 3.0	14.2 ± 1.2
	Kawana	2020	14.2 ± 0.3	52.2 ± 2.4	13.2 ± 1.5

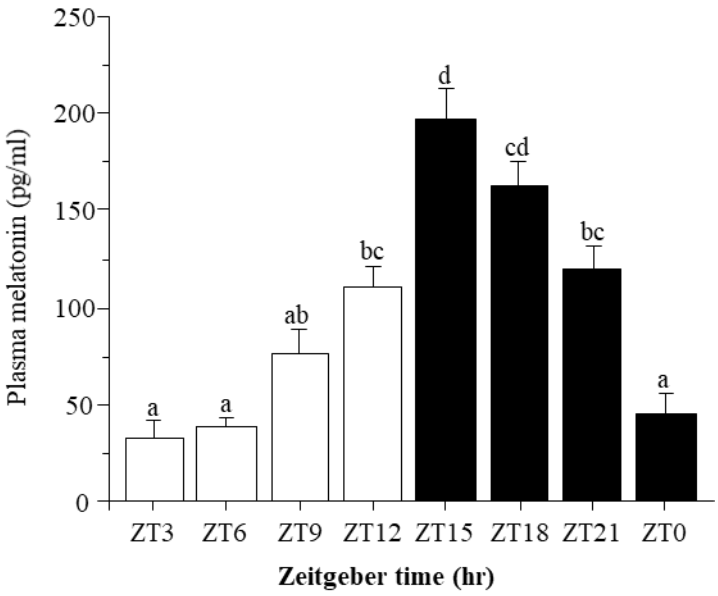

Fig. 5.2.1. Diurnal variations in the plasma melatonin levels in the grass puffer. Values are presented as mean ± standard error of the mean (SEM) (n = 8 in each time point). Different subscript of alphabets is statistically significant at $p < 0.05$.

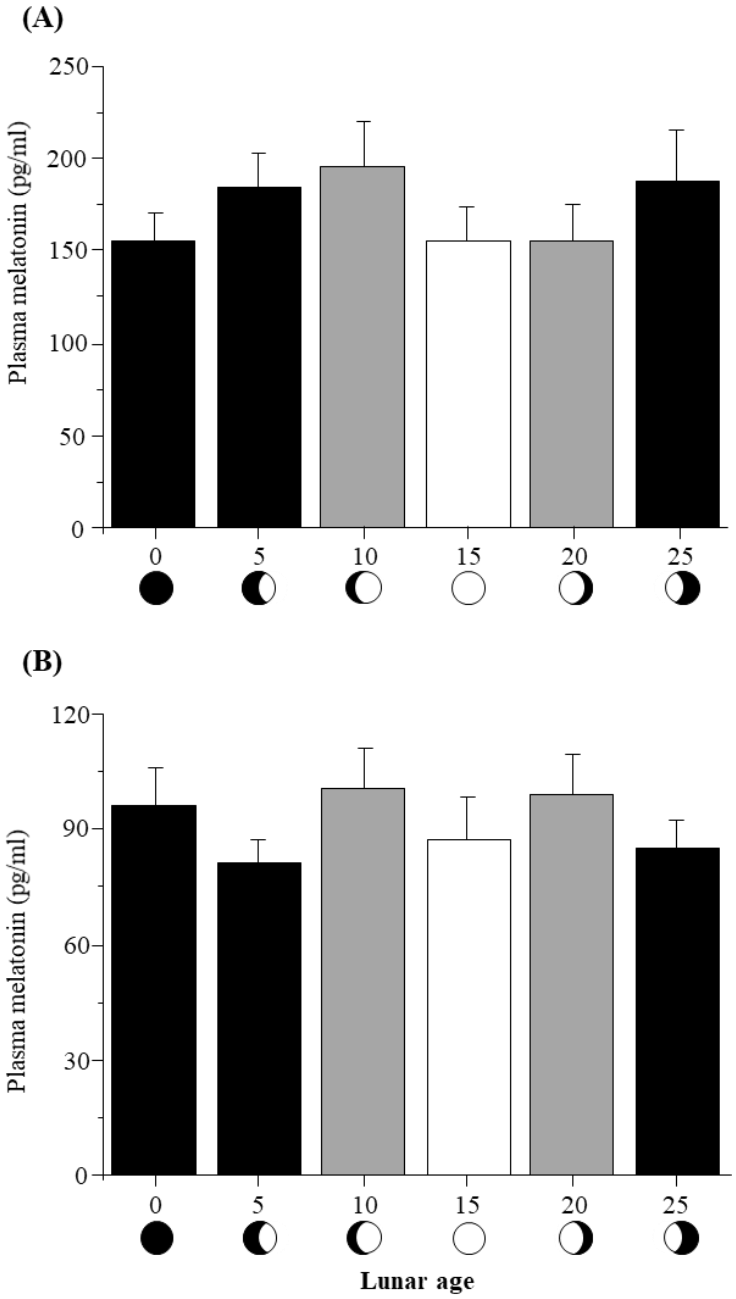

Fig. 5.2.2. Lunar age dependent variations in the plasma melatonin levels in 2017 (A) and 2018 (B) in the grass puffer during spawning season. Values are presented as mean ± standard error of the mean (SEM) (n = 8 in each time point). Lunar phase is expressed by circle shown below the X axis.

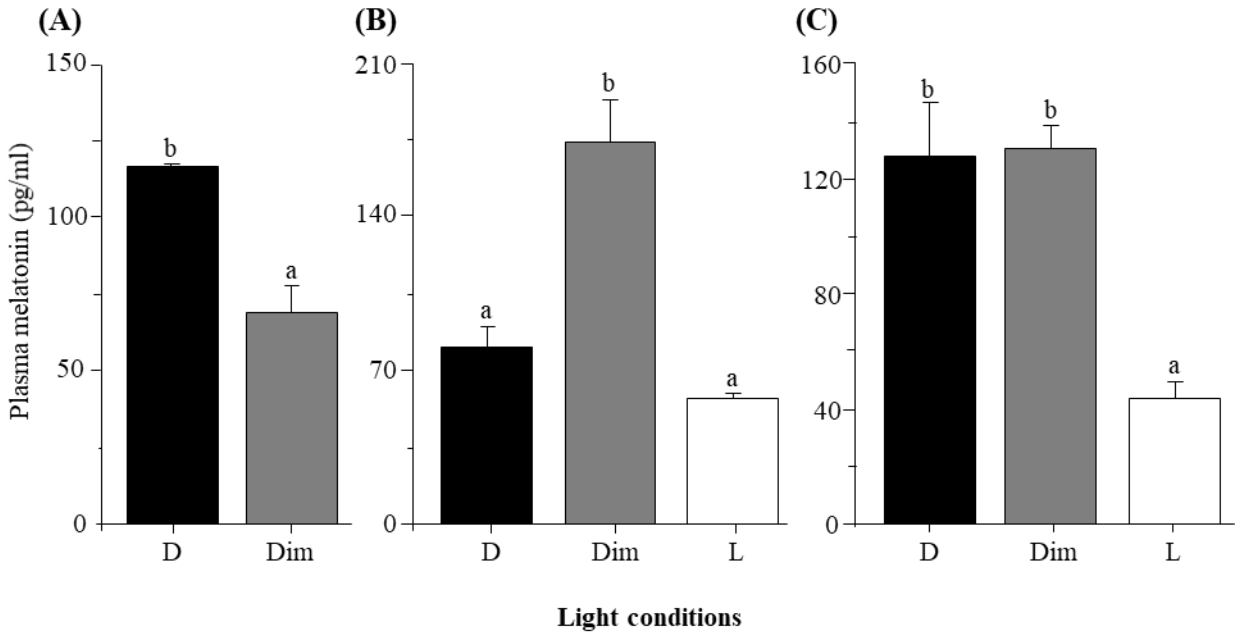

Fig. 5.2.3. Changes in the plasma melatonin levels in 2018 (A), 2019 (B) and 2020 (C) at different lighting conditions (D, control; Dim, dim light; L, light) in the grass puffer during spawning season after 6 h exposure of light at midnight (ZT 20). Values are presented as mean ± standard error of the mean (SEM) (n = 4−7 in each time point). Different subscript of alphabets is statistically significant at $p < 0.05$.

Chapter 6

General discussion and conclusions

This study was intended to clarify the role of kisspeptin and gonadotropin-inhibitory hormone (GnIH) in the regulation of periodic reproductive activities in grass and tiger puffers with special reference to the possible role of melatonin in lunar synchronicity. The results of the present study demonstrate that both kisspeptin and GnIH have a stimulatory effect on reproductive function through activating the gonadotropin-releasing hormone (GnRH)/gonadotropin (GTH) system in the grass puffer. It was also suggested that kisspeptin and GnIH are important in sexual maturation in the tiger puffer, followed a similar expression pattern in the hypothalamus-pituitary-gonadal (HPG) axis genes accompanying the grass puffer. In addition, the present results find out that melatonin has a stimulatory effect on the HPG axis in the grass puffer, suggesting its important role in the periodic regulation of reproduction in a photoperiod-dependent manner. However, the involvement of moonlight in the semilunar spawning via melatonin is not obvious in the grass puffer; rather melatonin signal may be involved in the semilunar oscillation via a circatidal clock mechanism, which warrants further investigation.

In Chapter 2, the administration of gpKiss2 significantly elevated the expression of *gnrh1* in the brain, *fshb* and *lhb* in the pituitary, and *kiss2r* in both organs in immature and mature fish, indicating the stimulatory role of kisspeptin in the GTH secretion through stimulating the GnRH1 secretion and by direct local action in the pituitary. It is now evident that in mammals kisspeptin has a stimulatory effect on reproductive functions through the stimulatory action on the GnRH release. However, in fish, the role of kisspeptin has been controversial: stimulatory, inhibitory or no effect has been reported depending on the species and gonadal conditions

(Kanda et al., 2013; Ogha et al., 2014; Kim et al., 2014; Tang et al., 2015; Nakajo et al., 2017). In addition, there are two kisspeptin forms (Kiss1 and Kiss2) and two types of kisspeptin receptors (Kiss1r and Kiss2r) in teleosts, and this increases the functional complexity of the kisspreptin system. Grass puffer has only a single pair of kisspeptin (Kiss2) and kisspeptin receptor (Kiss2r) and this neuropeptide has most probably hypophysiotropic function via both indirect neuroendocrine actin and direct local action. In the brain of grass puffer, Kiss2- and Kiss2r-immunoreactive (ir) cells are localized in the magnocellular preoptic nucleus pars magnocellularis (PMm) in the prepotic area (POA) (Rahman, 2020), which is one of the major hypothalamic nuclei that consist of hypophysiotropic neurons including GnRH1 neurons. So, the colocalization of GnRH1 neurons with Kiss2r is feasible and also close proximity of Kiss2 and GnRH1 neurons is indicative of functional interaction in stimulating the GTH secretion. It should be of considerable importance to determine the neuroanatomical structure of Kiss2 and GnRH1 neurons in the grass puffer.

In Chapter 3, the role of GnIH in the regulation of reproductive function in the grass puffer was examined by the administration of native GnIH peptides. Like kisspeptin, GnIH has been shown to have stimulatory and inhibitory effects on GTH secretion and reproductive function depending on fish species and gonadal conditions (Shahjahan et al., 2011, 2016; Moussavi et al., 2014; Di Yorio et al., 2016; Ando et al., 2018; Wang et al., 2019; Zhai et al., 2020). Previous studies in the grass puffer demonstrated the stimulatory effect of goldfish LPXRFamide peptide on the expression of the pituitary GTH subunit genes in vivo and in vitro (Shahjahan et al., 2011, 2016; Ando et al., 2018). In the present study, the administration of native GnIH-1 also stimulated the expression of the HPG axis genes in the mature grass puffer which further confirmed the stimulatory role of GnIH in this fish. On the other hand, the administration of gpGnIH-2 showed no effect on the expression of HPG axis genes in the regressed fish, suggesting that multiple GnIH peptides produced from the same precursor may have differential

effects on the regulation of the HPG axis depending on the types of peptide and reproductive stages. It was also shown that GnIH-ir and GnIH-R-ir cells are localized in the PMm in the grass puffer (Rahman, 2020), suggesting the co-localization of GnIH, Kiss2 and GnRH1 neurons. Considering that both Kiss2 and GnIH have a stimulatory role in the HPG axis, there might be functional interaction of these two neurohormones in the regulation of reproductive function in the grass puffer, and this warrants further investigation.

In Chapter 4, changes in the expression levels of the HPG axis genes at different reproductive stages were examined in a wild population of tiger puffer, which is closely related to grass puffer. The expressions of the genes encoding GnIH, GnIH-R and GnRHs in the brain and GTH subunits in the pituitary were significantly increased in the mature fish compared to the immature fish, especially in the females. Furthermore, the expression levels of these genes were drastically decreased in the post-ovulatory females. This expression dynamics of the HPG axis genes are comparable between grass and tiger puffers. Therefore, it is considered that both puffer species may share physiological and neuroendocrine mechanisms which underlie the similar periodic reproduction: both repeat group spawning in spring at certain coastal areas.

In Chapter 5, the role of melatonin in the regulation of HPG axis was examined by the melatonin administration, and possible involvement of moonlight in the melatonin signal that mediates lunar synchronicity in the spawning rhythm was investigated by the measurement of plasma melatonin levels with respect to daily and lunar age-dependent variations. Furthermore, fish were exposed to artificial dim light to examine the impact of moonlight on the synthesis of melatonin. The photoperiodic regulation of reproduction by melatonin has been well established in many vertebrates especially in seasonal breeders. The administration of melatonin induces or inhibits reproduction in animals depending on the species reproductive strategy. In the present study, augmented expression in case of *kiss2*, *kiss2r*, *gnih*, *gnih-r*, *gnrh1*, *gnrh3*, *fshb*, and *lhb* were found after melatonin administration in the mature fish,

indicating a stimulatory role of melatonin in the reproduction of grass puffer. In the mature fish, the plasma melatonin levels clearly showed daily variations with higher levels during nighttime. However, the augment levels at night did not vary according to the moonlight intensity. Furthermore, the responses in the plasma melatonin levels to the dim light conditions at midnight were not consistent in different years. Therefore, it seems unlikely that moonlight could affect the melatonin synthesis and thus function as an important entrainer in the semilunar spawning in this species. This may consistent with the fact that grass puffer is a semilunar spawner that repeats spawning on both new moon and full moon nights. In contrast to the findings of the present study, it has been reported in rabbitfish, a lunar spawner, the plasma melatonin levels vary according to the moon age with high levels at new moon night and low levels at full moon night (Takemura et al., 2004b). This may be due to the difference in the spawning rhythm as rabbitfish spawn once in a month, while grass puffer spawns twice a month on the day of new moon and full moon.

So, it is therefore important to find out the possible involvement of melatonin in the semilunar spawning in the grass puffer. One possible hypothesis may be in the involvement of melatonin in the tidal related rhythm because melatonin receptor gene expression displayed ultradian oscillations (Ikegami et al., 2015). As grass puffer repeats spawning before high tide on the day of only during spring tide, tidal oscillations and circatidal clock mechanism may be a crucial factor in the semilunar spawning in the grass puffer. Recent studies of transcriptome analyses on the diencephalon and pineal gland of the grass puffer demonstrated that more than 200 genes displayed ultradian oscillations in expression (unpublished data). So, there might be a circatidal clock in the brain of grass puffer. Additionally, in the pineal gland of grass puffer, a circadian clock gene (*per1b*) displayed a circadian variation in expression, indicating that there are two different clocks in the grass puffer (Ando et al., 2018). It has been proposed that beating reaction of circatidal (ca. 12.4 hours of cycle) and circadian (ca. 24 hours of cycle)

clocks can produce the semilunar rhythm: two oscillators only coincide once per semilunar month (ca. 14.7 days of cycle) (Bünning and Müller, 1961). Accordingly, it is considered that melatonin signal may be involved in the coordination of the tidal related rhythmicity and the semilunar spawning. Further studies are required to verify this hypothesis and the possible regulatory mechanism that involves circatidal clock and melatonin signal will unveil the currently unknown neuroendocrine system that regulates the semilunar spawning in the grass puffer.

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