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Correspondence between otolith microstructual changes and early life history events in *Anguilla marmorata* leptocephali and glass eels

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Abstract—To determine the exact correspondence between otolith characteristics and early life history events such as metamorphosis, coastal migration and recruitment to estuaries, both otolith microstructure and microchemistry analyses were applied to a sequential developmental series of samples, e.g. leptocephali, a metamorphosing larva, oceanic glass eels, and coastal glass eels. Total length and age were 10.1–50.7 mm and 22–137 d in leptocephali, 46.3 mm and 147 d in a metamorphosing larva, 47.8, 48.6 mm and 159, 160 d in oceanic glass eels, and 47.9–54.8 mm and 119–168 d in coastal glass eels. Checks at hatching and first feeding were observed in all specimens, but metamorphosis and freshwater checks were observed only in some specimens. It was confirmed that the abrupt drop in otolith Sr : Ca ratios and drastic increases of otolith increment widths in the metamorphosing larval stage correspond to the onset of metamorphosis, and the decrease after the peaks suggested the completion of metamorphosis, because the metamorphosing larva had no decrease in incremental widths. The relatively conserved Sr : Ca ratios decreased sharply in synchrony with the increasing increment widths. This study provides the first direct evidence that these drastic changes in otolith microstructure and microchemistry actually occur during metamorphosis, which has been only hypothesized.

Key words: *Anguilla marmorata*, leptocephalus, glass eel, otolith, metamorphosis, daily growth increments, Sr : Ca ratios, check

Introduction

The early life history of the catadromous anguillid eels is remarkable because their larva in the ocean, a transparent leaf-like "leptocephalus", does not resemble the body form of the juvenile and adult eels. During the metamorphosis process, the teeth are lost, the gut moves forward, the body thickens and changes from being laterally compressed to being eel-like round and cylindrical, and amazingly there is a decrease in body length (Otake 2003, Tanaka 2003). The early life history of anguillid leptocephali and glass eels has been the focus of a variety studies in recent years that have been designed to learn about the growth, metamorphosis, and migration of these unusual fish larvae. However, information about these aspects of the biology of leptocephali have been slow to accumulate primarily because they reach larger sizes than typical fish larvae and can avoid most standard size plankton nets. In addition, when they are captured they rarely stay alive due to their fragile body structure (Miller and Tsukamoto 2004). As a result of these factors, otolith microstructure has emerged as a useful technique to reveal details about the early life history of anguillid eels, which are very poorly known for most species worldwide.

There have been a few studies on the otolith microstructure of anguillid leptocephali (Castonguay 1987, Tsukamoto 1989, 1990, Lecomte-Finiger 1994, Arai et al. 2001a, Ishikawa et al. 2001) and a variety of studies on the otolith microstructure and microchemistry of their glass eels that were caught in coastal areas in both temperate (e.g. Tabeta et al. 1987, Tsukamoto 1989, Lecomte-Finiger 1992, Cheng and Tzeng 1996, Marui et al. 2001, Shiao et al. 2002) and tropical regions (e.g. Arai et al. 1999a, 2001b, 2002, Robinet et al. 2003). These studies have provided estimates of the larval growth rates, timing and duration of metamorphosis, and the apparent age of glass eels at recruitment. The otolith increments have been found to be deposited daily in the glass eel or elver stages after they have recruited to coastal areas in temperate anguillid species (Umezawa et al. 1989, Tsukamoto 1989, Martin 1995, Cieri and McCleave 2001) as

well as tropical ones (Arai et al. 2000, Sugeha et al. 2001). However, questions remain about whether there is continuous deposition during some part of the larval period in the temperate anguillid species that may experience extended periods of cold water temperatures (McCleave et al. 1998, Cieri and McCleave 2000).

Compared to collecting glass eels as they recruit to rivers, the earlier stages of anguillid species are more difficult to collect, and so there is much less information available about the oceanic larval stages between hatched leptocephali and glass eels before they reach coastal waters. This is especially true for metamorphosing larva and oceanic glass eels because much of the sampling effort has been focused on the spawning areas where only premetamorphic leptocephali can be collected.

The primary objectives of the present study were to examine the correspondence between developmental stages classified by external body morphology and the otolith microstructure during all stages of the marine early life history of the giant mottled eel, *Anguilla marmorata* (Quay and Gainard). This tropical species has the widest distribution of any of the 18 species or subspecies of anguillid eels worldwide (Ege 1939, Watanabe 2003) and appears to have at least five different populations throughout its range (Ishikawa et al. 2004). It is found from the southeast coast of Africa in the Indian Ocean, eastward through Madagascar and islands such as Reunion, northward through Indonesia and up to southern Japan, and through the tropical western Pacific, including the many Islands of the South Pacific. The only known spawning area is that of the northern population of *A. marmorata*, which spawns in the North Equatorial Current (NEC) region of the western North Pacific (WNP) and these larvae appear to recruit to areas from northern Indonesia to southern Japan (Ishikawa et al. 2004, Aoyama et al. 1999, Miller et al. 2002). In this study we examined the otolith microstructure and microchemistry of all the larval stages of *A. marmorata*, e.g. leptocephalus, metamorphosing larva, oceanic glass eel, and coastal glass eel, which were collected in the vicinity of the NEC spawning area, in the northern Indonesian Seas where some of the leptocephali from the NEC recruit, and coastal glass eels that recruited to the coast of Sulawesi Island, to study their otolith microstructure and microchemistry.

Materials and Methods

Sample collection and examination

Anguillid leptocephali, a metamorphosing larva and oceanic glass eels were collected during a cruise of the R/V Hakuho Maru of the Ocean Research Institute, University of Tokyo, in the WNP, in July 2002 (KH-02-2), and two cruises of the R/V Baruna Jaya VII of the Indonesian Institute of Science, in Indonesian waters around Sulawesi Island during May 2001 and October 2002 (BJ-01-1 and BJ-02-4). They were collected with a 3 m Isaacs Kidd Midwater Trawl (8.7 m^2 mouth opening; 0.5–1.0 mm mesh) fished down to 100–600 m. Coastal glass eels were collected with a dip net at the mouth of the Dumoga River, North Sulawesi Island, in June 1996 (Arai et al. 1999b).

After measuring the specimens to the nearest millimeter total length (TL), the morphological characters of all specimens except for the coastal glass eels were examined and they were identified the using their mtDNA (unpublished). The coastal glass eels were identified using their external morphology and sectional vertebral counts according to Ege (1939) and Tabeta et al. (1976). Some specimens were photographed using a hand held digital camera (Fig. 1c, d) or using a Nikon DMX1200 dissecting scope and a Nikon SMZ 1500 digital imaging system (Fig. 1a, b) and then several images were combined to create a whole body image.

Otolith preparation

The sagittal otoliths, the largest of 3 pairs of otoliths in the inner ear, were extracted from 49 specimens, embedded in epoxy resin (Epofix, Struers) and mounted on glass slides. The otoliths were ground to near the core using a grinding machine equipped with a diamond cup-wheel with 70 μm and 13 μm diamond paste (Discoplan-TS, Struers), and further polished to expose the core on an automated polishing wheel with 6 μm and 1 μm diamond paste (Planopol-V, Struers). Finally, they were cleaned in an ultrasonic bath and rinsed with deionized water, prior to being examined.

Otolith X-ray microscope analysis

For electron microprobe analysis, fourteen otoliths (5 leptocephali, 1 metamorphosing larva, 2 oceanic glass eels and 6 coastal glass eels) were Pt-Pd coated by high vacuum evaporator. Sr and Ca contents were measured along the longest axis of each otolith using a wavelength dispersive X-ray electron microprobe (JXA-733 or JXA-8900, JEOL). Calcite ($CaCO_3$) and strontianite ($SrTiO_3$) were used as standards. Accelerating voltage and beam current were 15 kv and 7.0 or 1.2 nA, respectively. The electron beam was focused on a point about 1 μm in diameter, with measurements spaced at 1 μm intervals. The averages of Sr and Ca concentrations pooled for every ten successive growth increments were used for the life-history transect analysis.

Otolith increment analysis

Following electron microprobe analysis, the otoliths were repolished to remove the coating, and all otoliths (28 leptocephali, 1 metamorphosing larva, 2 oceanic glass eels and 18 coastal glass eels) were etched with 0.05 M HCl and vacuum coated with Pt–Pd in an ion-sputterer for scanning electron microscope observations (SEM, Hitachi S-4500).

SEM photographs were used for the observation of otolith microstructure of each stage and counting the number of growth increments and measuring their widths. The average of every ten successive rings from the hatch check to the edge were used for otolith growth analysis.

Results

Morphological characters

Anguilla marmorata leptocephali have the typical leaf-like body shape and lack of external pigmentation that is characteristic of anguillid species (Fig. 1a, b), but these features changed drastically during metamorphosis into the glass eel stage (Fig. 1c, d). The metamorphic leptocephalus had no teeth, a thicker head, and the position of the anus and the origins of the dorsal and anal fins had moved anteriorly.

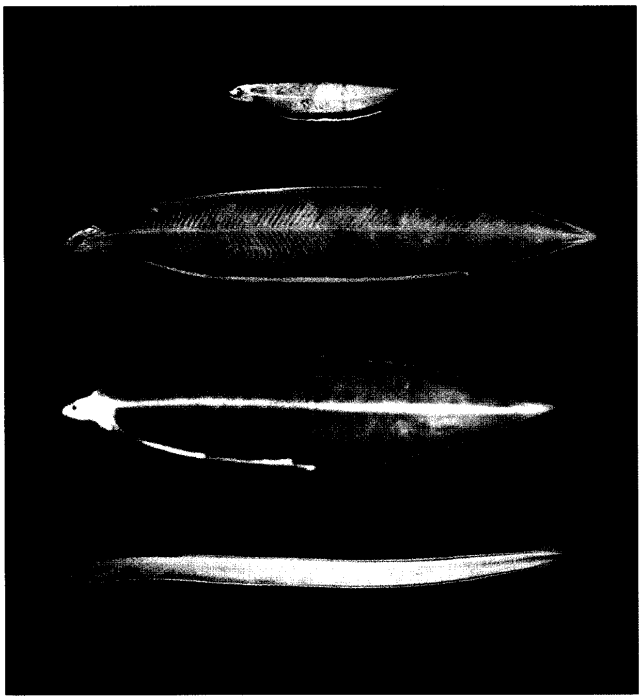

Fig. 1. Photographs of *Anguilla marmorata* larvae: a) 17.0 mm TL and b) 49.2 mm TL leptocephali from the North Equatorial Current region of the western North Pacific, and c) a 46.3 mm TL metamorphosing larva and d) a 47.8 mm TL oceanic glass eel collected in the Indonesian Seas.

After metamorphosis, the body shape of the oceanic glass eels caught over deep water in the Celebes Sea and in Tomini Bay showed the more juvenile-like shape of glass eels. The oceanic glass eels were classified at the IV stage based on the stages defined by Bertin (1956). The coastal glass eels of this species are typically at the VA stage (Arai et al. 1999b).

The proportional size or position of the major morphological features of *A. marmorata* larvae changed considerably during growth and metamorphosis. The ratios of preanal lengths to total lengths ranged from 64.9–85.5% (mean±SD: 75.5±4.1%) in leptocephali (N=28), 48.4% in the metamorphosing larva (N=1), to 40.5 and 54.4% in oceanic glass eels (N=2) (Table 1). This showed that the ratios of preanal lengths to total lengths became smaller during metamorphosis compared to leptocephali. The ratios of body depth to total lengths also became smaller during metamorphosis and were 14.4–24.4% (21.8±2.0%) in leptocephali, 25.7% in the metamorphosing larva, and 7.4 and 8.6% in oceanic glass eels (Table 1). The ratios of head lengths to total lengths were 7.4–9.7% (8.5±0.6%) in leptocephali, 8.8% in the metamorphosing larva, and 8.4% oceanic glass eels (Table 1), indicating that these ratios did not change very much during the metamorphic process.

Otolith microstructure

The otoliths of small leptocephali were round, but they became more oval shaped after they had grown larger in the metamorphosing larva and the glass eels. The maximum otolith radius ranged from 23.22 to 112.81 μm in leptocephali (10.1–50.7 mm TL), 122.22 μm in the metamorphosing larva (46.3 mm TL), 153.56 and 160.33 μm in the oceanic glass eels (47.8, 48.6 mm TL), and 134.00 to 156.00 μm in the coastal glass eels (47.9–54.8 mm TL).

The primordium formed a dark hole and the surrounding core was observed as a deeply etched cavity in the center of the otolith (Fig. 2). A hatch check (HC) was observed near the outer edge of the core of the otoliths in all of the specimens. The mean diameter of the HC was 10.03±1.47 μm (range: 7.11–13.41 μm). Continuous increments were observed from the HC to the otolith edge in all development stages (Fig. 3). The first feeding check (FFC) was observed outside the crystalline crown surrounding the otolith core in all specimens (Fig. 2). The mean distance between the HC

Table 1. Morphological characteristics of leptocephali, a metamorphosing larva, oceanic glass eels, and coastal glass eels of *Anguilla marmorata*. TL: total length, PAL: preanal length, BD: body depth, and HL: head length.

Development stage	N	TL (mm)	PAL/TL (%)	BD/TL (%)	HL/TL (%)	Age at metamorphosis (days)	Total age (days)
Leptocephalus	28	10.1–50.7	64.9–85.5	14.4–24.4	7.4–9.7	—	22–137
Metamorphosing larva	1	46.3	48.4	25.7	8.8	145	147
Oceanic glass eel	2	47.8, 48.6	40.5, 54.4	7.4, 8.6	—, 8.4	143, 147	159, 160
Coastal glass eel	18	47.9–54.8	—	—	—	96–147	119–168

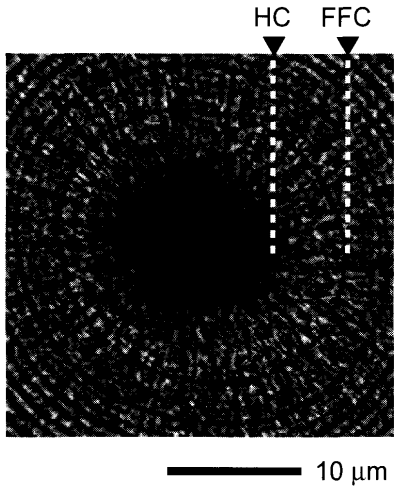

Fig. 2. SEM photograph of the central core region of an *Anguilla marmorata* leptocephalus showing the hatching check (HC) and the first feeding check (FFC).

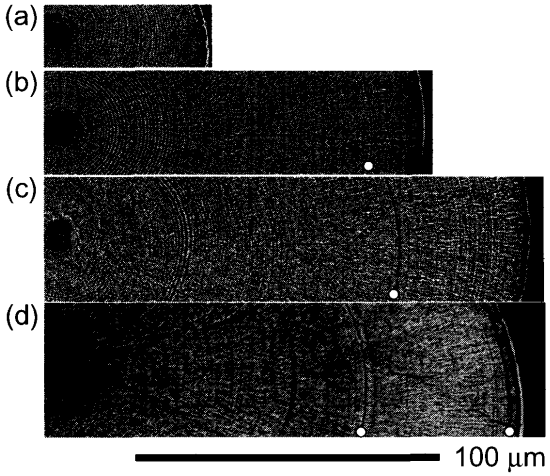

Fig. 3. SEM photographs of the otoliths of all four larval stages of *Anguilla marmorata* showing the metamorphosing check (MC; first circle) and the freshwater check (FWC; second circle)

and FFC was 0.56±0.11 μm (0.40–0.80 μm).

The metamorphosing check (MC) was etched deeper than other rings, and was observed in the metamorphosing larva (N=1), one of the two oceanic glass eels (N=2), and seven of the coastal glass eels (N=18), thus totally this check was seen in 45% of the specimens (N=20). These checks appeared approximately when the width of the otolith increments shifted from narrow to wide (±5 rings) (Fig. 3b, c, d). These drastic increment changes were observed at about an age of 145 d in the metamorphosing larva, 143 and 147 d in oceanic glass eels, and 96 to 147 d in coastal glass eels (Table 1). A freshwater check (FWC) according to Kawakami et al. (1998) was observed in five of the coastal glass eels (28%, N=18) (Fig. 3d). Furthermore, specimens with a FWC were those that had a MC, indicating that they might have physiological characteristics to form checks easily.

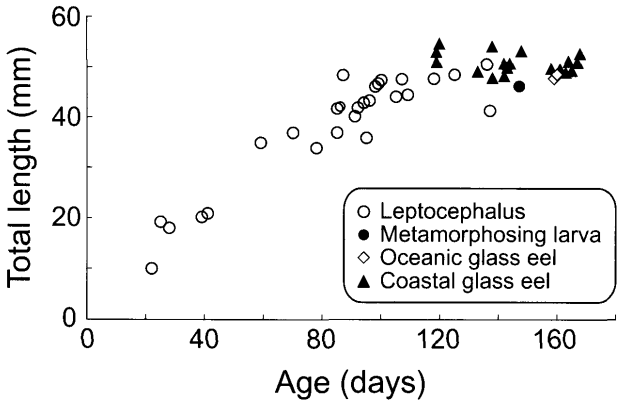

Fig. 4. Plots of the ages and total lengths of four development stages, leptocephalus (open circle), metamorphosing larva (closed circle), oceanic glass eel (diamond), and coastal glass eel (triangle) of *Anguilla marmorata*.

Age and total length

The relationship between age and total length showed that leptocephali of *A. marmorata* grew linearly up to ages of about 100 d (ca. 45 mm TL) when growth rates appeared to reach a stationary phase (Fig. 4). The size of leptocephali appeared to reach a plateau showing their full-grown size at about 50 mm TL. The metamorphosing larva, oceanic glass eels and coastal glass eels showed similar total lengths even though their ages increased up to a maximum age of 168 d in this study.

Otolith growth pattern and Sr : Ca ratio

The pattern of change in otolith increment widths and change in Sr : Ca ratios along the life history transect from the core to the edge of the four different stages showed a variety of patterns (Fig. 5). The otolith increment widths increased between the hatch check and from 20 to 50 d, and showed first small peaks (mean±SD: 1.12±0.24 μm, range: 0.72–1.75 μm) in all development stages except for 5 of the youngest leptocephali (10.1–21.0 mm TL). After the peak, the otolith increment widths decreased in all stages. The widths increased sharply in the metamorphosing larva, oceanic glass eels, and coastal glass eels. Second large peaks (2.59±0.24 μm, 2.10–2.96 μm) were observed from 110 to 160 d in oceanic glass eels and coastal glass eels, which decreased sharply just after the peaks.

The pattern of Sr : Ca ratios also changed according to the development stage (Fig. 5). The Sr : Ca ratios in the otolith cores were relatively low (mean±SD: 9.16±1.21, range: 7.43–10.80), and generally increased from the core toward the edge in all stages. The Sr : Ca ratios decreased after each peak (16.73±1.81, 13.24–19.03) in the metamorphosing larva, oceanic glass eels, and coastal glass eels at 90–140 d, depending on the specimens. The timing of the abrupt increase in increment width and the drop in the Sr : Ca ratio coincided exactly in all the metamorphosing larva, oceanic

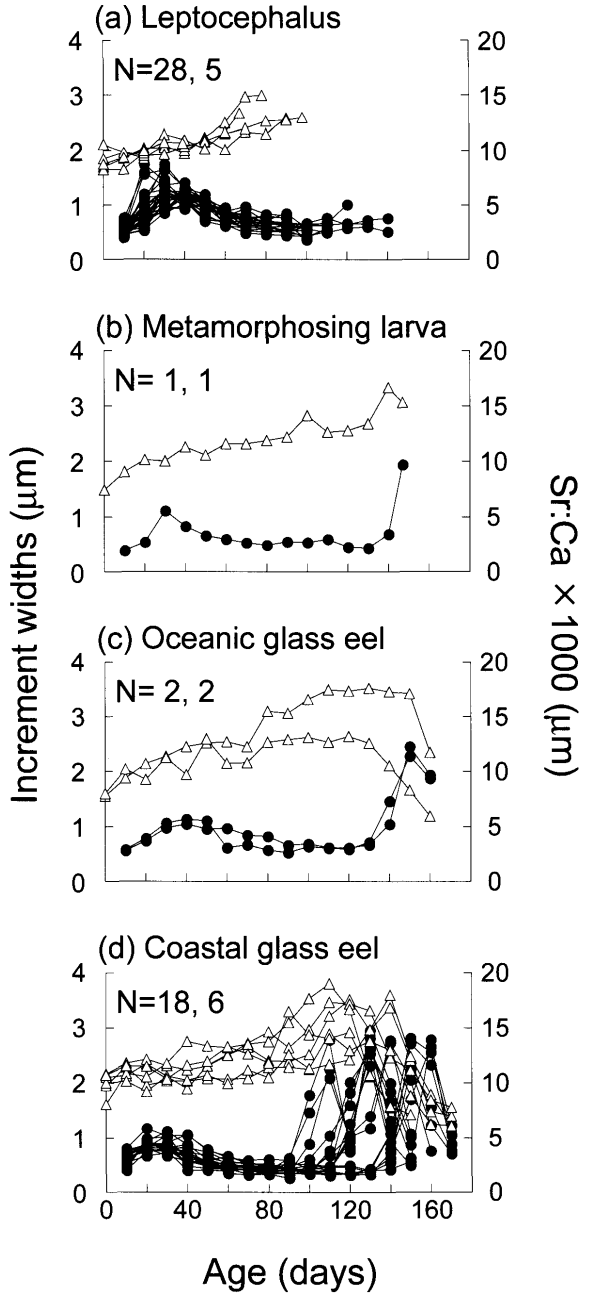

Fig. 5. Plots of the Sr : Ca ratios measured at 1 μm intervals (triangles) and otolith increment widths averaged for every 10 increments (circles) along transects from the core to the edge of the otoliths of the four larval stages of *Anguilla marmorata*. The sample shown are the number of specimens used for increment widths and Sr : Ca ratios analysis, respectively.

glass eels except for one, and coastal glass eels. The exception that did not show exact correspondence between the Sr decreases and the increased increment widths may have resulted from technical problems associated with obtaining the increment widths as an average value of ten consecutive increments, or from the Sr : Ca ratio measurement transects shifting off the same exact transect of the increments measurements along the otolith radius.

Discussion

This is the first study of the early life history of anguillid larvae that examined individuals all the way from their hatching area (ocean) to their growth habitats (estuarine and freshwater) by examining the development stages of leptocephalus, metamorphosing larva, oceanic glass eels, and coastal glass eels. The genetically identified leptocephali of this study showed that the body shape and lack of any pigmentation of *Anguilla marmorata* leptocephali is similar to the other longfin species that have been described (Schmidt 1912, Mochioka 2003, Miller and Tsukamoto 2004). The process of metamorphosis in *A. marmorata* appears to be the same as those described for temperate species (Schmidt 1912, Otake 2003, Tanaka 2003), but the size at metamorphosis of *A. marmorata* and of their glass eels (46.3–54.8 mm TL) is smaller than the 60–75 mm range of temperate species of glass eels (Jespersen 1942, Kleckner et al. 1985, Tsukamoto and Umezawa 1990). Other tropical species also have smaller sized glass eels of about 44.0–54.6 mm in *A. celebesensis*, 45.5–52.3 mm in *A. bicolor bicolor*, and 44.4–54.4 mm in *A. bicolor pacifica* (Arai et al. 1999b, Marui et al. 2001).

The otolith characteristics of the *A. marmorata* specimens examined in this study were similar to other otolith studies on anguillid larvae. Checks may be formed in otoliths when there is perturbation or stress to the fish (Campana and Neilson 1985), and *A. marmorata* had four different checks that have been described in other anguillid species (Fig. 6). Both the HC and FFC were confirmed in all specimens without reference to developmental stages in this study. The HC is thought to form by the physiological and environmental changes that occur after hatching (Umezawa et al. 1989). The FFC typically is formed at about a 10 days after the hatch, and is thought to form when leptocephali begin to feed. The timing of this check has been confirmed in the otoliths of reared larvae of *A. japonica* (Shinoda et al. 2004), and it has been observed in the otoliths of a variety of anguillid species such as *A. anguilla* (Lecomte-Finiger 1992, Wang and Tzeng 1998), *A. australis* (Arai et al. 2001b, Marui et al. 2001), *A. bicolor* (Arai et al. 2001b, Marui et al. 2001), *A. celebesensis* (Arai et al. 2001b), *A. dieffenbachii* (Marui et al. 2001), *A. japonica* (Cheng and Tzeng 1996), *A. marmorata* (Budimawan 1997, Arai et al. 2001b, Marui et al. 2001), and *A. rostrata* (Wang and Tzeng 1998). These studies have shown that there is little difference between individuals and species in these characteristics, which suggests that the period of development when they utilize their yolk reserves and their feeding ecology during later stages may be similar among individuals and species of anguillid leptocephali.

The MC has been observed in temperate species such as glass eels of *A. japonica* (Cheng and Tzeng 1996), and the

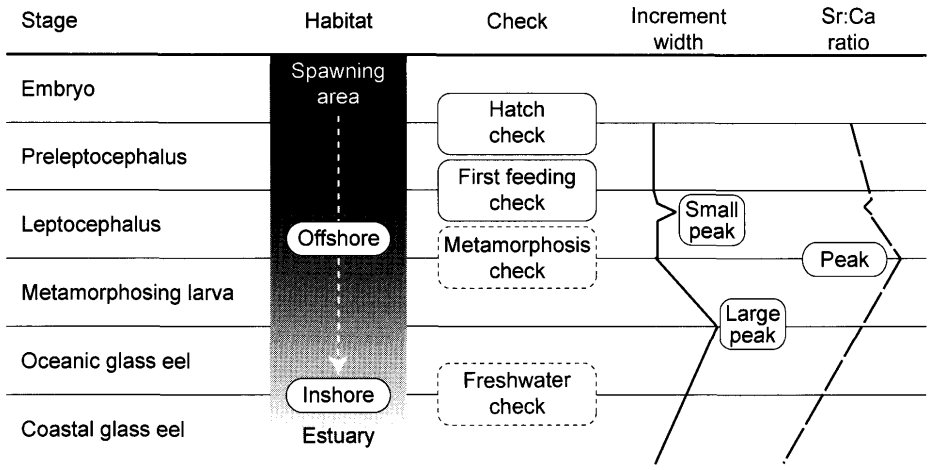

Fig. 6. The sequence of the otolith events in the early life history of *Anguilla marmorata* larvae as they migrate from the spawning area to their estuarine recruitment areas, showing the various checks and patterns of changes of otolith increment widths and Sr : Ca ratios that occur in their otoliths.

FWC has been observed in temperate species such as the New Zealand glass eels of *A. dieffenbachii* and *A. australis* (Marui et al. 2001). It was reported in these studies that all the analyzed samples had each of these particular checks. In our study, however, the MC and FWC were observed in only some oceanic glass eels (MC: 50%) or coastal glass eels (MC: 39%, FWC: 28%), and it was reported that only 7% of some tropical eels such as *A. biclor* pacifica and *A. celebesensis* in Indonesia had a FWC (Marui et al. 2001). These could suggest that it may be more difficult for tropical eels to form these checks than for temperate eels, possibly because of the differences in the water temperatures they experience or their shorter larval durations. Alternatively, there may be individual differences in the process of check formation, for example, with checks only being formed in individuals that experienced a strong stress from some endogenous or exogenous factors during early feeding, metamorphosis, or entering freshwater. However, there remains a small possibility that the visibility of these checks might be reduced by technical problems.

There have been several studies on the otolith microstructure and microchemistry of *A. marmorata* coastal glass eels, but this study also examined these characteristics in leptocephali and glass eels in the ocean. The pattern of change in otolith growth increments was roughly divided into four phases (Fig. 6) in this study and in previous studies on *A. marmorata* glass eels (Arai et al. 2001b, 2002, Marui et al. 2001). The first phase showed an increase in otolith increment widths, and the second phase showed a decrease after the small peak and a longer period of low values. These two phases were observed in all development stages. The third phase showed a rapid increase followed by the drastic decrease of the fourth phase. The third phase was observed in all development stages except for the leptocephalus stage, but the fourth phase of decrease in otolith increment widths after the large peak was only seen in the two glass eel stages. Furthermore, Sr : Ca ratios in the otoliths were roughly divided into two periods (Fig. 6). The first period had a tendency of slight increases in all specimens corresponding to the first and second phases of increment widths, and the second period showed a drastic decrease, which corresponded to the third and fourth phases of increment widths that was observed in all development stages except for the leptocephalus stage.

The first small peak may be affected by the rapid growth of the leptocephalus from 20 to 40 days after hatching. In contrast, during the period of the second large peak, the larval somatic growth is stopped or decreased because metamorphosis is beginning. These increases of otolith increments were observed not only in anguillid species but also other larvae of the Anguilliformes (Lee and Byun 1996, Tsukamoto and Okiyama 1993). These common changes suggest that the drastic increase of otolith increments are controlled by the contribution of hormonal factors such as thyroid hormones. Exogenous thyroxine has been found to accelerate the advancement of metamorphosis in some species of leptocephali of the Elopomorpha, such as *Conger myriaster* (Kitajima et al. 1967, Yamano et al. 1991) and *Megalops cyprinoides* (Shiao and Hwang 2004). Thus, these increment width increases may be controlled by physiological factors rather than by the experienced temperature or feeding.

Consistent patterns of changes in otolith increment widths and Sr : Ca ratios were seen in the different development stages, and these patterns did not appear to change with the growth of the larvae. Therefore, these changes may be able to be used as an indicator for defining the beginning and end of metamorphosis in anguillid eels. The beginning of metamorphosis has been suggested to occur at the time of the rapid increases in otolith increments and decreases of Sr : Ca

ratios, and the end of metamorphosis by decreases of Sr : Ca ratios (Otake et al. 1994, Arai et al. 1997, 2000, Marui et al. 2001; Fig. 6). Anguilliform leptocephali use glycosaminoglycans (GAG) as an energy storage material in their body (Pfeiler 1984) and have a unique physiology compared to other fish larvae (Pfeiler 1999). GAG is highly compatible with Sr and as a result, the Sr level is high in the body (Otake et al. 1994). They utilize the GAG and lipids during the physiological changes of metamorphosis (Pfeiler 1991). The decreases in Sr : Ca ratios may also be associated with decreasing Sr levels during metamorphosis in anguillid eels, because *Conger myriaster* leptocephali also have been confirmed to have similar changes in Sr : Ca ratios during metamorphosis (Otake et al. 1997).

Up to now, there has been little direct confirmation that other processes such as otolith reabsorption (Cieri and McCleave 2000) don't occur during metamorphosis. However, the analysis of the otoliths of metamorphosing larvae and oceanic glass eels during this study provide the best confirmation that the drastic changes in increment width and Sr : Ca ratios do actually occur during metamorphosis in anguillid eel larvae, with no evidence of reabsorption, as has also recently been found for tarpon leptocephali (Shiao and Hwang 2004). The numbers of metamorphosing larvae and oceanic glass eels were relatively few in this study, so further examination of a series of specimens before and after metamorphosis is needed to validate the detailed relationship between otolith characters and metamorphosis of anguillid eels and to reveal any individual differences.

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