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Authors: Inoue, Koji, and Takei, Yoshio

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Diverse Adaptability in *Oryzias* Species to High Environmental Salinity

Koji Inoue* and Yoshio Takei

Ocean Research Institute, the University of Tokyo,
Nakano, Tokyo 164-8639, Japan

ABSTRACT—The genus *Oryzias* containing freshwater (FW) and seawater (SW) species is a potential model for studying mechanisms of osmotic adaptation. In this study, we compared SW adaptability of four *Oryzias* species, *O. javanicus*, *O. dancena*, *O. latipes* and *O. marmoratus* inhabiting different osmotic environments. SW adaptability was evaluated at several stages of the lifecycle: (i) survival rates of adult fish after transfer from FW to 50%SW or SW, (ii) spawning ability in FW and SW, (iii) fertilization rates in FW and SW, and (iv) hatching rates in FW, 50%SW and SW. Results obtained agreed with the natural habitat of each species: *O. javanicus*, which inhabits SW or brackish water (BW), is fully adaptable to both SW and FW at all the stages examined. The BW species *O. dancena* also revealed high SW adaptability except for the hatching rate. *O. marmoratus*, confined in FW, exhibited low SW adaptability at all stages examined while *O. latipes*, another FW species, was adaptable to SW at most stages examined. Based on these results, the role of SW adaptability to the distribution area of each species is discussed.

Key words: *Oryzias*, adaptation, hatching, fertilization, distribution

INTRODUCTION

The genus *Oryzias* consists of more than 14 species including medaka (*O. latipes*), a species well known as a laboratory animal (Naruse *et al*., 1993; Naruse, 1996; Roberts, 1998). Each species of this genus reveals a specific pattern of geographic distribution; some species are endemic to freshwater (FW) lakes in an Indonesian Island, while others are distributed widely in FW, brackish water (BW) and seawater (SW) area of Asia (Naruse *et al*., 1993; Naruse, 1996; Roberts, 1998). Since such distribution patterns have hardly been altered by human activities because of low commercial value, *Oryzias* species offer interesting examples for studies of species diversity, evolution and ecology.

The genus *Oryzias* is also a potential model for studies of adaptation to hyper- and hypoosmotic environments because diverse osmotic adaptability among species is expected. In this paper, we compared the SW adaptability of four *Oryzias* species, *O. javanicus*, *O. dancena*, *O. latipes* and *O. marmoratus* inhabiting different osmotic envronments. *O. javanicus* is found in SW and BW of Thailand, the Malaysian Peninsula and Indonesian Islands (Iwamatsu *et al*., 1982; Naruse, 1996; Roberts, 1998). *O. dancena* (also known as *O. melastigma*) is widely distributed in BW and FW ranging from Pakistan and India to Burma and Thailand (Naruse, 1996; Roberts, 1998). *O. latipes* is distributed widely in FW of Japan, Korea and China (Naruse *et al*., 1993; Naruse, 1996) although it is sometimes found in BW (Miyamoto *et al*., 1986). *O. marmoratus* is endemic to Lake Towuti, a highland FW lake of Sulawesi Island, where Adrianichthid fish including *Oryzias* are highly diversified (Naruse *et al*., 1993; Naruse, 1996). The results showed that adaptability to SW at different life stages correlated with distribution area in their natural habitats.

MATERIALS AND METHODS

Fish

All experiments were carried out according to the guideline for animal experiments, Faculty of Science, University of Tokyo. *O. javanicus* sampled at Penan, Malaysia was obtained from Dr. J. Koyama of Kagoshima University, *O. dancena* was from Dr. K. Naruse of University of Tokyo, HNI strain of *O. latipes* was from Drs. A. Shima and A. Shimada of University of Tokyo, and *O. marmoratus* was from Drs. M. Sakaizumi of Niigata University and K. Naruse of University of Tokyo. All experiments were performed at 26°C. Aged tap water and natural water of North Pacific Ocean (approximately 35‰) were used as FW and SW, respectively. *O. javanicus* was bred and maintained in SW and other three species were in FW.

Survival rate of Adult fish

For each species, 24 individuals acclimated to FW or SW for

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* Corresponding author: Tel. +81-03-5351-6465;
FAX. +81-03-5351-6462.
E-mail: inouek@ori.u-tokyo.ac.jp

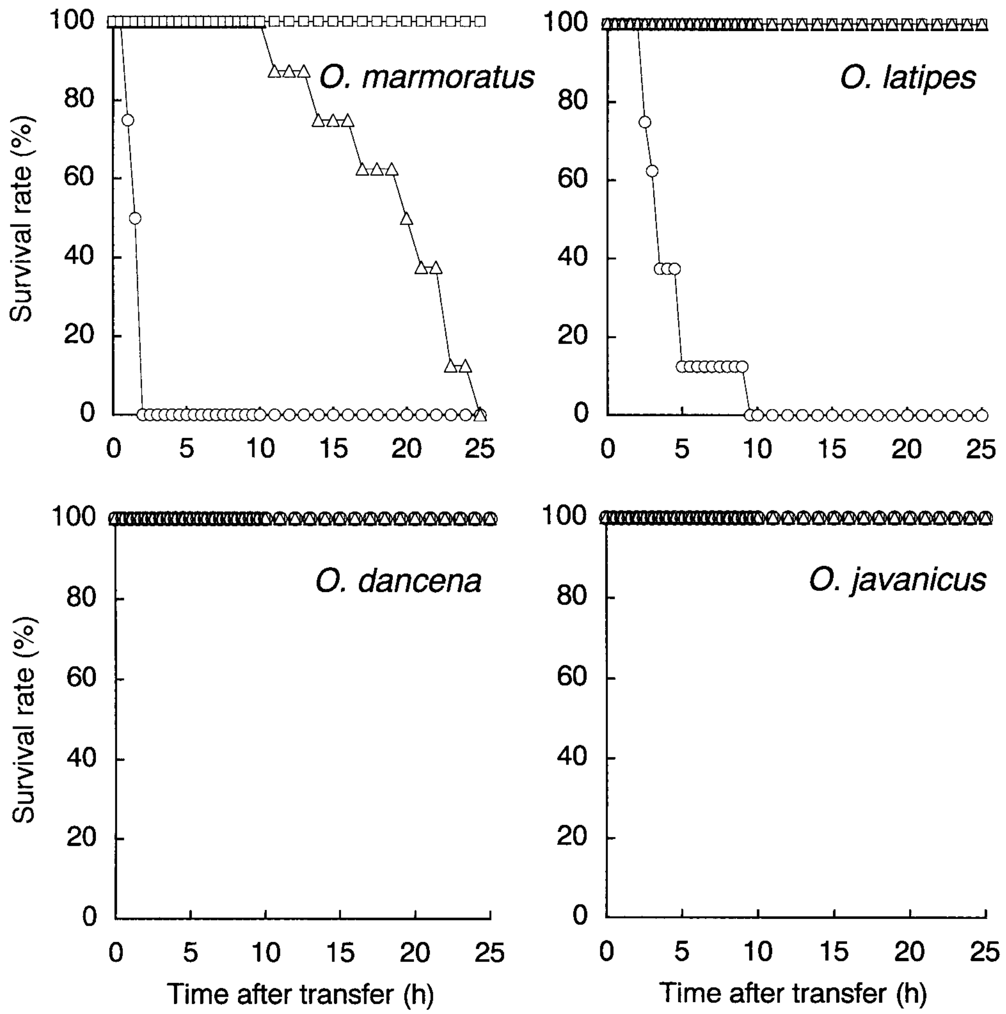

Fig. 1. Survival of four *Oryzias* species after direct transfer from freshwater (FW) to seawater (SW), 50% SW and FW (n=8). Circles, triangles and squares indicates survival rates in SW, 50% SW and FW, respectively. Note that all *O. dancena* and *O. javanicus* survived in SW, 50% SW and FW and thus the three symbols are piled up at same points.

2 wks were prepared. They are divided into 3 groups of 8 individuals and transferred directly into small plastic aquaria filled with 3L of FW, 50%SW and SW, respectively. The survival was monitored every 1 hr for 25 hr. The death of fish was defined as the stopped movement of the operculum. In *O. latipes*, an additional experiment was performed: 8 individuals that have been reared only in FW were transferred to SW after 24 hr acclimation to 50% SW and the survival was monitored everyday for 30 days.

Observation of spawning

Four adult male and 4 adult female of each species were maintained in a 18L aquarium filled with FW or SW under the controlled photoperiod of 14 hr light and 10 hr dark. Spawning was watched everyday for 1 hr from the start of the light period during which spawning always occurred.

Fertilization rate

Spawned eggs were collected from the abdomen of females of each species approximately 5 hr after the start of light period. Eggs in which cleavage progressed normally to 16–128 cell stage (Stages 6–8 of Iwamatsu, 1994) or more advanced stages were counted as fertilized. In our preliminary experiment, it was found that most unfertilized eggs have unbroken cortical alveoli just after

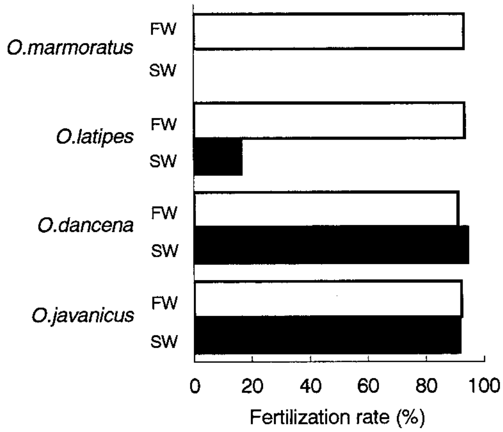

Fig. 2. Fertilization rates in eggs of four *Oryzias* species spawned in seawater and freshwater. n=86 for FW-spawned *O. marmoratus* eggs, n=242 and 173 for FW- and SW-spawned *O. latipes* eggs, n=101 and 52 for FW- and SW-spawned *O. dancena* eggs, and n=75 and 71 for FW- and SW-spawned *O. javanicus* eggs, respectively.

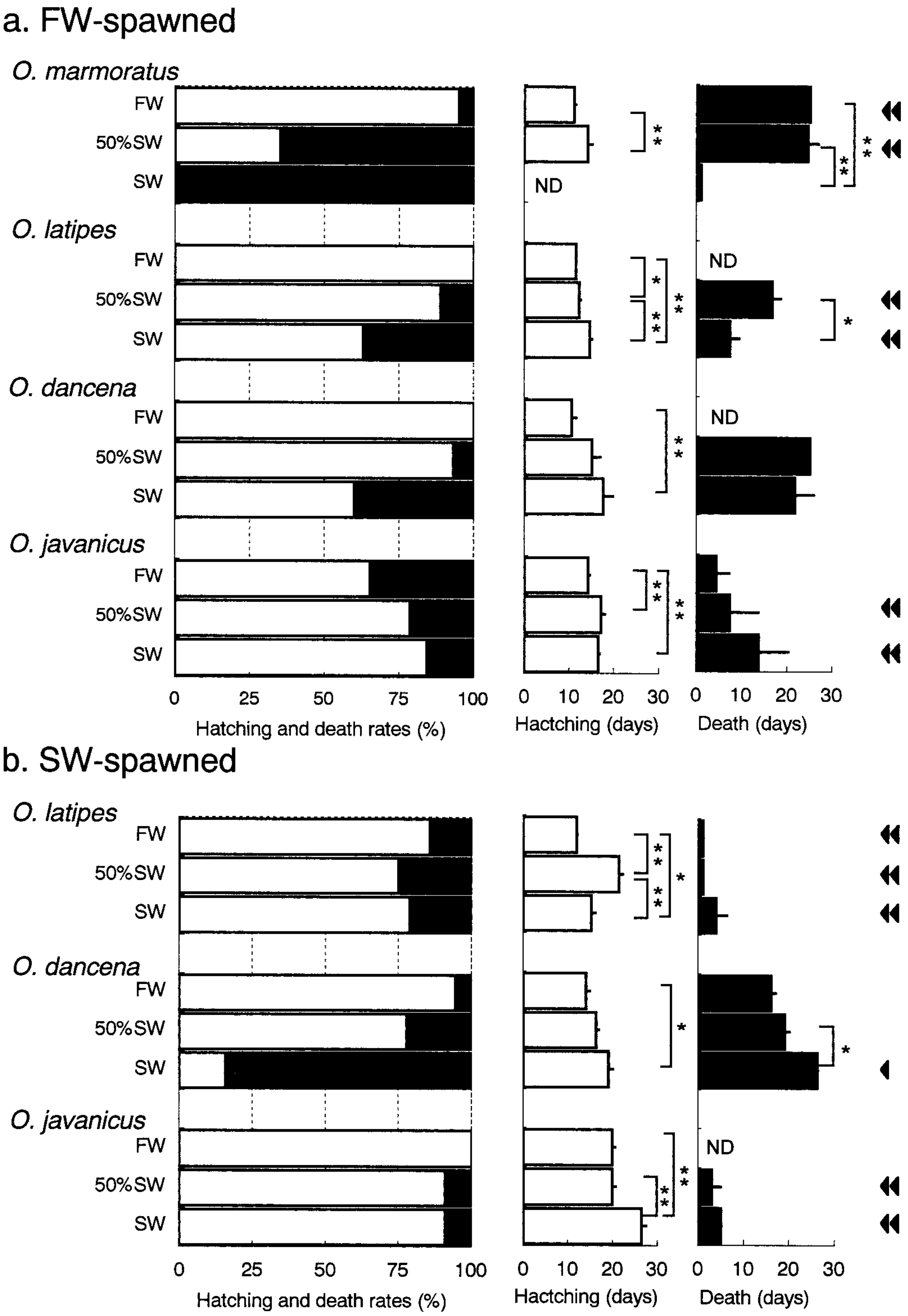

Fig. 3. Comparison of hatching and death of eggs of four *Oryzias* species cultured in freshwater (FW), 50% seawater (SW) and SW. Results using eggs spawned in FW and SW were shown in panels a and b, respectively. In the left graph of each panel, rates of embryos hatched out (open bars) and those of embryos died before hatching (solid bars) are shown. In the center graph, the time of hatching after fertilization in hatched embryos is shown as means±S.E.M. In the right graph, the time of death after fertilization in embryos died before hatching is shown as means±S.E.M. Significant differences among eggs cultured in FW, 50% SW and SW were indicated by asterisks. Significant differences between the time of hatching and that of died before hatching were indicated by arrowheads at the right end. Single and double asterisks and arrowheads represent p<0.05 and p<0.01, respectively. Numbers of fertilized eggs used for each group were 23–30, 22–40, 14–19, and 14–30 for *O. marmoratus, O. latipes, O. dancena*, and *O. javanicus*, respectively.

spawning. Although some of them are sometimes found activated by unknown stimuli a few hours after spawning, they are distinguishable from fertilized eggs because they do not reveal regular patterns of cleavage.

Sperm activity

The testis was isolated from 3 adult males of each species that had maintained in FW for more than 30 days after decapitation. It was divided into 2 pieces using forceps and each piece was put into a drop of FW and SW on a slide glass, respectively. Sperm cells were suspended by tearing the piece of the testis using forceps and the rate of motile sperm cells was estimated immediately after suspension according to the standard of Suquet *et al.* (1992) under the microscope (BX-51, Olympus, Tokyo).

Hatching rate

Fertilized eggs obtained in FW or SW were transferred to FW, 50% SW and SW about 5 hr after spawning. Eggs were incubated separately using 96-well culture plates at 26°C. Embryos that died before hatching and those that successfully hatched were counted everyday. Embryos in which collapse of the morphology or stop of the heartbeat was observed were regarded as dead.

To examine hatching ability separately from the difference in development, FW-spawned fertilized eggs of *O. javanicus*, *O. dancena* and *O. latipes* were collected about 5 hr after spawning and incubated in SW until the hatching of the first individual. After the hatching of the first fish, other embryos were divided into two groups, transferred into newly prepared FW and SW, respectively, and incubated at 28°C. Hatching was checked every 30–120 min for 24 hr.

Statistics

Results were expressed as means±S.E.M. Student's t-test was employed to test for significant difference in time of adult survival, hatching and death of embryos.

RESULTS

Survival of adult fish after direct transfer

All *O. javanicus* and *O. dancena* survived the transfer to 50% SW and SW (Fig. 1). In contrast, all *O. marmoratus* and *O. latipes* transferred to SW died within 2 hr and 9.5 hr, respectively. The average time of survival in SW was, 1.6±0.2 hr in *O. marmoratus* and 4.3±0.8 hr in *O. latipes*, respectively, which was significantly different from each other ($p<0.01$). *O. marmoratus* was not adaptable even to 50% SW; all fish died within 25 hr (19.3±1.7 hr in average) after transfer to 50% SW. On the other hand, *O. latipes* survived in 50% SW for more than 25 hr (Fig. 1) and no fish died after elongated exposure to 50% SW for up to 48 hr (data not shown). It was also found that prior acclimation of *O. latipes* in 50% SW for 24 hr allowed them to survive in SW for more than 30 days. SW-acclimated *O. javanicus*, *O. dancena* and *O. latipes* could survive the transfer back to FW.

Spawning and fertilization

O. latipes spawned eggs everyday while other three species spawned several times a week in FW. Most eggs of the four species were fertilized eggs (Fig. 2). *O. latipes*, *O. dancena* and *O. javanicus* also spawned eggs in SW as frequently as in FW. Most eggs of *O. dancena* and *O. javanicus* were fertilized as in FW. However, 83.9% of *O. latipes* eggs spawned in SW were unfertilized (Fig. 2).

The frequency of motile sperms of *O. javanicus* was more than 75% in FW and more than 50% in SW (classes 4 and 3 of Suquet *et al.*, 1992, respectively). The same result was also obtained in *O. dancena*. While the sperm of *O. latipes* and *O. marmoratus* was more than 75% motile in FW (class 4), no apparently motile sperm was found in SW (class 0).

Hatching

Eggs of *O. javanicus* spawned in FW revealed the highest hatching rate in SW followed by 50%SW and FW (Fig. 3a). In contrast, other three species revealed the highest hatching rates in FW. In all species, hatching tended to be delayed by the increase of salinity. Hatching rates of FW-

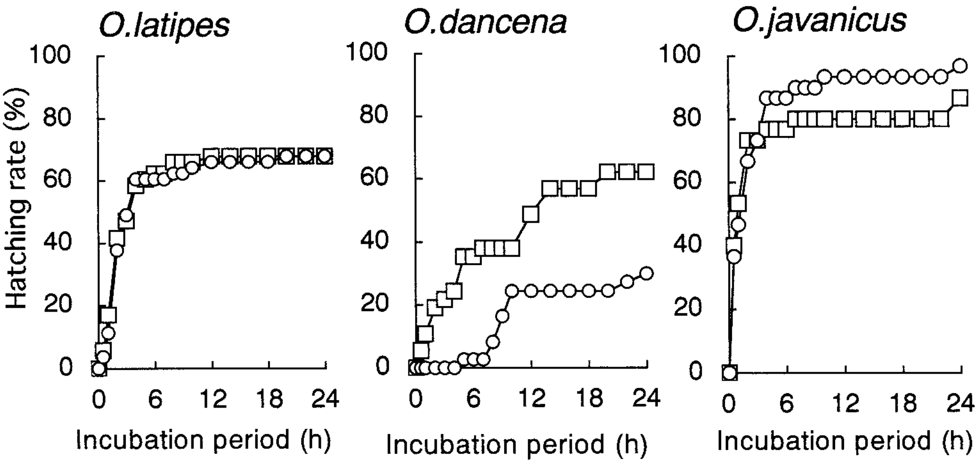

Fig. 4. Comparison of hatching rates of embryos of three *Oryzias* species in freshwater (FW) and seawater (SW). Fertilized eggs, spawned in FW, were cultured in SW until the hatching of the first embryo was observed and then equal number (n=53, 37, and 30 for *O. latipes, O. dancena*, and *O. javanicus*, respectively) of pre-hatching embryos were transferred into FW and SW. Hatching and death of embryos were checked every 30–120 min. Squares and circles indicate embryos transferred to FW and SW, respectively.

spawned *O. dancena* and *O. latipes* eggs in SW were almost the same, about 60%. *O. marmoratus* embryos died immediately after the transfer to SW and revealed a low hatching rate even in 50%SW.

Similar results were obtained with *O. latipes*, *O. dancena* and *O. javanicus* eggs spawned in SW (Fig. 3b). In this case, more than 90% of *O. javanicus* embryos hatched out in all salinity conditions. The hatching rate of SW-spawned eggs was higher than that of FW-spawned eggs in *O. latipes,* but it was lower in *O. dancena*.

To examine the hatching ability in SW separately from the difference of development, we cultured FW-spawned fertilized eggs of *O. javanicus*, *O. dancena* and *O. latipes* in SW until the hatching of the first embryo and then transferred to newly prepared SW and FW (Fig. 4). After transfer, the hatching of *O. javanicus* and *O. latipes* embryos immediately started both in FW and SW possibly by stimulus of the change of water or elevated temperature. The hatching rate of *O. latipes* 24 hr after the transfer was almost the same in SW and in FW and that of *O. javanicus* was slightly higher in SW than in FW. On the other hand, the hatching of *O. dancena* embryos delayed in SW and the hatching rate 24 hr after the transfer was higher in FW than in SW.

DISCUSSION

To expand the habitat from FW to the sea, it is indispensable for adult fish to have ability to adapt to hyperosmotic condition in SW. In addition, processes in early development such as the fertilization, embryonic development and hatching are influenced directly by ambient salinity in oviparous fish. Thus, it is necessary to adapt all such processes to complete the whole lifecycle in the sea.

Adult fish of *O. javanicus* and *O. dancena* revealed high adaptability to sudden changes of the ambient osmotic pressure in both directions, i.e., from FW to SW or vise versa, whereas *O. marmoratus* exhibited the lowest ability for SW adaptation among the four species (Fig. 1). The result of *O. latipes* was intermediate: Although this species failed to survive direct transfer to SW, it became adaptable after 24 hr acclimation in 50% SW. This result suggests that *O. latipes* is potentially euryhaline as suggested by previous reports (Yanagishima, 1957; Miyamoto *et al*., 1986; Haruta *et al*., 1991; Sakamoto *et al*., 2001) but lacks the adaptability to a sudden increase of the ambient osmotic pressure. This characteristic is especially interesting for studies of osmoregulatory mechanisms: Using this species, it becomes possible to study functions of fast-acting osmoregulatory factors such as natriuretic peptides and angiotensin II separately from slow-acting hormones such as cortisol and growth hormone (Takei, 1993; Takei and Hirose, 2002).

It is well known that the sperms of SW fish are activated when they are exposed to water with osmotic pressure or concentration of ions such as K^+ higher than the seminal plasma, while those of FW fish are activated by lower osmotic pressure or ion concentration (Morisawa and Suzuki, 1980; Billard and Cosson, 1992). We found in this study that the sperm of *O. dancena* and *O. javanicus* can be activated not only in FW but also in SW. These species offers a new model to study sperm activation mechanisms. In *O. latipes*, approximately 16% of eggs spawned in SW were fertilized (Fig. 2) although the sperm was immotile in SW. It is possible that a very small number of sperms are motile in SW, or the immotile but mature sperm might be transferred passively to the micropyle.

We also found that hatching rates in SW are different among species in this study (Figs. 3, 4). The hatching rate of FW-spawned eggs in SW was the highest in *O. javanicus*, followed by *O. latipes* and *O. dancena*, and the lowest in *O. marmoratus*. The hatching rate of SW-spawned eggs was the highest in *O. javanicus*, followed by *O. latipes* and then *O. dancena*. The low hatching rate of *O. marmoratus* in SW is obviously due to a low survival rate of embryos in SW, not due to the failure in hatching itself, because all embryos transferred in SW died within 1 day. The cause of the decease of hatching rate of *O. latipes* embryos in SW also seems to be the death of embryos before reaching the hatching stage because the average period for hatching is longer than that of death of embryos (Fig. 3). On the other hand, it seems that the decrease of hatching rate of *O. dancena* embryos in SW is due to the low ability of hatching in SW, i.e., embryos of this species developed successfully to hatching stage but failed to hatch out. This idea is supported by (i) the average time of embryo death longer than the average hatching time (Fig. 3), (ii) our microscopic observation, in which we found that *O. dancena* embryos that failed to hatch out died after the absorption of most of the yolk without apparent morphological abnormalities (data not shown), and (iii) the higher hatching rate in FW than in SW when embryos that had been cultured in SW until the hatching stage were transferred to FW or SW (Fig. 4). The mechanism of hatching has been extensively studied in *O. latipes*; it has been reported that two enzymes, low choriolytic enzyme (LCE) and high choriolytic enzyme (HCE), cooperatively function at the time of hatching (Yasumasu *et al*., 1997 for review). Although a possible cause of interspecific differences in hatching ability in SW is that the range of salt concentration in which hatching enzymes can function is different among species, information on relationship between activities of hatching enzymes and salinity is not available at present except for the report in *O. latipes* by Yamagami (1973). Molecular cloning of cDNAs encoding hatching enzymes of *Oryzias* species is now in progress to compare enzyme activities under the different salt concentration using recombinant techniques.

Results obtained in this study are consistent well with the natural habitat of each species (Table 1). *O. javanicus*, a BW and SW species, fulfilled all the requirements. We also found that the reproduction of this species became less active after a long culture in FW (unpublished observation). Thus, it seems that the lifecycle of this species has adapted to SW than to FW. *O. dancena* also fulfills most require-

Table 1. Adaptability to seawater in four *Oryzias* species

Species	*O. marmoratus*	*O. latipes*	*O. dancena*	*O. javanicus*
Geographic Distribution	Endemic to Lake Towuti in Sulawesi Island	Japan, Korea and China	Pakistan to Thailand	Malaysian Peninsula and Indonesian Islands
Habitat	FW	FW or BW	BW or FW	BW or SW
Immediate adaptation to SW	–	–	++	++
Slow adaptation to SW	–	++	++	++
Spawning in SW	N.D.	++	++	++
Fertilization in SW	N.D.	+	++	++
Sperm activation in SW	±	±	++	++
Embryonic development in SW	–	+	++	++
Hatching in SW	–	++	+	++

++, possible; +, possible in low rate; ±, hardly possible; –, impossible; N.D., no data.

ments to live in SW. It seems, however, more advantageous for this species to inhabit BW and FW than SW because the hatching system is not fully adapted to SW. *O. latipes* seems more suitable to live in FW than the above two species, which is consistent with the fact that the major habitat of this species is FW area. The current results showed that it became adaptable to SW after a short acclimation period in 50% SW and spawned normal eggs in SW although the fertilization rate is low. These results are consistent with the fact that this species is sometimes observed in a pond containing high concentration of salt (Yanagishima and Mori, 1957). *O. marmoratus*, found only in a FW lake, revealed

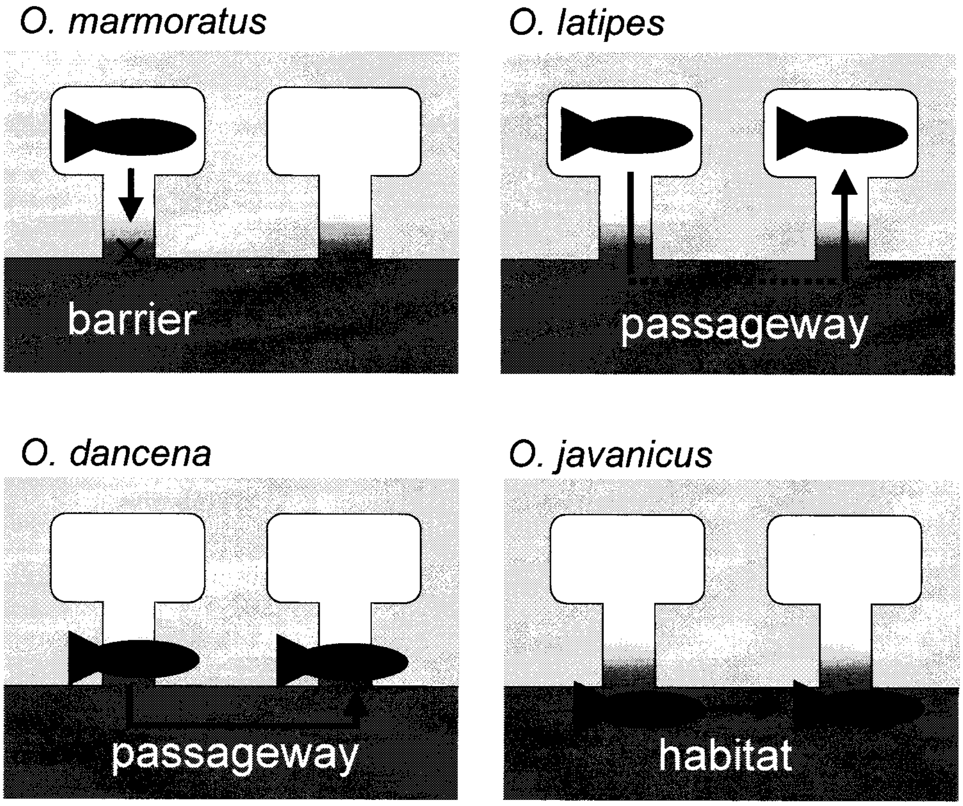

Fig. 5. Possible route of expansion of distribution area through the sea in four *Oryzias* species. White area and dark gray area represent FW and SW, respectively.

the poorest adaptability to SW at every stage of the lifecycle.

Adaptability to SW may be one of the accounts for the geographical distribution of each species. Myers (1938) and Darlington (1957) proposed a classification of FW fish into three categories based on salt tolerance; the primary division consisting of strict FW fish that cannot or do not enter the sea, the secondary division consisting of fishes that occur chiefly in FW but can enter the sea and survive there for a limited time, and the peripheral division consisting of fishes that are found in FW but connected closely with the sea. Darlington (1957) emphasized that SW is the most effective barrier to stop the dispersal of fishes in primary division and the present distribution of fishes in other divisions are often the result of dispersal through the sea. Although he classified *Oryzias* into the secondary division, results in the present study suggested that SW-tolerance of each species is too variable to put them into the same division. *O. marmoratus* is apparently a member of the primary division. While Lake Towuti has a river to the sea, the sea is merely a “barrier” to prevent the migration to other regions (Fig. 5). *O. latipes* is likely to be in the secondary division. While this species is essentially a FW species, results in this study suggested that it has enough adaptability to achieve the transition through the sea, which may be one of the causes of wide geographic distribution of this species. *O. dancena* seems to be at an intermediate position between the secondary and the peripheral divisions. For this species, the sea is not the best habitat but can be used as “passageway” to the next BW or FW area. *O. javanicus* can be classified into the peripheral division but is somewhat SW fish-like. The sea is a part of the “habitat” and it seems easy to expand the distribution area along the coast for this species.

Among the four species examined in this study, *O. latipes* is well known as a laboratory animal. This species has various advantages for experimental purposes: it is small in size, tough enough to be maintained in small aquarium in the laboratory, frequently spawn transparent eggs, and have relatively short generation time (Ozato and Wakamatsu, 1994; Ozato *et al*., 1989). We found that other three species, *O. javanicus*, *O. dancena* and *O. marmoratus* share such advantages with *O. latipes*. Thus, it seems possible to apply to these species experimental approaches including foreign gene transfer (Ozato *et al*., 1986; 1989; Inoue *et al*., 1990; 1992), mutagenesis (Shima and Shimada, 2001) and nuclear transplantation (Wakamatsu *et al*., 2001). We expect that the application of such experimental techniques to various *Oryzias* species would bring us new information to understand how fish has developed diverse mechanisms to adapt to hyper- and hypo-osmotic environments.

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