Original Article

Developmental changes in pituitary–thyroid axis, and formation of gonads in leptocephali and glass eels of Anguilla spp.

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SUMMARY: Pituitary, thyroid gland and gonads in leptocephali of Japanese eel Anguilla japonica (19.8–32.6 mm in total length), A. obscura (45.0 mm), and A. bicolor pacifica (49.5 mm) and those in glass eels of the Japanese eel were histologically and immunohistochemically examined in order to observe the developmental changes of these endocrine organs in the Anguillidae. The pituitary, consisting of adenohypophysis and neurohypophysis in Japanese eel leptocephali over 22.5 mm, did not contain thyroid stimulating hormone (TSH) immunoreactive cells. Such cells were, however, detectable in the more developed pituitaries of leptocephali of A. obscura and A. bicolor pacifica and in those of glass eels. Conversely, thyroxine (T4)-immunoreactive thyroid follicles could be detected in all specimens, both leptocephalic and glass eel. Only in glass eels, gonads were found in the body cavity, and these gonads harbored one or two primordial germ cells (PGC) per cross-section. Our results indicate that thyroid hormones (TH) production started prior to TSH production, and that TSH and TH are both secreted during the metamorphosis from leptocephalus to glass eel. Therefore, it is plausible that the TSH–TH axis is involved in the metamorphosis from leptocephalus to glass eel, but not in the early growth from preleptocephalus to leptocephalus.

KEY WORDS: glass eel, gonads, leptocephalus, metamorphosis, pituitary, thyroid gland, TSH–TH axis.

INTRODUCTION

The Japanese eel Anguilla japonica has a complex life history which, in spite of many years of intensive research, still is only partly known. It is assumed that after completing growth in freshwater habitats, adult Japanese eels migrate thousands of kilometers to breed in the spawning area, west of the Mariana Islands.1 After spawning and hatching of the eggs, preleptocephalus larvae develop into leptocephali, which drift with the current toward the coasts where the leptocephali metamorphose into glass eels.1–5 On entering freshwater, the glass eels pigment and live for 5–12 years as immature yellow eels before migrating back to the sea. Mature eels, fertilized eggs and preleptocephalus larvae, however, have not been captured from the wild, while only parts of the leptocephali’s oceanic life has unveiled. Much remains therefore to be discovered.

Although a very important transition, very little is known about the endocrine control of metamorphosis from the leptocephalus to the glass eel. Amphibian metamorphosis has been studied extensively, and it is clear that thyroid hormones (TH) play a very important stimulatory role in the initiation of this process.6–8 Recently, it has been shown that TH is involved in metamorphosis of some fish also. Thus, like amphibians TH concentrations increase rapidly just prior to the onset of metamorphosis in fish, such as flounder9–11 and conger eel.12 Moreover, metamorphosis of the flounder can be induced with exogenous TH treatment.13–15
Thyroid hormone secretion is induced by thyroid-stimulating hormone (TSH) released from the pituitary. Many peptide hormones are secreted by this gland, but the developmental stage at which the secretory cells of the different hormones appear in the pituitary of fish varies.\textsuperscript{15–30} For example, prolactin (PRL) and growth hormone (GH) cells were detected in the pituitary of the leptocephali of the Japanese eel, as little as 10 mm in total length, by immunohistochemistry.\textsuperscript{29} Prolactin and GH cells were also recognized in the pituitary of other species of Atlantic anguilliform leptocephali, the total length of which varied from 17 to 51 mm.\textsuperscript{30} However, the development of thyrotrophs in leptocephali has not been documented.

Therefore, in order to clarify the changes in the pituitary and thyroid gland during early development, especially during metamorphosis to the glass eel stage, and to determine the involvement of the TSH–TH axis during metamorphosis, we examined these endocrine organs in leptocephali and glass eels of several \textit{Anguilla}...
Development of endocrine organs in eel

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MATERIALS AND METHODS

Leptocephali of Japanese eel (19.8–32.6 mm in total length, TL, n = 10), Anguilla obscura (45.0 mm TL, n = 1) and Anguilla bicolor pacifica (49.5 mm TL, n = 1) were captured with plankton nets in the area west of the Mariana Islands during cruises of the research vessel Hakuhō-Maru (KH94-2, KH95-2, Ocean Research Institute, University of Tokyo) in 1994 and 1995. Glass eels of Japanese eel (56–61 mm TL, n = 10) were obtained from a commercial dealer. After measuring, specimens were fixed in Bouin’s solution, dehydrated through an ethanol series, and embedded in paraffin. Heads were cut sagittally at 5 μm thickness and sections stained immunohistochemically for TSH-β-subunit\(^3\) and thyroxine (T\(_4\)) (Chemicon Int. CA, USA) with the avidin–biotin–peroxidase complex (ABC) (Nichirei Corp., Tokyo, Japan) method. In brief, sections were deparaffinized, hydrated and incubated in 0.1% hydrogen peroxide (H\(_2\)O\(_2\)) containing methanol for 50 min to block endogenous peroxidase activities. Following washing in phosphate-buffered saline (PBS) for 10 min and incubation with normal goat serum for 45 min to reduce non-specific binding, sections were exposed to specific antisera against Japanese eel TSH-β-subunit or T\(_4\) overnight at 4°C. Sections were again washed with PBS, incubated with biotinylated antirabbit IgG for 2 h and treated with avidin–biotin–peroxidase complex for 45 min prior to staining with diaminobenzidine as substrate for 4–5 min. In addition to immunohistochemical staining, some sections were stained with Delafield’s hematoxylin and eosin (HE) or aldehyde fuchsin (AF). Body portions were cut transversely at 5 μm thickness and stained with HE.

For ultrastructural studies of the gonads, glass eels were fixed in a mixture of 1% glutaraldehyde-2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 day at 4°C, and postfixed with 1% osmium tetroxide for 2 h at room temperature. Specimens were dehydrated through a graded acetone series and embedded in Epon 812 epoxy resin (Oken, Tokyo, Japan). Ultrathin sections were cut transversely, double stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (H 7000 Hitachi) at 75 kV.
RESULTS

In Japanese eel leptocephali over 22.5 mm in length, the pituitary contained a recognizable adenohypophysis, while the neurohypophysis was detectable beneath the hypothalamus, growing gradually in size with increasing length. We did not find any structural differences among leptocephali between 22.5 and 32.6 mm TL (Fig. 1a–c). However, the pituitary of *A. obscura* and *A. bicolor pacifica* leptocephali appeared more flattened in shape (Fig. 2a,c), while the pituitary of glass eels extended even further (Fig. 1e), resembling that of the adults. Immunohistochemically, TSH cells could not be detected in *A. japonica* leptocephali (Fig. 1d), but few were perceived in the rostral pars distalis (RPD) of *A. obscura* leptocephalus (Fig. 2b). In *A. bicolor pacifica* leptocephalus TSH immunoreactive cells were more abundant (Fig. 2d), while many were seen in the glass eel stage (Fig. 1f).

In all leptocephali over 19.8 mm TL, thyroid glands, consisting of follicles located in the connective tissue of

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**Fig. 3** Sagittal sections of thyroid follicles of leptocephali and glass eel. (a) Leptocephalus of Japanese eel (19.8 mm) stained with AE. (b) Leptocephalus of Japanese eel (19.8 mm) immunostained with anti-T4. (c) Leptocephalus of *Anguilla obscura* (45.0 mm) stained with HE. (d) Leptocephalus of *A. obscura* (45.0 mm) immunostained with anti-T4. (e) Glass eel of Japanese eel (57 mm) stained with HE. (f) Glass eel of Japanese eel (57 mm) immunostained with anti-T4. Bar, 30 μm.
irregularly shaped nuclei, thought to be primordial steroid-producing cells, were observed. Those primordial steroid-producing cells were in contact with the PGC through desmosome structures (Fig. 5).

DISCUSSION

It has been reported that wild Japanese eel leptocephali of about 60 mm TL are in the early stages of metamorphosis. In this study, we used Japanese eel leptocephali up to 32.6 mm TL; larger specimens could not be acquired. However, we could obtain leptocephali of *A. obscura* (45.0 mm) and *A. bicolor pacifica* (49.5 mm), which were judged to be in a later developmental stage than those of Japanese eel leptocephali, based on, for instance, the structure of the pituitary.

Gonads could not be found in the developing body cavity of any of the leptocephali (Fig. 4a,b). However, in glass eels, a pair of gonads was found attached to both sides of the mesentery by a mesogonadium and wrapped by monolayered somatic cells (Figs 4c,5). The gonads contained one or two primordial germ cells (PGC) per cross-section (Fig. 4d). These PGC were large ovoidal cells, about 10 μm in diameter, with a large, round nucleus 7–8 μm in diameter. In the cytoplasm, mitochondria could be observed. The basal membrane was enveloped by four or five flattened somatic cells with large, irregularly shaped nuclei. Inside the basal membrane, and next to the PGC, cells with large and irregularly shaped nuclei, thought to be primordial steroid-producing cells, were observed. Those primordial steroid-producing cells were in contact with the PGC through desmosome structures (Fig. 5).

Fig. 4 Transverse sections through the body cavity of leptocephali and glass eel, and the gonads of glass eel. (a) and (b) Body cavity of leptocephalus of *Anguilla bicolor pacifica* (49.5 mm) stained with HE [(a) anterior region, (b) posterior region]. (c) Body cavity of glass eel of Japanese eel (59 mm) stained with HE. Arrowheads indicate gonads. (d) Gonad of glass eel of Japanese eel (59 mm) stained with HE. Bar, 30 μm.
Thyroid stimulating hormone cells tend to appear at roughly the same time as GH and PRL cells, while GTH cells were not recognized until the onset of maturation. In the preleptocephalus, however, only GH cells were detectable (Kagawa, unpubl. data), and our present work suggests that TSH cells do not appear until just before metamorphosis from leptocephalus to glass eel, much later than other fish. Therefore, it is deduced that GH plays a major role in the early development and growth from preleptocephalus to leptocephalus.

Thyroid follicles were present in small numbers in all leptocephali specimens more than 19.8 mm TL, while in the glass eel, thyroid follicles were larger and greater in number. Positive T4 immunoreaction was seen in all leptocephali and glass eels.

In the leptocephalus of *A. obscura*, the epithelial cells of thyroid follicles were classified as columnar, indicating high activity, while thyroid glands strongly reacted with anti-T4. Arguably, TH concentrations increase just prior to the onset of metamorphosis from leptocephalus to glass eel, thus playing a role in the induction of this event as already documented in other eels, the pituitary is morphologically similar to that of adults and contained many TSH immunoreactive cells. These observations imply that TSH-producing cells appear in the pituitary just prior to metamorphosis to glass eel.

The pituitary of small Japanese eel leptocephali (~10 mm TL) was reported to be a cell cluster or mass, located beneath the hypothalamus and barely distinguishable as a definitive organ. Immunohistochemically, PRL and GH cells could already be detected at this stage. Younger, preleptocephalic stages of the Japanese eel have not as yet been captured from the wild, but it is possible to produce preleptocephali artificially. In the pituitary of these preleptocephali, GH cells were recognized by immunohistochemistry after 4 days posthatching, while PRL cells could not be detected (Kagawa, unpubl. data). These results indicate that the pituitary had already appeared and started to function in the early preleptocephalus larva period (before 4 days after hatching).

In several freshwater fishes, including salmonids, tilapia and *Oryzias latipes*, GH and PRL cells can be recognized in the pituitary at hatching. In marine fish larvae, the appearance of GH and PRL cells seems more variable, occurring between prior to hatching and the yolk absorption stage. Thyroid stimulating hormone cells tend to appear at roughly the same time as GH and PRL cells, while GTH cells were not recognized until the onset of maturation. In the preleptocephalus, however, only GH cells were detectable (Kagawa, unpubl. data), and our present work suggests that TSH cells do not appear until just before metamorphosis from leptocephalus to glass eel, much later than other fish. Therefore, it is deduced that GH plays a major role in the early development and growth from preleptocephalus to leptocephalus.

Fig. 5  Electron micrograph of a gonad of glass eel of Japanese eel (59 mm). N, nucleus; M, mitochondria; SPC, primordial steroid-producing cell; D, desmosome; Bm, basal membrane (arrowhead). Bar, 3 μm.
fish and in amphibians. It is also speculated that TH production during this period is regulated by TSH, and that the TSH–TH axis is involved in the onset of metamorphosis.

In preleptocephali older than 3 days posthatching, thyroid follicles were detectable and reactive with anti-

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In conclusion, it is clear that in the eel, TH production starts well ahead of TSH production (in the preleptocephalus stage), that TSH production starts just prior to metamorphosis from leptocephalus to glass eel, and that both TSH and TH are secreted during this process. It is suggested that the TSH–TH axis is involved in metamorphosis from leptocephalus to glass eel, but not during early development to the leptocephalus stage.

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