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Physiological and Ultrastructural Changes in *Labeo rohita* (Hamilton-Buchanan) Fingerlings Exposed to Sublethal Acidic and Alkaline pH for Long Duration

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Abstract

Physiological changes were exhibited by fingerlings of Rohu, *Labeo rohita* Cyprinidae exposed to sublethal pH of 5.5 and pH of 9.0 for 30 days. At pH 5.5, elevated levels of haemoglobin, haematocrit, plasma cortisol and glucose with reduced levels of plasma chloride was observed on 7-day post exposure. Fish exposed to pH 9.0 showed similar changes in the physiological parameters after 21 days of exposure. Ultrastructural alterations of gills in fish at acidic pH were rugose lamellar surface, destruction of normal secondary lamellae, release of blood cells, increased mucous production and loss of microridged patterns on the epithelial cells of filaments. At pH 9.0 fusion of secondary lamellae occurred. The extent of alterations in blood parameters with the ultrastructural changes in gills of the exposed fish was greater at pH 5.5 than at pH 9.0. These results suggest that the fish are under greater physiological stress at the acidic pH.

Introduction

The pH of water is one of the most important water quality characteristics influencing the health of fishes. Fishes residing for a prolonged period in aquatic

habitat with acidic pH either below 6.5 or alkaline above 9.0 are subjected to stress affecting their growth and reproduction.

Effect of sublethal acidic pH on fish physiology was studied in Rainbow trout, *Salmo gairdneri* Salmonidae by Bam and Pottinger (1993), in Tilapia, *Oreochromis mossambicus* Cichlidae by Van Ginneken et al. (1997), and in *Tilapia mossambica* by Murthy et al. (1981). Changes in the various physiological parameters of *Labeo rohita* subjected to low and high pH for short duration was investigated by Dutta et al. (2003). Jagoe and Haines (1983) studied the alterations of gill morphology of yearling sunapee trout *Salvelinus alpinus* Salmonidae exposed to acute acid stress. To date Scanning Electron Microscopy (SEM) has also been used to study gills of stressed rainbow trout exposed to water of high iron content (Hart and Oglesby 1979), to heat shock (Jacobs et al. 1981), and to hexavalent chromium (Van der Putte and Part 1982). Also Munshi et al. (1996), Mallat (1985) and Adhikari et al. (1998) demonstrated alterations in the gill ultrastructure of fish exposed to malathion.

The present study was undertaken to assess the impact of sublethal acidic and alkaline pH on the physiology and gill morphology of *Labeo rohita*, a tropical Indian major carp. *L. rohita* is widely distributed in India and cultured extensively under a wide range of water pH conditions.

Materials and Methods

Hatchery bred fingerlings of *Labeo rohita* averaging 25.0 gm in weight and 120 mm in length were acclimatized in the laboratory in fibre glass tanks at a stocking density of 0.4 gml^{-1}. The measured water quality conditions were pH (7.75 - 7.9), temperature (25 - 27°C), alkalinity (130-150 mgl^{-1}), hardness (170-190 mgl^{-1}) and dissolved oxygen (5.5-6.0 mgl^{-1}) with 12hr light and 12hr darkness. All fish were fed tubifex worms *ad libitum*, one-fourth water was changed daily and excretory products were siphoned out. Water quality parameters were measured as per the methods given in APHA (1989).

For initiation of the experiment, healthy fish were subjected to pH 5.5 and pH 9.0 and maintained for 30 days along with control fish at the acclimation pH. Water pH of 5.5 was obtained by adding 10^{-1} N HCl solution to tap water and pH 9.0 by adding 10^{-1} N NaOH solution. Continuous aeration was given in control and experimental tanks. Fish were fed live tubifex worms daily but were fasted 24h prior to blood and tissue sampling. Subsamples of ten fish of each group were sacrificed after 7, 14, 21 and 30 days from the start of the experiment.

Blood and tissue sampling and analysis

To obtain blood of *L. rohita*, fingerlings were netted gently and rapidly anesthetized using MS222 (ethyl m-aminobenzoate methane sulphonate) at the dose of 60 mgl^{-1}. The fishes were immobilized within one minute of application (Das et al. 2002). Blood was collected from the caudal artery using 1ml syringes fitted with 24G needle or by caudal peduncle cut. Heparin was used as an anticoagulant. Immediately after collection blood was centrifuged at 3000 rpm for five minutes in cold and separated plasma was either used for analysis immediately or stored at –20°C for later analysis. Sampling procedure of netting, anesthesia and plasma storage was completed within 10 min to avoid influence of netting, combined with anesthesia on the basal cortisol levels (Franklin et al. 1990; Tanck et al. 2000). The methods employed for measuring the various blood parameters were haemoglobin (Hesser, 1960), haematocrit (Blaxhall and Daisely 1973), plasma glucose (Hyvarinen and Nikkila 1962) and plasma chloride (Schales and Schales 1941). Plasma cortisol was measured using a direct immunoenzymatic determination of cortisol kit manufactured by Equipar srl via G. Ferrari (21/N - 21047, Saronno, Italy) using a 96 well microtitre plate read by ELISA, microplate reader (Model EL311SX, Biotech Instruments Inc.) Statistical analysis of data for all physiological parameters are expressed as mean ± S.E. Statistical comparisons between experimental and control fish were made by student *t* test (Snedecor and Cochran, 1969). The probability levels at $P<0.05$ and $P<0.01$ were considered statistically significant. Gills from the control and experimental fish were removed quickly after 30 days of experimentation and fixed in 2.5% glutaraldehyde. After fixation of the gills at 4°C for 72 h, the gill filaments were repeatedly washed in 0.1 M phosphate buffer (pH 7.4), dehydrated in graded series of alcohols, alcohol-acetone mixture and finally in anhydrous acetone at room temperature. The tissues were critically point dried in Hitachi, gold coated in ion coater (Model no. IB/2, Ioncoater, Japan) and then studied under a scanning electron microscope (Model no. S530, Hitachi, Japan) at the University Sophisticated Instrumentation Centre, University of Burdwan, Burdwan, West Bengal, India.

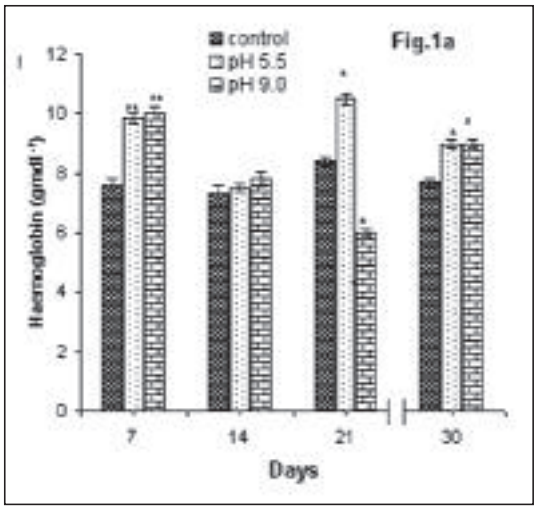
Results

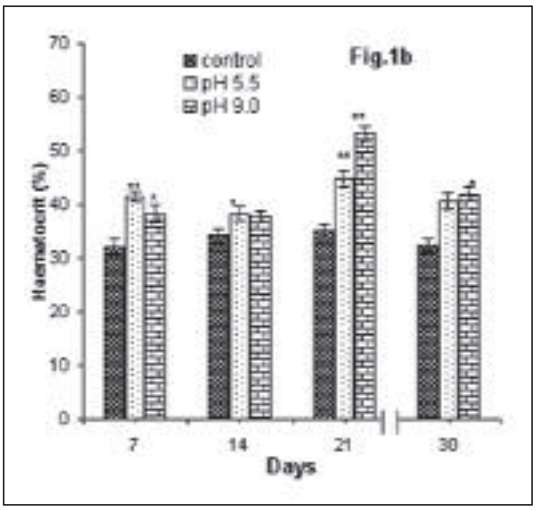
Fish exposed to pH 5.5 for 30 days

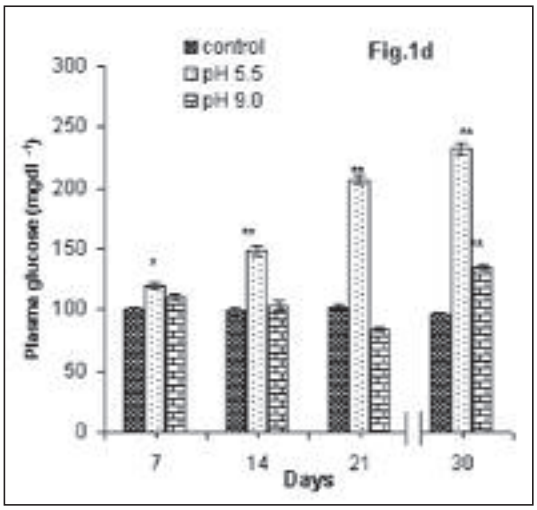
A significant rise in blood haemoglobin level was observed in fish exposed to pH 5.5 on day 7 ($P<0.01$), day 21 ($P<0.01$) and 30 day ($P<0.05$) (Fig.1a). Haematocrit value increased significantly on day 7 ($P<0.01$), day 14 ($P<0.05$) and day 21 ($P<0.01$)

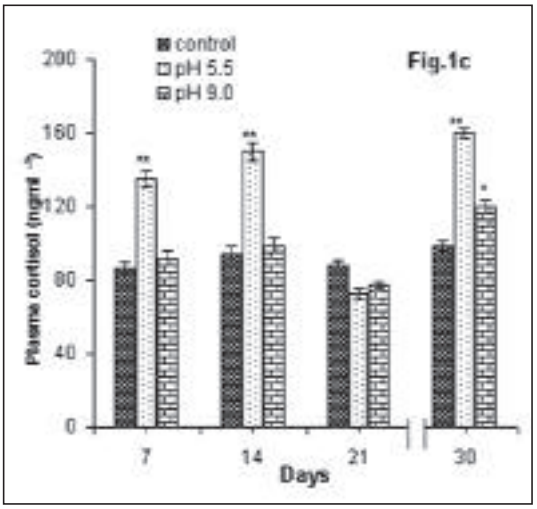
of exposure (Fig.1b).

Activation of the pituitary interrenal axis was indicated in *L. rohita* by the marked elevation in plasma cortisol level on day 7 ($P<0.01$), day 14 ($P<0.01$) and day 30 ($P<0.01$) (Fig.1c). That carbohydrate metabolism was affected was indicated by the continuous increase in plasma glucose on 7 ($P<0.05$), 14 ($P<0.01$), 21 ($P<0.01$) and 30 days ($P<0.01$) (Fig.1d). Pronounced hypochloremia was noticed from 7 days onwards till 30 days of exposure.









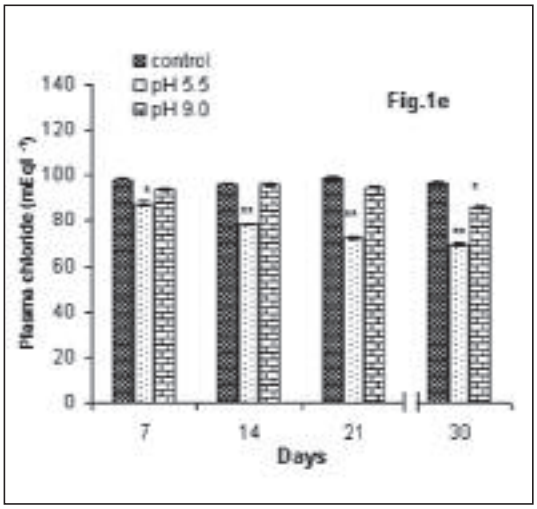


Fig. 1 (a-e): The changes in the physiological parameters of *Labeo rohita* fingerlings of control (pH 7.8) and subjected to pH (5.5 and 9.0) for 7, 14, 21 and 30 days. a. Haemoglobin, b. Haematocrit, c. Plasma cortisol, d. Plasma glucose, e. Plasma chloride. The values are given as Mean ± S.E., n=10, *P<0.05, **P<0.01.

Significant lowering of plasma chloride level at 7 ($P<0.05$), 14 ($P<0.01$), 21 ($P<0.01$) and 30 ($P<0.01$) days shows continuous disturbance of the acid base equilibrium (Fig.1e).

Fish exposed to pH 9.0 for 30 days

At pH 9.0 almost all measured parameters were affected after 30 days of exposure. The blood haemoglobin level increased in the fish attaining significant levels after day 7 ($P<0.01$), day 14 ($P<0.01$) and day 30 ($P<0.05$) (Fig.1a). Haematocrit level increased significantly after day 7 ($P<0.05$), day 21 ($P<0.01$) and day 30 ($P<0.05$) of exposure (Fig. 1b). Plasma cortisol did not vary much from controls on days 7,14 and 21 whereas, a significant rise in cortisol level was found after 30 days ($P<0.05$)(Fig.1c). Plasma glucose elevated markedly after 30 days ($P<0.01$) of exposure at pH 9.0, but no significant changes were observed from control after 7,14 and 21 days of exposure (Fig.1d). A significant decrease in plasma chloride level was observed after 30 days of exposure ($P<0.05$) (Fig.1e).

SCANNING ELECTRON MICROSCOPIC (SEM) STUDY OF GILLS

Control gills

In gills of control *L. rohita* fishes, primary lamellae appeared normal and mucous free, and uniform branching of secondary lamellae from primary lamellae was visible. SEM showed the presence of a definite microridged pattern of gill filament and mucous pores (Fig.2a).

Fish gills exposed to pH 5.5 for 30 days

SEM showed severe damage of gill structure after exposure for 30 days at low pH. The secondary lamellae severely deformed releasing RBC (Fig.2b). Microridges appeared fused with no definite pattern on the gill filament surface (Fig.2c). Corrugation of gill filament surface and mucous gland opening increased in size (Fig.2d).

Fish gills exposed to pH 9.0 for 30 days

Ultrastructural modifications manifested in the presence of rough surface of gill filaments was revealed by SEM study (Fig.2e). Fusion of secondary lamellae was also found (Fig.2f).

Fig. 2 (a-f): SEM photomicrographs showing the control and pH 5.5 and 9.0 exposed gill structure of *Labeo rohita* for 30 days

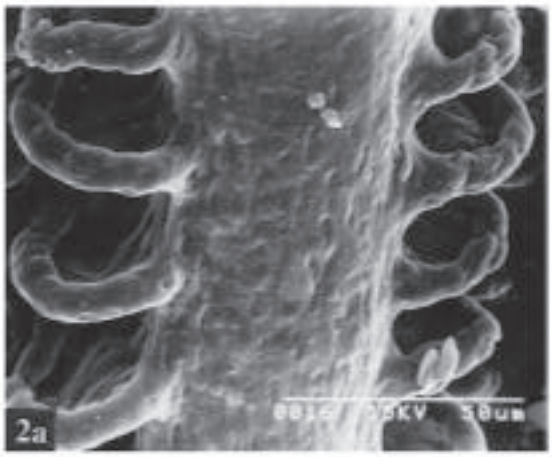


Fig. 2a: normal gills of *L. rohita* - Control gill filament showing the uniform branching of secondary lamellae

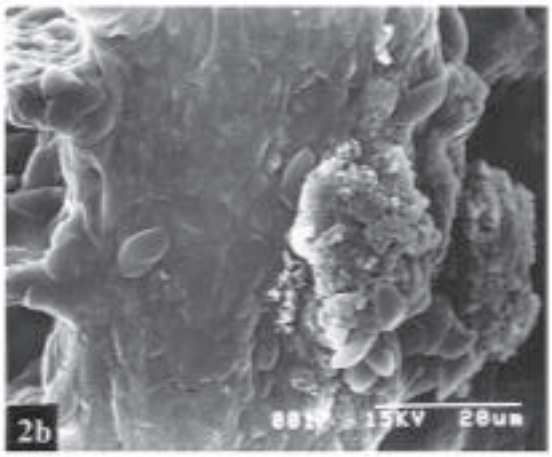


Fig. 2b: gills of *L. rohita* exposed to pH 5.5 - Severe damage of secondary lamellae with the release of RBC

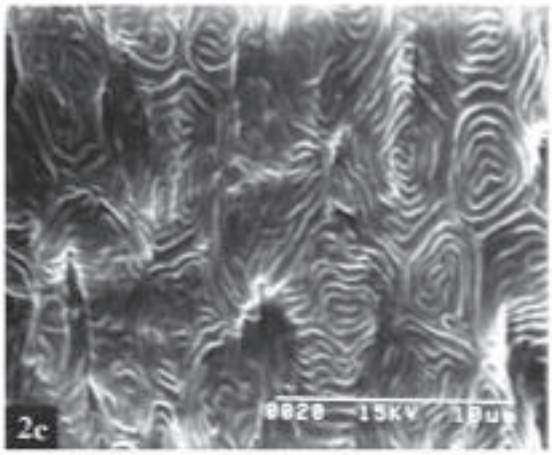


Fig. 2c: gills of *L. rohita* exposed to pH 5.5 - Fusion of microridges of gill filament affecting oxygen uptake

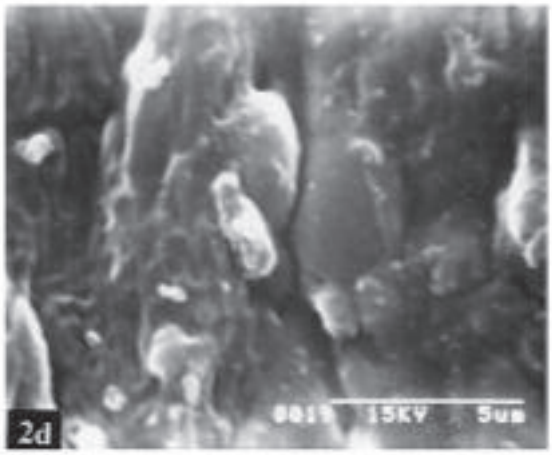


Fig. 2d: gills of *L. rohita* exposed to pH 5.5 - Corrugation of gill filament epithelia and increase in size of mucous gland opening

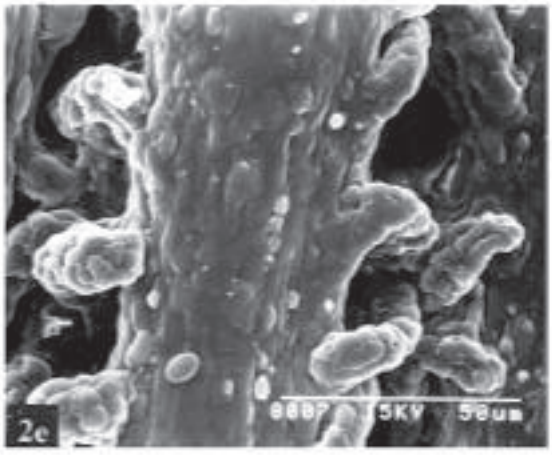


Fig. 2e: gills of *L. rohita* exposed to pH 9.0 - Rough surface of gill filament

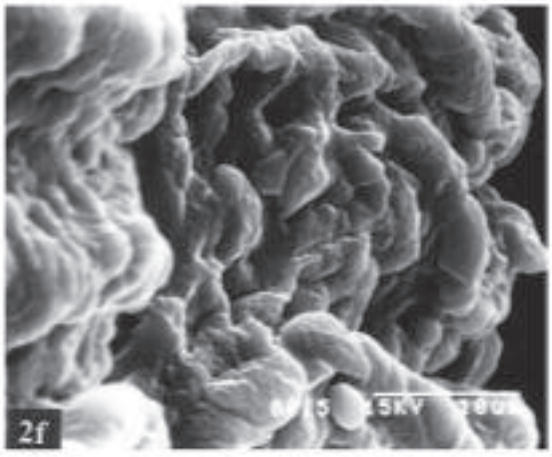


Fig. 2f: gills of *L. rohita* exposed to pH 9.0 - Swelling and fusion of secondary lamellae

Discussion

Fish exposed to pH 5.5 for 30 days

Increase in haemoglobin and haematocrit levels observed in the present study indicate haemoconcentration in blood of experimental fish. Contraction of plasma volume may have increased the haemoglobin level. Increase in haematocrit might be due to cellular swelling (Soivio and Nikinmaa 1981). Decrease in the pH in the aquatic environment causes acidosis (Packer and Dunson 1972), which induces a decrease in both the oxygen carrying ability of haemoglobin (Root effect) and its affinity to O_2 (Bohr effect). Thus, impairment of O_2 uptake or delivery appears to be a key toxic mechanism of lower pH (Spry et al. 1981). In company with these disturbances, elevation in haematocrit level may include increase in erythropoiesis in response to tissue hypoxia or transfusion of cells from spleen (Milligan and Wood 1982). Low chloride level was due to increased gill fluxes resulting in loss of chloride ions (McDonald and Wood 1981). Increased loss of ions may be catecholamine mediated which increases the permeability of gills (Wendelaar Bonga 1997). This corroborates the findings of Giles et al. (1984) and Jones et al. (1987) who found impaired osmoregulation in rainbow trout and Arctic char, *Salvelinus alpinus* following 22 days and 14 days of sublethal acid exposure respectively. The plasma glucose elevation was probably associated with high plasma cortisol levels. High cortisol levels are generally associated with glucose mobilization, stimulation of gluconeogenesis and protein catabolism (Leach and Taylor 1980, 1982). Exposure to sub-lethal acidity causes aberrant physiological functioning by the low pH syndrome manifested by (i) recruitment of erythrocytes from spleen (Milligan and Wood, 1982); (ii) increase in red blood cells size (McDonald and Wood, 1981); (iii) increase in level of haemoglobin (Nieminen et al. 1982); (iv) hyperglycaemia (Brown et al., 1983; Waiwood et al. 1992) and (v) an increase in cortisol levels (Brown et al. 1983).

The ultrastructural appearance of gills at pH 5.5 showed release of RBC from secondary lamellae, surface roughness, increased mucous production, progressive loss of microridge pattern and irregularity of gill epithelium. Jagoe and Haines (1983) observed swelling of lamellae with increased mucous production and shrinkage of the capillary systems in the gills of trout exposed to acid stress. Spry et al. (1981) reviewing the mechanisms of acid toxicity in fish suggested that the primary cause of death at very low pH is anoxia. In this study, change of microridge pattern, copious amount of mucous and released RBC from gill filament shows fish to be under considerable stress which affected oxygen uptake by reducing the surface area available for gas exchange and lengthening the distances over which respiratory exchange diffusion occurs. Similar appearance of the gill epithelium in Singhi, *H. fossilis* Heteropneustidae due to the exposure of malathion and plant extract have

been reported by Adhikari et al.(1998). The release of RBC is indicative of acute damage to the gills of fish because as opined by Adhikari et al. (1998) that the release of RBC by the fish indicate that it is unable to repair its wound. The hyperproduction of mucous may limit oxygen uptake by the gills. The observed increase in blood haemoglobin and haematocrit level may be due to increase in the oxygen carrying capacity of fishes due to greater demand by the tissues. Heavy mucous production by fish gills has been implicated in hypoxia (Gardner 1975). Changes in respiratory surfaces alter oxygen uptake which eventually creates physiological imbalances in the organism as indicated by high cortisol and glucose values in the blood of experimental fish.

Fish exposed to pH 9.0 for 30 days

Increase in haemoglobin and haematocrit level after day 7 may be due to reduction in the plasma volume or cellular swelling (Soivio and Nyholm 1973). Elevation in cortisol of plasma with enhanced blood glucose indicates stress. Loss of chloride ion reflects ionosmoregulatory dysfunction. This is probably due to increased gill permeability or internal redistribution of ions as suggested by Kirk (1974).

Fusion of secondary lamellae reduced the total available respiratory surface area of the gill resulting in decreased oxygen uptake (Adhikari et al. 1998) as observed in this study. The present study showed increased haemoglobin and haematocrit levels in order to compensate the required oxygen for various metabolic activities. SEM observations showed the presence of mucous droplets, rough surface of gill filament with blebbed epithelial cells of secondary lamellae. All these alterations indicate functional disturbance of the fish respiratory organ, although the alterations were not as pronounced as in the case of fish exposed to pH 5.5 for 30 days.

Conclusion

Alterations in blood parameters and gill ultrastructure indicated the fish to be under more physiological stress at pH 5.5 as the changes were more pronounced in the case of fish exposed to the acidic pH than at alkaline pH of 9.0 for 30 days. The observations can provide a guideline to a fishery manager for the health management programmes for fish grown under natural conditions. The data obtained and the changes observed in the gills can help to assess the physiological state of fish under similar conditions prior to a situation when the tolerance limit exceeds their limit and ultimately results in death.

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