# EFFECT OF ALTERED pH ON EMBRYOS AND TADPOLES OF THE FROG *MICROHYLA ORNATA*

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## ABSTRACT

Short term effects of altered pH on embryos and tadpoles of the frog *Microhyla ornata* were studied under laboratory conditions. Alterations in pH were made by using dilute hydrochloric acid and sodium hydroxide solutions.

Late gastrula stage embryos tolerated pH between 4 to 10.5, showing normal development and hatching. At pH 3 and below development was immediately arrested and the embryos were killed within a few hours. Between pH 3.2 and 3.6 there was gradual decrease in toxicity, however, development was often arrested at about tail-bud stage. At pH 3.8 there was only 20% mortality while survivors showed normal development.

In alkaline range, the maximum pH tolerated without any apparent ill effect on development and hatching was about 10.5. At pH 11.0 and above there was drastic effect on the embryos which caused immediate cell to cell disaggregation of the embryos within an intact vitelline membrane.

The effects of altered pH, especially acidic pH, were similar to those observed earlier with sodium chloride treatment. In either cases normal swelling of the embryonic perivitelline space was prevented.

There was no significant difference in the tolerance of the tadpoles to altered pH as compared to that of the embryos, however hind-limb stage tadpoles appeared to be slightly resistant to acidic pH and slightly more sensitive to alkaline pH.

## INTRODUCTION

Acid rains and acidic effluents are polluting aquatic bodies all over the world. Acidification of lakes and other waterbodies has been reported by several workers (Almer *et al.*, 1974; Wright *et al.*, 1976; Johnson, 1979). Effects of lake acidification on aquatic biota, especially fish, have been well documented (Beamish and Harvey, 1972; Lievestad and Muniz, 1976). Mortality of amphibian species due to acidification of habitats has also been observed (Pough, 1976; Pough and Wilson, 1977; Beebee and Griffin, 1977). Studies on toxicity of acidic bog waters and acidic mine drainages indicated that amphibians are sensitive to low pH (Porter and Hakanson, 1976; Saber and Dunson, 1978). In recent years laboratory studies have also been carried out to understand effects of low pH on amphibian development (Gosner and Black, 1957; Freda and Dunson, 1985 a,b; Clark and Hall, 1985; Beebee, 1986). There are, however, no data on any of the native amphibians regarding their tolerance to altered pH conditions. Since industrial activity generates acidic effluents and acid rains are not unlikely in the areas of high industrial activity, we have investigated short term effects of altered pH on different developmental stages of the frog *Microhyla ornata*.

## MATERIALS AND METHODS

Naturally fertilised eggs or tadpoles of the frog *M. ornata* were collected from unpolluted ponds around Pune. Embryos were manually dejellied and transferred to aged tap water until required. Solutions of different pHs were made by mixing dilute hydrochloric acid and sodium hydroxide in aged tap water. The pH was adjusted accurately using a pH meter. Gross pH range was prepared with a difference of 0.5 units. Based on earlier experiments a fine range of pHs was prepared with a difference of 0.2 units between the subsequent pHs to determine lethal and critical pH for the embryos. Embryos and other developmental stages were exposed to these solutions in 200ml or larger glass bowls. Usually 10 embryos were exposed per 100ml of solution but for larger tadpoles 200-400ml solution was taken for a batch of 10 tadpoles. Aged tap water with a pH of about 7.6 to 7.8 and total hardness as well as total alkalinity below 50 ppm (as calcium carbonate) was used as a control medium during the experiments. The temperature of the water varied between 22 to 26° Celsius. The embryos and tadpoles were observed periodically under stereozoom dissecting microscope. At least 30 embryos or tadpoles were exposed to each of the pH tested. The experiments were carried out for 96 hours. Tests of such short duration are routinely carried out to determine toxic effects of pollutants on fishes and other animals. Further, hatching takes place in about 48 hours in this frog and hence 96 hours of treatment gives idea about the effects on embryonic development, hatching as well as effects on freshly hatched tadpole. At least 80% solution from each experimental bowl was replaced by fresh solution of identical pH every 24 hours to keep the pH variation minimum. Mortality and other effects were noted after every 24 hours and the data was used to calculate LC50 as per the Reed-Muench method (Woolf, 1968). Measurements were taken using stage and ocular micrometer.

## RESULTS

The embryos kept as controls showed normal development and differentiation of head, trunk and tail region. At the end of first 24 hours, the embryonic perivitelline space (PVS) was considerably swollen. The embryos hatched as tadpoles at about 48 hours. At 96 hours all control tadpoles showed well developed head, eyes and tail region. Typical pigmentation also appeared on the dorsal side of the head. All such tadpoles were actively swimming by 96 hours.

In the gross acidic range (between pH 2.5 to 7.0) the embryos tolerated pH 4.0 and above without any apparent ill effect. At and below pH 3.0 there was immediate arrest of development and total mortality within few hours. Exposure of embryos to a fine range of pH between 3.0 to 4.0 showed that pH about 3.6 was critical pH. Upto pH 3.6 there was 100% mortality within 96 hours. Mortality was reduced at pH 3.8 and the embryonic development was also normal. Mortality data along with LC 50 values is given in Table 1A. Similarly, in alkaline range the embryos tolerated pH 10.5 and lower. However, at and above pH 10.8 the embryonic development was immediately arrested and there was immediate cell to cell disaggregation of the embryo. Exposure of the embryos to a fine range of alkaline pH between 10.0 to 11.0 indicated that critical alkaline pH was about 10.6 (see Table 1B). However, it may be pointed out that NaOH solutions were not very stable and did not maintain exact pH.

Measurement of diameter of the vitelline membrane (VM) was carried out at the beginning and at the end of first 24 hours of the experiment. The diameter of the VM in control embryos increased from about 1.4mm to about 4.3mm in 24 hours. (Figs. 1 and 2). The embryonic PVS thus increased considerably from late gastrula stage to late tail-bud stage under control conditions. In the embryos exposed to acidic pHs, the swelling of the PVS was significantly affected between pH 3.0 to 4.0. In the solution of pH 3.0 the embryonic

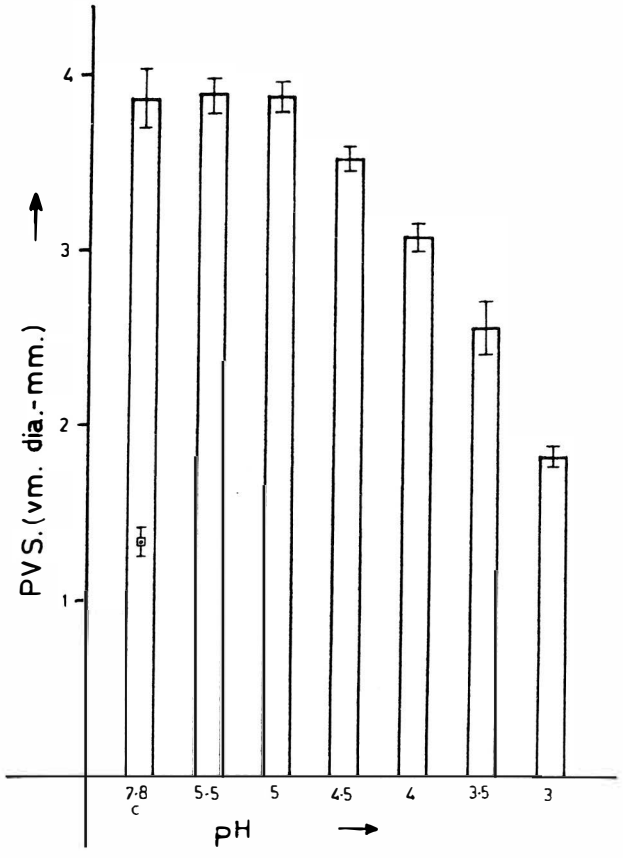


Fig. 1 Diameter of the perivitelline space of the embryos of *Microhyla ornata* after 24 hours of exposure of acidic pH. The mark inside the control (c) bars shows the diameter at the beginning of the experiment. Values listed are mean ± S.D.

PVS remained almost unaltered (see Fig. 1). Similarly, in the embryos exposed to alkaline pH, there was adverse effect on swelling of the PVS (see Fig. 2). Though the PVS was also reduced at pHs between 4.0 to 5.0 and 9.5 to 10.5, the reduction was not severe. Further the embryos developed normally and were able to stretch the VM making room for the

(A)

| Hours of Exposure | pH 3.0 | 3.2 | 3.4 | 3.6 | 3.8 | 4.0 | 'C' 7.8 | LC50 | 95% Confidence limits |
|---|---|---|---|---|---|---|---|---|---|
| 24 | 0 | 0 | 24 | 30 | 30 | 30 | 30 | 3.27 | 3.20-3.35 |
| 48 | 0 | 0 | 0 | 21 | 30 | 30 | 30 | 3.45 | 3.35-3.55 |
| 72 | 0 | 0 | 0 | 5 | 24 | 30 | 30 | 3.69 | 3.61-3.78 |
| 96 | 0 | 0 | 0 | 0 | 24 | 30 | 30 | 3.67 | 3.60-3.75 |

(B)

| Hours of Exposure | 'C' 7.8 | 10.0 | 10.2 | pH 10.4 | 10.6 | 10.8 | 11.0 | LC50 | 95% Confidence limits |
|---|---|---|---|---|---|---|---|---|---|
| 24 | 30 | 30 | 30 | 30 | 18 | 0 | 0 | 10.66 | 10.32-11.02 |
| 48 | 30 | 30 | 30 | 30 | 18 | 0 | 0 | 10.66 | 10.32-11.02 |
| 72 | 30 | 30 | 30 | 30 | 18 | 0 | 0 | 10.66 | 10.32-11.02 |
| 96 | 30 | 30 | 30 | 30 | 9 | 0 | 0 | 10.54 | 10.48-10.60 |

TABLE 1: Tables showing survival of embryos exposed to different pHs prepared using HCl (A) and NaOH (B). Number of embryos exposed at each pH was 30. Hours of exposure were counted from late gastrula stage onwards. 'C' indicates control.

development. This resulted in curling of only the tip of the tail, a deformity which was not observed after hatching. However, at pH 3.8 the development was normal but the PVS was considerably reduced and, due to lack of enough space, embryos developed with curled body axis. Such embryos were swimming abnormally after hatching. Hatching of the embryos was not prevented at any pH between 3.8 to 10.5, although slight delay in hatching was observed upto pH 5.0.

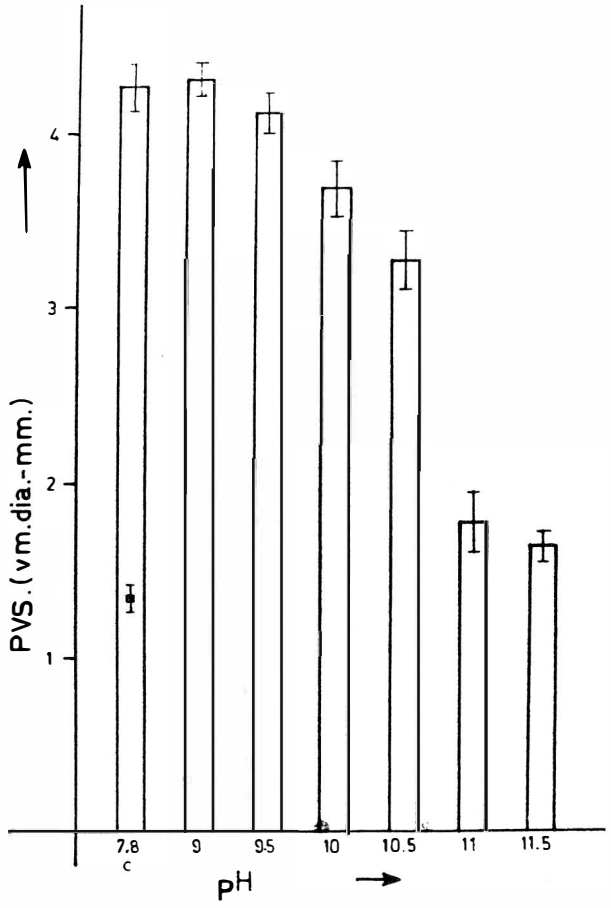


Fig. 2 Diameter of the perivitelline space of the embryos of *Microhyla ornata* after 24 hours of exposure to alkaline pH. The mark inside the control (c) bar shows the diameter at the beginning of the experiment. Values listed are mean ± S.D.

Under similar treatment conditions, 10 day old tadpoles did not show any significant difference in the tolerance to altered pH as compared to that of the embryos. Hind-limb stage and older tadpoles however tolerated pH 3.5 which was lethal for the embryos. At the same time, hind-limb stage tadpoles were killed at pH 10.5, a pH well tolerated by the embryos. Thus the hind-limb stage and older tadpoles were slightly more tolerant of acidic and more sensitive to alkaline pH as compared to the embryos.

Erratic swimming, just after the release of the tadpoles into the media, was observed at and below pH 3.5 and at and above pH 10.5. At pH 11 disintegration of the tail fin was observed prior to death of the tadpoles. Effects at pH 11.5 were very severe and the tadpoles were killed within 3 to 5 minutes. Thus the lethal acidic pH for older tadpoles was 3.0 while the lethal alkaline pH was less than 10.5.

## DISCUSSION

Gosner and Black (1957) defined lethal pH as the one which causes irreversible cessation of normal development. According to this definition, the lethal acidic pH for *Microhyla* was 3.0 and the lethal alkaline pH was 11.0. None of the embryos exposed to the lethal pHs could survive and continue development. Gosner and Black also defined critical pH range and minimum limiting pH. The critical pH range solutions cause high mortality; and in our experiments this range was 3.2 to 3.6 for acid and 10.8 to 11.0 for alkali used. Minimum limiting pH is said to be the pH that allows normal development of more than 50% of the exposed embryos. In our experiments minimum limiting acidic pH was 3.8 because 80% of the exposed embryos survived and showed normal development at this pH. Limiting alkaline pH was found to be about 10.5. These values are comparable with those reported for other amphibians by Gosner and Black (1957), as far as acidic pH is concerned. There are no comparable data regarding alkaline pH. In case of acidic pH, older tadpoles were found to be more tolerant than embryos. Similar observations have been reported by Beebee (1986), who found that in the case of the natterjack toad, embryos and early tadpoles were killed around pH 4.0 while older tadpoles survived even at pH 3.5.

Our observation regarding prevention of swelling of the PVS of the embryos exposed to low pH is consistent with the reports published on other amphibians (Gosner and Black, 1957; Salthe, 1965; Freda and Dunson, 1985a). Reduction of PVS also occurs with salt treatment (Salthe, 1965; Gosner and Black, 1957; Padhye and Ghate, 1986). It is known that slow flow of water into PVS is necessary for normal development in amphibians (Krogh, 1939). Reduction in PVS observed in our experiments is probably related to change in osmotic gradients across the egg membrane, the gradient that is stated to decide the size of the PVS (Holtfreter, 1943). However, in our experiments, reduction in PVS was not severe to cause any deformity in the embryos exposed to pH between 4.0 to 10.5. Also, there was no significant effect on the hatching process, though severe curling of body axis and reduction in hatching success has been reported in case of other amphibians (Gosner and Black, 1957; Pough and Wilson, 1977; Freda and Dunson, 1985a; Clark and Hall, 1985). It is interesting to note that Dunson and Connell (1982) found that the curling and other deformities do not occur, and embryos survive the exposure to low pH, if the VM is removed, indicating that the chief cause of mortality associated with curling defect is membrane disfunction. There being no direct effect on the embryo. They further hypothesised that low pH inhibited activity of hatching enzyme and (or) other mechanisms responsible for the enlargement of the PVS. Hatching enzyme, as pointed out by Freda and Dunson (1985a), is responsible for changes in the VM allowing it to expand in response to the osmotic uptake of water enlarging PVS; further, digestive action of the same enzyme weakens VM facilitating its rupture by muscular action of the embryo. Thus inhibition of this enzyme, which occurs after exposure to low pH or high salt concentrations, will have adverse effects.

Regarding toxic effects of low pH on amphibian development, it has been reported that disruption of ionic regulation is the major cause responsible for the mortality of embryos and tadpoles; loss of sodium has been shown to be one of the prominent effect (Freda and Dunson, 1984, 1985b). Loss of sodium has also been shown to be responsible for mortality of fishes exposed to low pH (Packer and Dunson, 1970). Why older tadpoles were found to be more tolerant of low pH as compared to embryos is inexplicable at present. Presumably, this tolerance was related to the development of functional kidneys and skin, the organs that can control ion influx and efflux.

Results presented here show consequences of short term exposure to altered pHs in *Microhyla ornata* embryos and tadpoles. Effects of long term exposure, from early development through metamorphosis, are being investigated.

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