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Ionoregulatory and Toxicological Responses of Stonefly Nymphs (Plecoptera) to Acidic and Alkaline pH

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Abstract. The acute toxicities of acidic and alkaline pH to nymphs of the stoneflies *Pteronarcys dorsata, P. proteus,* and *Tallaperla maria* were determined in 96-hr static bioassays. The acidic and alkaline 96-hr LC_{50} values were 2.8 to 3.3 and 12.1 to 10.3, respectively. Exposure to pH 3.0 for 72 hr or longer caused a significant loss of sodium from nymphs of *P. proteus*. Morphological changes, including distension of cuticular disk and increased number of vesicles, were observed in gill tissue from nymphs of *P. dorsata* exposed to pH 2.5 for 9 hr while minor changes were observed in nymphs exposed to pH 4.0 for 96 hr. Changes in gill tissue ultrastructure included an increase in number of vesicles and a decrease in number and size of mitochondria in nymphs exposed to alkaline pH of 11.75.

Changes in the pH of surface waters have occurred over the past 30 years due to increased combustion of fossil fuels. One source of this perturbation is ash effluents from coal-fired power plants (Cherry *et al.* 1979a, 1979b). In a survey of 14 coal ash effluents, Chu *et al.* (1978) found a pH range of 4.4 to 11.3. Most studies concerning extreme pH in aquatic systems have dealt with the effects on fish survival, reproduction, and physiology as reviewed by Fromm (1980), Haines (1981), and Alabaster and Lloyd (1980). Studies of pH effects on benthic macroinvertebrates have dealt mainly with acid mine drainage (Herricks and Cairns 1977; Letterman and Mitsch 1978; Moon and Lucostic 1979). Few laboratory studies have addressed the effect of pH on survival, reproduction, and physiology of aquatic insects (Stickney 1922; Bell and Nebeker 1969; Bell 1970, 1971; Strange *et al.* 1982). Although may effluents are alkaline, including coal ash, little is known about the effect of high pH (*e.g.*, 8–11) on aquatic receiving systems (Daye and Garside 1980; Cairns *et al.* 1972; Shaw 1981).

In the present study, 96-hr static bioassays were used to determine the toxicity of extreme pH to nymphs of three species of stoneflies. The effect of low pH on the regulation of major cations by *Pteronarcys proteus* was determined, and alterations in gill tissue ultrastructure were observed in nymphs of *P. dorsata* exposed to extreme pH.

Materials and Methods

Test Organisms

Test organisms were stonefly nymphs, *Tallaperla maria, P. dorsata, P. proteus. Tallaperla maria* and *P. proteus* were collected from Big Stony Creek at a site located 1 km west of the White Rocks Recreation Area in the Jefferson National Forest, Giles County, Virginia. The collection site was located on Route 635, ~30 km northeast of U.S. Route 460 at an elevation of 880 m above sea level. *Pteronarcys dorsata* was collected from the Little River, Montgomery County, Virginia. The collection site, at an elevation of 570 m above sea level, was located on Route 787, 2 km south of its intersection with Route 693 and ~15 km southwest of Christiansburg, Virginia. All organisms were collected with D-framed kick nets and placed in aerated stream water. Upon arrival in the laboratory, organisms were placed in 4-L plastic containers containing dechlorinated Blacksburg tap water and were acclimated to the specific test temperature and photoperiod regime for at least one week prior to experiments.

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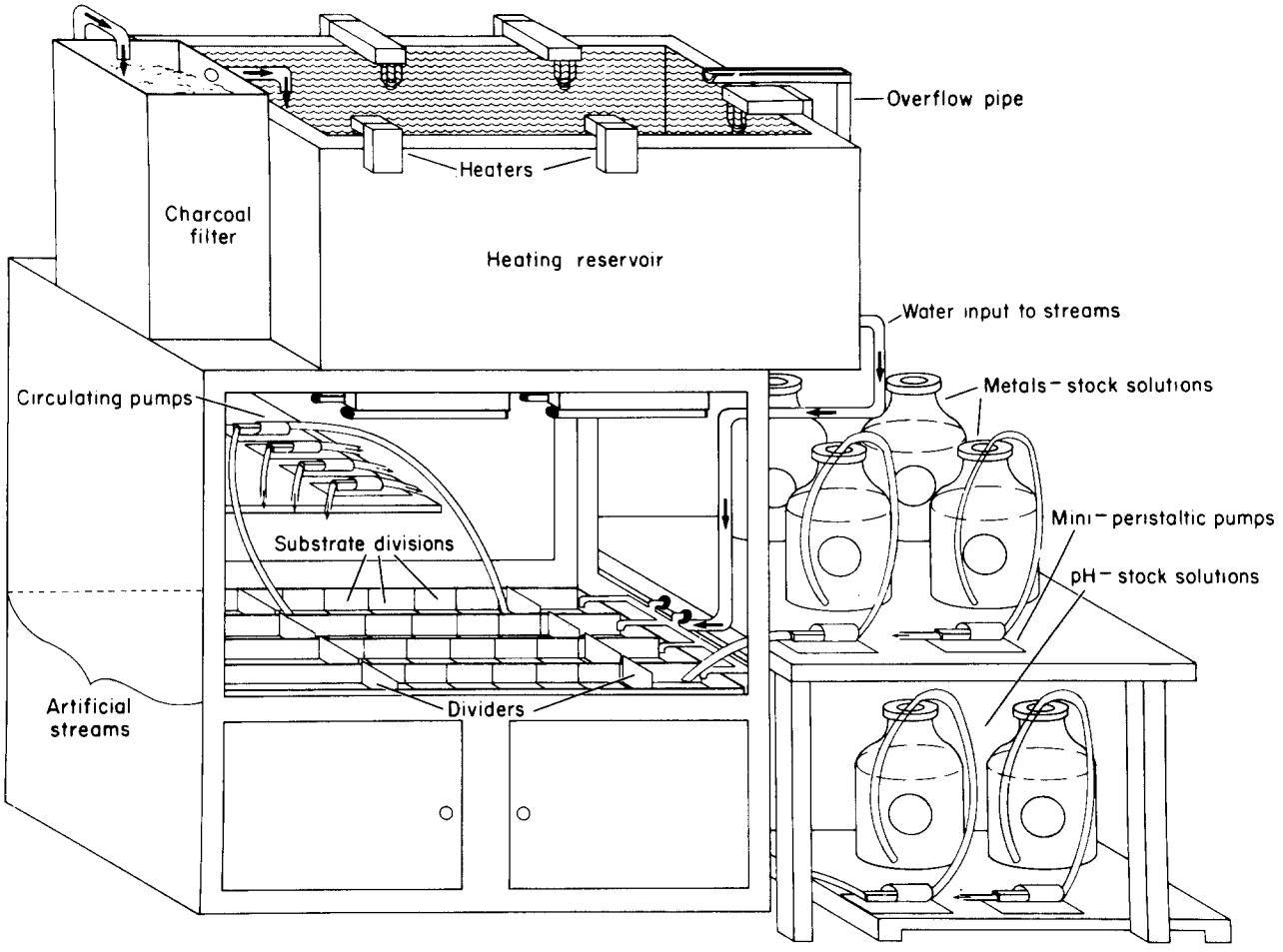

Fig. 1. Schematic of artificial stream system used to evaluate acidic pH stress on ion regulation

During acclimation, stoneflies were fed conditioned leaf material collected from Big Stony Creek, and, prior to any test, organisms were not fed for 48 hr.

pH Bioassays

Static 96-hr bioassays were conducted in 4-L cylindrical glass aquaria with magnetic stirrers to maintain a current. Ten organisms were placed in fiberglass screen cages (13 × 10 ×8 cm) and suspended in test solutions. Acidic and alkaline test solutions were prepared by adding 0.2 M HCl and 0.5 M NaOH, respectively, to 3 L of dechlorinated tap water. Target pH was obtained by monitoring the solution with an Orion 601A pH meter. Test solutions were monitored frequently (6–10 times daily) and were adjusted to maintain the target pH. Test pH of the alkaline bioassays varied ±0.15 pH units, while the acidic bioassays varied by ±0.10 pH units. Standard chemical and physical parameters were measured twice during each bioassay and included temperature, hardness, alkalinity, specific conductance, and dissolved oxygen, according to Standard Methods for the Examination of Water and Wastewater (American Public Health Association 1976). All tests were conducted at 20°C under a photoperiod regime of 16 hr light/8 hr dark.

Effect of pH on Ion Regulation

To determine the effects of low pH on ion regulation, nymphs of *P. proteus* were exposed to pH 3.0 for up to 5 days in artificial streams that received dechlorinated tap water at 1 L/min (Figure 1). The experimental stream was dosed with 0.5 M HCl to obtain a pH of 3.0. This pH was maintained automatically by a Fischer Model 604 pH regulator. Nymphs were placed in fiberglass screen cages containing washed cobble in either the experimental (pH 3.0) or control (pH 8.0) streams. After 6, 24, 48, 72, 96, and 120 hr of exposure, four nymphs were removed from each stream, rinsed three times with distilled water, and frozen in liquid nitrogen. Organisms were dried for 24 hr at 105°C and weighed to within 0.1 mg. Dried samples were placed in No. 980 Pyrex ignition tubes and ashed at 500°C in a muffle furnace for 12 hr. Ashed samples were digested in 5 to 10 ml of 1 HNO_3:1 HCl:2 H_2O (Baker Instru-Analyzed ultrapure acid) and analyzed for calcium (Ca), magnesium (Mg), potassium (K), and sodium (Na) using a Perkin-Elmer model 460 atomic absorption spectrophotometer.

Examination of Gill Tissue

Scanning and transmission electron microscopy (SEM and TEM) were used to determine the effect of pH on the gill tissue of *P.*

Table 1. The 96-hr LC_{50} values of three aquatic insects exposed to acidic and alkaline conditions in static laboratory bioassays

Insect species	Exposure	LC_{50}	95% Confidence limits	
			Lower	Upper
Pteronarcys dorsata	Acid	3.3	3.2	3.4
	Alkaline	12.1	12.0	12.2
Pteronarcys proteus	Acid	2.8	2.7	2.9
	Alkaline	12.1	11.9	12.2
Tallaperla maria	Acid	2.8	2.6	3.0
	Alkaline	10.4	10.1	10.5

dorsata. Nymphs were exposed to extreme pH for varying time periods. Following exposure, abdominal or thoracic tracheal gills were excised by making an incision at the gill base of the body wall. Excised gill tissue was fixed in 3% glutaraldehyde in phosphate buffer at pH 6.8. Tissue to be examined by TEM was post-fixed in 1% osmium tetroxide, dehydrated in an alcohol series, embedded in Spurr's resin for 24 hr, and hardened into plastic at 68°C. Gill tissue was sectioned with a diamond knife, placed on a coated copper grid, and post-stained with uranyl nitrate and lead citrate. Sections were viewed with a JEOL 100C transmission electron microscope. Samples examined by SEM were dehydrated in an alcohol series, dried in a critical point drier, and coated with gold-palladium in a sputter coater. Gill tissue was viewed with a JEOL JSM-35 scanning electron microscope.

Statistical Analysis

Determination of 96-hr LC50 values for acidic and alkaline pH exposures was performed with Finney's probit analysis in the statistical analysis systems (Barr *et al.* 1979). Differences in body burdens of Ca, Mg, K, and Na between control and low pH exposure groups were determined by an unpaired t-test (Barr *et al.* 1979).

Results

Acidic and Basic pH Bioassays

The three insect species were highly resistant to extreme acid and alkaline pH conditions under acute exposures (96-hr). The 96-hr LC50 values for the acidic conditions ranged from 3.3 for *P. dorsata* to 2.8 for *T. maria* and *P. proteus* (Table 1). *Pteronarcys dorsata* and *P. proteus* nymphs were highly resistant to the alkaline exposures with 96-hr LC50 values of 12.1. *Tallaperla* nymphs were less resistant to the alkaline conditions than the other two species with a 96-hr LC50 value of 10.4.

Effects of Low pH on Ion Regulation

When nymphs of *P. proteus* were exposed to low pH (3.0) for 120 hr, no significant ($\alpha = 0.05$) differences in the body burdens of Ca, Mg, or K were observed when compared to nymphs in dechlorinated tap water at pH 8.0 (Figure 2). However, significant ($\alpha = 0.05$) changes were observed in the Na body burden of the low pH exposure group as compared with the control organisms. The mean body burden of Na decreased with time for nymphs in the low pH water until 72 hr of exposure when the mean body burden was significantly lower than that of the control organisms. The body burden of Na remained below control levels throughout the rest of the exposure.

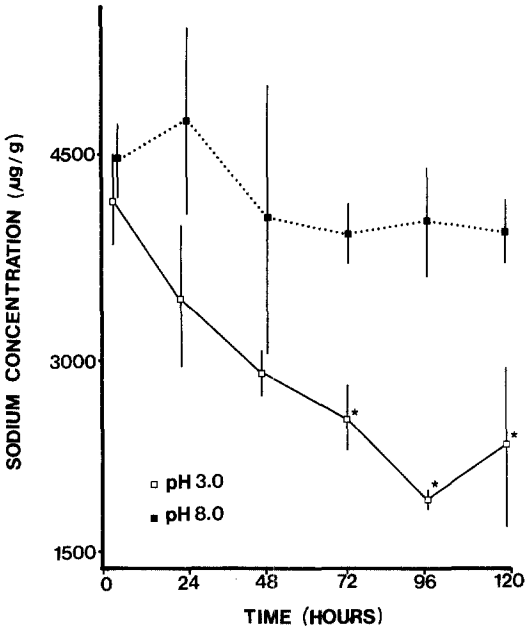

Fig. 2. The change in sodium body burden of *P. proteus* with time, when exposed to either control (pH 8) or low pH (pH 3). Asterisks represent significant ($\alpha = 0.05$) differences in sodium body burden ($n = 4$) when compared to the controls (pH 8.0) (mean ± SE)

Electron Microscopy

For *P. dorsata* nymphs, which have tracheal gills projecting from the ventral surface of the thoracic and first two abdominal segments, cup shaped, specialized osmoregulatory cells (chloride cells) were located (Figure 3). The gill filaments were covered by an external cuticle layer underlain by epithelial cells (Figure 4B). The hemocoel and numerous trachea and tracheoles were located inside the epithelial cells (Figure 4B). The osmoregulatory cells were located within the cuticle and were cup-like in structure (Figure 4A), being similar in shape to those of *Paragnetina media* described by Kapoor and Zachariah (1973a, 1973b). These cells were characterized by having a distinct cup-shaped

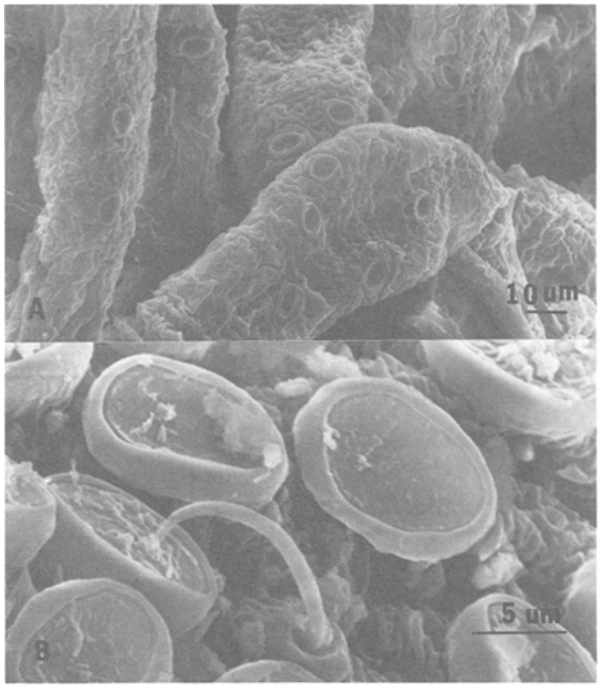

Fig. 3. (A) Scanning electron micrograph showing the osmoregulatory cells (OC) on the gills of *P. dorsata* (540×); (B) Enlargement of osmoregulatory (chloride) cell showing the surface of the cuticular disk (D) (2700×)

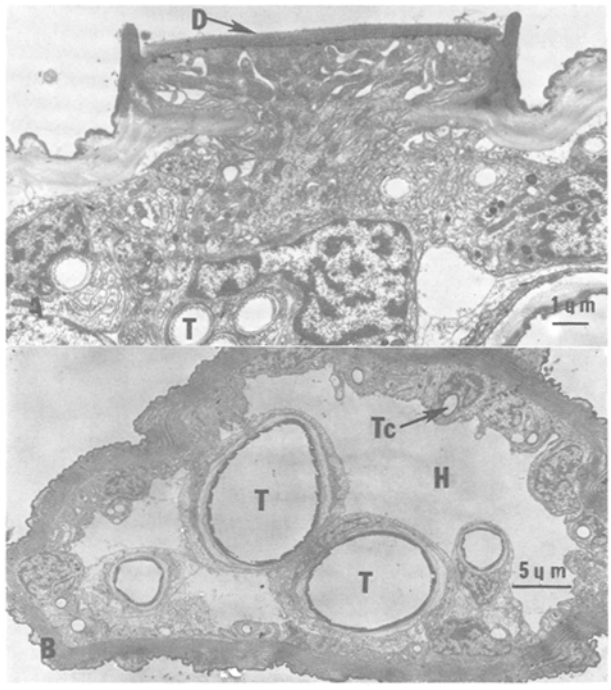

Fig. 4. (A) Control gill tissue from *P. dorsata* showing the osmoregulatory cell with cuticular disk (D), cuticle (C), mitochondria (M), and vesicles (V) (7300 ×); (B) Cross section of gill filament showing trachea (T), tracheoles (Tc), hemocoel (H), and cuticle (C) (1700×)

apical region which contained a cuticular disk with the cytoplasm having numerous mitochondria and small membrane bound vesicles (Figure 4A).

After exposure to alkaline conditions (pH 11.75 for 96 hr), several major changes were noted in chloride cell ultrastructure. Vesiculation (increased number of vesicles) was evident, and a decrease was observed in the number and size of mitochondria (M) (Figure 5).

Gill tissue from nymphs exposed to pH 2.5 for 9 hr showed a marked change in ultrastructure, with a distension of the cuticular disk and a large vacuole (V) formed beneath the disk (Figure 6A). In addition, the mitochondria were elongated and clumped. Exposure to pH 4.0 had some effect on the gill tissue and chloride cells after 96 hr (Figure 6B). Cellular ultrastructure of the chloride cell were normal (*i.e.*, there were numerous mitochondria [M] and a normal cuticular disk [CD], although there were an increased number of vacuoles [V]).

Discussion

In this study, 96-hr acidic LC_{50} values ranged from 2.8 for *T. maria* and *Pteronarcys proteus* to 3.3 for *P. dorsata*. Bell and Nebeker (1969) conducted 96-hr acidic bioassays with hydrochloric acid to determine the LC_{50} values for 10 aquatic insects. These values ranged from 1.5 for the caddisfly, *Brachycentrus americanus,* to 4.65 for the mayfly, *Ephemerella subvaria,* and a 96-hr LC_{50} of 4.25 for *P. dorsata*. This latter value was one pH unit higher than that observed for the same species in the present study. This difference may have been due to modifications in the test systems since Bell and Nebeker (1969) used a flow-through system and a static bioassay was used in the present study. Several authors have observed profound effects on hydrogen ion toxicity to fish due to Ca levels in the test water (Evans 1975; Oduleye 1975; McWilliams and Potts 1978; McDonald *et al.* 1980; Brown 1981). It is unlikely that the observed differences in toxicity were due to different Ca levels, since the water hardness in the two studies was similar (*i.e.*, difference of 52 to 45 mg/L as $CaCO_3$). The observed difference may be due to natural variability between the two populations of *P. dorsata* used.

From studies on the effects of low pH on fish, two possible modes of toxic action have been hypothesized. The first is that hydrogen ions damage gill tissue and disturb blood acid-base balance, thereby slowing the exchange of oxygen and carbon dioxide between environment and test organisms. The second is that increased hydrogen ion concen-

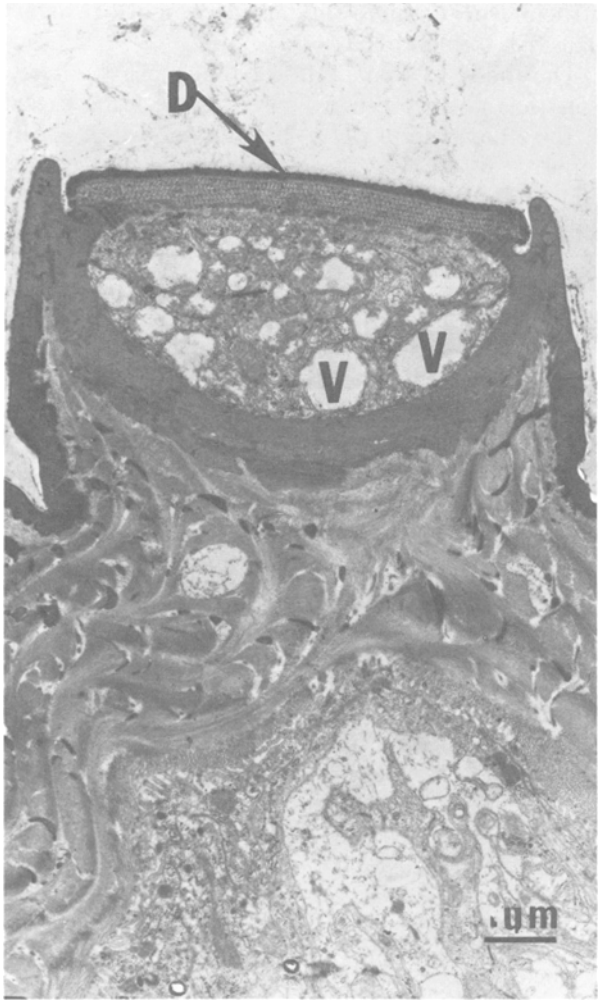

Fig. 5. Chloride cell from *P. dorsata* with cuticular disk (D) and vesicle (V) formation after exposure to pH 11.75 for 96 hr

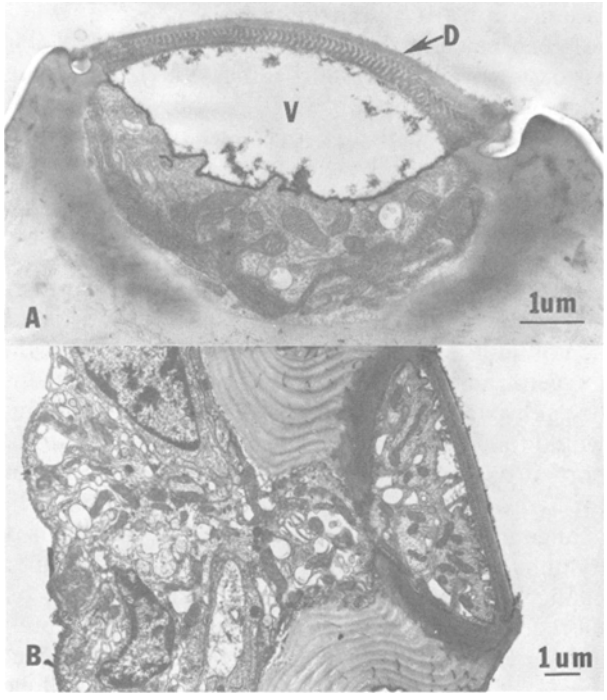

Fig. 6. (A) Osmoregulatory cell of *P. dorsata* exposed to pH 2.5 for 9 hr (12,000×); (B) Osmoregulatory cell from *P. dorsata* exposed to pH 4.0 for 96 hr (4500×)

tration disrupts ion regulatory abilities of the organism. Evidence exists to support both hypotheses (Fromm 1980; Alabaster and Lloyd 1980). Several authors have observed a great loss of Na and an inhibition of Na uptake when fish were exposed to low pH conditions (Parker and Dunson 1978; Fromm 1980; McWilliams 1980). A three-fold increase in Na loss was observed when brown trout, *Salmo trutta,* were exposed to pH 4.0 as compared to control fish in pH 7.0 water (McWilliams and Potts 1978). In the present study, a similar response was observed in *P. proteus* nymphs exposed to pH 3.0. In low pH water, a progressive loss of Na with time was observed until at 72 hr the Na concentrations of the exposed group were significantly lower than that of the control organisms (Figure 4). Evans (1975) hypothesized that the exchange of Na ions in fish is best correlated with the exchange of either hydrogen or ammonium ions. A similar mechanism may occur in aquatic insects.

In a laboratory study, Fiance (1978) measured the rate of uptake of radio-labeled Cl^{36} by three species of mayfly nymphs. No significant differences in the uptake rates were detected between the insects in the control (pH 6.3) and the low pH (4.0). The author concluded that low pH had no effect on the osmoregulatory functions of the insects. Kapoor (1980) observed a reduction in Na^+-K^+ ATP-ase activity from *Paragnetina media* when the enzyme was incubated in pH below 7.0 and above 7.5. Increased hydrogen ion concentration may reduce the ability of aquatic insects to obtain Na^+ from the environment by inhibiting this enzyme. From the data presented by other authors for fish and that presented in the present study, apparently the increased hydrogen concentrations (low pH) can have profound effects on the Na balance in both freshwater fishes and aquatic insects.

Examination of tracheal gills of *Pteronarcys dorsata* exposed to low pH provided further evidence that the mode of toxic action of hydrogen ions was due to changes in their ionoregulatory ability. Several investigators have observed an increased mucus layer in fish on the outer gill epithelial layer of acid exposed organisms. Accumulation of mucus was believed to interfere with the exchange of gases across the gill tissue to the blood (Daye and Garside 1980; Dively *et al.* 1977; Ultsch and Gros 1979). In addition, increased hydrogen ion content of the

blood caused by environmental exposure to low pH, can cause a decreased affinity of hemoglobin for oxygen (Fromm 1980; Haines 1981). No mucus accumulations were observed on gill surfaces of *P. dorsata* exposed to low pH. The outer chitonous layer surrounding the gill tissue may act as a protective layer that requires no mucus production. Once oxygen enters tracheal gills, it is transported through air filled trachea and tracheoles to the rest of the body, instead of a blood pigment as in fish. An increase in hydrogen ion concentration of the hemolymph should have little or no effect on the oxygen transport within the insect, since there is no oxygen-carrying blood pigment. This evidence would tend to rule out changes in uptake and transport of oxygen as a possible mode of toxic action for hydrogen ions in aquatic insects. Whereas little change in the general surface of the tracheal gills of nymphs exposed to low pH was observed, great changes were evident in the structure of the osmoregulatory cells of nymphs exposed to acutely lethal pH levels. In nymphs of *P. dorsata* exposed to pH 2.5 for 9 hr, vast deterioration of the cellular ultrastructure of the osmoregulatory cells was seen (Figure 6A). Similar changes were observed by Kapoor (1978) when nymphs of *Paragnetina media* were exposed to hypertonic solutions containing 1.2% NaCl. These changes imply a loss of ionoregulatory function that may explain why all organisms at pH 2.5 were dead after 48 hr. When the nymphs were exposed to pH 4.0 for 96 hr, only subtle changes were observed in the osmoregulatory cells. A strong correlation was evident between the degree of damage to the osmoregulatory cells and the acute toxicity of the target pH level.

The effects of alkaline pH on freshwater organisms have not been investigated extensively. Several fish species have been tested for their resistance to alkaline conditions. In general, salmonids have 96-hr LC_{50} values ranging from 9.0 to 10.0 pH units, depending on the species and water quality of the dilution water (Alabaster and Lloyd 1980). For non-salmonid fishes, the range of acute toxicity is from pH 9.5 to 11.0. Above pH 11.0, few, if any, fish can survive even in short-term exposures. The freshwater shrimp, *Paratya curvirostris,* had a 48-hr LC_{50} of 9.71 (Shaw 1981). Strange *et al.* (1982) found mosquito larvae, *Aedis dorsalis,* to survive in pH solutions up to 11.0. The three species of insects tested in the present study were all resistant to alkaline pH. *Pteronarcys dorsata* and *P. proteus* were extremely tolerant, exhibiting a 96-hr LC_{50} value of 12.1, while *T. maria* was less resistant (i.e., 96-hr LC_{50} value of 10.4). Apparently, from the data presented in the present study that, as in the case of acidic pH, stonefly nymphs are more resistant than fish to high pH.

The mode of toxic action of high pH (hydroxyl ions) has not been investigated to the same extent as the effects of low pH. Alabaster and Lloyd (1980) reported hydroxyl ions to affect both mucous and epithelial cells of brook trout (*Salvelinus fontinalis*) gills. High pH caused hypertrophy and separation of the surface layer from the pilaster cells. There was little detectable effect at high pH on the epithelial cells of the tracheal gills of *P. dorsata* which is probably due to the external cuticle layer of the gills. However, acutely lethal alkaline pH effects were evident in the chloride cells of nymphs (Figure 5). These results suggest that high pH caused loss of the ionoregulatory ability in the species tested. However, no attempt was made to document changes in the ionic composition of nymphs exposed to high pH, so no definitive conclusions can be made.

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