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**A part of the Japan Sea form of the threespine stickleback, *Gasterosteus aculeatus*,
spawns in the seawater tidal pools of western Hokkaido Island, Japan**

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Synopsis

The Japan sea form of the threespine stickleback, *Gasterosteus aculeatus*, was found to spawn in the seawater tidal pool of the Cape Benkei, western Hokkaido Island, Japan, in 2001 and 2003. Nest-guarding males, adult females and juveniles of this form were also observed in four tidal pools of the Capes Benkei, Kabuto and Kamui, in 2001–2003. Otolith Sr:Ca ratios in sticklebacks collected from these tidal pools were relative high from the core to the edge, suggesting they lived in seawater environments throughout its life cycle. These findings suggest that a part of the Japan Sea form's individuals in western Hokkaido Island have the potential to breed in the coastal sea and the life history style has evolved in dependence on the seawater environment.

Introduction

The threespine stickleback, *Gasterosteus aculeatus*, is widely distributed along coastal regions throughout the Northern Hemisphere. This fish can be found in freshwater, brackish water and seawater regions, and has been classified into both anadromous and freshwater types, based on its life cycle (Wootton 1984). In addition, a few studies reported several populations of *G. aculeatus* that bred in coastal regions such as estuary, salt marsh and tidal pool (Wootton 1984, Bell & Foster 1994). In Amory Cove, Québec, Canada, for example, *G. aculeatus* was found to breed in the seawater tidal pools (Coad & Power 1973). In Nova Scotia, Canada, threespine and white sticklebacks were also found to breed in seawater environments (Blouw & Hagen 1990). In North Atlantic, furthermore, *G. aculeatus* collected in the ocean, apart 500 km from the nearest land had nearly maturing gonads, suggesting the fishes would reach full breeding condition in the sea (Jones & John 1978). These marine populations belong to the European and eastern North American clade (Haglund et al. 1992).

In previous studies, however, several so-called marine populations, excluding from the European and eastern North American clade, spawned in freshwater and/or brackish water environments (e.g. Münzing 1963, Miller & Hubbs 1969, Borg 1985, Bañbura 1994). Furthermore, the other populations reported by several researchers regarded as marine populations by the reason of collection of only immature sticklebacks from open oceanic and/or coastal regions (e.g. Quinn & Light 1989, Peeke & Morgan 2000). From these facts, it has been believed that populations of *G. aculeatus* breeding in full seawater environments is distributed in only eastern North American region.

Recent allozyme analyses revealed that *G. aculeatus* around Japan comprised two genetically divergent forms (Nei's genetic distance $D = 0.482$), viz., the Japan Sea and Pacific Ocean forms (Higuchi & Goto 1996). The two forms have following different life history pattern: the Japan Sea form consists of anadromous and estuarine populations, whereas the Pacific Ocean form has anadromous, estuarine and freshwater life history features (Higuchi & Goto 1996, Higuchi et al. 1996, Arai et al. 2003a, b). Both forms were found to use freshwater and brackish water regions as breeding grounds, but not to use the seawater region (Ikeda 1933, Mori 1987, Ishikawa & Mori 2000, Kume et al. 2005). In this paper, we document a part of the Japan Sea form of *G. aculeatus* was found to breed in the seawater tidal pools, western Hokkaido Island, Japan, and estimate migratory history of this Japan Sea population by otolith Sr:Ca analyses.

Materials and methods

The present study was conducted at four seawater tidal pools in western Hokkaido Island, i.e., two tidal pools in the Cape Benkei (42°49'N, 140°11'E), and other pools in the Capes Kabuto (43°5'N, 140°27'E) and Kamui (43°20'N, 140°21'E) (Figure 1). One tidal pool of the Cape Benkei (BK1) inside the Benkei Port was located in a shallow open area with sand–rock bottom (Table 1). The other one (BK2) in inter-tidal zone consisted of rocky bottom partly covered with sand (Table 1). In rising tide, BK1 and BK2 were filled with seawater. Tidal pool in the Cape Kabuto (KB) was located in sandy bottom with vegetations, and had higher water temperature than other tidal pools in this study (Table 1), because of upwelling of hot spring water from the bottom. Tidal pool in the Cape Kamui (KM) was located in sandy bottom with both deeper open and

shallower vegetation areas (Table 1). There was no inflowing river in the vicinity (> ca. 2.3 km) of each tidal pool. No differences in salinity were found among these four tidal pools (Table 1).

In the field survey, spawning behaviour of the Japan Sea form of *Gasterosteus aculeatus* was observed at BK1 and KM from June to July in both 2001 and 2002. The matured adult and juvenile fishes were collected at four tidal pools using a dip net (2 mm in mesh size) from May to July in 2003 (Figure 1), and measured for standard length (SL) and total length (TL) to the nearest 0.05 mm using a caliper. In addition, categories of maturation in both sexes were recorded, following to Kume et al. (2005). To estimate their migratory histories by analyses of otolith Sr:Ca ratios, a part of specimens were preserved in 99% ethanol (Table 2, see below in details). Physical environments (salinity, water temperature, area and maximum water depth) in four tidal pools were measured in 2003 (Table 1). Salinity at both the surface and the bottom in each tidal pool was measured randomly at five points, repeating three times. Water temperature at both the surface and the bottom in each tidal pool was also measured randomly at five points.

We extracted sagittal otoliths from each fish, embedded them in epoxy resin (Struers, Epofix) and mounted them on glass slides. The otoliths were then ground to expose the core using a grinding machine equipped with a diamond cup-wheel (Struers, Discoplan-TS) and polished further with 6 and 1 μm diamond paste on an automated polishing wheel (Struers, Planopol-V). Finally, they were cleaned in ultrasonic bath and rinsed with deionized water prior to examination.

For electron microprobe analyses, all otoliths were Pt–Pd coated in a high vacuum evaporator. Otoliths from 23 specimens were used for life history transect analysis of Sr and Ca concentrations (Table 2), which were measured along a line down the longest

axis of each otolith from the core to the edge using wavelength dispersive X-ray electron microprobe (JEOL JXA-8900R), as described in Arai et al. (1997, 2003a, b). Calcite (CaCO₃) and strontianite (SrCO₃) were used as standards. The accelerating voltage and beam current were 15 kV and 1.2×10^{-8} A, respectively. The electron beam was focused on a point 2 μ m in diameter, with measurements spaced at 2 μ m intervals.

Results and discussion

In the field survey, the Japan Sea form of *Gasterosteus aculeatus* was found to breed in the seawater tidal pools, western Hokkaido Island (Figure 1). All tidal pools were located in high salinity environments (average 33.3–34.9‰, Table 1). The numbers of the adult Japan Sea form were observed at four tidal pools in 2001–2003. At BK1, one to five males were observed to build their nests every year. One or two nest-building males were also found at BK2 in 2003 and at KM in both 2001 and 2003. Furthermore, one or two females in grade 2 and/or one to three females in grade 3 (see Kume et al. 2005) were collected at four tidal pools in 2003. In both 2001 and 2003, a single pair was found to spawn at BK1. A small number of sneaking males were also found at BK1 only in 2001 (Kuwahara pers. obs.). Therefore, these findings suggest that a part of the Japan Sea form builds their nests and breeds in the seawater tidal pools year by year.

After spawning, several juvenile fishes were found at BK1 and KM in 2001 (≥ 10 fishes observed in both sites), at BK1 in 2002 (≥ 10 fishes observed), and at BK2 (25 fishes collected on 15–16 June and one fish on 17 July) and KB (91 fishes collected on 16 June) in 2003. In eastern Hokkaido Island, juveniles of the Japan Sea form migrate toward the sea when they reached ca. 25.0–30.0 mm

SL (Higuchi 1996, Kume unpubl. data). However, the juvenile fishes collected at BK2 (13.6 mm SL \pm 1.87 standard deviation, $n = 25$) and KB (21.5 mm SL \pm 1.59, $n = 91$) would not reach critical body size for migrating toward the sea from the tidal pools. These findings in the field survey suggest that individuals of the Japan Sea form collected from the tidal pools have the ability to survive in high salinity environments during the periods from maturation of adults to seaward-migration of juveniles in the next generation.

The otolith Sr:Ca ratios of the Japan Sea form collected from the seawater tidal pools, measured along a transect from the core to the edge, are shown in Figure 2. Higher otolith Sr:Ca ratios in fish indicate a time period in seawater and/or brackish water environments, while much lower rates indicate time spent in freshwaters (Arai 2002). The ratios of adult fishes were average $5.8 \pm 0.56 \times 10^{-3}$ (mean \pm SD) at BK1, 5.6×10^{-3} at KB and $5.7 \pm 0.49 \times 10^{-3}$ at KM (Figures 2a, b, c and Table 2). In addition, otolith ratios of juvenile fishes at KB were average $5.7 \pm 0.22 \times 10^{-3}$ (Figure 2d, Table 2). The otolith ratios of these Japan Sea forms were remarkably higher than those of freshwater types of *G. aculeatus*, just same as the freshwater resident of the Pacific Ocean form in the Otsuchi River, Iwate Prefecture of Japan ($1.5 \pm 0.19 \times 10^{-3}$) and Hariyo, *G. a. microcephalus*, in Gifu Prefecture of Japan ($0.85 \pm 0.04 \times 10^{-3}$) (Arai et al. 2003a, b), the latter being a land-locked type probably derived from the anadromous Pacific Ocean form (Ikeda 1933, Higuchi & Goto 1996). These otolith Sr:Ca data suggest that the Japan Sea form found in the tidal pools lived in brackish water and/or seawater environments throughout its life cycle.

Otolith Sr:Ca ratios of adult Japan Sea form collected from the Tokachi River ($5.6 \pm 0.15 \times 10^{-3}$) and a brackish lake, Lake Akkeshi ($6.3 \pm 0.87 \times 10^{-3}$), eastern Hokkaido Island, which were identified as anadromous populations based on their morphological

characteristics (Ikeda 1933, Higuchi & Goto 1996), revealed that these populations regarded as the estuarine life history type, living in relative high salinity environments throughout its life cycle (Arai et al. 2003a, b). The Sr:Ca ratios of estuarine Japan Sea forms were almost equivalent to those of sea-spawning Japan Sea forms found in this study. In Lake Akkeshi, furthermore, the greater part of the Japan Sea form migrated to breed in the brackish water environment (Kume et al. 2005). Therefore, the present results support the assumption by Arai et al. (2003a, b) that the greater part of the Japan Sea form's population collected from the Tokachi River and Lake Akkeshi were the estuarine type, not being the anadromous type.

In consequence, our field observations show that a part of Japan Sea forms in western Hokkaido Island breed in the seawater tidal pools. In addition, otolith microchemistry analyses in both adult and juvenile individuals of the Japan Sea form indicate that they probably live in brackish water and/or seawater throughout its life cycle. These facts suggest that the Japan Sea form in the tidal pools can attain to full maturation and breed in the sea. In contrast, previous studies demonstrated that the Japan Sea form utilized freshwater and brackish water environments as breeding grounds (Ikeda 1933, Mori 1987, Ishikawa & Mori 2000, Kume et al. 2005). Therefore, the Japan Sea form is supposed to have the potential for breeding in various salinity environments. In spite of that, no population with freshwater resident life history style has been found in the Japan Sea form (Higuchi & Goto 1996, Higuchi et al. 1996, Arai et al. 2003a, b). In rearing experiments, juvenile fishes of the Japan Sea form did not survive for more than two or three months in freshwater environments, being attacked by thyroid goiter (Hamada 1975, Honma et al. 1977, Sugisaki & Honma 1981, Yamada 2003). Thus, lack of freshwater populations of the Japan Sea form in natural waters may be attributed to their low freshwater adaptability

in young developmental period. In laboratory experiments, on the other hand, the anadromous adults of the Japan Sea form collected from the Shinano River, Niigata Prefecture, Japan, which breed in freshwater environments (Ikeda 1933, Mori 1987, Ishikawa & Mori 2000), was found to spawn in the seawater environment (Sugisaki & Honma 1981). This experimental data suggest that the Japan Sea form has originally high salinity tolerance in breeding period. The Japan Sea form is thought to have diverged from an ancestral stock of the Pacific Ocean form and attained high salinity tolerance during the period of isolation in the old Sea of Japan about 2 million years ago (Higuchi & Goto 1996). During Pleistocene, salinity in the Sea of Japan might have fluctuated from 20‰ to 34‰, based on salinity-balance model (Matsui et al. 1998).

The evolution of diadromous migration in fish can be generally explained by a difference in food availability for fishes between marine and freshwater habitats, namely the food availability hypothesis (Gross et al. 1988). *Gasterosteus aculeatus* is widely distributed at high latitudes (Wootton 1984), where the primary productivity in seawater habitats is higher than that in freshwater habitats. The Japan Sea form has anadromous and estuarine life history styles (Higuchi & Goto 1996, Arai et al. 2003a, b), the juveniles migrating to the sea with high productivity for growth and the adults returning to freshwater or brackish water with relatively low productivity for breeding. However, it may not be always benefited for the Japan Sea form's individuals to migrate for breeding into freshwater or brackish water environments: spawning migration from the sea to rivers may have an energy cost associated with further swimming, and the other cost may be physiological one which accompanies with osmotic and ionic regulation between different salinity environments. The occurrence of the sea-spawning population in

the Japan Sea form, therefore, may have been resulted from the site alteration from freshwater to seawater environment for not only growth but also breeding. These suggest that the life history style of the Japan Sea form has evolved in dependence on the seawater environment.

In the Pacific Ocean form, contrastingly, the freshwater residents are found in many lakes and streams (Bell & Foster 1994, Arai et al. 2003a, b). In fact, it is well known that, in *G. aculeatus* except for the Japan Sea form, the freshwater resident populations have frequently derived from the anadromous type (Mori 1990, Bell & Foster 1994, Higuchi et al. 1996). These suggest that the life history of the Japan Sea form might have evolved in different process from that of the other *G. aculeatus* taxa including the Pacific Ocean form with respect to salinity dependence. Therefore, the existence of sea-spawning populations within the Japan Sea form should provide an important insight into understanding the evolutionary process of the life history style in this form which is highly dependent on the seawater environment. Further comparative ecological and physiological studies of the Japan Sea form among a number of breeding populations from different salinity environments are needed to understand deeply the diversity of life history evolution in *G. aculeatus* complex.

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Table 1. Physical environments at four tidal pools where the breedings of the Japan Sea form of *Gasterosteus aculeatus* were found in 2003.

Location	Area (m ²)	Maximum water depth (cm)	Mean salinity (‰)	Mean water temperature (°C)	Substratum
BK1	20	20	34.9 (34–36)	22.3 (21.7–23.5)	Rock and sand
BK2	15	70	34.0 (30–35)	22.3 (21.0–23.1)	Rock and sand
Sea ^a			34.8 (34–36)	16.4 (16.4–16.5)	
KB	10	70	34.2 (31–36)	25.1 (22.2–28.0)	Rock and sand
KM	25	70	33.3 (32–34)	21.5 (21.4–21.6)	Rock and sand
Sea ^b			36.3 (36–37)	16.7	

^aWater collected from the Benkei Port.

^bWater collected from the Cape Kamui.

BK1: tidal pool inside the Benkei Port, BK2: tidal pool in inter-tidal zone of the Cape Benkei, KB: tidal pool in the Cape Kabuto, KM: tidal pool in the Cape Kamui (see text).

Numerals in parentheses indicate the range.

Table 2. Specimens of the Japan Sea form of *Gasterosteus aculeatus* used for otolith microchemistry analyses.

Location	Sampling date	Stage of fish	No. of fish examined	Total length (mm)		Sr:Ca ratios	
				Mean \pm SD	Range	Mean \pm SD	Range
BK1	15–16 June 2003	adult	6	72.0 \pm 2.18	66.2–79.1	5.8 \pm 0.56 $\times 10^{-3}$	5.1–
KB	16 June 2003	adult	1	62.6		5.6 $\times 10^{-3}$	
	16 June 2003	juvenile	9	23.7 \pm 0.346	22.1–25.4	5.7 \pm 0.22 $\times 10^{-3}$	5.4–
KM	17 June 2003	adult	7	74.6 \pm 2.18	62.9–77.3	5.7 \pm 0.49 $\times 10^{-3}$	5.2–

BK1: tidal pool inside the Benkei Port, KB: tidal pool in the Cape Kabuto, KM: tidal pool in the Cape Kamui (see text).

Legend of Figures

Figure 1. Sampling locations (circles) of the Japan Sea form of *Gasterosteus aculeatus* in western Hokkaido Island, Japan.

Figure 2. Typical changes in otolith Sr:Ca ratio along line transects from the core (0 μm) to the edge in the sagittal plane of sagittal otoliths of specimens of the Japan Sea form of *Gasterosteus aculeatus* collected from three localities in 2003 (Table 2). Figures show adult fishes from (a) BK1, (b) KB and (c) KM, and (d) juvenile fishes from KB (see text).



