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Effects of Acute Acid Stress on Hatching and Mortality of Hermaphroditic Teleost, *Rivulus marmoratus* (Cyprinodontiformes; Rivulidae)

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The effects of acute acid stress on hatching success and hatching period of laboratory-reared hermaphroditic fish *Rivulus marmoratus* were examined. The effects of acute acid toxicity on mortality was also determined in three life stages of this fish. There was a significant negative effect of acid stress on hatching performance in the *R. marmoratus* embryos. The hatching success was only 5% at pH 3.5 compared to over 78% at pH higher than 4.0. The hatching period was also delayed by low pH treatments. The larval and juvenile stages were more sensitive to acid toxicity on mortality than the adult stage, but larvae and juveniles showed similar sensitivity. The 96-h LC50 value was pH 3.8 in larval and juvenile stages and pH 3.3 in adult stage.

Acidification of water is thought to have a major impact on fish mortality and structure of their populations. The field and laboratory studies have shown a clear correlation between the acid stress and decline of fish population (Brown and Sadler, 1989; Turnpenny, 1989). The recruitment failure by embryo and larva mortalities is considered as a primary factor leading to gradual loss of fish stocks of aquarium as well as of wild population (Jagoe et al., 1984; Kwain and Rose, 1985).

The oviparous mangrove rivulus, *Rivulus marmoratus* (Fig. 1), inhabits estuaries throughout the Caribbean and is the only known vertebrate that exhibits natural functional hermaphroditism with internal self-fertilization (Harrington, 1961; Hughes, 1989). Consequently, a given group of individuals originating from one progenitor is genetically homogeneous (Kallman and Harrington, 1964; Laughlin et al., 1995). This is a useful feature for excluding the effects of differing genetic makeup among test animals. In addition to these genetic merits, this fish has several other desirable attributes as an experimental animal for educational and research purposes in fish biology. The generation time is only 4-6 months. Eggs are large enough (1.8 mm in diameter) to handle and develop normally at room temperature outside the body in 14 days, enabling us to examine detailed developmental sequence through their transparent chorion. This species has an extremely broad physiological tolerance, being euryhaline (fresh water to 35‰ salinity) and eurythermal (4–40°C). Thus, the fish are hardy and easy to breed in the laboratory.

The aim of this study is to determine the effects of low pH, a key parameter of acid stress, on hatching success and hatching period and mortality of three life stages of *R. marmoratus* as part of an aquatic toxicology study. This information may serve as a baseline in acid toxicity and in establishing this species as an experimental laboratory animal.

Materials and Methods

Animal and laboratory culture

Mangrove rivulus was bred and reared as described previously (Park and Kim, 1984; Park and Yi, 1989). Fish used in this study were from the 27th to 30th generations of a single progenitor. This progenitor originated from the stock of the Zoologisches Institut und Zoologisches Museum, University of Hamburg in 1981; the Hamburg stock was derived from Floridian wild ancestors in the mid 1970s. The progenitor from the Hamburg stock was bred for 30 generations at the Hanyang University until this study. The fish were housed in groups in 40-L glass tanks containing aerated water of 10±1‰ salinity. They were kept in an air-conditioned room at 25±1°C with a daily cycle of 14:10 light: dark. All fish were fed brine shrimp (*Artemia salina*) nauplii at 2-d intervals.

Acid stress on embryos

One day prior to test, 0.2 L of water at 10‰ salinity was

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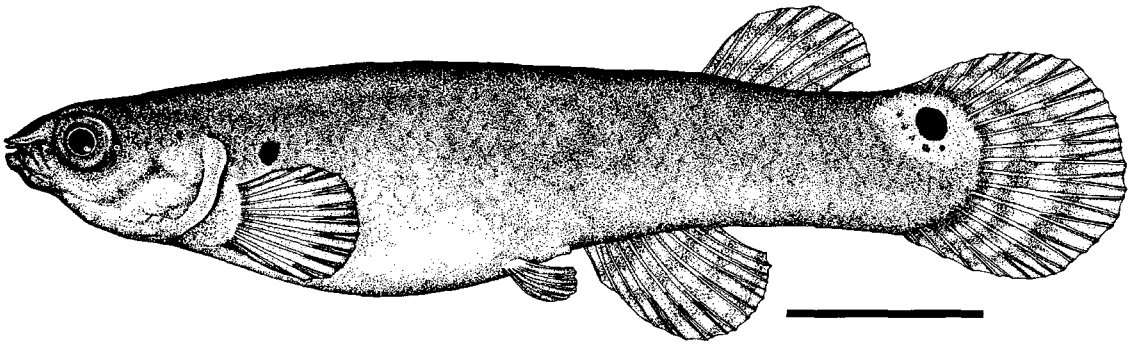

Fig. 1. External morphology of adult (4 months old) *Rivulus marmoratus*. A pair of round black spots on the caudal peduncle are caudal ocelli which are monotypic characteristics of this species. Scale bar = 1 cm.

placed in a 1-L transparent plastic bottle. The appropriate test pH of water was adjusted with H_2SO_4, and buffered with 0.01M N-2-hydroethylpiperazine-N-2-ethanesulfonic acid (HEPES) to guarantee a constant pH tested. The test water was saturated with oxygen by continuous aeration prior to loading the embryos. Fifty healthy embryos in the blastula stage were selected and placed in each bottle containing water at the appropriate test pH. The test containers were completely sealed and kept in an incubator at 25±0.1°C for 96 h. After 96 h incubation at the desired pH, the embryos were transferred to water of neutral pH until hatching. Hatching success was scored as a fraction of embryos that survived to hatching. Hatching period was also determined as the time between collecting of embryos and hatching. Duplicate exposures were carried out at each pH.

Acid stress on larva, juvenile, and adult

Acid stress on mortality was conducted for larval (7±2 days old) juvenile (30±2 days old) and adult (120±2 days old) stages. Test water was prepared like as for embryo tests. The 20-L plastic containers filled with 10 L of test water were used. Loading densities were maintained at 10 larvae/L, 5 juveniles/L, and 1 adult/L. Fifty healthy fish at each life stage were selected and loaded in each pH group. The fish were starved for 1 d prior to and during the tests. The criteria for death were lack of opercular movement and heart beats and response to mechanical stimulus over a period of 2 min. Duplicate tests were conducted at each pH.

Statistics

The 96-h LC5Os were calculated by probit analysis using Laboat Module Package, Version 4.0 (Innovation Programming Association, Princeton). Significant difference were assessed by Student's t-test at a significance level of $P<0.05$.

Results

There was a significant negative effect of acid stress on hatching success in the *R. marmoratus* embryos. Hatching success was only 5% at pH 3.5 compared to over 78% at pH higher than 4.0 (Table 1). Hatching period was also significantly affected by the 96-h acid stress. The hatching period was about 10 d longer with pH 3.7 treatment than pH 6.0 exposure, by which the hatching period was not delayed.

The larval and juvenile stages were more sensitive to acid stress than the adult stage. The 96-h LC50 value was pH 3.8 in larval and juvenile stages and pH 3.3 in adult stage. The mortality rates of larva and juvenile reached a level of about 60% at low pH (3.5) condition. The mortality rate of adult stage was only 5% at the same pH condition. However, there was no significant difference of mortality between larval and juvenile stages (Table 2). The mortality rates increased significantly in larvae and juveniles when exposed to pH 4.0 and below compared to pH 6.0. However, the mortality rate was increased in adults by treatments of water at pH 3.5 and below.

Table 1. Effects of acute acid stress on hatching success and hatching period of *Rivulus marmoratus* embryos

pH	N	Hatching success (%)[1]	Hatching period (day)[1]
7.0	100	78.5 ± 2.0	14.0 ± 1.5
6.0	100	78.0 ± 1.5	14.0 ± 1.0
5.0	100	79.0 ± 1.0	19.0 ± 5.7
4.0	100	78.0 ± 2.0	17.8 ± 2.3*
3.7	100	37.0 ± 8.5**	24.3 ± 5.4**
3.5	100	5.0 ± 1.2**	22.6 ± 1.6**
3.0	100	0.0	–

[1]Mean±S.D. Significantly different from corresponding values of pH 7.0 groups at $P<0.05$(*) or $P<0.01$(**).

Table 2. Effects of 96-h acid stress on mortality in three life stages of *Rivulus marmoratus*

Life stage	Age (days old)	N	Mortality (%)[1] at pH						
			2.7	3.0	3.5	3.7	4.0	5.0	6.0
Larva	7 ± 2	500	–	100 ± 0.0	58.2 ± 25.5	–	32.4 ± 8.5	1.8 ± 1.4	0.0
Juvenile	30 ± 2	500	–	100 ± 0.0	64.5 ± 8.5	–	25.8 ± 9.9	0.0	0.0
Adult	120 ± 2	500	99.5 ± 1.4	91.0 ± 1.4	5.5 ± 1.4	–	0.0	0.0	–

[1]Mean ± S.D. –; not tested.

Discussion

Several experimental studies revealed negative effects of acid stress on embryos of different fish species (Ingersoll et al., 1990; Oyen et al., 1991). Also when the effects of acid exposure through-out the life cycle was investigated, the embryo stage was found to be particularly sensitive in fish (Brown and Sadler, 1989). Low pH leads to denaturation of the hatching enzyme and subsequently to deformation of the embryos and high embryonic mortality as well as hatching delay in various fish species (Kwain and Rose, 1985; Ingersoll et al., 1990). This phenomenon was also observed from the amphibian embryos (Glos et al., 2003). Therefore, in embryo hatching problems may also result from decreased hatching enzyme activity at low pH.

Numerous laboratory studies have tested tolerance of fish species to acid stress (Marcus et al., 1986), but the sensitivity of *R. marmoratus* to acidification has not been determined previously. The present study revealed that *R. marmoratus* have higher tolerance to acid stress than most fish species studied previously. In most fish species, 96-h LC50 was reported ranging from pH 4.0 to pH 5.0 for early and adult life stages (Marcus et al., 1986; Holtze and Hutchinson, 1989) in contrast to pH 3.3 to pH 3.8 for different life stages of *R. marmoratus*. Fish mortality by acid stress have been thought to be associated with disturbance of water and ion balance, and may eventually lead to disruption of ion homeostasis (Heisler, 1989; Wilsom et al., 1999). The chloride cells in the gills, opercular epithelium, and skin of teleosts are known to play a key role in regulation of ion balance (Marshall, 1995). King et al. (1989) found that *R. marmoratus* has large numbers of well differentiated chloride cells in their gills and operculum. This may be one of the possible reasons why *R. marmoratus* is more tolerant to acid stress than other species.

Previous studies have shown that *R. marmoratus* is a useful laboratory animal for the study of carcinogenesis (Koenig and Chasar 1984; Park and Kim 1984; Park et al. 1993), mutagenesis (Park and Yi, 1989), teratogenesis (Park et al., 1992) and acute toxicity tests (Park et al., 1994). This fish is also a suitable model in studying the transition in the mode of fertilization (Kweon et al., 1998). Therefore, this result can serve as a baseline for breeding and rearing of this fish for an animal model in captivity in a suitable condition.

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