Sexual Difference in Acid Tolerance in Medaka *Oryzias latipes*

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Sexual differences in survival and changes in plasma ion levels after transfer from neutral water to acid water prepared with sulfuric acid were examined in medaka *Oryzias latipes*. In acid water at pH 3.5 and 3.8, survival of female fish was better than male. During exposure to acid water at pH 4.1, there was no mortality in either sex. Male fish showed a transient decrease in the plasma sodium level 24 h after transfer to pH 4.1, whereas there was no change in the female. The plasma level of sulfate was not affected in the female, but a significantly high level was consistently observed during the exposure in the male. In both sexes, the activity of gill Na^+ , K^+ -ATPase increased within 24 h after transfer to pH 4.1, and this high level was maintained for 1 month. Treatment with short day-photoperiod (8L: 16D) caused a cessation of spawning due to inactivation of the testis and ovary. Transfer to acid water caused a decline in plasma sodium only in male fish, but not in female even under a short photoperiod. These results indicate that female medaka are more tolerant to acidic environment, possibly with a greater osmoregulatory ability than male fish.

Key words: acid water, osmoregulation, sexual difference, medaka, Na^+ , K^+ -ATPase, sodium, calcium, sulfate

In freshwater fish, acidification of environmental water may result in acute damage followed by death or cause a variety of chronic physiological problems including failure of reproductive processes.^{1,2)} There is some consensus that the physiological problems of fish exposed to acid water is brought about a chain of events starting with a large increase in net sodium loss mainly from the gills.^{1,3)} In fish surviving after exposure to acid water, recovery of the decreased sodium influx and reduction of the sodium efflux have been observed.³⁾ Reduced pH of stream or lake water due to acid rain have not noted in any areas of Japan, because of chemical neutralization of loaded acid based on soil alkalinity.⁴⁾ However, the continuous deposition of acidic compounds observed in recent years may begin to affect the buffering capacity of soil and cause water acidification of some streams or lakes in future.

Reproductive failure of female fish under chronic acidic condition has been reported in several species.^{1,2)} On the other hand, there is little information on the ionic disturbance of male fish which seems to involve in reproductive failure in acidic environment. A sexual difference in adaptability to different salinities has been reported,^{5,6)} while that in adaptability to acidic environment is not known well.

The habitat of medaka *Oryzias latipes* is generally in swamps, ditches, and streams with low flow and heavy vegetation. Medaka are known to tolerate wide ranges of environmental temperature, salinity, and pH,^{7–10)} and spawn almost every morning from spring to autumn.¹¹⁾ The present study was undertaken to examine the possible sexual differences in acid tolerance which may involve in the reproductive failure in acidic environment using meda-

ka in spawning and degenerative phases controlled by artificial photoperiod. Survival rates in lethal acidic water (pH 3.5 and 3.8), changes in plasma ion levels and gill Na^+ , K^+ -ATPase activity in sublethal acidic water (pH 4.1) were analyzed in male and female fish.

Materials and Methods

Animals

The red variety of adult medaka *Oryzias latipes* were reared for successive generations in outdoor freshwater ponds at National Research Institute of Fisheries Science at Ueda. The original pH of water used in the present study was 7.6, and concentrations of sodium, calcium, and sulfate were 0.3, 0.4, and 0.1 mM, respectively. Concentrations of both aluminum and copper were 0.1 μM .

One month before the experiments, fish weighing 0.4–0.8 g, were transferred to indoor 200 l rectangular glass tanks containing fresh water at 25°C, and the pH adjusted to 7.0 with 0.1 N sulfuric acid by a pH controller (NPH-6800, Nissin, Tokyo) connected to a peristaltic pump (AC-2110, Atto, Tokyo) under a lighting regime of 14 h light and 10 h dark (14L: 10D). They were fed commercially-reared mosquito larvae. Experiments were carried out from March to August. Under these conditions, the fish spawned almost every morning.

Experimental Protocols

Survival in acid water: Fish were transferred directly from neutral water (pH 7.0) to 200 l rectangular glass tanks containing fresh water at 25°C, and the pH adjusted to 3.5, 3.8, or 4.1 by pH controllers as described above.

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Twenty males and twenty females were used for each experimental group. Mortality of the fish was recorded twice daily up to 7 days during the experiment.

Time-course of changes in plasma ion levels and gill Na^+ , K^+ -ATPase activity: Fish were reared in 200 l rectangular glass tanks containing fresh water at 25°C for one month. Water pH of the experimental tank was then reduced from 7.0 to 4.1 within 30 min by the addition of 0.1 N sulfuric acid. The water pH was then kept at 4.1 for one month by the pH controller. The control tank was kept at pH 7.0 during the experiment. At various intervals, about 2 μl of blood samples were collected directly from the caudal artery with a fine-tipped capillary tube as described by Jozuka and Adachi.⁹⁾ Plasma samples were immediately separated from the blood by centrifugation at $3,000 \times g$ for 5 min, and kept frozen at -80°C until analyses. Immediately after bleeding, gill filaments were cut from ceratobranchials to measure Na^+ , K^+ -ATPase activity as described by McCormick.¹²⁾ They were placed in 100 μl of ice-cold sucrose-EDTA-imidazole solution (150 mM sucrose, 10 mM disodium ethylenediamine tetraacetate, 50 mM imidazole, pH 7.3). They were then stored at -80°C and were analyzed within 3 months after sampling. After the experiment, concentrations of sodium, calcium, and sulfate at pH 4.1 were 0.9, 0.6, and 0.6 mM, respectively. Concentrations of aluminum and copper were 1.7 and 0.2 μM , respectively. At pH 7.0, concentrations of sodium, calcium, and sulfate were 0.7, 0.4, and 0.2 mM, respectively, and those of aluminum and copper were 0.2 and 0.1 μM , respectively.

Prevention of gonadal maturation: The other group of fish was reared in a tank at pH 7.0 under a lighting regime of 8 h light and 16 h dark (8L: 16D) for one month. They were then transferred to a tank containing water at pH 4.1 or 7.0, and sacrificed 24 h after the transfer as described above. After bleeding, gonads of more than 14 representative fish for each sex were dissected and fixed in Bouin's solution. They were then dehydrated through graded ethanols and embedded in paraffin. Serial sections were cut at 5 μm , mounted on gelatinized slides, and stained with Mayer's hematoxylin and eosin.

Measurements of Ions

Sodium and calcium concentrations were measured by flame atomic absorption spectrophotometry (AA-855, Nippon Jarrell-Ash, Kyoto). Aluminum and copper concentrations were measured by atomic absorption spectrophotometry with graphite furnace (Z-8200, Hitachi, Tokyo). The sulfate concentration was measured by ion-exchange chromatography (ICA-5000, Toa Electronics, Tokyo).

Measurement of Gill Na^+ , K^+ -ATPase Activity

Gill Na^+ , K^+ -ATPase activity was measured as the ouabain-sensitive hydrolysis of adenosine triphosphate (ATP) enzymatically coupled to an oxidation of nicotinamide adenine dinucleotide (reduced form, NADH).¹²⁾ The oxidation of NADH was directly measured in a microplate reader (MTP-120, Corona Electronics, Ibaragi). The protein concentration was measured by Protein Assay Kit (Bio-Rad, California) using bovine serum albumin as a standard.

Statistics

In the survival experiment, probit transformations of mortality were regressed on the log hours after exposure to acid water, and an analysis of variance with the hours as the variate was performed to detect a significance of difference in regression lines between the sexes as described by Groden and Lockwood.¹³⁾ Differences in plasma ion levels and gill Na^+ , K^+ -ATPase activity were analyzed by ANOVA followed by Duncan's multiple range test. Calculations were performed using a computer program, STATISTICA (Design Technologies Incorporation, Tokyo).

Results

Survival in Acid Water

The fish transferred to acid water at pH 3.5 and 3.8 stopped spawning immediately, and a significant progressive mortality was observed in both sexes for 7 days (Fig. 1). There was no mortality at pH 4.1, and the fish kept spawning daily. Regression analysis between the probit of the mortality and the log hours after transfer showed that the survival of female fish was better than of male at pH 3.5 ($F_{(1,7)}=16.175$, $P<0.05$) and 3.8 ($F_{(1,10)}=9.748$, $P<0.05$).

Time-course of Changes in Plasma Sodium Level and Gill Na^+ , K^+ -ATPase Activity

When exposed to acid water at pH 4.1 for 1 month, no mortality was seen in either male or female. The plasma sodium level in the male decreased significantly 24 h after transfer to acid water, but was restored after 7 days (Fig. 2). On the other hand, there was no significant change in plasma sodium of the female during the experiment. The plasma sulfate level in the male increased significantly 24 h after transfer, and this high level was maintained throughout the experiment, whereas no significant change was observed in the female. No change was seen in plasma calcium level of either sex. Activity of gill Na^+ , K^+ -ATPase was increased in acid water in both sexes, and the high level was maintained during the remainder of experiment

Fig. 1. Survival of male (open symbols) and female (closed symbols) medaka in acid water at pH 3.5, 3.8, and 4.1.

Fig. 2. Changes in plasma sodium, sulfate, and calcium levels of male and female medaka after exposure to acid water at pH 4.1 (○) or neutral water at pH 7.0 (●).

The vertical bars represent the mean ± SEM ($n=5-10$). **Significantly different from the level in neutral water at $P<0.05$ and $P<0.01$, respectively.

Fig. 3. Changes in gill Na⁺, K⁺-ATPase activity of male and female medaka after exposure to acid water at pH 4.1 (○) or neutral water at pH 7.0 (●).

The vertical bars represent the mean ± SEM ($n=5-7$). **Significantly different from the level in neutral water at $P<0.05$ and $P<0.01$, respectively.

Fig. 4. Light microscopic sections of gonads.

A, testis of a male reared under a lighting regime of 14L: 10D. B, testis of a male reared under 8L: 16D. C, ovary of a female reared under 14L: 10D. D, ovary of a female reared under 8L: 16D. Scale bar, 50 μm. S, sperms; SC, spermatocytes; YG, oocytes of yolk globule stage; YV, oocytes of yolk vesicle stage.

(Fig. 3).

Prevention of Gonadal Maturation

The testes of the male under 14L: 10D were filled with a large number of sperms (Fig. 4A). After the exposure to 8L: 16D for one month, sperms were still observed in

sperm ducts (Fig. 4B). The ovaries of the female reared under a long day-photoperiod had oocytes of the yolk globule stage (Fig. 4C). After the exposure to short days, a few oocytes of the yolk vesicle stage but no yolk globule stage were observed (Fig. 4D).

Figure 5 shows plasma levels of sodium, sulfate, and cal-

Fig. 5. Changes in plasma sodium, sulfate, and calcium levels of male and female medaka reared under 14L: 10D or 8L: 16D 24 h after exposure to acid water at pH 4.1 (hatched column) or neutral water at pH 7.0 (open column).

The vertical bars represent the mean $\pm$ SEM ($n=5-8$). **Significantly different from the level in neutral water at $P < 0.01$.

cium in the fish reared under 14L: 10D or 8L: 16D after exposure to acid water at pH 4.1 for 24 h. The male reared under 8L: 16D showed a significant decrease in plasma sodium and an increase in sulfate as the fish reared under 14L: 10D. The female reared under 8L: 16D showed no change in plasma sodium and sulfate levels as those reared under 14L: 10D. The calcium level of the female under 8L: 16D was significantly less than that under 14L: 10D ($P < 0.01$). However, there was no change in plasma calcium after transfer to acid water in both sexes even under 8L: 16D.

Discussion

This study reveals that female medaka are more tolerant to an acidic environment than male fish, possibly with a greater osmoregulatory ability. Female fish survived longer than male in lethal acid water at pH 3.5 and 3.8. Although both sexes survived well and kept spawning at pH 4.1, only the male showed a transient reduction in plasma sodium. These facts suggest that the sexual difference in acid tolerance is related to sodium-retaining ability and a cessation of spawning in lethal acid water. In immature brook trout *Salvelinus fontinalis*, no sexual difference was observed in changes of plasma ion levels after transfer to acid water at pH 4.5.¹⁴ Although no sexual difference in the hyperosmoregulatory ability in acid water has been

reported in fishes, an impairment of the hypoosmoregulatory ability in males of some salmonid species in seawater was observed in association with gonadal maturation.^{5,6,15} In the present study, the exposure of spawning medaka to a short photoperiod caused a cessation of spawning with regressed gonadal activity. Even under this condition, the male was less tolerant to acidic environment than the female, suggesting that the sexual difference of medaka in acid tolerance is not directly related to gonadal maturation. In salmonid fish, sex steroids are known to impair adaptability to seawater.^{16,17} While circulating levels of sex steroids were not measured in the present study, there is a possibility that sex steroids are involved in sexual difference in acid tolerance even in degenerative phase of gonadal maturation. Effects of sex steroids on acid tolerance must be clarified in future.

Both sexes of medaka showed an increase in gill Na^+ , K^+ -ATPase activity within 24 h after transfer to acid water. It is well known that Na^+ , K^+ -ATPase is mainly localized in gill chloride cells, playing an important role in excretion of excess salts in seawater.¹⁸⁻²⁰ Gill chloride cells are also likely to be involved also in the uptake of sodium and chloride ions in fresh water.²¹⁻²³ In Atlantic salmon *Salmo salar* and coho salmon *Oncorhynchus kisutch*, the exposure to acid water attenuated an increase of gill Na^+ , K^+ -ATPase activity which was accompanied by parr-smolt transformation.^{24,25} The absence of an increase in ATPase activity in acid water was discussed in relation to a disturbance of the plasma sodium balance. On the other hand, an increase in gill Na^+ , K^+ -ATPase was observed in rainbow trout *O. mykiss* after exposure to acid water, but not in largescale sucker *Catostomus macrocheilus*.²⁶ The difference in the response of ATPase activity to acid-exposure seems to coincide with the fact that the trout can regulate plasma ion levels better than the sucker in acid water. In the present study, gill Na^+ , K^+ -ATPase increased similarly in both sexes of medaka after transfer to acid water, whereas a transient reduction in the plasma sodium level was observed only in the male. In rainbow trout surviving after acid-exposure, McDonald *et al.*²⁷ have shown that the decreased rate of sodium influx exhibits only a small recovery, while the increased sodium efflux is markedly corrected to the control level, suggesting that sodium permeability of the gills reduces during adaptation to acid water. Our results suggest that an impairment of the ion balance or an increase in branchial sodium efflux was greater in male medaka than in female.

There are few reports on changes in the plasma sulfate level of fish after acid-exposure. Toxicity of water acidification by sulfuric acid has been compared to the effects of other acids in some species. The mean period of brook trout survival on exposure to acid water at pH 3.5 prepared by sulfuric acid was longer than that at the same pH prepared by hydrochloric acid.²⁸ In medaka, however, the toxicity of water acidification was essentially similar to those with hydrochloric, citric or oxalic acids.⁸ This implies that an excess of environmental sulfate is less toxic than a decrease in pH. Male medaka survived in acid water at pH 4.1 for one month in the present study, in spite of a chronic increase in the plasma sulfate level. However, female medaka could regulate their plasma sulfate level in acid water irrespective of the gametogenesis status. This

fact implies that female medaka have a system for regulating the plasma sulfate level which the male does not.

One of the possible reasons for the increased permeability of ions in acid water is leaching of Ca^{2+} away from the paracellular channels in the branchial epithelium.³⁾ Calcium in environmental water is important for survival of aquatic animals, and water acidification by acidic deposition is thought to be a problem especially in soft water ($[\text{Ca}^{2+}]$ below 0.2 mM).^{3,24,29)} On the other hand, even in hard water ($[\text{Ca}^{2+}]$ over 0.6 mM), reductions in plasma electrolyte levels are observed in brook trout.¹⁴⁾ Also in rainbow trout, Kakizawa *et al.*³⁰⁾ observed reductions in plasma electrolytes after exposure to acid water, prepared from Tokyo tap water at $[\text{Ca}^{2+}]$ about 0.6 mM, which seems to be a typical freshwater condition in Japan. In future, water acidification may occur in some area of Japan where the buffering capacity of soil is limited. Environmental trace metals including aluminum and copper in acid soft water cause osmoregulatory and respiratory disruptions.^{31,32)} In this study, an increased concentration of environmental aluminum was observed after the time-course experiment in acid water at pH 4.1, possibly dissolved from the surface of the glass tank. The level was still below that which causes acute toxicity in soft acid water ($\text{LD}_{50}=7.3 \mu\text{M}$ at pH 5.5 for brook trout).³¹⁾ Exposure to 7 μM aluminum in water at pH 5 induced a reduction in gill Na^+ , K^+ -ATPase activity in Atlantic salmon and rainbow trout.³²⁾ In this study, environmental aluminum does not seem to have disrupted gill Na^+ , K^+ -ATP activity. In tilapia *Oreochromis mossambicus* and carp *Cyprinus carpio*, acute acidification of soft water resulted in a severe decrease in the plasma ion levels, osmotic pressure and hormonal status, but gradual acidification caused only slight changes.^{33,34)} Environmental calcium, trace metals, and other factors such as the rate of acidification may interact with sexual difference in acid tolerance.

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