A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Role of melatonin on the regulation of reproductive system in the grass puffer, *Takifugu niphobles*

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Department of Marine Life Science

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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CONTENTS

국문요약

어류는 광주기 및 수온의 변화에 적응하여 종 특이적인 산란시기를 갖는다. 어류의 산란시기는 주된 생체리듬의 하나이며, 생체리듬은 다양한 생리현상을 조절한다. 멜라토닌은 생체리듬을 조절하는 주요 조절자로서, 어류의 번식생리에 영향을 미친다. 어류의 멜라토닌은 송과체와 망막에서 분비된다. 이 연구는 복섬의 번식특성과 멜라토닌의 역할에 대하여 조사하였다.

1. 복섬의 생식주기

복섬의 산란시기와 생식주기를 조사하기 위하여 월별 GSI 변화 및 생식소 발달 단계를 조사하였다. 암컷과 수컷 복섬의 GSI는 2월부터 4월까지 서서히 증가하기 시작하였고 난소는 다수의 유구기 난모세포를 가지고 있고, 정소는 정모세포와 정세포들을 가지고 있다. 이후 수컷과 암컷은 각각 5월과 6월에 가장 높은 GSI값을 보였고, 난소는 대부분 난황이 축적된 성숙한 난모세포들을 포란하였다. 정소는 정자로 가득하였다. 이후 GSI는 8월까지 급격히 감소하였으며, 난소에서는 산란 후의 잔존여포와 주변인기 난모세포들이 확인되고, 정소는 방정 후 비어있는 소엽 상태였다. 11월까지 암컷과 수컷 모두 낮은 GSI값을 유지하였으며, 12월부터 GSI가 서서히 증가하기 시작하였다. 이 시기에 난소에 주변인기 난모세포와 유구기 난모세포가 분포하고, 정소에서는 정원세포와 정모세포가 관찰되었다. 생식세포 발달 단계와 GSI 변화에 따른 복섬 암컷의 생식주기는 2월~3월까지의 성장기, 3월~6월까지의 성숙기, 6월과 7월의 산란기, 7월~10월의 휴지기 그리고 이듬해 1월까지 초기성장시기로 구분되며, 수컷의 생식주기는 암컷과 유사하게 나타났다. 이 결과 복섬의 산란시기는 6월과 7월로 추정된다. 광주기와 수온변화에 따른 생식세포 발달은 광주기가 길어지고 수온이 증가하는 시기에 일어나고 있다.

2. 생식주기에 따른 멜라토닌분비와 성성숙인자들의 발현 변화

복섬의 생식주기에 따른 멜라토닌분비와 성성숙인자들의 발현 변화를 탐색하기 위하여 망막멜라토닌의 일주기 리듬과 계절별 분비 변화를 조사하였다. 그리고 복섬의 성성숙시기 동안의 멜라토닌 합성효소의 발현리듬변화와 성숙관련인자들의 발현 변화를 조사하였다. 망막멜라토닌의 일주기 리듬 조사 결과, 멜라토닌 분비는 주로 야간에 증가하는 nocturnal 리듬을 보였다. 또한 망막 멜라토닌 분비는 복섬의 성숙기에 가장 활발하였다. 망막 멜라토닌합성효소인 *aanat1a* mRNA는 성숙기와 휴지기에 주야간 발현차이가 있으며, *aanat1b* mRNA는 성장기를 제외한 성숙기, 휴지기 그리고 초기성장기에 낮과 밤의 발현차이기 있었다. 송과체 멜라토닌 합성효소인 *aanat2* mRNA는 오직 성숙기에만 낮과 밤의 발현차이가 있다. 성성숙인자들의 발현 변화를 보면, *sbGnRH* mRNA는 암수 각각 성숙기와 휴지기에 유의적으로 높게 발현하였고, *chGnRH* mRNA는 성장기, *saGnRH* mRNA는 초기성장기에 높게 발현하였다. *Kiss2* mRNA와 *lpxrfa* mRNA는 암수 모두 초기성장기에 발현이 높았다.

이러한 실험 결과 복섬의 망막멜라토닌은 nocturnal 리듬을 갖으며, 성숙기에 분비가 활발하게 이루어짐을 확인하였다. 성성숙인자들은 생식소발달단계에 따라 특이적으로 발현하였으며, 초기성장기에 대부분 성숙관련 인자들이 높게 발현하는 경향을 확인하였다.

3. 멜라토닌과 광주기가 성성숙에 미치는 영향

멜라토닌이 복섬의 성성숙에 대한 영향을 알아보기 위하여 일련의 실험을 수행하였다. 첫 번째, 광주기 조건에서 단주기(9L:15D)와 장주기(15L:9D)가 멜라토닌합성효소의 발현리듬에 미치는 영향을 조사하였다. 망막에서 *aanat1a* mRNA는 모든 광주기 조건하에서 nocturnal 리듬을 보였고, 광주기 길이에 따라 peak time이 달랐다. *aanat1b* mRNA는 단주기 조건에서 특이적으로 주야간의 발현리듬이 없었고, 장주기조건에서 주간에 발현이 야간 보다 높았다. 뇌에서의 *aanat2* mRNA는 모든 광주기 조건에서 nocturnal 리듬을 보였으나, 그 리듬은 광주기 길이와 상관없이 일정하였다. 이 결과를 통하여 망막에서의 멜라토닌합성

효소는 *aanat1a* mRNA가 *aanat1b* mRNA보다 빛에 대한 멜라토닌분비에 관여하며, 송과체에서의 *aanat2* mRNA는 circadian 리듬에 따라서 멜라토닌 분비에 관여하는 것으로 생각된다.

두 번째, 멜라토닌과 성성숙인자들의 상관관계를 조사하기 위하여, 멜라토닌(1 mg/kg)을 매일 정오에 2주 동안 복강주사하였다. 멜라토닌 처리한 실험구의 뇌에서 *chGnRH* mRNA발현이 증가하였고, *sbGnRH* mRNA와 *saGnRH* mRNA는 발현차이가 없었다. *Kiss2* mRNA는 발현이 억제되는 반면, *lpxrfa* mRNA는 발현이 증가하였다. 뇌하수체에서 *GtHs* mRNA는 유의적인 차이가 없었다. 이 결과를 통하여 인위적인 멜라토닌의 처리는 *kiss2* mRNA의 발현 억제 및 *lpxrfa* mRNA의 발현을 증진시켜 멜라토닌이 성성숙을 억제 하는 것으로 생각된다.

세 번째, 광주기가 성성숙에 미치는 영향을 조사하기 위하여 각각 단주기와 장주기 조건에서 14주간 복섬을 사육하였다. 성성숙인자들의 발현 조사결과 오직 *kiss2* mRNA와 *lpxrfa* mRNA가 장주기 조건에서 단주기 조건보다 발현이 유의적으로 높았고, 다른 성성숙인자들의 유의적인 발현차이는 나타나지 않았다. GSI는 장주기 조건에서 유의적으로 높았으며, 생식소 발달단계는 단주기조건에서 유구기 난모세포들이 분포하는 성장기였으나, 장주기 조건에서는 성숙한 난모세포들이 분포하는 성숙기였다. 이를 통하여 광주기조절은 멜라토닌의 분비와 번식인자들의 발현을 조절하고, 이를 통하여 성성숙을 제어하는 것으로 생각된다.

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Chapter Ⅰ. Reproductive cycle in grass puffer *Takifugu niphobles*

Abstract

Fish have specific optimal spawning cycle depending on environmental factors such as water temperature and photoperiodic conditions. The aim of the present study was to investigate the reproductive cycle in the grass puffer, *Takifugu niphobles*, with annual photoperiod and water temperature. Fish were sampled once a month from February 2013 to January 2014, where we examined the monthly change in gonadosomatic index (GSI) and hepatosomatic index (HSI). In addition, we examined the phase of gonadal development based on histological observation. Day lengths of sampling site was longest in June while shortest in December. Female and male HSI levels increased from September to December. Female GSI levels showed a rapid increase from April to June, and decreased in July. Male GSI levels also increased from April and peaked in May, and then showed a rapid decrease until July. Based on histological findings, ovaries showed frequent yolk stage oocytes (YO) from April to June, meanwhile postovulatory follicles (POF) were identified in July. Spermatozoa (SZ) were found in the testis from May to July, meanwhile in July and August, we observed an empty cavity, reflecting an after-spermation testis. Based on the histological microscopy of ovarian and testicular development phase, we divided the reproductive cycle of the grass puffer into early growing, growing, maturing, and resting phases.

According to our experiment results, the main spawning season of grass puffer is suggested to be from June to July, and sexual maturation progressed with increased day length and water temperature. Our results suggest that sexual maturation in grass puffer was correlated with changes in the photoperiod and water temperature. Further studies are needed to investigate the processes involved in these environment-based effects on the reproductive mechanism.

1. Introduction

Animals have various biological rhythms influenced by the photoperiods due to the earth's revolution and rotation. Fish spawning cycle is one of such biological rhythms, which is influenced by annual photoperiod fluctuation and water temperature. These factors also determine sexual maturation in fish (Bromage et al., 1993; Biswas et al., 2004; Suquet et a., 2005; Targońska et al., 2014). Thus, fish spawning cycle is a reproductive strategy that is adapted to the environment. Photoperiod and water temperature signals affect the brain-pituitary-gonadal (BPG) axis, and regulate the fish reproductive endocrine system (Shimizu, 2003; Howell et al., 2003; de Almeida et al., Carrillo et al., 2009; 2011; Levy et al,. 2011; Andersson et al., 2013; Shao et al., 2013). Particularly, photoperiod signals are transmitted to the brain through the sense organ, retina, and the pineal organ. These photoperiod signals stimulate the production of numerous reproduction-related factors, including the gonadotropin-releasing hormone (GnRH) in the brain. GnRH is synthesized and secreted in the hypothalamus, and it is widely known as a typical reproductive factor in vertebrates, including fish. GnRH is a decapeptide, and plays a central role in the reproductive system. GnRH stimulates gonadotropin (GtH) synthesis and secretion in the pituitary gland. Two GtHs, follicle stimulating hormone (FSH) and luteinizing hormone (LH), induce sex-steroid hormone secretion in the gonads. These hormones affect the secretion of GnRH and GTH by feedback actions. Based on this, many studies are being conducted to regulate the reproductive endocrine system and spawning cycle in some fishes using photoperiod. In a study concerning female Eurasian perch *Perca fluviatilis*, an artificial short photoperiod treatment (17L:7D) provoked a delay in the onset of their reproductive cycle (Abdulfatah et al., 2011). The regulation of sexual reproduction by manipulation of photoperiod conditions was also reported in European sea bass *Dicentrarchus labrax L.* (Begtashi et al., 2004), Chinook salmon *Oncorhynchus tshawytscha* (Unwin et al., 2005), Atlantic cod *Gadus morhua* (Cowan et al., 2012) and olive flounder

Paralichthys olivaceus (Kim et al., 2013)

The grass puffer *Takifugu niphobles*, from the family Tetraodontidae, was used in this study. Grass puffer has a unique spawning behavior; spawning occurs forming a group at the beach during low-tide influenced by lunar cycles (Yamahira, 1994; Oh et al., 2000). Grass puffers are easy to collect, because spawning occurs periodically at the same place. Grass puffers are also appropriate for use as experimental animals because of easy handling owing to their small size.

The objective of this study was to examine the reproductive cycle in grass puffer in Jeju coastal water. In this study, we investigated the monthly changes of gonadosomatic index (GSI), hepatosomatic index (HSI) and changes in the gonadal development phase.

2. Materials & methods

2.1. Fish

Grass puffer were captured using the net from harbor of Shinhung, Jocheon, Jeju, South Korea (Fig. 1). The collected fish kept in Marin Science Institute, Jeju National University. Fish were reared under natural photoperiod and temperature conditions in indoor tank before starting experiment. The fish were fed commercial pellets (Daehan co., MP3, Busan, South Korea) twice a day. Grass puffer is sufficiently adapted and used in the following experiments.

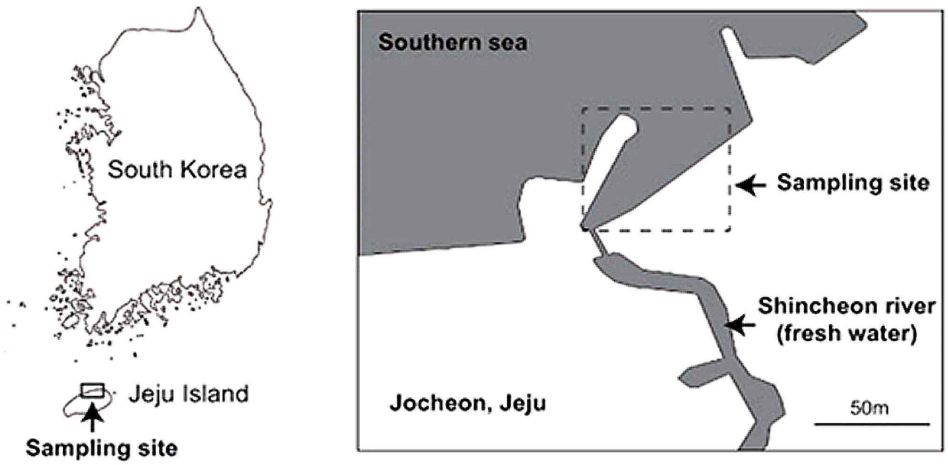

Fig. 1. Grass puffer sampling site. Fish captured from harbor of Shinhung, Jocheon, Jeju, South Korea.

2.2. Hepatosomatic index (HSI) and gonadosomatic index (GSI) of grass puffer

To investigate sexual maturation and gonadal development phase in grass puffer, fish (n=394, body weight 37.1±1.0 g, total length 14.3±0.3 cm) were sampled once a month from February 2013 to January 2014. The fish were anesthetized with 0.05% of MS-222 (Sigma, USA) before sampling and extracted liver with gonad tissues. Extracted liver and gonad tissues were weighted for calculation of the hepatosomatic index (HSI) and gonadosomatic index (GSI). For the histological analysis, extracted ovary and testis were immediately fixed in Bouin's solution.

2.2. Histological observation

Fixed gonad samples by Bouin's solution were dehydrated in a graded series of ethanol, embedded in paraffin and sectioned 5μm thickness. For histological observation, Slide were stained Gill's hematoxylin and 0.5% eosin. Microscopy of the ovary and testis development was performed using a light microscope (Olympus, Tokyo, Japan) with cellSens Standard software (Olympus, Tokyo, Japan).

2.7. Statistical Analysis

Calculated index of GSI and HSI levels were presented as mean ± standard error (SEM).

3. Results

3.1. Monthly changes of HSI and GSI

Monthly changes of HSI and GSI levels in grass puffer were shown compare with annual photoperiod fluctuation and water temperate changes in during all the experiment period (Fig. 2 and 3).

Photoperiod of February was approximately 11 h light and 13 h dark (11L:13D) and showed that the day length prolonged gradually to June (14.5L:9.5D). The peaked photoperiod in June was gradually shortened to December (9.5L:14.5D) but photoperiod again prolonged in January. The water temperature was increased from February (12.3℃) to August (27.5℃) after that, water temperature reversely decreased to January (15.7℃).

Female HSI levels was gradually decreased from February (7.5±0.4) to September (2.8±0.2) (Fig. 2). After that, HSI levels dramatically increased to December (20.4±2.3) and again decreased in January (13.7±1.6). Male HSI was maintained basal levels from February (8.4±0.5) to August (8.7±0.6) and steadily increased to December (19.1±2.5). In January, male HSI levels was decreased (16.8±1.6).

Grass puffer GSI levels showed similar annual change in both sexes (Fig. 3). Male and female GSI levels were gradually increased from February (male 1.6±0.2 and female 2.4±0.1) to April (male 3.8±0.7 and female 3.8±0.6) and significantly highest each at May (male 10.1±0.9) and June (Female 15.0±1.1). After that, each male and female GSI levels were rapidly decreased to August (female 1.1± 0.0, male 0.8±0.1) and showed again increased GSI levels in December (male 3.1±0.5 and female 4.2±0.3).

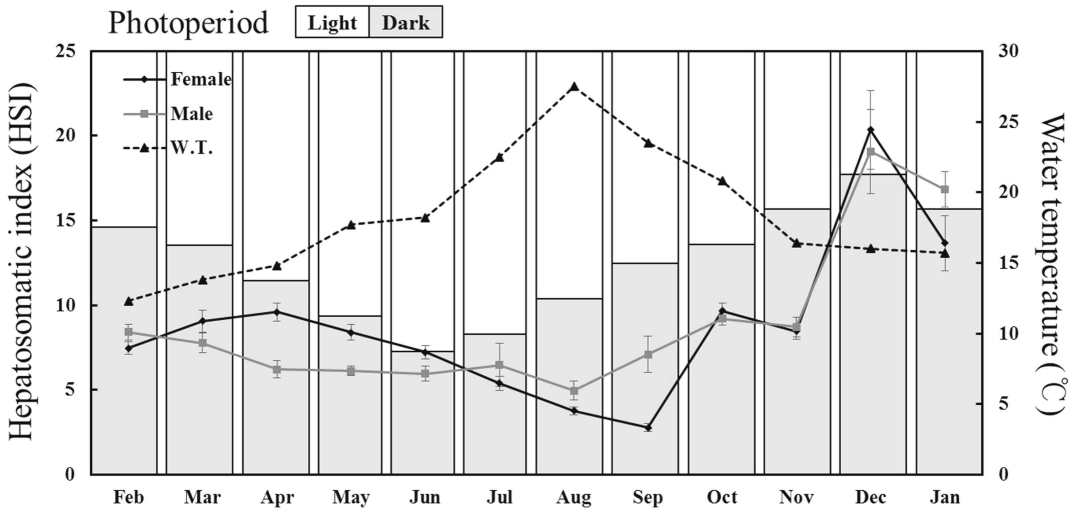

Fig. 2. Monthly changes of hepatosomatic index (HSI) in female (black line) and male (gray line) grass puffer. Monthly changes of water temperature (dash) and photoperiod (gray bars) showed in this figure during all the experimental period.

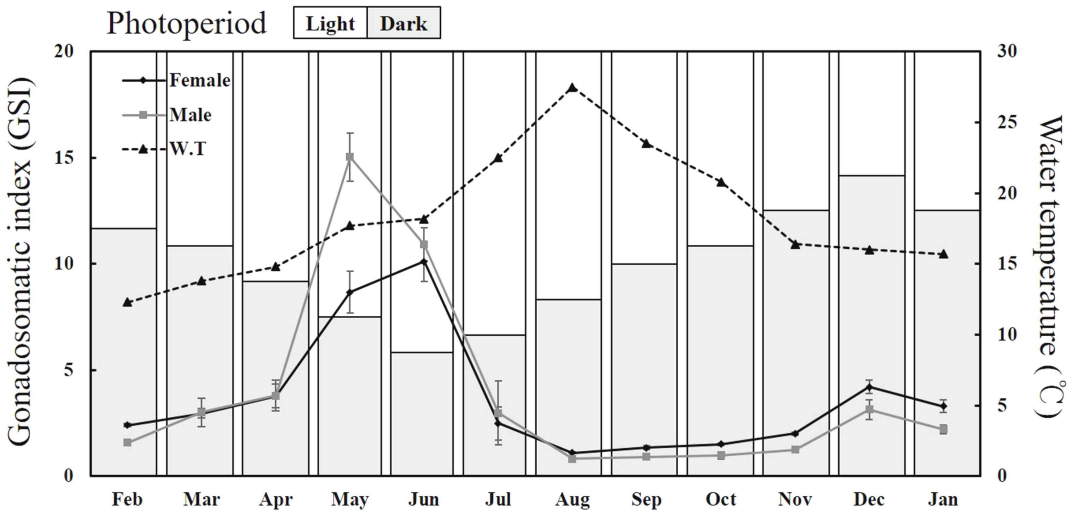

Fig. 3. Monthly changes of gonadosomatic index (GSI) in female (black line) and male (gray line) grass puffer. Monthly changes of water temperature (dash) and photoperiod (gray bars) showed in this figure during all the experimental period.

3.2. Gonadal development phase

Changes of ovary development phase are shown in Fig. 4. In February and March 2013, the ovaries showed growing phase and mainly observed oil-droplet stage oocytes (ODS) of 170 to 250 μm in diameter (Fig. 4A and 4B). The ovaries of mature phase were identified from April to June and that ovaries contained the yolk stage oocytes (YO) of 280 to 450 μm in diameter (Fig. 4C to 4E). In July, ovaries showed after spawning and observed postovulatory follicle (POF) (Fig. 4F). In August and September, the ovaries showed immature phase and contained mainly perinucleolus stage oocytes (PNS) of 20 to 100 μm in diameter (Fig. 4G and 4H). From October to January 2014, the ovaries were maintained early growing phase and observed PNS with ODS (Fig. 4I to 4L).

Changes of testicular development phase are shown Fig. 5. For testis of growing phase observed spermatogenesis from spermatogonia (SG) to spermatid (ST) in February to April (Fig. 5A to 5C). Spermatozoa (SZ) were first detected in mature phase testis from May and it filled in the testis until July (Fig. 5D to 5F). In August, for testis observed empty cavity after spermation (Fig. 5G) and testis were mainly comprised for spermatogonia in September (Fig. 5H). Early growing phase of testis were showed spermatogonia with spermatocyte (SC) from October to January 2014 (Fig 5I to 5L).

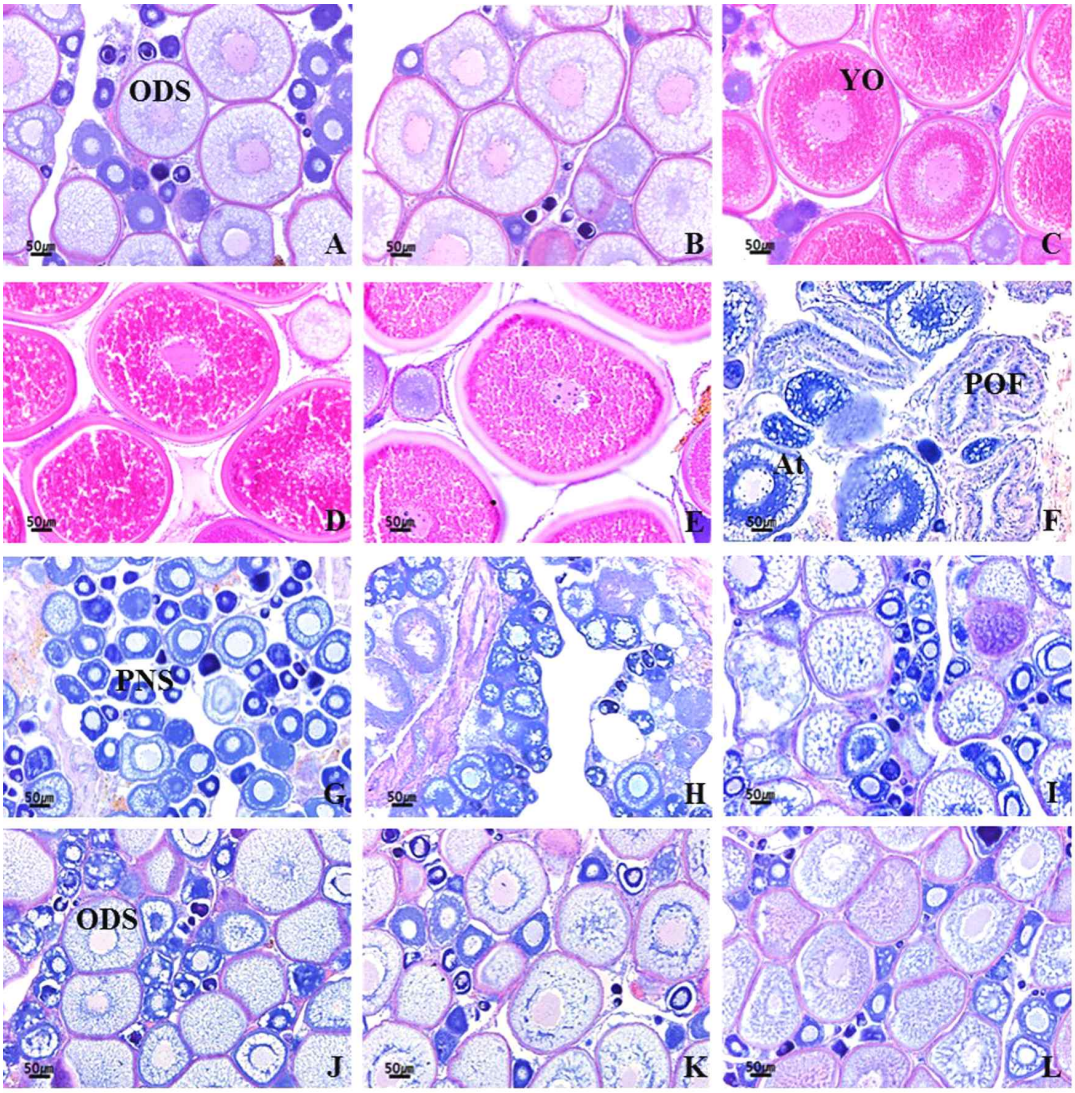

Fig 4. Photomicrograph of ovarian development phases of grass puffer from February, 2013 (A) to January, 2014 (L). Scale bar indicates 50 μm. At, atresia oocyte; PNS, peri-nucleolus stage; POF, postovulatory follicle oocyte; ODS, oil-droplet stage; YS, Yolk stage.

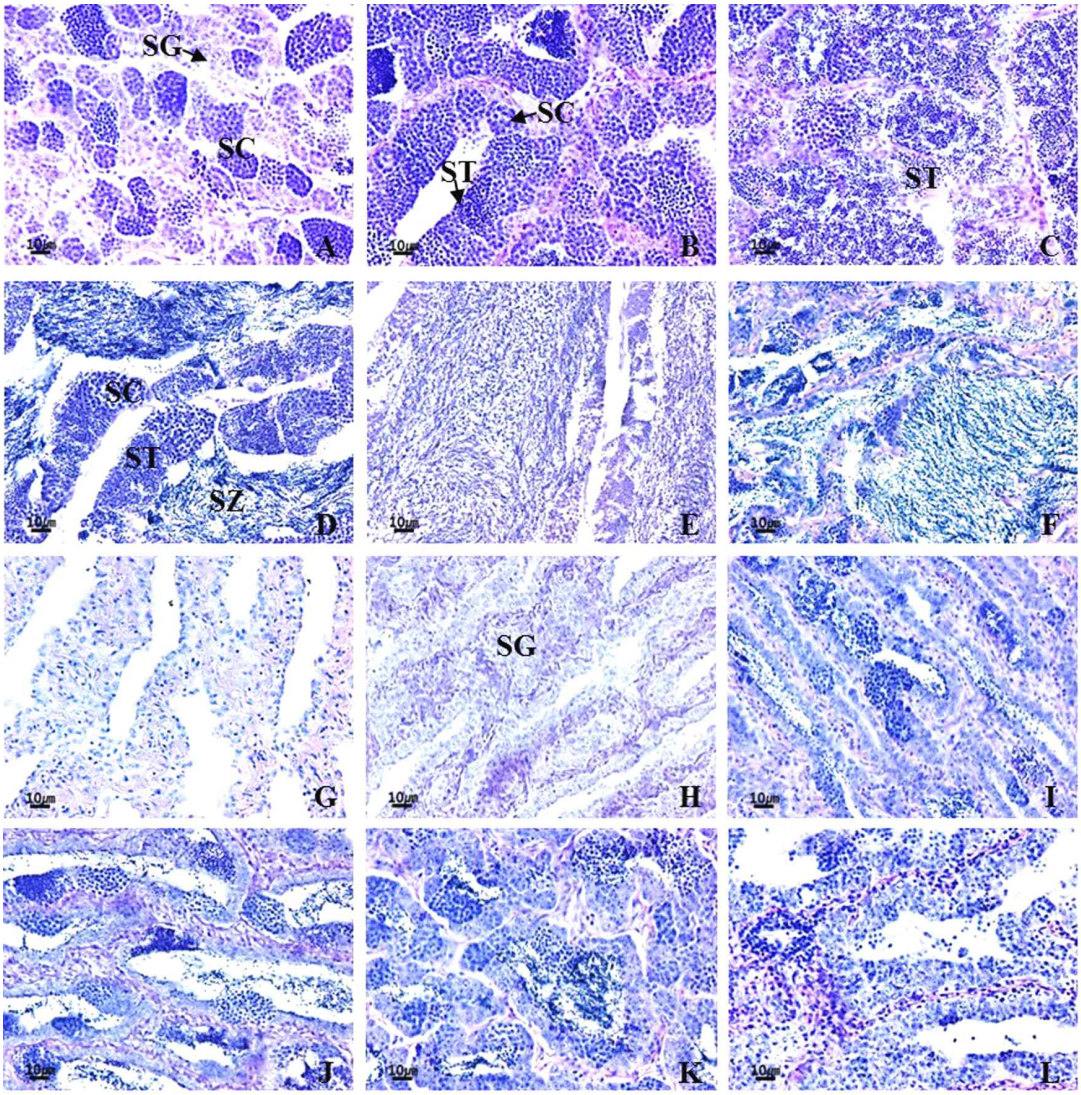

Fig. 5. Photomicrograph of testicular development phases of grass puffer from February, 2013 (A) to January, 2014 (L). Scale bar indicates 10 μm. SC, spermatocyte; SG, spermatogonia; ST, spermatid; SZ, spermatozoa.

4. Reproductive cycle of grass puffer

The ovarian and testicular developmental phase of grass puffer showed in Fig. 6A and B. Each gonadal development phase was divided into four phases, early growing phase, growing phase, maturing and spawning or ejaculating phase and resting phase.

In the female reproductive cycle investigation results (Fig. 6A), individuals of early growing phase were first observed in February. Numerous perinucleolus stage and some oil-droplet stage oocytes observed in this phase. This phase again observed from October after spawning season. Growing phase ovaries also observed in February and in this ovaries contained the numerous oil droplet stage oocyte. Number of perinucleolus stage oocytes were decreased than early growing phase. Individuals of maturing phase first observed in March and ovaries contained numerous yolk stage oocytes. Spawning phase ovaries appeared from May to July and in this ovaries observed postovulatory follicle. In resting phase ovaries contained mainly perinucleolus stage oocytes and this phase observed from July to December.

In the male reproductive cycle investigation results (Fig. 6B), individuals of early growing phase first observed in February. Early growing phase testis contained numerous spermatogonia and spermatocytes. Growing phase of testis showed numerous spermatocytes and spermatids. This testis phase was appeared from February to April before ejaculating phase and again appeared from November after spermation. Mature phased of testis showed from April to July and observed mainly spermatozoa in this period. The testicular lobules empty after spermation was observed in this period. In resting phase testis observed from August to January and contained mainly spermatogonia.

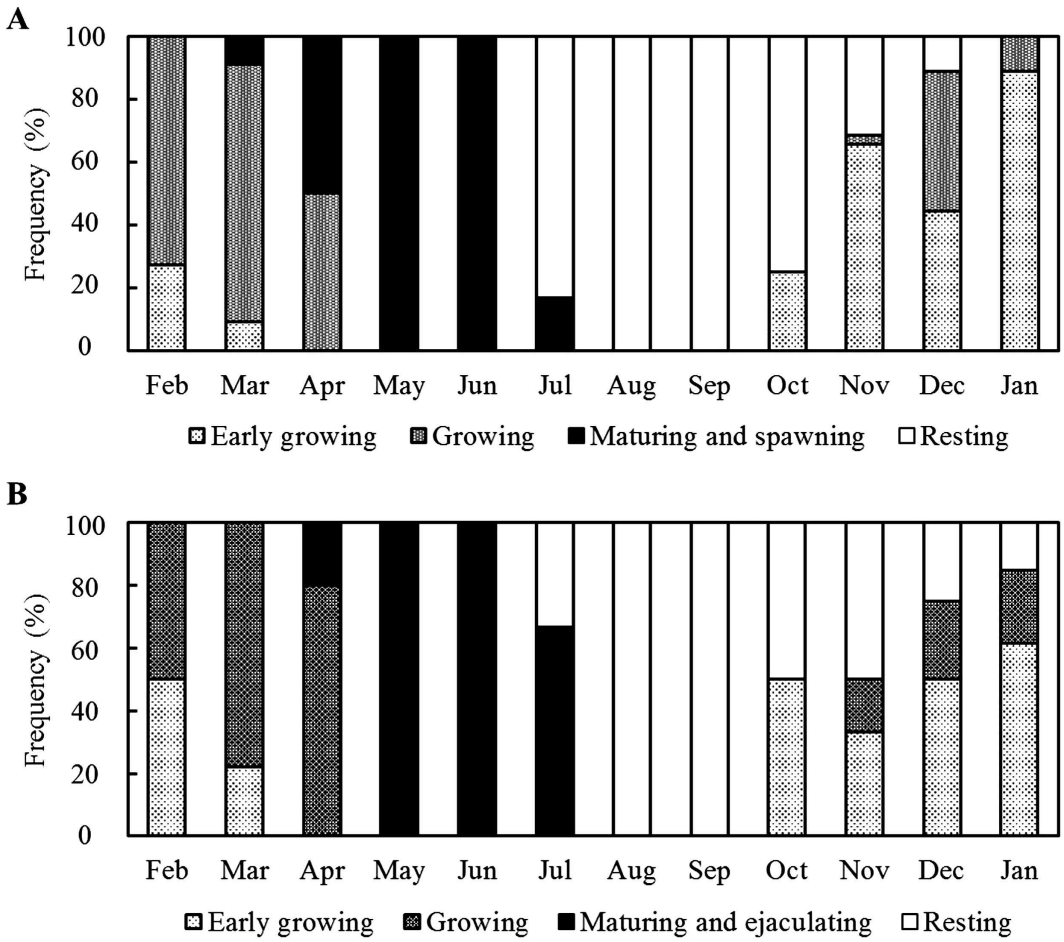

Fig. 6. Frequency of ovarian (A) and testicular (B) development phase in grass puffer from February, 2013 to January, 2014.

4. Discussion

Fishes have species-specific sexual maturation and spawning cycle determined by suitable environmental conditions (photoperiod, water temperature, and nutrient state). In this study, we examined the sexual maturation and spawning cycle of grass puffer based on the monthly gonadal developmental phase and processes under natural photoperiod and considering water temperature conditions.

Concerning sexual maturation of grass puffer, we examined the associations between monthly changes of GSI and HSI levels with changes in the photoperiod and water temperature. Females and males' GSI levels were steadily increased from February to April. In this period, gonadal development phase corresponded to a growing phase in both females and males. GSI levels of males and females showed a peak in May and June, respectively. During this period of increased GSI levels, the day length increased accordingly, showing the longest day length in June. In addition, water temperature was also steadily increased; with the highest values observed in August. The following reduction of GSI levels revealed until July, suggests that this period would correspond to the spawning season of grass puffer. During spawning season, photoperiod showed longer day-lengths, which started to shorten in July. After spawning season, GSI maintained basal levels until November. In this period, day length and water temperature tended to decrease gradually. Again, GSI levels increased from November to January. During this period, we observed the shortest day length (in December), meanwhile water temperature showed a continuous decline. Furthermore, based on histological analysis, changes in GSI levels were related to monthly gonadal developmental phase. According to the results of the ovarian developmental phase, mature oocytes were observed from April to June, and as a feature of post ovulation, POF were observed in July. According to the results of the testicular developmental phase, SZ in testes were observed from May to July, and empty internal testes after spermation were observed from August.

According to our results, grass puffer gonad developmental stages could be divided

in the following phases: growing phase (February to March), maturing and spawning phase (April to July), resting phase (August to October), and early growing phase (November to January), where the main spawning season is considered to be in June and July. Our results are consistent with prior studies of grass puffer spawning season from the coast Dolsan, Korea (Oh et al., 2000). In this results, grass puffer spawning season showd to be from May to July.

In addition, our study compared the monthly GSI levels changes of grass puffer with annual photoperiod fluctuation and water temperature. We found that sexual maturation occurred along with longer photoperiod and increased water temperature.

Photoperiod is known to play an important role in the timing of sexual maturation; in some fishes, sexual maturation has been regulated by means of photoperiod manipulation (Bromaga et al., 2001; Davies and Bromage, 2002; Begtashi et al., 2004; Cowan et al., 2012). On the other hand, water temperature was reported to be a key factor for final gamete maturation and ovulation (Anguis and Canavate, 2005). In a study conducted with salmonids, elevated temperature during gametogenesis induced gonadal steroid synthesis and vitellogenin production (Pankhurst and King, 2010). Our study suggests that grass puffer sexual maturation is stimulated by changes in the photic signal, from a short photoperiod to a long photoperiod, and that increased water temperature during sexual maturation affects gametogenesis. Further studies are required to better understand the association of annual photoperiod fluctuation and water temperature changes with reproduction.

We also observed a rapid increase in HSI levels from September to December, which corresponded to gonadal resting and early growing phases in both sexes of grass puffer. During this period, the day length was gradually shortening, and water temperature was gradually decreasing. The increase in HSI levels was suggested to occur due to energy storage in the liver, as a preparation for sexual maturation or for winter spend. Liver is a major endocrine organ, and affects many physiological processes, including growth, immune response, and sex maturation (Schulz et al., 2009; Dai et al., 2015; Li et al., 2015). Especially, vitellogenin synthesized by

gonadal estradiol-17β and affects the regulation of oocyte vitellogenesis (Nagahama et al., 1994). For this reason, the decrease in HSI levels observed in grass puffer during sexual maturation and spawning seasons, it is suggested to occur due to energy consumption in liver based on the gametogenesis induction in the gonads.

In conclusion, the main spawning season of grass puffer was in June and July, demonstrated by histological observations of ovarian and testicular developmental phase. In addition, we suggest that the regulation of grass puffer sexual maturation is influenced by annual photoperiod fluctuations and water temperature changes. However, deeper studies are needed in the future.

Chapter Ⅱ. Seasonal melatonin variations and reproduction-related genes expression in grass puffer

Abstract

Environmental changes stimulate the brain-pituitary-gonadal (BPG) axis, and may activate the reproductive system. In this BPG axis, the GnRH is synthesized in the hypothalamus, and consequently regulates the GtHs secretion from the pituitary. Recent studies, kisspeptin and LPXRFa peptide identified in the hypothalamus. Kisspeptin mainly plays a role in the production of GtH synthesis and release, but LPXRFa plays a role in the inhibition of GtH synthesis.

The aim of the present study investigate the seasonal melatonin secretion and reproductive-related genes expression during sexual maturation of the grass puffer, *Takifugu niphobles*. For this purpose, we first examined the ocular melatonin oscillation under natural photoperiod conditions. We observed that ocular melatonin increased during scotophase. The concentration of seasonal ocular melatonin was significantly higher in gonadal maturing phase compared with other gonadal development phases. The scotophase expression of ocular *aanat1* subtype genes and brain *aanat2* was increased mainly during maturing or resting phases. In addition, we investigated the reproductive-related genes expression in the brain under different gonadal development phases. In female, the expression of *sbGnRH*, *chGnRH* and *saGnRH* mRNA were significantly higher in resting, growing and early-growing phases; on the other hand, their expression in males were higher in maturing, growing and early-growing phases. The *kiss2* and *lpxrfa* mRNA expression in both sexes was significantly higher in early-growing phase compared with other gonadal development phases. In addition, seasonal *igf-1* expression differed between sexes; in females, it showed a peak during November, and in males, the peak was observed in February. According to our results, seasonal variation of ocular melatonin suggested effect upon sexual maturation in grass puffer. Brain reproductive-related factors suggested that each gene acts independently, according to different gonadal development phases. Further studies are needed to clarify the relationship between melatonin and reproductive-related genes.

1. Introduction

External photic signals, in particular changes in light/dark patterns during the day, affect melatonin synthesis and secretion in the pineal organ. Melatonin is an indole-derived hormone synthesized in the pineal organ in mammals, although in teleost fishes it is also synthesized in the retina. Melatonin is synthesized via the activation of arylalkylamine N-acetyltransferase (AANAT) enzyme; thus, nocturnal melatonin secretion depends on the activation of AANAT during scotophase. Teleost fishes have two AANAT genes (AANAT1 and AANAT2), which are mainly expressed in the retina and pineal organ (Coon et al., 1999). Recently, studies have identified the existence of two AANA1 genes (AANAT1a and AANAT1b) in the retina of some fish species (Coon and Klein, 2006). However, until now, studies concerning the role or function of both AANAT1a and AANAT1b genes have not been conducted. . In most teleost fish, the nocturnal secretion rhythm of melatonin has been confirmed and explored (Migaud et al., 2006; Seth and Maitra, 2010). For example, in the European sea bass *Dicentrarchus labrax* (Bayarri et al., 2004), and in the Mozambique tilapia *Oreochromis mossambicus* (Nikaido et al., 2008), studies have observed the plasma oscillation of melatonin levels in different light/dark photoperiod conditions. It is widely known that melatonin is involved in a variety of physiological actions, including reproduction (García-Allegue et al., 2001; Amano et al., 2004; Herrero et al., 2007; De Pedro et al., 2008; Maitra et al., 2013).

The fish reproductive system is based on a BPG axis, with various factors involved in sexual maturation. GnRH is synthesized in the hypothalamus, and has a major role in controlling the GtH secretion in the pituitary gland. In vertebrates, GnRH has two or three distinct forms, GnRH1, GnRH2 and GnRH3. The GnRH1 is known to have a species-specific form in vertebrates, including the sea bream GnRH (sbGnRH), pejerrey GnRH (pjGnRH), catfish GnRH (cfGnRH), among others. GnRH1 has an important role in gonadal development and in the induction of GtH in the pituitary gland (Kah et al., 2007). The chickens' GnRH2 is the GnRH-II

(chGnRH-II), and plays a role in sexual and feeding behavior (Volkoff and peter, 1999; Millar 2003). In salmons, the GnRH3 is the salmon GnRH (saGnRH), and its function its associated with reproductive behavior in some fishes (Ogawa et al., 2006). However, the role and regulation mechanisms for reproductive system of these three GnRHs in fishes are still unclear; although they are suggested to be different between fish species.

Recently, kisspeptin, a member of the RF-amide family, and its receptor (G-protein-coupled receptor 54, GPR54) attracted public attention based on their key role associated with reproduction control and the onset of puberty in mammals (Roa et al., 2008). In cichlid fishes, GPR54 expression was confirmed in GnRH neurons, suggesting that GnRH may be regulated by the GPR activation, which is activated by kisspeptin signals (Parhar et al., 2004). In teleost fishes, kisspeptin has revealed multiple forms (kiss1 and kiss2), isolated in species such as the zebrafish *Danio rerio* (Brian et al., 2008), fathead minnow *Pimephales promelas* (Filby et al., 2008), chub mackerel *Scomber japonicas* (Selvaraj et al., 2010) and the rohu *Labeo rohita* (Saha et al., 2016). According to recent reports, it is plausible that kisspeptin controls reproduction by means of PBG axis activation in fishes (Filby et al., 2008; Kim et al., 2013). For example, in the white bass *Morone chrysops*, chronic kisspeptin administration provoked advanced oocytes (Beck et al., 2012).

LPXRFamide peptide (LPXRFa) was a member of the RF-amide family and recently discovered in the hypothalamus of quails *Coturnix japonica* (Tsutsui et al., 2000). LPXRFa mainly plays a role in the inhibition of GtH synthesis and release. For example, studies concerning quails revealed the direct inhibition of GtH release and gonadal development by LPXRFa (Ubuka et al., 2006). In some fish species, such as the gold fish *Carassius auratus* and zebrafish, studies confirmed the regulatory role of LPXRFa in reproduction, particularly in GtH regulation (Zhang et al., 2010; Moussavi et al., 2011). However, information concerning LPXRFamide in teleost fish is still scarce, and with several inconsistencies among species-specific results (Biran et al., 2014).

The aim of this study is to identify annual variations of melatonin and reproductive-related factors during sexual maturation in grass puffers. In the present study, we investigated daily oscillations and seasonal variations of ocular melatonin in grass puffer. In addition, we identified of IGF-1 cDNA in the liver and investigated seasonal variations of reproductive-related genes (*GnRHs*, *kiss2*, *lpxrfa* and *igf-1*) and *aanats* genes expression.

2. Materials & methods

2.1. Experiment design

2.1.1. Daily oscillation of ocular melatonin

Experiment was performed twice in February (n=48, body weight 36.4±1.5 g, total length 12.5±0.2 cm) and May (n=56, body weight 31.2±0.9 g, total length 10.7±0.3 cm). In each month, fish were adapted under natural photoperiod condition and natural water temperature in indoor tank. In February, natural environment conditions showed approximately 11L and 13D (sunrise at 07:11 and sunset at 18:20) of photoperiod and water temperature was 12.3±0.1℃. Environmental conditions in May, photoperiod was approximately 14L and 10D (sunrise at 05:14 and sunset at 19:45) of photoperiod and water temperature was 12.3±0.1℃. Fish were reared in during 1 week after that anesthetized by MS-222 (Sigma, USA) and sampled total 8 times at 3 h intervals for 1 day by decapitation. The sampling at darkness time was conducted under red dim light, and retina was collected for measure of melatonin by time-resolved fluoroimmuno assay (TR-FIA). The collected samples were kept at -80℃ until the analysis.

2.1.2. Ocular melatonin and *aanats* genes variations during sexual maturation.

For this investigation, grass puffer captured and kept in indoor tank under natural photoperiod and water temperature. Fish (n=100, body weight 37.5±1.2 g, total length 12.2±0.2 cm) sampled total 4 time in three month intervals (February, May, August and November in 2013). Fish sampling was performed at Clock time (CT) 12 in during photophase and three times (CT21, CT24 and CT3) in during scotophase. In during scotophase, sampling performed under red dim light. Fish retina was extracted

and used the melatonin concentration and *aanat1* subtype genes expression measurement by time-resolved fluoroimmuno assay (TR-FIA) and real time-qPCR respectively. Also, fish brain was collected for investigation of *aanat2* mRNA expression by real time-qPCR respectively.

2.1.3. Seasonal variations of reproduction-related genes and *igf-1* expression.

For this investigation, grass puffer sampling was performed in the same manner as in the previous experiment (experiment design 2.1.2). However, fish sampling time was differently proceeded with previous experiment and only performed at midday (CT12). Grass puffer were separated the male (n=25, body weight 37.5±1.3 g, total length 12.6±1.4 cm) and female (n=25, body weight 42.6±1.7 g, total length 13.3±0.2 cm) through dissection and collected the brain and liver. Collected brain and liver tissues were performed in each reproduction-related genes (*GnRHs*, *kiss2* and *lpxrfa*) and *igf-1* gene expression analysis by real time-qPCR.

2.2. IGF-1 cDNA partial cloning and sequence analysis

For IGF-1 cDNA partial cloning, degenerated primer set were designed on the basis of regions of high identity from IGF-1 nucleotide sequences of tiger puffer *Takifugu rubirpes* (GenBank Accession Numbers: AB465576). cDNA was amplified by RT-PCR using a degenerate primer set (Table 1). PCR was performed initial denaturation at 94 ℃ for 5min and 30cycles each of denaturation (94 ℃ for 45 s), annealing (55 ℃ for 45 s), and extension (72 ℃ for 1 min). In the last cycle, the extension time was increased to 3min. PCR products were identified by electrophoresis on 1 % of agarose gel followed by ethidium bromide staining and UV transillumination. The desired size band of PCR products were purified using DNA purification system (Promega, Madison WI USA). Purified DNA fragments were cloned into the T-blunt vector (Solgent, Daejeon, Korea) and transformed.

Plasmid DNA was extracted according to the manufacturer's instructions of Wizard® SV 96 Plasmid DNA Purification System (Promega, Madison, WI, USA). Extracted plasmid DNA was sequenced using a high throughput DNA analysis system (Genotech, Korea). Fragment sequences of grass puffer IGF-1 cDNA was confirmed by BLAST analysis. Analysis of nucleotide sequences were performed using BLASTN (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA). Multiple alignment of amino acid sequences were analyzed using the Clustal Omega software. A phylogenetic tree was constructed by PHYLIP package (ver. 3.63, J. Felsenstein, University of Washington, Seattle, WA, USA).

Table 1. Degenerate primer sets used in IGF-1 cDNA partial cloning

Primers	Sequence (5'-3')	GenBank Accession No.
IGF-1-F	TCTCGCTACTGCTGTGCATC	AB465576
IGF-1-R	GCATTGCCTCGACTTGAGTT	

2.3. Tissue specific expression of *aanats* and *igf-1* genes

For analysis of tissue specific expression, grass puffer (n=3, body weight 61.3±15 g, total length 14.9±0.2 cm) brain was extracted and divided to six portions each olfactory lob (Of), telencephalon (Te), optic tectum (Op), diencephalon (Di), cerebellum (Ce) and medulla oblongata (Me). Further pituitary (Pt), retina (Re), gill (Gi), heart (He), liver (Li), kidney (Ki), spleen (Sp), intestine, testis (Tes), ovary (Ov) and muscle (Mu) also contained analysis of tissue distribution. Extracted all tissues were immediately froze at -80℃ and tissue distribution analysis of *aanats* and *igf-1* genes were performed using the real time-qPCR analysis.

2.4. Total RNA extraction and cDNA synthesis

Collected samples were absolutely homogenized with RNAiso Plus (Takara, Kyoto, Japan) reagent and for total RNA extracted following the manufacturer's protocol. For prevent genomic DNA contamination, extracted total RNA was treated using RQ1 RNase-Free DNase (Promega, Madison, WI, USA). Total RNA concentration were measured using the Nano Vue (GE Healthcare, Ver.1.0.1, UK) and cDNA was synthesized using the 500 ng of total RNA an A260/280 ratio of 1.8-2.0 with Transcriptor First strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany). Synthesized cDNA was used experiment of real time-qPCR analysis.

2.5. Real time-qPCR

For analysis of real time-qPCR, primer sets were designed using the National Center for Biotechnology Information (NCBI) and summarized in table 2. The real time-qPCR was assayed using the CFX^{TM} Real-time System (Bio-Rad, Hercules, CA, USA) and FastStart Universal SYBR Green Master (Roche Diagnostics) with 20 ng cDNA. The real time-qPCR cycling conditions were initial denaturation at 95℃ for

10 min, 40 cycles of 95℃ for 15 sec, 60℃ for 1 min, and last 60℃ for 1 min. Expression of genes in each brain and liver were normalized to amount of the internal control *β-actin* gene.

Table 2. Primer sets used in real time-qPCR.

Primers	Sequence (5'-3')	GenBank Accession No.
aanat1a-F	GTCGTCTCACTCTGTGTCCC	LC010909
aanat1a-R	GAAGCCCGACTTGCAGTAGA	
aanat1b-F	GCGAAGAGTTCCTGGTTCCC	LC010910
aanat1b-R	CGGACCGGGTAGAACATCTC	
aanat2-F	CAACTTCCTGGGCATGTGTC	LC010911
aanat2-R	CCTCCTGTGAAAGCCTCTCT	
sbgnrh-F	TGTCAGCACTGGTCCTATGG	AB531127
sbgnrh-R	GGTCCGGCTGTTCAGAATTT	
chgnrh-F	CCCTTTCAACCCCTCAGAGAT	AB531128
chgnrh-R	GGAGCTCTCTGGTTAAGGCA	
sagnrh-F	AGGATGATGGGGACGGGA	AB531129
sagnrh-R	CTTCTCTTTGGGTCGAAGCG	
kiss2-F	AAGAGTCCAACCCGTGTCTG	AB548304
kiss2-R	GGCTCTGCGGTAAATGAAGC	
lpxrfa-F	GAGGTTTGGGAGGTCTTGGA	AB566100-F
lpxrfa-R	TTCTGATGAGACTCTGGCCG	
igf-1-F	CAACAGGCTATGGCACCAAC	
igf-1-R	CTTAGGCGCTCTCGTCTTGT	
β-actin-F	GCCATCCTTCCTTGGTATGGA	
β-actin-R	GTCGTACTCCTGCTTGCTGA	

2.7. Ocular melatonin measurement

The levels of melatonin was measured by time-resolved fluoroimmuno assay (TR-FIA) according to the previous report (Takemura et al., 2004). Briefly, a 96-well plate (AGC Techno Glass) was coated with 50 mM carbonate buffer, pH 9.6 (100 ll/well), containing a melatonin-bovine serum albumin (BSA) conjugate (5 ng/ml) for 2 h at 24 ± 0.5℃ in an incubator (Sanyo). After three washes with DELFIA wash buffer, 50 ll of samples/standards (7.8-4000 pg/ml) and 50 ll of anti-melatonin (1:200,000 in assay buffer) were placed in the wells. The plate was incubated overnight at 4℃. After washing, 100 ll of the secondary antibody against rabbit immunoglobin Glabeled with europium (1:1000 in assay buffer) was added to the well and incubated at 24℃ for 1 h. After washing, 50 ll of DELFIA enhancement solution was added to the wells. The signal in each well was quantified using a time-resolved fluorometer (Arcus1234, Wallac, Oy, Finland). The composition of the assay buffer (pH 7.75) used in the present study was 0.05 M Tris, 0.9% NaCl, 0.5% BSA, (Sigma), 0.05% NaN3, 0.01% Tween 40, and 20 lM diethylenetriamine pentaacetic acid (DTPA, Kanto Chemicals, Tokyo, Japan). DELFIA washing buffer (pH 7.75) contained 0.05 M Tris, 0.9% NaCl, 0.1% NaN3, and 0.01% Tween 20.

2.8. Statistical analysis

All TR-FIA and real-time qPCR results expressed as mean ± standard error (SEM). Our experiment data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. All statistical differences among means were using the statistics 21.0 for windows (SPSS Inc.).

3. Results

3.1. IGF-1 cDNA partial cloning and phylogenetic analysis

Grass puffer IGF-1 partial sequence was isolated from the liver. IGF-1 partial sequence composed total 442 base pairs fragments (Fig. 1). The amino acid sequence homology of grass puffer IGF-1 investigated with other teleost fish. Grass puffer IGF-1 showed high homology with tiger puffer *Takifugu rubripes* IGF-1 (99.3%). Among the fish species, Siberian taimen *Hucho taimen* IGF-1 was showed very low amino acid similarity (76.4%) with grass puffer IGF-1 (Fig. 2).

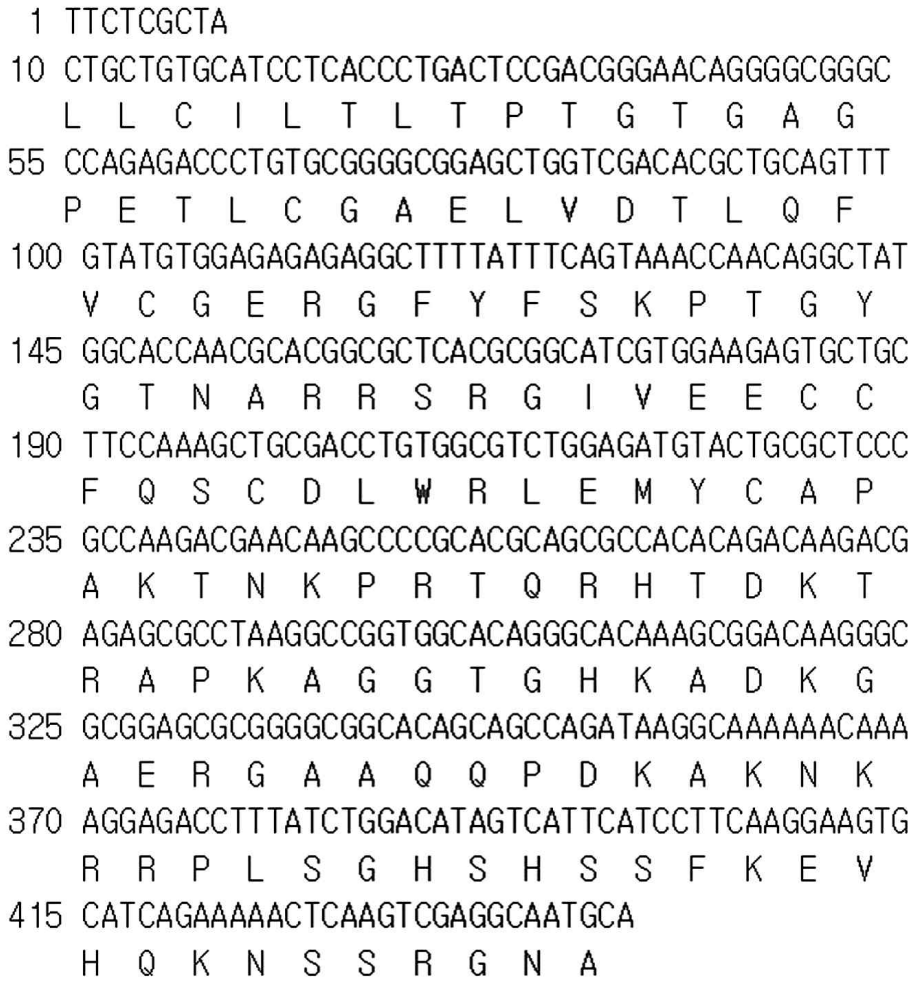

Fig. 1. The IGF-1 partial nucleotide and deduced amino acid sequence obtained from grass puffer liver. It was 442 base pairs long and encoding 144 amino acids.

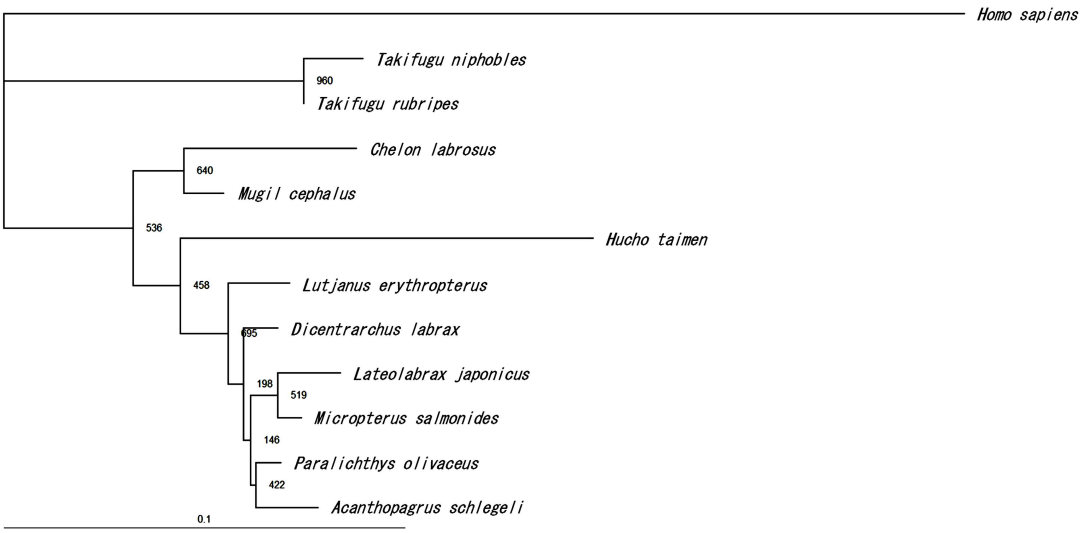

Fig. 2. Phylogenetic tree of grass puffer IGF-1 cDNA

3.2. Distribution of *igf-1*, *aanat1a*, *aanat1b* and *aanat2* genes

We investigated the distribution of the liver *igf-1,* ocular *aanat1a*, *aanat1b* and brain *aanat2* genes using the real time-qPCR (Fig. 3). In the results, *igf-1* mRNA was showed significantly highest expression in the liver (Fig. 4). The most significantly high expression of *aanat1a* was observed in the retina and secondarily high expression was detected in the liver (Fig. 5A). Beside, *aanat1b* mRNA was observed significantly only higher expression in the retina (Fig. 5B). On the other hand, *aanat2* mRNA expression was detected most tissues and significant high expression was detected in olfactory lob and retina (Fig. 5C).

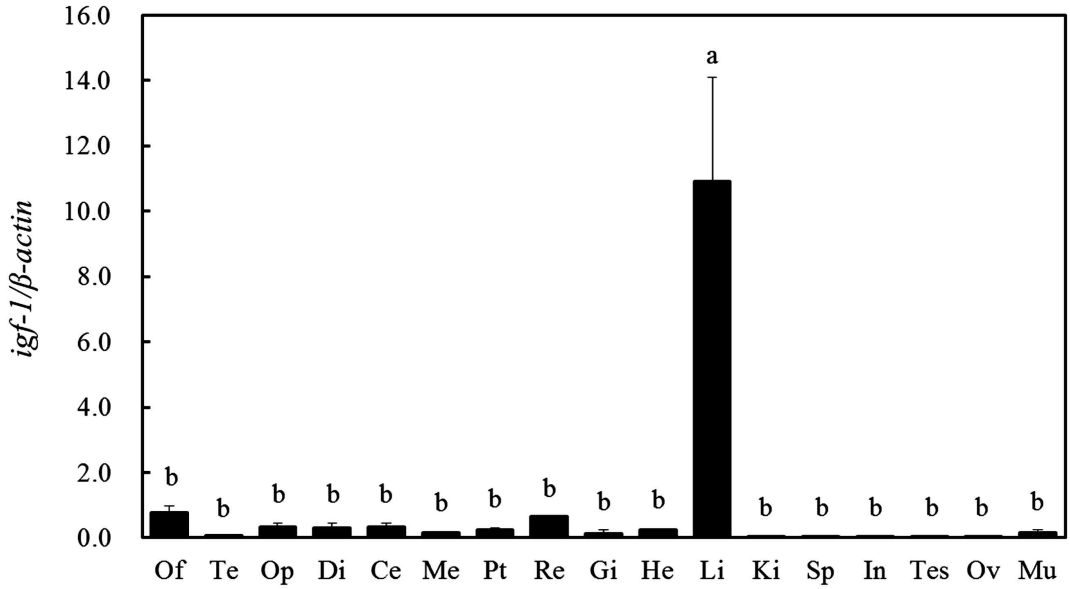

Fig. 3. Tissue specific expression of *igf-1* mRNA in the grass puffer by real time-qPCR. Of, olfactory lob; Te, telencephalon; Op, optic tectum; Di, diencephalon; Ce, cerebellum; Me, medulla oblongata; Pt, pituitary; Re, retina; Gi, gill; He, heart; Li, liver; Ki, kidney; Sp, spleen; In, intestine; Tes, testis; Ov, ovary; Mu, muscle. The different letters indicate statistically different values (P<0.05). Values are mean ± SEM.

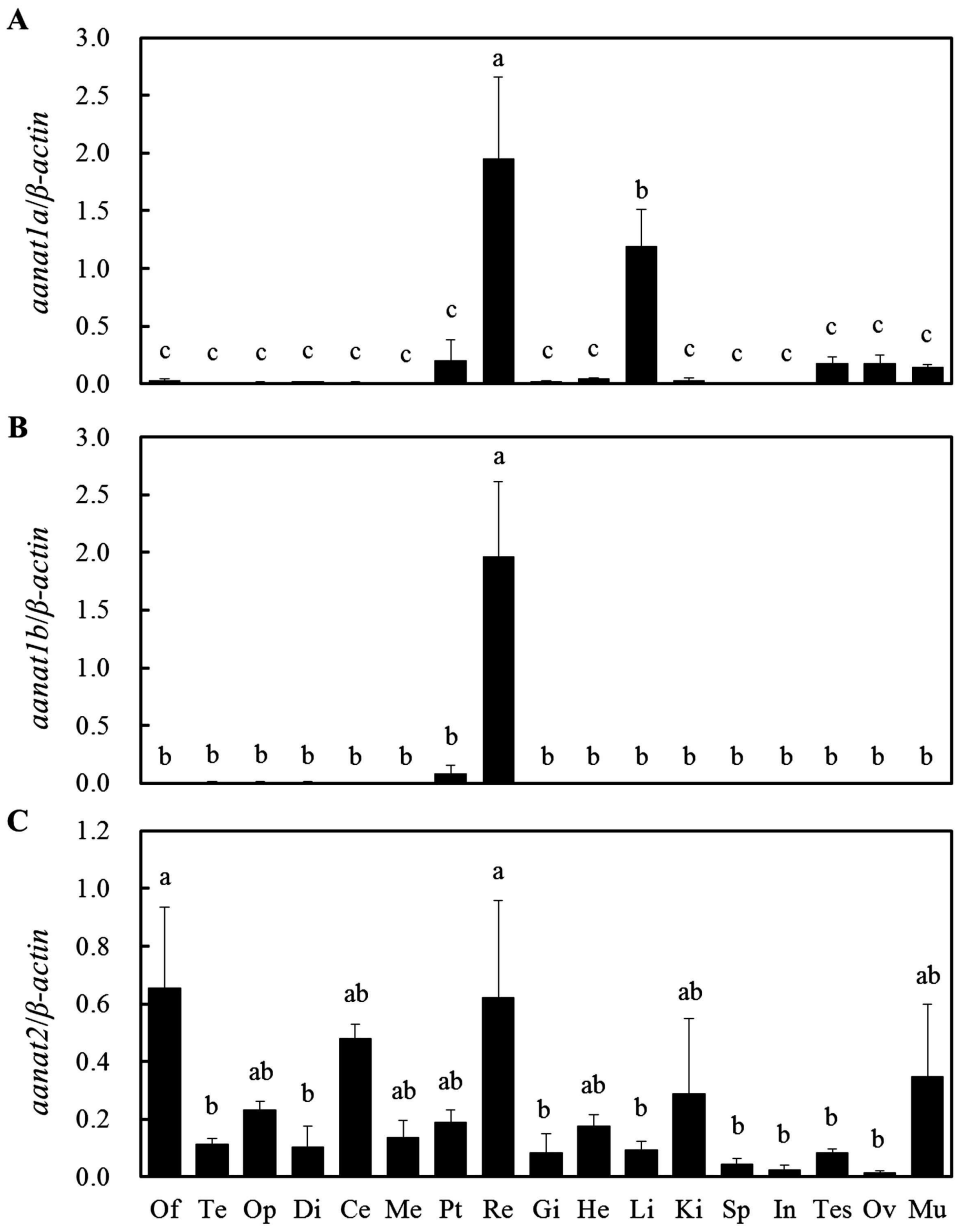

Fig. 4. Tissue specific expression of ocular *aanat1a* (A), *aanat1b* (B) and brain *aanat2* mRNA (C) in the grass puffer by real time-qPCR. Of, olfactory lob; Te, telencephalon; Op, optic tectum; Di, diencephalon; Ce, cerebellum; Me, medulla oblongata; Pt, pituitary; Re, retina; Gi, gill; He, heart; Li, liver; Ki, kidney; Sp, spleen; In, intestine; Tes, testis; Ov, ovary; Mu, muscle. The different letters indicate statistically different values (P<0.05). Values are mean ± SEM.

3.3. Daily oscillation of ocular melatonin

Daily oscillation of ocular melatonin was investigated under natural photoperiod conditions in February (11L:13D) and May (14L:10D) (Fig. 5). In February, ocular melatonin secretion observed nocturnal oscillation (Fig. 5A). Ocular melatonin was maintained the low levels from CT6 to CT18 and rapidly increased in after CT21. After that, melatonin levels rapidly decreased to CT24 and low levels continued until CT3. Alike nocturnal oscillation of ocular melatonin with in February was observed in May (Fig. 5B). Significant low melatonin levels observed from CT6 to CT18 and significant high melatonin levels observed at CT21. After that, melatonin levels decreased until CT3.

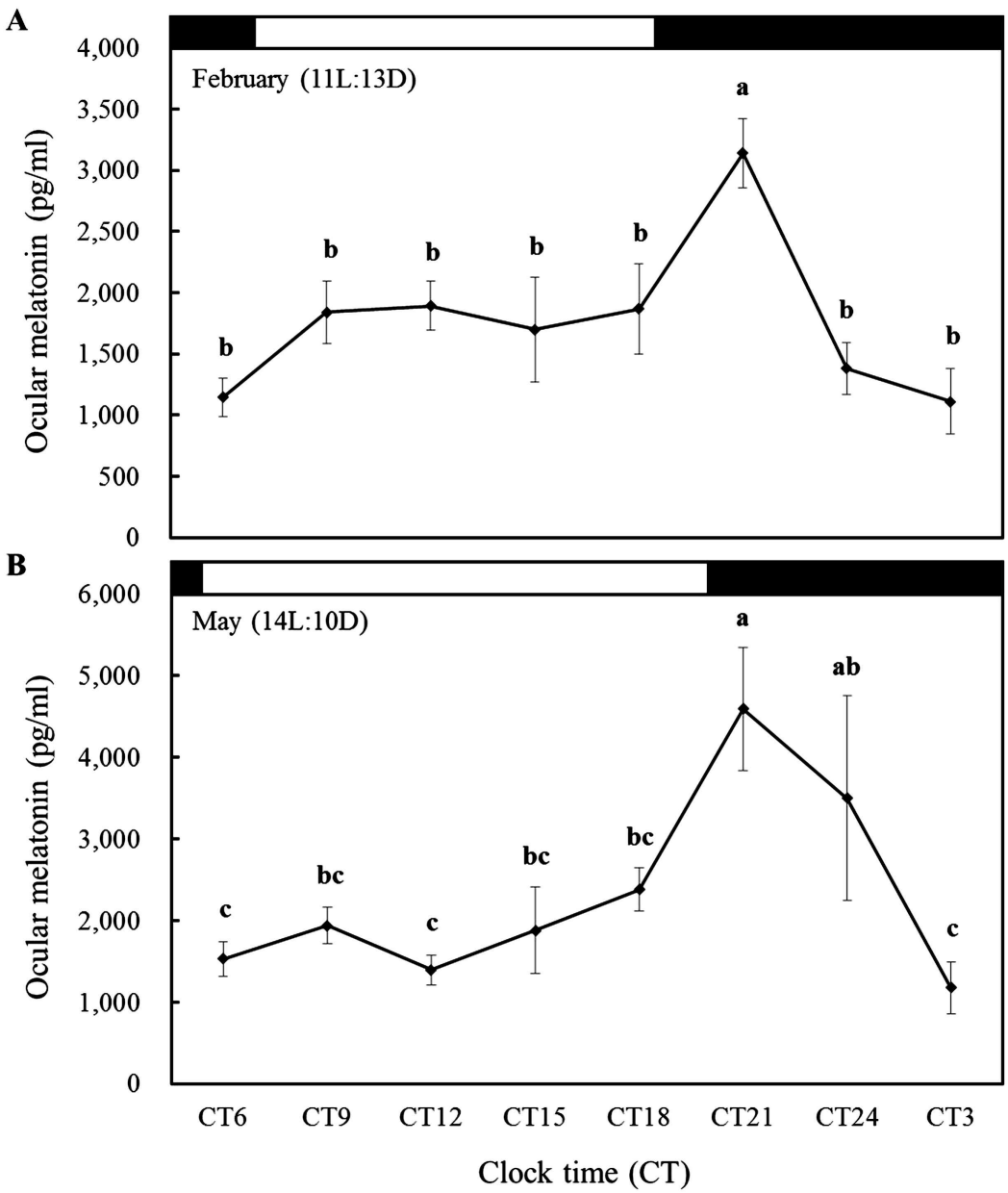

Fig. 5. Daily rhythms of ocular melatonin under natural photoperiod conditions in February (A) and May (B) from grass puffer. The different letters indicate statistically different values (P<0.05). Values are mean ± SEM.

3.4. Variations of ocular melatonin secretion during sexual maturation

We investigated the ocular melatonin secretion in during scotophase from each four gonadal development phases (Fig. 6). Ocular melatonin secretion showed different variation under each gonadal development phases. Ocular melatonin showed significantly low levels in growing phase (February) but significantly higher ocular melatonin levels showed in maturing phase (May). After that ocular melatonin levels gradually decreased and maintained low melatonin levels in early growing phase (November.)

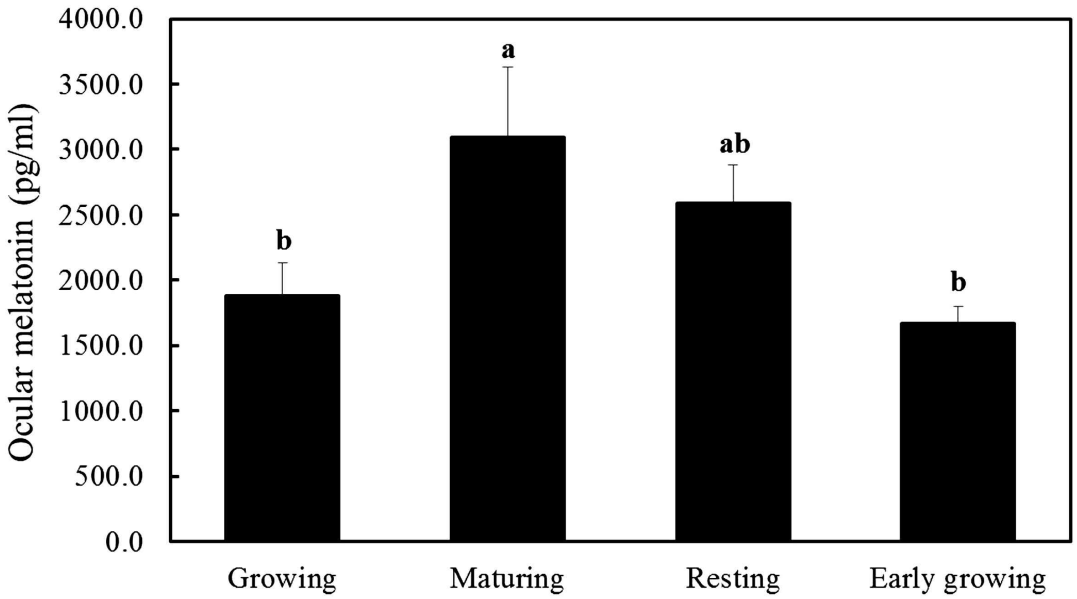

Fig. 6. Ocular melatonin variations in during growing (February), maturing (May), Resting (August) and early growing (November) of grass puffer. The different letters indicate statistically different values (P<0.05). Values are mean ± SEM.

3.5. Variations of *aanat* genes expression during sexual maturation

The variations of *aanat1a* and *aanat1b* mRNA expression in retina during sexual maturation are represented in Fig. 7 and Fig. 8. The *aanat1a* mRNA revealed more low expression levels than other seasons and showed no significant expression during at all times from growing phase (Fig. 7A). In maturing phase, *aanat1a* expression was very lower in midday and significantly higher at CT21 during photopase (Fig. 7B). Further, *aanat1a* expression was decreased at CT 24 but *aanat1a* expression was again increased at CT3. In resting phase, *aanat1a* mRNA showed more high expression levels than other gonadal development phases and showed clearly higher expression during at scotophase than at midday (Fig. 7C). *aanat1a* expression was significantly low at midday, but *aanat1a* expression was very higher at CT24 and CT3. In early growing phase, significant difference of *aanat1a* expression between midday and scotophase disappeared (Fig. 7D).

In the case of *aanat1b* expression, not showed significant expression during midday and scotophase in growing phase (Fig. 8A). In May, *aana1b* mRNA was showed more high expression during scotophase than midday (Fig. 8B). *aanat1b* mRNA was more low at CT12 and steadily increased from CT21 to CT3. In maturing, *aanat1b* mRNA also showed high expression in scotophase and significantly high expression was showed at CT24 (Fig. 8C). Early growing phase *aanat1b* mRNA expression was alike with *aanat1b* mRNA expression in maturing phase (Fig. 8D).

Seasonal brain *aanat2* mRNA expression was represented Fig. 9. In growing phase, brain *aanat2* mRNA expression showed no significant difference during at all times (Fig. 9A). However, significantly high expression of *aanat2* mRNA in scotophase than midday showed in maturing phase and peak expression time was at CT24 (Fig. 9B). In resting phase, significant different *aanat2* mRNA expression was disappeared but some increased tendency of *aanat2* expression observed at CT21 (Fig. 9C). In early growing phase, *aanat2* mRNA did not showed significant different expression,

but *aanat2* mRNA expression was slightly increased at CT21 and CT3 (Fig. 9D).

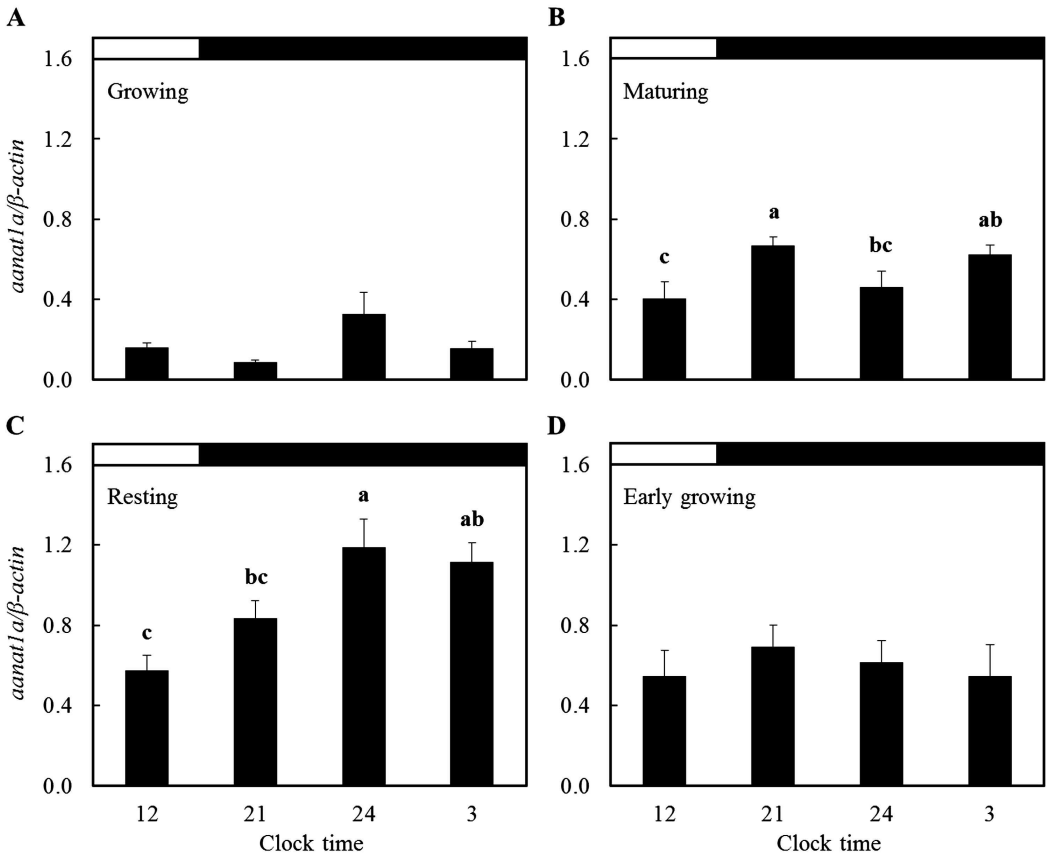

Fig. 7. Ocular variations of *aanat1a* mRNA expression during sexual maturation of grass puffer. (A) Growing phase. (B) Maturing phase. (C) Resting phase. (D) Early growing phase. The different letters indicate statistically different values ($P<0.05$). Values are mean ± SEM.

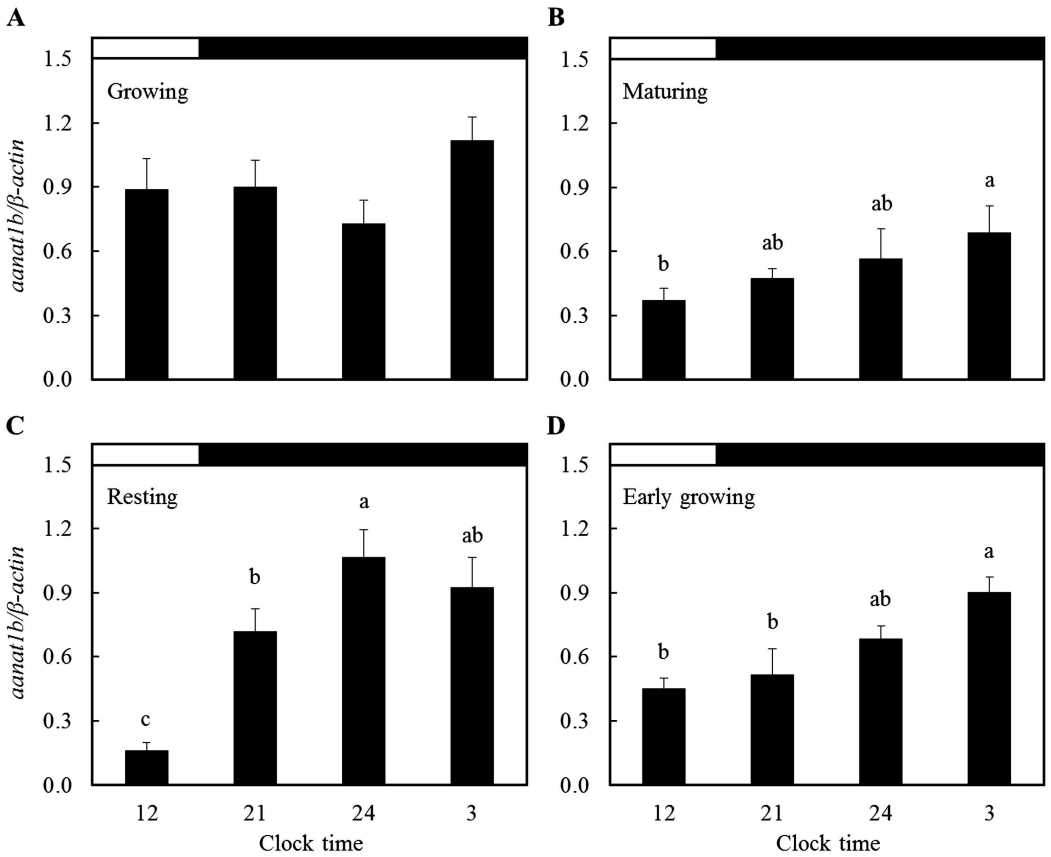

Fig. 8. Ocular variations of *aanat1b* mRNA expression during sexual maturation of grass puffer. (A) Growing phase. (B) Maturing phase. (C) Resting phase. (D) Early growing phase. The different letters indicate statistically different values ($P<0.05$). Values are mean ± SEM.

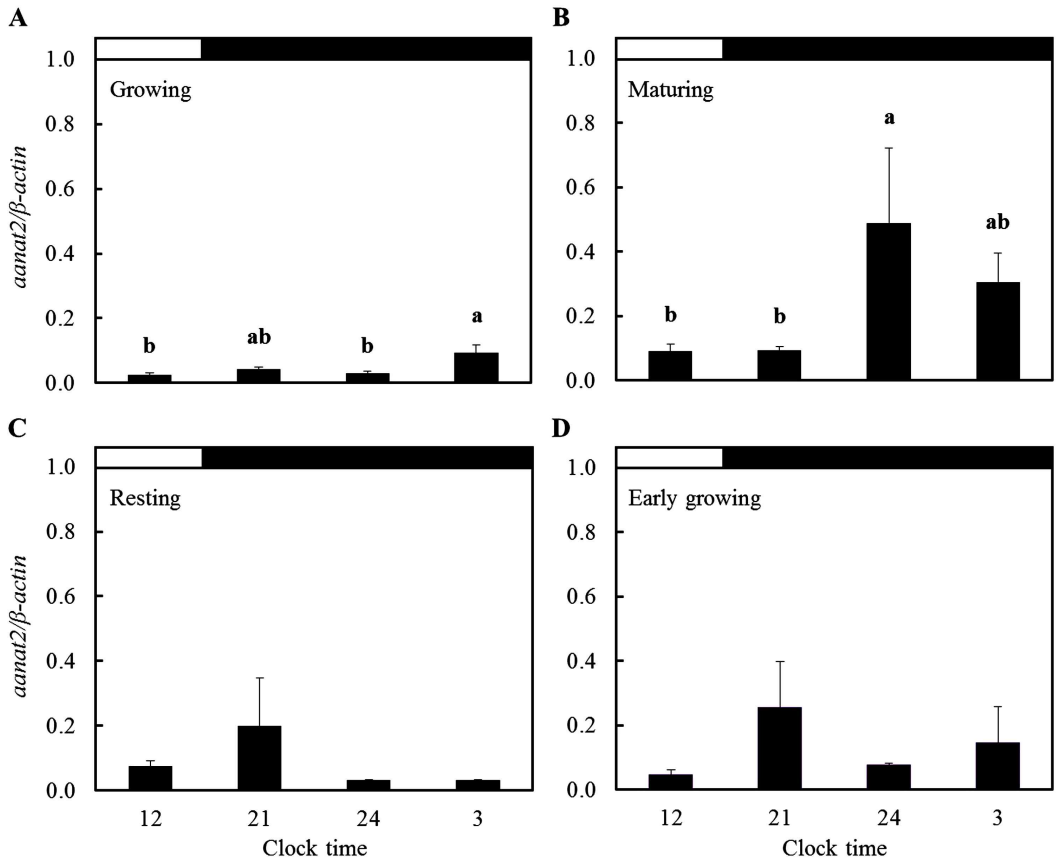

Fig. 9. Brian variations of *aanat2* mRNA expression during sexual maturation of grass puffer. (A) Growing phase. (B) Maturing phase. (C) Resting phase. (D) Early growing phase. The different letters indicate statistically different values ($P<0.05$). Values are mean ± SEM.

3.6. Seasonal expression of reproductive genes in the brain during sexual maturation

We profiled the expression change of reproduction-related genes in the brain during sexual maturation in the brain of the female and male grass puffer respectively (Fig. 10 and 11). Gonadal development phase was shown divided into four phases including, growing phase (February), maturing phase (May), resting phase (August) and early growing phase (November).

In female brain, *sbGnRH* mRNA observe low expression in growing phase and expression gradually increased to resting phase (Fig. 10A). After that *sbGnRH* expression rapidly decreased to early growing phase. The significantly highest expression of female *chGnRH* mRNA observed in growing phase and expression steadily decreased to resting phase (Fig. 10C). After that *chGnRH* expression increased again in early growing phase. Female *saGnRH* mRNA was maintained low expression levels from growing phase to resting phase and dramatic expression rise of *saGnRH* mRNA showed in early growing phase (Fig. 10E).

In Male brain, *sbGnRH* mRNA showed basal expression levels in growing phase and significantly high expression showed in maturing phase (Fig. 10B). Expression of male *chGnRH* mRNA was significantly peaked in growing phase and gradually decreased to early growing phase (Fig. 10D). Male *saGnRH* mRNA showed low expression in resting phase and inversely significant high expression showed in early growing phase (Fig. 10F).

Expression change of *kiss2* and *lpxrfa* mRNA in brain were showed Fig. 11. The female *kiss2* mRNA showed continuous basal expression from growing phase to resting phase but this expression was dramatically increased in early growing phase (Fig. 11A). Expression of male *kiss2* mRNA was low in growing phase and gradually increased to early growing phase (Fig. 11B). Female with male *lpxrfa* mRNA expression showed similar expression patterns and significantly peaked in early growing phase than other developmental phase (Fig. 11C and D).

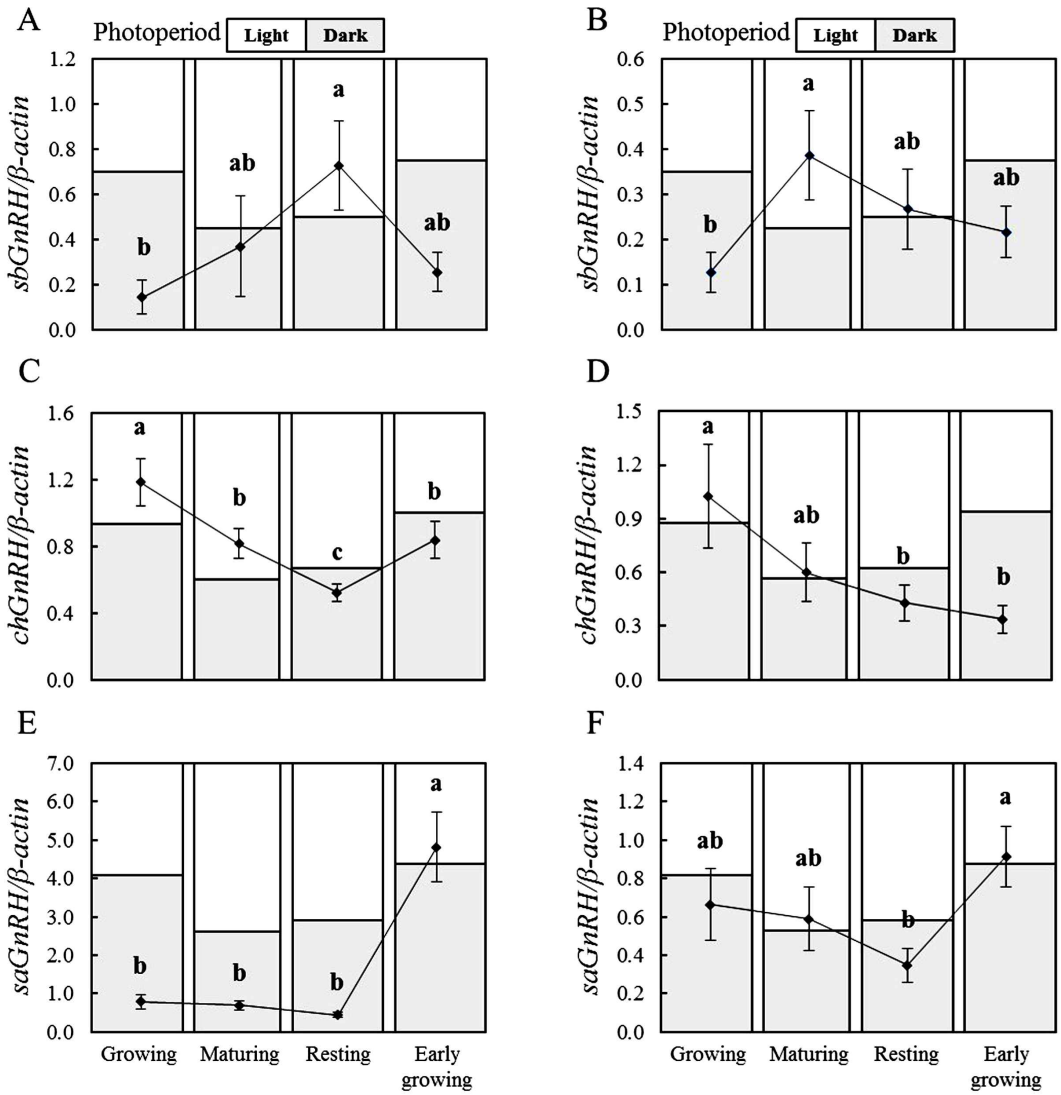

Fig. 10. Expression changes of *sbGnRH*, *chGnRH* and *saGnRH* mRNA in during growing (February), maturing (May), resting (August) and early growing (November) in the brain of female (A, C and E) and male (B, D and F) grass puffer. The different letters indicate statistically different values ($P<0.05$). Values are mean ± SEM.

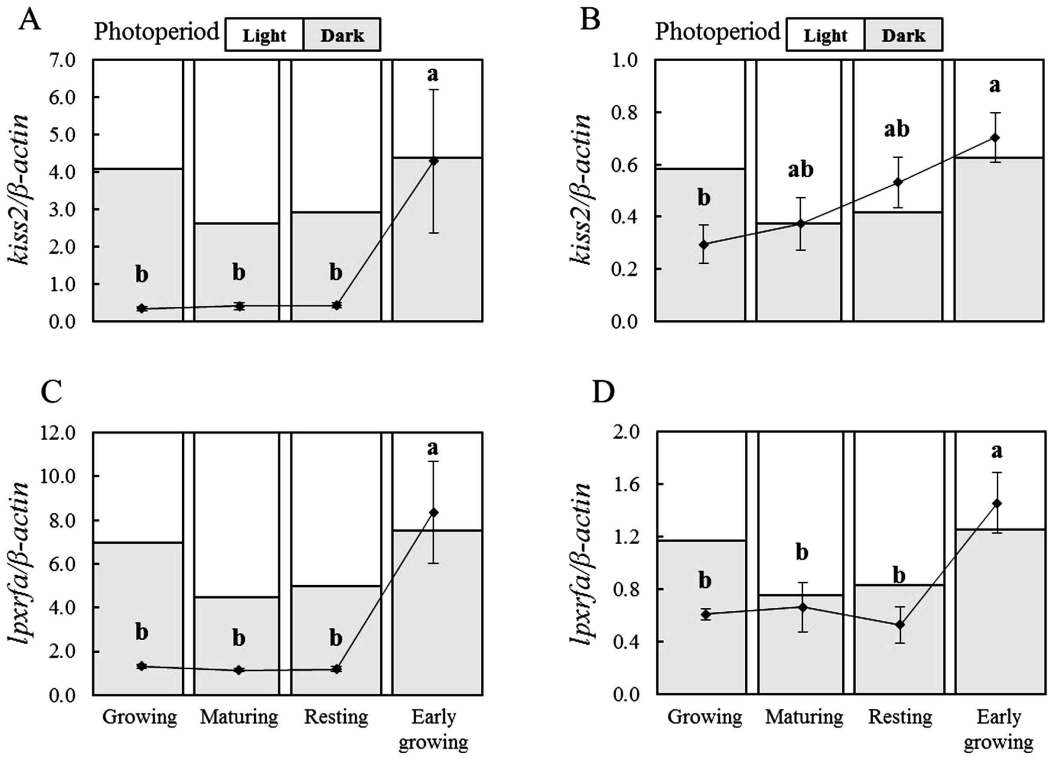

Fig. 11. Expression changes of *kiss2* and *lpxrfa* mRNA in during growing (February), maturing (May), resting (August) and early growing (November) in the brain of female (A and C) and male (B and D) grass puffer. The different letters indicate statistically different values ($P<0.05$). Values are mean ± SEM.

3.7. Expression variation of *igf-1* mRNA during sexual maturation in the liver

We investigated the expression change of *igf-1* mRNA during sexual maturation in the liver of grass puffer (Fig. 12). Female *igf-1* was showed basal expression from maturing phase to resting phase and expression rapidly increased in early growing phase (Fig. 12A). Male *igf-1* expression was peaked in growing phase and decreased to resting phase (Fig. 12B). After that, *igf-1* expression was showed again increase in early growing phase.

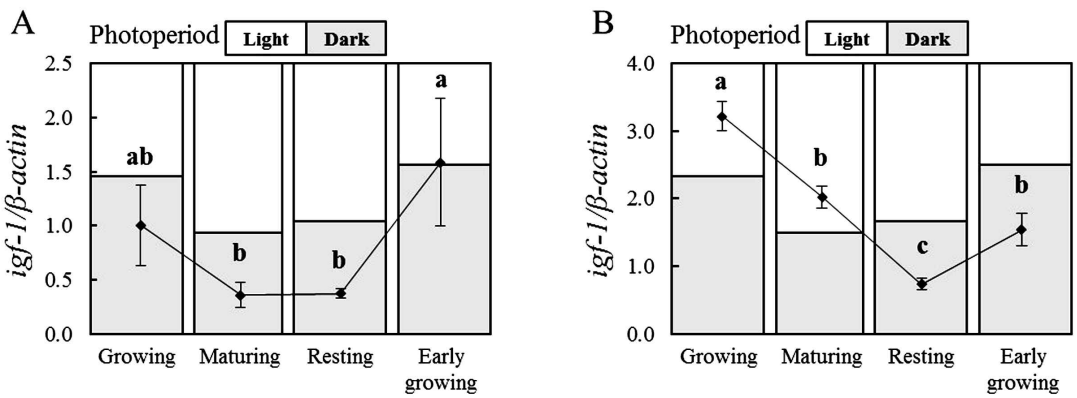

Fig. 12. Expression changes of *igf-1* mRNA in during growing (February), maturing (May), resting (August) and early growing (November) in the brain of female (A) and male (B) grass puffer. The different letters indicate statistically different values (P<0.05). Values are mean ± SEM.

4. Discussion

In the present study, we compared the seasonal melatonin variations with reproductive-related genes' expression during sexual maturation in grass puffer. For this study, we first performed the IGF-1 cDNA partial cloning in the liver of grass puffer. Partial nucleotide sequence of IGF-1 was composed of 442 bp and 144 amino acids. In addition, we examined the tissue distribution of *igf-1*, *aanat1a*, *aanat1b* and *aanat2* mRNA by means of real-time qPCR. In our result, *igf-1* mRNA mainly detected in the liver. Liver is the major site of IGF-1 production and it has been revealed in the common carp *Cyprinus carpio* (Vong et al., 2003), tilapia (Caelers et al., 2004), Chilean flounder *Paralichthys adspersus* (Escobar et al., 2011) and tongue sole *Cynoglossus semilaevis* (Ma et al., 2011). Teleost fishes have two AANAT genes in each retina (AANAT1) and in the pineal organ (AANAT2) (Falcónet al., 2007b). In addition, two AANAT1 genes (AANAT1a and AANAT1b) were recently identified (Coon and Klein, 2006). In our results, *anat1a* and *aanat1b* mRNA were detected to be significantly higher in the retina compared with other tissues. However, *aanat2* mRNA was detected in numerous peripheral tissues as well as in nervous tissues; this situation is similar to previous findings in rainbow trout, where *aanat2* was expressed in various peripheral tissues (Fernández-Durán et al., 2007). In contrast, *aanat2* mRNA was expressed only in the pineal organ in species such as the pike *Esox lucius*, trout *Oncorhynchus mykiss* (Coon et al., 1998) and zebrafish (Bégay et al., 1998). The bases of these different *aanat2* distributions between fish species is still unclear; thus, further studies are needed to explore the various physiological processes in which *aanat2* may be involved in peripheral tissues.

In our study, we investigated the ocular melatonin oscillation of grass puffer under natural photoperiod conditions. Ocular melatonin secretion showed nocturnal oscillation in February and May. Previous findings in the oikawa *Zacco platypus* described an ocular melatonin daily oscillation under light and dark conditions, where melatonin oscillation disappeared under constant light (LL) or constant dark (DD)

conditions (Iigo et al., 1997). However, melatonin concentration was higher under DD conditions compared with LL. This ocular melatonin oscillation described in oikawas was also described in the wrasse *Halichoeres tenuispinnis* (Iigo et al., 1997) and in the sea grass rabbitfish *Siganus canaliculatus* (Rahman et al., 2004). These results indicated that ocular melatonin is regulated by light presence or absence.

On the other hand, we investigated the variations of ocular melatonin secretion during sexual maturation of grass puffer. As described in chapter 1, the sexual maturation of grass puffer could be divided into four phases: growing, maturing, resting and early-growing phase. We found that ocular melatonin showed significantly higher levels during maturing phase. In previous studies concerning teleost fishes, melatonin was reported to vary according to different reproductive seasons. For example, in the major carp *Catla catla*, seasonal serum melatonin concentration showed peak levels during the post-spawning phase, and lower levels during the spawning phase (Maitra et al., 2005). In the same study, melatonin administration during pre-spawning and spawning phase provoked an antigonadal effect. In contrast, other studies showed that melatonin administration during preparatory phase provoked a precocious maturation of ovary. In three-spined stickleback *Gasterosteus aculeatus*, seasonal changes of brain melatonin examined in during sexual maturation (Sokolowska et al., 2004) and proved to have antigonadal effects via melatonin injection (Borg and Ekström,1981). Even though these experiments were conducted assessing both brain and serum, it was plausible to suggest that melatonin is involved in fish reproduction. However, the influence of melatonin on reproduction relies on the different effects of melatonin, which depends on the gonadal development phase and fish species. Therefore, our results suggest that the seasonal ocular melatonin variation has an effect in fish reproductive whether negative or positive.

In addition to ocular melatonin, we investigated changes in the expression of *aanat1* subtype and *aanat2* genes in each retina and in brain during sexual maturation. We observed induced ocular *aanat1a* mRNA expression during scotophase in maturing and resting phases. However, the expression of *aanat1a* mRNA during

scotophase in early-growing and growing phases was low, and *aanat1a* expression showed slight increase. When comparing gonadal developmental phases, *aanat1a* mRNA expression was more activated during the resting phase. The expression of nocturnal ocular *aanat1b* mRNA was increased in most gonadal development phases, excepting in the growing phase. On the other hand, we observed nocturnal expression of *aanat2* mRNA in brain during growing and maturing phases, although this nocturnal expression disappeared during resting and early-growing phases. Furthermore, the brain expression of *aanat2* mRNA was more activated during maturing phase, compared with other gonadal development phases. These results suggest that ocular and brain melatonin were mainly activated during the maturing phase. In this study, we investigated the seasonal reproductive-related genes expression in the brain of grass puffer. For sexual maturation and spawning, the activation of numerous reproductive factors in the brain are needed, including the activation of GnRH and kisspeptin. In females of species such as the red sea bream *Pagrus major* (Okuzawa et al., 2003), barfin flounder *Verasper moseri* (Amano et al., 2008), spotted halibut *Verasper variegatus* (Xu et al., 2012) and the European sea bass *Dicentrarchus labrax* (alvarado et al., 2013), the *sbGnRH* mRNA expression increases during the maturing phase of the ovary, and has a peak during spawning season. However, other species such as the grass rockfish *Sebastes rastrelliger* showed higher sbGnRH levels during the post-spawning phase compared with the maturing phase (Collins et al., 2001), and chub mackerel showed significantly higher *sbGnRH* mRNA levels during post-spawning season (Selvaraj et al., 2012). In these latter results, Collins et al. (2001) suggested that the accumulation of sbGnRH at the end of the reproductive cycle reflects a diminished activity along the BPG axis. Our study showed a similar pattern of *sbGnRH* expression; the *sbGnRH* of grass puffer females gradually increased from the growing to the resting phase. In males, *sbGnRH* expression rapidly increased from the growing to the mature phase. Therefore, we suggest that *sbGnRH* mRNA expression also affects gametogenesis and spawning in grass puffer.

The chGnRH is known to be involved in spawning behavior. Previous studies of *chGnRH* expression in the chub mackerel showed no difference between gonadal phases (Selvaraj et al., 2012). These results indicate that *chGnRH* expression may have not effect upon the gonadal developmental phases. However, in the goldfish *Carassius auratus,* studies confirmed a stimulated expression of *chGnRH* mRNA at the beginning of spawning behavior (Canosa et al., 2008). Our results showed a high expression of brain *chGnRH* mRNA during the growing phase. These results concerning *chGnRH* expression suggest an relevant relationship with gonadal developmental phases. However, in teleost fishes, the potential association between gonadal development and chGnRH remains unclear. According to previous results from gold fishes, food intake decreased along with chGnRH administration (Matsuda et al., 2008). Therefore, the activation of chGnRH is suggested to affect fishes' behavior. We require further studies to clarify the mechanisms and effects of *chGnRH* expression in the grass puffer.

The saGnRH has been also implicated in the reproductive behavior of fishes. Previous experiments with male tilapia *Oreochromis niloticus* showed that saGnRH immunoneutralization suppressed nest-building and aggressive behaviors (Ogawa et al, 2006). Our results showed high levels of *saGnRH* expression during the early-growing phase, both in females and males. This *saGnRH* expression is considered to be not associated with reproductive behavior, although it is considered to be associated with early gametogenesis. Previous studies observed saGnRH neuronal fibers in the pituitary of goldfish and masu salmon *Oncorhynchus masou*, describing the potential ability of saGnRH to induce GtH secretion (Kobayashi et al., 1997). Our results showed and increased *saGnRH* expression during early-growing phase; therefore, we suggest that *saGnRH* expression its involved with early gametogenesis in grass puffer. However, further studies are needed to explore the correlation between *saGnRH* expression and GtH secretion.

It has been recently recognize the essential role of kisspeptin in the control of puberty and reproduction in teleost fishes. In the European sea bass, the expression

of *kiss1* mRNA showed no difference between different testicular stages; however, *kiss2* mRNA expression increased during immature stage (Alvarado et al., 2013). On the other hand, in female sea bass, *kiss1* and *kiss2* mRNA expression increased during the maturation-ovulation stage. In chub mackerel, the expression of *kiss2* mRNA was significantly higher before the onset of meiosis in the testis (males), and before the onset of vitellogenesis in oocytes (females). (Ohga et al., 2015). Our results showed similar seasonal expression of *kiss2* mRNA; the expression of *kiss2* mRNA had a single peak during early-growing phase in the ovary, suggesting that *kiss2* acts in the initial development of the oocytes. However, we found that seasonal *kiss2* expression appeared somewhat differently in grass puffer males. The *kiss2* expression in males was low during the growing phase; after that, it gradually increased until the early-growing phase. Therefore, *kiss2* mRNA is suggested to affect early spermatogenesis, meanwhile the gradual increase of *kiss2* mRNA during maturation and spawning phases may reflect its role in spermatogenesis and spermation.

The LPXRFa, belong to the RFamide peptide family, it is widely known its role as a common inhibitor of GtH secretion in birds and mammals (Tsutsui et al., 2000; Clarke et al., 2009). However, this latter role of GtH-inhibitor is not always present in fish species. In goldfish, the inhibition of LH secretion was provoked by LPXRFa i.p injection (Zhang et al., 2010). However, LPXRFa showed the induction of FSH and LH secretion in the sockeye salmon and tilapia (Amano et al., 2006; Biran et al., 2014). In our results, seasonal expression of *lpxrfa* mRNA was significantly higher during the early-growing phase in both females and males grass puffers. However, our experiments did not verify the correlation between LPXRFa and GtH, because we did not identify the seasonal GtH expression. However, the peak of lpxrfa mRNA during the early-growing phase suggests that it may be involved in the regulation of GtH secretion associated with gonadal development. Further studies are needed to clarify the effect of LPXRFa on reproduction processes.

We also investigated seasonal changes of *igf-1* expression in the liver. Previous studies on some fish species report the effect of IGF-1 on GtH stimulation; for example, in the European eel *Anguilla Anguilla*, IGF-1 treatment stimulated the secretion of LH from the pituitary gland (Huang et al., 1998). *In vitro* experiments with the rainbow trout *Oncorhychus mykiss* described the secretion of FSH and LH after IGF-1 administration during gametogenesis (Weil et al., 1999). In our results with grass puffer, we found that *igf-1* seasonal expression in both females and males were high during the growing phase (November and February), decreasing during spawning seasons. Therefore, we suggest that the observed changes in seasonal *igf-1* expression are associated with gonadal development by means of GtH stimulation in the pituitary gland.

Consequently, we observed daily oscillation of ocular melatonin under natural photoperiod conditions. Ocular melatonin secretion was mainly increased during the gonadal maturing phase, meanwhile the expression of ocular *aanat1a* and brain *aanat2* increased during the maturing and resting phases. These results suggest that melatonin is involved with reproduction processes in the grass puffer. On the other hand, brain reproductive-related factors are thought to affect differently according to gonadal development phases. However, our results did not explore direct relationships between melatonin and growth-related genes. Therefore, the clarification of these potential relationships between melatonin and reproductive-related genes would need to be addressed in further studies.

Chapter Ⅲ. Effect of melatonin on sexual maturation in grass puffer *Takifugu niphobles*

Abstract

Recent studies in several fish species confirm the important role of melatonin in regulating the seasonal reproductive cycle. The melatonin produced in the retina and in the pineal organ is regulated by annual changes in the photoperiodic oscillation; these changes in melatonin production consecutively regulate the reproductive endocrine system. The aim of this study was to reveal the effect of melatonin in reproductive endocrine system of the grass puffer. We investigated the *aanat* genes oscillation under different photoperiod conditions. In addition, we conducted melatonin injections to assess the associations between melatonin and reproduction-related genes. Finally, we investigated the regulation of sexual maturation by means of treatments with different artificial photoperiod conditions. In the investigation of the oscillation of *aanat* genes under different photoperiodic conditions (short day photoperiod, SD and long day photoperiod, LD), we observed nocturnal oscillation of *aanat1a* and *aanat2* mRNA in retina and brain under different photoperiodic conditions. Ocular *aanat1a* showed different expression peak-times depending on different photoperiodic conditions; brain *aanat2* mRNA expression showed unchanged oscillation under each SD and LD photoperiodic conditions. In the melatonin injection to assess the associations between melatonin and reproduction-related genes, we observed a significant induction of *chGnRH* mRNA in the brain. We also observed a significant inhibition in the expression of *kiss2* mRNA, and an increasing expression of *lpxrfa* mRNA. Finally, in the regulation of sexual maturation by means of treatments with different artificial photoperiod conditions for 14 weeks, we observed a significant inhibition of GSI levels under SD group, compared with than in LD group. Based on histological findings during the growing phase, the ovaries obtained from the SD group presented initial ovary developmental phase, meanwhile the ovaries from the LD group presented a maturing phase. The expression of *kiss2* and *lpxrfa* mRNA was inhibited in the SD group. These results are considered to be associated with the melatonin-effect due to the SD treatment. The SD treatment was design to increase

the secretion of melatonin in retina and in the pineal organ; this melatonin would have suppressed the reproductive-related factors.

1. Introduction

In fishes, light (photoperiod, intensity and spectra) is a main regulator of several physiological phenomena including growth, feeding, migration and reproduction (Boeuf & Le Bail, 1999; Migaud et al., 2010; Kim et al., 2016). Particularly, photoperiod is a key factor in the regulation of reproduction in fishes (Hansen et al., 2001; Howell et al., 2003; Zhu et al., 2014). Recent studies concerning the control of sex maturation in fishes have explored associations between photoperiod and reproductive endocrine system. In an experiment with the olive flounder *Paralichthys olivaceus*, an artificial long-day (15L:9D) treatment was followed by the inhibition of GtHs expression in the pituitary gland, and ovarian development showed no progress compared with the short-day treatment (9L:15D). (Kim et al., 2013). In masu salmons *Oncorhynchus masou* under two different conditions of short-photoperiod (8L:16D) and long-photoperiod (16L:8D), a short-photoperiod treatment induced sexual maturation, meanwhile long-photoperiod treatment delayed sexual maturation (Amano et al., 1995). Until now, it is accepted that the photoperiodic regulation of sexual maturation is affected by melatonin secretion, and the regulation of the reproductive endocrine system by melatonin has been already reported in teleost fish (Mayer et al., 1997; Amano et al., 2000; Falcón et al., 2007a; Falcon et al., 2010). Melatonin is synthesized in the pineal organ and in the retina, depending on the presence or absence of light. In general, melatonin tends to have a nocturnal secretion rhythm, and secretion period depends on the duration of scotophase, which changes according to seasonal photoperiod fluctuations (Randall et al., 1995). This fluctuation of melatonin secretion according to photoperiod changes affects the reproductive endocrine system in fishes. Melatonin implants in the eel *Anguilla anguilla* have shown to induce GtHs expression in the pituitary gland, and the release of sexual steroid hormones from the gonads (Sébert et al., 2008). However, recent studies in sea bass reported the inhibition of GnRH expression in the brain after intraperitoneal injection (i.p) of melatonin (Servili et al., 2013). Therefore, the effects of melatonin

in fishes' reproduction it is suggested to be species-specific, related to different development processes, habitats and climate. However, most studies concerning fish melatonin have focused in the pineal organ or plasma levels, whereas studies focusing in the role of fish ocular melatonin upon physiological process such as reproduction are still very scarce.

The aim of this study was to reveal the effect of melatonin in reproductive endocrine system of the grass puffer. For this, we investigated the *aanats* genes oscillation in the retina and brain under different photoperiod conditions. In addition, we conducted melatonin injections to assess the associations between melatonin and reproductive-related genes. Finally, we investigated the regulation of sexual maturation by means of treatments with different artificial photoperiod conditions.

2. Materials & methods

2.1. Effect of artificial photoperiod regulation in *aanat* genes expression in retina and brain

Grass puffer (n=96, body weight 57.4±0.9 g, total length 14.0±0.1 cm) were acclimated for 7 days in indoor tank under natural water temperature (18.6±0.1℃) and artificial 12 h light and 12 h dark (12L:12D) photoperiod conditions. During the acclimated period, fish were fed commercial pellets (Daehan co., MP3, Busan, South Korea) twice a day. Acclimated fish were separated into two groups, artificial long day group (n=48, 15 h light and 9 h dark, LD) and short day group (n=48, 9 h light and 15 dark, SD). Under each photoperiodic conditions, fish were adapt in during 7 days and feed was provided until 6 days. After that, fish were sampled at 3 hours intervals for 1 day at zeitgeber time (ZT) 3, 6, 9, 12, 15, 18, 21 and 24. Sampling were performed on anesthetized state by MS-222 and fish extracted the retina and brain tissues in fish. Collected samples were immediately stored at -80℃ until the analysis.

2.2. Effect of melatonin in reproduction-related genes expression

For research of relationship between melatonin and reproduction-related genes, experiment was performed under natural photoperiod and water temperature conditions (17.8±0.1℃) in indoor tank. Grass puffer was fed commercial pellets (Daehan co., MP3, Busan, South Korea) twice a day and adapted during a 1 week. After that, fish were separated the two groups, saline group (n=6, body weight 26.8±1.4 g, total length 10.9±0.2 cm) and melatonin group (n=6, body weight 27.1±2.2 g, total length 11.1±0.3 cm). Melatonin group was injected melatonin (Sigma, 1 mg/kg) by intraperitoneal injection (i.p) and saline group was injected the only vehicle solution (0.6% of saline). The melatonin and vehicle solution injection were processed during

the 2 weeks and fish sampling was performed 1 hour after i.p in last day of experiment. Fish brain and pituitary tissues were collected for compare of reproductive genes expression. Sampling was performed on anesthetized state by MS-222 and collected tissues were immediately kept at -80℃ until the real time-quantitative PCR (qPCR) analysis.

2.3. Control of sexual maturation by artificial photoperiod regulation

Grass puffer (n=48, body weight 31.3±1.3 g, total length 12.±0.2 cm) were sufficiently adapted under natural water temperature (15.6~19.9℃) and photoperiod conditions in indoor tank. Fish were fed commercial pellets (Daehan co., MP3, Busan, South Korea) twice a day. After acclimation, fish were reared each other different artificial photoperiodic conditions, long day (15 h light and 9 h dark, LD) and short day (9 h light and 15 dark, SD), for 14 weeks. Fish was selected only female through dissection and sampled on the anesthetized state by MS-222. Fish brain, pituitary tissues immediately froze at -80℃ until analyzed by real-time qPCR and ovary tissue was weighted for calculation of the GSI. Calculated ovaries were immediately fixed in Bouin's solution and ovarian development stage was analyzed using the histological methods. Fixed ovary samples were dehydrated in a graded series of ethanol, embedded in paraffin and sectioned 5 μm thickness. For histological observation, Slide were stained Gill's hematoxylin and 0.5% eosin. Microscopy of the ovarian development was performed using a light microscope (Olympus, Tokyo, Japan) with cellSens Standard software (Olympus, Tokyo, Japan).

2.4. Real time-qPCR

Brain and pituitary samples were absolutely homogenized with RNAiso Plus (Takara, Kyoto, Japan) reagent before total RNA extraction. Total RNA was extracted according to manufacturer's protocol and treated using the RQ1 RNase-Free DNase (Promega, Madison, WI, USA) for avoid genomic DNA contamination. For cDNA synthesis, concentration of total RNA was measured by the Nano Vue (GEHealthcare, Ver.1.0.1, UK). The 500 ng of total RNA an A260/280 ratio of 1.8-2.0 was synthesized using the PrimeScript RT reagent Kit (Takara Bio Inc, Otsu, Japan). The synthesized cDNA was used in the real time-qPCR. For real time-qPCR analysis, primer sets of reproduction-related genes and *aanat* genes were designed using the isolated gene sequence of grass puffer in the National Center for Biotechnology Information (NCBI) web site (Table 1). Real time-qPCR was conducted by CFX^{TM} Real-time System (Bio-Rad, Hercules, CA, USA) with 20 ng of cDNA using the with Eva green premix PCR kit (abm, Canada). The real time-qPCR amplification conducted by initial denaturation at 95℃ for 10 min, 40 cycles of 95℃ for 15 sec, 60℃ for 1 min, and last 60℃ for 1 min. The *β-actin* gene was used as an internal control.

Table 1. Primer sets used in real time-qPCR

Primers	Sequence (5'-3')	GenBank Accession No.
aanat1a-F	GTCGTCTCACTCTGTGTCCC	LC010909
aanat1a-R	GAAGCCCGACTTGCAGTAGA	
aanat1b-F	GCGAAGAGTTCCTGGTTCCC	LC010910
aanat1b-R	CGGACCGGGTAGAACATCTC	
aanat2-F	CAACTTCCTGGGCATGTGTC	LC010911
aanat2-R	CCTCCTGTGAAAGCCTCTCT	
sbGnRH-F	TGTCAGCACTGGTCCTATGG	AB531127
sbGnRH-R	GGTCCGGCTGTTCAGAATTT	
chGnRH-F	CCCTTTCAACCCCTCAGAGAT	AB531128
chGnRH-R	GGAGCTCTCTGGTTAAGGCA	
saGnRH-F	AGGATGATGGGGACGGGA	AB531129
saGnRH-R	CTTCTCTTTGGGTCGAAGCG	
kiss2-F	AAGAGTCCAACCCGTGTCTG	AB548304
kiss2-R	GGCTCTGCGGTAAATGAAGC	
lpxrfa-F	GAGGTTTGGGAGGTCTTGGA	AB566100-F
lpxrfa-R	TTCTGATGAGACTCTGGCCG	
fsh-F	GTAACGGCGACTGGACCTAT	AB543564
fsh-R	CATGTCCCCATTGAAGCGAC	
lh-F	CTGCATCACCAAGGACCCAG	AB543563
lh-R	AAGGTGCAGTCGGATGTGTT	
β-actin-F	GCCATCCTTCCTTGGTATGGA	
β-actin-R	GTCGTACTCCTGCTTGCTGA	

2.6. Statistical Analysis

All data were showed as mean±standard error (SEM) and considered significantly different at $P<0.05$. Statistical analysis of *aanat* genes expression were analyzed by one-way ANOVA followed by Duncan's multiple range test. Results of reproduction-related genes expression and GSI levels was analyzed by independent-sample t-test. All statistical differences among means were using the statistics 21.0 for windows (SPSS Inc.).

3. Results

3.1. Change of *aanat1a*, *aanat1b* and *aanat2* mRNA oscillation in each retina and brain under different photoperiodic conditions

The expression of ocular *aanat1a* and *aanat1b* mRNA showed different oscillation respectively in SD or LD conditions (Fig. 1 and 2). In SD conditions, *aanat1a* was showed low expression levels in during photophase (ZT 2~ZT8) but *aanat1a* levels significantly peaked at ZT14 in during scotophase (Fig. 1A). After that, *aanat1a* expression was decreased to ZT23. In LD condition, *aanat1a* maintained low expression in during photophase (ZT2~ZT17), but it was rapidly increased at ZT20 and returned back to a lower levels at ZT23 (Fig. 1B). In each photoperiod conditions, *aanat1a* showed different peak time, but each *aanat1a* peak time was included in the scotophase.

In SD condition, *aanat1b* expression was did not showed a specific daily oscillation between photophase and scotophase (Fig. 2A). In LD conditions, *aanat1b* oscillation was represented reverse oscillation pattern compared with *aanat1a* oscillation and more increased in during photophase (ZT2~ZT14) than scotophase (ZT17~ZT23) (Fig. 2B).

In the brain, *aanat2* mRNA was showed similar oscillation under SD condition with LD condition (Fig. 3). In each photoperiodic conditions, *aanat2* mRNA was significantly peaked at ZT17 during scotophase and showed unchanging oscillation by length of photophase and scotophase.

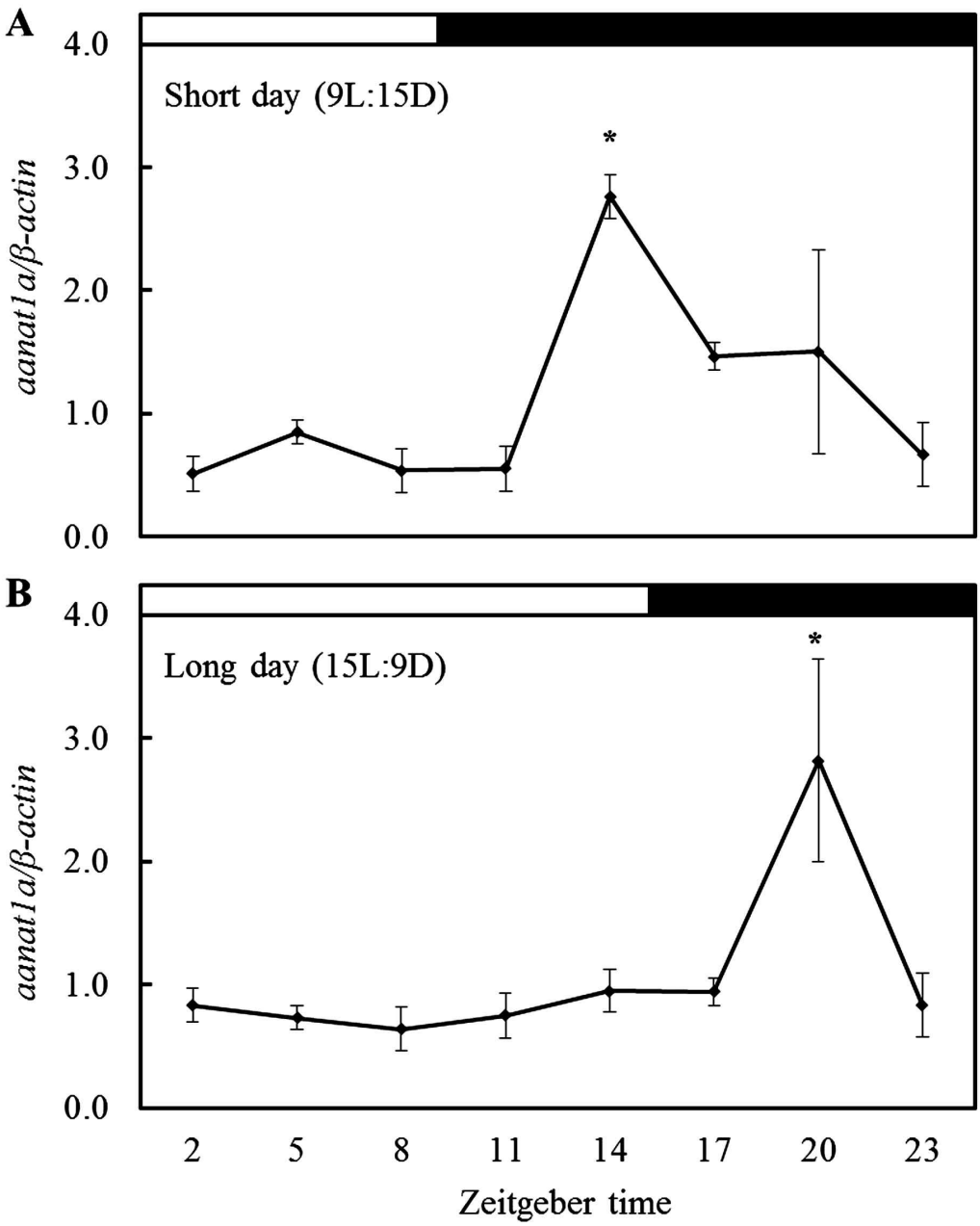

Fig. 1. Daily oscillation of *aanat1a* mRNA in retina under artificial different photoperiodic conditions. Photoperiodic conditions was divided to short day (9L:15D, A) and long day (15L:9D, B). Asterisk represents statistical difference at $P<0.05$. Values are mean ± SEM.

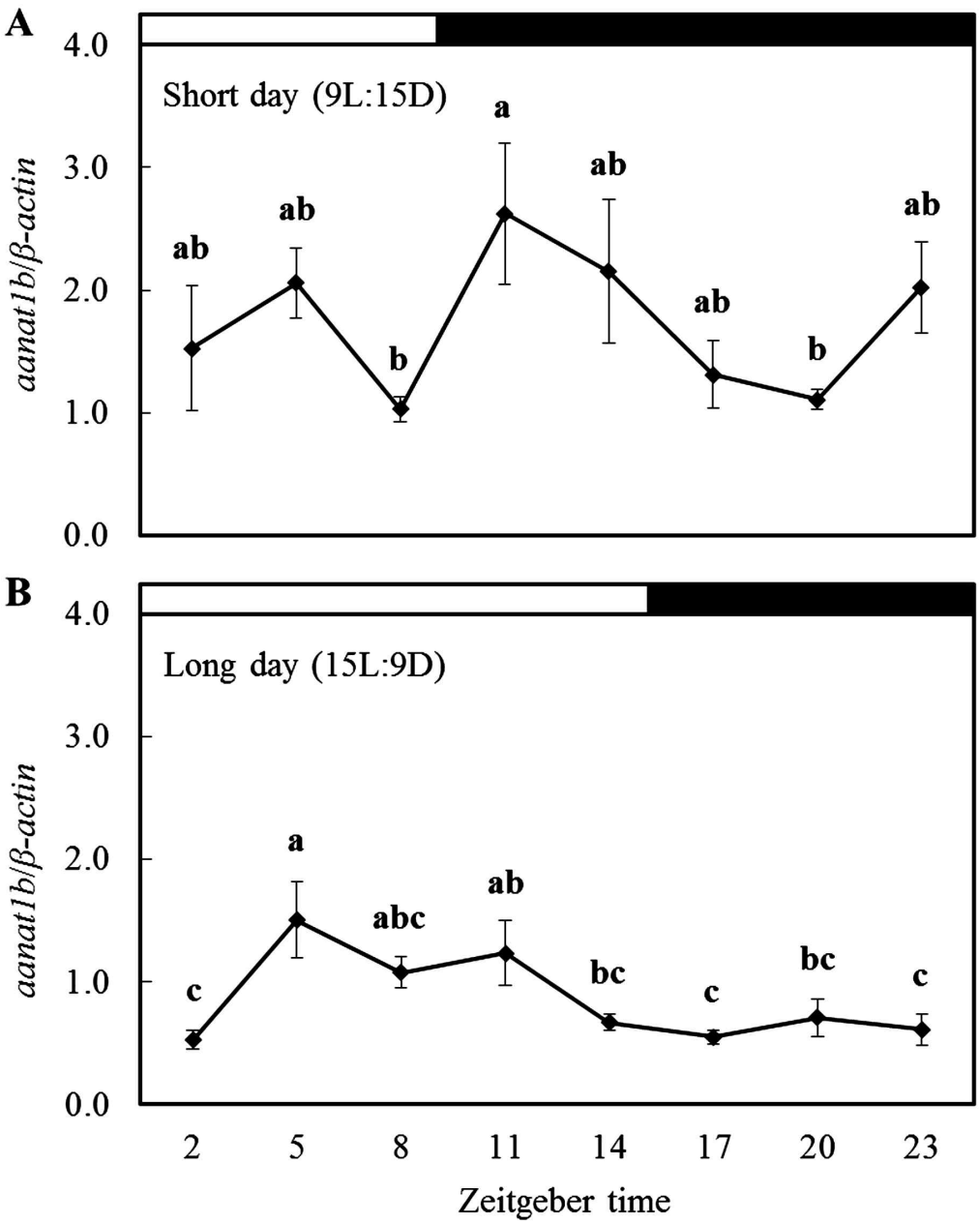

Fig. 2. Daily oscillation of *aanat1b* mRNA in retina under artificial different photoperiodic conditions. Photoperiodic conditions was divided to short day (9L:15D, A) and long day (15L:9D, B). The different letters indicate statistically different values (P<0.05). Values are mean ± SEM.

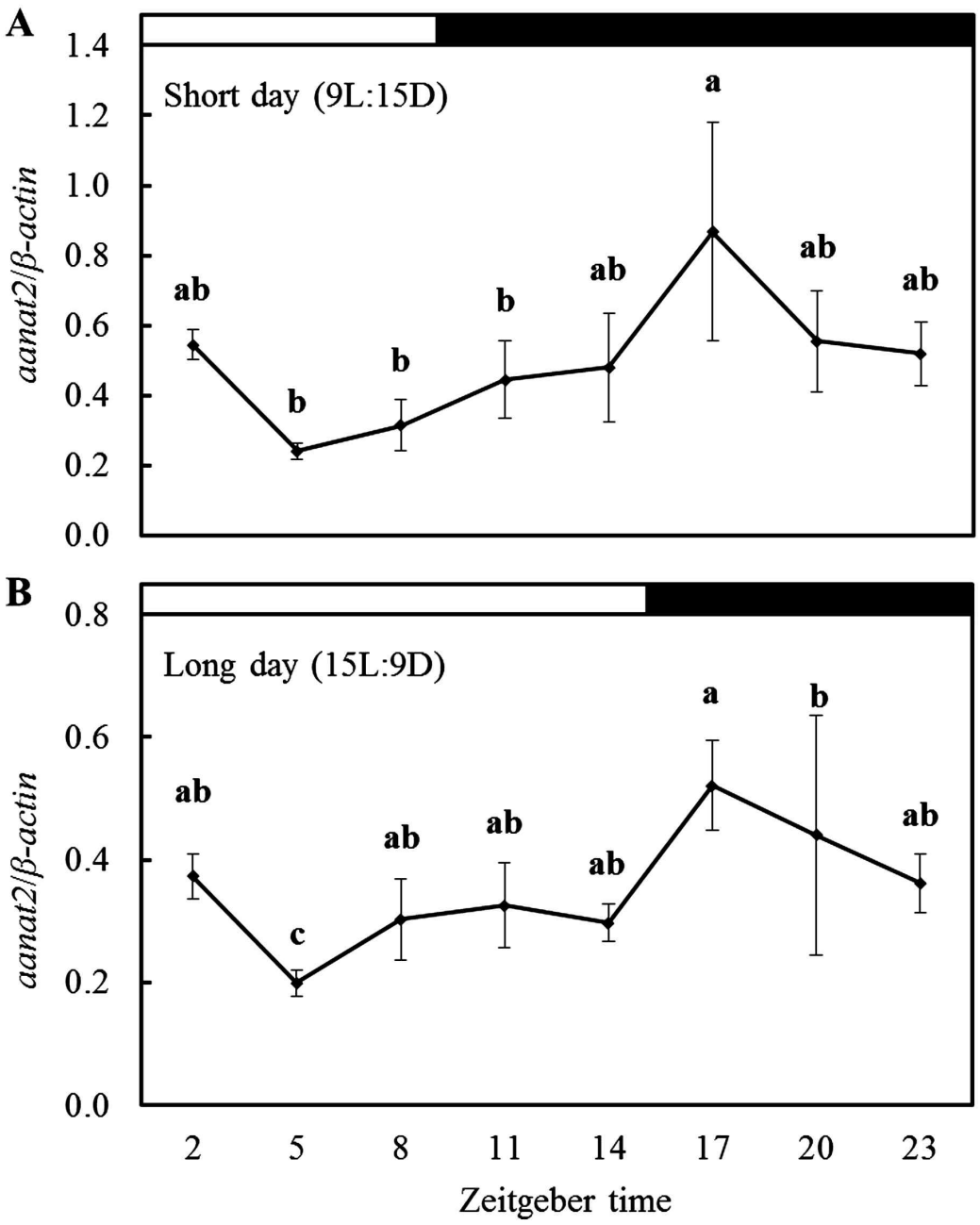

Fig. 3. Daily oscillation of *aanat2* mRNA in brain under artificial different photoperiodic conditions. Photoperiodic conditions was divided to short day (9L:15D, A) and long day (15L:9D, B). The different letters indicate statistically different values (P<0.05). Values are mean ± SEM.

3.2. Relations between melatonin with reproduction-related genes expression

The expression of reproduction-related genes in the brain (*sbGnRH*, *chGnRH*, *saGnRH*, *kiss2* and *lpxrfa*) and pituitary (*fshβ* and *lhβ*) by melatonin i.p were analyzed using the real time-qPCR. In the brain, *sbGnRH* expression was showed no significant difference between control group and melatonin group (Fig. 4A). The *chGnRH* mRNA expression was significantly more increased in melatonin group than control group (Fig. 4B). Expression of *saGnRH* mRNA did not showed significant difference between melatonin group and control group, but *saGnRH* mRNA just showed decreasing trend in melatonin group than control group (Fig. 4C).

In the brain of melatonin groups, *kiss2* mRNA was showed significant reduction of expression than control group (Fig. 5A). However, *lpxrfa* mRNA expression was more inducted in melatonin group than control group (Fig. 5B).

In the pituitary, *fshβ* and *lhβ* expression were no different between control group and melatonin group (Fig. 6).

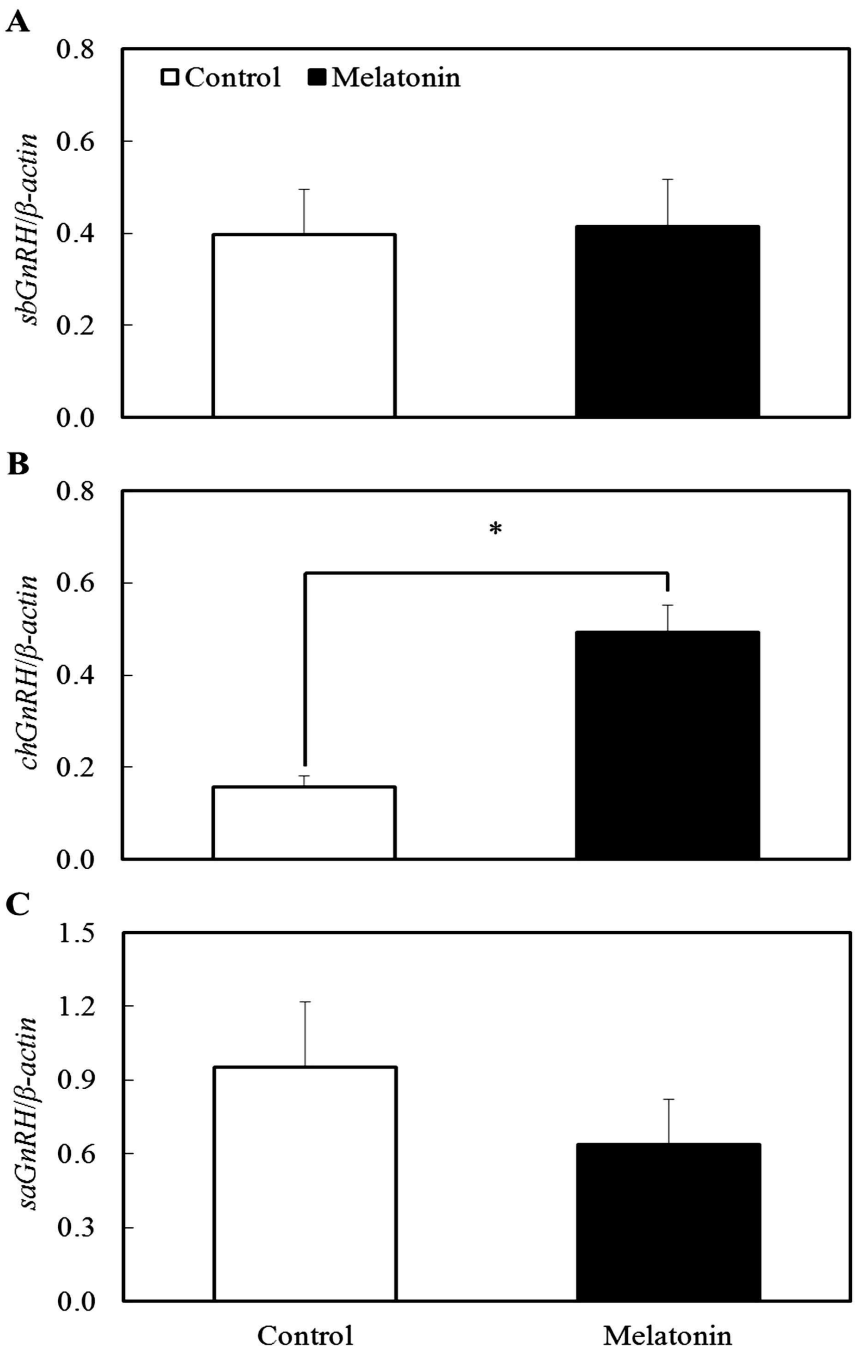

Fig. 4. The *sbGnRH* (A), *chGnRH* (B) and *saGnRH* (C) expression in the grass puffer brain by after 2 weeks of melatonin i.p. Asterisk represents statistical difference at $P<0.05$. Values are mean ± SEM.

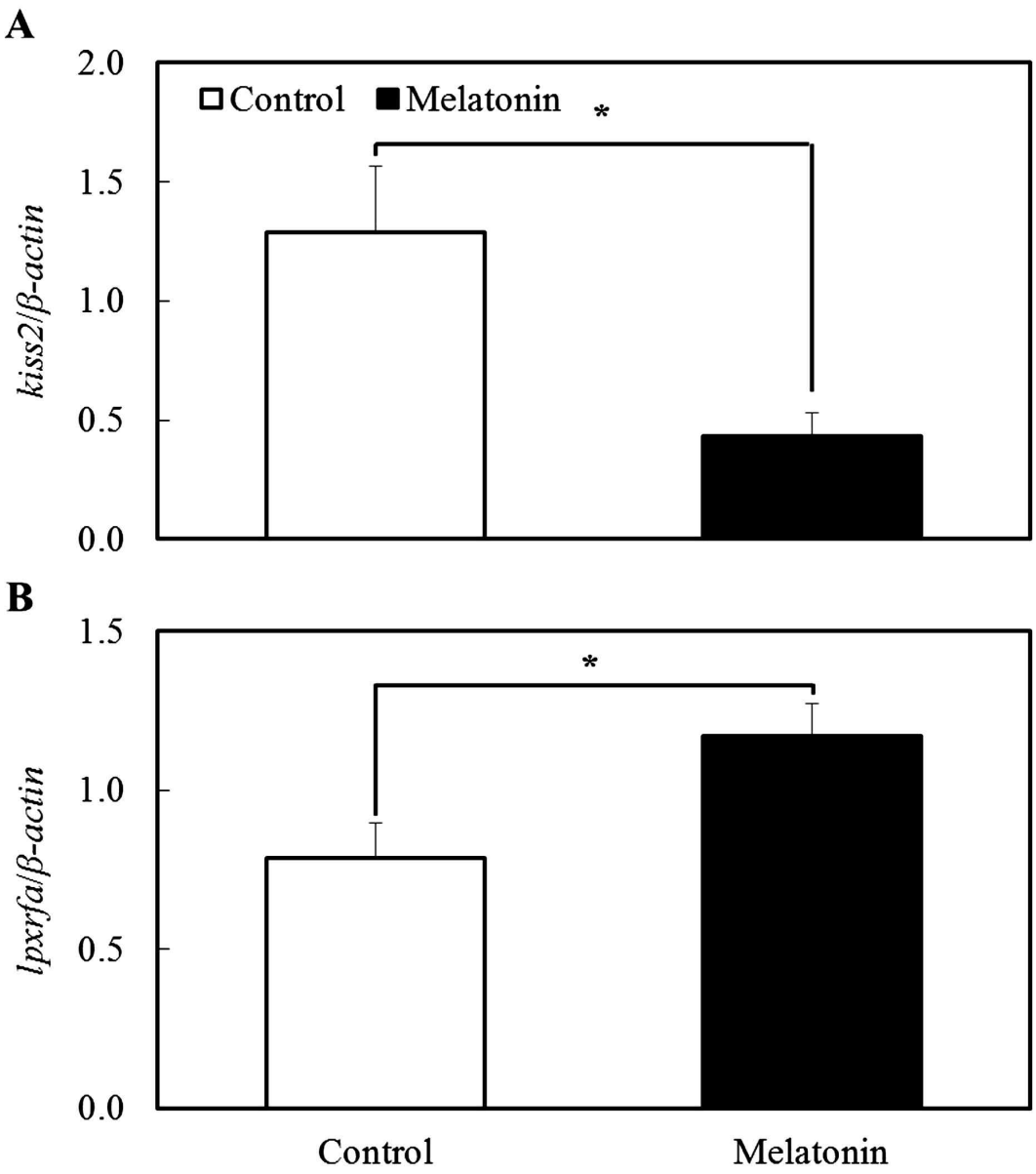

Fig. 5. The *kiss2* (A) and *lpxrfa* (B) expression in the grass puffer brain by after 2 weeks of melatonin i.p. Asterisk represents statistical difference at $P<0.05$. Values are mean ± SEM.

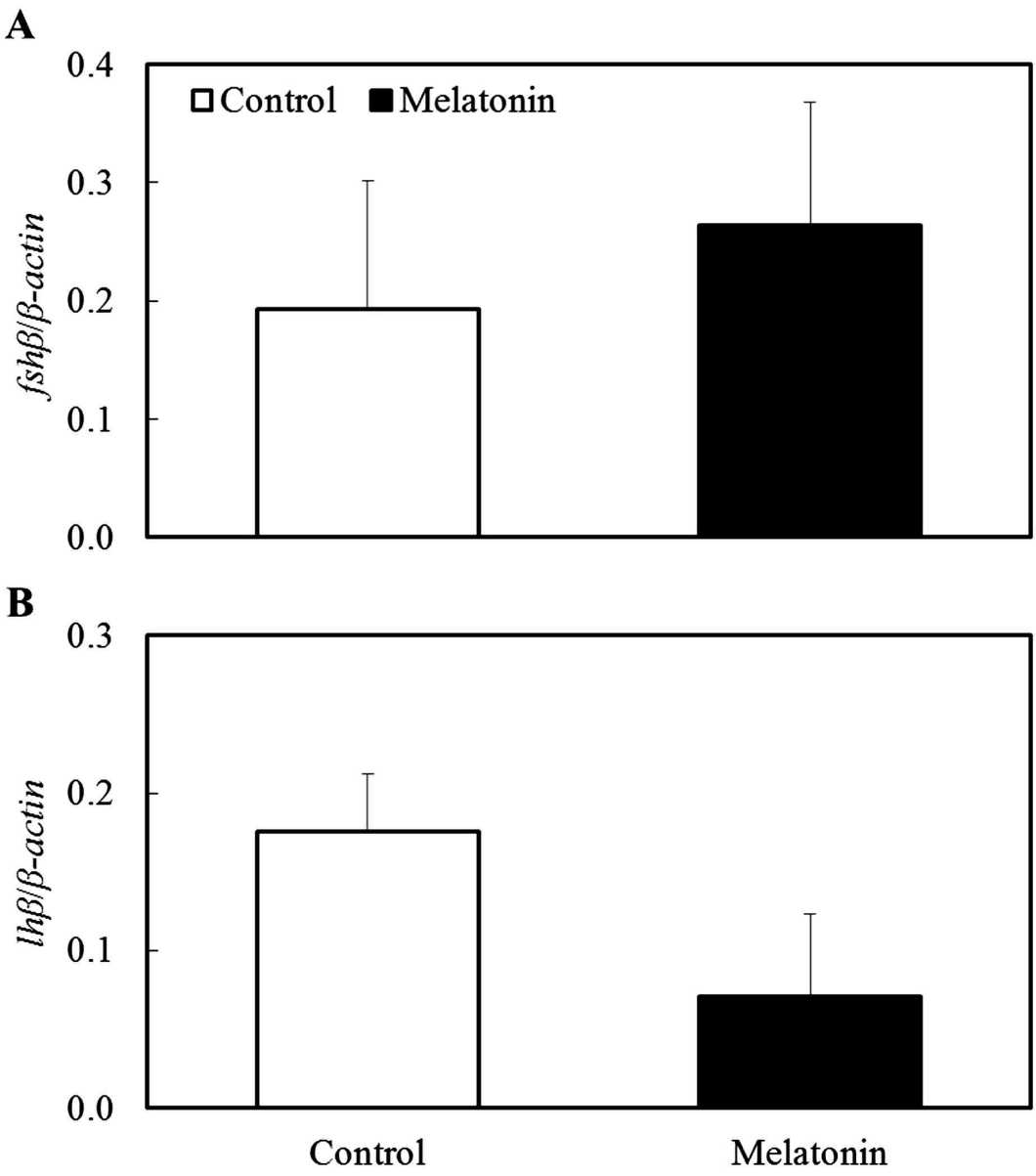

Fig. 6. The *fshβ* (A) and *lhβ* (B) expression in the grass puffer pituitary by after 2 weeks of melatonin i.p. Values are mean ± SEM.

3.3 Regulation of Sex maturation in grass puffer by artificial photoperiodic control

The female grass puffer reared in each different photoperiodic conditions, SD and LD, in during 14 weeks. The GSI levels of SD group was no different with GSI levels of 14 weeks ago but GSI levels of LD group was significantly induced than GSI levels of SD group (Fig. 7).

After 14 weeks, we investigated expression of three *GnRH*, *kisse2* and *lpxrfa* mRNA in brain and *fshβ* and *lhβ* in pituitary gland (Fig. 8 to 10). Three *GnRHs* genes expression in brain showed did not significant difference between SD ad LD groups (Fig. 8A to C). However, brain *kiss2* and *lpxrfa* mRNA were showed more inhibition of expression in SD group brain than LD group (Fig. 9A and B). Pituitary *fshβ* mRNA showed did not significant expression between SD and LD groups but *fshβ* mRNA tended to decrease in SD group (Fig. 10A). Pituitary *lh*β mRNA showed no difference between SD and LD groups (Fig. 10B).

Grass puffer ovarian development change in during experiment period was examined using the histological method (Fig. 11). The initial group ovary was observed some perinucleolus stage oocytes (PNS) and numerous oil-droplet stage oocytes (ODS) (Fig.11A). After 10 weeks, ovary of SD group was comprised numerous ODS and showed similar ovary development phase with initial group (Fig. 11B). However, ovary of LD group was observed many number of yolk stage oocytes (YS) and showed more mature phase ovary compared with LD group (Fig. 11C).

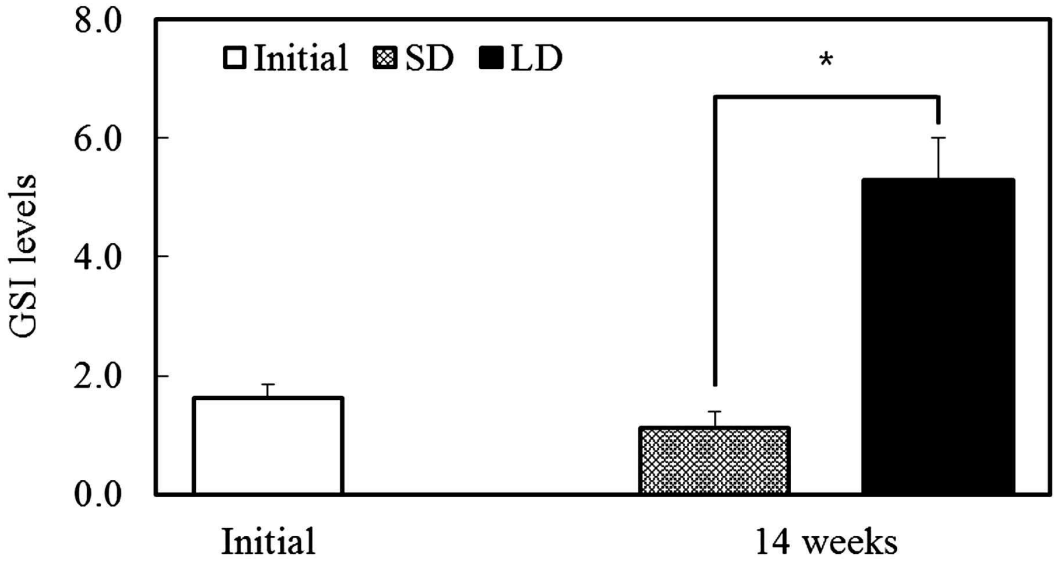

Fig. 7. Changes of female grass puffer GSI levels under short photoperiod (9L:15D) and long photoperiod (15L:9D) conditions. Asterisk represents statistical difference at $P<0.05$. Values are mean ± SEM.

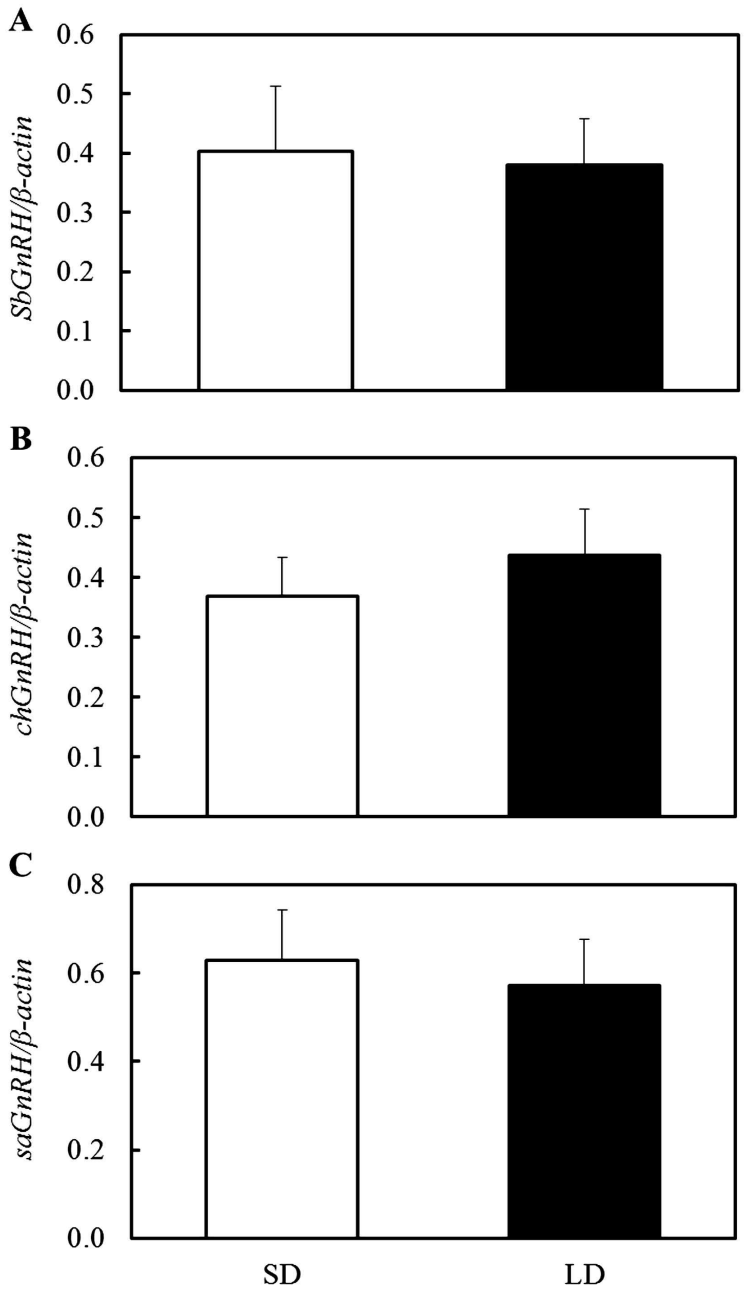

Fig. 8. Effect of photoperiod regulation during 14 weeks in the mRAN expression of *sbGnRH* (A), *chGnRH* (B) and *saGnRH* (C) in the brain of female grass puffer. Values are mean ± SEM.

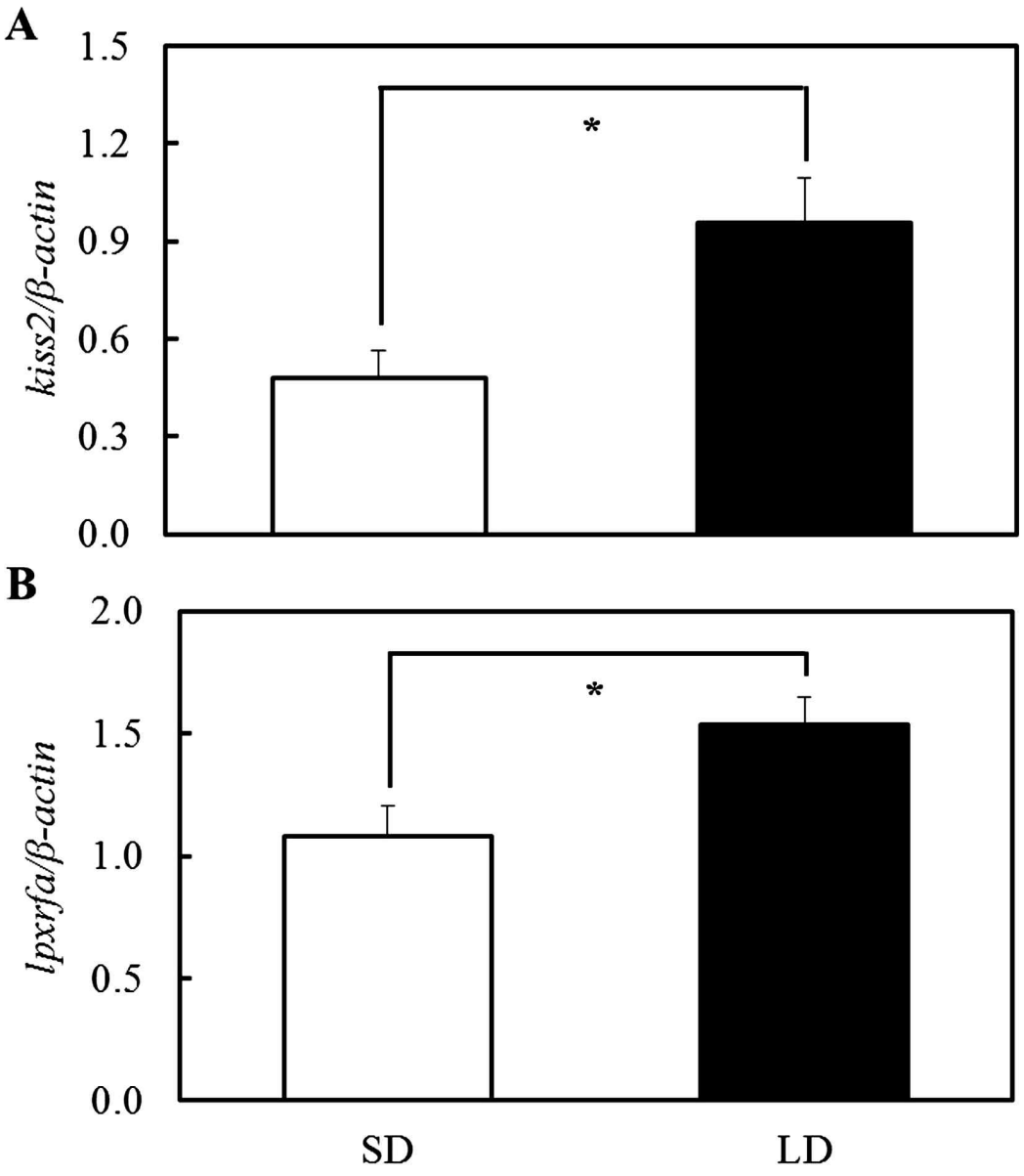

Fig. 9. Effect of photoperiod regulation during 14 weeks in the mRAN expression of *kiss2* (A) and *lpxrfa* (B) in the brain of female grass puffer. Asterisk represents statistical difference at $P<0.05$. Values are mean ± SEM.

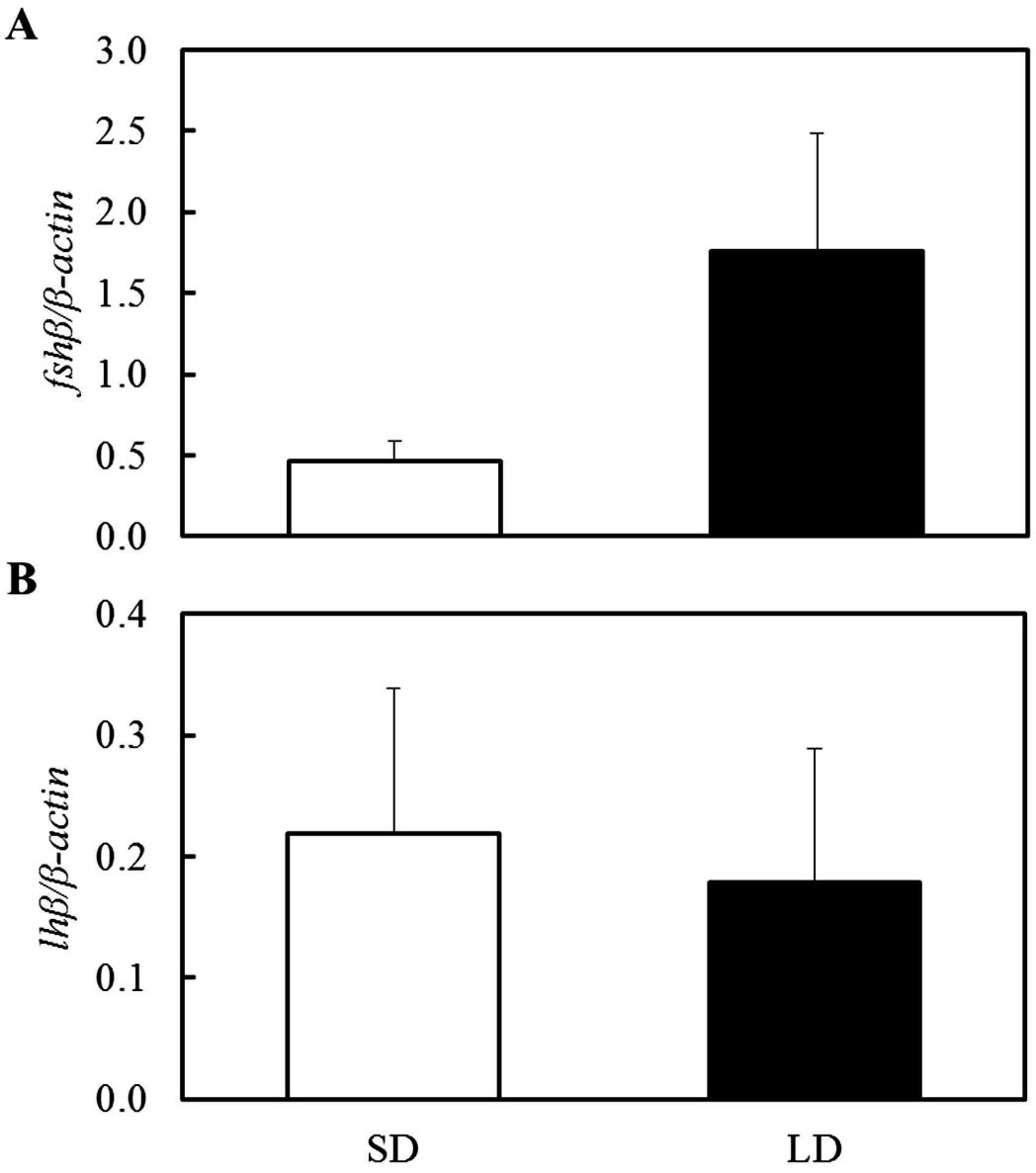

Fig. 10. Effect of photoperiod regulation during 14 weeks in the mRAN expression of *fshβ* (A) and *lhβ* (B) in the pituitary gland of female grass puffer. Values are mean ± SEM.

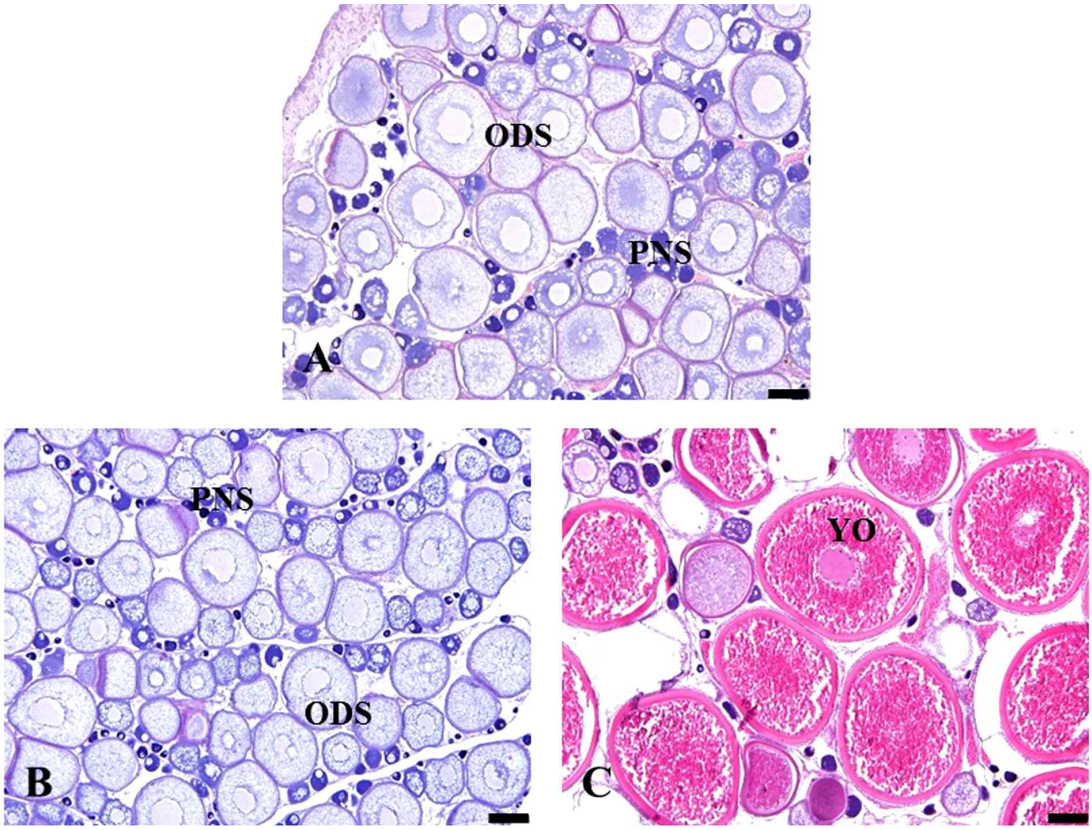

Fig. 11. Changes of ovarian developmental phase under short photoperiod (9L:15D) and long photoperiod (15L: 9D) conditions. Scale bar indicates 100 μm. PNS, peri-nucleolus stage; ODS, oil-droplet stage; YS, Yolk stage.

4. Discussion

In this study, we investigated the effect of artificial changes in the photoperiod upon the reproductive endocrine system of grass puffer. For this study, we explored the ocular *aanat1* subtype genes and brain *aanat2* gene oscillation under different photoperiod conditions. In addition, we conducted melatonin injections to assess the associations between melatonin and reproductive-related genes. Finally, we investigated the regulation of sex maturation by means of treatments with different artificial photoperiod conditions.

To explore the photoperiod effect in the expression of *aanat* genes, we conducted an analysis of *aanat1a*, *aanat1b* and *aanat2* mRNA oscillations in retina and brain under artificial short-day and long-day photoperiod conditions. In our study, ocular *aanat1a* expression revealed a nocturnal oscillation in both SD and LD photoperiod conditions, with a significantly peaked expression at ZT14 and ZT20 in both SD and LD conditions. The peak-times of *aanat1a* mRNA expression in both treatments were observed approximately 5 h after the lights were turn off, although the length of *aanat1a* expression differed between the two light conditions. However, in SD condition *aanat1b* mRNA did not show a daily expression, and expression peak-time also differed with *aanat1a* mRNA. In LD condition, *aanat1b* mRNA presented a diurnal rhythm. Melatonin increasing-time varied depending on photophase and scotophase length. Therefore, according to our results, ocular *aanat1a* mRNA was more sensitive in recognizing changes of external light signals compared with *aanat1b* mRNA, and the length of the *aanat1a* mRNA expression reflected the length of the photophase and scotophase. These results concerning the changes of *aanat1a* mRNA expression are similar to previous studies in the wrasse *Halichoeres tenuispinnis*, which presented nocturnal ocular melatonin oscillation under light and dark conditions, and the length of the increasing-time of ocular melatonin was modified by photoperiodic manipulation (Iigo et al., 2003). In addition, wrasse observed the induction and reduction of ocular melatonin when exposed to acute light

or dark conditions, respectively; this suggests that ocular melatonin is regulated by photic environment. Furthermore, previous studies concerning daily serum melatonin oscillation under different photoperiod conditions (DD, 8L:16D, 12L:12D and 16L:12D) in common dentex *Dentex dentex*, reported a change in melatonin increasing-time in different photoperiod conditions, excepting in DD condition (Pavlidis et al., 1998).

On the other hand, in our results, brain *aanat2* mRNA showed nocturnal oscillation under SD and LD conditions. Migaud et al. (2007) studied the photic regulation of the melatonin production system, describing at least three different melatonin production systems in different teleost species. The first system presented melatonin production in the pineal organ as a direct response to light and dark independently of the retina. In a second system, retina and pineal organ participated in the production of melatonin during scotophase. Finally, in the last system, melatonin was produced in retina as a response to light and dark.

In our results, we detected nocturnal oscillation of ocular *aanat1a* mRNA and brain *aanat2* mRNA; thus, it suggests that the melatonin in grass puffer is produced in both pineal organ and retina. However, the peak-time of *aanat2* mRNA presented no differences between photoperiodic conditions, suggesting a circadian oscillation. This circadian oscillation of melatonin secretion has been reported for some teleost fishes (Iuvone et al., 2005; Iigo et al., 2006). For example, nocturnal melatonin oscillation of zebrafish *Danio rerio* maintained during five day in constant darkness conditions (Cahill, 1995). These fishes have a circadian melatonin oscillation and high secretion during scotophase, mediated by a circadian clock. However, the circadian oscillation of melatonin in the pineal organ has not been confirmed in salmonid species (e.g., rainbow trout *Oncorhynchus mykiss*, masu salmon and sockey salmon *Oncorhynchus nerka*) (Coon et al., 1998; Iigo et al., 2007). Even thought our study did not investigate the circadian oscillation of *aanat* genes, we observed that only the brain *aanat2* mRNA oscillation maintained stable under different photoperiodic conditions between SD and LD. Therefore, we suggest that the ocular

aanat1a mRNA oscillation is controlled by photoperiod-signals, while the brain *aanat2* mRNA oscillation is independently regulated by the circadian clock. Further studies are needed to clarify and explore the circadian oscillation of ocular *aanat1* and brain *aanat2* expressions.

The effect of melatonin on the reproduction endocrine system has been investigated by means of the regulation of photoperiod and melatonin administration in some teleost fishes (Maitra at al., 2001; Ghosh and Nath, 2005; Renuka et al., 2010). In zebrafish, *kiss1*, *kiss2* and *saGnRH* mRNA expressions in the brain were significantly increased after melatonin administration, which also induced follicle maturation (Carnevali et al., 2011). In the Atlantic croaker *Micropogonias undulates*, intraventricular administration of melatonin in the preoptic area was followed by a significant increase of LH release in the pituitary gland, suggesting a melatonin-derived effect in the hypothalamus and pituitary gland (Khan and Thomas, 1996). Killifish *Fundulus heteroclitus* studies report after melatonin exposure an increase in the expression of *GnRH* and LH receptor (*lhr*) mRNA both brain and ovaries (Lombardo et al., 2014). This suggests that *lhr* mRNA was directly regulated by melatonin, or by a gnrh-stimulated LH release in the pituitary gland that increased its gonadal receptor. These results suggested a positive effect of melatonin in fish reproduction. In contrast, an inhibition role of melatonin was reported in the sea bass, which were exposed to melatonin (via implantation or injection) (Servili et al., 2013; Alvarado et al., 2015). After melatonin exposure, kisspepatin and GnRHs genes showed an inhibition of expression. In the masu salmon, melatonin administration showed a reduction of GnRH and LH content in the pituitary gland (Amano et al., 2004). In these results, showed inhibitory effect on the *kiss1*, *kiss2*, *saGnRH* and *sbGnRH* mRNA expression by melatonin treatment in the brain. Thus, melatonin presented an inhibitory effect on the reproduction in sea bass and masu salmon.

Our results concerning melatonin activation showed similarly with those of the sea bass. To investigate the association between melatonin and reproductive-related genes, we conducted a 2-weeks analysis of genes-expression, preceded by a melatonin i.p

injection in grass puffer. We observed that, among the three types of *GnRH* from the brain, only *chgrh* mRNA presented a significant difference between melatonin group and control group; thus, we assume that *chGnRH* mRNA expression was stimulated by melatonin treatment. Although we did not detect any significant difference in the expression of *sbGnRH* and *saGnRH* mRNA between groups, we did detect a tendency to decrease in *saGnRH* mRNA expression within the melatonin group. On the other hand, we observed that both treatments (melatonin and control groups) had significant differences in *kiss2* and *lpxrfa* mRNA expressions; however, they presented an opposite trend. Melatonin treatment was followed by an inhibition effect in the *kiss2* mRNA expression, meanwhile *lpxrfa* mRNA expression was stimulated by melatonin. In the pituitary gland, *fshβ* and *lhβ* showed not difference in their expression due to melatonin treatment. The *lhβ* expression was partially reduced in the melatonin group compared with the control group, but this difference was insignificant. Even thought our results did not show difference in the expression of *GnRH* and *gth*, s*aGnRH* expression showed a slight reduction induced by the melatonin treatment. Furthermore, our results confirmed the inhibition effect upon *kiss2* mRNA and stimulation effect upon *lpxrfa* mRNA induced by melatonin treatment. Kisspeptin is a main regulation element in reproduction, which has a positive stimulation effect on sex maturation and puberty (Kanda et al., 2008; Roa et al., 2008 In contrast, LPXRFa is widely known to have a negative effect in sexual maturation of vertebrates, including some fishes (Tsutsui at al., 2010; Zhang et al 2010). Our results of decreased *kiss2* mRNA and increased *lpxrfa* mRNA expression suggests that melatonin acts as an inhibitor of sexual maturation in grass puffer. Even though *fshβ* and *lhβ* expression were not directly affected by melatonin, we suggest that they may be indirectly affected, due to the regulation effect of melatonin upon *kiss2* and *lpxrfa* mRNA, which possibly influences *fshβ* and *lhβ* expression. Therefore, further studies are required to assess the association between melatonin and GtHs.

Here, we investigated the regulation of sexual maturation by means of treatments

with different photoperiod conditions. Studies focused on the control of sexual maturation via artificial manipulation of photoperiod have been already reported in some fish species, such as the masu salmon (Amano et al., 1995), gilthead seabream *Sparus aurata* , Nile tilapia *Oreochromis niloticus* (kissil et al., 2001), Atlantic cod *Gadus morhua* (Almeida et al., 2011) and olive flounder (Kim et al., 2013), in which long photoperiod conditions inhibited or delayed sex maturation, due to the inhibition of GnRH and GtH expression, or decreases in the blood concentration of sex steroid hormones. These phenomenons of GtH or sex steroid hormones inhibitions are thought to originate in the reduction of melatonin secretion in retinal or pineal organs, due to short lengths of the scotophases. On the other hand, melatonin may have a positive effect in the reproductive system of some fish species. However, our results gainsay these melatonin positive effects upon fish reproduction. Grass puffer were left 14 weeks under SD (9L:15D) and under LD (15L:9D) conditions; after that, in each photoperiod condition we observed different GSI levels, the expression of reproductive-related genes, and the ovarian development phase. In the SD group, GSI levels maintained stable; however, GSI levels of the LD group significantly increased. Concerning th reproductive-related gens analysis, three *GnRH* mRNA showed no difference in their brain expression between SD and LD groups; however, *kiss2* and *lpxrfa* mRNA were decreased in the SD group. Interestingly, this inhibited expression of *lpxrfa* presents a conflict with our previous results of increased *lpxrfa* mRNA expression induced by melatonin treatment. These results suggest that acute melatonin treatment directly activated the *lpxrfa* expression in order to suppress sexual maturation. However, long-term short photoperiod treatment would consistently induce the melatonin secretion, and decrease *lpxrfa* mRNA, due to negative feedback action of continuous increased melatonin. But the potential feedback-action between melatonin and *lpxrfa* gene is still unclear, and further deeper studies are needed. On the other hand, in pituitary gland, *fshβ* and *lhβ* mRNA expression did not present significant differences between groups; however, *fshβ* mRNA expression was mildly decreased in SD group compared with the LD

group. Finally, in LD group we observed ovaries containing mature oocytes; however, in the SD group, ovaries did not advanced in their sexual maturation. Therefore, our results suggest a negative action of melatonin in grass puffer reproduction.

We investigated the association between photoperiod manipulation and reproductive endocrine system in grass puffer. Grass puffer ocular *aanat1a* mRNA showed a change in its expression according to photoperiod manipulation, and SD treatment induced a longer length of high *aanat1a* mRNA expression, compared with LD treatment. Therefore, ocular *aanat1a* mRNA expression suggests that it is regulated by photoperiod manipulation. Furthermore, melatonin treatment directly inhibited *kiss2* and stimulated *lpxrfa* mRNA expression. Furthermore, long-term SD treatment suppressed gonadal development via inhibiting the expression of *kiss2* and *fshβ* genes. Therefore, the melatonin production, regulated by photoperiod manipulation, is suggested to negatively affect sexual maturation through the inhibition of reproductive-related genes in the grass puffer.

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감사의 글

2008년 5월 10일. 아무런 목표의식 없이 단순한 마음에 시작했던 연구실 생활이 어느덧 학사과정과 석사과정을 마치고.. 2016년 6월 27일. 이제는 박사과정의 끝자락에서 이렇게 감사의 글을 쓰고 있습니다. 불과 얼마 전까지 박사학위발표를 준비하면서 항상 불안해하고 걱정이 많았던 시간을 지내왔지만 학위발표를 마친 지금은 그동안의 힘들었던 시간이 저에게는 무엇과도 바꿀 수 없는 특별한 시간이었으며, 힘든 시간 뒤에 느낄 수 있는 성취감은 무엇보다도 소중한 경험이었습니다. 참으로 다양한 경험을 할 수 있었습니다. 기쁘고 행복한 순간도 있었던 반면, 힘들고 포기하고 싶었던 순간 역시 있었습니다. 하지만 그럴 때 마다 위로해주고 넘어지지 않도록 뒤에서 저의 등을 밀어주셨던 많은 분들이 계셨기에 지금의 제가 있을 수 있다고 생각합니다. 항상 감사한 마음을 잊지 않겠습니다.

참으로 부족하고 철이 없는 제자였습니다. 그럼에도 교수님께서는 끝까지 저의 손을 놓지 않으셨고 결국 여기까지 제자를 지도해주셨습니다. 이영돈 교수님. 진심으로 감사드립니다. 교수님께서 해주셨던 많은 조언들을 잊지 않고 실천 할 수 있는 제자가 되기 위하여 최선을 다하여 노력하겠습니다. 그리고 저의 부족한 학위논문을 심사하기 위하여 제주도까지 내려오셔서 아낌없는 조언을 해 주셨던 권준영 교수님. 항상 열정적인 모습으로 학생들을 가르치시고 모두가 자신들의 제자인 듯 학생들의 미래를 진심으로 걱정해주시는 여인규 교수님, 이경준 교수님, 김기영 교수님. 진심으로 감사의 인사를 드립니다. 또한 대학의 발전과 인재 양성에 힘쓰시는 학과 교수님들과 강원도립대학교 김형배 교수님, 부경대학교 백혜자 교수님께도 감사의 말씀을 드립니다.

연구실 생활을 하면서 많은 인연들을 만났으며, 이러한 인연들 덕분에 많은 어려움을 극복하여 지금의 자리까지 도달 할 수 있었습니다. 저의 연구실 생활 처음부터 박사과정을 마무리하고 있는 지금까지도 한결같은 모습으로 연구실의 살림을 꾸려나가고 있는 “CR”의 대표 이치훈 선배님. 얼마 전까지 같이 연구실에서 생활하다가 지금은 부산에서 자신의 뜻을 위하여 열심히 노력 중인 허상우 선배님. 결국에는 그 어려운 붉바리 종묘를 만들어낸 부문수 선배님. 아무런 목표가 없던 박사과정에 목표를 제시해 주셨던 허성표 선배님. 저에게 연구실과의 인연을 이어주셨던 류용운 선배님. “Fish Bone”의 대박을 위해 노력중인 이승현 선배님. 석사학위논문을 준비하고 있는 석사과정 삼총사 오승보 후배님, 전은정 후배님, 김은수 후배님. 붉바리 종묘를 위해 이곳 제주도까지 내려와 열심히 일하는 선문대 커플 조성관 후배님, 김범규 후배님. 학사과정 졸업을 위해 즐겁고

흥미로운 연구실 생활을 보내게 될 최송희 후배님. 웬만한 남자보다도 일 잘하고 앞으로의 길이 창창한 이지은 후배님. 연구실 생활기간에 밀려 억울한 막내생활을 하고 있는 이진영 후배님. 그리고... 비록 지금은 옆에 계시지 않지만 학문에 대한 열정과 함께 항상 후배들에게 많은 조언을 아끼지 않으셨던 김성훈 선배님. 연구실 선·후배님들과의 특별한 인연이 있었기에 특별하고 소중한 연구실 생활을 할 수 있었습니다. 모두 진심으로 감사드립니다. 지금은 각자의 길을 걷고 계시지만 저의 연구실 생활 초기에 후배들의 든든한 맏형이 되어주셨던 임봉수 선배님, 송영보 선배님. 그리고 저의 석사과정을 책임져 주셨던 박용주 선배님. 다른 연구실임에도 지식서비스를 아끼지 않으셨던 정형복 선배님. 선배님들의 가르침을 잊지 않겠습니다.

연구실 생활에 있어서 항상 학생들의 편의를 제공해주신 해양과학연구소의 강태연 선생님, 진창경 선생님, 김승필 선생님, 김선희 이모님, 윤태석 이모님, 지금은 학교에 계시는 변수철 선생님께도 감사의 말씀을 드립니다. 또한 RIS 사업단의 강창협 팀장님과 김정아 선생님, 이보람 선생님께도 감사의 말씀을 드립니다.

제대로 된 직장도 없이 기약 없는 학위과정을 옆에서 묵묵히 지켜봐주고 응원해줬던 나의 반쪽 순림이.. 지금까지 해주지 못했던 것 그 이상으로 더욱 잘 해주기 위해 최선을 다하여 노력하겠습니다. 항상 사랑하고 고맙습니다.

아무리 힘들어도 함께라면 소주 한 잔에 모두 털어버릴 수 있었던 늘 고마운 나의 친구들..일일이 언급하지는 못하지만 모두들에게 고마운 마음을 전합니다.

집에 제대로 찾아가지도 못하지만, 그래도 첫 째 아들이라는 이유 하나만으로 묵묵히 지금까지의 생활을 뒷바라지 해 주시고 언제나 믿음을 주셨던 아버지, 어머니. 형을 위해 많은 것을 희생하면서도 언제나 믿고 따라주던 내 동생. 앞으로 더욱 효도하고 믿을 수 있는 집안의 장남이 될 수 있도록 노력하겠습니다. 우리 가족 언제나 사랑하고 또 사랑합니다.

지금까지 부족했던 저를 아껴주시고 사랑해 주시는 모든 분들에게 다시 한번 감사의 말씀을 드리며, 그 분들에게 받은 사랑을 돌려드릴 수 있는 사람이 될 수 있게 노력하겠습니다. 모두 감사하고 사랑합니다.