Doctoral Thesis of Philosophy

Studies on Environmental Regulation of Iodothyronine Deiodinase Genes in Tropical Fish

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Wambiji Nina Nawanjaya

Fish Physiology
Marine and Environmental Sciences
Graduate School of Engineering and Science
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Supervisor: Professor Akihiro Takemura

We, the undersigned, hereby, declare that we have read this thesis and we have attended the thesis defense and evaluation meeting. Therefore, we certify that, to the best of our knowledge this thesis is satisfactory to the scope and quality as a thesis for the degree of Doctor of Philosophy in Fish Physiology under Marine and Environmental Sciences, Graduate School of Engineering and Science, University of the Ryukyus.

THESIS REVIEW & EVALUATION COMMITTEE MEMBERS

(**Chairman) Akihiro Takemura**

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Abstract

Iodothyronine deiodinases play a role in regulating thyroid hormone (TH) dependant processes in vertebrates. The aim of this study was to clone and characterize the cDNA of type II (D2) and type III (D3) iodothyronine deiodinases of the goldlined spinefoot *Siganus guttatus*, and to examine involvement of environmental factors in mRNA expression of D2 and D3 in the goldlined spinefoot and the sapphire devil *Chrysiptera cyanea*. D2 of the goldlined spinefoot (SgD2) had 1013 bp that encoded a protein with 270 amino acids (aa) without the SECIS element. D3 of this fish (SgD3) yielded 1492 bp (269 aa) and a SECIS element between nucleotides 1267–1367. The deduced amino acid sequences of SgD2 and SgD3 showed high homology with those of fish in D2 and D3 clusters. Abundance of SgD2 and SgD3 mRNA in several tissues were expressed with an increase at 12.00 h and a decreased at 24.00 h. High expressions of these genes were observed in the brain and liver. Food deprivation suppressed the expression of SgD2, but not SgD3. The mRNA levels of SgD2 and SgD3 in the liver at 25 °C were higher than those reared at 20 and 30 °C. These results suggest that exogenous factors influence the mRNA levels of these genes in the liver and that transcription of the genes in certain tissues is partially regulated in a circadian manner. 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 SgD2 mRNA abundance during the subjective night. A single injection of melatonin lowered SgD2 mRNA abundance within 3 h. Collectively, it appears that hypothalamic SgD2 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 SgD2 mRNA expression during the daytime.

Effects of food availability and photoperiod on mRNA abundance of type-II (CcD2) and type-III (CcD3) iodothyronine deiodinase in the sapphire devil's brain were also examined. Fish out of breeding season were acclimated to LD=12:12 and then transferred to long (LD14:10; LP) and short (LD=10:14; SP) photoperiods with or without feeding for 1 week at 27 °C. The abundance of CcD2 and CcD3 mRNA in the brain was measured using quantitative real-time polymerase chain reaction (qPCR). Under LD=12:12, no day/night change occurred in CcD2 and CcD3 mRNA abundance. Both genes' abundance in the fed and unfed fish was higher at 12.00 h

than at 24.00 h under LP. Under SP, there was no day-night difference in CcD2 mRNA for the fed and unfed fish, although its expression was higher in the unfed fish than the fed fish. On the other hand, abundance of CcD3 mRNA in the fed fish, but not unfed ones, was higher at 12.00 h than at 24.00 h under SP. These results are indicative of an interaction between photic stimuli and nutritional status on expressions of D2 and D3 mRNA in the hypothalamus and liver of these two fish. Since these are possible environmental factors affecting reproductive performance in fish, it is suggested that local concentrations of thyroid hormones change in accordance with aquatic environments which fish live in.

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To a phrase that will forever be etched in my mind

'Ganbatte kudasai'

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General Introduction

Iodothyronine deiodinases are enzymes that belong to the family of selenoproteins with catalytic properties and responsible for deiodination of thyroid hormone (TH) (St. Germain and Galton, 1997). This deiodination process involves a stepwise removal of iodide atoms from the outer- and inner-rings of TH and their metabolites. They also uniquely contain selenium, which acts as a cofactor in the deiodinase activities. This selenium is in the form of a rare amino acid, selenocysteine (Köhrle, 1999, 2000), which is recognized as the 21st amino acid in ribosome-mediated protein synthesis (Berry and Larsen, 1995; Stadtman, 1996). Iodothyronine deiodinase activates 3,5,3,5′-tetraiodothyronine (T_4), a prohormone secreted from the thyroid follicles (Köhrle, 1999). Conversion from T_4 to 3,5,3′-triiodothyronine (T_3), an active form of 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), an inactive metabolite, 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; type I (D1 or Dio1, EC1.97.1.10) has ORD and IRD, while type II (D2 or Dio2, EC1.97.1.10) and type III (D3 or Dio3, EC1.97.1.11) have only ORD and IRD, respectively (Köhrle, 1999). Activation and inactivation of iodothyronine deiodinases causes changes in TH levels in neural and peripheral tissues which have an impact on various physiological functions in vertebrates.

Recently, it was reported in higher vertebrates that photoperiodic stimuli triggers the initiation and termination of reproductive activity and that iodothyronine deiodinases in the hypothalamic region act as a mediator of photoperiodic stimuli (Yoshimura et al., 2003; Watanabe et al., 2004; Yasuo et al., 2005; Yasuo et al., 2006, 2007a, b; Revel et al., 2006). For instance, long-day conditions up-regulate D2 mRNA expression and induce an increase in local T_3 levels in the mediobasal hypothalamus (MBH) of the Japanese quail *Cortunix japonica*, a long-day breeder (Yoshimura et al., 2003). Expression of D2 mRNA mediates a thyrotrophin (TSH) receptor-cyclic AMP (cAMP) signaling pathway (Nakao et al., 2008) and,

afterwards, stimulates the reproductive endocrine axis through the release of gonadotropin-releasing hormone from its neurons (Okamura, 2008).

Melatonin is an indoleamine hormone and its synthesis in the pineal gland and fluctuates daily with an increase during nighttime and a decrease during daytime (Reiter, 1993). This hormone plays a role in transduction of external day-night changes in peripheral and neural tissues (Falcon et al., 2010). Information of day length is also expressed as the signal of melatonin; the duration of the melatonin signal lasts longer under short-day conditions and shorter under long-day conditions (Yasuo et al., 2007b). It is found that melatonin affects D2 mRNA expression in mammals (Watanabe et al., 2004; Yasuo et al., 2007a) and avian (Yoshimura et al., 2003). Since synthesis of this hormone in the pineal gland is regulated by a circadian manner (Cahill, 2002), it is likely that photoperiodic signal transduction is involved in a clock-dependent local conversion of TH in MBH through expression of D2 mRNA.

Unlike in the case of higher vertebrates, reproduction of teleost fishes seems to be regulated by several environmental factors including photoperiod and temperature as proximate cues. Rhythmic changes in these two environmental factors are notable in the temperate waters. On the other hand, the tropical monsoon climate brings periodic changes in rainfalls, direction of current and winds, and primary productivity (Johannes, 1978), although less variation of temperature and photoperiod occurs in this area. Thus, these may be regarded as local environmental variables (Pankhurst and Porter, 2003). There may be diverse systems to transduce changes in environmental factors and to initiate and terminate reproductive activity. However, to date, there is no experimental evidence of involvement of iodothyronine deiodinases in the reproductive activities of teleost fishes. This may be partially due to the perception and utilization of multiple environmental factors involved in the reproductive activity of fish (Bromage et al., 2001).

The goldlined spinefoot (formerly named as golden rabbitfish or orange-spotted spinefoot) *Siganus guttatus*, originates from the tropics and is widely distributed in the eastern Indian and western Pacific oceans (Woodland, 1990). In Okinawa, Japan (26°42'N, 127°52'E),

the reproductive season of this species lasts for two months between June and July (Rahman et al., 2000a, b). On the other hand, the same species adapted to the tropical monsoon climate has two reproductive seasons from March to May and from September to November (Sri Susilo et al., 2009). In the former case, the reproduction of this species starts with the increase in photoperiod and temperature. In the latter case, the reproductive peaks coincided with changing phases between the dry and rainy season. On the other hand, the sapphire devil *Chrysiptera cyanea*, is a reef-associated species belonging to the family Pomecentridae and widely distributed in the eastern Indian and Western Pacific oceans (Allen, 1991). Its reproductive season lasts for five months from April to August in Okinawa (Bapary et al., 2009). It was reported that the gonadal development of this species can be controlled by long-day conditions within suitable temperature ranges (Bapary et al., 2010) and food availability (Bapary et al., 2011). Therefore, these species are ideal models for studying effects of multiple environmental factors on mRNA expression of iodothyronine deiodinases.

The purpose of this study is to examine how proximate and permissive environmental factors have an impact on the mRNA abundance of iodothyronine deiodinases in tropical fishes. In Chapter I, D2 and D3 cDNAs of the goldlined spinefoot were cloned from the brain and characterized. The tissue distributions and expression levels of D2 and D3 mRNA abundance were examined using reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR), respectively. These provide basic molecular information for further experiments in the following chapters. Effects of photoperiod, temperature, and food availability on D2 and D3 mRNA abundance in the liver and hypothalamus of goldlined spinefoot were examined in Chapter II and III, respectively. Involvement of melatonin and circadian clock in D2 and D3 mRNA abundance was also evaluated in these chapters. Similar approaches were carried out using the sapphire devil to confirm diverse effects of environmental factors on D2 and D3 mRNA abundance in Chapter IV because the reproduction of this species can be completely controlled by artificial conditions (Bapary et al., 2010, 2011).

Extensive studies have been carried out in teleost fishes on importance of TH in physiological aspects of growth, development and reproduction (Leatherland, 1982; Eales and Brown, 1993; St. Germain and Galton, 1994; Sanders et al., 1997; Mol et al., 1998). Little attention is paid to cellular transduction of TH signals, although iodothyronine deiodinases are key mediators between external information and endogenous stimuli. This study contributes to basic biology by elucidation of molecular mechanisms on transduction of environment stimuli to endogenous stimuli information as well as to aquaculture promotion by artificial control of reproduction and growth in fish.

Chapter I

Cloning and characterization of iodothyronine deiodinase genes (D2 and D3) in the goldlined spinefoot

1.1. Introduction

The enzymes responsible for the TH deiodination processes with catalytic properties are iodothyronine deiodinases, which belong to the family of selenoproteins (St. Germain and Galton 1997; Bianco et al., 2002). Three types of iodothyronine deiodinases – D1 (Dio1), D2 (Dio2) and D3 (Dio3) – have so far been identified in mammals (Leonard and Visser, 1986; Croteau et al., 1996; Davey et al., 1999), birds (Gereben et al., 1999), amphibians (Davey and Becker, 1995), and teleosts (Eales, 1985; Mol et al., 1993; Orozco et al., 1996, 1997;Valverde et al., 1997; Sanders et al., 1999; Klaren, 2005). They are all selenoproteins and characterized by the presence of an essential selenocysteine residue within the catalytic domain with a premature stop codon (TGA) in the open reading frame (ORF) and a selenocysteine insertion signal (SECIS) in the 3' untranslated region (UTR) of the cDNA (Berry et al., 1991; Mandel et al., 1992; St. Germain and Galton 1994; Becker et al., 1995; Croteau et al., 1995, 1996; Davey et al., 1995; Larry and Berry, 1995; Salvatore et al., 1995; Toyoda et al., 1995; Sanders et al., 1997; Valverde et al., 1997; Van der Geyten et al., 1997;1996a, b).

Deiodination processes by activities of ORD and IRD are tissue-specific and regulate intracellular TH availability and disposal (Galcía-G et al., 2004). In mammals, D1 with ORD and IRD activities is highly expressed in the liver, kidney, and thyroid gland (Visser, 1996, Sanders et al., 1997, St. Germain and Galton, 1997; Mol et al., 1998). D2 with only ORD activity is expressed predominantly in the brain, pituitary, and brown adipose tissue, thyroid gland, skeletal muscle and heart (Croteau, 1996; Salvatore et al., 1996a, b; Larry and Berry, 1995). D3 with only IRD activity is mainly found in brain, skin, liver, placenta, and fetal tissues (St. Germain et al., 1994; Becker et al., 1995; Croteau et al., 1995; Salvatore et al., 1995). According to diverse physiological roles of TH, it is possible that expression patterns of iodothyronine deiodinases are different among tissues (Van der Geyten et al., 2005). Compared with extensive studies in higher vertebrates, molecular information on iodothyronine deiodinase genes is very limited in fish. The molecular cloning of the genes has been carried out in mummichog *Fundulus heteroclitus* (Valverde et al., 1997; Orozco et al., 2002), Nile tilapia *Oreochromis niloticus* (Sanders et al., 1999), rainbow trout *Onchorynchus*

mykiss (Sambroni et al., 2001), brownbanded shark *Chiloscyllium punctatum* (Martinez et al., 2008), gilthead seabream *Sparus auratus* (Klaren, 2005) and Senegalese sole *Solea senegalensis* (Isorna et al., 2009).

D2 is a key enzyme in regulating TH action and responsible for mediation of local deiodination of T_4 (Leonard and Visser, 1986; Berry and Larsen, 1992). On the other hand, D3 also acts as a modulator of intracellular TH levels by the inactivation of T_3. Therefore, it is necessary to pay attention to D2 and D3, if effects of environment factors on expression of iodothyronine deiodinases are examined. The aim of this chapter was to clone the cDNAs of D2 and D3 in the goldlined spinefoot using 3'- and 5'- rapid amplification of cDNA ends (RACE) methods. Tissue distribution and day-night difference in D2 and D3 mRNA were also evaluated using qPCR methods. In this study, the D2 and D3 of the goldlined spinefoot were named as SgD2 and SgD3, respectively.

1.2. **Materials and Methods**

1.2.1. Fish

Juvenile goldlined spinefoot *Siganus guttatus* with a body mass of 0.08 – 0.15 g were collected from the mangrove estuary of the Teima River, Northern Okinawa, Japan, during daytime under low tide around the new moon in July and August by using nets with a small mesh size. They were reared in holding tanks (capacity 5 MT) with constant aerated seawater flow at ambient temperature and natural photoperiodic conditions at Sesoko Station (26^{o}42´N, 127^{o}52´E), Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan. They were fed daily at 10.00 h with commercial pellets (EP1 and then EP2; Marubeni Nisshin, Tokyo, Japan). Immature fish with a mean body mass of 200 ± 0.5 g (age 1) and mature fish with a mean body mass of 346 ± 0.5 g (age 3 and 4) were used in the present study (Fig. 1.)

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

1.2.2. Sample collections for molecular cloning and tissue expression

The fish were transferred in October 2008 to outdoor polyethylene tanks (capacity 300 L) with running seawater at 25 ± 1.0 ^{o}C under natural photoperiodic conditions and acclimated to the rearing conditions with a fixed food provision (commercial pellets) at 10.00 h for 1 week. For sampling, fish were taken from the tanks at 12.00 h, anesthetized with 0.01% of 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan), and immediately killed by decapitation. The whole brain from mature fish (n = 3) was collected for the initial isolation of SgD2 and SgD3 cDNA fragments from the goldlined spinefoot.

For the tissue distribution of SgD2 and SgD3 mRNA, the brain, retina, gill, heart, kidney, liver, spleen, gonad, and skin were collected at 12.00 h (n = 7) and 24.00 h (n = 7) from immature fish that had been acclimated under the same conditions. The samples were immediately immersed in RNA*later*® (Applied Biosystems, Foster City, CA, USA) at 4 °C and then stored at –20 °C until further analysis. During the dark period, samples were collected under a dim red light.

1.2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from the tissues using the TriPure Isolation Reagent (Roche Applied Science, Hague Road, IN) according to the manufacturer's instructions. When necessary, the samples for the real-time qPCR were treated with deoxyribonuclease (RT grade; Nippon Gene, Tokyo, Japan) at 37 °C for 15 min to avoid contamination with genomic DNA. The amount of RNA was measured at 260 and 280 nm, and samples with an absorbance ratio (A_{260}/A_{280}) of 1.8 – 2.0 were used for complementary DNA (cDNA) synthesis. cDNA was reverse-transcribed from 500 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) for qPCR according to the manufacturer's instructions. The first strand cDNA was synthesized from 1000 ng of total RNA using PrimeScript^{1st} strand cDNA Synthesis kit (Takara Bio, Otsu, Japan) for molecular cloning.

1.2.4. Cloning of iodothyronine deiodinase genes

For cloning of iodothyronine deiodinase genes in the goldlined spinefoot, SgD2 and SgD3 cDNA fragments were amplified using degenerate oligonucleotide primers that were designed on the basis of the highly conserved regions of the target genes using Primer3 software (Whitehead Institute/Massachusetts Institute of Technology, Boston, MA, USA) (Table 1). Oligo-nucleotide primers were designed on the basis of the D2 sequences of the tiger puffer *Takifugu rubripes* (GenBank accession no. AB360768), bastard halibut *Paralichthys olivaceus* (AB362422), and mummichog (FHU70869), while on the basis of D3 sequences of the Nile tilapia (Y11111), tiger puffer (AB360769), and bastard halibut (AB362423) (Table 1). The PCR was performed in 25 µl of sample with GoTaq Green Master Mix (Promega, Madison, WI) under the following cycling conditions: 1 cycle of initial denaturation for 2 min at 94 °C; 35 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and 72°C for 1 min and 1 cycle of 72 °C for 3 min. The PCR products were separated on a 1% agarose gel with an appropriate molecular weight marker, stained with ethidium bromide, and visualized under UV illumination (ATTO, Tokyo, Japan). When the PCR products of the predicted sizes were obtained, these were purified using the Wizard SV Gel and PCR Clean-up System kit (Promega). The purified amplicons (233 and 281 bp for SgD2 and SgD3 respectively were then ligated and cloned into the pGEM T-easy Vector (Promega). Plasmid DNA was then purified using the DNA Wizard Plus SV Miniprep DNA Purification System (Promega) and sequenced. RACE was carried out using the SMART RACE cDNA Amplification kit (Clontech Laboratories, Mountain View, CA, USA) according to the manufacturer's instructions. On the basis of the sequence of the partial cDNA fragments described above, the specific primers and nested primers for the RACE of SgD2 and SgD3 sequences were designed for the 5´- and 3´-ends, respectively (Table 1). RACE reactions in the first PCR were performed using the Universal Primer A Mix (UPM) and the gene-specific primers in a three-step touchdown PCR program: (1) 5 cycles of 94 °C for 30 s and 72 °C for 3 min; (2) 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; (3) 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72°C for 3 min. Nested PCR was performed using the 50-fold diluted first PCR

products as a template with the Nested Universal Primer A (NUP) and each gene-specific nested primer at the following cycling conditions: 94 °C for 2 min; 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 1 min; a final step of 72 °C for 3 min (Park et al., 2007). The cDNA fragments amplified by RACE were cloned into the pGEM-T Easy vector and then sequenced. To confirm that the sequences determined by 5'- and 3' RACE were derived from the same transcripts, we amplified cDNA containing the entire coding region by PCR with primers localized in the 5'- and 3' UTR regions (Table 1).

1.2.5. Sequence analysis

The new nucleotide sequences were deposited in GenBank (Accession number GU372962 for SgD2 and GU385469 for SgD3). The homology based on GenBank database was performed for the nucleotide and deduced amino acid sequences using the BLAST program. (http://ncbi.nlm.nih.gov/BLAST). Multiple alignments of full length nucleotide and deduced amino acids for phylogenetic analysis were performed using the full length deiodinase sequences of several vertebrates by the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The ORF regions were found through (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A phylogenetic tree was constructed by the neighbor-joining method, using PRODIST and CONSENSE program from the PHYLIP package (Ver 3.63, by J. Felsenstein, University of Washington, Seattle, WA, USA) through neighbor-joining method and Treeview (Win32) for plotting the tree. Iodothyronine deiodinase sequences (with their accession numbers) were retrieved from GenBank for alignment and phylogenetic analysis. To construct a strict consensus tree, bootstrap trials were run. SECISearch 2.19 was used to search and identify SECIS elements in nucleotide sequences on the basis of their primary sequences, secondary structures and predicted free energy criteria (http://genome.unl.edu/SECISearch.html). For the analysis of protein sequences and structures, the ExPASy (Expert Protein Analysis System) proteonomics server of the Swiss Institute of Bioinformatics (SIB) was used http://expasy.org/.

1.2.6. Quantitative real-time PCR (qPCR)

The expression levels of SgD2 and SgD3 mRNA were assessed using the CFX96™ Real-Time System C1000™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The forward and reverse primers for qPCR (SgD2-qPCR-F and SgD2-qPCR-R for SgD2, SgD3-qPCR-F and SgD3-qPCR-R for SgD3, and *β*-actin-qPCR-F and *β*-actin-qPCR-R for *β*-actin) were designed as shown in Table 1. *β*-actin mRNA levels in the same sample were determined using qPCR to normalize the expression data according to (Park et al., 2007). The qPCR reaction mixture (10 µl) 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 cDNA template, and 2.4 µl RNase-free water. The following PCR cycling conditions were used: initial denaturation at 95 °C for 30 s; 40 amplification and denaturation cycles at 95 °C for 5 s annealing and elongation at 60 °C for 34 s. To ensure the specificity of the PCR amplicons, melting curve analysis was carried out by raising the temperature of the sample slowly from 60 – 95 °C until the final step of the PCR. The expression levels of SgD2, SgD3 and *β*-actin mRNA were measured in triplicate. Data were normalized relative to the mean expression level of each gene and analyzed using the normalized gene expression ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen, 2001).

1.2.7. Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). Normality was tested using the Kolmogorov–Smirnov method. Student's *t*-test was used to analyze the statistical differences between two sets of data. Probability of $P < 0.05$ was considered to be statistically significant.

1.3. Results

1.3.1. Cloning and properties of SgD2 and SgD3

The present RACE analyses of SgD2 cDNA yielded 1013 bp with a 5′ UTR of 179 bp, an open reading frame (ORF) of 810 bp and an incomplete 3′ UTR region of 21 bp. (Fig. 2). The predicted amino acid sequence was 270 residues long. The ORF was interrupted by an in-frame TGA codon at position 591 that was likely to encode for selenocysteine (Sec). SECISearch 2.19 was used to search for SECIS element in the nucleotide sequence of this study. However, the sequence did not contain a SECIS element in the 3′ UTR. This seems to be due to failure of cloning of the entire 3′ non coding region.

The present RACE analyses of SgD3 cDNA yielded 1492 bp with a 5′ UTR of 150 bp, an ORF of 804 bp and a 3′ UTR of 335 bp. The Sec residue was at position 504. The predicted amino acid sequence of SgD3 was 269 residues long, which showed a consensus polyadenylation signal (AATAAA) at 1145 bp upstream of the poly (A) tail (Fig. 3). A SECIS element was found between nucleotides 1267 – 1367 (Fig. 4).

1.3.2. Sequence analysis of SgD2 genes

Regarding the amino acid sequence, SgD2 showed a high similarity with D2 from several teleosts, such as the gilthead seabream *Sparus aurata* (90%), bastard halibut (85%), medaka *Oryzias latipes* (83%), mummichog (85%), *Danio rerio* (75%), and tiger puffer (86%). It showed a moderately high similarity with D2 from the rat *Rattus norvegicus* (69%), rabbit *Oryctolagus cuniculus* (66%), mouse *Mus mus* (67%), cow *Bos taurus* (65%), human *Homo sapiens* (65%), and (66%) to chicken *Gallus gallus*. Less similarity was observed when sequence was compared with goat *Capra hircus* (Fig. 5).

1.3.3. Sequence analysis of SgD3

Amino acid sequence comparison with D3 of other teleosts showed that SgD3 showed high similarity of 90% with gilthead seabream and Nile tilapia, 84% with walleye *Sander vitreus* (80%) with bastard halibut, and 79% with Atlantic halibut *Hippoglossus hippoglossus*, tiger puffer (78%). It had equally high similarities with D2 from other vertebrates like 50% with pig *Sus scofa*, human (51%), rhesus monkey *Macaca mullata* (51%), rat (50%), African clawed frog *Xenopus laevis* (57%), western clawed frog *Silurana tropicalis* (58%), chicken (59%) and sheep *Ovis aries* (52%). Less similarity was observed when sequence was compared with the cow (25%) (Fig. 6).

An unrooted phylogenetic tree was constructed from the amino acid sequences of vertebrate iodothyronine deiodinases. This phylogenetic tree confirmed that the sequences of D2 and D3 were grouped within their respective clusters. In particular, they were categorized into the fish D3 groups, respectively (Fig. 7).

1.3.4. Tissue distribution of SgD2 and SgD3 genes

The tissue distribution of SgD2 and SgD3 was examined at 12.00 h and 24.00 h using qPCR (Fig. 8). SgD2 and SgD3 mRNA were detected in all of the tissues tested. A comparison of the expression of SgD2 mRNA in various tissues at 12.00 h revealed high expression in the liver, brain, skin, and spleen (Fig. 8A). High levels of SgD3 mRNA were observed in the brain, retina, liver, and skin (Fig. 8B). Day – night differences in the abundance of SgD2 and SgD3 mRNA were observed. The expression levels of SgD2 in the brain, heart, liver, gonad, and were significantly higher at 12.00 h than at 24.00 h. A significantly higher expression of SgD3 mRNA during the daytime was also observed in the brain, retina, gills, heart, liver, gonad, and skin. Negligible levels of SgD2 mRNA were observed in the gonads and heart, while the smallest amount of SgD3 mRNA was detected in the kidney followed by the gills and heart at 24.00 h (Fig. 8A, B).

1.4. Discussion

The first step of this study was the cloning and characterization of cDNA encoding type II (SgD2) and type III (SgD3) iodothyronine deiodinases of the goldlined spinefoot, since they activated or deactivated TH and have multiple effects in physiology of living organisms. All their ORF's contained an in-frame TGA stop codon that was characterized by the presence of selenocysteine hence belonging to the selenoenzyme family. The incorporation of an essential selenocysteine residue within the catalytic domain requires the presence of a premature stop codon (TGA) in the ORF and a SECIS element located in the 3′ UTR of the cDNA (Berry et al., 1991). The SECIS element in the 3′ UTR of SgD3 was located between nucleotide positions 1267 and 1367. Failure to identify a SECIS element in SgD2 may have been due to the sub-cloning of fragments without a 3′ UTR and poly (A) signal in this study. The difficulty in obtaining such a fragment may be partially attributed to the occurrence of long introns; it has been reported in mammals that the 3′ UTR of D2 has long introns (8.1 – 8.5 kb) within two exons (Celi et al., 1998). Similar incomplete sequences have been reported for D2 of the Senegalese sole (Isorna et al., 2009) and mummichog (Valverde et al., 1996) and considered to be due to either the lack of an extended 3´ UTR (up to 7.5 kb in length) including the SECIS structure (Davey et al., 1999) or the fragment being a splice variant (Isorna et al., 2009). The full sequence of mummichog D2 cDNA was later cloned and a SECIS element was found within the 4652 bp region with an intron divided by a 4.8 kb exon (Orozco et al., 2002). These cDNAs show significant sequence homology to mammals, birds and amphibians including the in-frame TGA codon, which codes for selenocysteines.

qPCR also revealed a tissue specific difference with SgD2 mRNA highly expressed in the liver, retina, brain, and skin while SgD3 mRNA was highly expressed in the liver, skin, brain, spleen, and gonads although the expression of both genes was, to some extent, detected in all of the tissues tested. These results suggest that the organs and tissues with a high expression of SgD2 and SgD3 play a role in metabolism of TH. Exceptionally high SgD3 mRNA levels were detected in the retina, followed by the brain. The transcription pattern of

both genes was different in the respective tissues, as has been observed in some higher vetebrates (Wagner et al., 2003; Darras et al., 1998; Becker et al, 1997) and fishes (Picard-Aitken et al., 2007; Sanders et al., 1999, Itoh et al., 2010) where the regulation was related to growth and development, hormonal treatment, thyroid status, pollution biomarkers, and food availability. A simultaneous comparison of D2 and D3 mRNA levels has been carried out in walleye using reverse transcription-PCR; D2 mRNA abundance in the liver was significantly higher than in all other tissues, while D3 mRNA was highly expressed in the liver and whole eye, followed by the brain, gills, and skin (Picard-Aitken et al., 2007). In terms of deiodination activities, high levels of T_4ORD and T_4IRD were observed in the liver and brain, respectively, of the blue tilapia *O. aureus* (Mol et al., 1997), salmonids (Eales et al., 1993), and Atlantic cod *Gadus morhua* (Cyr et al., 1998). In rainbow trout under physiological conditions, the predominant deiodinase pathways in the brain were observed to autoregulate T_3 levels through the degradation of T_4 and T_3, while the liver generated T_3 (Frith and Eales, 1996). A positive correlation between hepatic D2 activity and plasma T_3 levels has been found in the Nile tilapia (Van der Geyten et al., 1998) and red drum *Sciaenops ocellatus,* (Leiner et al., 2000). This situation appears to vary among vertebrates; D1 inactivation and D3 activation coincidentally occur in the mammalian liver (Chopra, 1980; Chopra et al., 1981; O'Mara et al., 1993), while the activation of hepatic D3 is one of the main factors responsible for decreasing plasma T_3 levels in chicken (Darras et al., 1998).

To the best of our knowledge, daily variations of iodothyronine deiodinase transcript levels have only been reported in the late metamorphic stages of the Senegalese sole; D3 transcript levels in larval homogenates were measured using qPCR, and they were observed to significantly increase from zeitgeber time (ZT), ZT7 to ZT12 and then decrease from ZT12 to ZT24 (Isorna et al., 2009). The present study clearly shows that the abundance of SgD2 and SgD3 mRNA in several tissues was higher at 1200 h than at 24.00 h. In contrast to T_4, little or no daily variation in plasma T_3 levels was reported in certain teleosts (Gomez et al., 1997; Leiner and MacKenzie 2003). However, the plasma levels of total triiodothyronine (TT_3) increased during the scotophase in juvenile Atlantic salmon parr (*Salmo salar*) when they

were reared under photoperiodic conditions (LD=8:16) in winter, while in spring the TT_3 levels were higher in smolts, but there was no daily rhythm. The opposite effect was observed for total thyroxine in parrs and smolts (Ebbesson et al., 2008). The daily fluctuation of plasma T_4 and T_3 levels was also reported in juvenile red drum with an increase during the photophase in fish fed 1 h before lights off, dusk-fed fish, and dawn-fed fish kept under photoperiod conditions (12L:12D) at 23 °C (Leiner et al., 2000). Similar increases in plasma TH levels during the photophase were observed in goldfish *Carassius auratus* reared under (LD=12:12) (Spieler and Noeske, 1981) and in channel catfish reared under natural photoperiodic conditions in July (Loter et al., 2007). Leiner and MacKenzie (2001) found a free-running circadian rhythm of circulating T_4 levels in the juvenile red drum that were reared under constant photoperiodic conditions with and without feeding. This finding implies that an endogenous circadian clock regulates TH levels. Concurrent variations of T_3 with T_4 in certain teleost species may mean that the activity of D2 and D3 is regulated by the circadian system and influences the intercellular and extracellular levels of TH.

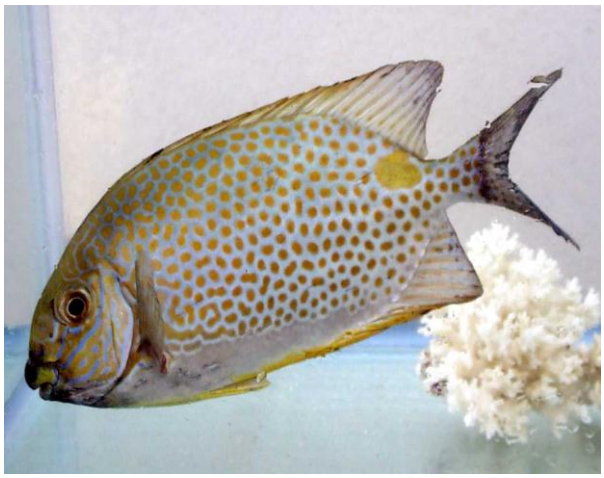

Fig. 1. Picture of the experimental fish-an immature goldlined spinefoot, *Siganus guttatus*.

```
1   GGAGCTAACCAGGAAACACCTGAAACATCGCATTCATTCTCTGCGCAAAGTCTGGAGTAC
61  TCCGCGGAGCACAGCAACAACTATGAGCGGAGTCACTAAGAGCGGCGACTCAGCCGTGCG
121 CGTCATGAATGACACTCAGAAATAAACTGTCTGCGCTCGCTGAAACCCATAAAAGAAAA**A**

181 **TG**GGCATGGCGAGCGAGGATCTGCTCGTCACACTCCAGGTACTGCCTGGTTTCTTTTCGA
    **M**  G   M   A   S   E   D   L   L   V   T   L   Q   V   L   P   G   F   F   S
241 ACTGCCTGTTTCTGGCCCTGTACGACTCTGTGGTGCTGGTGAAGCGCGTCGTGTCGCTGC
    N   C   L   F   L   A   L   Y   D   S   V   V   L   V   K   R   V   V   S   L
301 TCAGCTGCTCCAGGTCCGCCCGCTCCGGAGAGTGGCGCCGCATGCTGACCTCTGCCGGGC
    L   S   C   S   R   S   A   R   S   G   E   W   R   R   M   L   T   S   A   G
361 TGCGCTCCATCTGGAACAGCTTCCTGCTAGATGCCTACAAGCAGGTCAAACTTGGTTGCG
    L   R   S   I   W   N   S   F   L   L   D   A   Y   K   Q   V   K   L   G   C
421 AAGCACCCAACTCCAAATTGGTGAAAGTGCCAGAAGGCTCTCGGTGGAGCACCACTATCA
    E   A   P   N   S   K   L   V   K   V   P   E   G   S   R   W   S   T   T   I
481 GTGACAGTAGTGTGCCACCTGATGCCAGGATTCGAAACAGAGATGAATGCCACCTCCTGG
    S   D   S   S   V   P   P   D   A   R   I   R   N   R   D   E   C   H   L   L
541 ATTTTGAATCATCAGATCGCCCTCTGGTGGTCAACTTTGGCTCAGCCACC**TGA**CCCCCCT
    D   F   E   S   S   D   R   P   L   V   V   N   F   G   S   A   T  **Sec** P   P
601 TCATCAGCCACCTGCCAGCTTTCCGGCAGTTGGTTGAGGACTTCAGCGATGTGGCGGATT
    F   I   S   H   L   P   A   F   R   Q   L   V   E   D   F   S   D   V   A   D
661 TCCTGTTAGTGTACATTGATGAGGCTCATCCATCGGATGGCTGGGTGGCTCCACCTATGG
    F   L   L   V   Y   I   D   E   A   H   P   S   D   G   W   V   A   P   P   M
721 GCTCTTGCTCATTTAGTGTTCGGAAACATCAGAGTCTGGAGGAGAGGCTTAGAGCAGCAC
    G   S   C   S   F   S   V   R   K   H   Q   S   L   E   E   R   L   R   A   A
781 GCAAACTCATTGAGCACTTTTCTCTGCCACCTCAGTGTCAGCTGGTGGCTGACTGCATGG
    R   K   L   I   E   H   F   S   L   P   P   Q   C   Q   L   V   A   D   C   M
841 ACAACAATGCAAATGTGGCCTATGGTGTGTCCAATGAACGTGTGTGTATAGTACAACAGA
    D   N   N   A   N   V   A   Y   G   V   S   N   E   R   V   C   I   V   Q   Q
901 GAAAGATTGCCTACCTGGGGGGTAAGGGGCCTTTTTTTTACAATCTGAGGGAGGTGCGGC
    R   K   I   A   Y   L   G   G   K   G   P   F   F   Y   N   L   R   E   V   R
961 AGTGGCTGGAACAAACCTATGGTAAACGG**TAG**GAGTCTTGAAGGACAAACACT
    Q   W   L   E   Q   T   Y   G   K   R  **\***
```

Fig. 2. Nucleotide and deduced amino acid sequence of *S. guttatus* iodothyronine deiodinase type II (SgD2) sequence of the full length cDNA clone. The complete mRNA spans 1013 bp with a 5´ untranslated region (UTR) of 179 bp, an open reading frame of 810 bp (270 aa) and an incomplete 3´ UTR of 21 bp. The Sec residue is denoted by Sec in bold at position 591. The start and stop codons are indicated in bold with the stop codon denoted by an asterisk. The GenBank accession number is GU372962.

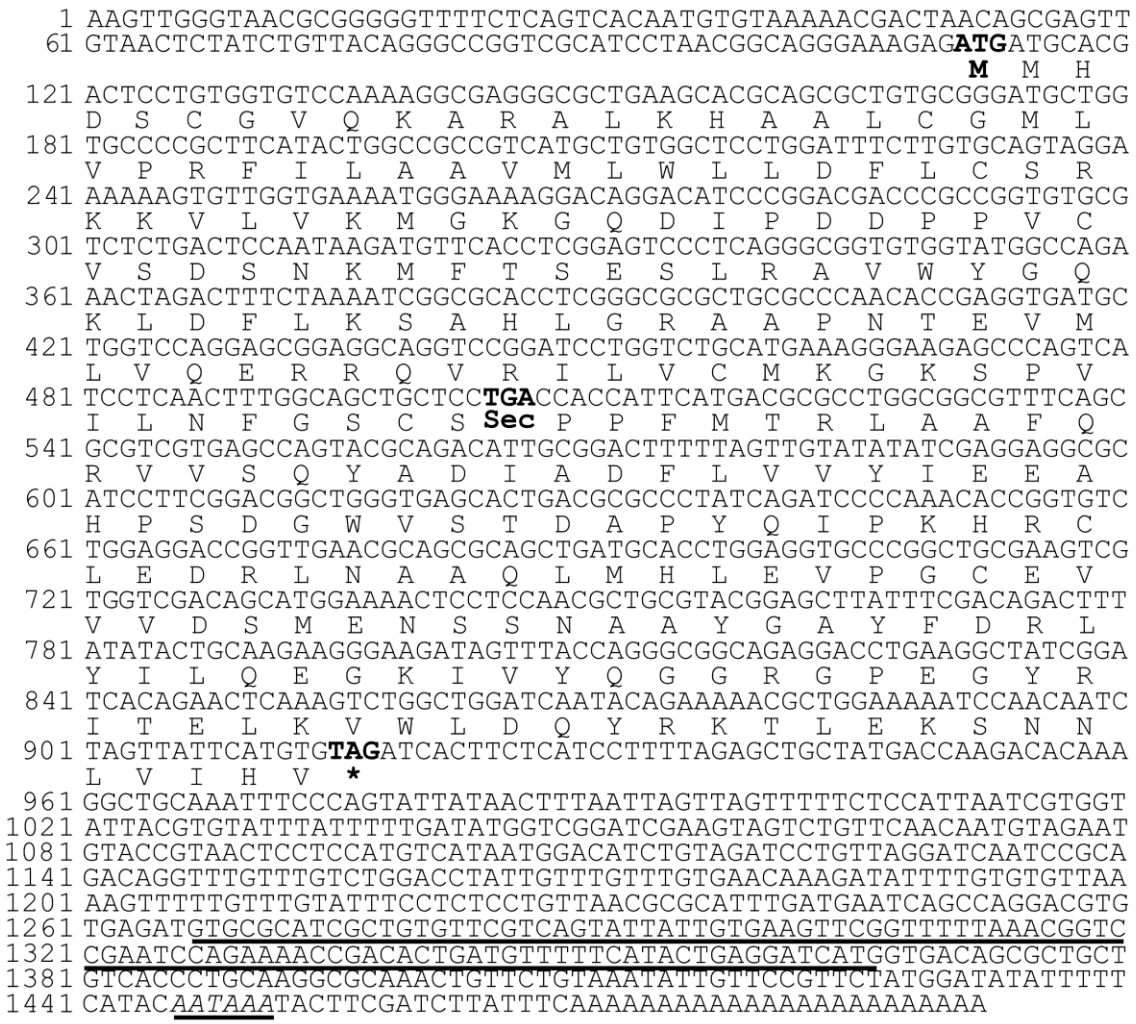

Fig. 3. Nucleotide and deduced amino acid sequence of the *S. guttatus* iodothyronine deiodinase type III (SgD3) sequence of the full length cDNA clone. The complete mRNA spans 1492 bp with a 5′ untranslated region (UTR) of 150 bp, an ORF of 804 bp (269 aa) and a 3′ UTR of 335 bp. The Selenocysteine residue (Sec) in bold was at position 504. The start and stop codons are indicated in bold, with the stop codon denoted by an asterisk. The polyadenylation signal (AATAAA) is in italics and underlined at position 1145 bp upstream of the poly (A) tail. The GenBank accession number is GU385469.

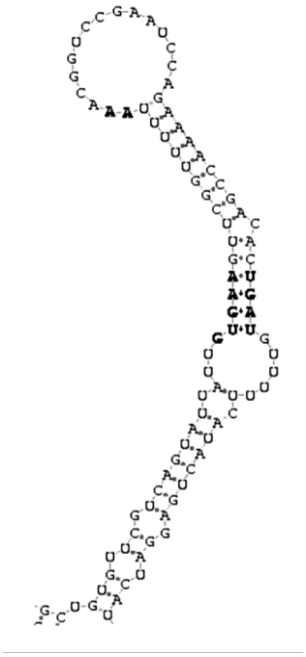

Fig. 4. The putative SECIS element determined by the SECISearch ver. 2.19 in the 3´ untranslated region is underlined between nucleotides 1267–1367.

```
PoD2            MGMASEDLLVTLQILPGFFSNCLFLVLYDSVLLVKRVVALLSSSRSGGSGEWRRMLTSAG 60
HtD2            ------------------------------------------------------------
FrD2            MGMASEDLLITLQILPGFFSNCLFLALYDSVVLVRRVVSRLSCSRSAGPKEWRPMLTSAG 60
SgD2            MGMASEDLLVTLQVLPGFFSNCLFLALYDSVVLVKRVVSLLSCSRSARSGEWRRMLTSAG 60
FhD2            MGSASEDLLVTLQILPGFFSNCLFLALYDSVVLVKRVVALLSRSRSAGCGEWRRMLTSEG 60
CcD2            MGTAGEDLLVTLQILPGFFSNCLFLALYDSVVLLKRAVSLLSCSRSAGCGEWRRMLTSEG 60
OlD2            MGSAGAELLVTLQILPGFFSNCLFMALYDSVVLLKRVVSLLSCSRSVSCGEWRRMLTSAG 60
DrD2            MGLLSVDLLVTLQILPGFFSNCLFFVLYDSIVLVK--RVVSLLSCSGSTGEWQRMLTTAG 58
BtD2            MGILSVDLLITLQILPVFFSNCLFLALYDSVILLKHVVLLLSRSKSTR-GQWRRMLTSEG 59
OcD2            MGILSVDLLITLQILPVFFSNCLFLALYDSVILLKHVVLLLSRSKSTR-GEWRRMLTSEG 59
HsD2            MGILSVDLLITLQILPVFFSNCLFLALYDSVILLKHVVLLLSRSKSTR-GEWRRMLTSEG 59
MmD2            MGLLSVDLLITLQILPVFFSNCLFLALYDSVILLKHVALLLSRSKSTR-GEWRRMLTSEG 59
GgD2            MGLLSVDLLITLQILPVFFSNCLFLALYDSVILLKHMVLFLSRSKSAR-GEWRRMLTSEG 59

PoD2             LRSIWNSFLLDAYKQVKLGCEAPDSKVVKVPDGPWCSSNISNVTNVPTGARMRNGDECRL 120
HtD2            ------------------------------------MGNKTNGPLGPRIRNEEECHL 21
FrD2            LRSIWNSFLLDAYKQVKLGCEAPNSKLVKVPDGARWSS-INNITNMLPGASLCNGIECHL 119
SgD2            LRSIWNSFLLDAYKQVKLGCEAPNSKLVKVPEGSRWST-TISDSSVPPDARIRNRDECHL 119
FhD2            LRSIWNSFLLDAHKQVKLGCEAPNSKVVKVPDGPRWS-----STVVPCGSRIQAGGECRL 115
CcD2            LRSIWNSFLLDAYKQVKLGCEAPNSKVVKVPDGPRWSSAISSTTSVPCGAKIQNGDECRL 120
OlD2            LRAIWNSFLLDAYKQVKLGCEAPNSKVVKVPESPRWSSSIKSMTSVPRGARAQTGDECRL 120
DrD2            VRSIWNSFLLDAYKQVKLGEAAPNSKVVKVTG-----------INRCWSISGKTHNQCHL 107
BtD2            MRCIWKSFLLDAYKQVKLGEDAPNSSVVHVSSPEGGDT-----SGNGAQEKTVDGTECHL 114
OcD2            LRCVWKSFLLDAYKQVKLGEDAPNSSVVHVSSPEAGAG-----AGNGAQDKTADGAECHL 114
HsD2            LRCVWKSFLLDAYKQVKLGEDAPNSSVVHVSSTEGGDN-----SGNGTQEKIAEGATCHL 114
MmD2            LRCVWNSFLLDAYKQVKLGEDAPNSSVVHVSNPESGN--------NYASEKTADGAECHL 111
GgD2            LRCVWNSFLLDAYKQVKLGGEAPNSS VIHIAKGNDGSN-----S-SWKSVGGKCGTKCHL 113
                                                                    *:*

PoD2            LDFGSSDRPLVVNFGSATUPPFISHLPAFRQLVEDFSDVADFLLVYIDEAHPSDGWVAPP 180
HtD2            LDFETSDRPLVVNFGSATUPPFISHLPSFGQLVEDFSDVADFLLVYIDEAHPSDGWVAPP 81
FrD2            LDFESSNRPLVVNFGSATUPPFISHLPAFRQLVEDFSDVADFLLVYIDEAHPSDGWKAPP 179
SgD2            LDFESSDRPLVVNFGSATUPPFISHLPAFRQLVEDFSDVADFLLVYIDEAHPSDGWVAPP 179
FhD2            LDFESSDRPLVVNFGSATUPPFISHLPAFRQLVEDFSDVADFLLVYIDEAHPSDGWVAPQ 175
CcD2            LDFESSDRPLVVNFGSATUPPFISHLPAFRQLVEDFSDVADFVLVYIDEAHPSDGWVAPP 180
OlD2            LDFESSDRPLVVNFGSATUPPFISHLPAFRQLVEDFSDVADFLLVYIDEAHPSDGWVAPQ 180
DrD2            LDFESPDRPLVVNFGSATUPPFISQLPVFRRMVEEFSDVADFLLVYIDEAHPSDGWVGPP 167
BtD2            LDFASPERPLVVNFGSATUPPFTNQLPAFSKLVEEFSSVADFLLVYIDEAHPSDGWAVPG 174
OcD2            LDFASSERPLVVNFGSATUPPFTSQLPAFRXLVEEFSSVADFLLVYIDEAHPSDGWAVPG 174
HsD2            LDFASPERPLVVNFGSATUPPFTSQLPAFRKLVEEFSSVADFLLVYIDEAHPSDGWAIPG 174
MmD2            LDFASAERPLVVNFGSATUPPFTRQLPAFRQLVEEFSSVADFLLVYIDEAHPSDGWAVPG 171
GgD2            LDFANSERPLVVNFGSATUPPFTSQLSAFSKLVEEFSGVADFLLVYIDEAHPSDGWAAPG 173
                *** ..:***************  :*. *  :**:**.****:*************  *
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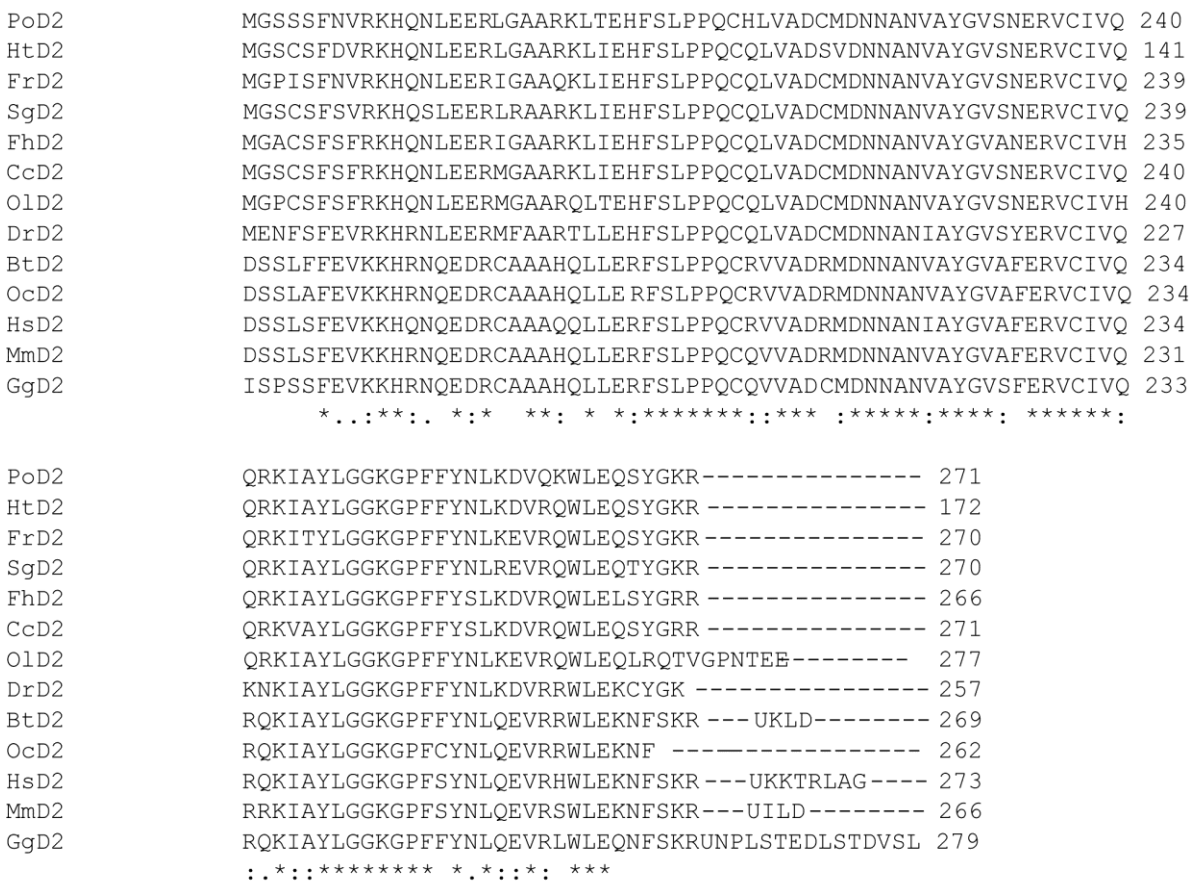

Fig. 5. Alignment of the cDNA deduced amino acid sequences of type II iodothyronine deiodinase (D2) proteins of with accession numbers as follows; *Siganus guttatus* (SgD2, ADB46158), *Chrysiptera cyanea*, (CcD2, ADD82413), *Halichoeres trimaculatus*, (HtD2, ADH94050), *Paralichthys olivaceus* (PoD2, BAG15907), *Takifugu rubripe*s (FrD2, BAG12772), *Oryzias latipes* (OlD2, NP_001129993), *Fundulus heteroclitus* (FhD2, AAB39651), and *Danio rerio* (DrD2, NP_997954), *Oryctolagus cuniculus* (OcD2, ABO21650), *Mus mus* (mmD2, NP_034180), *Gallus gallus* (GgD2, NP_989445), *Bos taurus* (BtD2, NP_001010992) and *Homo sapiens* (HsD2, NP_054644) was prepared by ClustalW2 program. The selenocysteine residues in the catalytic centre are indicated by U. Consensus amino acids are indicated by asterisks, full colons denotes two or three identical residues respectively.

```
PoD3            ---------MARALKHAALCLMLLPRFLLAAVMLWLLDFLCIRKKVLLKMGERQDG----47
CcD3            -MDDTDGVQMARALKHAALSLMLLPRFLLAAVMLWLLDFLCIRKKVLLKMGERQDG----55
FrD3a           MMLDSCGVQMATALKHAALCLMLLPRFLLTAVMLWLLDFSCIRRKVLLQMGQRQKS----56
HtD3            MMHDSWGVQKARALKHAALCLMLMPRFVLAAVMLWLLDLLCIRKKVVVKM----------50
SgD3            -MHDSCGVQKARALKHAALCGMLVPRFILAAVMLWLLDFLCSRKKVLVKMGKGQDI---- 55
StD3            -MLHCAGPHTSKLLKQVAACCLLLPRFLLTALMLWLLDFQCIRRRVLVTAREESSPE ---56
XlD3            -MLHCAGPHTGKLVKQVAACCLLLPRFLLTGLMLWLLDFQCIRRRVLLTAREESTAE ---56
GgD3            -MLHSLGAHTLQLLTQAAACILLFPRFLLTAVMLWLLDFLCIRKKMLTMPTAEEAAGAGE 59
MmD3            -MLRSLLLHSLRLCAQTASCLVLFPRFLGTAFMLWLLDFLCIRKHFLGRRRRGQPEPEVE 59
HsD3            -MLRSLLLHSLRLCAQTASCLVLFPRFLGTAFMLWLLDFLCIRKHFLGRRRRGQPEPEVE 59
RnD3            -MLRSLLLHSLRLCAQTASCLVLFPRFLGTAFMLWLLDFLCIRKHFLRRRHPDHPEPEVE 59
OvaD3           -MLRSLLLHSLRLCAQTASCLVLFPRFLGTAFMLWLLDFLCIRKHLLGRRRRGQPEIEVE 59
SsD3            -MLHSLLLHSLRLCAQTASCLVLFPRFLGTACMLWLLDFLCIRKHLLGRRRRGEPETEVE 59
                            :.* . :*.***: :. ******: * *::.:

PoD3            ---------PDDPPVCVSDSNKMFTLESLRAVWYGQRLDFFKSAHLGRAAPNTEVVLVQE 98
CcD3            ---------SDDPPVCVSDSNKMFTLESLRAVWYGQKLDFLKSAHLGHAAPNTEVVLVEE 106
FrD3a           ---------PDDPPVCVSDSNRMFTLESLGAVWYGQKLDFFKSAHLGSAAPNTEVMLVQE 107
HtD3            ----------------------FTLESLRAVWYGQKLDFLKSAHLGSAAPNTEVMLVQE 87
SgD3            ---------PDDPPVCVSDSNKMFTSESLRAVWYGQKLDFLKSAHLGRAAPNTEVMLVQE 106
StD3            ---------LEDPPLCVSDSNRMCTVESLRAVWHGQKLDYFKSAHLGSSAPNTEVVMLEG 107
XlD3            ---------HEDPPLCVSDSNRMCTVESLRAVWHGQKLDYFKSAHLGCSAPNTEVVMLEG 107
GgD3            GP------PPDDPPVCVSDSNRMFTLESLKAVWHGQKLDFFKSAHVGSPAPNPEVIQLDG 113
MmD3            LNSEGEEVPPDDPPICVSDDNRLCTLASLKAVWHGQKLDFFKQAHEGGPAPNSEVVLPDG 119
HsD3            LNSEGEEVPPDDPPICVSDDNRLCTLASLKAVWHGQKLDFFKQAHEGGPAPNSEVVLPDG 119
RnD3            LNSEGEEMPPDDPPICVSDDNRLCTLASLKAVWHGQKLDFFKQAHEGGPAPNSEVVRPDG 119
OvaD3           LNSDGEEVPPDDPPVCVSDDNRLCTLASLRAVWHGQKLDFFKQAHEGGPAPNSEVVLPDG 119
SsD3            LNSDGDEVPPDDPPICVSDDNRLCTLASLRAVWHGQKLDFFKQAHEGGPAPNSEVVLPDG 119
                                *  ** ***:**:**::*.** * .***.**:  :

PoD3            GRQVRILDCMKGKRPLILNFGSCSUPPFMTRLAAFQRVVSQYADIADFLVVYIEEAHPSD 158
CcD3            RKQVRILDCMKGKRPLILNFGSCSUPPFMARLAAFQRVVSQYADIADFLVVYIEEAHPSD 166
FrD3a           RRQVRILDCMKGKRPLILNFGSCSUPPFMTRLAAFQRVVQQYADIADFLVVYIEEAHPSD 167
HtD3            RRQVRILDCMKGKRPLILNFGSCSUPPFMTRLAAFQRIVRQYEDIADFLVVYIEEAHPSD 147
SgD3            RRQVRILVCMKGKSPVILNFGSCSUQPFMTRLAAFQRVVSQYADIADFLVVYIEEAHPSD 166
StD3            RRLCRILDFSQGKRPLVVNFGSCTUPPFMARLQAYRRLAAQHVGTADFLLVYIEEAHPSD 167
XlD3            RRLCKILDFSQGKRPLVVNFGSCTUPPFMARLQAYRRLAAQHVGIADFLLVYIEEAHPSD 167
GgD3            QKRLRILDFARGKRPLILNFGSCTUPPFMARLRSFRRLAAHFVDIADFLLVYIEEAHPSD 173
MmD3            FQSQRILDYAQGNRPLVLNFGSCTUPPFMARMSAFQRLVTKYQRDVDFLIIYIEEAHPSD 179
HsD3            FQSQHILDYAQGNRPLVLNFGSCTUPPFMARMSAFQRLVTKYQRDVDFLIIYIEEAHPSD 179
RnD3            FQSQRILDYAQGTRPLVLNFGSCTUPPFMARMSAFQRLVTKYQRDVDFLIIYIEEAHPSD 179
OvaD3           FQNQHILDYARGNRPLVLNFGSCTUPPFMARMSAFQRLVTKYQRDVDFLIIYIEEAHPSD 179
SsD3            FQNQHILDYARGNRPLVLNFGSCTUPPFMARMSAFQRLVTKYQRDVDFLIIYIEEAHPSD 179
                 :  :**   :*.:::*****:* ***:*: :::*:. :.   .***::*********
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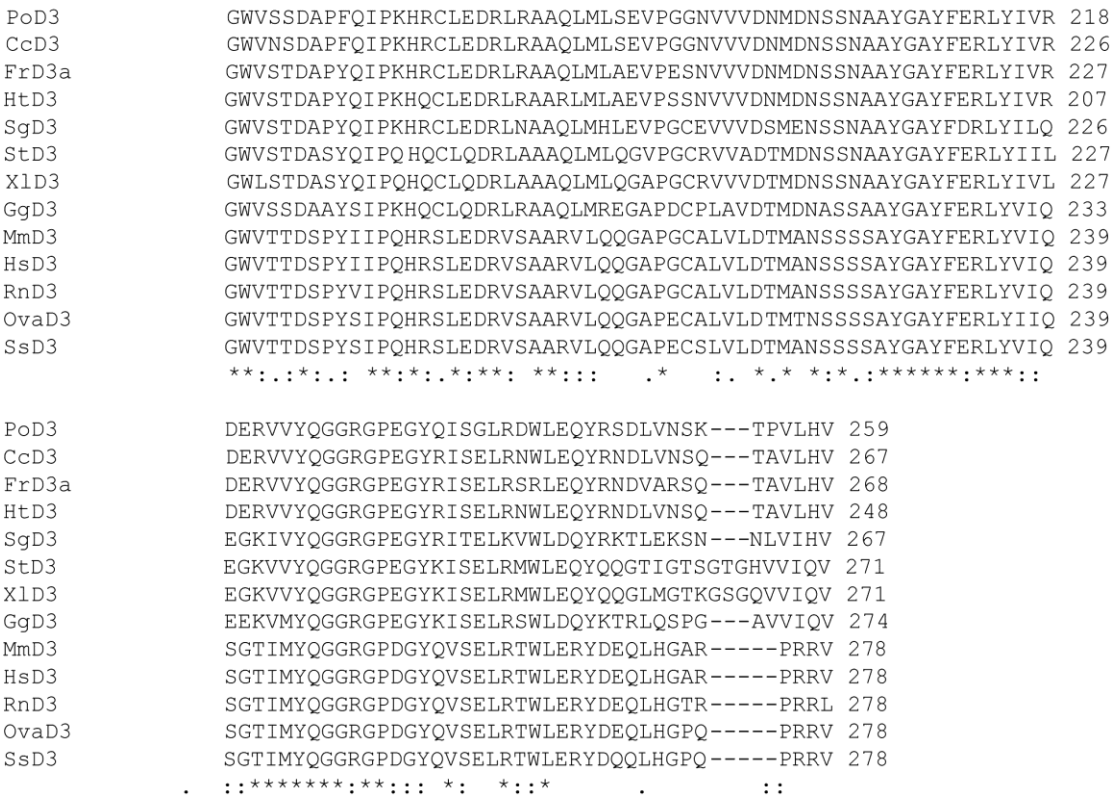
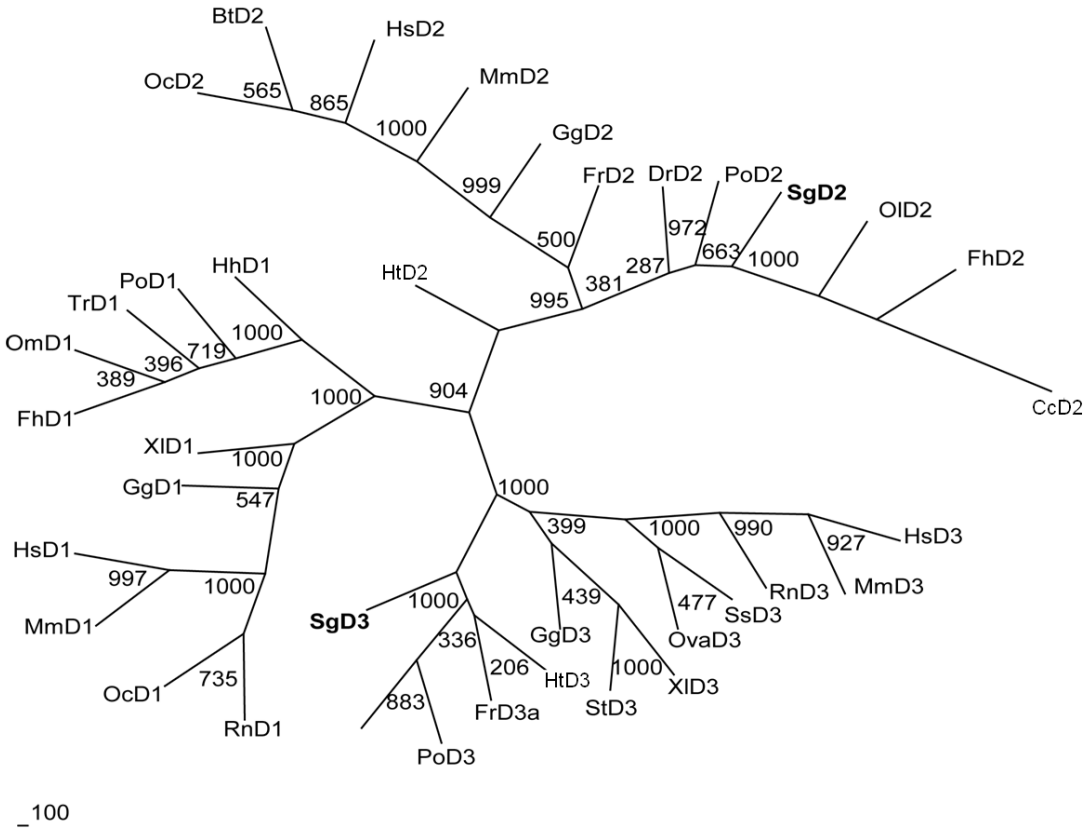


Fig. 6. Alignment of the deduced amino acid sequences of D3 proteins with their Accession numbers as follows; *Siganus guttatus* (SgD3, ADC92287), *Chrysiptera cyanea,* (CcD3, ADD82415), *Halichoeres trimaculatus,* (HtD3, ADC92286), *Paralichthys olivaceus* (PoD3, BAG15908), *Takifugu rubripe*s (FrD3, BAG12773), *Macaca mullata* (MmD3, NP_001116121), *Gallus gallus* (GgD3, NP_001116120), *Homo sapiens* (HsD3, NP_001353), *Rattus norvegicus* (RnD3, NP_058906), *Xenopus laevis* (XlD3, NP_001081332), *Silurana tropicalis*, (StD3, NP_001107139), *Sus scrofa* (SsD3, NP_001001625), was prepared by ClustalW2 program. The selenocysteine residues in the catalytic centre are indicated by U. Consensus amino acids are indicated by asterisks, full colons denote two or three identical residues respectively.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HsD2
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PoD2
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OlD2
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972
663
1000
FhD2
287
HtD2
381
995
HhD1
PoD1
TrD1
1000
OmD1
719
396
389
FhD1
904
CcD2
1000
XlD1
1000
GgD1
547
1000
399
1000
990
927
HsD1
HsD3
997
1000
RnD3
MmD3
MmD1
SgD3
1000
439
477
SsD3
GgD3
OvaD3
336
206
HtD3
1000
XlD3
OcD1
735
883
FrD3a
StD3
RnD1
PoD3
_100

Fig.7. Phylogenetic tree of iodothyronine deiodinase amino acid sequences built using PHYLIP package (Ver. 3.63) and Treeview (Win32), with 1000 bootstrap trials through neighbor-joining method. This phylogenetic analysis compares the two deduced amino acid sequences of goldlined spinefoot with other known iodothyronine deiodinases retrieved from GenBank with their respective accession numbers. The names denote the genus and species names; For D1 sequences; *Xenopus laevis* (XlD1, NP_001089136), *Gallus gallus* (GgD1, NP_001091083), *Rattus norvegicus* (RnD1, NP_067685), *Macaca mulatta* (MmD1, NP_001116124), *Oreochromis niloticus* (OnD1, CAA71995), Hippoglossus hippoglossus (HhD1, ABI93488), *Oryctolagus cuniculus* (OcD1, ABO21318), *Homo sapiens* (HsD1, NP_000783), *Fundulus heteroclitus* (FhD1, AAO31952), *Takifugu rubripes* (TrD1, NP_001129616), *Paralichthys olivaceus* (PoD1, BAG15906). For D2 sequences; *Siganus guttatus* (SgD2, ADB46158), *Chrysiptera cyanea* (CcD2, ADD82413), *Halichoeres trimaculatus* (HtD2, ADH94050), this study, *Fundulus heteroclitus* (FhD2, AAL62449), *Takifugu rubripes* (TrD2, BAG12772), *Danio rerio* (DrD2, NP_997954), *Homo sapiens* (HsD2, NP_054644), *Mus mus* (MmD2, AAI25386), *Gallus gallus* (GgD2, NP_989445), *Oryzias latipes* (OlD2, NP_001129993), *Oryctolagus cuniculus* (OcD2, ABO21650) while for D3 sequences they were *Xenopus laevis* (XlD3, NP_001081332), *Gallus gallus* (GgD3, NP_001116120), (SgD3, ADC92287), *Chrysiptera cyanea* (CcD3, ADD82415), *Halichoeres trimaculatus*, (HtD3, ADC92286), *Takifugu rubripes* (TrD3, BAG12774), *Sus scrofa* (SsD3, NP_001001625), Ovis aries (OaD3, NP_001116122), *Rattus norvegicus* (RnD3, NP_058906), *Homo sapiens* (HsD3, NP_001353) and *Macaca mulatta* (MmD3, NP_001116121). Sequences from *S. guttatus*, are shown in bold.

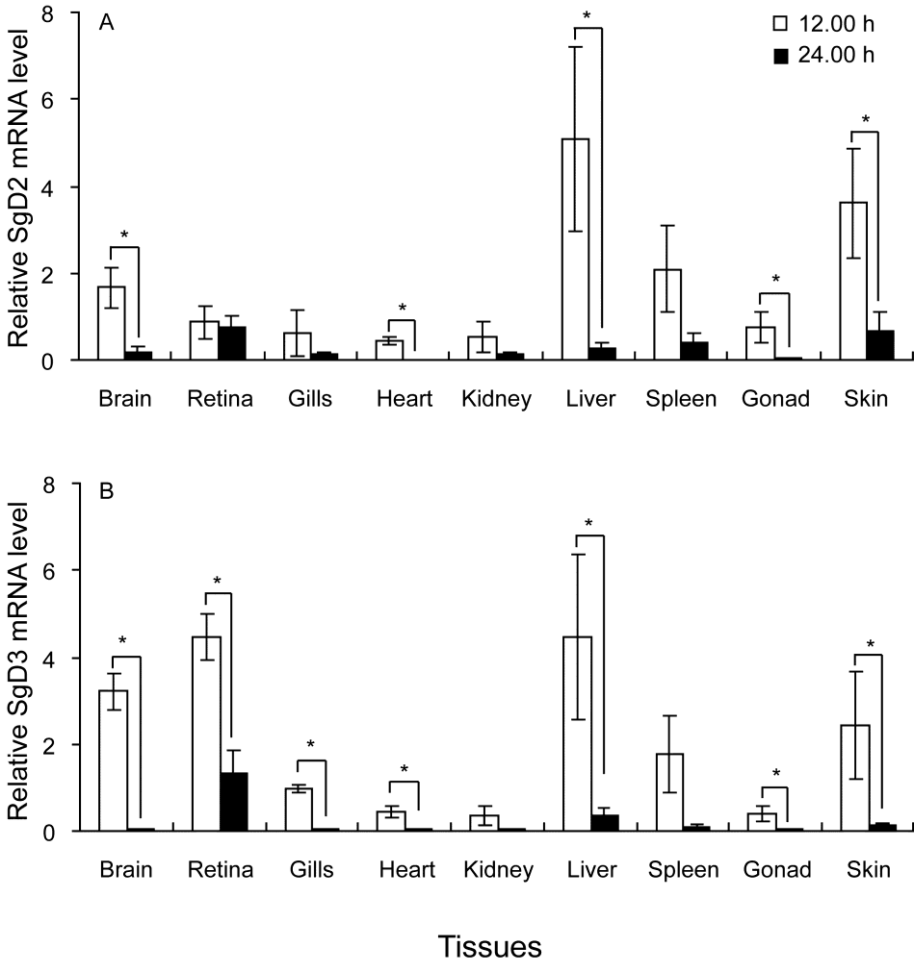


Fig. 8. Tissue distribution of iodothyronine deiodinase gene abundance. SgD2 (A) and SgD3 (B) mRNA expression in immature goldlined spinefoot kept under natural conditions for 1 week and sampled at 12.00 h (white bars) and at 24.00 h (black bars). Data represents means ± SEM, $n = 7$ per group. Asterisks indicate significant differences (Student's *t*-test, $P < 0.05$)

Table 1. Primer sequences for cDNA cloning and expression of iodothyronine deiodinases in goldlined spinefoot.

Primers	Sequence
Oligonucleotide primers	
SgD2-F2	5′-CGCTCCATCTGGAACAGYTT-3′
SgD2-R1	5′-CTGATGAAKGGGGGTCAGGT-3′
SgD3-F1	5′-CGGTGTGCGTCTCBGACTCY-3′
SgD3-R2	5′-RTGCTTGGGGATCTGATASG-3′
Cloning primers	
SgD2-GSP2	5′-CCGAGCCAAAGTTGACCACCAGAG-3′
SgD2-NGSP2	5′-TGTAGGCATCCAGCAGGAAGCTGTT-3′
SgD2-F1-GSP1	5′-CGCCCAACTCCAAAGTGGTGAAGGT-3′
SgD2-F3-NGSP1	5′-CCTCTGGTGGTCAACTTTGGCTCAG-3′
SgD3-GSP2	5′-CGCAATGTCTGCGTACTGACTCACG-3′
SgD3-NGSP2	5′-CGGTCTCTTCCCTTTCATGCAGTCC-3′
SgD3-GSP1	5′-GTGTGGTACGGCCAGAAACTGGACT-3′
SgD3-NGSP1	5′-CGTCGTGAGCCAGTACGCAGACATT-3′
qPCR primers	
SgD2-qPCR-F	5′-GATCTGCTCGTCACACTCCA-3′
SgD2-qPCR-R	5′-TTCACCAGCACCACAGAGTC-3′
SgD3-qPCR-F	5′-GTGTGCGTCTCTGACTCCAA-3′
SgD3-qPCR-R	5′-GATGCGCCGATTTTAGAAAG-3′
β-actin-qPCR-F	5′-TCCTCCCTGGAGAAGAGCTA-3′
β-actin-qPCR-R	5′-CAGGACTCCATACCGAGGAA-3′
End to end primers	
E2E-SgD2-F1	5′-GGGGAGAGCTCTTGCGGCCC-3′
E2E-SgD2-R1	5′-AGTGTTTGTCCTTCAAGACTCCTACCG-3′

Chapter II

Effects of internal and external factors on the SgD2 and SgD3 mRNA expression in the liver of the goldlined spinefoot

2.1. Introduction

The liver is the primary organ in which the deiodination activities of TH occur in certain teleost fishes (MacLatchy and Eales, 1992; Mol et al., 1993). A deiodination assay using radiolabeled iodine has demonstrated the high level of activity of low-K_m T_4ORD (the functional equivalent of D2) in the liver of many teleost fishes (Frith and Eales, 1996; Mol et al., 1998; Cyr et al.,1998; Orozco et al., 2000; Moore VanPutte et al., 2001; Galcía-G et al., 2004). It was found that plasma T_3 levels were highly correlated to T_4ORD activity in the liver in the Atlantic salmon suggesting that this organ is a major source of circulating T_3 in teleosts (Morin et al., 1993). On the other hand, D3-like activity was also reported in the liver of salmonids (Johnston and Eales, 1995; Specker et al., 2000), sturgeon *Acipencer fulvescens* (Plohman et al., 2002), walleye (Pitcard-Aitken et al., 2007), American plaice *Hippoglossoides platessoides* (Adams et al., 2000), and Nile tilapia (Sanders et al., 1999). Feeding Nile tilapia and rainbow trout with T_3-supplemented food resulted in an increase in D3 activity in the liver and gills but not the brain, kidney and gills (Finnson and Eales, 1999; Mol et al., 1999; Van der Geyten et al., 2005) while rainbow trout immersed in T_4 treated solution also resulted in induced D3 activity in the brain, liver and retina (Plate et al., 2002). These findings for D2 and D3, therefore, indicate that the alternation of hepatic iodothyronine deiodinase activity impacts on the TH-based status in certain peripheral organs.

Acclimation to low temperature conditions decreased D2 activity in the liver of the Atlantic cod (Cyr et al., 1998). The enzymatic activities of D2 and D3 were also affected by the nutritive and stress conditions of fish (Van der Geyten et al., 1998, Walpita et al., 2007). In addition, D2 activity in the liver responded to sex steroids and pituitary hormones (Cyr et al., 1988; MacLatchy and Eales, 1988, 1990). Therefore, the hepatic deiodination processes are directly or indirectly affected by endogenous and exogenous factors (Eales and Brown, 1993). To date, most studies have concentrated on effects of environmental factors on deiodinase enzyme activities with fewer molecular approaches evaluating the effects of such factors on the expression of iodothyronine deiodinase genes in fish, although those genes have been fully cloned and characterized in Nile tilapia (Sanders et al., 1997) and rainbow trout

(Sambroni et al., 2001). The aim of this chapter was to assess the molecular characteristics of iodothyronine deiodinases in the liver of the goldlined spinefoot using quantitative real-time PCR (qPCR). Since TH is closely related to reproductive and nutritive conditions, understanding of D2 and D3 status in the liver may lead improvement of aquaculture of this species.

2.2. Materials and methods

2.2.1. Fish

Juvenile goldlined spinefoot with a body mass of 0.08 – 0.15 g were caught using small-mesh nets from the mangrove estuary of the Teima River, Northern Okinawa, Japan, during daytime, at low tide, around the time of the new moon in July and August. They were reared under natural photoperiodic conditions in holding tanks (capacity 5 MT) containing constantly aerated flowing seawater at ambient temperature at the Sesoko Station (26°42′ N 127°52′ E), Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan. The fish were fed daily at 10.00 h with commercial pellets (EP1 and then EP2; Marubeni Nisshin, Tokyo, Japan). Immature fish with a mean body mass of 200 ± 0.5 g (age 1) and mature fish with a mean body mass of 346 ± 0.5 g (age 3 and 4) were used in the study.

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

2.2.2. Daily/circadian variations of D2 and D3 mRNA abundance in the liver

Fish were transferred from outdoor polyethylene tanks (capacity 0.5 MT) to outdoor polyethylene tanks (capacity 300 L) and reared under ambient temperature and running seawater with a fixed food provision at 10.00 h for 1 week under natural photoperiodic

conditions. The fish were taken from the tanks at 12.00 and 24.00 h, anesthetized with 0.01% of 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan), and immediately killed by decapitation. The liver was taken from the fish (n = 8), weighed and approximately 50 g immediately immersed in RNA*later* (Applied Biosystems, Foster City, CA, USA) at 4 °C and then stored at -20 °C until further analysis. During the dark period, samples were collected under a dim red-light. Since the day/night variations of both deiodinases among the tissues were higher during the day than at night, we subsequently collected all our samples at 12.00 h.

Fish under constant dark (DD) and constant light (LL) 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 and D3 mRNA levels between subjective day and night. The samples were processed as above. The sample collection during the dark period was conducted under a dim red light of less than 1 lx on the bench.

2.2.3. Hormone treatment

Immature fish (12 individuals per aquarium) were transferred to 2 glass aquaria (60 L capacity) with running seawater. Melatonin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ethanol/saline solution (5:95 v/v) and injected intraperitoneally (IP) at a dose of 500 ng/g body weight (the experimental group). The fish in the control group were injected IP with vehicle. At 1 h after injection, the liver (n = 6) was sampled from the fish of each group and treated according to the methods described above.

2.2.4. Effects of food deprivation and temperature on gene expression

For the food deprivation experiment, immature fish we retransferred to two polyethylene tanks (capacity 300 L) containing running seawater at 25 ± 1.0 °C under natural photoperiodic conditions. The fish (n = 7) in one tank were fed daily at 10.00 h with commercial pellets at 5% of their body mass, while the fish (n = 7) in the other tank were not fed after the initiation

of the experiment. After rearing under these conditions for 1 week, the fish from each tank were anesthetized and sacrificed at 12.00 h. After weighing, blood was collected from the caudal vein using heparinized syringes and centrifuged at 10,000 g for10 min at 4°C to obtain plasma samples that were stored at -20 °C until their glucose levels could be measured. The liver was then taken from the body cavity and weighed. Pieces of the liver were immersed in RNAlater at 4 °C and stored at -20 °C until analysis. The hepatosomatic index (HSI) was calculated using the following formula: HSI = (liver mass/body mass).

For the temperature experiment, immature fish (8 fish per aquarium) were transferred to three glass aquaria (capacity 60 L) with running seawater at 25 °C, maintained under LD = 12:12 by placing a fluorescent lamp (20 W) above each aquarium that provided illumination at 1200 lx (light intensity 2.23 W/m2), which was measured using a quantum photo radiometer (model HD 9021, Delta OHM, Padova, Italy). After acclimation for 1 week, the temperature of each aquarium was gradually changed using temperature control system with a programmable set point to 20 °C (lowest in winter), 25 °C (temperature during spawning season), and 30 °C (highest in summer). The fish were fed with commercial pellets daily at 10.00 h at 5% of their body mass. One week after rearing the fish under these conditions, samples were collected as mentioned above.

2.2.5. Measurement of glucose levels

Plasma glucose levels were determined using the Glucose CII Test Wako kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions.

2.2.6. Extraction of RNA and cDNA synthesis

Total RNA was extracted from the tissues using the Tri-Pure Isolation Reagent (Roche Applied Science, Hague Road, IN) according to the manufacturer's instructions. When necessary, the samples for qPCR were treated with deoxyribonuclease (RT grade; Nippon Gene, Tokyo, Japan) at 37 °C for 15 min to avoid contamination with genomic DNA. The

amount of RNA was measured at 260/280 nm, and samples with an absorbance ratio (A_{260}/A_{280}) of 1.8 – 2.0 were used for cDNA synthesis. cDNA was reverse-transcribed from 1000 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) for qPCR according to the manufacturer's instructions.

2.2.7. qPCR

Liver D2 and D3 mRNA expression levels were assessed using a Real-Time System C1000 thermal cycler (Bio-Rad, Hercules, CA). The forward and reverse primers for the qPCR (SgD2-qPCR-F and SgD2-qPCR-R for SgD2; SgD3-qPCR-F and SgD3-qPCR-R for SgD3; *β*-actin-qPCR-F and *β*-actin-qPCR-R for *β*-actin) were designed as shown in Table 1. The qPCR reaction mixture (10 µL) contained 5 µL Express SYBR GreenER qPCR Supermix Universal (Invitrogen, Carlsbad, CA), 0.3 µM forward primer, 0.3 µM reverse primer, 2 µL cDNA template, and 2.4 µL RNase-free water. The following PCR cycling conditions were used: initial denaturation at 95 °C for 30 s; 40 amplification and denaturation cycles at 95 °C for 5 s annealing and elongation at 60 °C for 34 s. To ensure the specificity of the PCR amplicons, a melting curve analysis was carried out by raising the temperature of the sample slowly from 60 to 95 °C until the final step of the PCR. The expression levels of SgD2, SgD3, and *β*-actin mRNA were measured in triplicate. Data were normalized relative to the mean expression level of each gene and analyzed using the normalized gene expression ($2^{-\Delta\Delta C}{}_{T}$) method (Livak and Schmittgen, 2001).

2.2.8. Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). Normality was tested using the Kolmogorov–Smirnov method. Student's *t*-test and the Mann–Whitney *U* test were used to analyze the statistical differences between two sets of data. A one-way analysis of variance (ANOVA) was performed for the temperature experiment. Probabilities of $P < 0.05$ were considered to be statistically significant.

2.3. Results

2.3.1. Circadian variation in SgD2 and SgD3 mRNA

As suggested in Chapter I, there were daily variations of SgD2 and SgD3 abundance in the liver: their levels were significantly higher during daytime than during nighttime (Fig. 8). When the fish were reared under DD conditions, SgD2, but not SgD3 mRNA abundance was significantly higher ($P < 0.05$) at CT6 than at CT18 (Fig. 9A). No changes in both gene expressions were noted under LL conditions (Fig. 9A, B).

2.3.2. Melatonin treatment

The effect of melatonin injection on SgD2 and SgD3 mRNA levels was evaluated in the liver. A two-way ANOVA revealed no effect of melatonin administration in both SgD2 and SgD3 mRNA expression levels. The mRNA levels of both genes did not significantly change at 1 h after injection, although there was a tendency for the levels of SgD2 mRNA to decrease within 3 h (Fig. 10A, B).

2.3.3. Food deprivation

The effects of food deprivation on HSI and glucose were examined at 12.00 h. Following food deprivation, HSI and plasma glucose levels significantly decreased (Fig. 11a, b). The effects of food deprivation on SgD2 and SgD3 mRNA expression in the liver at 12.00 h are shown in Fig. 12. Food deprivation significantly lowered the abundance of SgD2 mRNA ($P < 0.05$) (Fig. 12A) but not of SgD3 (Fig. 12B).

2.3.4. Temperature

Effects of SgD2 and SgD3 mRNA expression were examined in the liver for the range of water temperatures encountered in the habitat of the goldlined spinefoot (Fig. 13).

Temperature significantly affected the expression of SgD2 and SgD3 mRNA ($P < 0.01$). The levels of SgD2 transcription were significantly higher ($P < 0.01$) at 25 °C than at 20 °C and 30 °C (Fig. 13A). A similar temperature effect was observed for SgD3 mRNA levels ($P < 0.01$) (Fig. 13B).

2.4. Discussion

As shown in Chapter I, there were day-night differences in mRNA expression of SgD2 and SgD2. The present chapter clearly shows that the abundance of SgD2 changes in the liver of fish reared under DD conditions, suggesting that its transcription is regulated by a circadian manner. One possibility is that the daily variations of these genes are regulated by melatonin, an indoleamine hormone that is synthesized mainly in the pineal organ and retina, and is considered to be a transducer of photoperiodic information to the peripheral tissues (Bromage et al., 2000). In the goldlined spinefoot, the plasma levels of this hormone exhibit a restricted daily variation with an increase during the scotophase and a decrease during the photophase (Takemura et al., 2006). The present study failed to show changes in SgD2 and SgD3 mRNA levels in the liver after the injection of melatonin. Although it appears that the duration of hormone treatment was too short to exert a hormonal effect, this may indicate that melatonin is not directly related to the expression of these genes in the liver. The other possibility is that pituitary hormones are involved in the daily variations of these genes in the liver. Regarding this, it is of value that the mRNA levels of growth hormone (GH) fluctuate daily with an increase during night time in the pituitary gland of the goldlined spinefoot (Ayson and Takemura, 2006) because GH is involved in the hepatic 5´-monodeiodinase conversion of T_4 to T_3 in the rainbow trout *Salmo gairdneri* (MacLatchy and Eales, 1990; MacLatchy et al., 1992) and eels *Anguilla anguilla* (de Luze and Leloup, 1984). Since melatonin modulates GH secretion from the pituitary gland (Falcón et al., 2003), it is likely that melatonin is indirectly related to the alternation of hepatic D2 activity through the action of GH (Reddy et al., 1999; Leatherland and Lin, 2001).

The present study showed that food deprivation lowered plasma glucose and hepatic glycogen levels, similarly to channel catfish *Ictalurus punctatus* (Shoemaker et al., 2003). These results suggest that stored nutrients in the liver are mobilized after food deprivation. Concomitant with these metabolic changes, the mRNA abundance of hepatic SgD2 decreased after food deprivation. It was reported that hepatic D2 activity decreases after starvation, and concurrent increases in plasma T_3 levels and hepatic D2 activity were observed after refeeding (Van der Geyten et al., 1998). Food deprivation lowered the plasma TH levels of Nile tilapia (Toguyeni et al., 1996; Van der Geyten et al., 1998), rainbow trout (Flood and Eales, 1983), and red drum (Leiner et al., 2000). This suggests that nutrition impacts on gene transcription via the intracellular and extracellular levels of TH. On the other hand, SgD3 mRNA abundance did not change after food deprivation. This seems to be comparable to the case of starved tilapia, in which D3 activity was decreased in the gill, brain, and liver (Van der Geyten et al., 1998).

The present study demonstrated that SgD2 and SgD3 mRNA levels were affected by temperature; the highest expression levels of both genes were observed in fish reared under an intermediate temperature (25 °C), but not at lower (20 °C) or higher (30 °C) temperatures. Since the present study mimics the minimum winter and maximum summer temperatures, the abundance of these genes in the liver is likely to reflect their physiological responses. In this regard, there was a crucial effect of temperature on growth of the juvenile marbled spinefoot *S. rivulatus*, a relative species to the goldlined spinefoot; when the fish were reared at 17 °C, 22 °C, 27 °C, and 32 °C, significantly greater growth was observed in the fish reared at 27 °C and 32 °C. In addition, the specific growth rate was higher in fish reared at 27 °C than at 32 °C (Saoud et al., 2008). A similar temperature effect was observed in the reproductive activity of the sapphire devil, a common species in coral reefs (Bapary et al., 2009). Overall, these results show that tropical fish have a suitable range of temperature for their physiological state including the deiodination activities in the liver.

It is concluded that the abundance of SgD2 and SgD3 mRNA in fish is subject to endogenous and exogenous factors they are exposed to. Walpita et al. (2007) reported that the

intravenous injection of dexamethasone resulted in a decrease of the enzymatic activity and mRNA levels of D3, and in an increase in the enzymatic activity and mRNA levels of D3 in the Nile tilapia. Since handling the fish also stimulated D3 enzymatic activity and mRNA within 6 h (Walpita et al., 2007), it is likely that stress has a negative impact on the deiodination processes through the direct and indirect action of stress hormones. Further studies are needed to elucidate the involvement of stress in the alternation of SgD2 and SgD3 mRNA levels in the goldlined spinefoot.

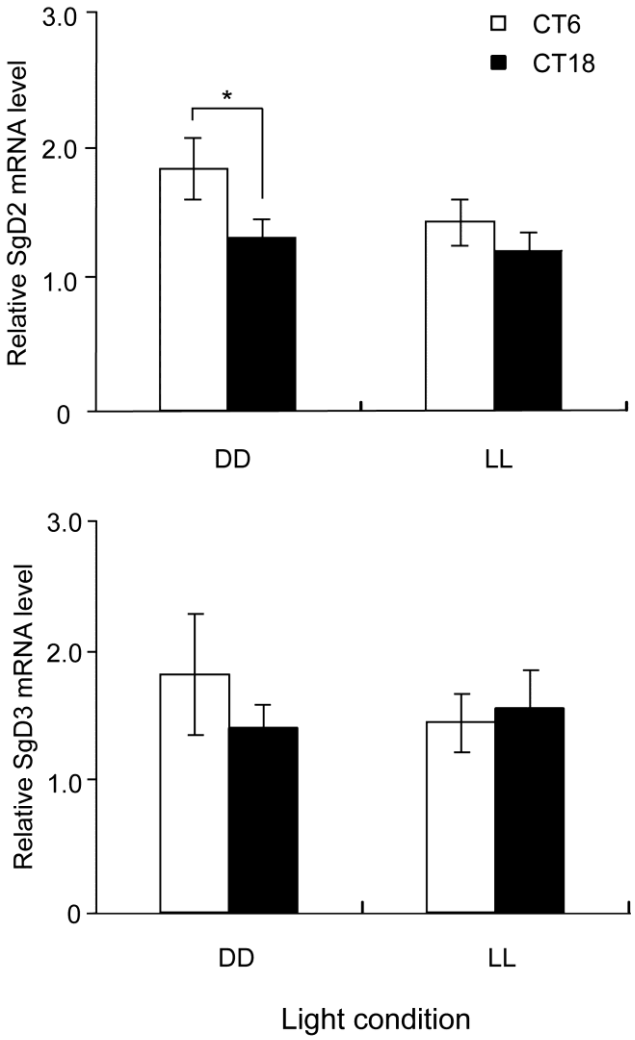


Fig. 9. Effect of constant dark and light conditions on iodothyronine deiodinase gene abundance in the liver of the goldlined spinefoot. SgD2 (A) and SgD3 (B) mRNA expression levels after 1 week exposure to constant dark (DD) and constant light (LL) conditions. White and black bars represent CT6 (n = 5) and CT18 (n = 5), respectively. Data are expressed as means ± SEM. Asterisks indicate significant differences (Mann–Whitney U-test, $P < 0.05$).

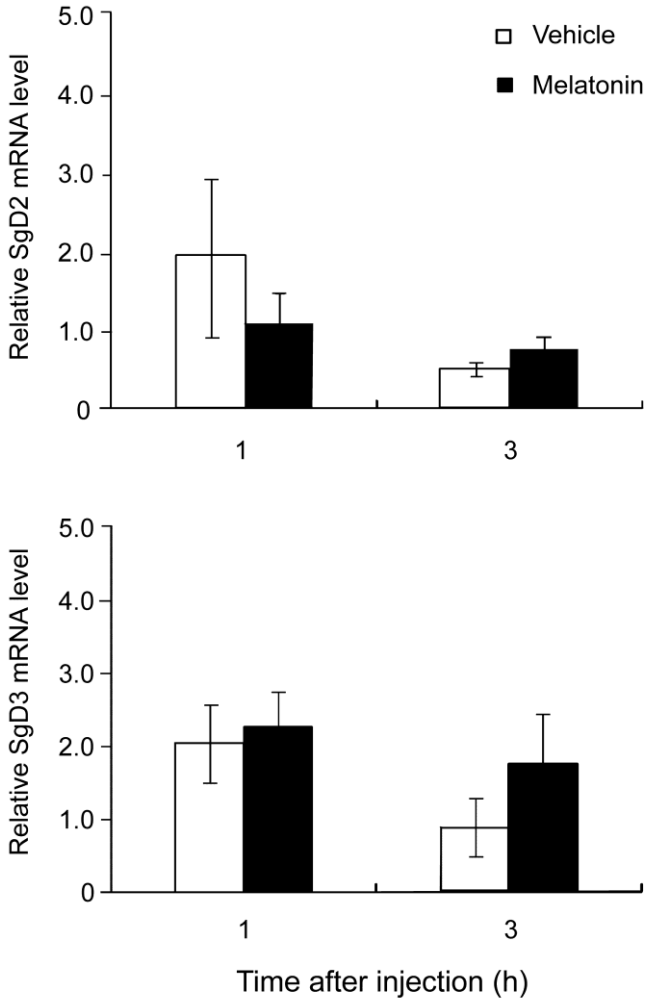


Fig. 10. Effect of melatonin injection on iodothyronine deiodinase gene abundance in the liver of the goldlined spinefoot. SgD2 (A) and SgD3 (B) mRNA expression levels in fish treated with saline with melatonin at a dose of 500 ng/g body mass to experimental group (n = 12), while saline without melatonin to control group (n = 12). Fish livers were sampled within 3 (n = 6 per group) and 12 h (n = 6 per group) after injection. White and black bars represent control and experimental group, respectively. Data represent means ± SEM.

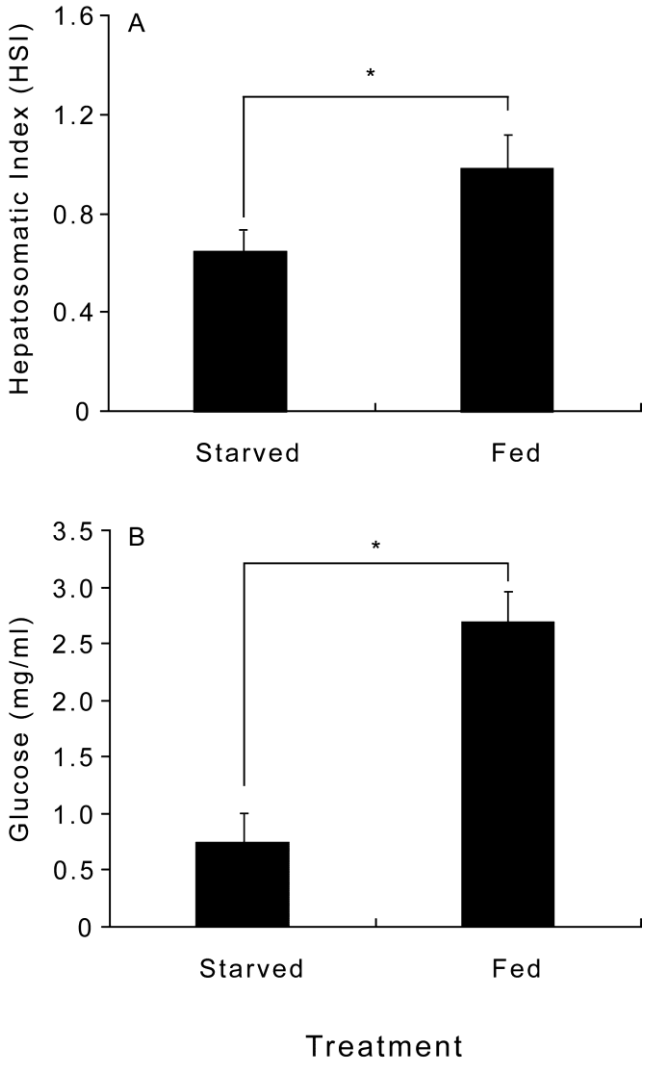


Fig. 11. Effect of food deprivation on nutritive parameters of goldlined spinefoot. Hepatosomatic index (A) and plasma glucose levels (B) in fed and unfed fish (n = 7 per group) after a 1 week experimental period. The livers were collected from the fish at 12.00 h. Data are given as the mean ± SEM. Asterisk indicate significant differences (Student's t –test, $P < 0.05$).

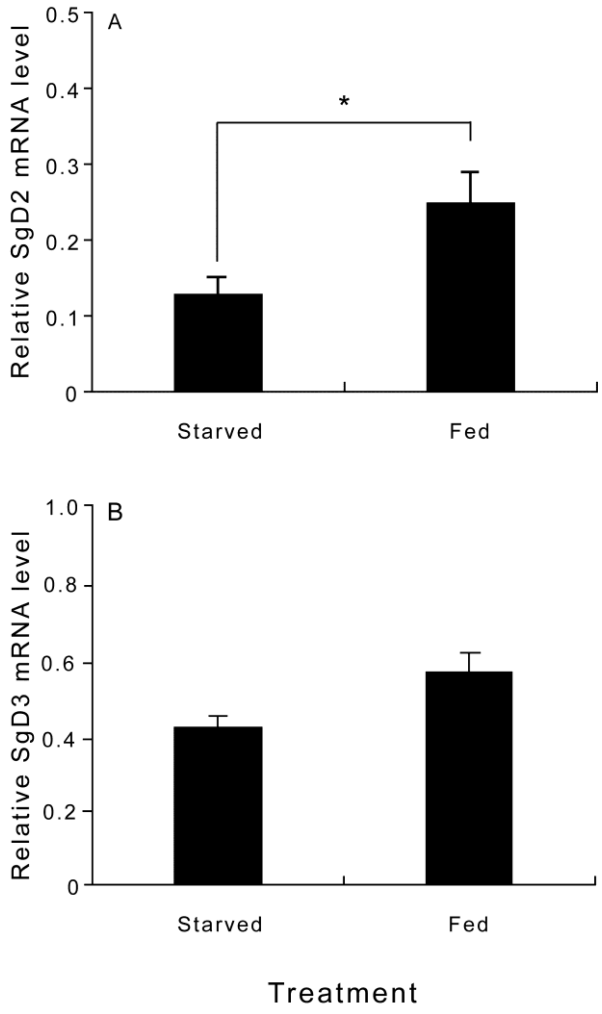


Fig. 12. Effect of food deprivation on iodothyronine deiodinase gene abundance in the liver of the goldlined spinefoot. SgD2 (A) and SgD3 (B) mRNA expression levels in fish reared under fed (n = 16) and unfed (n = 16) conditions for 2 weeks. The samples (n = 8 per sampling time) were collected at 12.00 h. Data represent means ± SEM. Asterisks indicate significant differences (Student's t-test, $P < 0.01$).

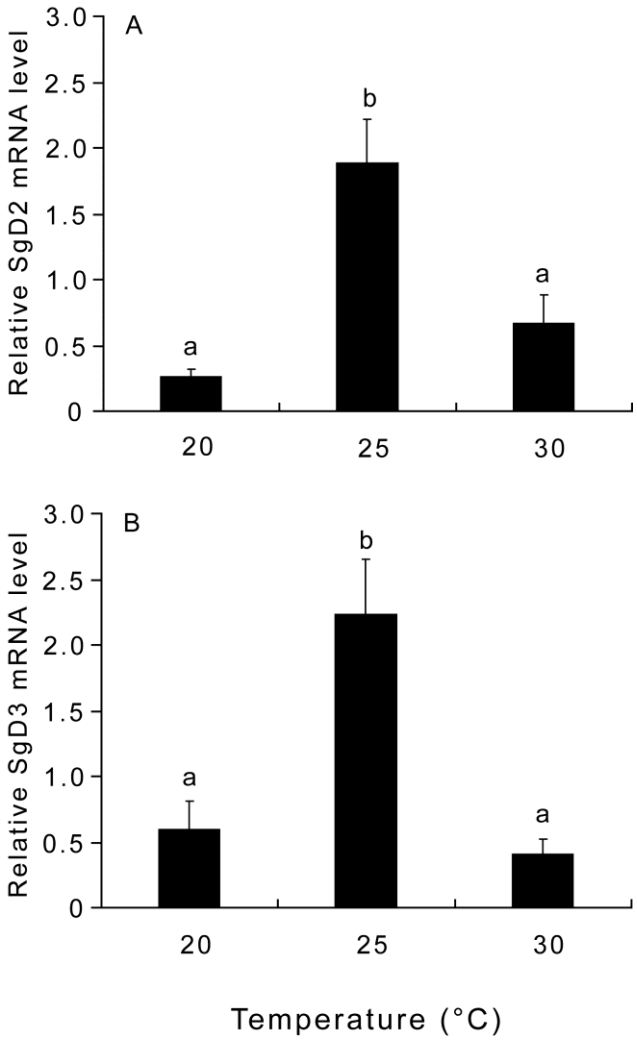


Fig. 13. Effect of chronic temperature regimes on iodothyronine deiodinase gene abundance in the liver of goldlined spinefoot. SgD2 (A) and SgD3 (B) mRNA expression levels in fish kept at 20, 25, and 30 °C under LD=12:12 for 1 week. The livers were collected from the fish at 12.00 h then SgD2 and SgD3 mRNA abundance measured by qPCR. Data are given as the mean ± SEM, n = 7 per group. Different letters indicate significant differences (one-way ANOVA, $P < 0.01$).

Chapter III

Effects of external and internal factors on mRNA expression of SgD2 in the brain of the goldlined spinefoot

3.1. Introduction

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, 2007a) and birds (Yoshimura et al., 2003; Yasuo et al., 2005; Watanabe et al., 2007). 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, 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., 2007b), 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.

Long-day conditions are also the principal factors initiating gonadal development in teleost fishes including the Indian major carp, *Catla catla* (Dey et al., 2005), Senegal sole (Vera et al., 2007), and sapphire devil (Bapary et al., 2009). Since there is experimental evidence that TH plays a cooperative role in gonadal development and function and are positively correlated with reproductive cycles in fish (Bromage et al., 2001). For example ovarian T_4 concentrations have been shown to increase during vitellogenesis and oocyte final maturation but decreased during embryogenesis in the viviparous rockfish *Sebastes inermis* (Kwomn et al., 1999). Therefore, it is likely that like in higher vertebrates, photoperception in the hypothalamic area is involved in initiation and termination of reproductive activity. In some cases, however, changes in temperature accelerated 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). 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).

In the previous chapter, high expressions of SgD2 and SgD3 were confirmed in the brain, suggesting that TH plays a role in central nerves systems through activation and inactivation of iodothyronine deiodinases. The aim of the present study was to obtain basic information on changes in D2 and D3 mRNA abundance in the brain – specifically in the hypothalamic area – of the goldlined spinefoot to help understand how environmental cues affected their physiology. The fish were experimentally reared under different conditions to elucidate the effects of light related factors (day/night, circadian and photoperiod involvement). Food availability and melatonin were also assessed because periodic changes in primary production are a principal environmental factor in the tropical monsoon climate (Johannes, 1978).

3.2. Materials and methods

3.2.1. Animals

Goldlined spinefoot 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. They 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 5 MT) with a constant aerated 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.

3.2.2 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 0.01% of 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, which is the basal area of the diencephalon, medulla oblongata, the olfactory bulbs, optic tectum and the telencephalon (Fig. 13). The samples were immediately immersed in RNA*later*® (Applied Biosystems, Foster City, CA), refrigerated at 4 °C overnight and then stored at –20 °C until further analysis.

A study was conducted to determine the daily variations in hypothalamic D2 mRNA expression levels. Fish were reared in 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 under natural photoperiodic conditions. Since the experiments were carried out in September, the day length was approximately 12 hours. The fish (n = 8 per sampling time) were captured from the outdoor polyethylene tanks at 18.00, 24.00, 06.00, 12.00, and 18.00 h, anesthetized in 0.01% of 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan), and immediately killed by decapitation. Fish under DD 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 1 week and 2 weeks respectively. After the whole brain was removed from the fish, the hypothalamus was separated and immediately immersed in RNA*later*® (Applied Biosystems, Foster City, CA, USA), refrigerated at 4 °C overnight, and stored at –20 °C until further analysis. The sample collection during the dark period was conducted under a dim red light of less than 1 lx on the bench.

3.2.3. 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) 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.

3.2.4. 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 (20W) 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 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 20W 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.

3.2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from respective brain parts using TriPure Isolation Reagent (Roche Applied Sciences, Indianapolis, IN), 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 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) for the reverse-transcription polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR), according to the manufacturer's instructions.

3.2.6. RT-PCR

The cDNA from respective brain parts was amplified with RT-PCR in a reaction mixture (total volume 25 µL) containing 12.5 µL of GoTaq Green master mix (Promega, Madison, WI), 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′-CACAAAGATAAGCCGGGAAG-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., 2011a). 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 sec, 58 °C for 45 sec, 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.

3.2.7. 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′-GATCTGCTCGTCACACTCCA-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., 2011a). The qPCR reaction mixture (10 µL in total) contained 5 µL Express SYBR® GreenER™ qPCR Supermix Universal (Invitrogen, Carlsbad, CA), 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 sec, 40 cycles of amplification including denaturing at 95 °C for 5 sec, and annealing and extension at 60 °C for 34 sec. 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).

3.2.8. 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.

3.3. Results

3.3.1. Distribution and variation in SgD2 in the brain

SgD2 mRNA was detected in the entire brain (Fig. 14). 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 (Fig. 15).

Under natural photoperiodic conditions, SgD2 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. 16). Under DD conditions, SgD2 mRNA abundance was significantly higher ($P < 0.05$) at CT6 than at CT18 (Fig. 17) both after 1 week and 2 weeks respectively.

3.3.2. 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 SgD2 mRNA abundance after 3 h, but not 12 h later (Fig. 18).

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

The effects of changes in photoperiod, temperature, and food availability on SgD2 mRNA abundance were assessed. The fish were acclimated to LD=16:8 (long-day) or LD=8:16 (short-day) conditions, and SgD2 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. 15). A significant difference ($P < 0.05$) in SgD2 mRNA abundance was observed in the fish maintained under long-day conditions, and SgD2 mRNA expression was significantly ($P < 0.05$) higher at 12.00 h than at 24.00 h. In contrast, no daily variations in SgD2 mRNA expression were observed in the fish kept under short-day conditions (Fig. 19).

Figure 20 shows the effect of water temperature on SgD2 mRNA expression in the hypothalamic area. Fish were reared at 20, 25, and 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 SgD2 mRNA.

The effect of food availability on SgD2 mRNA expression in the hypothalamic area was also examined. When D2 mRNA abundance was compared between 12.00 h and 24.00 h, SgD2 mRNA expression was significantly ($P < 0.01$) higher at 12.00 h than at 24.00 h. Day–night differences in SgD2 mRNA gene expression were not significant in fish supplied with sufficient food (Fig. 21). The hepatosomatic index of the food-limited fish was significantly lower than that of fish provided with sufficient food (Fig.11).

3.4. Discussion

The present study shows widespread distribution of SgD2 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), 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 SgD2 mRNA abundance in the goldlined spinefoot were observed with significantly higher SgD2 mRNA levels at 12.00 than at 06.00 or 24.00 h. A difference in SgD2 mRNA abundance between the subjective day and night was also confirmed when the fish were reared under DD 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 SgD2 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 the rhythm-generating enzyme of melatonin biosynthesis. As a result, under LL 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 DD conditions (Takemura et al., 2006). Because we showed that administering melatonin lowers hypothalamic SgD2 mRNA abundance during the 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 SgD2 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., 2007b), suggesting that melatonin

acts as a signal transducer controlling the photoperiodic response by regulating D2 mRNA expression in the MBH (Yasuo et al., 2007b). 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).

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 SgD2 mRNA abundance were examined in the present study, nutritive status, but not temperature, had an impact on fluctuating SgD2 mRNA abundance. This result indicates that in addition to photic responsiveness, hypothalamic SgD2 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.

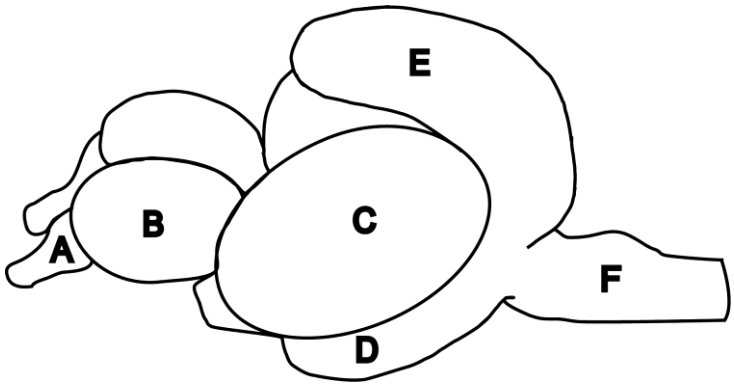


Fig. 14. Schematic representation of the goldlined spinefoot brain used for RT-PCR analysis of SgD2 gene expression. The brain was separated into six parts; part A; olfactory bulbs, part B; telencephalon, part C; optic tectum, part D; hypothalamus, part E; cerebellum, part F; medulla oblongata.

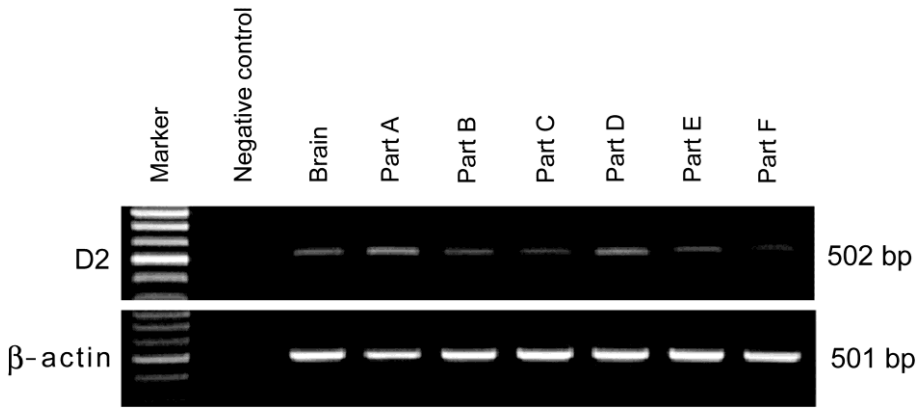


Fig. 15. SgD2 mRNA expression in different brain regions. The whole brain of the fish reared under natural conditions was collected at 12.00 h. The brain was separated into six parts (see Fig. 14). Expression levels of D2 mRNA were detected using RT-PCR and normalized by *β*-actin. The negative controls contained no cDNA templates. D2 and *β*-actin had 502 bp and 501 bp, respectively.

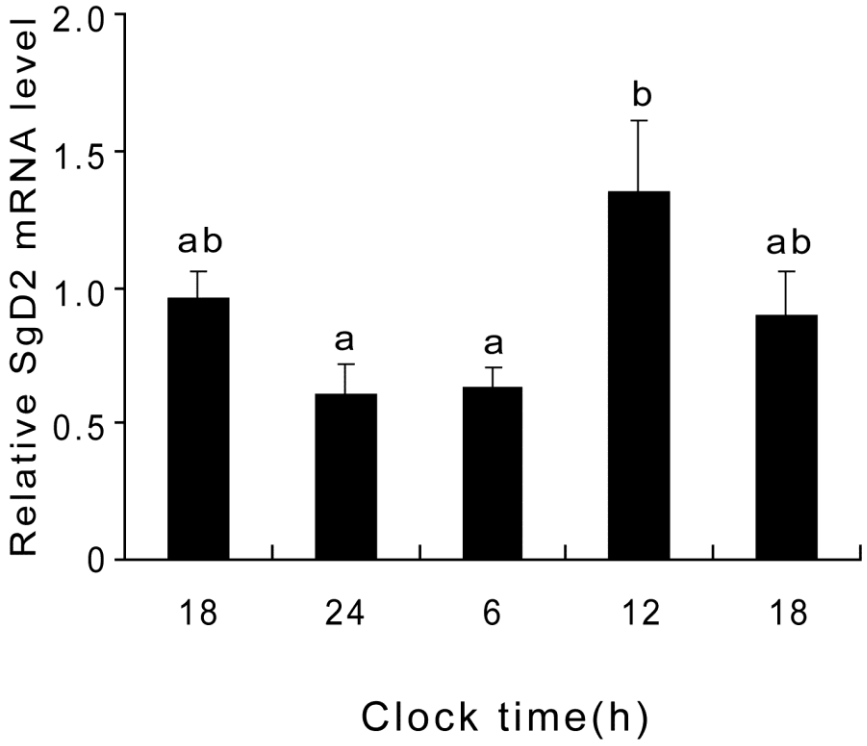


Fig. 16. Daily rhythm of SgD2 mRNA expression in the hypothalamic area of goldlined spinefoot. The fish (n = 8 per each sampling time) were reared under natural conditions and sampled at intervals of 6 h within a 24 h cycle. Data represent means ± SEM. Different letters on each column indicate significant differences (one-way ANOVA followed by Bonferroni-type multiple t-tests, $P < 0.05$).

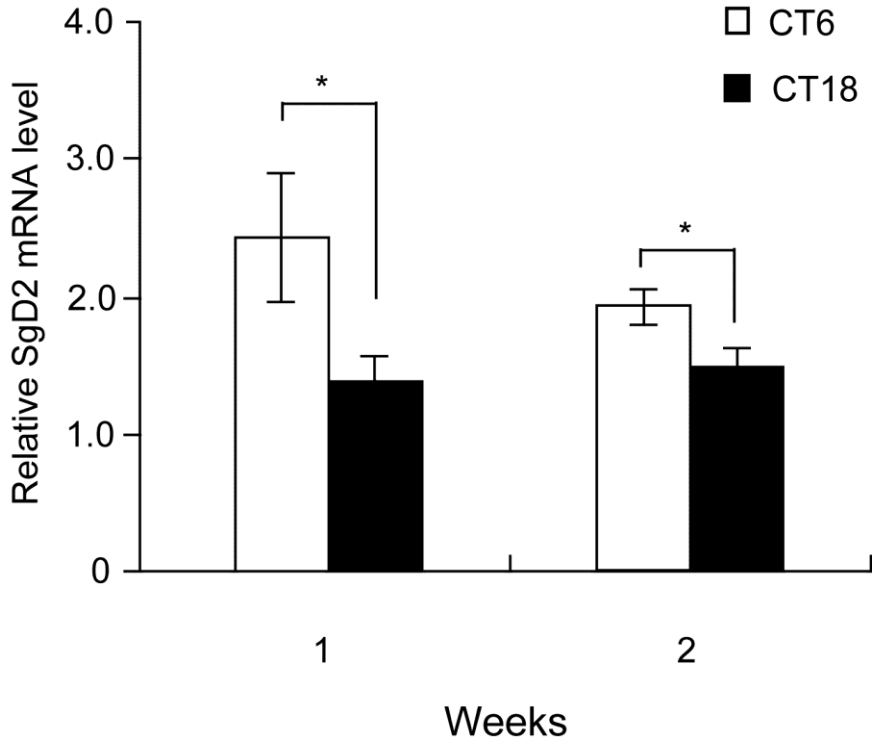


Fig. 17. Changes in SgD2 mRNA expression levels in the hypothalamic area of goldlined spinefoot at 1 and 2 weeks after exposure to constant dark (DD) conditions. White and black bars represent CT6 (n = 5) and CT18 (n = 5), respectively. Data are expressed as means ± SEM. Asterisks indicate significant differences (Mann–Whitney U-test, $P < 0.05$).

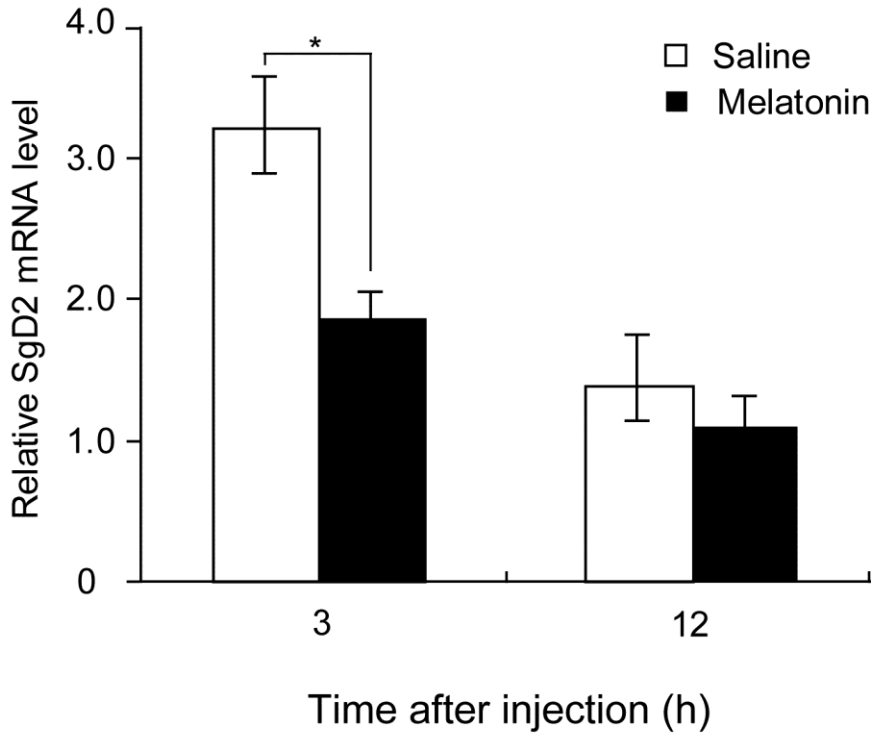


Fig. 18. Effect of melatonin injection on SgD2 mRNA expression in the hypothalamic area of goldlined spinefoot. Saline with melatonin at a dose of 500 ng/g body mass was given to the fish of experimental group (n = 12), while saline without melatonin to the fish of control group (n = 12). Fish were sampled within 3 (n = 6 per group) and 12 h (n = 6 per group) after injection. White and black bars represent control and experimental group, respectively. Data represent means ± SEM. Asterisks indicate significant differences (two-way ANOVA followed by Tukey's HSD test, $P < 0.05$).

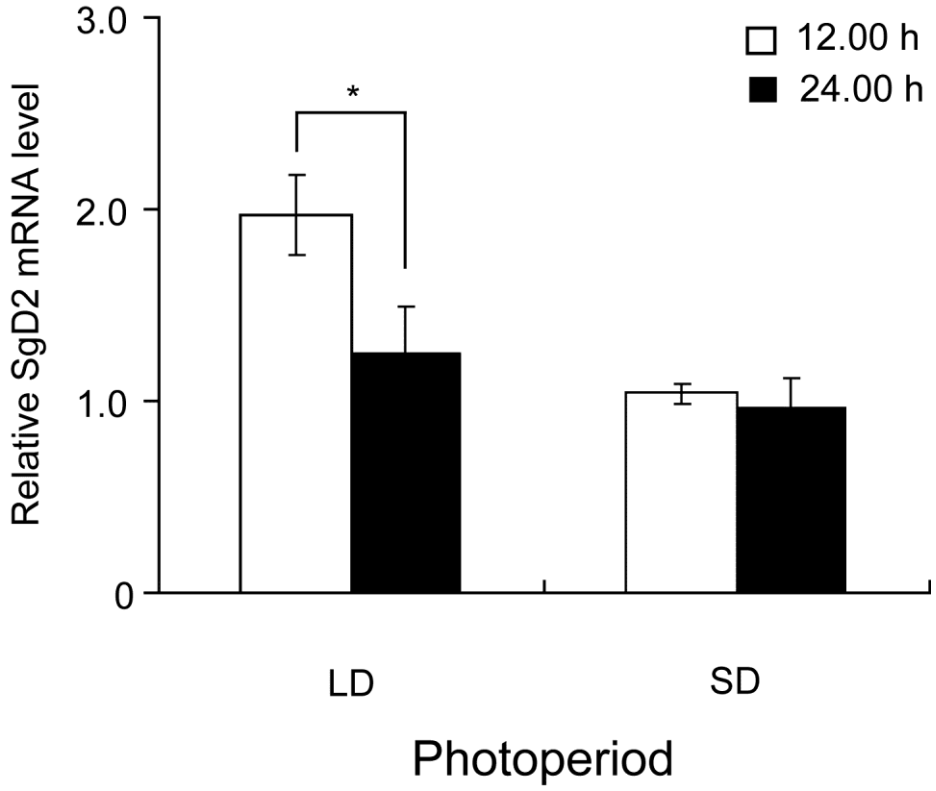


Fig. 19. Effect of artificial photoperiods on SgD2 mRNA expression in the hypothalamic area of goldlined spinefoot. Fish were reared under conditions of long-day (LD, n = 12) or short-day (SD, n = 12) within a duration of one week. The samples were collected at 12.00 h (white bars, n = 6) and 24.00 h (black bars, n = 6). Data represent means ± SEM. Asterisk indicates significant differences (Student's t-test, $P < 0.05$).

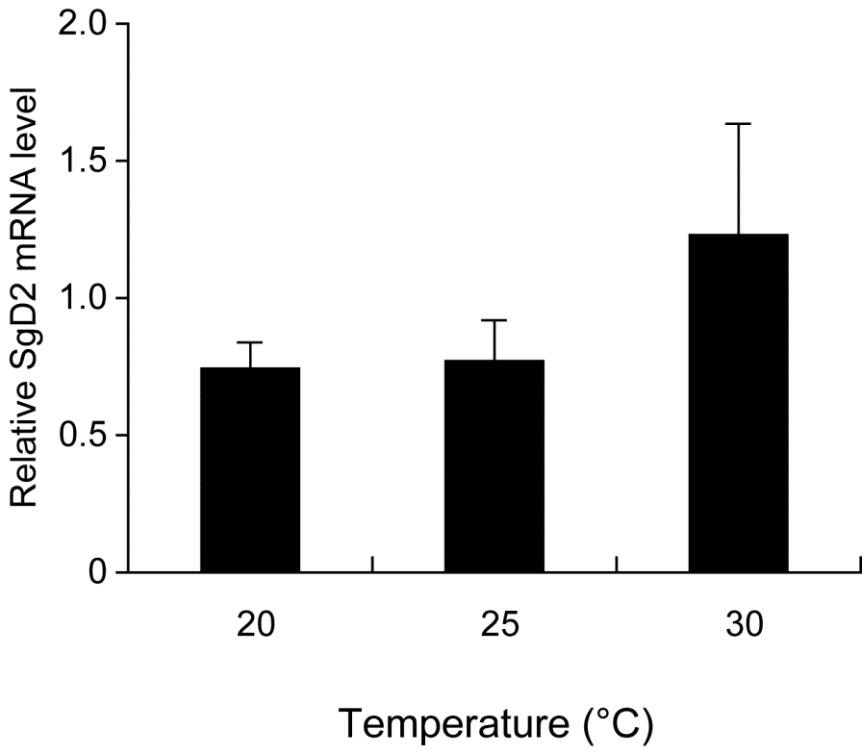


Fig. 20. Effect of different temperatures on SgD2 mRNA expression in the hypothalamic area of goldlined spinefoot. The fish (*n* = 8 per group) were reared under temperatures at 20, 25, and 30 °C for 2 weeks. The samples were collected at 12.00 h. Data represent means ± SEM.

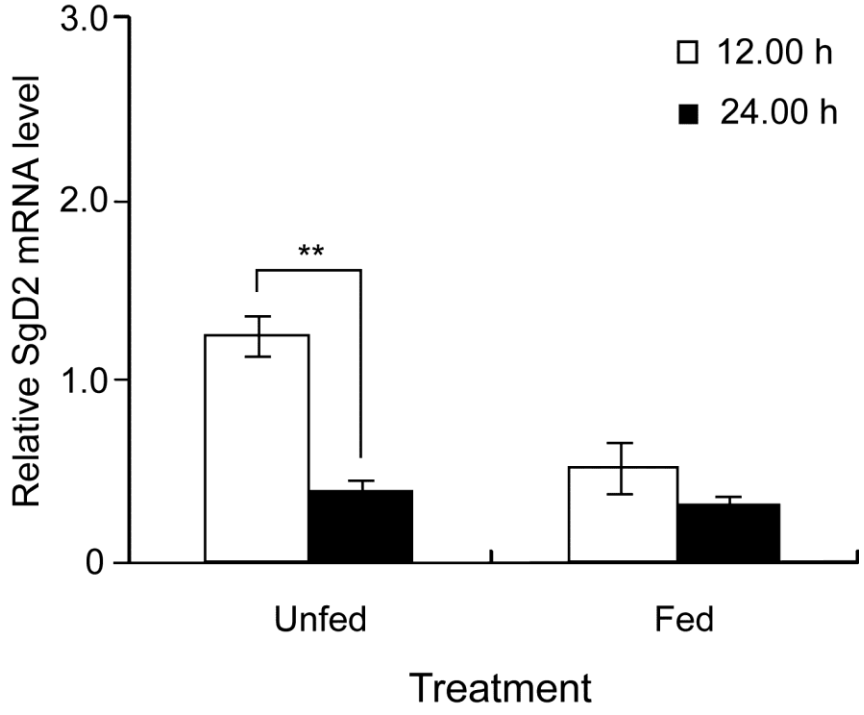


Fig. 21. Effect of food deprivation on SgD2 mRNA expression in the hypothalamic area of goldlined spinefoot. The fish were reared under fed (n = 16) and unfed (n = 16) conditions for 2 weeks. The samples (n = 8 per sampling time) were collected at 12.00 h (white bars) and 24.00 h (black bars). Data represent means ± SEM. Asterisks indicate significant differences (Student's t-test, $P < 0.01$).

Chapter IV

Effects of nutrition and photoperiod on iodothyronine deiodinase gene expression in the brain of a tropical damselfish, sapphire devil *Chrysiptera cyanea*

4.1. Introduction

Fish can perceive changes in environmental factors in their habitat and consequently alter physiological functions. Reproductive processes are accelerated when proximate environmental factors are within suitable range. It is known that photoperiod has a fundamental efficacy in regulation of annual reproductive cycle in most fish in temperate waters. Utilization of long-day conditions for the initiation of gonadal development occurs in particular fish belonging to orders Perciformes; goldfish (Gillet et al., 1978), three-spine stickleback *Gasterosteus aculeatus* (Baggerman, 1972, 1980; Borg et al., 2004), cyprinids (Indian major carp (Dey et al., 2005), soleidae Senegal sole (Vera et al., 2007), Nile tilapia (Campos-Mendoza, 2004) and Pomecentridae sapphire devil, (Bapary et al., 2009). The same conditions suppress development of gonads in certain salmonids e.g. masu salmon (Amano et al., 1995b, 1999, 2000; Berrill et al., 2000), gilthead seabream (Kissil et al., 2001) and ayu,*Plecoglossus altivelis* (Chang et al., 1992). In other studies conducted on rainbow trout have shown that exposure to both long then short photoperiods can delay or advance maturation and spawning (Davies et al., 1999). Uniquely in sticklebacks which rely on LP for gonadal maturation when administered aromatase inhibitors resulted in suppressed spermatogenesis but activated GTH and Leydig cells which accordingly stimulated maturation in males under SP (Borg et al., 2004). Because of less variation of photoperiod, on the other hand, perception and utilization of environmental factors seems to be different in fish in tropical waters. Johannes et al. (1978) noted that there are at least four possible environmental factors – temperature, plankton productivity, rainfall, and speed of prevailing currents and winds – to ensure reproductive success and larval survival. More recently, Pankhurst and Porter (2003) indicated importance of changes in local temperature and social events as proximate factors entraining reproductive activity in teleost fish. On the other hand, it was reported that the reproductive pattern of green damselfish *Abudefduf abdominalis* is influenced by food availability, which varies with stream discharge in Hawaii (Tyler and Stanton, 1995). It remains unknown what factors fish in tropical origin perceive and how fish

transduce these factors into endogenous signals, although there may be signaling systems according to factors among a variety of fish species in this region.

Recently, attention has been paid to a local role of TH in the hypothalamic area regarding the initial step to transduce changes in day-length in higher vertebrates (Yasuo et al., 2005; Yasuo and Yoshimura, 2009; Yoshimura et al., 2003). It was reported in avian that expression pattern of D2 with the activity of ORD alters after shifting photoperiodic conditions in avian (Ono et al., 2009, Yasuo et al., 2005; Yoshimura et al., 2003) and mammals (Watanabe et al., 2004; Ono et al., 2009; Revel et al., 2006; Hanon et al., 2008). On the other hand, D3 activity seems to be related to transduction of photoperiodic stimuli in short-day breeders (Yasuo et al., 2005, 2009; Barrett et al., 2007). The change in D2 consequently increases the local levels of T_3. Yoshimura et al. (2003) suggested that this increase is related to activation of GnRH neuron. It is known that D2 activity is influenced by nutrient status; (Diano et al., 1998; Coppola et al., 2005). Furthermore, photoperiod coupled with the nutritive status of fishes further influenced and altered the activity in TH ultimately controlling their biological rhythms (Boujard and Leatherland, 1992) through the stimulation of various peptides and hormones in the brain (Lin et al., 2001; Schwartz et al., 2000). These findings suggest the hypothesis that photoperiod and nutritive status act as proximate factors. Thus changes in these factors alter the expression levels of iodothyronine deiodinase genes in teleost fish of tropical origins.

The sapphire devil is a reef-associated species belonging to family Pomecentridae. It is widely distributed in the eastern Indian and Western Pacific oceans (Allen, 1991). Bapary et al. (2010) reported that gonadal development can induce in this species out of the breeding season after transferring to long-day conditions within a suitable temperature range. In addition, the reproductive performance during the breeding season alters food availability; gonadal development was suppressed in the unfed fish but restored immediately after giving a suitable amount of food (Bapary et al., 2011). Therefore, it is likely that photoperiod and food availability integrally regulate the reproduction of this fish. The aim of the present study was to examine change in abundance of iodothyronine deiodinase genes in the brain of the

sapphire devil at the initial stage of environment alternation. We reared fish under combination of long or short-day conditions and fed and unfed conditions and measured mRNA expression levels of D2 and D3 using qPCR (Wambiji et al., 2011a). In this study, the D2 and D3 of the sapphire devil were named as CcD2 and CcD3, respectively.

4.2. Materials and methods

4.2.1. Animals and experimental design

The sapphire devil used in this study were collected from the reef lagoon in Bise (26^{o}70'N, 127^{o}87'E), Northern Okinawa, Japan, during daytime low tide by using nets. They were transferred to Sesoko Station (26^{o}42 'N, 127^{o}52' E), Tropical Biosphere Research Centre, University of the Ryukyus, Okinawa, Japan, and reared in holding tanks (capacity 60 L) at constant aerated seawater flow under ambient temperature and natural photoperiodic conditions. Commercial pellets (Fry Feed Kyowa CP1000, Scientific Feed Laboratory, Tokyo, Japan) were given to the fish daily at 10:00 h. The present experiments were carried out in using eighty fish with body mass of 1.86 ± 0.16 g in November, when the fish were out of the breeding season (Fig. 22).

The fish (n = 20 per each aquarium) were accommodated into 4 aquaria (capacity 60 L) with running seawater at 27 °C. They were acclimated for one week under LD=12:12 and fed daily with the commercial pellets at 10:00 h. Afterwards, the light regime of 2 aquaria was switched to LD=14:10 (LP; turn on at 05.00 h and turn off at 19.00 h) and of other 2 aquaria to LD=10:14 (SP; turn on at 07.00 h and turn off at 17.00 h). Fish from one aquarium of LP (LP-WF group) and SP (SP-WF group) were fed with commercial pellets daily at 5% of body mass at 10.00 h. Fish from the other aquarium of LP (LP-UF group) and SP (SP-UF group) were not fed. Light was provided by a white fluorescent lamp (20W; GEX, Higashi-Osaka, Japan) and illuminance was set at 900 lx (light intensity: 2.0 W/m^2) near the water surface. Samples collection during the dark period was undertaken under a dim red light.

At 1 week after they were kept under these conditions, fish (n = 4 per each time) were netted randomly from the aquaria at 12.00 h and 24.00 h, anaesthetized with 0.01% of 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan). After measuring total length (TL) and body mass (g), they were killed by decapitation. The whole brain was then dissected out, immediately immersed in RNA*later*® (Applied Biosystems, Foster City, CA, USA), refrigerated at 4° C overnight, and then stored at –20 °C until total RNA extraction was performed. The liver and gonad were also taken from the body cavity and weighed; gonadal mass (GW) and liver mass (LW). Condition factor (K) =body mass x100/ length^3) were calculated.

4.2.2. *In situ* hybridization

In situ hybridization was performed on 7 μm sections, which were deparaffinized, washed in PBS, permeabilized in TE buffer containing proteinase K (50 μg/mL) for 30 min, post-fixed in 4% paraformaldehyde for 10 min at 4 °C and washed again with phosphate-buffered saline (PBS). Sections were acetylated for 20 min with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0. Then they were washed in hybridization buffer (50% formamide, 10% dextran sulfate, 2 × sodium chloride–sodium citrate buffer (SSC) containing 0.01% salmon sperm DNA). The hybridization probes were prepared from following primer sets by labeling with digoxigenin using a DIG-High Primer DNA Labeling and Detection Starter Kit (Roche Diagnostics GmbH, Mannheim, Germany): D2, 5′- TCCGTGGTGCTGCTGAAGCG -3′ and 5′- GGCTCCACACGGGACACTGG -3′, which amplify fragment of 243 bp; D3, 5′- CCAAATGGCGAGGGCGCTGA -3′ and 5´- TGGCCAAGGTGCGCGGATTT -3′, which amplify fragment of 261 bp. Hybridization was carried out in hybridization solution with 0.1 μg/mL D2 and D3 mRNA probe at 72 °C for 6 h. After hybridization, slides were washed three times for 10 min with 2× SSC at room temperature. All procedures for immunological detection of D2 and D3 mRNA were using DIG Nucleic Acid detection kit (Roche Diagnostics GmbH). Sections were washed with 100 mM Tris–HCl, pH 7.5, containing 150

mM NaCl (Tris-buffered saline, TBS), blocked with blocking buffer (10% blocking reagent, 90% maleic acid) for 30 min, and then incubated with alkaline-phosphatase-conjugated anti-DIG antibody (1:500) for 60 min. After washing with TBS, sections were incubated overnight with a color solution with 0.16 mg/mL bromo-4-chloro-3- indolyl-phosphate (BCIP) and 0.33 mg/mL nitro-blue tetrazolium salt (NBT), which were dissolved in 200 mM Tris–HCl, pH 9.2, containing 100 mM NaCl and 50 mM $MgCl_2$. The reaction was stopped by washing sections with 100 mM Tris–HCl, pH 7.5, containing 1 mM EDTA. After washing with 1% diethyl pyrocarbonate (DEPC) treatment distilled H_2O, sections were mounted using glycerol mounting solution prior to observations using a light microscopy.

4.2.3. Extraction of RNA and cDNA synthesis

RNA was isolated from brain samples using TriPure Isolation Reagent (Roche Applied Sciences, Hague Road, IN) according to the manufacturer's instructions. To avoid contamination from genomic DNA, the samples were treated with Deoxyribonuclease (RT grade; Nippon Gene, Tokyo, Japan) at 37 °C for 15 min. RNA concentration was determined using the Thermo Scientific NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). After measuring wavelengths at 260 and 280 nm, the samples with absorbance ratio ($A_{260}/_{280}$) between 1.8 and 2.0 were used for cDNA synthesis. cDNA synthesis was performed with 1000 ng of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

4.2.4. qPCR

D2 and D3 cDNA in the brain were analyzed on CFX96™ Real-Time System C1000™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The primer sets and housekeeping genes used are given in Table 1. The D2 and D3 mRNA levels are expressed relative to those of β-actin and measured in triplicate according to Park et al. (2007). Data were normalized

relative to the mean expression level of each gene and analyzed using the normalized gene expression ($2^{-\Delta\Delta C_T}$) method. The qPCR reaction mixture (10 µL in total) containing 5 µL Express SYBR® GreenER™ qPCR Super mix Universal (Invitrogen, Carlsbad, CA), 0.3 µM forward primer, 0.3 µM reverse primer, 2 µL cDNA template, and 2.4 µL RNase-free water. The following PCR cycling conditions were used: 95 °C for 30 sec and 39 cycles of 95 °C for 5 sec, 60 °C for 34 sec. Specificity of PCR amplicons was determined by adding melting curve analysis by raising the temperature of the sample slowly from 60–95 °C until the final step of the PCR.

4.2.5. Statistical analysis

All data was expressed as the mean ± standard error of the mean (SEM). Day-night differences in D2 and D3 mRNA levels of fish reared under LD=12:12 were compared by Student's *t*-test. For comparisons of groups, two-way analysis of variance (ANOVA) was used and followed by Tukey's HSD test.

4.3. Results

4.3.1. Localization of D2 and D3 mRNA in brain tissue

Above results showed that both D2 and D3 were major deiodinase subtypes functioning in thyroid hormone homeostasis of brain tissue. Thus, the localizations of these two mRNAs within brain tissue were investigated by *in situ* hybridization. As shown in Fig. 23, both D2 and D3 signals were clearly detected in the preoptic of hypothalamus region. Interestingly, D3 had a stronger signal than D2 (Fig. 23E, G) in these regions which were two times higher than those of D2 (Fig. 23A, C). No signal was detected in the sense control. This result strongly suggests that the T_3 level in hypothalamus of sapphire devil may be determined mainly by thyroid inactivating enzyme, D3. In situ hybridization was examined on sagittal sections (20 µm)

4.3.2. Effects of photoperiod and nutritive status on iodothyronine deiodinase genes

Figure 23 shows changes in condition factor of the fed and unfed fish reared either under LP or SP. After rearing under SP for 7 days, the condition factor of the fed and unfed fish decreased significantly ($P < 0.05$) (Fig. 24A). On the other hand, while they were reared under LP, the condition factor of the fed fish did not change, while that of the unfed fish significantly decreased ($P < 0.05$) (Fig. 24B).

Day-night differences in CcD2 and CcD3 mRNA expression in the brain of the sapphire devil under LD=12:12. When compared their abundance between 12.00 h and 24.00 h, no day-night differences were observed in mRNA abundance of both genes (Fig. 25). After shifting the light regime to LP, day-night difference in CcD2 mRNA abundance appeared in the fed fish, but not in the unfed fish; its abundance was significantly higher ($P < 0.01$) at 12.00 h than at 24.00 h (Fig. 26A). Similarly, there was significant difference in D3 mRNA abundance in the fed fish (Fig. 26B). On the other hand, when the fish were reared under SP, the day-night difference in CcD2 mRNA abundance was not evident. However, D2 mRNA abundance was significantly different between the fed and unfed fish ($P < 0.01$, two-way ANOVA); it was higher in the unfed fish than the fed fish (Fig. 27A). The abundance of D3 mRNA of the fed fish under SP was significantly higher at 12.00 h than at 24.00 h. This difference was not observed in the unfed fish (Fig. 27B).

4.4. Discussion

The present study demonstrates that the mRNA expression levels of iodothyronine deiodinases (D2 and D3) in the brain of the sapphire devil are differentially affected by changes in several factors, on which the fish are facing in their habitats. We focused on two possible factors – photoperiod and food availability – in coral reefs from the sub-tropic to tropic zone because this species distributes widely (Allen, 1991). Alternation of photoperiod from LD=12:12 to LD=14:10 (LP), but not to LD=10:14 (SP), resulted in an increase in D2 mRNA abundance, suggesting that LP stimulates translation of these genes in the whole brain

of the sapphire devil. Daily variations of key parameters in relation to thyroid hormone metabolism were reported in the Senegalese sole, which were during metamorphosis under LP (approximately LD=16:8); D2 activity was higher at midday (ZT7) and daytime (ZT12) than at nighttime (ZT19 and ZT24) and a peak of D3 mRNA abundance appeared at ZT12 in the specimen at days 21 post-fertilization (Isorna et al., 2009). It was suggested that thyroid hormone system becomes active during daytime at late metamorphosis, (Isorna et al., 2009), although it is not known where the enzymes were expressed in this species. Photostimulation of D2 mRNA expression in the brain has been reported in temperate avians (Yoshimura et al., 2003; Steinman et al., 2008) and mammals (Kalsbeek et al., 2005; Yasuo et al., 2007); *in situ* hybridization revealed that D2 mRNA expression was induced in the basal tuberal hypothalamus (BTH) of the Japanese quail, after exposing to long-day (LD=18:6) but not to short-day (LD8:16) (Yoshimura et al., 2003). In the same species, on the other hand, D3 mRNA expression in BTH decreased after exposing to long-days and increased under short-days (Yasuo et al., 2005). This opposite pattern between D2 and D3 means an increase in T_3 levels. In fact, local content of T_3 level in the mediobasal hypothalamus (MBH) increased under long-day conditions, although plasma levels of T_3 did not change under long and short-day conditions (Yoshimura et al., 2003). Unlike those cases in avian, D3 mRNA expression in the brain was higher at daytime than at nighttime under both LP and SP in the sapphire devil, suggesting that metabolism of thyroid hormones with D3 is not affected by shift of photoperiod. Since day–night difference in D2 mRNA abundance appears only in LP, it is likely that photoperiodic regulation of local content of T_3 in the brain occurs according to changes in the expression pattern of D2.

It was reported that after short-term fasting for 3 days under LD=12:12, mRNA abundance of D2 elevated in the 3rd ventricular tanycyte layer of male Sprague-Dawley rats (Diano et al., 1998). It was also reported that D2 mRNA expression decreased under LP (LD=16:8) but increased under SP (LD=8:16) in the same layer of Siberian hamster, if they were starved for 2 days (Herwig et al., 2009). In this species, SP, but not LP, induced D3 mRNA expression and this expression was lowered by starvation (Herwig et al., 2009). These

reports demonstrate that food availability has an impact on the regulation of iodothyronine deiodinases in the hypothalamus of rodents and that there is an obvious interaction between photoperiod and food availability on D2 and/or D3 transcription in the hypothalamus (Diano et al., 1998; Chan et al., 2005; Coppola et al., 2007; Herwig et al., 2009). In the present study, we also found interaction between these two factors in fish; in the fish reared without food under SP, D2 mRNA abundance increased while day-night difference evident in D3 mRNA abundance in the fed group disappeared in the unfed group.

Bapary et al. (2011) reported that in the sapphire devil, the cycle of ovarian development and spawning is arrested and ovaries were occupied by immature oocytes, when restricted food was given to the fish. Giving an appropriate amount of food to the arrested fish resulted in rapid return of their reproductive performance. Since photostimulation is required to induce the ovarian development of the sapphire devil (Bapary et al., 2009), two factors – photoperiod and food availability – are involved in initiation and termination of gonadal situation. From the aspect of this thought, the present study does not support the theory in the case of short-days. Therefore, improvement of food availability is partially involved in this. It is concluded that iodothyronine deiodinase transcription levels in the brain of the sapphire devil are influenced by photoperiod and food availability. It is likely that other factors such as temperature are related to the expressions of D2 and D3 mRNA because rearing the goldlined spinefoot at different temperatures altered them (Wambiji et al., 2011a). Because of technical limitation with qPCR, we could not determine which parts of the brain D2 and D3 mRNA are expressed with changes in environmental factors. It would be necessary to evaluate these mRNA levels with quantitative *in situ* hybridization.

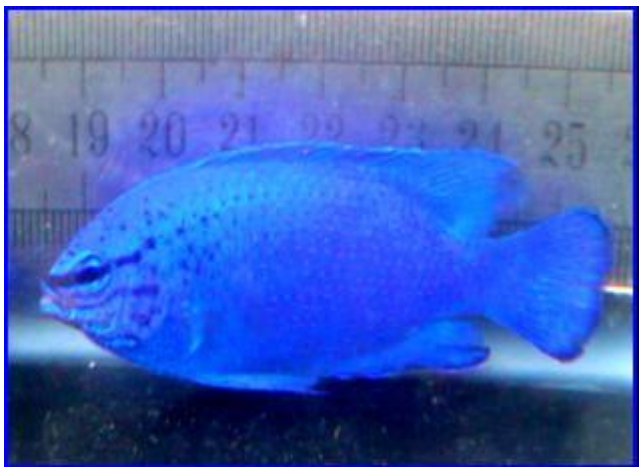


Fig. 22. Picture of the experimental fish –a mature sapphire devil**,** *Chrysiptera cyanea***.**

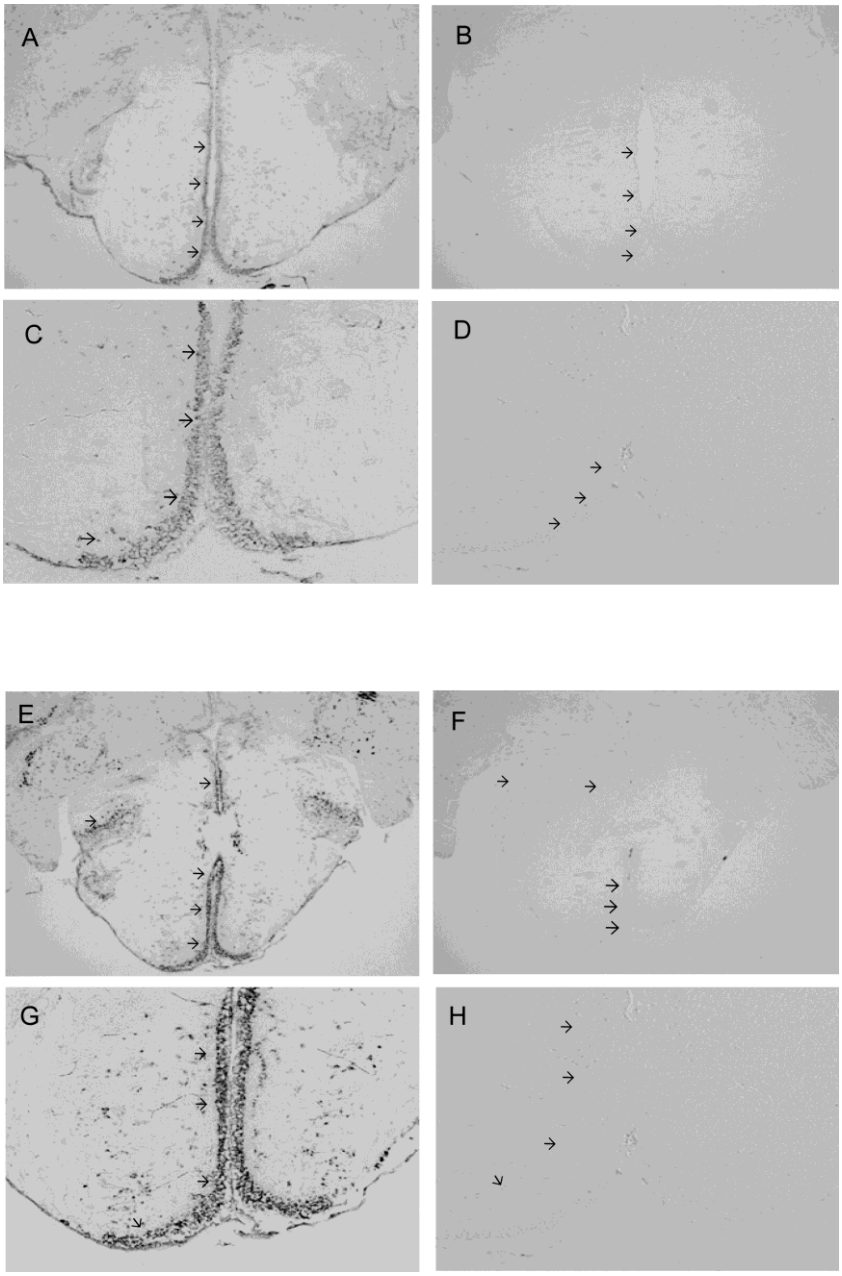


Fig. 23. Localization of iodothyronine genes in the sapphire devil brain detected by in situ hybridization. CcD2 (A and C, antisense probe) and CcD3 (B and D sense probe) mRNA expression were detected in the mediobasal hypothalamus area of the brain. In situ hybridization was examined on sagittal sections (7µm). Arrows indicate similar positions between sense and antisense areas. No labeling was detected in the sense probe.

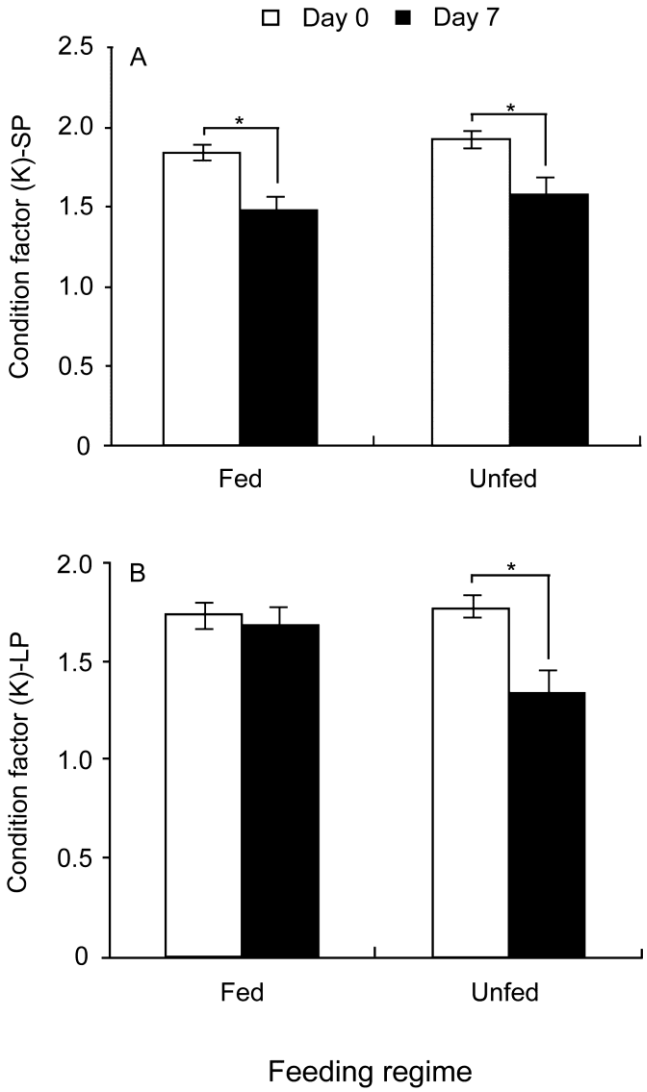


Fig. 24. Changes in condition factor under different photoperiodic and feeding conditions. Fish exposed to SP (A) and LP (B) under fed and unfed conditions for a duration of 1 week. Data represents means ± SEM; $n = 6$ per group. Asterisks indicate significant differences (two -way ANOVA, Tukey's HSD test, $P < 0.05$).

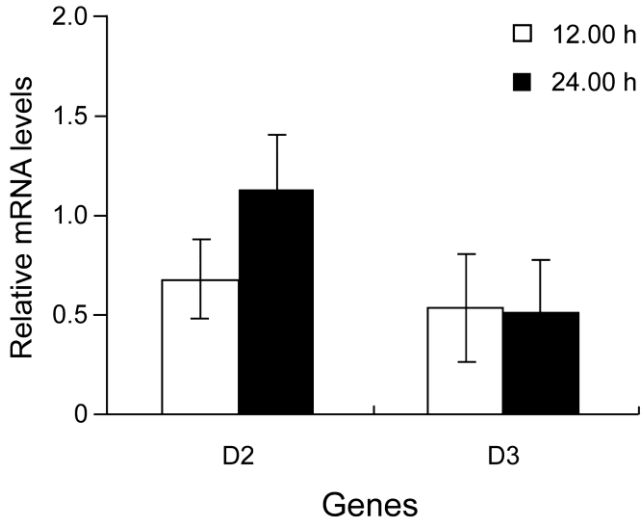


Fig. 25. Effect of day-night variations on CcD2 and CcD3 mRNA expression in the brain within a 24 hour cycle in immature sapphire devil kept under natural conditions. Data represents means ± SEM; *n* = 8 per group.

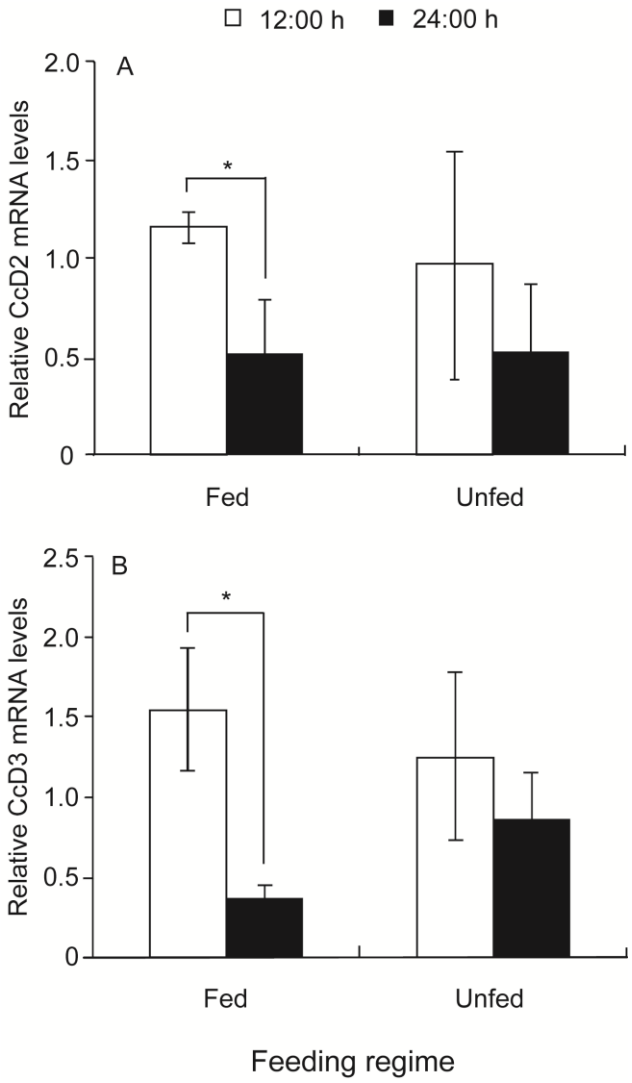


Fig. 26. Effect of artificial long photoperiod (LP) and different feeding conditions on iodothyronine deiodinase in the brain of the sapphire devil. CcD2 (A) and CcD3(B) mRNA expression in fish kept under LD=12:12 for 1 week then later transferred to LD=14:10 and LD=10:14 and fed to satiation (experimental group) and unfed (control group) and sampled 12.00 h and 24.00 h feeding after a 1 week treatment period. Data represents means ± SEM; *n* = 6 per group with significant differences indicated by asterisks (two way ANOVA, Tukey's HSD test, $P < 0.05$).

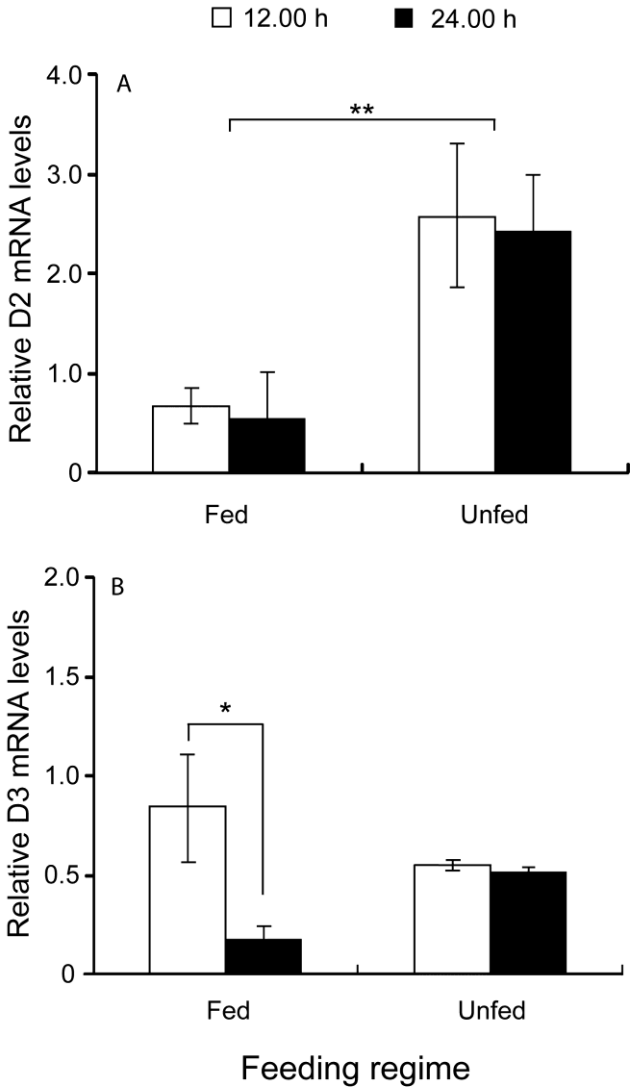


Fig. 27. Effect of artificial short photoperiod (SP) and different feeding conditions on iodothyronine deiodinase in the brain of the sapphire devil. CcD2 (A) and (B) CcD3 mRNA expression in fish kept under LD=12:12 for 1 week then later transferred to LD=14:10 and LD=10:14 and fed to satiation (experimental group) and unfed (control group) and sampled 12.00 h and 24.00 h feeding after a 1 week treatment period. Data represents means ± SEM; *n* = 6 per group with significant differences indicated by asterisks (two- way ANOVA, Tukey's HSD test, $P < 0.05$).

Table 2. Primer sequences for cDNA expression of iodothyronine deiodinases in sapphire devil (*Chrysiptera cyanea*).

Primers	Sequence
CcD2-Real-2F	5′-TGTGAGGCGCCCAACTCCAAA-3′
CcD2-Real-2R	5′-AGAGGGCGGTCTGACGATTCA -3′
CcD2-Real-1F	5′-AAAGTGGTGAAGGTGCCCGAC-3′
CcD2-Real-2R	5′-TGGCCGAGCCAAAGTTGACCA-3′
CcD3-Real-F	5′-TCAAATCCGCGCACCTTGGC-3′
CcD3-Real-R	5′-GCCATGAATGGCGGTCAGGA-3′
Cc*β-actin* -F	5′-TACCACCATGTACCCTGGCATC-3′
Cc*β-actin* -R	5′-TACGCTCAGGTGGAGCAATGA -3′

General Conclusions

Proximate and permissive environmental factors have an impact on the mRNA abundance of iodothyronine deiodinases goldlined spinefoot (*Siganus guttatus*) and sapphire devil (*Chrysiptera cyanea*) tropical fishes. They provide the gates for iodothyronine deiodinase activities as has been shown in the present study. Many experimental studies have provided valuable information by illustrating the diversity of physiological processes associated with presence of thyroid hormone levels in neural and peripheral tissues in vertebrates. Despite this diversity, an underlying theme becomes apparent; limitations are inherent in the case of the role and mechanisms of deiodinases in teleosts. These findings in the laboratory are valuable in elucidating physiological roles of deiodinases in different fish habitat.

In Chapter 1, type II and III iodothyronine deiodinases of the goldlined spinefoot, *Siganus guttatus*, were successfully cloned and characterized. Phylogenetic analysis showed that the deduced amino acid sequences of SgD2 and SgD3 showed high homology with the D2 of other vertebrates. In addition, they were clustered together with other teleosts. Highly conserved catalytic regions within the core active site surrounding a selenocysteine amino acid among the species were noted. It is likely that all known deiodinases have evolved from a single ancestral selenocysteine-containing protein. They also advance the fact that the deiodinase family of selenoproteins is highly conserved in structure and function in vertebrates. qPCR confirmed varying widespread expression levels of both genes in the neural (brain) and peripheral tissues (retina, gills, heart, kidney, liver, spleen, gonads, and skin) indicative of tissue-specific physiological functions. High expression levels in the brain and liver suggested the involvement of T3 homeostasis. Changes in light/dark cycles influenced the expression SgD2 and SgD3 mRNA levels with increases during daytime, suggesting that local TH levels and related physiological processes change daily. This is the first report showing daily variations of deiodinase genes in the tissues in teleost fishes.

In Chapter II, the liver, due to its high expression of SgD2 and SgD3 levels in Chapter I and it being reported to be the primary organ for deiodination of TH. Changes in light/dark cycles influenced the expression SgD2 and SgD3 mRNA levels with an increase at 12.00 h

and a decrease at 24.00 h. Food availability induced abundance of SgD2 mRNA, but not SgD3 mRNA. Temperatures mimicking spring showed 25 °C to be the optimal temperature for both gene expressions while 20 °C (winter) and 30 °C (summer) suppressed their expression levels. These results suggest that exogenous factors influence the mRNA levels of iodothyronine deiodinase genes in the liver and that transcription of the genes in certain tissues is partially regulated by melatonin in a circadian manner.

In Chapter III, the abundance of SgD2 mRNA in the brain of the goldlined spinefoot was widely expressed in various brain regions by using RT-PCR analysis. SgD2 mRNA 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. The abundance of hypothalamic SgD2 mRNA was higher at 12.00 h than at 06.00 h or 24.00 h. Rearing fish under DD conditions resulted in a decrease in SgD2 mRNA abundance during the subjective night. A single injection of melatonin lowered SgD2 mRNA abundance within 3 h. Long photoperiod LD=16:8 also revealed a day-night variation with daytime having higher expression levels. Collectively, it appears that hypothalamic SgD2 mRNA abundance is regulated by the circadian system and/or melatonin. The deiodinase expression levels in the hypothalamus of fish reared at 20 ^{o}C, 25 ^{o}C, and 30 ^{o}C were tolerant to this wide range since no differences in SgD2 mRNA abundance were observed. However, food deprivation caused an inverse relationship, whereby SgD2 mRNA was up regulated in the hypothalamus during the daytime unlike the suppression in the liver daytime as shown in Chapter II. These results suggest that photoperiodic and nutritive conditions affect hypothalamic SgD2 mRNA expression in the goldlined spinefoot.

In Chapter IV, the effects of food availability and photoperiod on mRNA abundance of CcD2 and CcD3 in the brain of sapphire devil, *Chrysiptera cyanea* were clear during the initial stages of environmental alternation. Sapphire devil lacked a daily rhythm in both SgD2 and SgD3 expression. *In situ* hybridization revealed high expression in preoptic area of the hypothalamus of both CcD2 and CcD3. Photoperiod affected both deiodinase expression levels. Photoperiod affected both deiodinase expression levels. Long photoperiod (LP) only

favoured high abundance of both genes in the fed fish group with higher levels at 12.00 h than at 24.00 h. This proved that both photoperiod and food availability are crucial factors for deiodinase expression under LD. Under SP, food availability was the driving factor since regardless of time; unfed fish had higher expression levels of CcD2 mRNA than the fed fish. On the other hand, abundance of CcD3 mRNA in the fed fish, but not unfed fish group, increased during daytime than at nighttime under both LP and SP, suggesting that D3 is not affected by shift of photoperiod. These results indicate that there is an interactive effect of photic stimuli and nutritional status on the expressions of CcD2 and CcD3 mRNA in the brain of sapphire devil.

This study contributes to basic biology by elucidation of molecular mechanisms. It reveals that on transduction of environmental exogenous (light, temperature, food availability) stimuli to endogenous (neurotransmitters) stimuli information is mediated by iodothyronine deiodinases action by fish in any particular habitat. This information will be crucial for the promotion of aquaculture by artificial control of reproduction and growth in fish.

It is known that physiological processes including growth and reproduction are regulated by subtle changes in local concentrations of TH levels which are driven by iodothyronine enzyme action in target tissues through the deiodination process. In the hypothalamus, TH *vis a vis* the deiodinase enzymes target thyroid releasing hormone (TRH) neurons which is secreted by the hypothalamus. TRH promotes the release of thyroid stimulating hormone (TSH) secretion by the anterior pituitary gland which stimulates TH secretion by the thyroid gland. Excess levels of TH activate a negative feedback loop whereby TH inhibits TRH secretion which in turn reduces TSH secretion in the pituitary and TH secretion from the thyroid follicles. TH are released into the blood supply and transported to target tissues. Specifically in the liver T_4 is deiodinated to T_3 by D2.

These processes are dependent on two possibilities; namely when the fish are exposed to either adequate or adverse environmental conditions which may promote proper physiology or stressful conditions respectively as shown in figures 28A, B. In rabbitfish it is clear that the fish perceive light through their pineal organs which are known to secret melatonin hormone,

an indoleamine that determines day length. In the brain, both deiodinases had high levels during the day than at night.

1. Under adequate physiological conditions

In some teleosts and in this case goldlined spinefoot and sapphire devil, it is clear that the fish possibly perceive light through their pineal organs that act as photoreceptors and pacemakers unlike in mammals who perceive light through the retina. Goldlined spinefoot possess photoreceptive cells and melatonin hormone secreting cells though which melatonin's cyclic secretion with low daytime and high nighttime levels occurs in nature. Melatonin is also the chemical signal entrained to the light-dark cycles within 24 hours and a synchronizer of seasonal rhythms in the annual cycle in accordance with the day length of the environment. The light-dark cycle rhythm is also entrained to circadian clocks neurally and peripherally that produce melatonin rhythmically and are controlled by clock genes. Melatonin acts through G-protein coupled receptors located in those tissues that synchronize both brain and peripheral clocks. It is synthesized from tryptophan absorbed from the blood, which is decarboxylated to produce serotonin which is methylated by *hydroxyindole-O-methyl transferase* (HIOMT) to produce melatonin. Therefore, light perception, adequate food and optimal temperarutes promote good physiological conditions which stimulate hypothalamic pathways in the brain. This enables the fish to predict and anticipate environmental changes within their habitats.

In the liver, metabolic pathways are stimulated through anabolism and catabolism by increasing synthesis and degradation of macromolecules important for energy input and expenditure.

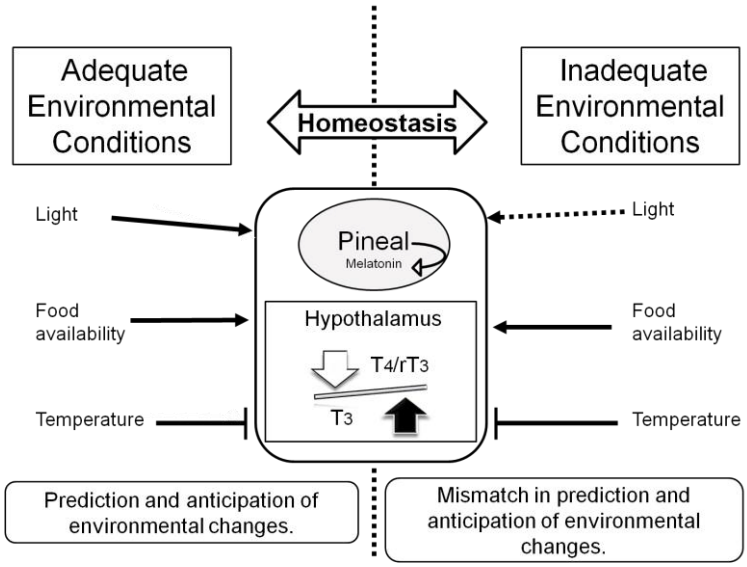
2. Under inadequate and stressful conditions

Concurrently, deiodinase expression was hampered when fish were subjected to inadequate food supplies, light/ dark cycles and photoperiod coupled with extreme high or low temperatures. This resulted in fish with bad physiological conditions which inhibited their hypothalamic pathways or negatively impacted on the metabolic activities in the liver. This would contribute to a mismatch in the prediction and anticipation of environmental changes in their habitats. It is likely that starvation creates a negative energy balance arising from the

energy input and expenditure causing an inverse relationship between the hypothalamus and the liver. Several hormones including cortisol, glucagon and epinephrine may indirectly be involved in the synthesis of macromolecules in the livers of these two fish. It is suggested that the central nervous system has the ability to autoregulate thyroid status and maintain T_3 availability within physiological levels in the brain (Kalsbeek et al., 2005) while peripherally in the liver, both production and degradation of thyroid hormones possibly contributes T_3 that brings about homeostasis between the two tissues. This is facilitated by the interactions of T_3 with its receptors in the nucleus of the target cells and influenced by intracellular concentrations of both T_3 and the receptors. In addition, since TH also belongs to steroid – thyroid receptor super family which include melatonin receptors, it is expected that their pathways are somehow interconnected.

Lately, reproductive activities have been linked to the expression of deiodinases in the brain of seasonal breeders like birds, goats, sheep and hamsters albeit in a species-specific manner. This process coupled by melatonin regulation (Revel et al., 2006) is considered to control reproduction in mammals indirectly by regulating gonadotropin hormones while deiodinases seem to induce GnRH secretions in birds (Yoshimura, 2010). It can be assumed that since D2 increased under long photoperiods and optimum temperature, teleost reproductive cycles too may follow suit as those of higher vertebrates.

A.



B.

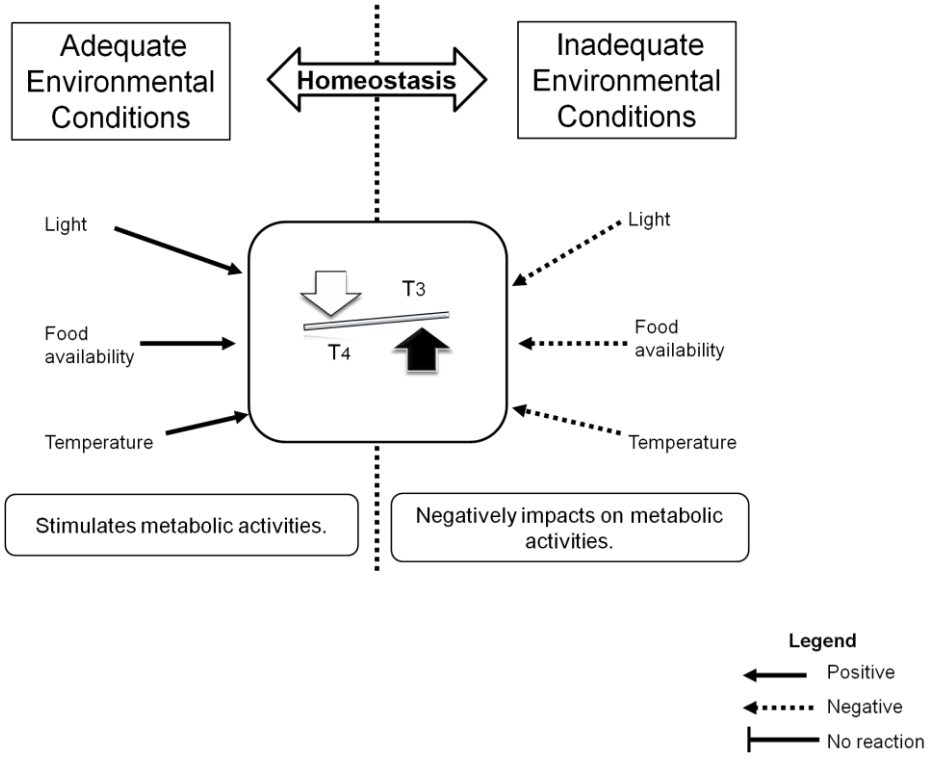


Fig. 28. Schematic diagram of possible regulatory roles of iodothyronine deiodinase genes (D2 and D3) in (A) the brain and (B) the liver of two tropical fish.

Future Research Plans

Iodothyronine deiodinases have shown that they are regulated by environmental cues like light, temperature and food availability albeit differently within the two genes and also the respective target tissues. From novel studies conducted on higher vertebrates, the unmistaken role of initiating reproduction has been confirmed but it is still unclear in fish. Given that deiodinases act upon TH which act in synergy with different hormones or cell signaling agents and their metabolic pathways are interconnected with many thyroid-mediated metabolic actions; it is likely that other hormones or neural stimuli effectiveness will be enhanced. Therefore coupled with the diversity in the neuroendocrine control on reproduction consideration should be given to:-

1. Investigations that will link photoperiod to expression of gonadotropins, steroid and melatonin hormones in these fishes.

2. Immunohistochemistry and *in situ* hybridization techniques to determine localization of site-specific deiodinase expression and understand their roles.

3. Understanding the roles of additional hormones (insulin, serotonin and tryptophan) in relation to the deiodinase pathway since the correlation between food availability and deiodinases has been remarkable.

4. Examining the genetic drift between fish living in subtropical and tropical regions.

5. Assessing the involvement of deiodinases in reproductive activities in rabbitfish still unknown.
6. The determination of thyroid hormone activity and levels in target tissues of these two fish.

References

Abesamis, R.A., Russ, G.R., 2010. Patterns of recruitment of coral reef fishes in a monsoonal environment. Coral Reefs. 29, 911–921.

Adams, B.A., Cyr, D.G., Eales, J.G., 2000. Thyroid hormone deiodination in tissues of American plaice, *Hippoglossoides platessoides*: characterization and short-term responses to polychlorinated biphenyls (PCBs) 77 and 126. Comp. Biochem. Physiol. 127C, 367–378.

Allen, G.R., 1991. Damselfishes of the world. Mergus Publishers, Melle, Germany. 271 pp.

Amano, M., Hyodo, S., Kitamura, S., Ikuta, K., Suzuki, Y., Urano, A., Aida, K., 1995a. Salmon GnRH synthesis in the preoptic area and the ventral telencephalon is activated during gonadal maturation in female masu salmon. Gen. Comp. Endocrinol. 99, 13–21.

Amano, M., Hyodo, S., Kitamura, S., Ikuta, K., Suzuki, Y., Urano, A., Aida, K., 1995b. Short photoperiod accelerates preoptic and ventral telencephalic salmon GnRH synthesis and precocious maturation in underyearling male masu salmon. Gen. Comp. Endocrinol. 99, 22–27.

Amano, M., Iigo, M., Ikuta, K., Kitamura, S., Yamada, H., Yamamori, K., 2000. Roles of melatonin in gonadal maturation of underyearling precocious male masu salmon. Gen. Comp. Endocrinol.120, 190–197.

Amano, M., Ikuta, K., Kitamura, S., Aida, K., 1999. Effects of photoperiod on salmon GnRH mRNA levels in brain of castrated underyearling precocious male masu salmon. Gen. Comp. Endocrinol. 115, 70–75.

Baggerman, B., 1972. Photoperiodic responses in the stickleback and their control by a daily rhythm of photosensitivity. Gen. Comp. Endocr. 3, 466–476.

Baggerman, B., 1980. Photoperiodic and endogenous control of the annual reproductive cycle in teleost fishes. In Environmental Physiology of Fishes (Edited by Ali M. A.), pp. 533–567. Plenum Press, New York.

Bapary, M.A.J., Fainuulelei, P., Takemura, A., 2009. Environmental control of gonadal development in the tropical damselfish *Chrysiptera cyanea*. Mar. Biol. Res. 5, 462–469.

Bapary, M.A.J., Takemura, A., 2010. Effect of temperature and photoperiod on the reproductive condition and performance of a tropical damselfish *Chrysiptera cyanea* during different phases of the reproductive season. Fisheries Sci. 76, 769–776.

Bapary, M.A.J., Amin, M.N., Takemura, A., 2011. Food availabiliy as a possible determinant for initiation and termination of reproductive activity in the tropical damselfish *Chrysiptera cyanea* during reproductive season. Mar. Biol. Res. *In press.* DOI:10.1080/17451000.2011.605146.

Barrett, P., Ebling, F.J.P., Schuhler, S., Wilson, D., Ross, A.W., Warner, A., Jethwa, P.H., Boelen, A., Visser, T.J., Ozanne, D.M., Archer, Z.A., Mercer, J.G., Morgan, P.J., 2007. Hypothalamic thyroid hormone catabolism acts as a gatekeeper for the seasonal control of body mass and reproduction. Endocrinology 148, 3608–3617.

Becker, K.B., Schneider, M.J., Davey, J.C., Galton, V.A., 1995. The type III 5-deiodinase in *Rana catesbeiana* tadpoles is encoded by a thyroid hormone-responsive gene. Endocrinology 136, 4424–4431.

Becker, K.B., Stephens, K.C., Davey, J.C., Schneider, M.J., Galton, V. A., 1997. The type 2 and type 3 iodothyronine deiodinases play important roles in coordinating development in *Rana catesbeiana* tadpoles. Endocrinology 138, 2989–2997.

Bégay, V., Falcón, J., Cahill, G.M., Klein, D.C., Coon, S.L., 1998. Transcripts encoding two melatonin synthesis enzymes in the teleost pineal organ: circadian regulation in pike and zebrafish, but not in trout. Endocrinology 139, 905–912.

Berrill, I.K., Porter, M.J.R., Smart, A., Mitchell, D., Bromage, N.R., 2003. Photoperiodic effects on precocious maturation, growth and smoltification in Atlantic salmon, *Salmo salar*. Aquaculture 222, 239–252.

Berry, M.J., Banu, L., Chen, Y., Mandel, S.J., Kieffer, J.D., Harney, J.W., Larsen, P.R., 1991. Type I iodothyronine deiodinase is a selenocysteine-containing enzyme. Nature 349, 438–440.

Bianco, A.C., Larsen, P.R., 2005. Cellular and structural biology of the deiodinases. Thyroid. 15, 777–786.

Bianco, A.C., Salvatore, D., Gereben, B., Berry, M.J., Larsen, P.R., 2002. Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. Endocr. Rev. 23, 38–89.

Borg, B., Bornestaf, C., Hellqvist, A., Schmitz, M., Mayer, I., 2004. Mechanisms in the photoperiodic control of reproduction in the stickleback. Behaviour 141, 1521–1530.

Boujard, T., Leatherland, J.F., 1992. Circadian rhythms and feeding time in fishes. Environ. Biol. Fish. 35, 109–131.

Bromage, N.R., Porter, M., Randall, C., 2001. The environmental regulation of maturation in farmed fish with special reference to the role of photoperiod and melatonin, Aquaculture 197, 63–98.

Cahill, G.M., 2002. Clock mechanisms in zebrafish. Cell Tissue Res. 309, 27–34.

Campos-Mendoza, A., McAndrew, B.J., Coward, K., Bromage, N., 2004. Reproductive response of Nile tilapia (*Oreochromis niloticus*) to photoperiodic manipulation; effects on spawning periodicity, fecundity and egg size. Aquaculture 231, 299–314.

Celi, F.S., Canettieri, G., Yarnall, D.P., Burns, D.K., Andreoli, M., Schuldiner, A.R., Centanni, M., 1998. Genomic characterization of the coding region of the human type II 5' deiodinase gene. Mol. Cell. Endocrinol. 141, 49–52.

Chang, C.F., Hu, H.J., Tang, H.C., 1992. Effects of photoperiod and temperature on testicular development in male ayu, *Plecoglossus altivelis*. Environ. Biol. Fishes 34, 309–314.

Chopra, I.J., 1980. Alterations in monodeiodination of iodothyronines in the fasting rat: effects of reduced nonprotein sulfhydryl groups and hypothyroidism. Metabolism 29, 161–167.

Chopra, I.J., Wiersinga, W., Frank. H., 1981. Alterations in hepatic monodeiodination of iodothyronines in the diabetic rat. Life Sci. 28, 1765–1776.

Coppola, A., Hughes, J., Esposito, E., Schiavo, L., Meli, R., Diano, S., 2005. Suppression of hypothalamic deiodinase type II activity blunts TRH mRNA decline during fasting. FEBS Letters. 579, 4654–4658.

Croteau, W., Whittemore, S.L., Schneider, M.J., St. Germain, D.L., 1995. Cloning and expression of a cDNA for a mammalian type III iodothyronine deiodinase. J. Biol. Chem. 270, 16569–16575.

Croteau, W., Davey, J.C., Galton, V.A., St. Germain, D.L., 1996. Cloning of the mammalian type II iodothyronine deiodinase. A selenoprotein differentially expressed and regulated in human and rat brain and other tissues. J. Clin. Invest. 98, 405–417.

Cyr, D.G., Eales, J.G., 1988. *In vitro* effects of thyroid hormones on gonadotropin-induced estradiol-17β secretion by ovarian follicles of rainbow trout, *Salmo gairdneri.* Gen. Comp. Endocrinol. 69, 80–87.

Cyr, D.G., Idler, D.R., Audet, C., McLeese, J.M., Eales, J.G., 1998. Effects of long-term temperature acclimation on thyroid hormone deiodinase function, plasma thyroid hormone levels, growth, and reproductive status of male Atlantic cod, *Gadus morhua.* Gen. Comp. Endocrinol. 109, 24–36.

Darras, V.M., Mol, K.A., Van der Geyten, S., Kühn, E.R., 1998. Control of peripheral thyroid hormone levels by activating and inactivating deiodinases. Ann. N.Y. Acad. Sci. 839, 80–86.

Davey, J.C., Becker, K.B., Schneider, M.J., St. Germain, D.L., Galton, V.A., 1995. Cloning of a cDNA for the type II iodothyronine deiodinase. J. Biol. Chem. 270, 26786–26789.

Davey, J.C., Schneider, M.J., Becker, K.B. Galton, V.A., 1999. Cloning of a 5.8kb cDNA for a mouse type 2 deiodinase. Endocrinology, 140, 1022– 1025.

Davies, B., Bromage, N., 2002. The effects of fluctuating seasonal and constant water temperatures on the photoperiodic advancement of reproduction in female rainbow trout *Oncorhynchus mykiss*. Aquaculture 205, 183–200.

Davies, B., Bromage, N., Swanson, P., 1999. The brain–pituitary–gonadal axis of female rainbow trout *Oncorhynchus mykiss:* effects of photoperiod manipulation. Gen. Comp. Endocrinol. 115, 155–166.

de Luze, A., Leloup, J., 1984. Fish growth hormone enhances peripheral conversion of thyroxine to triiodothyronine in the eel (*Anguilla anguilla* L.). Gen. Comp. Endocrinol. 56, 308–312.

Dey, R., Bhattacharya, S., Maitra, S.K., 2005. Importance of photoperiods in the regulation of ovarian activities in Indian major carp *Catla catla* in an annual cycle. J. Biol. Rhythms 20, 145–158.

Diano, S., Naftolin, F., Goglia, F., Horvath, T.L., 1998. Fasting-induced increase in type II iodothyronine deiodinase activity and messenger ribonucleic acid levels is not reversed by thyroxine in the rat hypothalamus. Endocrinology 139, 2879–2884.

Eales, J.G., 1985. The peripheral metabolism of thyroid hormones and regulation of thyroidal status in poikilotherms. Can. J. Zool. 63, 1217–1231.

Eales, J.G., Brown, S.B., 1993. Measurement and regulation of thyroidal status in teleost fish. Rev. Fish Biol. Fish 3, 299–347.

Eales, J.G., MacLatchy, D.L., Sweeting, R.M., 1993. Thyroid hormone deiodinase systems in salmonids, and their involvement in the regulation of thyroidal status. Fish Physiol. Biochem. 2, 313–321.

Ebbesson, L.O.E., Björnsson, B.T., Ekström, P., Stefansson, S.O., 2008. Daily endocrine profiles in parr and smolt Atlantic salmon. Comp. Biochem. Physiol. 151A, 698–704.

Falcón, J., Besseau, L., Fazzari, D., Attia, J., Gaildrat, P., Beauchaud, M., Boeuf, G., 2003. Melatonin modulates secretion of growth hormone and prolactin by trout pituitary glands and cells in culture. Endocrinology 144, 4648–4658.

Falcón, J., Besseau, L., Sauzet, S., Buf, G., 2007. Melatonin effects on the hypothalamo-pituitary axis in fish. Trends Endocrinol. Metab. 18, 81–88.

Falcón, J., Migaud, H., Muñoz-Cueto, J.A., Carrillo, M., 2010. Current knowledge on the melatonin system in teleost fish. Gen. Comp. Endocrinol. 165, 469–482.

Fekete, C., Freitas, B.C.G., Zeöld, A., Wittmann, G., Kádár, A., Liposits, Z., Christoffolete, M.A., Singru, P., Lechan, R.M., Bianco, A.C., Gereben, B., 2007. Expression patterns of WSB-1 and USP-33 underlie cell-specific posttranslational control of type 2 deiodinase in the rat brain. Endocrinology 148, 4865–4874.

Finnson, K.R., Eales, J.G., 1999. Effect of T_3 treatment and food ration on hepatic deiodination and conjugation of thyroid hormones in rainbow trout *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 115, 379–386.

Flood, C.G., Eales, J.G., 1983. Effects of starvation and refeeding on plasma T_4 and T_3 levels and T_4 deiodination in rainbow trout, *Salmo gairdneri*. Can. J. Zool. 61, 1949–1953.

Frith, S.D., Eales, J.G., 1996. Thyroid hormone deiodination pathways in brain and liver of rainbow trout, *Oncorhynchus mykiss.* Gen. Comp. Endocrinol. 101, 323–332.

García-G, C., Jeziorski, M.C., Valverde-R. C., Orozco, A. 2004. Effects of iodothyronines on the hepatic outer-ring deiodinating pathway in killifish. Gen. Comp. Endocrinol. 135, 201–209.

Gereben, B., Bartha, T., Tu, H.M., Harney, J.W., Rudas, P., Larsen, P.R., 1999. Cloning and expression of the chicken type 2 iodothyronine 5'-deiodinase. J. Biol. Chem. 274, 13768–13776.

Gereben, B., Goncalves, C., Harney, J.W., Larsen, P.R., Bianco, A.C. 2000. Selective proteolysis of human type 2 deiodinase: a novel ubiquitin-proteasomal mediated mechanism for regulation of hormone activation. Mol. Endocrinol. 14, 1697–1708.

Gillet, C., Breton, B., Billard, R., 1978. Seasonal effects of exposure to temperature and photoperiod regimes on gonad growth and plasma gonadotropin in goldfish (*Carassius auratus*). Ann. Biol. Anita. Biochem. Biophys. 18, 1045–1049.

Gomez, J.M., Boujard, T., Boeuf, G., Solari, A., Le Bail, P.Y., 1997. Individual diurnal plasma profiles of thyroid hormones in rainbow trout (*Oncorhynchus mykiss*) in relation to cortisol, growth hormone, and growth rate. Gen. Comp. Endocrinol. 107, 74–83.

Hernández-Rauda, R., Miguez, J.M., Ruibal, C., Aldegunde, M., 2000. Effects of melatonin on dopamine metabolism in the hypothalamus and the pituitary of the rainbow trout, *Oncorhynchus mykiss*. J. Exp. Zool. 287, 440–444.

Herwig, A., Wilson, D., Logie, T.J., Boelen, A., Morgan, P.J., Mercer, J.G., Barrett, P.I., 2009. Photoperiod and acute energy deficits interact on components of the thyroid hormone system in hypothalamic tanycytes of the Siberian hamster. Am. J. Physiol. Regul. Integr. Comp. Physiol. 296, 1307–1315.

Isorna, E., Obregon, M.J., Calvo, R.M., Vázquez, R., Pendón, C., Falcón, J., Muñoz-Cueto, J.A., 2009. Iodothyronine deiodinases and thyroid hormone receptors regulation during flatfish (*Solea senegalensis*) metamorphosis. J. Exp. Zool. 312, 231–246.

Itoh, K., Watanabe, K., Wu, X., Suzuki, T., 2010. Three members of the iodothyronine deiodinase family, dio1, dio2 and dio3, are expressed in spatially and temporally specific patterns during metamorphosis of the flounder, *Paralichthys olivaceus.* Zool. Sci. 27, 574–580.

Johannes, R.E., 1978. Reproductive strategies of coastal marine fishes in the tropics. Environ. Biol. Fish. 3, 65–84.

Johnston, C.E., Eales, J.G., 1995. Effects of acclimation and assay temperature on outer- and inner-ring thyroxine and 3,5,3'-triiodo-L-thyronine deiodination by liver microsomes of rainbow trout, *Oncorhynchus mykiss*. J. Exp. Zool. 272, 426–434.

Kalsbeek, A., Buijs, R.M., van Schaik, R., Kaptein, E., Visser, T.J., Doulabi, B.Z., Fliers, E., 2005. Daily variations in type II iodothyronine deiodinase activity in the rat brain as controlled by the biological clock. Endocrinology 146, 1418–1427.

Kissil, G.Wm., Lupatsch, I., Elizur, A., Zohar, Y., 2001. Long photoperiod delayed spawning and increased somatic growth in gilthead seabream (*Sparus auratus*). Aquaculture 200, 363–379.

Klaren, P.H.M., Haasdijk, R., Metz, J.R., Nitsch, L.M.C., Darras, V.M., Van der Geyten, S., Flik, G., 2005. Characterization of an iodothyronine 5´-deiodinase in gilthead seabream (*Sparus auratus*) that is inhibited by dithiothreitol. Endocrinology 146, 5621–5630.

Köhrle, J., 1999. Local activation and inactivation of thyroid hormones: the deiodinase family. Mol. Cell. Endocrinol. 151, 103–119.

Köhrle, J., Jakob, F., Contempre, B., Dumont, J.E., 2005. Selenium, the thyroid, and the endocrine system. Endocr. Rev. 26, 944–984.

Kwonm, J.Y., Chang, Y.J., Sohn, Y.C., Aida K., 1999. Plasma and ovarian thyroxine levels in relation to sexual maturation and gestation in female *Sebastes inermis*. 54, 370–379.

Kyte, J., Doolittle, R.F., 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157,105–132.

Larsen, P.R., Berry, M.J., 1995. Nutritional and hormonal regulation of thyroid hormone deiodinases. Annu. Rev. Nutr. 15, 323–352.

Leatherland, J.F., 1982. Environmental physiology of the teleostean thyroid gland: a review. Environ. Biol. Fish 7, 83–110.

Leatherland, J.F., 1994. Reflections on the thyroidology of fishes: from molecules to humankind. Guelph Ichthyology Reviews. 7, 1–67.

Leatherland, J.F., Reddy, P.K., Yong, A.N., Leatherland, A., Lam, T.J., 1990. Hepatic 5'-monodeiodinase activity in teleosts in vitro, a survey of thirty-three species. Fish Physiol. Biochem. 8, 1–10.

Leiner, K.A., Han, G.S., MacKenzie, D.S., 2000. The effects of photoperiod and feeding on the diurnal rhythm of circulating thyroid hormones in the red drum, *Sciaenops ocellatus.* Gen. Comp. Endocrinol. 120, 88–98.

Leiner, K.A., MacKenzie, D.S., 2001. The effects of photoperiod on growth rate and circulating thyroid hormone levels in the red drum, *Sciaenops ocellatus*: evidence for a free-running circadian rhythm of T_4 secretion. Comp. Biochem. Physiol. 130A, 141–149.

Leiner, K.A., MacKenzie, D.S., 2003. Central regulation of thyroidal status in a teleost fish: nutrient stimulation of T_4 secretion and negative feedback of T_3. J. Exp. Zool. 298A, 32–43.

Leonard, J.L., Farwell, A.P., 1997. Thyroid hormone-regulated actin polymerization in brain. Thyroid 7,147–151.

Leonard, J.L., Köhrle, J., 1996. Intracellular pathways of iodothyronine metabolism. In, Braverman LE, Utiger R (eds) The Thyroid. Lippincott-Raven, Philadelphia, pp 125–161.

Lin, X., Volkoff, H., Narnaware, Y., Bernier, N.J., Peyon, P., Peter, R.E., 2000. Review. Brain regulation of feeding behavior and food intake in fish. Comp. Biochem. Physiol. 126, 415–434.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. Methods 25, 402–408.

Loter, T.C., MacKenzie, D.S., McLeese, J., Eales, J.G. 2007. Seasonal changes in channel catfish thyroid hormones reflect increased magnitude of daily thyroid hormone cycles. Aquaculture 262, 451–460.

MacLatchy, D.L., Eales, J.G., 1988. Short-term treatment with testosterone increases plasma 3,5,3'-triiodo-L-thyronine and hepatic L-thyroxine 5'-monodeiodinase levels in arctic charr, *Salvelinus fontinalis.* Gen. Comp. Endocrinol. 71, 10–16.

MacLatchy, D.L., Eales, J.G., 1990. Growth hormone stimulates hepatic thyroxine 5'-monodeiodinase activity and 3, 5, 3'-triiodothyronine levels in rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol. 78, 164–172.

MacLatchy, D.L., Eales, J.G., 1992. Properties of T_4 5'-deiodinating systems in various tissues of the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol 86, 313–322.

Mandel, S.J., Berry, M.J., Kieffer, J.D., Harney, J.W., Warne, R.L., Larsen, P.R., 1992. Cloning and in vitro expression of the human selenoprotein, type I iodothyronine deiodinase. J. Clin. Endocrinol. Metab. 75, 1133–1139.

Martínez, L.M., Orozco, A., Villalobos, P., Valverde-R. C., 2008. Cloning and characterization of a type 3 iodothyronine deiodinase (D3) in the liver of the chondrichtyan *Chiloscyllium punctatum.* Gen. Comp. Endocrinol. 156, 464–469.

Mol, K., Kaptein, E., Darras, V.M., de Greef, W.J., Kühn, E.R., Visser, T.J., 1993. Different thyroid hormone-deiodinating enzymes in tilapia (*Oreochromis niloticus*) liver and kidney. FEBS Lett. 321,140–144.

Mol, K.A., Van der Geyten, S., Burel, C., Kühn, E.R., Boujard T., Darras, V.M., 2004. Comparative study of iodothyronine outer ring and inner ring deiodinase activities in five teleostean fishes. Fish Physiol. Biochem. 253–266.

Mol, K.A., Van der Geyten, S., Burel, C., Kühn, E.R., Boujard, T., Darras, V.M., 1998. Comparative study of iodothyronine outer ring and inner ring deiodinase activities in five teleostean fishes. Fish Physiol. Biochem. 18, 253–266.

Mol, K.A., Van der Geyten, S., Darras, V.M., Visser T.J., Kühn. E.R., 1997. Characterization of iodothyronine outer ring and inner ring deiodinase activities in the blue tilapia, *Oreochromis aureus*. Endocrinology 138, 1787–1793.

Mol, K.A., Van der Geyten, S., Kühn, E.R., Darras, V.M. 1999. Effects of experimental hypo- and hyperthyroidism on iodothyronine deiodinases in Nile tilapia, *Oreochromis niloticus*. Fish Physiol. Biochem. 20, 201–207.

Moore VanPutte, C.L., MacKenzie, D.S., Eales, J.G., 2001. Characterization of hepatic low-Km outer-ring deiodination in red drum (*Sciaenops ocellatus*). Comp. Biochem. Physiol. 128 B, 413–423.

Morin, P.P., Hara, T.J., Eales, J.G., 1993. Thyroid hormone deiodination in brain, liver, gill, heart and muscle of Atlantic salmon (*Salmo salar*) during photoperiodically-induced parr-smolt transformation. I. Outer- and inner-ring thyroxine deiodination. Gen. Comp. Endocrinol. 90, 142–156.

Nakao, N., Ono, H., Yamamura, T., Anraku, T., Takagi, T., Higashi, K., Yasuo, S., Katou, Y., Kageyama, S., Uno, Y., Kasukawa, T., Iigo, M., Sharp, P.J., Iwasawa, A., Suzuki, Y., Sugano, S., Niimi, T., Mizutani, M., Namikawa, T., Ebihara, S., Ueda, H.R., Yoshimura, T., 2008. Thyrotrophin in the pars tuberalis triggers photoperiodic response. Nature 452, 317–323.

O'Mara, B.A., Dittrich, W., Lauterio, T.J., St Germain, D.L., 1993. Pretranslational regulation of type I 5´-deiodinase by thyroid hormones and in fasted and diabetic rats. Endocrinology 133, 1715–1723.

Okamura, H., 2008. Brain comes to light. Nature 452, 294–295.

Orozco, A., Jeziorski, M.C., Linser, P.J., Greenberg, R.M., Valverde-R, C., 2002. Cloning of the gene and complete cDNA encoding a type 2 deiodinase in *Fundulus heteroclitus*. Gen. Comp. Endocrinol. 128, 162–167.

Orozco, A., Linser, P.J., Valverde-R, C., 2000. Kinetic characterization of outer-ring deiodinase activity (ORD) in the liver, gill and retina of killifish *Fundulus heteroclitus*. Comp. Biochem. Physiol. B 126, 283–290.

Orozco, A., Silva, E.J., Valverde-R, C., 1997. Rainbow trout liver expresses two iodothyronine phenolic ring deiodinase pathways with the characteristics of mammalian types I and II 5´-deiodinases. Endocrinology 138, 254–258.

Orozco, A., Valverde-R, C., 2005. Thyroid hormone deiodination in fish. Thyroid 15, 799–813.

Orozco, A., Villalobos, P., Valverde-R, C., 2002. Environmental salinity selectively modifies the outer-ring deiodinating activity of liver, kidney and gill in the rainbow trout. Comp. Biochem. Physiol. 131, 387–395.

Park, Y.J., Park, J.G., Hiyakawa, N., Lee. Y.D., Kim, S.J., Takemura, A., 2007. Diurnal and circadian regulation of a melatonin receptor, MT1, in the golden rabbitfish, *Siganus guttatus*. Gen. Comp. Endocrinol. 150, 253–262.

Park, Y.J., Park, J.G., Kim, S.J., Lee, Y.D., Rahman, M.S., Takemura, A., 2006. Melatonin receptor of a reef fish with lunar-related rhythmicity: cloning and daily variations. J. Pineal Res. 41, 166–174.

Parmentiern, E., Lecchini, D., Lagardere, F., Vandewalle, P., 2004. Ontogenic and ecological control of metamorphosis onset in a carapid fish, *Carapus homei*: experimental evidence from vertebra and otolith comparisons. J. Exp. Zool. 301, 617–628.

Peterman, R.M., Bradford, M.J., 1987. Wind speed and mortality rate of a marine fish, the northern anchovy (*Engraulis mordax*). Science 235, 354–356.

Picard-Aitken, M., Fournier, H., Pariseau, R., Marcogliese, D.J., Cyr, D.G., 2007. Thyroid disruption in walleye (*Sander vitreus*) exposed to environmental contaminants: cloning and use of iodothyronine deiodinases as molecular biomarkers. Aquat. Toxicol. 83, 200–211.

Plate, E.M., Adams, B.A., Allison, W.T., Martens, G., Hawryshyn, C.W., Eales, J.G., 2002. The effects on thyroxine or a GnRH analogue on thyroid hormone deiodination in the olfactory epithelium and retina of rainbow trout, *Oncorhynchus mykiss*, and sockeye salmon, *Oncorhynchus mykiss nerka*. Gen. Comp. Endocrinol. 127, 59–65.

Plohman, J.C., Dick, T.A., Eales, J.G., 2002. Thyroid of lake sturgeon, *Acipenser fulvescens* II. Deiodination properties, distribution, and effects of diet, growth, and a T_3 challenge. Gen. Comp. Endocrinol. 125, 56–66.

Rahman, M.S., 2004. Effects of moonlight exposure on plasma melatonin rhythms in the seagrass rabbitfish, *Siganus canaliculatus*. J. Biol. Rhythms, 19, 325–334.

Rahman, M.S., Takemura, A., Takano, K., 2000a. Annual changes in ovarian histology, plasma steroid hormones and vitellogenin in the female golden rabbitfish, *Siganus guttatus* (Bloch). B. Mar. Sci. 67, 729–740.

Rahman, M.S., Takemura, A., Takano, K., 2000b. Annual changes in testicular activity and plasma steroid hormones in the golden rabbitfish *Siganus guttatus* (Bloch). Fisheries. Sci. 66, 894–900.

Reddy, P.K., Leatherland, J.F., 1994. Does the time of feeding affect the diurnal rhythms of plasma hormone and glucose concentration and hepatic glycogen content in rainbow trout? Fish Physiol.Biochem.13, 133–140.

Reddy, P.K., Leatherland, J.F., 1995. Influence of the combination of time of feeding and ration level on the diurnal hormone rhythms in rainbow trout. Fish Physiol. Biochem. 14, 25–36.

Reiter, R.J., 1993. The melatonin rhythm: both a clock and a calendar. Experientia 654–663.

Revel, F.G., Saboureau, M., Pévet, P., Mikkelsen, J.D., Simonneaux, V., 2006. Melatonin regulates type 2 deiodinase gene expression in the Syrian hamster. Endocrinology 147, 4680–4687.

Salvatore, D., Low, S.C., Berry, M., Maia, A.L., Harney, J.W., Croteau, W., St. Germain, D.L., Larsen, P.R., 1995. Type 3 iodothyronine deiodinase, cloning, *in vitro* expression, and functional analysis of the placental selenoenzyme. J. Clin. Invest. 96, 2421–2430.

Salvatore, D., Bartha, T., Harney, J.W., Larsen, P.R., 1996a. Molecular biological and biochemical characterization of the human type 2 selenodeiodinase. Endocrinology. 137, 3308 –3315.

Salvatore, D., Tu, H., Harney, J.W., Larsen, P.R., 1996b. Type 2 iodothyronine deiodinase is highly expressed in human thyroid. J. Clin. Invest. 98, 962–968.

Sambroni, E., Gutieres, S., Cauty, C., Guiguen, Y., Breton, B., Lareyre, J.J., 2001. Type II iodothyronine deiodinase is preferentially expressed in rainbow trout (*Oncorhynchus mykiss*) liver and gonads. Mol. Reprod. Dev. 60, 338–30.

Sánchez-Vázquez, F.J., Zamora, S., Madrid, J.A., 1995. Light-dark and food restriction cycles in sea bass: Effect of conflicting zeitgebers on demand-feeding rhythms. Physiol. Behav. 58, 705–714.

Sanders, J.P., Van der Geyten, S., Kaptein, E., Darras, V.M., Kühn, E.R., Leonard, J.L., Visser, T.J., 1997. Characterization of a propylthiouracil-insensitive type I iodothyronine deiodinase. Endocrinology 138, 5153–5160.

Sanders, J.P., Van der Geyten, S., Kaptein, E., Darras, V.M., Kühn, E.R., Leonard, J.L., Visser, T.J., 1999. Cloning and characterization of type III iodothyronine deiodinase from the fish *Oreochromis niloticus*. Endocrinology 140, 3666–3673.

Saoud, I.P., Mohanna, C., Ghanawi, J. 2008. Effects of temperature on survival and growth of juvenile spinefoot rabbitfish (*Siganus rivulatus*). Aquac. Res. 39, 491–497.

Schwartz, M.W., Woods, S.C., Porte, D. Jnr., Seeley, R.J., Baskin, D.G., 2000. Central nervous system control of food intake. Nature. 404, 661–671.

Shoemaker, C.A., Klesius, P.H., Lim, C., Yildirim, M., 2003. Feed deprivation of channel catfish, *Ictalurus punctatus* (Rafinesque), influences organosomatic indices, chemical composition and susceptibility to *Flavobacterium columnare*. J. Fish. Dis. 26, 553–561.

Specker, J.L., Eales, J.G., Tagawa, M., Tyler, WA III., 2000. Parr-smolt transformation in Atlantic salmon: thyroid hormone deiodination in liver and brain and endocrine correlates of change in rheotactic behavior. Can. J. Zool. 78, 696–705.

Spieler, R.E., Noeske, T.A., 1981. Timing of a single daily meal and diel variations of serum thyroxine, triiodothyronine and cortisol in goldfish *Carassius auratus*. Life Sci. 28, 939–2944.

Sri Susilo, E., Harnadi, L., Takemura, A., 2009. Tropical monsoon environments and the reproductive cycle of the orange-spotted spinefoot *Siganus guttatus*. Mar. Biol. Res. 5, 179–185.

Srinivasan, M., Jones, G.P., 2006. Extended breeding and recruitment periods of fishes on a low latitude coral reef. Coral Reefs 25, 673–682.

St Germain, D.L., Galton, V.A., 1997. The deiodinase family of selenoproteins. Thyroid 7,655–668.

St. Germain, D.L., Schwartzman, R.A., Croteau, W., Kanamori, A., Wang, Z., Brown, D.D., Galton, V.A., 1994. A thyroid hormone-regulated gene in *Xenopus laevis* encodes a type III iodothyronine 5-deiodinase. Proc. Natl. Acad. Sci. USA 91, 7767–7771 (Erratum, p 11282).

Stadtman, T.C., 1996. Selenocysteine. Annu. Rev. Biochemistry. 65, 83–100.

Takemura, A., Rahman, M.S., Nakamura, S., Park, Y.J., Takano, K., 2004a. Lunar cycles and reproductive activity in reef fishes with particular attention to rabbitfishes. Fish. 5, 317–328.

Takemura, A., Sri Susilo, E., Rahman, M.S., Morita, M., 2004b. Perception and possible utilization of moonlight intensity for reproductive activities in a lunar-synchronized spawner, the golden rabbitfish. J. Exp. Zool. 301A, 844–851.

Takemura, A., Rahman, M.S., Park, Y.J., 2010. External and internal controls of lunar-related reproductive rhythms in fishes. J. Fish Biol. 76, 7–26.

Takemura, A., Ueda, S., Hiyakawa, N., Nikaido, Y., 2006. A direct influence of moonlight intensity on changes in melatonin production by cultured pineal glands of the golden rabbitfish, *Siganus guttatus*. J. Pineal Res. 40, 236–241.

Toguyeni, A., Baroiller, J.F., Fustier, A., Le Bail, P.Y., Kühn, E.R., Mol, K.A., Fauconneau, B., 1996. Consequences of food restriction on short-term growth variation and on plasma circulating hormones in *Oreochromis niloticus* in relation to sex. Gen. Comp. Endocrinol. 103, 167–175.

Toyoda, N., Harney, J.W., Berry, M.J., Larsen, P.R., 1995. Identification of critical amino acids for 3,3',5'-triiodothyronine deiodination by human type I deiodinase based on comparative functional-structural analyses of the human, dog and rat enzymes. J. Biol. Chem. 269, 20329–20334.

Tyler, W.A., III, Stanton, F.G., 1995. Potential influence of food abundance on spawning patterns in a damselfish, *Abudefduf abdominalis*. Bull. Mar. Sci. 57, 610–623.

Valverde-R, C., Croteau, W., LaFleur, Jr. G.J., Orozco, A., St. Germain, D.L., 1997. Cloning and expression of a 5'-iodothyronine deiodinase from the liver of *Fundulus heteroclitus*. Endocrinology 138, 642–648.

Van der Geyten, S., Sanders, J.P., Kaptein, E., Darras, V.M., Kühn, E.R., Leonard, J.L., Visser, T.J. 1997. Expression of chicken hepatic type I and type III iodothyronine deiodinase during embryonic development. Endocrinology 138, 5144–5152.

Van der Geyten, S., Mol, K.A., Pluymers, W., Kühn, E.R., Darras. V.M., 1998. Changes in plasma T_3 during fasting/refeeding in tilapia (*Oreochromis niloticus*) are mainly regulated through changes in hepatic type II iodothyronine deiodinase. Fish Physiol. Biochem. 19, 135–143.

Van der Geyten, S., Van den Eynde, I., Segers, I.B., Kühn, E.R., Darras, V.M., 2002. Differential expression of iodothyronine deiodinases in chicken tissues during the last week of embryonic development. Gen. Comp. Endocrinol. 128, 65–73.

Van der Geyten, S., Byamungu, N., Reyns, G.E., Kühn, E.R., Darras, V.M. 2005. Iodothyronine deiodinases and the control of plasma and tissue thyroid hormone levels in hyperthyroid tilapia (*Oreochromis niloticus*). J. Endocrinol. 184, 467–479.

Vera, L.M., De Oliveira, C., López-Olmeda, J.F., Ramos, J., Mañanós, E., Madrid, J.A., Sánchez-Bázquez, F.J., 2007. Seasonal and daily plasma melatonin rhythms and reproduction in Senegal sole kept under natural photoperiod and natural or controlled water temperature. J. Pineal Res. 43, 50–55.

Visser, T.J., 1996. Pathways of thyroid hormone metabolism. Acta Med. Austriaca 23, 10–16.

Wagner, M.S., Morimoto, R., Dora, J.M., Benneman, A., Pavan, R., Maia, A.L., 2003. Hypothyroidism induces type 2 iodothyronine deiodinase expression in mouse heart and testis. J. Mol. Endocrinol. 31, 541–550.

Walpita, C.N., Grommen, S.V.H., Darras, V.M., Van der Geyten, S., 2007. The influence of stress on thyroid hormone production and peripheral deiodination in the Nile tilapia *Oreochromis niloticus*. Gen. Comp. Endocrinol. 150, 18–25.

Wambiji, N., Park, Y.J., Kim, S.J., Hur, S.P., Takeuchi, Y., Takemura, A., 2011b. Expression of type II iodothyronine deiodinase gene in the brain of a tropical fish, *Siganus guttatus*. Comp. Biol. Physiol. *Article in press.* doi:10.1016/j.cbpa.2011.03.023.

Wambiji, N., Park, Y.J., Park, J.G., Kim, S.J., Hur, S.P., Takeuchi, Y., Takemura, A., 2011a. Expression patterns of type II and III iodothyronine deiodinase genes in the liver of the goldlined spinefoot, *Siganus guttatus.* Fisheries Sci. 77, 301–311.

Watanabe, M., Yasuo, S., Watanabe, T., Yamamura, T., Nakao, N., Ebihara, S., Yoshimura, T., 2004. Photoperiodic regulation of type 2 deiodinase gene in Djungarian hamster: possible homologies between avian and mammalian photoperiodic regulation of reproduction. Endocrinology 145, 1546–1549.

Watanabe, T., Yamamura, T., Watanabe, M., Yasuo, S., Nakao, N., Dawson, A., Ebihara, S., Yoshimura, T., 2007. Hypothalamic expression of thyroid hormone-activating and-inactivating enzyme genes in relation to photorefractoriness in birds and mammals. Am J. Physiol. Regul. Integr. Comp Physiol. 292, 568–572.

Woodland, D.J., 1990. Revision of the fish family Siganidae with descriptions of two new species and comments on distribution and biology. Indo-Pacific fishes, Vol 19. Honolulu: Bernice Puahu Bishop Museum.136 pp.

Yasuo, S., Watanabe, M., Nakao, N., Takagi, T., Follett, B.K., Ebihara, S., Yoshimura, T., 2005. The reciprocal switching of two thyroid hormone-activating and -inactivating enzyme genes is involved in the photoperiodic gonadal response of Japanese quail. Endocrinology 146, 2551–2554.

Yasuo, S., Nakao, N., Ohkura, S., Iigo, M., Hagiwara, S., Goto, A., Ando, H., Yamamura, T., Watanabe, M., Watanabe, T., Oda, S., Maeda, K., Lincoln, G.A., Okamura, H., Ebihara, S., Yoshimura, T., 2006. Long-day suppressed expression of type 2 deiodinase gene in the mediobasal hypothalamus of the Saanen goat, a short-day breeder: implication for seasonal window of thyroid hormone action on reproductive neuroendocrine axis. Endocrinology 147, 432–440.

Yasuo, S., Watanabe, M., Iigo, M., Nakamura, T.J., Watanabe, T., Takagi, T., Ono, H., Ebihara, S., Yoshimura, T., 2007a. Differential response of type 2 deiodinase gene expression to photoperiod between photoperiodic Fischer 344 and nonphotoperiodic Wistar rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292, 1315–9.

Yasuo, S., Yoshimura, T., Ebihara, S., Korf, H-W., 2007b. Temporal dynamics of type 2 deiodinase expression after melatonin injections in Syrian hamsters. Endocrinology 148, 4385– 4392.

Yasuo, S., Yoshimura, T. Ebihara, S., Korf, H., 2009. Melatonin transmits photoperiodic signals through the MT1 melatonin receptor. J. Neurosci. 29, 2885–2889.

Yasuo, S., Yoshimura, T., 2009. Comparative analysis of the molecular basis of photoperiodic signal transduction in vertebrates. Integr. Comp. Biol. 49, 507–518.

Yoshimura, T., 2010. Neuroendocrine mechanism of seasonal reproduction in birds and mammals. Anim. Sci. J. 81, 403–410.

Yoshimura, T., Yasuo, S., Watanabe, M., Iigo, M., Yamamura, T., Hirunagi, K., Ebihara, S., 2003. Light-induced hormone conversion of T_4 to T_3 regulates photoperiodic response of gonads in birds. Nature 426, 178–181.