Comparative Biochemistry and Physiology, Part A 160 (2011) 447–452

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Expression of type II iodothyronine deiodinase gene in the brain of a tropical spinefoot, *Siganus guttatus*

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ARTICLE INFO

Article history:
Received 12 December 2010
Received in revised form 28 March 2011
Accepted 28 March 2011
Available online 2 April 2011

Keywords:
Circadian
Food availability
Monsoon
Photoperiod
Rabbitfish
Real-time PCR

ABSTRACT

Type II iodothyronine deiodinase (D2) converts 3,5,3′,5′-tetraiodothyronine to 3,5,3′-triiodothyronine and is involved in regulating thyroid hormone-dependent processes in various tissues. D2 mRNA expression in the mediobasal hypothalamus is affected by photoperiod, which influences reproductive processes in temperate birds and mammals. We examined whether D2 mRNA is expressed in the hypothalamus (located in the forebrain within the diencephalon area) and whether its abundance is affected by day length, temperature, or food availability in the tropical spinefoot, *Siganus guttatus*, which is endemic to tropical monsoon areas. The reverse transcription-polymerase chain reaction (RT-PCR) revealed that D2 mRNA is expressed in various brain regions. The abundance of hypothalamic D2 mRNA was higher at 12.00 h than at 06.00 h or 24.00 h. Rearing fish under constant dark conditions resulted in a decrease in D2 mRNA abundance during the subjective night. A single injection of melatonin lowered D2 mRNA abundance within 3 h. Collectively, it appears that hypothalamic D2 mRNA abundance is regulated by the circadian system and/or melatonin. No differences in D2 mRNA abundance were observed, when fish were reared at 20, 25, and 30 °C. However, food deprivation stimulated D2 mRNA expression during the daytime. These results suggest that photoperiodic and nutritive conditions affect hypothalamic D2 mRNA expression in *S. guttatus*.

1. Introduction

Iodothyronine deiodinase activates 3,5,3′,5′-tetraiodothyronine (T_4), a major hormone of the thyroid gland that is secreted mainly from the thyroid follicles (Köhrle, 1999). Conversion from T_4 which is a prohormone to 3,5,3′-triiodothyronine (T_3), an active form of thyroid hormones (TH), is mediated by outer-ring deiodination (ORD). ORD and inner-ring deiodination (IRD) are also involved in the inactivation of T_4 to 3,3′,5′-triiodothyronine (rT_3) and of T_3 and rT_3 to 3,3′-diiodothyronine (3,3′-T_2). To date, three types of iodothyronine deiodinases have been identified and cloned in several teleosts (Valverde-R et al., 1997; Sanders et al., 1999; Klaren et al., 2005); type I (D1; EC 1.97.1.10) has ORD and IRD activities, whereas types II (D2; EC 1.97.1.10) and III (D3; EC 1.97.1.11) have ORD and IRD activity, respectively (Köhrle, 1999).

Recently, D2 has been associated with photoperiodic regulation of reproductive activity in the brain of mammals (Watanabe et al., 2004; Revel et al., 2006; Yasuo et al., 2006, 2007) and birds (Yoshimura et al., 2003; Yasuo et al., 2005). Watanabe et al. (2004) found that in the Djungarian hamster, *Phodopus sungorus*, D2 mRNA is expressed within the ependymal cell layer lining the infralateral walls of the third ventricle and the cell-clear zone overlying the tuberoinfundibular sulcus, and it fluctuates by increasing under long-day conditions and decreasing under short-day conditions. Yoshimura et al. (2003) also showed that D2 mRNA expression is stimulated in the mediobasal hypothalamus (MBH) of the Japanese quail, *Cortunix japonica*, under long-day, but not short-day conditions. Furthermore, melatonin administration lowered D2 mRNA expression under long-day conditions in the Djungarian hamster (Watanabe et al., 2004), the Syrian hamster, *Mesocricetus auratus* (Yasuo et al., 2007), and the Japanese quail (Yoshimura et al., 2003). These findings suggest that the perception and transduction of photic information and TH metabolic processes based on photoperiodism in the hypothalamic area play an important role in initiating and terminating reproductive activity during appropriate seasons in seasonal breeding animals.

To date, no experimental evidence indicates whether iodothyronine deiodinases in the hypothalamic area are involved in the reproductive activities of teleost fishes. Diverse mechanisms regulating fish reproduction may make experimental approaches and analyses difficult, as multiple environmental factors are perceived and utilized for reproductive activity in fish (Bromage et al., 2001). For example, long-day conditions are the principal factors initiating gonadal development in the Indian major carp, *Catla catla* (Dey et al., 2005), Senegal sole, *Solea senegalensis* (Vera et al., 2007), and sapphire

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doi:10.1016/j.cbpa.2011.03.023

devil, *Chrysiptera cyanea* (Bapary et al., 2009). In some cases, changes in temperature accelerate gonadal development processes (Vera et al., 2007; Bapary et al., 2009). These findings indicate that fish fundamentally use photoperiodism and utilize additional factors – temperature, social interaction, and food availability – to adapt to diverse environments (Takemura et al., 2010).

The goldlined spinefoot (formerly named the golden rabbitfish or orange-spotted spinefoot), *Siganus guttatus*, is endemic to the tropics and is widely distributed in the eastern Indian and western Pacific oceans (Woodland, 1990). The reproductive season of this species lasts for two months from June through July (Rahman et al., 2000a,b) in Okinawa, Japan (26°42′N, 127°52′E). In these subtropical waters, increases in water temperature and photoperiod are the likely cues that initiate reproductive activity. In contrast, in tropical waters with less variation in photoperiod and water temperature, the monsoon climate is the driving factor in the same species (Sri Susilo et al., 2009). The aim of the present study was to obtain basic information on changes in D2 mRNA abundance in the brain – specifically in the hypothalamic area – of the goldlined spinefoot, which were experimentally reared under controlled light and temperature conditions. Food availability was also assessed because periodic changes in primary production are a principal environmental factor in the tropical monsoon climate (Johannes, 1978).

2. Materials and methods

2.1. Animals

Goldlined spinefoot (*S. guttatus*, Perciformes, Siganiae) fry (0.08 to 0.15 g) were collected using hand nets from the mangrove estuary of the Teima River, northern Okinawa, Japan, during daytime low tide around the new moon in July and August, 2007. The fish were transferred to Sesoko Station (26°42′N, 127°52′E), Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan, and reared in holding tanks (capacity 5MT) with a constant seawater flow under ambient temperature and natural photoperiodic conditions. Commercial pellets (EP1 and EP2; Marubeni Nisshin, Tokyo, Japan) were given to the fish daily at 10.00 h. Immature fish with an average body mass of 200 ± 0.5 g were used in the present study.

All experiments were conducted in compliance with the Animal Care and Use Committee guidelines of the University of Ryukyus and with the regulations for the care and use of laboratory animals in Japan.

2.2. Distribution and daily/circadian variations of D2 mRNA abundance in the hypothalamic area

The fish were transferred to outdoor polyethylene tanks (capacity 0.5 MT) with ambient temperature and running seawater and were acclimated to the rearing conditions with a fixed food provision at 10.00 h for 1 week. Fish ($n = 5$) were captured from the tanks at 12.00 h, anesthetized in 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan), and immediately killed by decapitation. The entire brain was removed and separated into several parts including the cerebellum, hypothalamus that is the basal area of the diencephalon, medulla oblongata, olfactory bulb, optic tectum, and telencephalon (Fig. 1). The samples were immediately immersed in RNA*later*® (Applied Biosystems, Foster City, CA, USA), refrigerated at 4 °C overnight, and stored at −20 °C.

A study was conducted to determine the daily variations in hypothalamic D2 mRNA expression levels. Fish were reared under natural photoperiodic conditions. Since the experiments were carried out in September, the day length was approximately 12 h. The fish ($n = 8$ per sampling time) were taken from the outdoor polyethylene tanks at 18.00, 24.00, 06.00, 12.00, and 18.00 h. Fish under constant dark conditions were kept in indoor tanks (capacity 0.5 MT) with running seawater. The samples ($n = 5$ per sampling time) were collected at circadian time (CT) 6 and CT18 to compare relative D2 mRNA levels between subjective day and night. After the whole brain was removed from the fish, the hypothalamus was separated and treated using the above-mentioned procedures. The sample collection during the dark period was conducted under a dim red light of less than 1 lx on the bench.

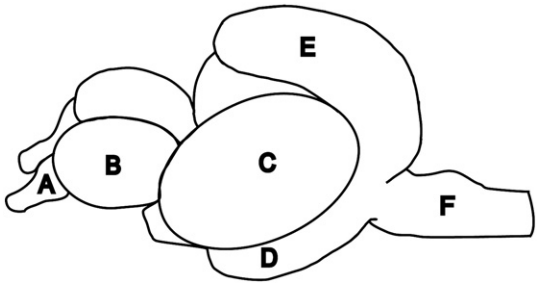

Fig. 1. Schematic representation of the goldlined spinefoot brain used for RT-PCR analysis of D2 gene expression. The brain was separated into six parts; part A; olfactory bulb, part B; telencephalon, part C; optic tectum, part D; hypothalamus, part E; cerebellum, part F; medulla oblongata.

2.3. Melatonin administration

Fish were transferred to two 60 L glass aquaria with running seawater at 25 ± 1 °C under natural photoperiodic conditions, and acclimated for 1 week. We prepared and administered melatonin to the fish according to Hernández-Rauda et al. (2000). Briefly, melatonin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ethanol and diluted in saline. At 12.00 h, melatonin was injected intraperitoneally into the fish ($n = 12$) at a dose of 500 ng/g body mass (experimental group), whereas saline without melatonin was injected into control fish ($n = 12$) (control group). At 3 ($n = 6$ per group) and 12 h ($n = 6$ per group) after injection, the whole brain was sampled from the anesthetized fish, and the dissected hypothalamic area was processed as described above.

2.4. Alternation of photoperiod, temperature, and nutritive status

For the experiment on photoperiodic conditions, fish ($n = 12$ per aquarium) were acclimated to two 60 L aquaria with running seawater at 25 ± 1 °C, which were set at LD = 16:8 (long-day; lights on at 04.00 h and off at 20.00 h) and LD = 8:16 (short-day;lights on at 08.00 h and off at 16.00 h). A fluorescent bulb (20 W) with 900 lx illuminance at water surface was set on each aquarium, which was measured using a quantum photoradiometer (Model HD 9021, Delta OHM, Padova, Italy). The fish were fed daily with commercial pellets at 10.00 h. After the fish acclimated to the conditions for 1 week, hypothalamic samples were collected at 12.00 h ($n = 6$)and 24.00 h ($n = 6$) and processed as described above.

For the experiment on different temperature conditions, fish ($n = 8$ per aquarium) were transferred to three 60 L aquaria with running seawater at 25 ± 1 °C under natural photoperiodic conditions. After a one week acclimation period, the temperatures of each aquarium were adjusted gradually to 20 °C (lowest in winter), 30 °C (highest in summer), and 25 °C (temperature during peak spawning season). Temperature was controlled using heat pumps with a temperature-control system and programmable set points that maintained the desired temperatures irrespective of the surroundings. This was achieved by attaching sensors to the bottoms of the water baths in which the aquaria were placed. Photoperiod was set at LD = 12:12 with a 20 W fluorescent bulb. Illuminance on the aquaria was maintained at 1200 lx. The fish were fed with commercial pellets daily at 10.00 h. Samples from the hypothalamic area were collected

at 12.00 h after the fish acclimated under these conditions for 1 week and processed as described above.

A nutritive status experiment was conducted in October, 2009. The fish were transferred to two 300 L polyethylene tanks with running seawater at 25 ± 1 °C under natural photoperiodic conditions (day length was 11 h 42 min). Fish in one tank ($n = 16$), but not in the other tank, were fed daily at 10.00 h with commercial pellets at 5% of body mass and maintained under these conditions for 2 weeks. After anesthetization, hypothalamic samples were collected at 12.00 h ($n = 8$) and 24.00 h ($n = 8$) and processed as earlier described.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from respective brain parts using TriPure Isolation Reagent (Roche Applied Sciences, Indianapolis, IN, USA), according to the manufacturer's instructions. To avoid genomic DNA contamination, the samples were treated with deoxyribonuclease (RT grade; Nippon Gene, Tokyo, Japan) at 37 °C for 15 min. RNA purity was measured at 260/280 nm, and the samples with absorbance of 1.8–2.0 were used for complementary DNA (cDNA) synthesis.

The cDNA was reverse-transcribed from 1000 ng total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) for the reverse-transcription polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR), according to the manufacturer's instructions.

2.6. RT-PCR

The cDNA from respective brain parts was amplified with RT-PCR in a Green master mix (total volume 25 μL) containing 12.5 μL of GoTaq® DNA polymerase (Promega, Madison, WI, USA), 1 μL of cDNA template, 0.25 μL of each primer, and 10.5 μL of nuclease free water. RT-PCR was performed with two specific primer sets designed from the cloned D2 sequence (GenBank accession number, **GU372962**): 5′-CACAAAGA-TAAGCCGGGAAG-3′ (forward) and 5′-AGGCATCTAGCAGGAAGCTG-3′ (reverse) for D2 as well as 5′-CCTGACAGAGCGTGGCTACT-3′ (forward) and 5′-TTGCTGATCCACATCTGCTG-3′ (reverse) for β-actin. The same primer sets were used in the previous study, in which gene expression of D2 and β-actin was determined in the liver of the goldlined spinefoot (Wambiji et al., in press). The amplicons generated using the primers had expected product sizes of 502 and 501 bp, respectively. The PCR cycle was programmed as follows: 1 cycle of 94 °C for 2 min, 28 cycles of 94 °C for 45 s, 58 °C for 45 s, 72 °C for 1 min and one last cycle of 72 °C for 3 min. The PCR products were electrophoresed on 1% agarose gel and visualized as fluorescent bands at 502 bp and 501 bp, respectively. Reactions without sample cDNA were considered to be negative controls.

2.7. qPCR

Hypothalamic D2 mRNA expression levels were assessed using a CFX96™ Real-Time System C1000™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The primer sets were 5′-GATCTGCTCGT-CACACTCCA-3′ (forward) and 5′-TTCACCAGCACCACAGAGTC-3′ (reverse) for D2 and 5′- TCCTCCCTGGAGAAGAGCTA-3′ (forward) and 5′-CAGGACTCCATACCGAGGAA-3′ (reverse) for β-actin, which were previously designed (Wambiji et al., in press). The qPCR reaction mixture (10 μL in total) contained 5 μL Express SYBR® GreenER™ qPCR Supermix Universal (Invitrogen, Carlsbad, CA, USA), 0.3 μM forward primer, 0.3 μM reverse primer, 2 μL 5-fold diluted cDNA template, and 2.4 μL RNase-free water. The following PCR cycle conditions were used: initial denaturation of 95 °C for 30 s, 40 cycles of amplification including denaturing at 95 °C for 5 s, and annealing and extension at 60 °C for 34 s. The specificity of the PCR amplicons was determined by conducting a melting-curve analysis by raising the temperature of the sample slowly from 60 to 95 °C until the final step of the PCR. D2 and β-actin mRNA expression was measured in triplicate according to Park et al. (2007). β-actin was used as the reference gene to normalize the expression data using the normalized gene expression $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

2.8. Statistical analysis

All data were expressed as the mean ± SEM. Comparisons were conducted using a one-way and two-way analysis of variance (ANOVA), followed by Bonferroni's and Tukey's HSD post hoc tests, respectively. The Student's *t*-test and Mann–Whitney *U*-test were also used to analyze the statistical differences between two sets of data.

3. Results

3.1. Distribution and variation in D2 mRNA

D2 mRNA was detected in the entire brain (Fig. 2). When the brain was separated into several parts, the abundance of this gene was detected in all parts. No reaction occurred in the control sample.

Under natural photoperiodic conditions, D2 mRNA abundance varied daily with a significant increase ($P<0.05$) at 12.00 h compared with 24.00 h and 06.00 h (Fig. 3). Under constant dark conditions, D2 mRNA abundance was significantly higher ($P<0.05$) at CT6 than at CT18 (Fig. 4).

3.2. Melatonin treatment

Melatonin was given to the fish at 12.00 h, and D2 mRNA expression was determined at 3 h and 12 h after hormone administration. A two-way ANOVA revealed that the effect of melatonin administration, but not time, was significant ($P<0.05$). Melatonin administration was significantly ($P<0.05$) associated with lower D2 mRNA abundance after 3 h, but not 12 h later (Fig. 5).

3.3. Effect of altered photoperiod, temperature, and food availability

The effects of changes in photoperiod, temperature, and food availability on D2 mRNA abundance were assessed. The fish were acclimated to LD = 16:8 (long-day) or LD = 8:16 (short-day) conditions, and D2 mRNA abundance in the hypothalamic area was compared between 12.00 h and 24.00 h because a significant difference in this gene abundance was observed in these two points (see Fig. 3). A significant difference ($P<0.05$) in D2 mRNA abundance was observed in the fish maintained under long-day conditions, and D2 mRNA expression was significantly ($P<0.05$) higher at 12.00 h than at 24.00 h. In contrast, no daily variations in D2 mRNA expression were observed in the fish kept under short-day conditions (Fig. 6).

Fig. 7 shows the effect of water temperature on D2 mRNA expression in the hypothalamic area. Fish were reared at 20, 25, and

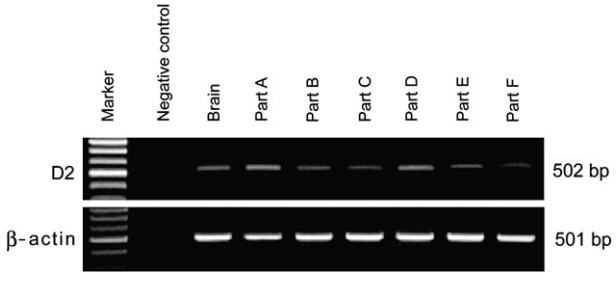

Fig. 2. D2 mRNA expression in different brain regions. The whole brain of the fish reared under natural conditions was collected at 12.00 h. The brain was separated into six parts (see Fig. 1). Expression levels of D2 mRNA were detected using RT-PCR and normalized by β-actin. The negative controls contained no cDNA templates. D2 and β-actin had 502 bp and 501 bp, respectively.

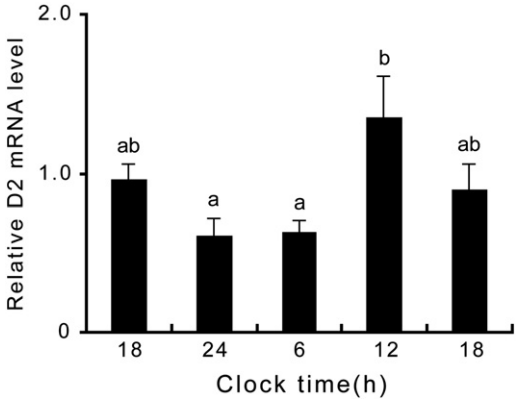

Fig. 3. Daily rhythm of D2 mRNA expression in the hypothalamic area of goldlined spinefoot. The fish ($n=8$ per each sampling time) were reared under natural conditions and sampled at intervals of 6 h within a 24 h cycle. Data represent means ± SEM. Different letters on each column indicate significant differences (one-way ANOVA followed by Bonferroni-type multiple *t*-test, $P<0.05$).

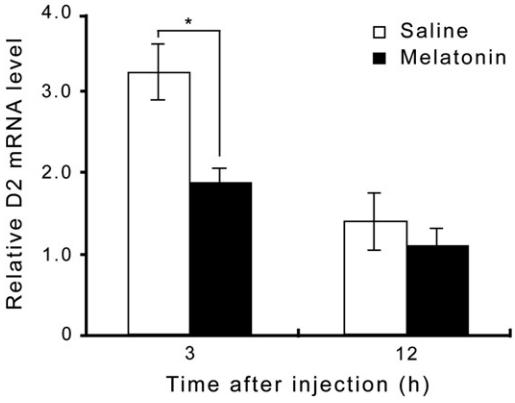

Fig. 5. Effect of melatonin injection on D2 mRNA expression in the hypothalamic area of goldlined spinefoot. Saline with melatonin at a dose of 500 ng/g body mass was given to the fish of experimental group ($n=12$), while saline without melatonin to the fish of control group ($n=12$).Fish were sampled within 3 ($n=6$ per group) and 12 h ($n=6$ per group) after injection. White and black columns represent control and experimental group, respectively. Data represent means ± SEM. Asterisks indicate significant differences (two-way ANOVA followed by Tukey-HSD test, $P<0.05$).

30 °C, which mimicked water temperatures in the winter, spring, and summer habitats of this species, respectively. No significant differences were noted in the abundance of hypothalamic D2 mRNA.

The effect of food availability on D2 mRNA expression in the hypothalamic area was also examined. When D2 mRNA abundance was compared between 12.00 h and 24.00 h, D2 mRNA expression was significantly ($P<0.01$) higher at 12.00 h than at 24.00 h. Day–night differences in D2 mRNA gene expression were not significant in fish supplied with sufficient food (Fig. 8). The hepatosomatic index of the food-limited fish was significantly lower than that of fish provided with sufficient food (data not shown).

4. Discussion

The present study shows widespread distribution of D2 mRNA expression in the brain of the goldlined spinefoot. Similar widespread distribution of this gene has been reported in the brain of avian (Gereben et al., 1999) and mammals (Croteau et al., 1996; Kalsbeek et al., 2005; Fekete et al., 2007). It is suggested that the central nervous system has the ability to autoregulate thyroid status and maintain T_3 availability within physiological levels (Kalsbeek et al., 2005).

Day–night differences in hypothalamic D2 mRNA abundance in the goldlined spinefoot were observed with significantly higher D2 mRNA levels at 12.00 than at 06.00 or 24.00 h. A difference in D2 mRNA abundance between the subjective day and night was also confirmed when the fish were reared under constant dark conditions. These results suggest that the D2 expression is regulated, in part, by the circadian system. Kalsbeek et al. (2005) reported daily variations in D2 activity in the cortex, hypothalamic area, and pituitary and pineal gland of the Wistar rats with intact suprachiasmatic nuclei (SCN), and these were abolished in SCN-lesioned rats, although the daily rhythmic pattern of enzyme activity was different among brain tissues. These results suggest that oscillation of D2 activity in the neural tissues is regulated by a master biological clock, as in mammalian species. However, the existence of a master biological clock, such as the SCN, has not been demonstrated in teleosts. It is possible that an additional oscillatory system may exist for D2 mRNA in the goldlined spinefoot. One possibility is that the pineal organ indirectly participates in D2 mRNA abundance in the hypothalamic area because the pineal organ of some teleosts directly perceives environmental light–dark cycles and shares a function as a circadian oscillator (Bromage et al., 2001; Cahill, 2002). Furthermore, this organ secretes melatonin, which is an indoleamine hormone and a transducer of photic and circadian information to neural and peripheral tissues (Bromage et al., 2001). The temporal organization of melatonin production in pineal organs is not identical in fish. In most species such as pike, *Esox lucius* and zebrafish, *Danio rerio*, the endogenous clock in the pineal organ contributes to the rhythmic component of melatonin synthesis by driving a rhythm in the mRNA expression of arylalkylamine *N*-acetyltransferase (AANAT), which is

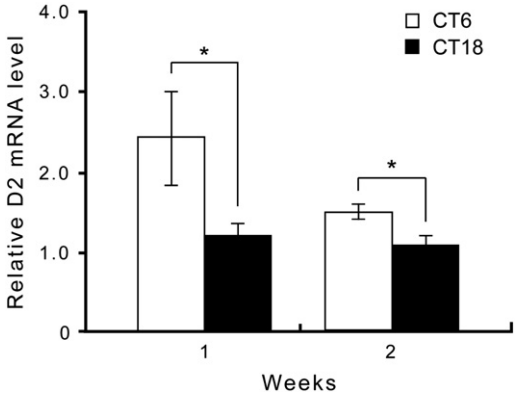

Fig. 4. Changes in D2 mRNA expression levels in the hypothalamic area of goldlined spinefoot at 1 and 2 weeks after exposure to constant dark conditions. White and black columns represent CT6 ($n=5$) and CT18 ($n=5$), respectively. Data are expressed as means ± SEM. Asterisks indicate significant differences (Mann–Whitney *U*-test, $P<0.05$).

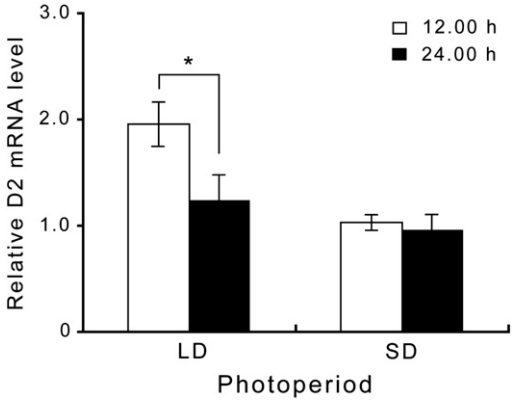

Fig. 6. Effect of artificial photoperiods on D2 mRNA expression in the hypothalamic area of goldlined spinefoot. Fish were reared under conditions of long-day (LD, $n=12$) or short-day (SD, $n=12$) within a duration of one week. The samples were collected at 12.00 h (white column, $n=6$) and 24.00 h (black column, $n=6$). Data represent means ± SEM. Asterisk indicates significant differences (Student's *t*-test, $P<0.05$).

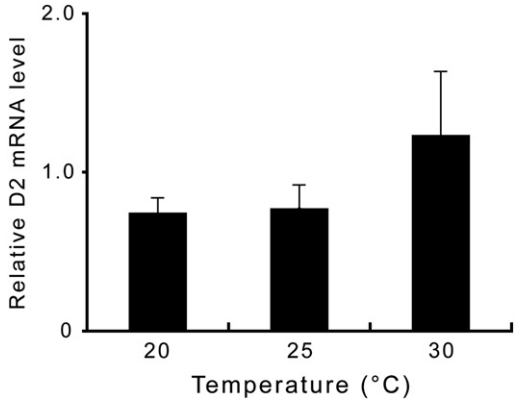

Fig. 7. Effect of different temperatures on D2 mRNA expression in the hypothalamic area of goldlined spinefoot. The fish ($n=8$ per group) were reared under temperatures at 20, 25, and 30 °C for 2 weeks. The samples were collected at 12.00 h. Data represent means ± SEM.

the rhythm-generating enzyme of melatonin biosynthesis. As a result, under constant light and darkness, melatonin release from the pineal organ in such species follows a circadian pattern. However, 'clock-regulated' melatonin rhythm generating systems are absent in the pineal organ of rainbow trout and other salmonids. Clock-driven changes in AANAT mRNA do not play a role in melatonin synthesis in the trout pineal organ, where light appears to turn off the activity of AANAT without changing mRNA levels (Bégay et al., 1998). In the case of the goldlined spinefoot, plasma melatonin levels exhibit daily fluctuations, with an increase during nighttime and a decrease during daytime (Takemura et al., 2004). Additionally, melatonin secretion from cultured pineal organs fluctuated under constant dark conditions (Takemura et al., 2006). Because we showed that administering melatonin lowers hypothalamic D2 mRNA abundance during daytime, melatonin secreted by the pineal organ may regulate mRNA abundance in this brain region. An alternative possibility is that photoreceptors adjacent to the hypothalamic area directly participate in the alternation of D2 mRNA expression, as vertebrate ancient *long* (VAL) opsin has been cloned from the brain of the goldlined spinefoot (Takeuchi, Park, Takemura, unpublished data). However, further studies are needed to clarify this possibility, as there is no direct evidence of the involvement of these photoreceptors in D2 gene expression.

Yoshimura et al. (2003) found in the Japanese quail, a temperate bird, that single light pulses within the photo-inducible phase specifically induce D2 mRNA expression in the mediobasal hypothalamus (MBH) including in the nucleus hypothalamicus posterior medialis, the infundibular nucleus, and the median eminence. Furthermore, exposing the Japanese quail to long-day conditions resulted in D2 gene expression in the infundibular nucleus and the median eminence. *In situ* hybridization analyses revealed that D2 mRNA expression in the MBH of the Syrian hamster is induced under long-day conditions and that its expression is suppressed by pinealectomy and melatonin treatment (Revel et al., 2006). A similar effect of melatonin administration on D2 mRNA expression was reported in the Djungarian hamster (Watanabe et al., 2004) and Syrian hamster (Yasuo et al., 2007), suggesting that melatonin acts as a signal transducer controlling the photoperiodic response by regulating D2 mRNA expression in the MBH (Yasuo et al., 2007). Since we found that exposure of the fish to long-day conditions rather than short-day conditions resulted in a large day–night difference in D2 mRNA abundance, it is likely that photoresponsiveness in the hypothalamic area of the goldlined spinefoot is similar to that of mammals and birds. Although we did not assess seasonal changes in melatonin levels, it is generally accepted that melatonin secretion in teleosts is partially related to the seasonally changing pattern of day length (Bromage et al., 2001); the dark-phase duration is reflected by a nocturnal increase in plasma melatonin (Vera et al., 2007).

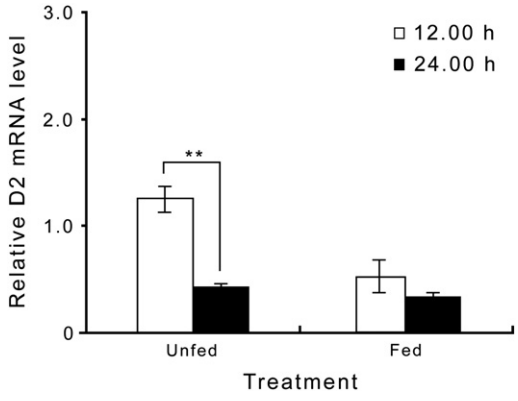

Fig. 8. Effect of food deprivation on D2 mRNA expression in the hypothalamic area of goldlined spinefoot. The fish were reared under fed ($n=16$) and unfed ($n=16$) conditions for 2 weeks. The samples ($n=8$ per sampling time) were collected at 12.00 h (white column) and 24.00 h (black column). Data represent means ± SEM. Asterisks indicate significant differences (Student's *t*-test, $P<0.01$).

Unlike higher vertebrates such as avian and mammals (Yasuo and Yoshimura, 2009), it is likely that D2 mRNA abundance in the hypothalamic area is not only affected by photoperiod, but also by other environmental factors. Photoperiod and water temperature are crucial factors controlling the seasonal reproductive cycle of fishes in the temperate zone, which involves the initiation and termination of gonadal development (Bromage et al., 2001). The environmental factors controlling the seasonal reproductive cycle seem to be different in fishes originating from tropical waters. In the case of the goldlined spinefoot, for example, the annual reproductive activity in the coral reefs of Okinawa, Japan (26°42′N, 127°52′E), extends for 2 months beginning in June when temperature and photoperiod are both increasing (Rahman et al., 2000a,b). The same species at the Karimunjawa Archipelago, Indonesia (05°83′S, 110°46′E), has two reproductive seasons: from March to May and from September to November, which correspond to transitions between the rainy and dry seasons (Sri Susilo et al., 2009). The latter case appears to indicate the importance of the tropical monsoon for initiating and terminating the seasonal reproductive cycle of the goldlined spinefoot (Takemura et al., 2010). The involvement of additional environmental factors related to the monsoons and the control of reproduction has been reported in tropical fishes (Tyler and Stanton, 1995; Srinivasan and Jones, 2006). Johannes (1978) proposed that temperature, plankton productivity, rainfall, and speed of prevailing currents and winds are possible factors synchronizing the seasonal reproductive activity of coastal marine fishes in the tropics. When the effects of possible factors related to the tropical monsoons on D2 mRNA abundance were examined in the present study, nutritive status, but not temperature, had an impact on fluctuating D2 mRNA abundance. This result indicates that in addition to photic responsiveness, hypothalamic D2 mRNA abundance changes with particular factors faced by these fish in their aquatic environment. Herwig et al. (2009) reported that D2 mRNA expression in the hypothalamus (in the third ventricular tanycyte layer) of the Siberian hamster was induced in response to 48 h of starvation under short-day conditions, suggesting that food deprivation increases the deiodinase enzyme responsible for local T_3 synthesis in the rodent hypothalamus. In addition to photoperiodic regulation, the local metabolic system may act in response to nutritive status through deiodinase processes of thyroid hormones, as possible metabolic indicators of growth or energetic status, such as leptin, growth factors, cortisol, and growth hormone, interact with the hypothalamic–pituitary mechanisms (Bromage et al., 2001).

In conclusion, we clearly demonstrated that photic and nutritive stimuli are related to D2 mRNA expression in the hypothalamic area of

the goldlined spinefoot. These factors may play a role in triggering reproductive processes in tropical fishes. In fact, although long-day conditions are crucial for gonadal development, food availability alters its acceleration and deceleration in tropical damselfish (Bapary and Takemura, 2010). Because there is complexity in aquatic environments, it cannot be ignored that the apparent activity of iodothyronine deiodinases is concealed by allelic factors. Further studies using immunohistochemistry and/or *in situ* hybridization are needed to examine localization of iodothyronine deiodinases adjacent to gonadotropin-releasing hormone neurons as well as their site-specific roles.

Acknowledgment

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and a Joint Research Project under the Japan–Korea Basic Scientific Cooperation Program from JSPS to AT.

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