Coastal Marine Science 38(1): 1–7, 2015

Profiles of thyroid hormones and mRNA expression for thyroid stimulating hormone in Japanese eel during downstream migration

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Received 11 November 2013; Accepted 7 May 2014

Abstract — To understand the roles of thyroid hormones in downstream migration of anguillid eels, we examined the profiles of serum thyroid hormones (thyroxine, T4 and triiodothyronine, T3) and thyroid stimulating hormone β-subunit (TSHβ) mRNA expression in relation to habitat use, silvering, and seasonal changes. Female Japanese eels, *Anguilla japonica*, were collected in the brackish Hamana Lake and its freshwater rivers from July to December. The habitat use history of the eels was determined by otolith analysis of strontium and calcium concentrations. Thyroid hormone levels were measured by enzyme immunoassay and expression levels of TSHβ mRNA were determined by real-time quantitative PCR analysis. There were no consistent differences in the thyroid hormones or TSHβ expression levels between different habitats or seasonality. In addition, there were no or weak significant differences in thyroid hormones levels and TSHβ mRNA expression levels between migrant and other eels, although thyroid hormones have been shown to regulate endocrine control of downstream migratory behavior in some fishes. These findings suggest that thyroid hormones have play a minor role in downstream migration of anguillid eels.

Key words: Japanese eel, silvering, thyroid hormones, thyroid stimulating hormone, downstream migration

Introduction

The Japanese eel, *Anguilla japonica*, is a catadromous fish with a complex life cycle. The eels spawn in the seamount area of the southern part of the West Mariana Ridge in the western North Pacific (Tsukamoto et al. 2011). Larvae drift in the North Equatorial Current and Kuroshio Current toward the coasts of East Asia (Shinoda et al. 2011). The growth phase begins in the continental waters and glass eels become yellow eels. After a 4–12 year growth period (Sudo et al. 2013), eels undergo secondary metamorphosis from yellow to silver eels, which is also known as silvering (Aoyama and Miller 2003), and begin their spawning migration. They then switch from a resident life in freshwater habitats to migratory life in the ocean.

Thyroid hormones, thyroxine (T4) and triiodothyronine (T3), are essential for development, growth, and metabolism in vertebrates (Gorbman 1963), and are regulated by thyroid stimulating hormone, which is a pituitary glycoprotein hormone. It is well known that thyroid hormones are involved in metamorphosis in amphibians (Kanamori and Brown 1996, Tata 2006) and several fishes, such as the Japanese flounder, *Paralichthys olivaceus* (Inui and Miwa 1985) and tilapia, *Ocreochromis mossambicus* (Okimoto et al. 1993). Thyroid hormones have also been implicated in the migration of some fishes. For example, juveniles of Atlantic salmon, *Salmo salar*, exhibited a marked surge in blood T4 just before downstream migration (Hoar 1988, Boeuf 1993). It has been reported that T4 played an important role for the initiation of upstream migration in ayu, *Plecoglossus altivelis* (Tsukamoto et al. 1988). Similarly, it is also assumed that thyroid hormones are involved in the downstream migration of freshwater eels and the silvering i.e., the body color change as a preparatory adaptation for oceanic migration.

To clarify the roles of thyroid hormones in the migration of eels, it is necessary to determine their profiles in wild eels during migration. In the Japanese eel, the serum thyroxine (T4) and thyroid stimulating hormone β-subunit (TSHβ) mRNA expression levels increased during silvering, suggesting that the thyrotropic axis is involved in silvering (Han et al. 2004). In the European eel, *Anguilla anguilla*, TSHβ mRNA levels did not change and showed weak or no variations in plasma thyroid hormones (Aroua et al. 2005), indicating no or moderate changes in the thyrotropic axis during silvering. Thus, the role of the thyrotropic axis during silvering in anguillid eels is still controversial, and information about migration is lacking. To obtain a better understanding

of the roles of thyroid hormones in downstream migration and silvering, more elaborate sampling design is needed, taking into consideration the ecological features of eels. Eels are euryhaline fish that can spend most of their life as yellow eels in water of different salinity (Tsukamoto et al. 1998, Tsukamoto and Arai 2001, Daverat et al. 2006). Silvering of eels only occurs just before the migratory season, which is in late autumn to early winter in East Asia. Therefore, it is necessary to consider habitat use, the timing of migratory behavior, and seasonality.

In the present study, we determined profiles of plasma thyroid hormones and mRNA expression of TSHβ, in female Japanese eel. Eels were caught from both the brackish lake and its freshwater inlets, and strontium calcium ratios (Sr : Ca ratios) in otolith, which reflects the environmental ambient salinity (Campana 1999), were examined to determine the salinities they had experienced. In addition, eels were sampled in both pre-migratory (July and September) and migratory seasons (October and December) to examine seasonal changes.

Materials and methods

Fish collection and study area

This study was conducted on female Japanese eels, collected in Hamana Lake and its inlets (Hana River, Miyakoda River, Egawa River, Ima River, and Irideohta River) (Fig. 1). Hamana Lake is a brackish lake with relatively high salinity ranging from 22 to 33 psu depending on the tide, and all inlets are small freshwater streams. In Hamana Lake, eels were captured by commercial fishermen using set nets and/or eel pots in July and from September to November 2007. In rivers, eels were collected by electro-fishing from September to November 2007.

Morphometric measurement and organ sampling

Immediately after collection, the live eels were quickly transported to the laboratory and divided into yellow eels and silver eels according to the silvering index for the Japanese eel (Okamura et al. 2007). Then, they were anesthetized with 0.08% 2-phenoxyethanol. After measuring total length (TL) and body weight (BW), blood samples were taken from the bulbus arteriosus using heparinized syringes. After centrifugation at 3300 g for 20 min at 4°C, plasma was collected and stored at −20°C until use for physiological analysis. Then, eels were sacrificed by decapitation while still anesthetized, and then the pituitaries were quickly removed and kept in RNAlater solution (Ambion) for molecular biological analysis. After collection of the sagital otoliths, the gonads were dissected and then weighed. Sex was determined by visual inspection of gonad morphology. Gonadosomatic index (GSI) was calculated using the following formula:

$$\text{GSI}=\text{gonad weight}/\text{BW}\times100$$

Otolith microchemistry and habitat determination

The extracted otoliths were embedded in epoxy resin (Struers) and ground to expose the core along the anterior-posterior direction in the frontal plane using a grinding machine equipped with a diamond cup-wheel (Struers). They were further polished with OP-S liquid (Struers), cleaned with distilled water and ethanol, and dried in an evaporator prior to examination. For electron microscopy analysis, otoliths were coated with Pt-Pd by a high vacuum evaporator. Life history transect analysis for Sr and Ca concentrations in

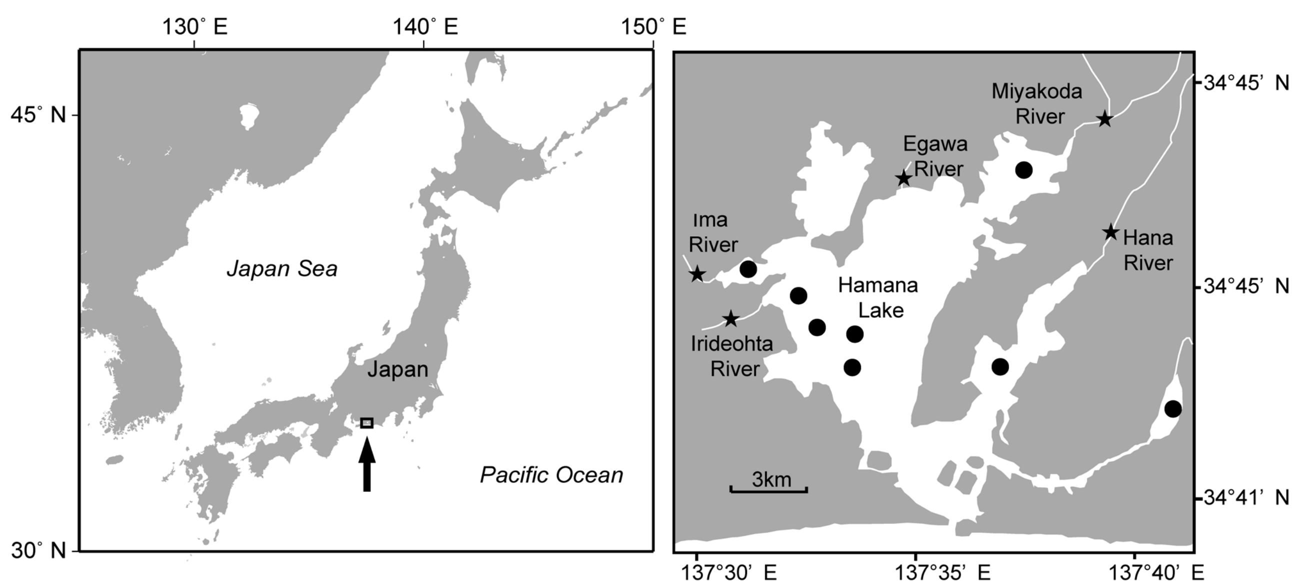

Fig. 1. Map of the sampling area in Hamana Lake system, Shizuoka Prefecture, Japan. Lake sampling sites (●), and river sampling sites (★) are shown.

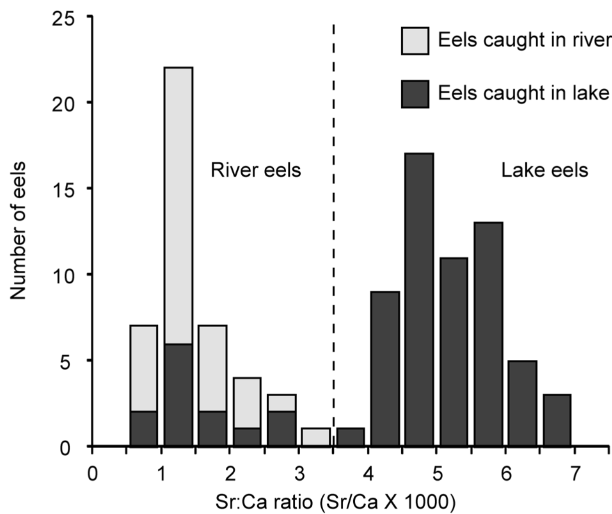

Fig. 2. Frequency distribution of the mean values of Sr : Ca ratio outside the elver mark in the otolith of the Japanese eels examined in this study.

all specimens was carried out from the core to the edge using a wavelength-dispersive X-ray electron microprobe (JXA-8900R; JEOL), as described previously (Tsukamoto and Arai 2001). $CaCO_3$ and $SrTiO_3$ were used as standards. The acceleration voltage and beam current were 15 kV and 12 nA, respectively. The electron beam was focused on a point 10 mm in diameter, with measurements spaced at 10 μm intervals.

To determine the habitat use of the eels, we calculated the average Sr : Ca ratios for the values outside the elver mark. As the frequency distribution of the mean values of the Sr : Ca ratio showed a bimodal distribution (Fig. 2), they were grouped into two categories based on the mean otolith Sr : Ca ratio: "river eels"(Sr : Ca$<3.5\times10^{-3}$), which inhabited a freshwater river after upstream migration; and "lake eel" (Sr : Ca$\geq3.5\times10^{-3}$) which stayed in brackish Hamana Lake. River eels caught in the brackish Hamana Lake were separated and defined as "migrants" in this study, because it was apparent that they had recently migrated down from a freshwater river to the brackish lake.

Measurement of thyroid hormones

T3 and T4 levels in fish were determined by enzyme immunoassay kit (KAPDB4220 for T3, KAPDB4240 for T4, DIA Source). Aliquots of 20 μL of the plasma were directly applied into each well of the assay system according to manufacturer's instruction. A microwell plate reader (MPR A4iII; Tosoh) was used for measurement of optical density. The minimum detectable levels of T3 and T4 were 0.2 and 10 ng/ml, respectively.

Quantitative real-time PCR for TSHb

Total RNA was isolated from each individual pituitary using a Quick Gene RNA Tissue Kit S II (Fujifilm) according to the manufacturer's protocol. The extracted total RNA was treated with deoxyribonuclease I (RNase-Free DNase set; Qiagen). Total RNA concentrations were measured by a spectrophotometry (Gene Spec III; Hitachi). First-strand cDNA was synthesized from 500 ng of total RNA from an individual pituitary with Random 6mer primer and Oligo dT primer using PrimeScript RT reagent kits (Takara), according to the manufacturer's protocol.

Table 1. Sequences of the primers used in real-time PCR.

Gene	Primer	Sequence
TSHβ	TSHβ fw	5'-TGA ACG AAG CAG GCA TCC A -3'
	TSHβ rv	5'- GGA GAG AAC CTG TCC TGC TAA CA -3'
ARP	ARP fw	5'- GTG CCA GCT CAG AAC ACT G -3'
	ARP rv	5'- ACA TCG CTC AAG ACT TCA ATG G -3'

The assay for TSHβ and acidic ribosomal phosphoprotein P0 (ARP) mRNA expression was set up using a 7300 Real-Time PCR System (Applied Biosystems). Target gene mRNA expression was quantified by measuring the threshold cycle value. This threshold cycle occurred at the most linear portion of the logarithmic phase of the PCR curves allowed determination of the standard concentration. Serial dilutions of plasmid DNA were made to produce standard curves that ranged from 10^2 to 10^7 copies, and the correlation coefficients were >0.99 for all curves. Quantitative real-time PCR was performed in 20 μL of reaction mixtures containing 1/50 aliquot of first-strand cDNA solution synthesized from 500 ng of total RNA. Primers were chosen with the assistance of the computer program Primer Express Software v2.0 (Applied Biosystems). Table 1 shows the oligonucleotide sequences of each primer (Table 1). SYBR Premix ExTaqII (Takara) was used according to the manufacturer's protocol with a primer concentration of 0.4 μM. The amplification procedure consisted of 95°C for 10 s followed by 40 cycles of 95°C for 5 s and 60°C for 31 s, and finally 95°C for 15 s and 60°C for 1 min as a dissociation reaction to confirm the amplification specificity. Each sample was analyzed in duplicate PCR amplifications. The final output was expressed as the copy number of the target mRNA per ARP mRNA.

Statistical Analysis

Hormone levels and mRNA expression levels are presented as the means±standard error of the mean (SEM). Differences among the mean values were examined by Mann-Whitney *U*-test or Kruskal-Wallis test followed by Steel-Dwass multiple comparison tests. Statistical analyses were performed using Excel Stat 2008 software (SSRI). In all analyses, $P<0.05$ was taken to indicate statistical significance.

Results

Body size and GSI of eels

Eel Specimens collected in this study were all female.

Table 2. Morphometric parameters (mean±SD) and silvering index of the Japanese eels used in this study.

River eel							
Season	Pre-migratory season	Migratory season					
Month	September	October			November		December
Silvering stage	Yellow	Yellow	Silver		Yellow	Silver	Yellow
Migratory	-	-	-	Migrant	-	Migrant	-
n	10	8	4	8	5	5	4
TL (cm)	67.1 ± 5.7	60.8 ± 5.2	73.6 ± 8.7	78.3 ± 6.0	58.3 ± 3.6	75.3 ± 2.9	54.1 ± 5.3
BW (g)	500 ± 153	319 ± 85	791 ± 328	801 ± 199	276 ± 69	686 ± 63	214 ± 104
EI	1.61 ± 0.18	4.67 ± 0.62	6.04 ± 0.34	7.39 ± 0.71	5.02 ± 0.18	6.96 ± 1.02	4.39 ± 0.45
GSI	0.99 ± 0.47	0.82 ± 0.59	1.46 ± 0.65	2.36 ± 0.56	0.67 ± 0.15	2.74 ± 0.58	0.81 ± 0.12

Lake eel								
Season	Pre-migratory season		Migratory season					
Month	July	September	October		November		December	
Silvering stage	Yellow	Yellow	Yellow	Silver	Yellow	Silver	Yellow	Silver
n	17	9	11	7	4	5	1	5
TL	66.9 ± 5.9	65.0 ± 2.9	63.3 ± 7.9	71.7 ± 4.5	55.3 ± 4.2	66.1 ± 2.6	62.1	67.8 ± 7.8
BW	474 ± 126	458 ± 90	375 ± 138	603 ± 87	239 ± 61	488 ± 57	344	526 ± 172
EI	4.83 ± 0.77	4.81 ± 0.61	4.60 ± 0.94	6.23 ± 0.55	4.41 ± 0.61	6.55 ± 0.74	6.31	7.07 ± 0.53
GSI	0.77 ± 0.18	0.75 ± 0.21	0.64 ± 0.23	1.59 ± 0.31	0.63 ± 0.08	1.66 ± 0.56	0.77	3.29 ± 0.95

Table 3. Comparison of thyroid hormone and TSH*β* expression between lake eels, river eels, and migrants.

Season	Pre-migratory season	Migratory season	
Silvering stage	Yellow eel	Yellow eel	Silver eel
Comparison type	River vs Lake	River vs Lake	River vs Lake vs Migrant
T4	ns (p=0.09)	River> Lake (p<0.01, 1.5 times higher)	ns (p=0.66)
T3	ns (p=0.09)	ns (p=0.39)	ns (p=0.20)
TSHβ	ns (p=0.54)	ns (p=0.10)	Lake > Migrant (p<0.05, 1.4 times higher)

ns: not siginificant

The morphometric parameters of eels used in this study are summarized in Table 2. The total length of river eels ranged from 51.1 to 85.6 cm with a mean±SD of 67.3±9.7 and body mass was 138–1221 g with a mean of 518±263. The total length and body mass of lake eels ranged from 46.1 to 81.5 cm with a mean±SD of 65.6±6.6 and 129 to 832 g with a mean of 456±141, respectively. The mean GSI were higher in silver eels than in yellow eels in both river and lake eels.

Thyroid hormones and TSH*β* mRNA expressions of eels

The results of comparisons between river and lake eels are summarized in Table 3. During the pre-migratory season, there were no significant differences in all three hormone levels between lake eels and river eels. In the migratory season, T4 levels of yellow eels were nearly 1.5 times higher in river eels than in lake eels, and TSH*β* mRNA levels of silver eels were 1.4 times higher in lake eels than migrants.

In river eels, T4 levels were significantly higher in migratory season than in pre-migratory season (Fig. 3). In lake eels, there were no significant differences in T4 levels between pre-migratory and migratory seasons. In both river and lake eels, there were no significant seasonal differences in T3 or TSH*β* expression levels.

In river eels, T4 levels were significantly higher in yellow eels than in migrants and silver eels, whereas there was no significant difference between yellow and silver eels in lake eels (Fig. 3). For T3 levels in both river eels and lake eels, no significant differences were observed between yellow and silver eels. TSH*β* mRNA levels were significantly higher in silver eels than yellow eels for lake eels, while there was no significant difference between each type of river eels.

Discussion

In the present study, we determined the changes in plasma thyroid hormones levels and pituitary TSH*β* mRNA expression levels: associated with habitats, seasonality, and silvering. There were no consistent difference in the thyroid hormone or TSH*β* expression levels between different habitats and seasons. In the pre-migratory season, there were no significant differences in all three hormones between brackish water lake eel and freshwater river eels (Table 3). There were also no significant seasonal changes in thyroid hormone and TSH*β* mRNA expression except for T4 in river eels. These results suggested that thyroid hormones were relatively stable for habitat transition and seasonality, except for T4 levels in river yellow eels.

There were no differences in T4 or T3 during silvering in lake eels or in T3 in river (Fig. 3). TSH*β* mRNA levels increased during silvering in lake eels, while there was no significant difference in TSH*β* mRNA expression between yellow and silver river eels. The profiles of TSH*β* expression determined in this study were consistent with previous studies on the Japanese eel (Han et al. 2004) and the European eel (Aroua et al. 2005). Aroua et al. (2005) also reported that 3-month treatment of yellow eels with T3 did not induce any silvering-related changes such as enlargement of eye size and

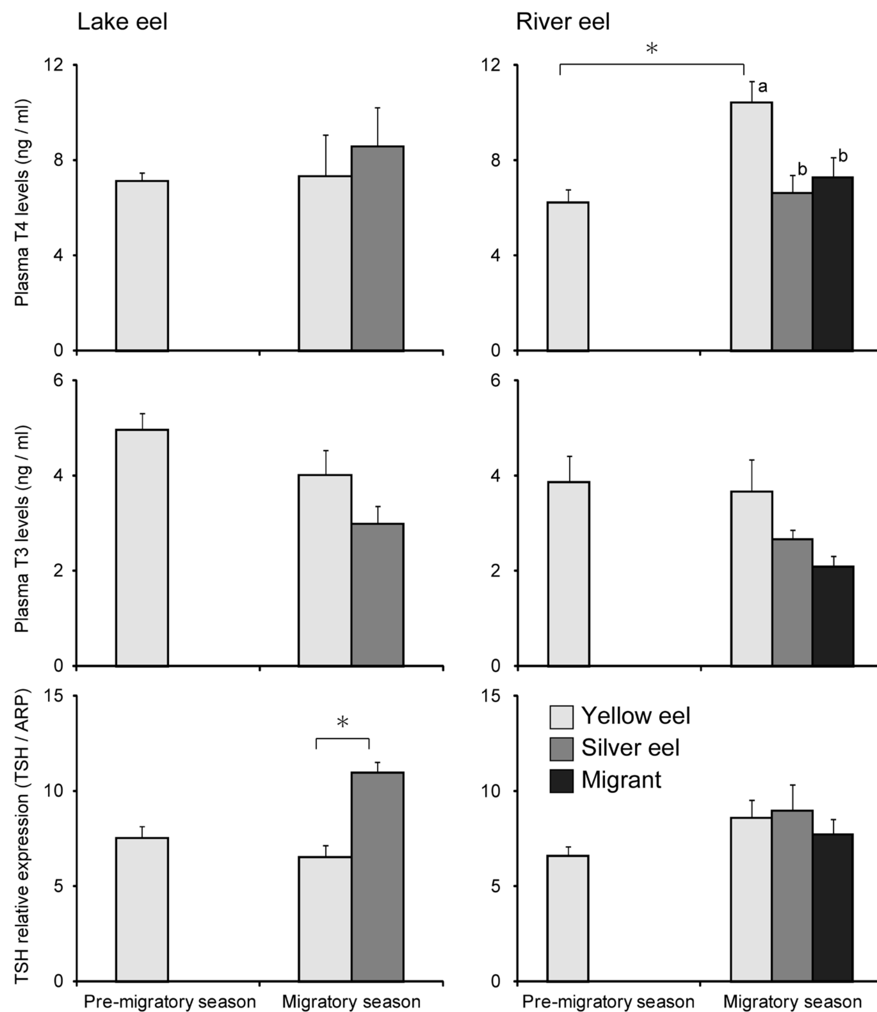

Fig. 3. Changes in thyroid hormone and TSHβ mRNA expression levels for each silvering stage of Japanese eels from both the river and lake. White, gray, and black bars indicate yellow eels, silver eels, and migrants, respectively. Data are presented as means±SE. Different letters and asterisks above the histograms indicate statistically significant differences ($P<0.05$).

regression of the digestive tract. These finding imply that thyrotropic axis may not be related to silvering of eels. For river eels, yellow eels exhibited higher T4 levels than silver eels and migrants. River yellow eels caught in the migratory season also had higher T4 levels than those in the pre-migratory season. T4 is known to have various functions, such as regulation of metabolic rate (Lanni et al. 2001, Leary et al. 1996), salinity tolerance (Schreiber and Specker 1999), olfactory cellular proliferation(Plate et al. 2002), and sexual maturation (Tagawa et al. 1994), other than metamorphosis or silvering. Therefore, it is difficult to hypothesize a reason for the higher T4 level of river yellow eel caught in the migratory season. To clarify this, more precise experiments related to T4 are needed.

To determine the roles of thyroid hormones on downstream migrations of eels, it is necessary to determine which individuals have begun to migrate and which have not. In this study, we used otolith microchemistry to detect downstream migrants. Otolith microchemistry revealed that the eels with low Sr : Ca ratios ($Sr:Ca<3.5\times10^3$) in their otoliths appeared in the brackish Hamana Lake from October to November, were defined as migrants. In general, there are two patterns of eel movement from freshwater river to estuaries, which include habitat shifts from freshwater to brackish water, and downstream migration at the onset of spawning migration (Yokouchi et al. 2012). In female Japanese eel, habitat shifts have been found to occur before age 5+, with most individuals at age 3+, when their body size is below 60 cm (Tzeng et al. 2002, Daverat et al. 2006). In the present study, the body size of migrants was above 60 cm. Therefore, it is apparent that the movement of all migrants was not a habitat shift, but rather a downstream migration.

Thyroid hormones are known to regulate endocrine control of downstream migratory behavior in salmon. For example, thyroid hormones play a central role in the regulation of smoltification that transforms river-dwelling parrs into migratory smolts that are adapted to the marine environment (McCormick et al. 1998). During smoltification, the T4 surge

is associated with a suite of changes that occur during downstream migration, including the acquisition of negative rheotaxis (Specker et al. 2000). However, there were no or weak significant differences in plasma T4 and T3 levels between migrant and other eels in this study. This may be related to the very different life history stages of eels and salmon, and the differences in spawning motivation between eels, which migrate for spawning, and salmon, which migrate for feeding and growth. Thus, it is hypothesized that thyroid hormones play an important role in fish migration at the juvenile stage, the purpose of which is mainly for growth. In the case of the upstream migration of juvenile ayu (Tsukamoto et al. 1988), fish with a high level of plasma T4 swam upstream (migrant) in an experimental stream, while fish with a low level of T4 swam downstream or remained in the stream where they were released (non-migrant). Correlations between migratory behavior and plasma thyroid hormones levels were also observed in the Atlantic charr, *Salvelinus alpines* (Hogasen and Prunet 1997) and the Atlantic cod, *Gadus morhua* (Comeau et al. 2000, 2001). In addition, Edeline et al. (2005) also reported that T4 treatment significantly increased both upstream and downstream movements of glass eels, suggesting that migration of glass eels may be promoted by increasing thyroid status, irrespective of the current direction. In elvers, upstream climbers, which climbed in an experimental tank, had higher T4 and T3 levels compared with inactive elvers (Imbert et al. 2008). These fishes, including eels, migrate for feeding and growth mainly at the juvenile stage.

The migrants in this study also did not exhibit higher TSHβ mRNA level than other fish group. This result suggested that the thyrotropic axis may not be activated during downstream migration, which is at the onset of spawning migration in eels. It is still unclear which hormones regulate downstream migration of anguillid eels. However, it has recently been reported that androgens may be related to downstream migration of eels. For example, 11-KT level was markedly increased in the Japanese eel during downstream migration (Sudo et al. 2011a), and a gradual water temperature decrease, simulating the temperature conditions during autumn migratory season, induced elevation of 11-KT (Sudo et al. 2011b). In addition, androgen administration appeared to induce silvering-related changes, such as oocyte-growth, enlargement of the eyes, degeneration of the digestive tract and development of the swim bladder (Rohr et al. 2001, Aroua et al. 2005, Sudo et al. 2012), while thyroid hormone treatment did not induce such changes (Aroua et al. 2005). These results suggest that the key hormones for eel downstream migration were not thyroid hormones but androgens, although thyroid hormones may have some effects on silvering (Han et al. 2004).

In conclusion, we examined the roles of thyrotropic hormones in downstream migration of female Japanese eels, by determining habitat use and migratory history of each eel from otolith microchemistry. No or moderate variations in plasma thyroid hormones levels and pituitary mRNA expression levels were found during downstream migration. Therefore, we suggest that thyroid hormones may have a minor role in the onset of spawning migration in eels. Behavioral experiments with hormone treatments are needed to confirm whether thyroid hormones motivate the downstream migration of eels.

Acknowledgements

We thank to M Oya for assistance and encouragement with many aspects of the study. This work was supported in part by Grants-in-Aid for Scientific Research (no. 21-228005) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The first author was supported by Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists (No. 20-4652).

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