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Otolith strontium : calcium ratios in a freshwater stingray, *Himantura signifer* Compagno and Roberts, 1982, from the Chao Phraya River, Thailand

Tsuguo OTAKE[1]*, Toshiaki ISHII[2] and Sho TANAKA[3]

[1] *International Coastal Research Center, Ocean Research Institute, The University of Tokyo, Akahama, Otsuchi, Iwate 028–1102, Japan*
** E-mail: otake@wakame.ori.u-tokyo.ac.jp*
[2] *Laboratory for Radioecology, National Institute of Radiological Sciences, Isozaki, Hitachinaka, Ibaraki 311–1202, Japan*
[3] *Department of Marine Sciences and Technology, Tokai University, Orido, Shimizu, Shizuoka 424–8610, Japan*

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Abstract—Otolith microchemistry was examined in a freshwater stingray, *Himantura signifer*, collected from a freshwater region of the Chao Phraya River, Thailand. Otoliths of *H. gerarudii* and *H. imbricata* from the estuary at the mouth of the Chao Phraya River, and *H. imbricata* from the sea adjacent to the river mouth were also examined. Otolith Ca concentration was almost constant among the three species, regardless of their habitat environments. In contrast, Sr concentration and the Sr : Ca ratio differed considerably among the species, those of the freshwater species being at the lowest levels and that of the marine population of *H. imbricata* the highest. Intraspecific variation in Sr concentrations and Sr : Ca ratios found in *H. imbricata* from estuarine and marine environments suggested that those levels varied according to the habitat. Otolith Sr concentrations and Sr : Ca ratios of male freshwater stingrays increased with body growth, whereas those of female otoliths decreased temporarily to the lowest level in 250–300 mm disk width (mature) specimens. The findings suggested that *H. signifer* from freshwater regions enter a brackish or seawater environment at some time in their life history. The marked fluctuations in Sr concentration and Sr : Ca ratio in female otoliths may be related to presumed reproductive behavior, such as ceasing migration across a saline boundary during the reproductive period and giving birth in a freshwater region of the river, or may have been affected by changes in physiology and elemental metabolism associated with reproduction.

Key words: *Himantura signifier*, freshwater stingray, otolith, Sr : Ca ratio, migration

Introduction

It is well known that freshwater elasmobranchs are broadly distributed in tropical rivers and lakes. Four families (Carcharhinidae, Pristinidae, Potamotrygonidae, Dasyatidae), including ten genera, have been reported as occurring in freshwater far beyond the influence of tidal reaches (Compagno and Cook 1995). The greatest number of species occur in the Atlantic drainages in South America, with lesser pockets of diversity and endemism in West Africa, southeastern Asia, southern China, Indonesia, Papua New Guinea, the Philippines and Australia. Family Dasyatidae, having the greatest degree of adaptations to freshwater of any living elasmobranchs, includes species ranging from possible stenohalious, adapting well to freshwater environments, to eurihalious, being distributed in marine inshore and estuarine waters, river mouths and up river freshwater regions (Compagno and Cook 1995). Dasyatid stingrays from rivers far beyond the limit of tidal reaches in Indonesia (Hirano 1978) and West Africa (Hirayama and Kitamura 1987) have been shown to have much higher plasma osmolarity, and ion and urea concentrations than potamotrygonid stingrays, obligate freshwater stingrays from South America (Thorson et al. 1967, 1978, Ogawa and Hirano 1982). These findings suggested that such freshwater Dasyatid species might have a relatively short history in freshwater habitats or retain a link with marine habitats, i.e. possibly migrating between freshwater and salt water. However, little is known of the life history of these freshwater stingrays, let alone any other freshwater elasmobranchs. Understanding of the life history of each freshwater species, in particular the possibility of individual diadromy, is indispensable to an understanding of the adaptations required for invasion of freshwater environments.

Recently, otolith microchemistry, such as the concentration ratio of strontium to calcium (Sr : Ca ratio), has been demonstrated as fluctuating (associated with ambient salinity or large differences in Sr concentration) between freshwater and sea water in teleost fishes, and has been used successfully for reconstructing individual migration histories in diadromous fishes (Kalish 1990, Rieman et al. 1994, Secor et al. 1995, Limburg 1995, Otake and Uchida 1998, Tsukamoto et al. 1998, Tsukamoto and Arai 1998, Katayama et al. 2000, Arai and Miyazaki 2001, Arai et al. 2002, 2003a, b, c, 2004, Goto and Arai 2003, Chang et al. 2004). Differing from teleost otoliths, those of elasmobranchs represent aggregations of otoconia, fine calcium carbonate particles (Popper and Fay 1977, Mulligan and Gauldie 1989, Mulligan et al. 1989, Lychakov et al. 2000). However, the formation mechanism and elemental composition of such otoliths is still largely unknown. Nevertheless, characteristics of elemental composition, such as Sr : Ca ratios, should be affected by ambient water environments. Accordingly, microchemical analyses should provide information on individual diadromy, although they cannot reveal migration history along a life-history transect because of the lack of growth increments, daily or yearly, in elasmobranch otoliths.

The present study addresses the relationship between otolith Sr : Ca ratios and ambient water environments in elasmobranchs, being the first microchemical study of elasmobranch otoliths. This should allow the disclosure of individual migration history between fresh and salt water, and an insight into the adaptation and evolution of freshwater elasmobranchs. A considerable number of *Himantura signifer* Compagno and Roberts, 1982, a Dasyatid stingray from the freshwater region of the Chao Phraya River, Thailand, were collected during an expedition conducted in 1996–1997 for freshwater elasmobranchs occurring in that river (Tanaka 1999). *Himantura gerrarudii* (Gray 1851) and *H. imbricata* (Bloch and Schneider 1801) were also collected from the river mouth estuary and immediately offshore at this time. Otolith Sr : Ca ratios were examined and the implications of the findings regarding individual migration history of freshwater elasmobranchs discussed.

Materials and Methods

A total of 20 *Himantura signifer*, comprising of 10 males (160–339 mm disk width) and 10 females (181–364 mm disk width), was used for the study (Table 1). All of the specimens were collected from the Chao Phraya River at Chai Nat, a freshwater region some 200 km from the river mouth. Salinities of the bottom, mid- and surface waters at the sampling sites were all 1.0 psu. After morphometric measurements were taken, according to Bigelow and Shroeder (1953), right and left otic complexs were dissected out from each specimen and preserved in 99% ethanol until microchemical analysis. Otoliths contained in each otic complex were removed as completely as possible and weighed to 0.1 mg after drying for 2 h at 50°C. Dried otolith samples were dissolved with 2 ml conc. nitric acid (high purity: Tama pure A-100, Tama Chemicals Ltd.) on a hot plate at 150°C

Table 1. Ca and Sr concentrations and Sr : Ca ratios of stingrays collected from freshwater and estuarine regions of the Chao Phraya River, and directly offshore from the river mouth.

Species	No. of specimens	Sex	Disk width range (mm)	Environment of collection site	Ca concentration Mean±SD (μg/g dry weight)	Sr concentration Mean±SD (μg/g dry weight)	Sr : Ca ratio	Discrimination factor Mean±SD
Himantura signifer	10	Male	160–339	Freshwater	303000±43900	600±115	2.06±0.57	0.56±0.15
"	10	Female	181–364	Freshwater	339000±40000	550±122	1.64±0.45	0.44±0.12
Himantura gerarudii	6	Male	150–392	Fstuary	309000±70200	4740±938	15.46±1.16	—
"	3	Female	131–186	Estuary	258000±29700	3590±239	14.07±1.98	—
Himantura imbricata	2	Male	168, 176	Estuary	369000, 326000	5760, 5200	15.61, 15.98	—
"	17	Male	164–190	Sea	305000±41700	5780±581	19.03±0.93	1.07±0.05

until dry. They were subsequently diluted with 1 N nitric acid to a volume of 50 ml, adding 1000 ppm yttrium (SPEX Industries Inc.) to each sample as an internal standard. Ca and Sr concentrations of all samples were measured using a Shimadzu ICPQ-1012W inductively-coupled plasma atomic emission spectrometer (ICP-AES). Six samples were further measured for Al, Ba, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, V and Zn by the same method, using SPEX XSTC-331 as a standard.

For comparison with the above, otoliths of nine *H. gerarudii*, comprising six males (150–392 mm disk width) and three females (131–186 mm disk width), and two male *H. imbricata* (168 and 176 mm disk width) collected from the estuary at the Chao Phraya River mouth, plus those of 17 *H. imbricata* (all males, 164–190 mm disk width) from the sea off the Chao Phraya River mouth were examined (Table 1). No data was available for environmental aspects of each sampling site. The otoliths of all of the specimens were treated for ICP-AES analysis using the same procedure as for *H. signifer*. Ca and Sr concentrations were measured for all specimens, and the additional 12 elements (mentioned above) analyzed for one example of *H. gerarudii* and five of marine *H. imbricata*.

The Sr : Ca ratio, expressed as Sr concentration in wt%×1000/Ca concentration in wt%, was calculated for each individual. A discrimination factor (Sr : Ca ratio in organism/Sr : Ca ratio in environment) was also calculated for *H. signifer* from freshwater and *H. imbricata* from the sea, thereby elucidating the discrimination ability of Sr in the process of Ca precipitation into the otolith in each species. The discrimination factor corresponds to the observed ratio (OR ratio), the first proposed use being by Comar et al. (1957) for denoting the discrimination observed for the two elements from an environmental state to within an organism. Ca and Sr concentrations in the water at the sampling sites in the Chao Phraya River averaged 22.7 ppm (range: 22.4–22.9 ppm) and 0.096 ppm (range: 0.091–0.10 ppm), respectively, the Sr : Ca ratio averaging 4.2 (range: 4.0–4.5). There were no differences in those concentrations among surface and bottom waters at each sampling site. Concentrations of each element in the seawater environment (Ca: 417.6 ppm, Sr: 7.404 ppm) are from Quinby-Hunt and Turekian (1983).

Results

Otolith Ca and Sr concentrations of *H. signifer* ranged from 244000 to 383000 μg/g dry weight (321000± 44900 μg/g dry weight: mean±SD) and from 355 to 697 μg/g dry weight (580±119 μg/g dry weight), respectively. Concentration ratios of Sr to Ca (Sr : Ca ratios) ranged from 0.93 to 2.78 (1.85±0.54: mean±SD). The calcium concentration in female otoliths (339000±40000 μg/g dry weight) was slightly higher than those in males (303000±43900 μg/g dry weight) and the Sr concentration (550±122 μg/g dry weight) and Sr : Ca ratios (1.64±0.45) in females lower than those in males (600±115 μg/g dry weight; 2.06±0.57), although there were no significant differences in either of the concentrations or the concentration ratio between female and male otoliths ($p>0.05$) (Table 1). Fig. 1 shows the relationship between disk width, and elemental concentrations and otolith Sr : Ca ratios in females and males. Otolith Ca concentration tended to decrease slightly as disk width increased in both sexes (Fig. 1A). Sr concentration was low in smaller individuals (disk width under 200 mm) and was relatively constant in larger individuals (over 200 mm disk width) (Fig. 1B). In female otoliths, Sr concentration temporarily decreased in specimens of 250–300 mm disk width, a significant phenomenon. Otolith Sr : Ca ratios showed a distinct positive relationship with disk

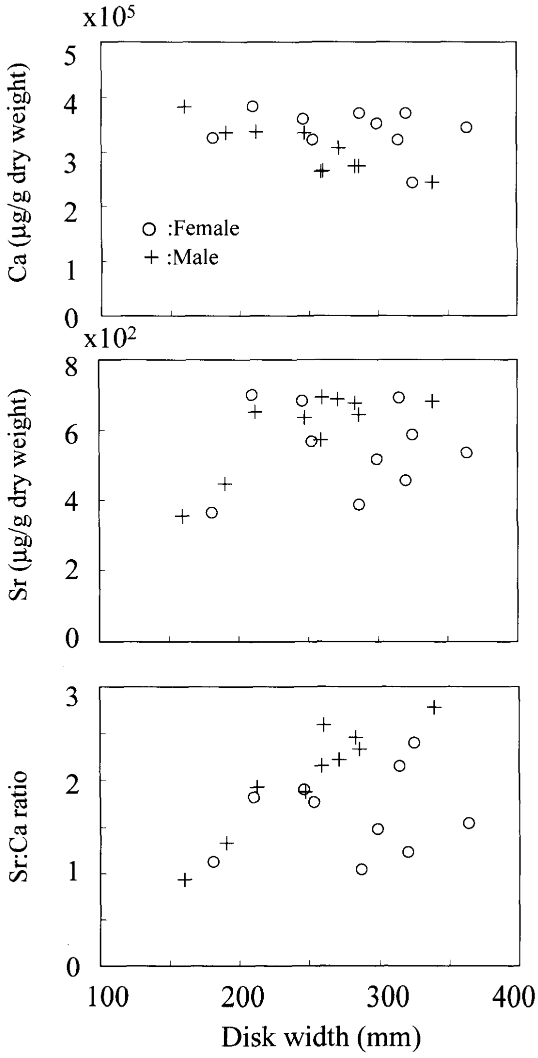

Fig. 1. Plots of otolith Ca (A) and Sr (B) concentrations and Sr : Ca ratios (C) against disk width of each individual of *Himantura signifer* collected from a freshwater region of the Chao Phraya River, Thailand.

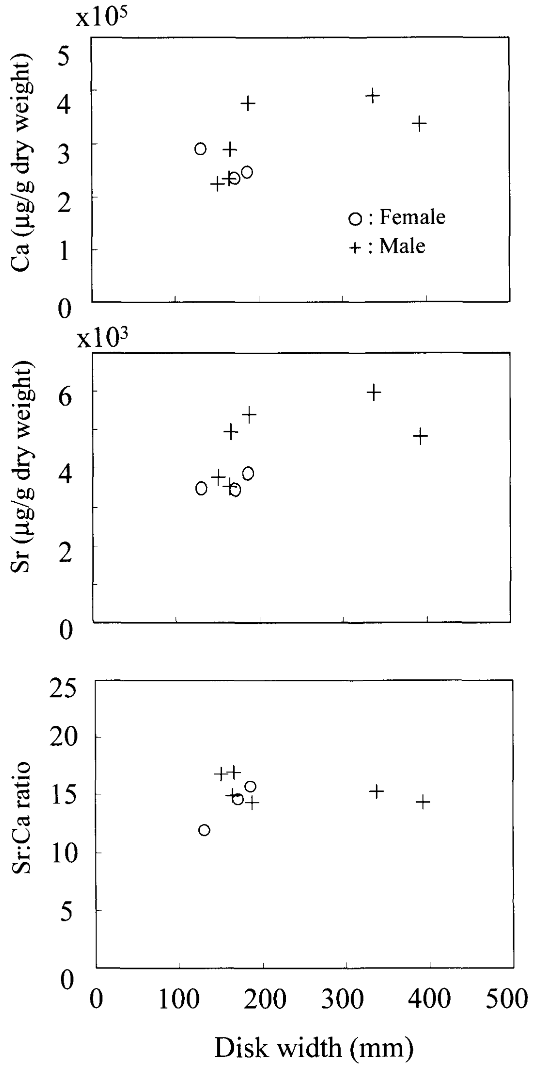

Fig. 2. Plots of otolith Ca (A) and Sr (B) concentrations and Sr : Ca ratios (C) against disk width of each individual of *Himantura gerarudii* collected from the estuary at the mouth of the Chao Phraya River, Thailand.

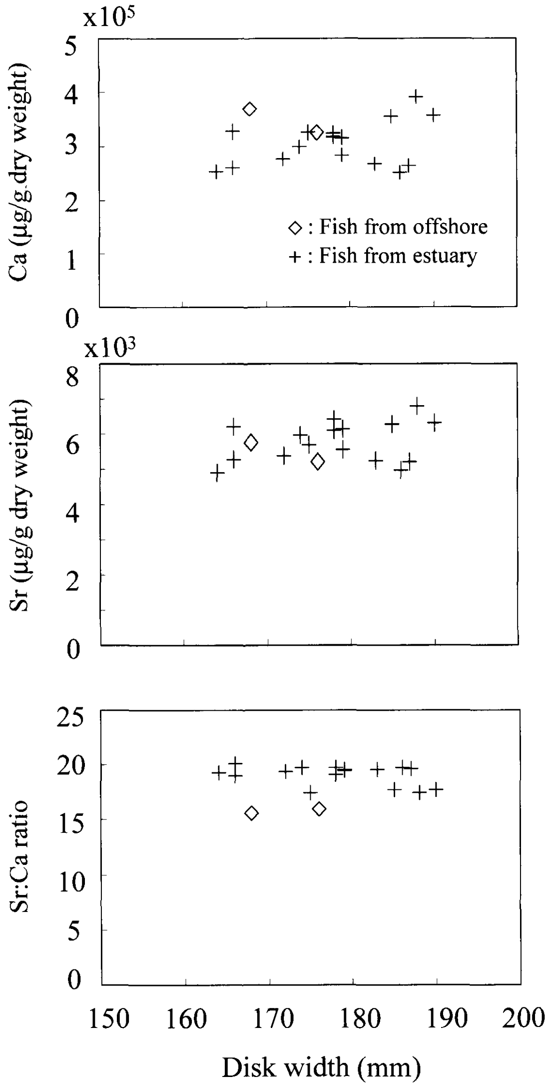

Fig. 3. Plots of otolith Ca (A) and Sr (B) concentrations and Sr : Ca ratios (C) against disk width of each individual of *Himantura imbricata* collected from the estuary at the mouth of the Chao Phraya River and directly offshore from the river mouth.

width in males (Fig.1C). The minimum ratio of 0.93 was found in the smallest individual (160 mm disk width) and the maximum (2.78) in a male of 339 mm disk width. Unlike in males, the ratios in female otoliths fluctuated markedly with increase in disk width, associated with fluctuations in Sr concentration. The ratio of the smallest individual (181 mm disk width) was 1.12, increasing with individual body growth with a marked decrease to a minimum level at 250–300 mm disk width. The minimum ratio recorded was 1.04 for an individual of 287 mm disk width and the maximum (2.40) for an individual of 325 mm disk width.

Calcium concentrations in otoliths of the brackish water stingray, *H. gerrarudii*, averaged 292000±62900 µg/g dry weight (males: 309000±70200 µg/g dry weight, females: 258000±29700 µg/g dry weight, Table 1), ranging from 225000 to 390000 µg/g dry weight (Fig. 2), which was not significantly different from the concentrations in the otoliths of the freshwater stingrays ($p>0.05$). In contrast, Sr concentrations (4360±945 µg/g dry weight; males: 4740±938 µg/g dry weight, females: 3590±239) and Sr : Ca ratios (15.00±1.52; males: 15.46±1.16, females: 14.07±1.98) were much higher than in the latter. Two *H. imbricata* collected from estuarine waters also showed higher concentrations of Sr (5200, 5760 µg/g dry weight) and higher Sr : Ca ratios (15.98, 15.61) than the freshwater species (Figs. 3, 4), Ca concentrations of the former being 369000 and 326000 µg/g dry weight. Ca and Sr concentrations in otoliths of marine *H. imbricata* averaged 305000±41700 µg/g dry weight and 5780±581 µg/g dry weight, respectively (Table 1, Fig. 3). Ca concentrations were at a similar level to those of brackish and freshwater stingrays, whereas Sr concentrations were significantly higher than in the brackish water inhabitants, including the same species ($p<0.05$). The average Sr : Ca ratio, 19.03±0.93, was also significantly higher than recorded for the brackish water inhabitants ($p>0.05$) (Fig. 4).

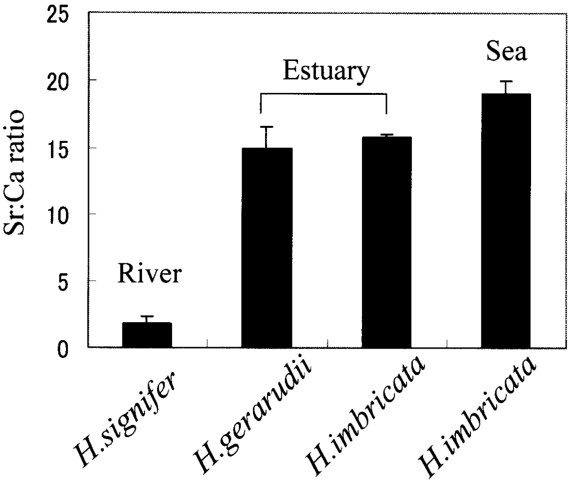

Fig. 4. Mean otolith Sr : Ca ratios of *Himantra signifer*, and *H. gerarudii* and *H. imbricata*, from freshwater and estuarine regions of the Chao Phraya River, respectively, and *H. imbricata* from directly offshore from the river mouth. Vertical bars indicate SD.

There were no distinct relationships between disk width and the above concentrations or Sr : Ca ratios in both the estuarine and marine stingrays (Figs. 2, 3), unlike in the freshwater stingray.

The discrimination factor of Sr uptake by the freshwater stingray, *H. signifer*, was 0.44±0.12 (range: 0.28–0.65) in females and 0.56±0.15 in males (range: 0.25–0.75) (Table 1), there being no significant difference between sexes ($p>0.05$). The discrimination factor in the marine stingrays, *H. imbricata* (males only) was 1.07±0.05 (range: 0.98–1.14), significantly higher than in the freshwater stingrays ($p<0.05$), indicating that the marine species-otoliths had the same Sr : Ca ratio as seawater.

Concentrations of 12 elements other than Ca and Sr in otoliths are shown in Table 2. Only Fe concentration in the otolith differed significantly between freshwater *H. signifer* and marine *H. imbricata* ($p<0.05$). Zn and Ba concentrations in otoliths of the freshwater stingrays were also higher than in the latter, although the differences were not significant.

Discussion

Otolith Sr : Ca ratios were distinctly different among species from different environments (i.e. fresh, brackish and salt water), although those differences might have been subject to some interspecific variation. However, the ratios of *Himantura imbricata* from the estuary were significantly lower than in the same species from the sea, the level in the former being similar to that of the other brackish species. This indicates that differences in otolith Sr : Ca ratios are associated with ambient water salinity or directly with Sr concentration in ambient water, as in otoliths of teleost fishes.

Size-related variations in otolith Sr : Ca ratios were ob-

Table 2. Concentrations of twelve elements in otoliths of stingrays collected from freshwater, estuarine and marine habitats.

Species	No. of specimens	Disk width range (mm)	Collection site	Elemental concentration Mean±SD (μg/g dry weight) Al	Ba	Cd	Cr	Cu	Fe	K	Mg	Mn	Na	V	Zn
Himantura signifer	6	181–253	Freshwater	0.043 ±0.016	0.012 ±0.011	0.005 ±0.001	0.006 ±0.030	0.014 ±0.00	0.013 ±0.001	0.380 ±0.006	0.47 ±0.30	0.001 ±0.003	0.42 ±0.30	0.011 ±0.019	0.04 ±0.03
Himantura gerarudii	1	131	Estuary	0.022	0.0029	0.024	0.0043	0.006	0.002	—	0.11	0.0018	—	0.002	0.003
Himantura imbricata	5	166–178	Sea	0.0380 ±0.011	0.0064 ±0.0089	0.0040 ±0.020	0.0055 ±0.0035	0.012 ±0.005	0.0033 ±0.0010	0.4302 ±0.115	0.59 ±0.21	0.0018 ±0.0007	0.325 ±0.17	0.003 ±0.001	0.006 ±0.003

served in both sexes of the freshwater stingray, *H. signifer*. Since the ratios in specimens from both the estuary and the sea showed no marked relationship with disk width, the change in the ratios found in otoliths of the freshwater species does not seem to be associated with individual growth. The fluctuations in otolith Sr : Ca ratios in freshwater stingrays suggests that they enter estuarine or marine waters at some time of their life history. Otake (1999) reported that blood serum composition and rectal gland histology of *H. signifer* from the freshwater region of the Chao Phraya River indicated incomplete adaptation for a freshwater environment, suggesting possible diadromy or a relatively short history of invasion into a freshwater habitat. Those observations are consistent with the present results of otolith microchemistry. Furthermore, the smallest individual, collected soon after birth (Tanaka and Ohnishi 1999), possessed the lowest ratio for otolith Sr : Ca ratio as well as the lowest Sr concentration, suggesting that pregnant *H. signifer* stays in the freshwater region during the reproductive period and gives birth in a completely freshwater environment, although we have no data available to show whether or not any relationship in otolith elemental composition exists between maternal fish and embryos. Unlike in males, female otoliths markedly decreased in Sr : Ca ratios at 250–300 mm disk width. Tanaka and Ohnishi (1999) reported that *H. signifer* in the Chao Phraya River matured at 250–260 mm disk width. The coincidence of those sizes suggests the possibility that the temporal decrease in otolith Sr : Ca ratios has some relationship with reproductive behavior, or physiology and elemental metabolism associated with reproduction. Kalish (1989, 1991) has suggested an affect of gonad maturation on otolith Sr : Ca ratios and Mugiya and Tanaka (1995) presented data on the negative effects of injected 17β-estradiol on the incorporation of Sr into otoliths in goldfish. Furthermore, since female *H. signifer* is thought to give birth in completely freshwater environments, it is plausible that they interrupt any migration across a salinity boundary during the reproductive period. Dasyatid stingrays are viviparous with a long gestation period. Such physiological and behavioral changes associated with reproduction seem to have a strong effect on otolith elemental composition. Since the otoliths of elasmobranchs exhibit no available age characters, otolith microchemistry cannot reconstruct the diadromous history of individuals. However, distinctive fluctuations in otolith microchemistry as found in freshwater *H. signifer* suggests that otolith microchemistry can reveal certain individual life history events. The mechanism of otolith formation in elasmobranchs should be examined for a better understanding of life history details obtained from otolith microchemistry.

The discrimination factor of the marine stingray, *H. imbricata*, was about 1.0, much higher than those of marine teleost fishes (0.10–0.37, Ueda et al. 1973), indicating that Sr was not discriminated against in the process of uptake of Ca from ambient water and precipitation onto the otolith in the former. Conversely, the marine stingray has a much lower ability in regard to Sr–Ca discrimination than marine teleost fishes. The labyrinth of elasmobranchs is known to open to the exterior via an endolymphatic duct arising from the apex of the sacculus, differing from that in teleost fishes (Tester et al. 1972). Such a morphological characteristic may strongly affect the elemental composition of endolymph and consequent otolith elemental deposit under a hypertonic environment in elasmobranchs. The factor for the freshwater stingray, *H. signifer*, was 0.44, a similar value to that for freshwater salmonid fishes such as *Salmo gairdneri*, landlocked *Onchorhynchus masou* and *Salvelinus pluvius*, with factors of 0.44, 0.47 and 0.56, respectively (Ishii unpublished data). The large difference in discrimination factor between marine and freshwater stingrays suggests some degree of the variability in the elemental precipitation kinetics for elasmobranch otoliths due to environmental factors, not withstanding the existence of interspecific differences in discrimination ability.

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