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# Dynamics of Reproductive Hormones during Downstream Migration in Females of the Japanese Eel, *Anguilla japonica*

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The profiles of sex steroids (estradiol-17 $\beta$ , testosterone and 11-ketotestosterone) and the mRNA levels of gonadotropins (luteinizing hormone and follicle-stimulating hormone) were investigated before and after downstream migration in females of the Japanese eel species *Anguilla japonica*, which were collected in the brackish Hamana Lake and its inlet freshwater rivers. Eels were separated into three groups using otolith microchemistry: 'migrants' that grew in the inlet rivers and then made a downstream migration to Hamana Lake mainly in October and November; 'non-migrant' yellow eels caught in rivers during the same season; and 'residents,' which were yellow eels caught in rivers in August. Sex steroid levels, especially those of testosterone and 11-ketotestosterone, were higher in migrants than in non-migrants and residents. Real-time quantitative PCR analysis indicated that mRNA levels of luteinizing hormone (LH)  $\beta$ -subunits were significantly higher in migrants than in other groups, whereas those of follicle-stimulating hormone  $\beta$ -subunits did not show significant changes during downstream migration. The high levels of these hormones during downstream migration raise the question about if they also play a role in motivating the migratory behavior of eels.

**Key words:** silver eel, downstream migration, otolith microchemistry, androgen, gonadotropins

## INTRODUCTION

The Japanese eel, *Anguilla japonica*, is a catadromous species that spawns in the seamount area of the southern part of the West Mariana Ridge in the western North Pacific (Tsukamoto, 2006). Its larvae are transported to East Asia (Tsukamoto, 1992) where the juveniles grow in continental waters. After a 5–10 year growth period in fresh-water habitats (Tzeng et al., 2000), the eels metamorphose from yellow eels during the growth phase to silver eels in the migratory phase, and begin their downstream migration towards the ocean for spawning in autumn to early winter (Aoyama and Miller, 2003). They then drastically switch their resident life in continental waters to a migratory life in the ocean. Many studies have suggested that downstream migration of eels is triggered by various environmental factors, such as lunar cycle (Lowe, 1952), river flow (Haro, 2003), or atmospheric depression (Okamura et al., 2002). Although there is information on environmental conditions that cause downstream migration in eels (Haro, 2003; Bruijs and Durif, 2009), the endocrinological mechanisms involved

in eel downstream migration are largely unknown (Han et al., 2003a, b).

Endocrine regulation has been thought to play a major role in motivating downstream migratory behavior in diadromous fishes, including both 'anadromous' and 'catadromous' species (Baggerman, 1960). In anadromous fishes, such as salmonids that grow in the sea and reproduce in fresh water, reproductive hormones have the potential to suppress their downstream migratory behavior. In the juvenile masu salmon (*Oncorhynchus masou*), an anadromous species, testosterone (T) is known to suppress downstream migratory behavior (Munakata et al., 2000). In contrast, in catadromous fishes such as freshwater eels, reproductive hormones may facilitate downstream migration of adult eels, because the downstream migration occurs at the onset of the spawning migration. Profiles of reproductive hormones during the downstream migration are one of the most important types of information in attempting to gain a better understanding of the function of reproductive hormones in the downstream migration of eels.

When studying downstream migratory eels, two different stages of eels, yellow (non-migrants) and silver eels (presumably migrants), have been examined (Lokman et al., 1998; Han et al., 2003a, b; Aroua et al., 2005), as various external and internal morphological changes have been

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observed during the metamorphosis from yellow to silver eels (Aoyama and Miller, 2003; Han et al., 2003c; Durif et al., 2005). These changes are thought to be adaptations for their oceanic spawning migration (Kleckner, 1980; Pankhurst and Lythgoe, 1982; Pankhurst and Lythgoe, 1983; Tsukamoto, 2009). However, not all eels that migrate downstream show the obvious external characteristics of silver eels (Vøllestad et al., 1986; Haro, 2003; Winter et al., 2006). In addition, silver eels that are prevented from migration for some reason may regress back to yellow eels (Durif et al., 2009). Thus, silvering is not a completely accurate measure to determine which eels have actually started their migration, so it is necessary to determine directly which individuals have begun to migrate and which have not. In the case of eels caught in estuarine habitats, otolith microchemistry, which reflects the environmental ambient salinity, would be an effective tool to determine if an eel has migrated out of fresh water (Radtke and Shafer, 1992; Otake et al., 1994; Tsukamoto et al., 1998; Tsukamoto and Arai, 2001; Arai et al., 2004). This is possible because the strontium:calcium (Sr:Ca) ratios in otoliths is positively correlated to ambient salinity (Campana, 1999). For example, if silver eels caught in brackish or sea water areas have low Sr:Ca ratios in the peripheral part of their otoliths, it would be apparent that they have recently migrated downstream from a freshwater river habitat to the sea/brackish water habitat.

In the present study, otolith microchemistry techniques were used to distinguish the female downstream migrants of the Japanese eel, and their reproductive hormone profiles were investigated for the first time during downstream migratory behavior in order to help understand the mechanism underlying initiation of downstream migration of anguillid eels.

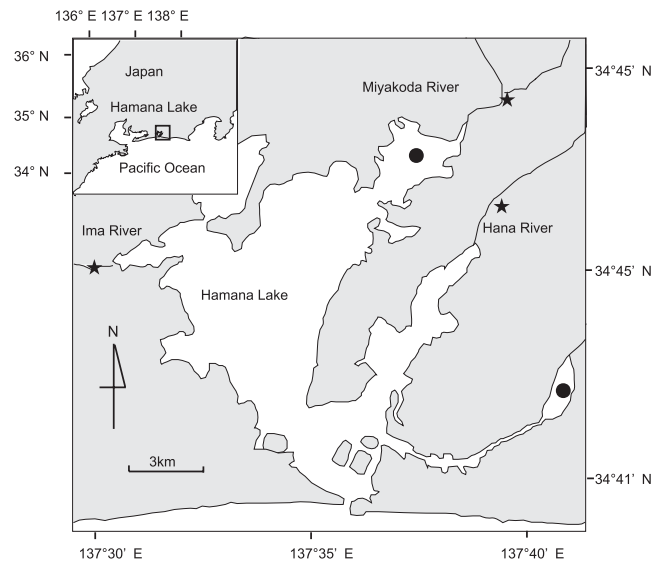
## MATERIALS AND METHODS

### Eels sampling and experimental design

Wild Japanese eels were sampled monthly from May to November by commercial fishermen using fyke nets and eel pots in Hamana Lake, central Japan (Fig. 1; Table 1). Three to 10 eels were randomly selected from the catches of eels in the lake each month. In this study, only female eels were used for analysis, as the nets used in the lake collect relatively few males due to their mesh size; we focused on only on females for which unbiased samples could be obtained. Hamana Lake is a brackish lake with relatively high salinity ranging from 22 to 33 psu depending on tide, and is connected to the Pacific Ocean only by the Imakireguchi Channel (200 m wide and 7 m deep). In the inlet rivers of the lake (Hana River, Ima

River, Miyakoda River), eels were collected by electro-fishing in August and October 2006 (Fig. 1; Table 2).

First, we measured the levels of three sex steroids and the mRNA expression of gonadotropins (GTH; follicle stimulating hormone: FSH, luteinizing hormone: LH)  $\beta$ -subunits to examine differ-



**Fig. 1.** Map of the sampling areas in Hamana Lake and its inlet rivers in Shizuoka Prefecture of Japan. Lake sampling sites (●), and river sampling sites (★) are shown.

**Table 1.** Biometrical parameters (mean  $\pm$  SD) and silvering index of the estuarine eels. TL, total length; BW, body weight; K condition factor; OI, ocular index; GSI, gonadosomatic index; HIS, hepatosomatic index; DSI, digestive tract index.

Month	May	June	July	August	September	October	November		
Silvering stage	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Silver	Yellow	Silver
n	4	5	3	9	9	1	1	1	3
TL	58.7 $\pm$ 2.5	59.8 $\pm$ 3.9	62.7 $\pm$ 1.3	59.3 $\pm$ 4.7	64.9 $\pm$ 2.7	63	66.8	69.3	65.4 $\pm$ 4.9
BW	249 $\pm$ 86	291 $\pm$ 68	296 $\pm$ 43	329 $\pm$ 101	462 $\pm$ 140	418	455	466	427 $\pm$ 74
K	1.21 $\pm$ 0.26	1.34 $\pm$ 0.09	1.20 $\pm$ 0.19	1.54 $\pm$ 0.15	1.64 $\pm$ 0.17	1.67	1.53	1.4	1.53 $\pm$ 0.16
OI	3.80 $\pm$ 0.49	4.17 $\pm$ 0.18	4.40 $\pm$ 0.43	4.64 $\pm$ 0.39	5.04 $\pm$ 0.32	5.43	5.57	4.84	6.51 $\pm$ 1.69
GSI	0.57 $\pm$ 0.15	0.79 $\pm$ 0.20	0.93 $\pm$ 0.30	0.64 $\pm$ 0.17	0.74 $\pm$ 0.18	0.86	1.94	0.67	2.22 $\pm$ 0.48
HIS	1.13 $\pm$ 0.23	1.22 $\pm$ 0.56	0.79 $\pm$ 0.12	0.91 $\pm$ 0.17	1.09 $\pm$ 0.19	1.29	0.97	0.78	1.20 $\pm$ 0.16
DSI	2.58 $\pm$ 0.31	1.91 $\pm$ 0.62	1.09 $\pm$ 0.24	2.33 $\pm$ 0.64	1.78 $\pm$ 0.46	2.7	1.09	1.96	0.84 $\pm$ 0.38
Age	4–6	4–6	5–7	4–8	4–8	5	6	6	4–12

**Table 2.** Biometrical parameters (mean  $\pm$  SD), age range and silvering index of the river eels. TL, total length; BW, body weight; K condition factor; OI, ocular index; GSI, gonadosomatic index; HIS, hepatosomatic index; DSI, digestive tract index.

Migratory type	Resident		Non Migrant		Migrant	
	August	October	October	September	October	November
Silvering stage	Yellow	Silver	Silver	Yellow	Yellow	Silver
n	6	5	5	1	2	6
TL	54.2 $\pm$ 4.5	59.1 $\pm$ 6.4	59.1 $\pm$ 6.4	70.5	65.1 / 60.1	69.7 $\pm$ 7.4
BW	230 $\pm$ 74	331 $\pm$ 162	331 $\pm$ 162	598	508 / 360	597 $\pm$ 212
K	1.41 $\pm$ 0.17	1.52 $\pm$ 0.36	1.52 $\pm$ 0.36	1.71	1.84 / 1.66	1.71 $\pm$ 0.10
OI	4.90 $\pm$ 0.56	4.70 $\pm$ 0.39	4.70 $\pm$ 0.39	4.87	6.71 / 6.94	6.25 $\pm$ 0.43
GSI	0.83 $\pm$ 0.47	0.93 $\pm$ 0.48	0.93 $\pm$ 0.48	0.91	1.74 / 1.94	2.21 $\pm$ 0.37
HIS	1.23 $\pm$ 0.16	1.45 $\pm$ 0.31	1.45 $\pm$ 0.31	1.52	1.53 / 1.30	1.23 $\pm$ 0.26
DSI	3.14 $\pm$ 1.00	3.01 $\pm$ 0.78	3.01 $\pm$ 0.78	1.48	1.52 / 0.88	0.56 $\pm$ 0.19
Age	–	–	–	7	7 / 9	6–9

ences in these parameters among downstream migrants collected in the lake during the migration season (September to November), non-migrants that remained in the rivers (October), and resident yellow eels collected in the river during non-migratory season (August) (Table 2). Second, we examined the seasonal changes in reproductive hormones in the estuarine eels collected in the lake throughout fishing season (May to November), which had not migrated upstream into the inlet rivers, but had stayed in the lake after recruitment into the lake as glass eels (Table 1). Otolith microchemistry was used to determine if eels caught in the lake were downstream migrants (Silver eels that had just entered the lake from freshwater during their migration) or estuarine eels (residents in the lake that were likely migrating silver eels when caught in October and November), so that sex steroids and GTH mRNA expression levels could be compared between migrating and non-migrating eels.

### Morphometric and biological measurements

Eels were classed as yellow or and silver, according to a silvery index (Okamura et al., 2007), anesthetized with 0.08% 2-phenoxyethanol, and then the following external measurements were made on all eels: total length (TL), body weight (BW), horizontal and vertical eye diameter (Dh and Dv). 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 being used for physiological analysis. Eels were sacrificed by decapitation while still anesthetized, and then pituitaries were quickly removed and kept in RNA later solution (Ambion) for molecular biological analysis. After the collection of the sagittal otoliths, the ovaries, liver, and digestive tract were dissected, and then weighed. Condition factor (K), ocular index (OI), gonado-somatic index (GSI), hepato-somatic index (HSI), and digestive tract-somatic index (DSI) were calculated using the following formulae, respectively:

$$K = 100 \times BW / TL^3$$

$$OI = [(Dh + Dv)^2 / 4 \times \pi] / TL$$

$$GSI = \text{gonad weight} / BW \times 100$$

$$HSI = \text{liver weight} / BW \times 100$$

$$DSI = \text{digestive tract weight} / BW \times 100$$

Gonads were fixed in Bouin's solution, dehydrated in an ethanol series and embedded in paraffin. They were then cut in thin sections (thickness, 5 µm), rehydrated, and stained with hematoxylin and eosin. Maturation stages of the oocytes were determined according to Yamamoto et al. (1974).

### Otolith microchemistry and migratory types

The extracted otoliths of the 50 eels caught in Hamana Lake were embedded in epoxy resin (Struers, Epofix) 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, Discoplan-TS). They were further polished with OP-S liquid (Sturiers), cleaned using distilled water and ethanol, and dried in an evaporator prior to examination. For electron microscope analysis, otoliths were Pt-Pd-coated by a high vacuum evaporator. These otoliths were used in 'life history profile' analysis of Sr and Ca concentrations, which were measured in a line along the longest axis of each otolith from the core to the edge using a wavelength-dispersive X-ray electron microprobe (JEOL JXA-8900R), as described previously (Tsukamoto and Arai, 2001). CaSiO<sub>3</sub> and SrTiO<sub>3</sub> were used as standards. The accelerating voltage and beam current were 15 kV and 12 nA, respectively. The electron beam was focused on a point 10 µm in diameter, with measurements spaced at 10 µm intervals.

We calculated the average Sr:Ca ratios for the values outside

the elver mark, and grouped these specimens into the two categories of 'estuarine eels' (Sr:Ca  $\geq 3.0 \times 10^{-3}$ ) and 'river eels' (Sr:Ca  $< 3.0 \times 10^{-3}$ ), based on their mean otolith Sr:Ca ratios to enable statistical comparisons among eels with different habitat use histories.

In order to characterize the biometrical features and the reproductive hormone profiles during downstream migration, river eels caught in the brackish Hamana Lake were defined as 'migrants'. We also divided eels that were caught in the river into two groups depending on the collection seasons: 'residents' collected in August, and 'non-migrants' collected in October during the downstream migratory season. 'Residents' were in their growth phase before the migratory season, while non-migrants were the eels that did not migrate even during the migratory season (October).

### Age determination

Following electron microprobe analysis, otoliths were repolished to remove the coating, etched with 1% HCl, and thereafter stained with 1% toluidine blue. The age of specimens was determined by counting the number of blue-stained transparent zones outside the elver mark, following the method of Nagieć and Bahnsawy (1990).

### Immunoassays of E2, T, and 11-KT

The concentrations of E2, T, and 11-KT were measured in plasma samples by enzyme-linked immunosorbent assay according to the methods of Asahina et al. (1995). The intraassay and interassay coefficients of validation were 4.0% and 4.6% (n = 5), respectively. The sensitivity of this assay was 5.0 pg per well (0.1 ng / ml).

### Real-Time PCR assays of mRNA levels for FSH $\beta$ and LH $\beta$ subunits

Total RNA was isolated from each individual eel pituitary using a Quick Gene RNA Tissue Kit S II (Fujifilm) according to the manufacturer's protocol. The extracted total RNA was then treated with deoxyribonuclease I (RNA-Free DNase set; QIAGEN). Total RNA concentrations were measured using a spectrophotometer (Gene Spec III; Hitachi). First-strand cDNA was synthesized from 500 ng of total RNA of the individual pituitary with random 6 mer primer using ExScript RT reagent kits (Takara) according to the manufacturer's protocol.

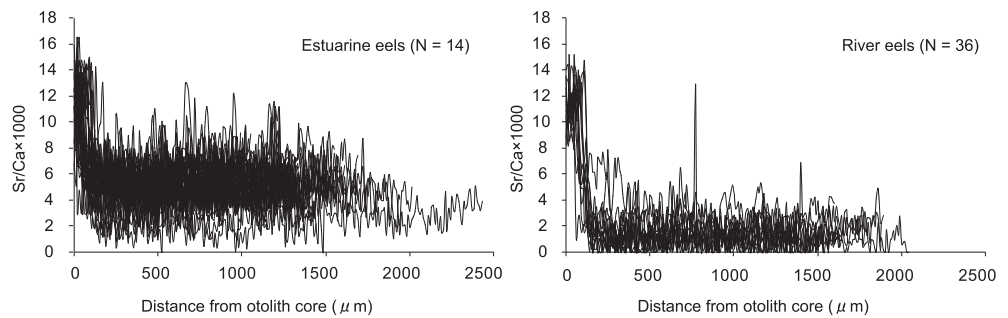
The assays for FSH $\beta$  and LH $\beta$  subunits and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene (Okubo et al., 1999) were set up using a 7300 Real Time PCR System (Applied Biosystems). Real-time PCR was performed in 20 µL of reaction mixture containing cDNA synthesis from 500 ng of total RNA with ABI 7300 (TAKARA SYBR). Table 3 shows the oligonucleotide sequences of the primers FSH $\beta$ , LH $\beta$ , and GAPDH used in the study. 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. Each sample was analyzed in duplicate PCR amplifications. The final output was expressed as the copy number of the target mRNA per that of GAPDH mRNA.

**Table 3.** Sequence and reference numbers for the primers used in Real-Time PCR.

Target	Primer	Sequence(from 5' to 3')	Reference
FSH	FSH fw	TCT CGC CAA CAT CTC CAT CTC	Suetake et al. (2002)
	FSH rv	AGG CGG TGG TGT TGA AGG T	
LH	LH fw	GGT CAC TGC ATC ACC AAG GA	Suetake et al. (2002)
	LH rv	CAC ACG CGC TGG TAC ACC	
GAPDH	GAPDH fw	CGA CTT CAA TGG AGA CAC CC	Okubo et al. (2002)
	GAPDH rv	CAC AAA GTG GTC ATT CAG TGC A	

### Statistical analysis

Each biometrical parameter was presented as the mean  $\pm$  standard deviation (SD). Hormone levels and mRNA expression levels were presented as the mean  $\pm$  standard error (SEM). Comparisons of means were analyzed with Man-Whitney U-tests or Kruskal-Wallis tests followed by Steel-Dwass multiple comparison tests, using Stat-View software and R software, respectively.



**Fig. 2.** Plots of the otolith Sr:Ca ratios along profiles from the core (0  $\mu\text{m}$ ) to the edge of the otoliths of eels (estuarine eels and river eels) collected in Hamana Lake.

## RESULTS

### Occurrence of silver eels

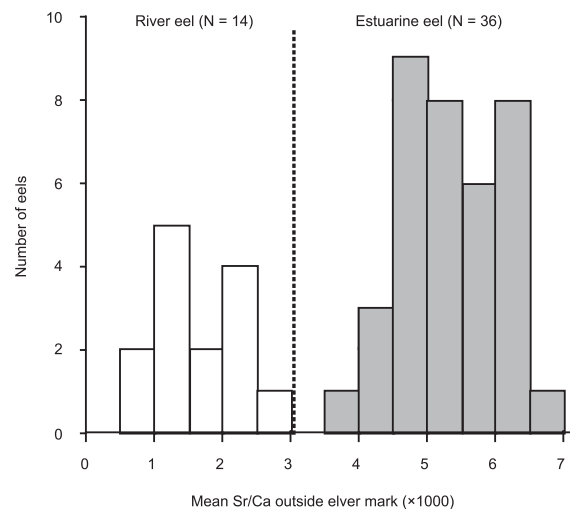
From May to November 2006, the 50 female eels collected in Hamana Lake included 15 silver eels that occurred only in October and November, and another 35 yellow eels that were caught throughout the fishing season (Tables 1, 2). In rivers, six female yellow eels were caught in August and five female yellow eels in October. Eels caught in Hamana Lake included both yellow and silver eels, but those in rivers were all yellow eels (Table 2). The total length of eels caught in Hamana Lake ranged from 532 to 816 mm, with a mean  $\pm$  SD of  $638 \pm 59$  mm, and that of eels caught in rivers ranged from 501 to 656 mm, with a mean of  $564 \pm 57$ . The body weight of eels caught in Hamana Lake ranged from 171 to 966 g, with a mean of  $413 \pm 155$  g, and that of eels caught in rivers ranged from 155 to 597 g, with a mean of  $276 \pm 127$  g.

### Sr:Ca ratio

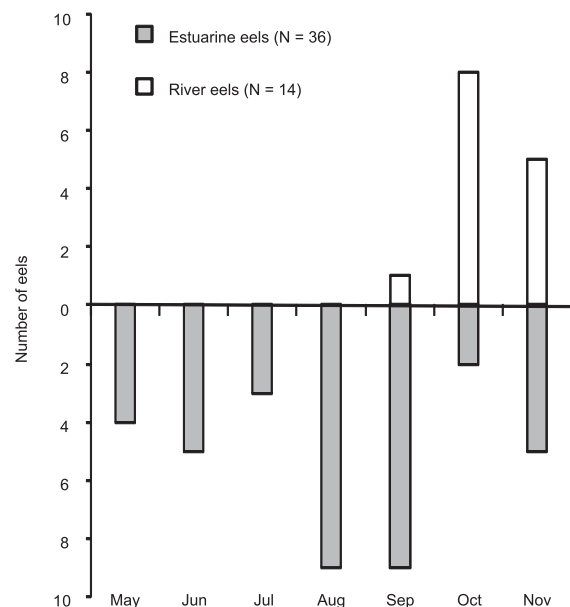
The Sr:Ca ratios of all 50 otoliths of eels collected in Hamana Lake had a peak of high values at the center of the otolith inside the elver mark that correspond to the larval period in the ocean (Fig. 2). Outside the high Sr otolith core, Sr:Ca ratios showed a large variation among different otoliths (Fig. 2) with the mean values ranging from  $0.9$  to  $6.6 \times 10^3$ . Two types of migratory histories were clearly distinguishable (Fig. 3): river eels, which inhabited a freshwater river after upstream migration at the elver stage, and estuarine eels, which stayed in Hamana Lake. Among the 50 eels examined in the otolith Sr:Ca analysis, 72% ( $n = 36$ ) of them were classed as estuarine eels and 28% ( $n = 14$ ) as river eels. The river eels were mainly at the silver stage, and appeared have to exhibited downstream migratory behavior in September to November, which are the months of the downstream migration season of this species in the Hamana Lake drainage system (Yokouchi et al., 2009), while estuarine eels occurred throughout sampling months (Fig. 4). This suggested that river eels moved downward to the lake not for feeding, but only for their spawning migration.

### Biometrical parameters of river eels

Residents ( $n = 6$ ) and non-migrants ( $n = 5$ ) caught in rivers were all yellow eels, whereas migrant river eels caught in the lake comprised both yellow ( $n = 3$ ) and silver eels ( $n = 11$ ) (Table 2). No significant difference was observed in any biometrical parameters between residents and non-migrants (Table 2,  $P > 0.05$ ). The mean GSI was significantly higher



**Fig. 3.** 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.



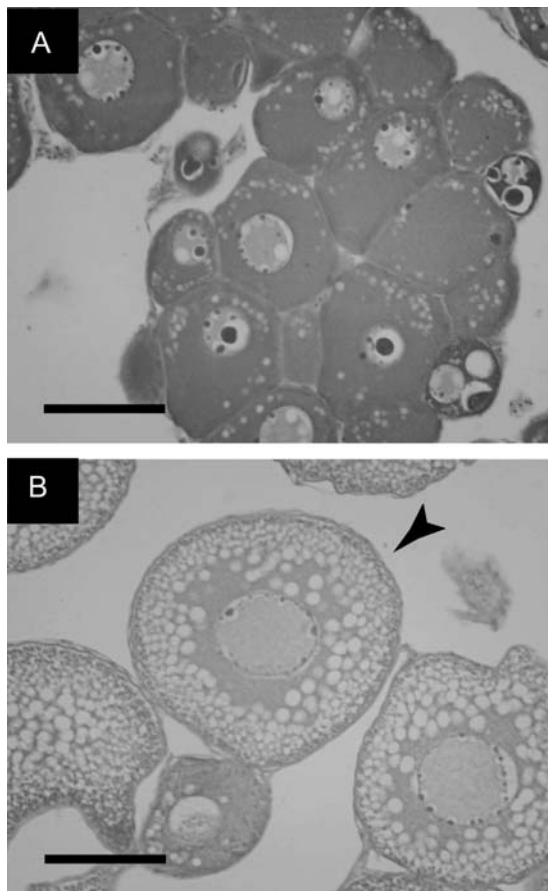
**Fig. 4.** The numbers of estuarine eels and silver eels found among the eels examined from Hamana Lake using their otolith Sr:Ca ratios for each month in this experiment.

in migrants than in non-migrant river eels ( $P < 0.001$ ). The GSI of migrants ranged from 0.91 to 2.77, with a mean  $\pm$  SD of  $2.12 \pm 0.49$ , and that of non-migrants ranged from 0.55 to 1.76, with a mean of  $0.98 \pm 0.48$ . Mean K and OI were also significantly higher in migrants than non-migrants ( $P < 0.05$ ). In contrast to GSI, DSI was significantly lower in migrants than in other eels. The DSI of migrants ranged from 0.31 to 1.52, with a mean of  $0.74 \pm 0.37$ , and that of non-migrants ranged from 1.69 to 3.63, with a mean of  $3.01 \pm 0.78$ . The age of migrant river eels ranged from 6–9, with a mean of  $7.38 \pm 1.12$ . The oocytes of migrants were all in the primary yolk globule stage, while those of non-migrants and residents were in the perinucleolus or early oil drop stages (Fig. 5).

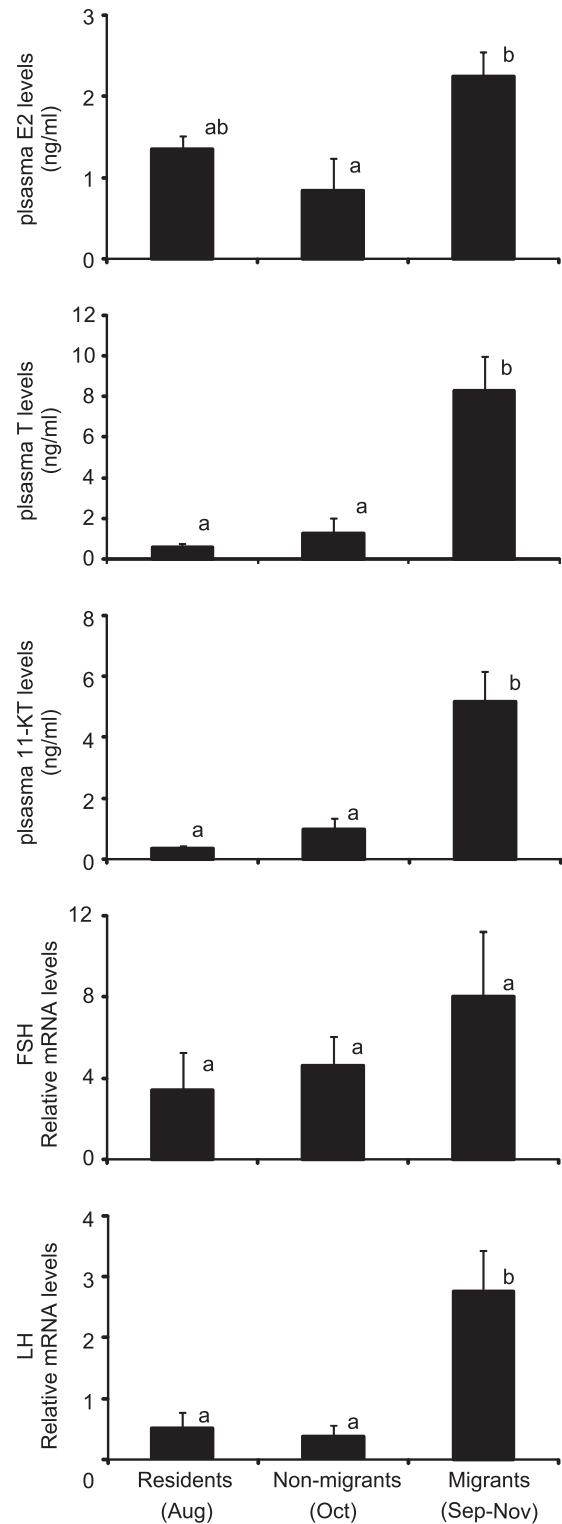
### Reproductive hormone profiles of river eels

Plasma E2 levels were slightly but significantly higher in migrant than non-migratory and resident river eels (Fig. 6,  $P < 0.05$ ). A strong difference in plasma T and 11-KT levels was observed between migrants and non-migrants ( $P < 0.001$ ), while no statistical difference was observed between non-migrants and residents ( $P > 0.05$ ). Plasma T and 11-KT concentrations were approximately 6-fold and 5-fold higher, respectively, in migrants compared to non-migrants. For FSH $\beta$  mRNA levels, no significant difference was observed

between eel groups ( $P > 0.05$ ). In contrast, a significant increase in LH $\beta$  mRNA levels was observed in migrants ( $P < 0.001$ ).



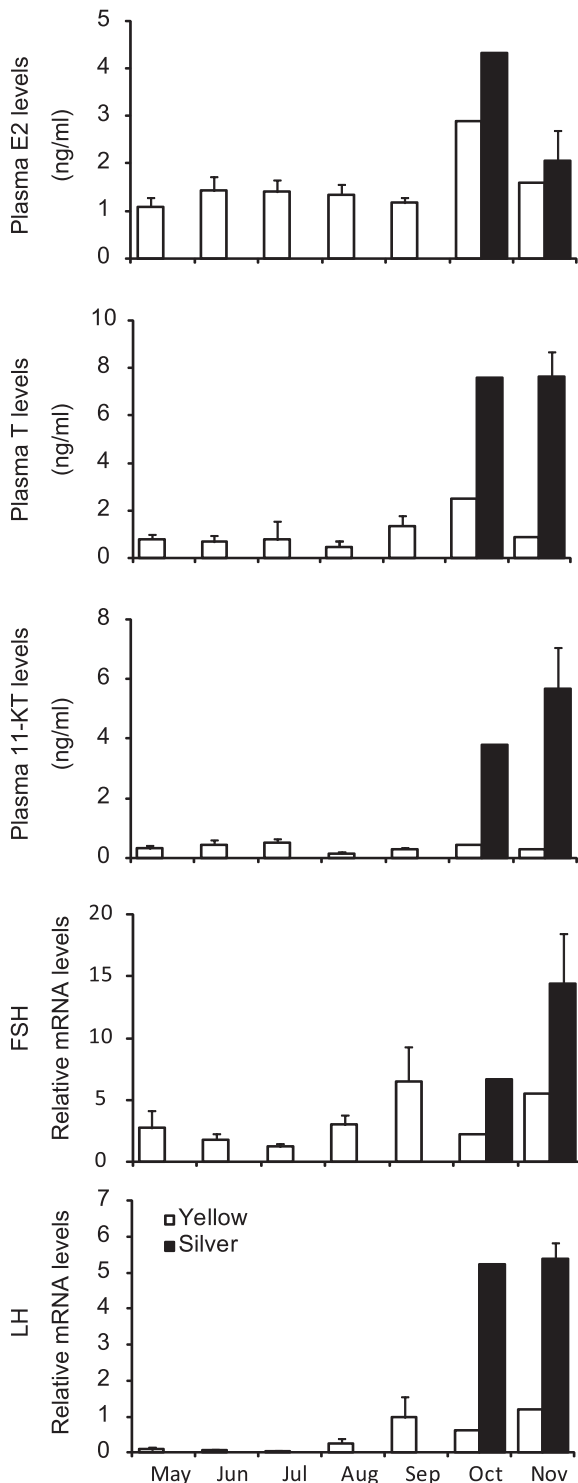
**Fig. 5.** Light micrographs of oocytes in river eels. A: a early oil droplet stage of non-migrant B: a primary yolk globule stage of a migrant. Arrow shows the cortical alveolus. Scale bar: 100  $\mu$ l.



**Fig. 6.** Reproductive hormone levels (mean  $\pm$  SE) of residents, non-migrants and migrants of Japanese eels from Hamana Lake. Statistically significant differences between groups are indicated by different letters ( $P < 0.05$ ).

### Biometrical parameters and reproductive hormones profiles of estuarine eels

The estuarine eels that were collected in Hamana Lake and categorized by otolith Sr:Ca ratio could be separated into two groups from their body color, as being either yellow or silver eels. Yellow estuarine eels were caught during all



**Fig. 7.** Reproductive hormone levels (mean  $\pm$  SE) of estuarine yellow and silver eels collected from May to November 2006.

seasons (Table 1). In contrast, silver eels only appeared in the samples of eels in Hamana Lake during October and November (Table 1). Silver eels had significantly higher GSI values than yellow eels ( $p < 0.01$ ). The GSI of silver eels ranged from 1.91 to 2.44, with a mean  $\pm$  SD of  $2.07 \pm 0.25$ , and that of yellow eels ranged from 0.36 to 1.21, with a mean of  $0.72 \pm 0.20$ . In contrast to GSI, DSI was lower in silver eels than yellow eels ( $P < 0.001$ ). The DSI of silver eels ranged from 0.40 to 1.28, with a mean of  $0.85 \pm 0.40$ , and that of estuarine yellow eels ranged from 0.85 to 2.70, with a mean of  $2.02 \pm 0.64$ . The age of silver eels ranged from 4 to 12 years, with a mean  $6.80 \pm 3.03$ , and that of yellow eels ranged 4 to 8 years, with a mean  $5.52 \pm 1.26$ . The oocytes of silver were in the primary yolk globule stage or oil drop stage, while those of yellow eels were in the perinucleolus or early oil drop stages.

In yellow eels caught in the lake, none of the sex steroids assayed in the present study showed significant changes from May to November (Fig. 7). However, T and 11-KT were clearly higher in silver eels collected in October/November than the yellow eels in during the whole fishing season (May–November) ( $P < 0.001$ ). Plasma E2 levels were also higher in silver eels than yellow eels ( $P < 0.05$ ). Neither FSH $\beta$  nor LH $\beta$  mRNA levels showed significant changes from May to November in yellow eels. In silver eels, however, LH $\beta$  mRNA levels showed remarkably higher values than those of yellow eels ( $P < 0.001$ ), and FSH $\beta$  mRNA levels were also higher ( $P < 0.05$ ). The hormone levels observed in the silver eels that had been inhabiting the lake and never migrated upstream, were similar to those of the migrants that grew in rivers and migrated downstream in to the lake (Figs. 6, 7).

## DISCUSSION

### Measurement of reproductive hormones during downstream migration

In this study, we used otolith microchemistry techniques to estimate the habitat use history of Japanese eels in the Hamana Lake system and so we were able to compare the reproductive hormone profiles between non-migrants and migrant eels during downstream migration. Endocrine control is thought to be important for initiation of migration, with various hormones potentially being involved (Baggerman, 1960). For example, thyroid hormones such as thyroxin and triiodothyronin, are recognized as the factors that trigger the downstream and upstream migration of anadromous and amphidromous fishes (Grau et al., 1981; Tsukamoto et al., 1988; Iwata, 1995; Iwata et al., 2003). Growth hormone, cortisol, and prolactin are also involved in regulation of downstream migration in salmonid fishes (Dickhoff et al., 1997; Nagae et al., 1994; Prunet et al., 1988; Young et al., 1989). In anguillid eels, the eels start downstream migration so that they can reproduce in the ocean. Therefore, it is possible that reproductive hormones could be involved in downstream migratory behavior.

Otolith microchemistry revealed that the eels with low Sr:Ca ratios ( $\text{Sr:Ca} < 3.0 \times 10^{-3}$ ) in their otoliths that appeared in the brackish Hamana Lake from September to November, were “migrants” that had high reproductive hormone levels compared to the non-migrants. In general though, there are two patterns of eel movement from fresh-

water rivers to estuaries, which include habitat shifts from fresh water to brackish water, and downstream migration at the start of their long spawning migration (Daverat et al., 2006). In female Japanese eels, habitat shifts have been found to occur before age class 5+, with most individuals switching at age class 3+, when their body size is below 600 mm (Tzeng et al., 2002; Daverat et al., 2006). In our study, the ages of migrants were more than 5+, and their body size was above 600 mm (Table 2). Therefore, it is apparent that the movement of all the migrants was not a habitat shift, but rather a downstream migration. Furthermore, most migrants had never entered into the brackish Hamana Lake before their downstream migration (Fig. 2). This suggests that these river eels with high reproductive hormones had just finished their downstream migration in the river and were moving through the estuarine habitat of the lake to start their oceanic spawning migration.

### Morphological characters of migrants and non-migrants

In this study, the migrants from freshwater inlets that were collected in the lake consisted of both silver eels and yellow eels, while no silver eels were collected in the freshwater habitats by electro-fishing techniques. It is worth noting that otolith microchemistry clearly showed that at least some downstream migration may have occurred in the yellow stage. This suggests that the prediction of individual downstream migration based solely on body coloration may be difficult in some cases. However most migrants (78.6%) were at the silver stage and only three migrants at the yellow stage were found, so the silvering index primarily reflected the migratory behavior in river eels caught in the lake. As far as we know, this is the first actual confirmation of the relationship between the silvering index and the completion of downstream migration using the otolith microchemistry technique.

Significant differences in both external and internal morphologies were observed between migrants and non-migrants. Migrants had developing gonads, large eye sizes, and degenerated digestive tracts (Table 2). In contrast, yellow eels caught in fresh water rivers (non-migrants and residents) showed immature gonads and thick digestive tracts. Thus, it was suggested that non-migrants that were collected during October were not going to migrate that particular year. External morphological changes, such as silvering and enlargement of the eyes, have been proposed to be a preparation for the adaptation to the marine environment (Pankhurst, 1982; Fontaine et al., 1995). Previous studies have indicated that external morphology and gonadal development were changed concomitantly (Han et al., 2003c; Durif et al., 2005; van Ginneken et al., 2007). This synchronous change of morphology and gonadal development was also observed in most of the migrants in the present study with some exception. Therefore, to start downstream migration, both external morphological changes and gonadal development were important conditions.

### Sex steroid profiles during downstream migration

We revealed that all the sex steroids assayed in the study were significantly higher in migrants than in non-migrants. Especially, T and 11-KT levels were clearly higher in migrants than in other groups of eels. Migrants were clearly more

mature and their morphological characteristics were different from those of non-migrants and residents. These suggest that androgen (T and 11-KT) has the ability to induce ovarian development and silvering synchronously. Indeed, chronic treatment with testosterone alone can stimulate the brain-pituitary-ovary axis of Japanese silver eel to induce ovarian development (Lin et al., 1991). In addition, T induced an increase in eye size and a reduction of the digestive tract in European eels (Aroua et al., 2005). Administration of 11-KT also induced silvering-related changes in immature female *A. australis* (Rohr et al., 2001).

Because these hormones also have the potential ability to influence the behavior of some fishes (Borg, 1994), we speculate that androgens could affect not only silvering and maturation, but also downstream migratory behavior. For example, androgens are involved in sexual behavior teleost fishes (Munakata and Kobayashi, 2010), so they may also be able to influence the behavior of eels, such as by providing motivation to migrate. In European eels, androgen was found to stimulate brain dopaminergic systems, which are thought to be related to reproduction and various behaviors (Weltzien et al., 2006). Although other hormones, such as thyroid hormone in salmonids, are thought to stimulate migratory behaviors (Grau et al., 1981; Hutchison and Iwata, 1998), there are some reports about androgen having an influence on migratory behavior in masu salmon. For instance, precocious males of masu salmon, which had relatively high plasma androgen levels, did not show downstream migratory behavior (Aida et al., 1984; Machidori and Katou, 1984; Kiso, 1995). In addition, androgen administration inhibited downstream migration in Atlantic salmon (Berglund et al., 1994) and masu salmon (Munakata et al., 2000). It is notable that any effect of androgens on downstream migration would be clearly contrasting in the two diadromous fishes with opposite migratory patterns; in catadromous adult Japanese eels, androgens increased during downstream migration, while they inhibited migration in anadromous juvenile masu salmon (Munakata et al., 2000). 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.

### Gonadotropin $\beta$ -subunit expression profiles during downstream migration

In the present study, we also focused on the expression of gonadotropins (LH and FSH)  $\beta$  subunits, because gonadotropins are involved in the control of sex steroids and reproduction and are also related to behavior (Munakata and Kobayashi, 2010). This study found that FSH $\beta$  mRNA expression in migrants whose oocytes had started vitellogenesis, was at a similar level as non-migrants with pre-vitellogenic stage oocytes. It is speculated that FSH would have an effect in both the pre-vitellogenic and vitellogenic stages, although there is no data about FSH profiles during early ovarian development. In an in vitro experiment, recombinant Japanese eel FSH stimulated E2 secretion in a dose-dependent manner, and was involved in vitellogenesis through the stimulation of E2 production in Japanese eels (Kamei et al., 2006). These ovaries were around mid-vitellogenic stage (Kamei et al., 2006).



We also showed that migrants not only exhibited high expression levels of FSH, but also high LH $\beta$  mRNA levels, compared to non-migrants. Histological observation revealed that migrants were at the vitellogenic stage, while non-migrants were in the pre-vitellogenic stage. Recently, it was reported that both FSH and LH receptor mRNA expression significantly increased in experimentally matured eels, which had begun vitellogenesis (Jeng et al., 2007). Therefore, it hypothesized that both FSH and LH are needed to induce ovarian vitellogenesis.

In conclusion, using otolith microchemistry to determine the habitat use and migratory history of each eel, we obtained profiles of reproductive hormones during the last part of the downstream migration of female Japanese eels. The plasma androgen (T and 11-KT) levels and LH $\beta$  mRNA levels were found to be increased significantly both in river eels migrating towards the sea, and in estuarine silver eels that were likely also beginning their spawning migration. Future, behavioral experiments with hormone treatments are needed to reveal whether these hormones motivate the onset of downstream migration of anguillid eels.

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