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Morphological changes and otolith growth during metamorphosis of Japanese eel leptocephali in captivity

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Abstract—To know the morphological changes in body shape and otolith growth during metamorphosis of anguillid eel larvae, we observed the process of metamorphosis in artificially reared leptocephali of the Japanese eel *Anguilla japonica* that were reared at four different temperatures, 15, 20, 25 and 30°C for 30 days. After the onset of metamorphosis, total length and relative preanal length decreased following the progress of metamorphosis. Relative body depth showed almost constant values at the early metamorphosis stage, but showed a rapid decrease at the late metamorphosis stage. Survival of the 11 leptocephali of each experimental group held at four different temperatures showed the highest rate of 81.8% at 25°C. Similarly, the number of leptocephali that completed metamorphosis to be glass eels was greatest at 25°C (5 specimens), suggesting that the temperature of 25°C facilitated the metamorphosis and survival of leptocephali. Otoliths drastically grew fast after the initiation of metamorphosis and deposited a mean width of 47.0 μm during the 30 days of the experimental period, while otoliths of non-metamorphosed leptocephali showed only a slight mean increase of 11.2 μm during the same one-month period. These results are the first demonstration of morphological change and otolith deposition during the metamorphosis process in anguillid eels.

Key words: *Anguilla japonica*, leptocephalus, metamorphosis, morphology, otolith increment formation, survival, water temperature

Introduction

Larval metamorphosis is one of the most significant events in the early life history of anguillid eels because it is accompanied by both drastic morphological and ecological changes (Otake 2003). The eel larvae called leptocephali, have a unique body form adapted to pelagic life in the open ocean (Miller 2009), and metamorphose into glass eels with transparent cylindrical form that is adapted to benthic life in coastal, estuarine and freshwater habitats of continental waters during their juvenile and pre-adult stages. However, knowledge about eel metamorphosis is limited. Even for the Japanese eel *Anguilla japonica* that is a relatively well-studied species among anguillid eels in the world, only several larvae have been collected to date (Tsukamoto et al. 2003, Otake et al. 2006). Therefore, the external morphological changes during metamorphosis are not well known.

Otolith daily increments are known to form in many fishes (Campana 2005) and daily periodicity in otolith increments has been confirmed in the Japanese eel at different developmental stages such as preleptocephali (pre-feeding larvae) (Umezawa et al. 1989), small leptocephali (Shinoda et al. 2004) and glass eels (Tsukamoto 1989, Fukuda et al. 2009), but they have not been validated to form in the stage of metamorphosing larvae. Clear differences have been pointed out between the spawning times of the Atlantic eels estimated from larval sampling at sea and from otolith analysis of recruiting glass eels (McCleave et al. 1998, McCleave 2008). Based on this discrepancy, and the smaller size of some American eel *Anguilla rostrata* glass eel otoliths compared to leptocephali of the same species, it was hypothesized that the eel leptocephali might absorb the otolith increments or stop deposition of daily increments during metamorphosis (Cieri and McCleave 2000). Studies on other species of elopomorphs have shown that otolith growth continues during metamorphosis (*e.g.* Chen and Tzeng 2006, Powles et al. 2006). However, no one has validated this hypothesis with experimental evidence using anguillid eels.

Recent rapid progress in aquaculture techniques has enabled us to study leptocephalus biology in laboratories (Tanaka et al. 2003, Kagawa et al. 2005). Using this technology, phototaxis, buoyancy, salinity and temperature tolerances of Japanese eel leptocephali have been studied (*e.g.* Okamura et al. 2007, 2009, Kurokawa et al. 2008, Tsukamoto et al. 2009, Yamada et al. 2009). Thus in this

study, we aimed to examine the morphological change in body shape and the amount of otolith growth during metamorphosis from the leptocephalus to the glass eel stage in artificially reared Japanese eels that were maintained at four different water temperatures in the laboratory for 30 days.

Materials and Methods

Fish and experimental design

Feminized Japanese eels reared from glass eels were artificially matured in the IRAGO Institute, Aichi, Japan, by administrating a saline suspension of chum salmon *Oncorhynchus keta* pituitary (20 mg/kg body weight) once a week following the methods of Yamamoto et al. (1974). The females were paired with males that were matured by human chorionic gonadotropin (HCG) injection. After spawning, fertilized eggs were maintained in seawater with a salinity of 34.5 at 22°C and hatched larvae were kept for 8 days in a 500 L polycarbonate tank until they had a functional mouth. Afterwards, larvae were fed five times per day for eight hours from 9:00 to 17:00 with the slurry-type diet mainly composed of shark egg, soybean peptide and krill extract (Tanaka et al. 2003). Since metamorphosis from leptocephalus to glass eel usually started around 250 days after hatching in this rearing method, we used a total of 44 leptocephali at an age of 257 days after hatching, which appeared to be near their maximum size just before metamorphosis or just after the onset of metamorphosis. They were randomly divided into four groups ($n=11$ for each), and each group was separately contained in bowl-type 5 L aquaria. The water temperature of each aquarium was gradually shifted for 18 hours from a rearing temperature of 22°C to four different water temperatures of 15, 20, 25 and 30°C. After this temperature acclimation, otoliths of all larvae were marked by an otolith marking technique with alizarin complexone (ALC) of 100 ppm/L for six hours following Tsukamoto (1988). Afterwards they were maintained at each fixed temperature for 30 days until the end of the experiments, while being fed with the same diet three times per day from 9:00 to 17:00. The number of survivors in each aquarium was counted every second day and the survival rate (%) was calculated for each aquarium.

Measurements of morphometric characteristics

After ALC immersion, larvae were anesthetized by 150 ppm MS222 (Tricaine Methanesulfonate) and their total length (TL: maximum body length), preanal length (PAL: distance between anus and snout), body depth (BD: maximum body depth) were measured to the nearest 0.1 mm under a dissecting microscope. All larvae in an aquarium could be individually identified based on their developmental stage and small scars or deformities. On every second day after ALC treatment, larvae were transferred individually to a small rectangular water tank without anesthesia and were photographed to observe the sequential changes in TL, PAL and BD during metamorphosis using ImageJ software (National Institute of Health, Bethesda, MD, USA), which was a technique used to reduce the burden of being exposed to anesthesia. At the end of the experiment, all larvae were anesthetized and the same morphological characters were measured again. Total myomeres (TM) were counted and then specimens were preserved in 95% ethanol for otolith analysis.

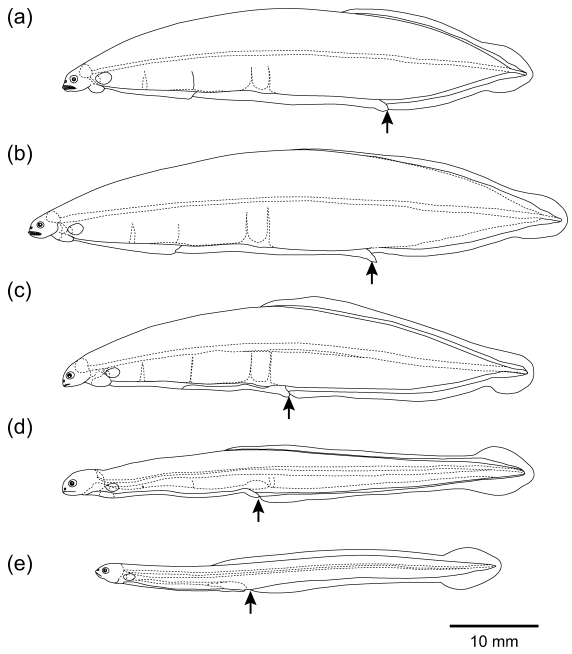

Fig. 1. Early development of the Japanese eel before and after metamorphosis in captivity. (a) leptocephalus stage at maximum size just before metamorphosis, (b) early metamorphosis stage, (c) mid point of metamorphosis, (d) late metamorphosis stage and (e) glass eel stage just after metamorphosis. Arrow in each panel shows position of the anus. The position of anus moves anterior according to the advancement of metamorphosis.

The ontogenetic developmental stages of Japanese eels were classified by the morphological indices of proportion, PAL/TL and BD/TL (Fig. 1). If following Mochioka (2003), larvae with PAL/TL of $>70\%$ would be categorized as the leptocephalus stage before the onset of metamorphosis, with fish (juvenile) with BD/TL of $<10\%$ being at the glass eel stage after the completion of metamorphosis, and larvae with PAL/TL of $\leq 70\%$ and BD/TL of $\geq 10\%$ being at the metamorphosis stage. In the present study, however, since we used artificial larvae reared in the laboratory from the egg stage, we conventionally defined the onset of metamorphosis as the timing of the beginning of decrease in TL. The metamorphosis stage was further divided into early and late metamorphosis stages by the mid point of the PAL/TL=50%. These morphological developmental stages were used for describing the

developmental stages of each individual and in relation to the otolith analyses.

Otolith preparation and observation

Sagittal otoliths were extracted from each individual that survived throughout the experiment. They were embedded in epoxy resin (Epofix, Struers) and mounted on glass slides. These samples were ground to expose the core using a grinding machine equipped with a diamond cup-wheel (Discoplan-TS, Struers). The surface was polished with colloidal silica suspension (OP-S, Struers) on a polishing wheel until the primordium was revealed.

The position of the fluorescent ALC mark was determined under UV light using an optical microscope (Optiphoto-2, Nikon) fitted with a fluorescence attachment (EFD2, Nikon) and the otolith microstructure was photographed using a digital camera (Digital camera DXM1200F, Nikon) (Fig. 2). The width of otolith growth between ALC marks at the start of experiment and the otolith edge at the end of the experiment was measured along the longest radius of the otolith.

Subsequently the otoliths of a total of six glass eels (n=4 at 25°C and n=2 at 30°C), which completed metamorphosis during the 30-day experiment, were vacuum coated with Pt-Pd in an ion-sputter for measurement of the change of Sr and Ca concentrations during metamorphosis by an Electron Probe Micro Analyzer (EPMA, JXA-8900R, JEOL). Strontianite ($SrTiO_3$) and Calcite ($CaCO_3$) 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 of 1 μm diameter, with measurements at 1 μm interval. The correspondence of otolith increments and Sr:Ca ratios was not possible due to the unclear increments in these otoliths of reared fish.

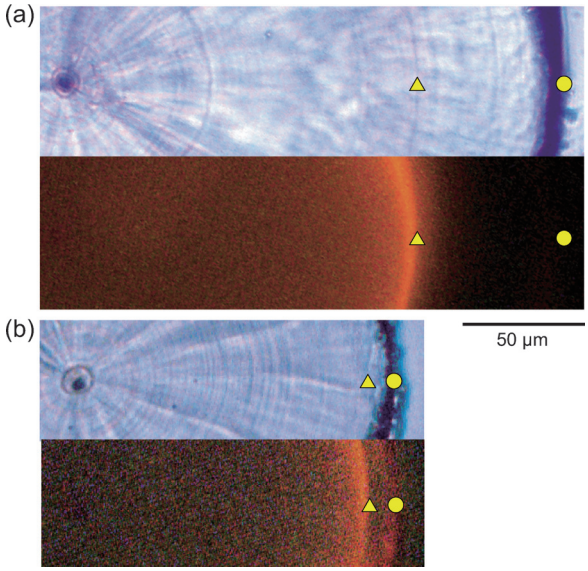

Fig. 2. (a) Otolith microstructure of glass eels of the Japanese eel marked with alizarin complexone (ALC) at the onset of metamorphosis and thereafter reared at 25°C for 30 days. Triangles show ALC marks at the timing of ALC treatment, and dots are the otolith edges. Upper panel shows an ordinary light microscope photograph and lower panel, the same otolith photographed under UV light showing the position of the ALC mark. (b) Otolith microstructure of the specimen at the leptocephalus stage that was also marked with ALC and reared for 30 days under the same condition with the specimen of (a). This specimen did not start to metamorphose during the experimental period.

Statistical analysis

Differences in initial values of TL, PAL/TL and BD/TL of the leptocephali among four temperature treatments (15, 20, 25 and 30°C) were examined by Kruskal-Wallis tests. Differences in survival curves among temperature treatments were tested using a logrank test. To test the effect of metamorphosis on the otolith growth, the otolith growth width deposited at 25°C during the 30 days of the experimental period was compared by Mann-Whitney U-test between groups before and after the start of metamorphosis (n=4 and n=5, respectively). To estimate the effect of water temperature on otolith growth of the leptocephali, differences in the width of otolith material deposited during 30 days were tested by Kruskal-Wallis among 20, 25 and 30°C. The experimental group at 15°C was excluded due to the small sample size of leptocephali (n=2) in this analysis.

Results and Discussion

Morphological changes during metamorphosis

There was no significant difference in initial values of TL, PAL/TL and BD/TL among the larvae in the four temperature groups (P>0.05) (Table 1). All larvae that started metamorphosis stopped feeding, although food was continuously provided every day. In a representative fish that under-

Table 1. Total length (TL), preanal length/total length (PAL/TL), and body depth/total length (BD/TL) of the reared leptocephali used in the study. Measurement was made just after the acclimation by each experimental temperature at the beginning of the 30-day rearing experiment. Top: mean±SD, Bottom: range. *n*: number of specimens examined.

Water temperature	*n*	TL (mm)	PAL/TL (%)	BD/TL (%)
15°C	11	48.2± 6.9	67.8± 9.2	17.9± 2.9
		36.3 – 60.3	44.9 – 74.9	13.1 – 22.8
20°C	11	51.2± 6.1	70.4± 2.4	18.1± 2.1
		38.3 – 62.0	66.1 – 73.4	15.1 – 21.1
25°C	11	50.7± 4.2	64.0±11.5	16.9± 3.3
		45.2 – 56.8	41.8 – 73.5	10.2 – 20.2
30°C	11	49.0± 6.3	62.9±12.0	15.7± 2.7
		36.3 – 59.7	42.1 – 72.7	9.3 – 18.6

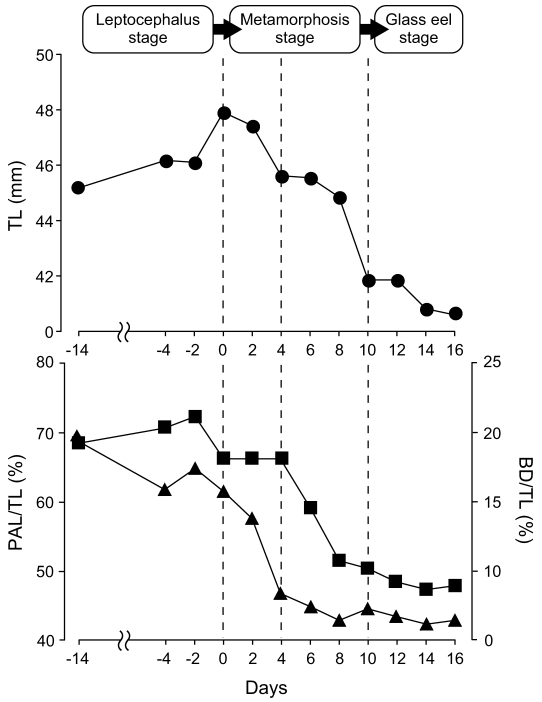

Fig. 3. Typical profiles of morphological change during metamorphosis in Japanese eels reared at 25ºC during the 30-day experiment. Changes in total length (TL, circles), preanal length/total length (PAL/TL, triangles), and body depth/total length (BD/TL, squares) are shown.

went metamorphosis in the experimental group with the 25°C temperature treatment (Fig. 3), the TL started to decrease after the leptocephalus reached its maximum size (48 mm in the specimen of Fig. 3). At the same time, PAL/TL decreased from about 60%, while BD/TL (18%) did not change during the early metamorphosis stage before the PAL/TL became 50%. The BD/TL started to decrease drastically during the late metamorphosis stage when the larval body became round, especially at the head and tail parts. After metamorphosis finished, the mean±SD of PAL/TL and BD/TL became constant at 42.0±5.0% and 8.4±1.2%, respectively, but TL continued to decrease even after completion of metamorphosis. The proportion of morphological characters of the glass eels in this study was not greatly different with those of wild glass eels (Mochioka 2003), suggesting the metamorphosis observed here was normal.

Survival

The survival rates at the end of the experimental period presented a wide range with values of 27.3, 72.7, 81.8 and 72.7% at four different temperatures of 15, 20, 25 and 30°C, respectively (Fig. 4). There was a significant difference in survival curves among the four temperature treatments (P=0.047). The survival rate at 15°C rapidly decreased to 63.6% within the first week, finally resulting in the lowest value at the end of the experiment, suggesting that 15°C would be out of the range for appropriate temperature for the

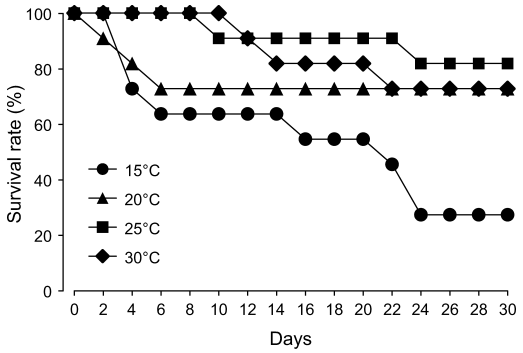

Fig. 4. The cumulative survival rates of the Japanese eel larvae reared at four different temperatures of 15, 20, 25 and 30°C during the 30-day experiment.

healthy development of this species. In fact, the large size leptocephali of the Japanese eel were collected near the sea surface shallower than 50 m during nighttime where the water temperature was higher than 28°C (Kajihara et al. 1988).

Metamorphosis

The number of leptocephali that had already started metamorphosis (early or late meatamorphosis stages) at the beginning of experiment because of acclimation to each experimental temperature was 1, 0, 3 and 3 at 15, 20, 25 and 30°C, respectively (Fig. 5). The number of individuals that progressed into metamorphosis during the experiment were 2, 0, 7 and 3 at 15, 20, 25 and 30°C, respectively. Further, the number of individuals that completed metamorphosis to glass eels during the experimental period of 30 days was 1, 0, 5 and 3 at 15, 20, 25 and 30°C, respectively. These results strongly suggested that 25°C would be the most appropriate temperature for the metamorphosis of eel leptocephali as well as for survival as discussed above. There were three individuals in the present study, one each at 15, 25 and 30°C, that died at the glass eel stage after the completion of metamorphosis.

The duration of metamorphosis from the beginning of the early metamorphosis stage to the end of the late metamorphosis stage was approximately 10 days at 25°C and appeared to be longer at 15°C and shorter at 30°C. The duration of metamorphosis at 25 and 30°C in the present study was shorter than that of wild-caught glass eels in Japan (2–4 weeks) (Kawakami et al. 1999, Shinoda and Tsukamoto 2009). This difference might appear to be caused by lower water temperatures experienced by wild metamorphosing leptocephali. During daytime they likely migrate down to deeper depths below the thermocline in the Kuroshio Current during diel vertical migration (Otake et al. 2006), while the experimental fish that metamorphosed within a short time were reared under constantly higher temperatures of 25 or 30°C.

It is noteworthy that the temperature shift for 18 hours from the rearing temperature at 22°C to each targeted experimental temperature might have triggered the onset of metamorphosis, because some individuals may have already begun to progress into metamorphosis before the start of experiment, especially at 25 and 30°C, although almost no larva was observed to start metamorphosis before the temperature acclimation. There was one individual that had started metamorphosis at 15°C, while no larvae did at 20°C. The rapid and sharp rise in temperature might have a more significant effect to trigger metamorphosis than the decrease in temperature. It is necessary to verify this phenomenon in future studies.

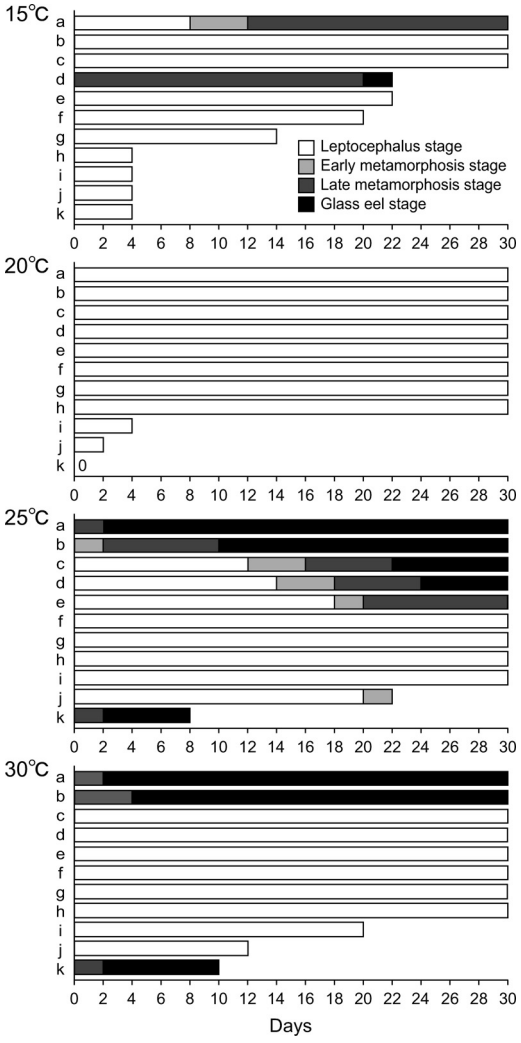

Fig. 5. Individual processes of early development through metamorphosis in Japanese eels reared at four different temperatures, 15, 20, 25 and 30°C during the 30-day experiment. Eleven leptocephali at around maximum size just before metamorphosis were kept in each temperature group at the beginning of the experiment.

Otolith growth

The otolith sizes were different between leptocephali and glass eels despite these specimens being the same age (Fig. 6). The mean±SD of otolith radius and diameter of leptocephali were 129±18 μm (range: 95–162 μm) and 226±35 μm (161–323 μm), respectively, and those of glass eels were 234±35 μm (196–283 μm) and 389±49 μm (329–438 μm). Those of two metamorphosing leptocephali were 162 and 195 μm for radius, and 308 and 333 μm for diameter.

The otolith growth widths of the surviving glass eels including two metamorphosing leptocephali were much larger (mean±SD: 47.0±30.8 μm, range: 17–112 μm) than those of non-metamorphosed leptocephali (11.2±6.2 μm, 3–28 μm) during the 30-day experimental period (Table 2). Even at the same temperature at 25°C, the otolith growth widths of the four surviving glass eels plus one metamorphosing larva were significantly larger than those of non-metamorphosed leptocephali ($P<0.05$), indicating that otoliths drastically grew fast after the initiation of metamorphosis. This is strong evidence that anguillid eels deposit wider otolith increments in the wild even during metamorphosis. This result obtained in the laboratory was also consistent with the observation in wild-caught eel larvae (Otake et al. 1994, Otake 2003, Kuroki et al. 2005).

The otolith growth widths of the leptocephali reared at 15°C seemed smaller than those reared at other higher temperatures (Table 2), although there was no significant difference in the otolith widths deposited during the experimental period by leptocephali reared at 20, 25 and 30°C ($P>0.05$). The apparently reduced amount of otolith deposition suggests that temperature condition could affect otolith growth in the leptocephali as well as in glass eels (Umezawa et al. 1989, Fukuda et al. 2009). Such information about otolith formation obtained from growth-phase leptocephali, metamorphosing larvae and glass eels reared at different temperatures is useful to understand the processes of otolith deposition and for the interpretation of otolith microstructure for ageing.

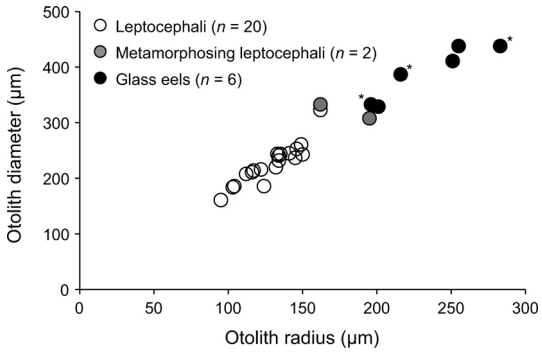

Fig. 6. Relationship between otolith radius and diameter for all Japanese eels (n=28) that survived throughout the 30-day experiment. Three otoliths of glass eels that had irregular shapes (asterisks) are also included.

Table 2. Otolith increment widths deposited by the Japanese eel larvae of various developmental stages that were reared at four different temperatures, 15, 20, 25 and 30°C during the 30-day experiment. Top: mean±SD, Bottom: range, and in case of $n<4$, actual values are shown. *n*: number of specimens.

Water temperature	15°C		20°C		25°C		30°C		Total	
Development during the experiment	*n*	Width (μm)	*n*	Width (μm)	*n*	Width (μm)	*n*	Width (μm)	*n*	Width (μm)
From leptocephalus to larger leptocephalus	2	5, 6	8	10.3±6.9 3–21	4	9.0±2.4 6–11	6	15.7±7.1 9–28	20	11.2±6.2 3–28
From leptocephalus to metamorphosing leptocephalus*	1	17	0	—	1	28	0	—		
From leptocephalus to glass eel	0	—	0	—	2	26**,112**	0	—	8	47.0±30.8 17–112
From metamorphosing leptocephalus* to glass eel	0	—	0	—	2	29,56	2	44**,64		

*including both early and late metamorphosis stage larvae
**irregular shaped otoliths

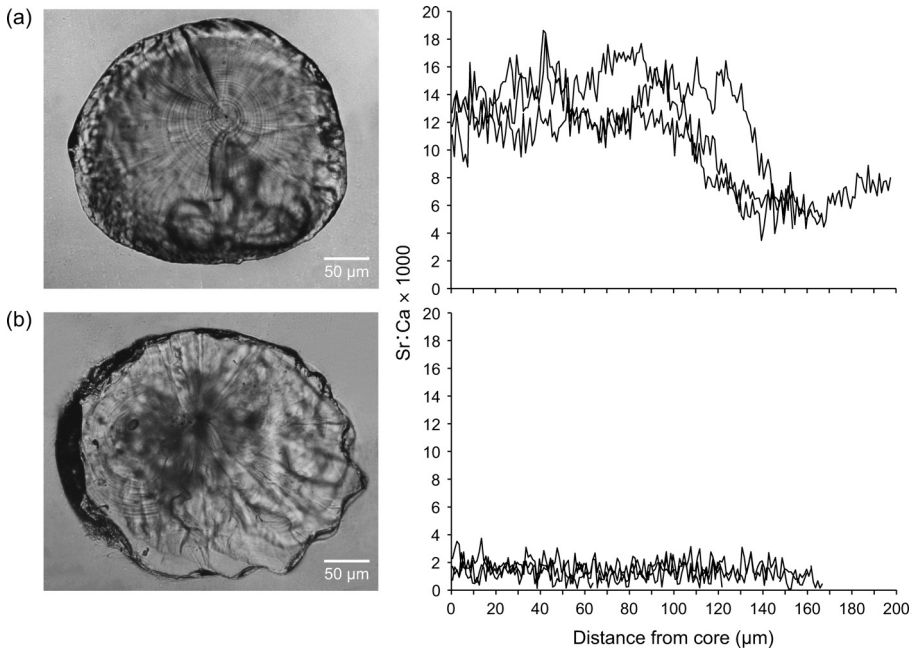

Fig. 7. (a) Normal otolith and (b) irregular shaped otolith under an optical microscope (left) and the profiles of Sr : Ca ratios (right) of from the otolith core to the edge in glass eels that completed metamorphosis during the 30-day experiment.

Another interesting observation of the present study was that among the otoliths of the six glass eels examined, three of them had different shaped otoliths with more transparent and crystalline appearances than the other three otoliths with normal shapes. The Sr : Ca ratios of normal otoliths showed high values before metamorphosis and gradually decreased to the otolith edge in synchrony with the start of metamorphosis (Fig. 7a). This change in Sr : Ca ratio was consistent with those generally observed in anguillid eels (Otake et al. 1994, Kuroki et al. 2005) and other anguilliform fishes (Correia et al. 2003, Ling et al. 2005). On the contrary, the three otoliths with irregular shapes showed lower Sr : Ca ratios throughout the otolith transects (Fig. 7b). The presence of irregular regions in anguillid otoliths was also reported in adult European eels *Anguilla anguilla* (Tzeng et al. 2004). Irregular shaped otoliths were reported more frequently in reared fishes than wild ones (Zhang et al. 1995, Sweeting et al. 2004, Ma et al. 2008), as was the case in the present study. The contrasting Sr : Ca profiles between normal and irregular otoliths suggests that the aragonite in the irregular otoliths was replaced by vaterite as has been reported in other fishes (Gauldie 1986, Tomás and Geffen 2003).

Conclusions

Here we examined the morphological change, survival, metamorphosis and otolith formation during metamorphosis

of the Japanese eel at different temperatures in the laboratory. The most significant finding in the study was that the anguillid eel leptocephali deposited otolith material even during the process of metamorphosis, and the amount of material deposited during metamorphosis was greater than during the leptocephalus stage or glass eel stages. Another finding is that 25°C appears to be an appropriate temperature to facilitate both survival and metamorphosis. This information would give many insights for the improvement of mass production techniques of artificially reared glass eels in aquaculture and can contribute to the conservation of the species whose resources are now rapidly decreasing.

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