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Adaptations of sodium balance to low pH in a sunfish (*Enneacanthus obesus*) from naturally acidic waters

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Summary. Net sodium flux (J_{net}), sodium influx (J_{in}), and sodium efflux (J_{out}) were measured in two sunfish, *Enneacanthus obesus* (acid-tolerant) and *Lepomis gibbosus* (less acid-tolerant), during 24 h exposure to soft water of pH's 4.0 and 3.5. *E. obesus* exhibited a mild transitory disturbance at both pH's caused by inhibited J_{in} and slightly stimulated J_{out}. Body and plasma ion concentrations of *E. obesus* were measured weekly during exposures for 5 weeks to acidified artificial soft water (ASW). Body sodium concentration declined 30% during 2 weeks exposure to pH 3.5, but no further during the next three weeks. Exposure to pH 4.0 had no effect on body sodium concentration during the entire 5 weeks. Plasma sodium concentration declined 15% over a 3 week period at pH 3.5; there was no further change in the next two weeks. Plasma potassium concentrations, which were measured after 4 and 5 weeks at pH's 5.8 and 3.5 in ASW, were not significantly different. In a separate two week long experiment, plasma sodium concentration of *E. obesus* in ASW was correlated with pH between pH's 3.5 and 7.5. This effect was mainly due to increases above pretreatment levels at pH 4.5 and above. Increased ambient sodium and calcium concentrations had no effect on body sodium concentration of *E. obesus* at pH 5.8, but mitigated the effects of exposure to pH 3.5. Increased calcium concentrations up to 25 μ*M* at pH 3.5 increased body sodium concentration, but higher concentrations had no additional effect. Body potassium concentration and body water concentration of *E. obesus* were linearly related to body sodium concentration under a wide variety of external conditions. This suggests the presence of a mechanism by which *E. obesus* regulates plasma sodium levels and body fluid compartments in response to sodium loss. In contrast to *E. obesus*, *L. gibbosus* showed larger sodium losses at low pH resulting from greater acceleration of J_{out}; those exposed at pH 3.5 died in less than 12 h. *L. gibbosus* also had reduced body and plasma sodium concentrations at pH 4.5 and below; those at pH 4.0 were the lowest. Body potassium concentration of *L. gibbosus* was reduced in those fish exposed to pH 4.0 and below, but body water was increased. Thus there are striking differences in the ability to regulate ion and water balance at low pH between an acid-tolerant specialist (*E. obesus*) and a less acid-tolerant generalist (*L. gibbosus*).

Introduction

Swamps and bogs are among the harshest aquatic environments in North America. Typically they are extremely dilute, low in dissolved oxygen, darkly stained with humic substances, and acidic. Thus it is not surprising that such rigorous environments have greatly reduced species diversity (Rahel 1984). There are 70 species of fish native to all of New Jersey while only 16 are found in the blackwater Pine Barrens, which encompass most of the southern half of the state (Hastings 1979). Although many factors may influence species diversity in such environments, one of the most important abiotic factors is likely to be low pH. Many fish are intolerant of low pH's, while others, although more tolerant, will avoid low pH's if possible (Graham and Hastings 1984).

It is thought that the primary cause of death at lethally low pH's is disruption of ionic regulation (see review by McDonald 1983). In acidic waters active uptake of sodium is inhibited, while

Abbreviations: ASW artificial soft water; *WBM* wet body mass; *DBM* dry body mass

diffusive efflux is greatly accelerated, resulting in death when about 50% of body sodium is lost (Packer and Dunson 1970, 1972). At pH's slightly above toxic levels, there are many sublethal effects, including depressed plasma ion levels (McDonald and Wood 1981), slowed growth (Menendez 1976), unequal sex ratios of young (Rubin 1985), and inhibited oogenesis (Ruby et al. 1977). Tolerance to low pH differs enormously between species (Dunson et al. 1977; Grande et al. 1978).

Graham and Hastings (1984) concluded that out of 6 habitat variables, the banded sunfish, *Enneacanthus obesus,* was most closely associated with acidic, humic stained waters. They also reported collecting individuals from waters as low as pH 3.70. In New Jersey, the distribution of *E. obesus* is limited almost exclusively to the acidic Pine Barrens (Graham and Hastings 1984). Sweeney (1972) described several morphological adaptations of *Enneacanthus* to life in swamps, including small size, a rounded caudal fin, and dark vertical bands. Our laboratory tests indicate that *E. obesus* also has striking physiological specializations for living in blackwaters; 6 out of 10 fish survived 3 weeks exposure to pH 3.3 after a gradual stepwise lowering of the pH to 3.3 over one week! This appears to establish *E. obesus* as one of the most tolerant fish known. A similarly acid tolerant tetra of South American rivers, *Cheirodon axelrodi,* could survive prolonged exposures to pH 3.50 (Dunson et al. 1977). In Japan *Tribolodon hakonensis* is common in the extremely acidic (pH 3.5), volcanic Lake Osoresan-ko (Mashiko 1940; Mashiko et al. 1973). The most acid tolerant of the salmonids, the brook trout, has a lower lethal limit of about pH 3.75 (Packer and Dunson 1970).

Although it is well known that *E. obesus* is characteristic of acidic swamp waters, the mechanism by which it maintains sodium balance in soft-waters of such low pH has not been studied previously. Thus the purpose of this investigation was to examine the physiological adaptations that allow *E. obesus* to osmoregulate at low pH's. Special attention was devoted to determination of the effects of prior exposure (acclimation) at low pH on body and plasma sodium concentration, of variation in water sodium and calcium concentrations on body sodium regulation at low pH, and of the effects of low pH on water balance. In addition, comparisons will be made with the less tolerant but related centrarchid *Lepomis gibbosus* in order to assess which physiological responses by *E. obesus* may be most critical in adapting to life in solute-poor, acidic waters.

Materials and methods

Experimental animals. Banded sunfish ($N=455$) were collected from ponds in Burlington County, New Jersey. *L. gibbosus,* which are not found in the acidic Pine Barrens but are found in surrounding, less acidic impoundments, were used in several parallel experiments for comparison with *E. obesus.* All *L. gibbosus* were collected from Deep Lake, Monroe Co., Pennsylvania which is a mountaintop lake with extremely soft water (approximately 30 μM Ca). Deep Lake may be affected by acid rain (Arnold, unpublished observation); pH's as low as 4.0 have been recorded several times since 1982. *L. gibbosus* are very abundant although stunted in Deep Lake, but other species are absent even though surrounding buffered lakes support large populations of such fish as yellow perch and bluegills. This population of *L. gibbosus* is a good group for comparison with *E. obesus* because these fish have demonstrated by their long term survival and reproduction in Deep Lake a certain degree of acid tolerance, yet the species is not found in the acidic waters of the Pine Barrens. All fish used in the analysis were greater than 0.5 g in mass. They were kept in all-glass aquaria at room temperature (20–22 °C), in aerated artificial soft water (ASW: pH 5.8, 44 μM NaCl, 26 μM $CaCl_2$, 25 μM KCl). ASW was used in all experiments. ASW was chosen because its ionic concentrations were very similar to the dark water from which the fish were collected, but it did not contain any of the undefined humic compounds. At present it does not seem likely that the humic acids would have any specific effect on ionic regulation in the sunfish. Freda and Dunson (1985) found no differences in J_{in} and J_{out} in larvae of *Rana pipiens* tested in ASW and bog water of the same pH. Fish were fed commercial trout chow ad lib. until one day before testing, and were unfed during all experiments.

Sodium fluxes. Net sodium flux (J_{net}) and sodium influx (J_{in}) of *E. obesus* were measured over a 1 h period at pH 5.8 (control), and after 1, 3, 6, 12, and 24 h exposure to either pH 4.0 or 3.5. They were also measured in *L. gibbosus* over 12 h at pH 4.0 and over 6 h at pH 3.5. Five fish were placed in individual 280 ml chambers (1 fish per chamber) fed continuously by a 150 l recirculating, flow through system 24 h prior to the first (control) flux period. Chambers of this size were chosen because they were the minimum size that would still allow for control of pH drift during the flux periods. Similar methods were used successfully to study the effects of copper on ion balance by Lauren and McDonald (1985). For flux measurements, the flux chambers were isolated from the recirculating system, 100 µl $^{24}NaCl$ (12 kBq/100 µl) was added to each chamber, and a 10 ml bath sample was taken from each chamber. One hour later, another 10 ml sample was taken from each chamber and the chambers were flushed of the isotope and reconnected to the recirculating system. Each 10 ml sample was assayed for gamma radiation using a Beckman Biogamma II gamma counter, and for sodium concentration with a Perkin Elmer model 303 Atomic Absorption Spectrophotometer. Immediately following the control flux period the pH of the water was lowered with dilute sulfuric acid to the desired pH. The process of lowering the pH took 15–20 min, and the low pH exposure period was said to begin when the desired pH was reached. The pH of the water was checked regularly during the 24 h test period, and was adjusted as needed.

J_{net} was calculated from the difference between the initial and final sodium concentration of the water in the flux chambers. J_{in} was calculated from the disappearance of the ^{24}Na from the water using the equation given by McDonald et al. (1983):

$$J_{in} = \frac{(\ln Q^*_{out0} - \ln Q^*_{out1})\, Q_{out}}{t \cdot M}$$

where Q^*_{out0} and Q^*_{out1} are the total cpm in the flux chambers at the beginning and the end of the flux period, respectively. Q_{out} is the average amount of Na in the flux bath during the flux period, t is the time in hours, and M is the mass of the fish in kg. This equation is equivalent to the one compartment equation commonly used (Robertson 1983). Sodium efflux (J_{out}) was calculated by subtracting J_{net} from J_{in}.

Body sodium. The effect of a 5 week exposure to sublethal pH's on body sodium concentrations was examined. On day zero, 9 fish were removed from the holding tank (ASW) and analyzed for body sodium concentration as follows. The fish were weighed, dried at 100 °C for 24 h, weighed again, and dissolved in concentrated nitric acid. The resulting solutions were diluted with distilled water and analyzed for sodium with a Varian Techtron model 1280 Atomic Absorption Spectrophotometer (air/propane flame). The remaining 75 fish were then divided randomly among three 40 l aquaria containing ASW adjusted to either pH 5.8 (control), 4.0, or 3.5. pH's were adjusted daily with sulfuric acid and water was changed weekly. Once a week, for 5 weeks, 5 fish were removed from each aquarium, weighed, and analyzed for body sodium concentration. All body sodium concentrations were expressed as μmol/g wet body mass unless otherwise noted. Shearer (1984) demonstrated that while the amount of sodium per gram dry body mass decreased dramatically as size increased in the rainbow trout, sodium per gram wet body mass remained unchanged.

Plasma sodium. In a separate but parallel experiment, the effect of a 5 week exposure to pH's 5.8 (control), 4.0, and 3.5 on plasma sodium concentrations was investigated. On day zero, 5 fish were removed from the holding tank and a blood sample was taken from each after decapitation behind the operculum. Blood was collected from the dorsal blood vessel with a heparinized capillary tube. The blood sample was centrifuged, hematocrit was measured, and the plasma was diluted for sodium analysis. Blood samples were taken once a week for five weeks from five fish in each pH treatment. By the second day of the test period all of the fish at pH 3.5 had died. The pH 4.0 tank was lowered to 3.5 that day and remained there for the duration of the experiment; no further deaths occurred. In all subsequent experiments prolonged exposure to pH 3.5 was preceded by a 3 day pre-exposure to pH 4.0 as a precautionary measure. pH's were adjusted daily, and the water in each tank was changed weekly.

Ambient cation effects. The interaction of water sodium and calcium concentrations and pH in control of body sodium concentration in *E. obesus* was examined by exposing sunfish to high and low sodium and calcium concentrations at pH's 5.8 and 3.5. They were exposed to two levels of sodium chloride, 45 μ*M* (low) and 450 μ*M* (high), and calcium chloride, 10 μ*M* (low) and 250 μ*M* (high). Potassium chloride was 25 μ*M* in all tests. At each pH, 40 fish were randomly divided among the 4 possible sodium and calcium concentrations in 40 l aquaria. Ten pre-treatment fish were removed from the holding tanks at the start of each exposure period to detect any differences in initial body sodium concentrations due to variation in starting times at the two pH's. Aquarium pH's were adjusted daily and the water in each tank was changed twice weekly. After two weeks the fish were removed, weighed, and analyzed for body sodium concentration.

Two additional tests were performed to more closely examine the interaction between calcium and pH in body sodium regulation. In the first, calcium and pH were varied at constant levels of sodium chloride (45 μ*M*) and potassium chloride (25 μ*M*). One hundred twenty fish were equally divided among 6 calcium chloride concentrations (0, 15, 25, 125, 625, and 1250 μ*M*) at pH's 5.8 and 3.5. Five additional fish each were placed in 0 and 1250 μ*M* calcium chloride at the two pH's to study the effects on plasma sodium concentration. All fish were removed after two weeks, and those to be analyzed for body sodium were weighed and processed as before, while blood samples were taken from the remainder.

In the second experiment, 15 sunfish each were placed in four 20 l aquaria containing ASW adjusted to pH's 7.5, 5.8, 4.5, or 3.5. Fifteen additional fish were sampled from the holding tanks prior to test exposures for pre-treatment body (10) and plasma (5) sodium concentrations. ASW was adjusted to pH 7.5 with dilute potassium hydroxide. pH's were adjusted daily and the water in each tank was changed weekly. After exposure for two weeks, 10 fish were removed from each tank, weighed and analyzed for body sodium concentration. The remaining 5 fish in each tank were removed, blood samples were taken, and the plasma was analyzed for sodium concentration.

For comparison with *E. obesus*, 60 *L. gibbosus* were divided among 6 ASW filled 40 l aquaria adjusted to pH's 5.8, 4.5, 4.25, 4.0, 3.75, or 3.5. Ten additional fish were removed from the holding tank prior to exposure to estimate pre-treatment ion levels. pH's of the test aquaria were adjusted twice daily with dilute sulfuric acid. Fish were removed at death or after one week. Upon removal, blood samples were taken from each fish and analyzed for plasma sodium and potassium concentration. The fish was then weighed, dried, and analyzed for body sodium concentration as before.

Statistical analysis. All results are reported as means ± SE. Significant differences between means were determined by either a Student's t-test, or analysis of variance and multiple comparisons (Bonferroni method) with an overall significance level of $P < 0.05$. Flux means were compared to zero using a one-sample t-test. Hematocrit and body water values were arcsine transformed before comparisons (Sokal and Rohlf 1969). An F-test was used to delineate significant linear relationships.

Results

Sodium fluxes

The effects of water at pH 4.0 on J_{net}, J_{in}, and J_{out} of *E. obesus* and *L. gibbosus* are shown in Fig. 1. Control *E. obesus* were in sodium balance; J_{net} was not statistically different from zero (Fig. 1). After a one-hour exposure to pH 4.0, J_{net} became slightly, but significantly negative (−65 μmol/kg·h). No further measurements of J_{net} were statistically different from zero. *L. gibbosus* were in positive sodium balance (336 μmol/kg·h) during the control period, but became negative (−419 μmol/kg·h) after a 1 h exposure to pH 4.0 ASW. J_{net} gradually approached zero as exposure to pH 4.0 continued, but it was still significantly negative after 12 h. An analysis of variance with multiple comparisons comparing test J_{net} to control for both species showed that all test J_{net} for

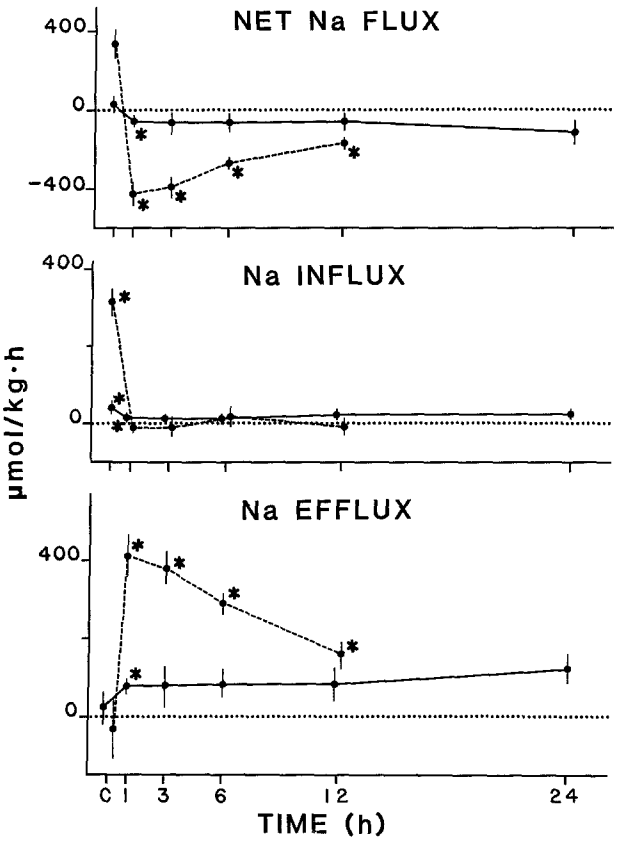

Fig. 1. Sodium fluxes at pH 4.0 of *E. obesus* (solid line) and *L. gibbosus* (dashed line). Control at pH 5.8 indicated by *C* on the abscissa. Values are means ± SE ($n=5$) and are slightly offset horizontally where they overlap to facilitate comparisons. Wet mass = 10.0 ± 1.4 g for *E. obesus* and 7.3 ± 0.6 g for *L. gibbosus*. Asterisks indicate significant differences from zero

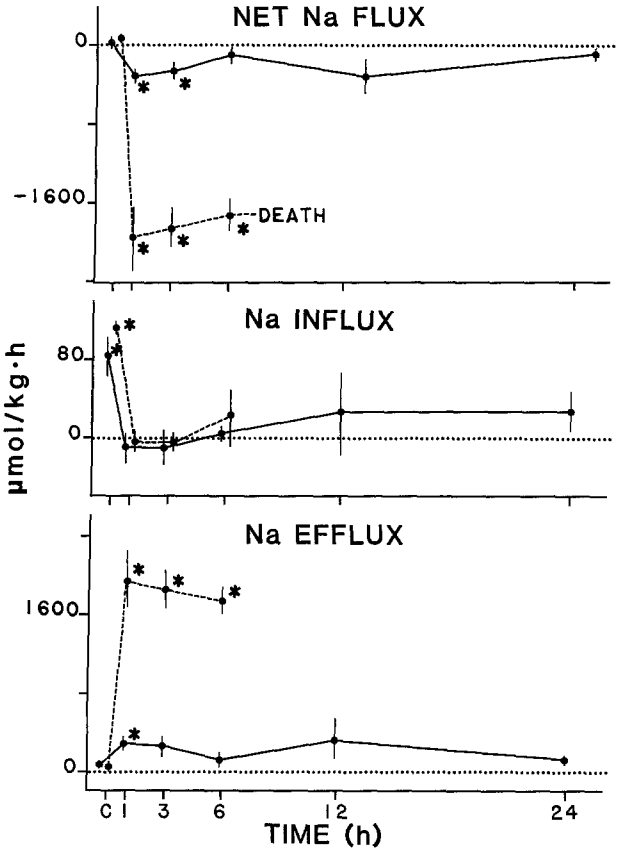

Fig. 2. Sodium fluxes at pH 3.5 of *E. obesus* (solid line) and *L. gibbosus* (dashed line). Control at pH 5.8 indicated by *C* on the abscissa. Values are means ± SE ($n=5$) and are slightly offset horizontally where they overlap to facilitate comparisons. Wet mass = 7.8 ± 0.7 g for *E. obesus* and 8.2 ± 0.7 g for *L. gibbosus*. Asterisks indicate significant differences from zero

L. gibbosus were significantly lower than control; none were different for *E. obesus*. J_{net} of *L. gibbosus* was significantly lower than *E. obesus* during all but the 12 h test period.

J_{in} of *E. obesus* was significantly positive (45 µmol/kg·h) during the control period (Fig. 1). It declined upon exposure to pH 4.0, but was still positive (17 µmol/kg·h) after 1 h; no other measurements were significantly different from zero. J_{in} of *L. gibbosus* was significantly positive (305 µmol/kg·h) during the control period, but appeared to be fully inhibited upon exposure to pH 4.0 ASW. An analysis of variance and multiple comparisons indicated that all test J_{in} for *L. gibbosus* were lower than controls.

J_{out} of *E. obesus* was not significantly different from zero during the control period, but it became significantly positive (82 µmol/kg·h) after 1 h exposure to pH 4.0 (Fig. 1). J_{out} was not different from zero during any other period except at 24 h. J_{out} of control *L. gibbosus* was not different from zero, but after 1 h exposure to pH 4.0 it rose to 415 µmol/kg·h. It gradually declined but remained elevated (158 µmol/kg·h) after 12 h. All test J_{out} for *L. gibbosus* were significantly greater than controls. J_{out} of *L. gibbosus* was significantly greater than that of *E. obesus* during all except the 12 h test period.

The effects of pH 3.5 ASW on J_{net}, J_{in}, and J_{out} of *E. obesus* and *L. gibbosus* are shown in Fig. 2. J_{net} of *E. obesus* became significantly negative (−299 µmol/kg·h) after a 1 h exposure to pH 3.5 (Fig. 2). J_{net} was still significantly negative after 3 h exposure, but not in later determinations. Exposure of *L. gibbosus* to pH 3.5 resulted in a massive decline in J_{net} to −1973 µmol/kg·h. There was some minor recovery over 6 h, but all 5 fish died prior to the 12 h flux period. All test J_{net} of *L. gibbosus* were different from controls. J_{net} of *L. gibbosus* was significantly lower than *E. obesus* during all test periods.

J_{in} of both species showed similar responses

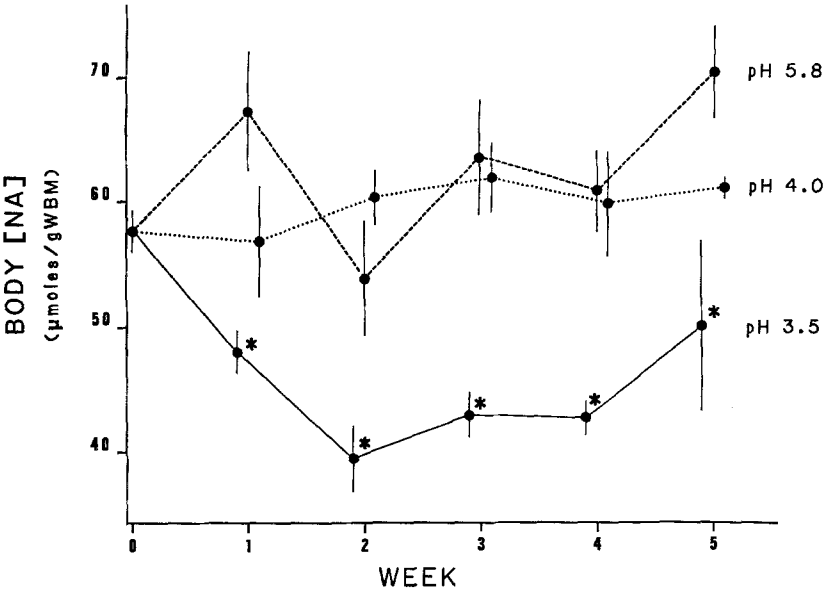

Fig. 3. Chronic changes in body sodium concentration (μmol/g wet body mass) of unfed *E. obesus* at pH's 5.8 (control), 4.0, and 3.5 in ASW. Values are means ± SE and are slightly offset horizontally at each weekly sampling interval to facilitate comparisons ($n = 5$ for each sample). Wet mass = 3.5 ± 1.6 g

to pH 3.5 ASW (Fig. 2). Control J_{in} of both species were significantly positive, and they were both fully inhibited throughout the exposure (Fig. 2).

J_{out} of *E. obesus* increased significantly to 287 μmol/kg·h upon exposure to pH 3.5, but was still less than that of *L. gibbosus* at pH 4.0 (Figs. 1, 2). J_{out} appeared to gradually decline between 1 and 24 h; no further measurements were different from zero. Upon exposure to pH 3.5, J_{out} of *L. gibbosus* accelerated enormously to 1969 μmol/kg·h. It declined slightly during the 6 h prior to death. All test J_{out} for *L. gibbosus*, but not *E. obesus*, were significantly higher than controls. J_{out} for *L. gibbosus* was significantly higher than for *E. obesus* during all test periods.

If J_{in} is zero, then it would be expected that no change occurs in the amount of radiotracer in the bath. However, because there is random error inherent in the counting procedure, it would be predicted that about half of the time $Q^*_{out1} > Q^*_{out0}$, and the other half $Q^*_{out1} < Q^*_{out0}$. Thus, observed J_{in} values should be randomly distributed around 0, and on average half of the observed values should be negative. As a result, mean J_{in} could be negative. Since J_{out} is calculated from the difference between J_{in} and J_{net}, it too could be negative. Negative values of mean J_{in} and J_{out} were observed, but as expected, none were significantly different from 0.

Body and plasma sodium

The effects of chronic exposure to pH's 4.0 and 3.5 on body sodium concentration are illustrated in Fig. 3. Body sodium concentration of fish at pH 3.5 was significantly lower than that at pH 5.8 after one week ($P < 0.025$), and had declined 30% after two weeks; it did not decline further during the next 3 weeks of exposure. No disturbance in body sodium was seen in fish at pH 4.0. It is interesting to note that although body sodium concentration did not change during the 5 week exposure to pH 5.8 it was quite variable, ranging from 53 to 68 μmol/g wet body mass. The effects of 5 weeks of exposure to ASW at pH 5.8 or 3.5 ASW on plasma sodium concentration are shown in Fig. 4. While plasma sodium concentration did not change during the first week of exposure to pH 3.5, it did decline significantly below controls to 104 m*M* after 3 weeks at pH 3.5. There was no further change during weeks 4 and 5. Plasma potassium concentration was measured at both pH's at 0, 4 and 5 weeks; there were no differences between treatments (pooled mean = 4.0 ± 0.2 m*M*).

A more detailed examination of the effects of varying pH on body and plasma sodium concentration of fish in ASW is presented in Table 1. Body sodium concentrations of fish exposed to pH 3.5 were significantly less than those of pre-treatment fish sampled from holding tanks at the beginning of the experiment, but those of fish exposed to higher pH's were unaffected. Plasma sodium concentration increased above pre-treatment levels for all pH's except 3.5, where there was no change. There was a direct relation between external pH and the plasma sodium concentration at two weeks (Plasma Na = 103.6 + 4.8 pH, $r^2 = 0.73$, $P < 0.01$).

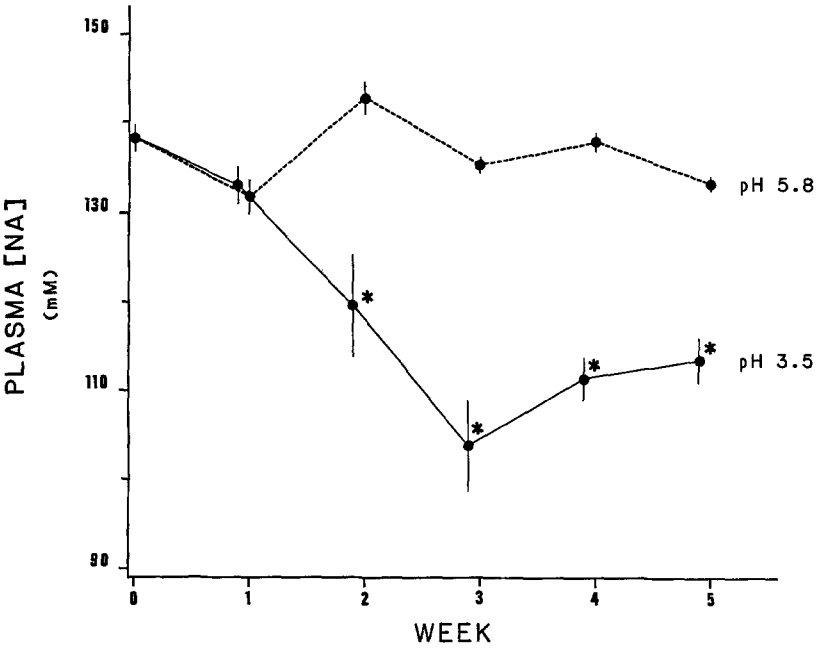

Fig. 4. Plasma sodium concentration of unfed *E. obesus* during chronic exposure (5 weeks) to pH's 5.8 and 3.5 in ASW. Values are means ± SE ($n=5$ for each sample)

Table 1. Body and plasma sodium concentrations of *E. obesus* exposed to pH's 7.5, 5.8, 4.5, and 3.5 for two weeks in ASW

Treatment	Wet body mass g(n)	Body [Na] μmol/g WBM	Plasma [Na] m*M* (*n*)
Pre-treatment	1.2±0.1 (9)	50.1±1.2	120.7±1.2 (5)
pH 7.5	1.9±0.3 (9)	49.8±0.6	137.5±1.0 (4)*
pH 5.8	1.6±0.2 (9)	49.6±0.7	131.6±2.0 (5)*
pH 4.5	2.0±0.3 (6)	53.9±0.7	128.6±1.4 (5)*
pH 3.5	1.3±0.1 (8)	45.0±1.3*	116.2±0.9 (4)

Pre-treatment fish were removed from ASW (pH 5.8) holding tanks prior to experimental exposures. Values are means ± SE. Asterisk indicates significant difference from pre-treatment ($P<0.05$); WBM = wet body mass

Results from exposure of *L. gibbosus* to low pH are presented in Table 2. All fish exposed to pH's 3.5 and 3.75 died in less than one day. Only 2 out of 10 fish in water at pH 4.0 died during the one week exposure; all fish at higher pH's survived the exposure period. Those exposed to pH 4.5 and below exhibited depressed body and plasma sodium concentrations. Paradoxically, those that lived one week at pH 4.0 had lower body and plasma sodium concentrations than those that died in less than a day at pH's 3.5 and 3.75. Body potassium concentration was lower in fish exposed to pH 4.0 and below; it was lowest at pH 4.0. Body water and plasma potassium concentration were elevated

Table 2. Body and plasma sodium and potassium concentration, body water content, and hematocrit of *L. gibbosus* exposed to various pH's in ASW for one week or until death

pH	Wet body mass g(n)	Body [Na] μmol/g WBM	Body [K] μmol/g WBM	Body water % WBM	Plasma [Na] m*M*	Plasma [K] m*M*	Hematocrit %
Pre-treatment	5.6±0.3 (10)	61.0±1.4	76.9±1.1	73.7±0.4	147.3±2.4	4.3±0.6	36.3±1.5
5.8	6.9±0.9 (10)	57.8±1.4	78.8±1.5	73.8±0.3	148.2±2.3	5.4±0.3	39.1±1.7
4.5	8.5±1.2 (10)	55.8±1.3*	73.8±1.1	73.6±0.3	141.1±1.6	4.1±0.7	61.3±5.2*
4.25	7.3±0.7 (10)	48.1±1.0*	72.4±1.3	73.9±0.3	130.2±2.1*	3.8±0.4	66.5±3.1*
4.0	6.2±0.5 (8)	32.5±0.9*	54.3±1.0*	76.5±0.5*	90.3±7.3*	6.9±1.2*	68.8±2.0*
3.75	8.6±1.0 (10)	37.6±2.0*	62.6±2.0*	77.3±0.5*	100.2±3.7*	7.6±0.3*	66.8±3.6*
3.5	9.2±0.9 (10)	44.3±1.3*	64.9±1.0*	76.2±0.3*	103.9±3.5*	9.3±0.3*	49.8±3.3*

Pre-treatment fish were removed from ASW (pH 5.8) holding tanks prior to experimental exposures. Fish exposed to pH's 3.5 and 3.75 died in less than one day. Values are means ± SE. Asterisk indicates significant difference from pre-treatment ($P<0.05$); WBM = wet body mass

Table 3. Effect of two weeks exposure to high and low sodium and calcium concentrations (constant potassium concentration) at pH's 5.8 and 3.5 on body sodium and water concentration of *E. obesus*

Treatment	Wet body mass g(n)	Body [Na] μmol/g WBM	Body water % WBM
pH 5.8			
Pre-treatment	1.3±0.1 (8)	46.9±1.1	76.8±0.1
LowNa, LowCa	2.1±0.4 (5)	49.9±1.2	77.6±0.4
LowNa, HighCa	1.4±0.2 (6)	50.5±0.6	77.7±0.3
HighNa, LowCa	1.6±0.4 (6)	53.1±1.4*	78.5±0.3*
HighNa, HighCa	1.3±0.2 (6)	50.7±0.2	77.6±0.2
pH 3.5			
Pre-treatment	3.2±0.3 (10)	58.2±1.7	77.6±0.4
LowNa, LowCa	3.8±0.4 (2)	39.1±2.8*	75.8±1.0
LowNa, HighCa	2.9±0.2 (9)	48.3±1.7*	74.9±0.4
HighNa, LowCa	0.6±0.1 (6)	47.4±2.3*	76.2±0.4
HighNa, HighCa	0.8±0.1 (9)	52.2±1.9	75.9±0.3

Pre-treatment fish were removed from ASW holding tanks prior to experimental exposures. All values are means±SE. Asterisk indicates significant difference from pre-treatment ($P<0.05$); WBM = wet body mass

at pH 4.0 and below, and hematocrit was significantly higher at pH's 4.5, 4.25, 4.0 and 3.75.

The interactive effects of changing water pH, and sodium and calcium concentration on body sodium concentration of *E. obesus* are reported in Table 3. Body sodium concentrations of pre-treatment fish (sampled from the holding tanks prior to test exposures) were significantly different; those sampled later (for the test at pH 3.5) were greater. Body sodium concentration of the HighNa, LowCa treatment was significantly greater than pre-treatment at pH 5.8 ($P<0.05$), while the others were not different. In contrast, body sodium concentrations of 3 out of 4 treatments at pH 3.5 were significantly less than pre-treatment levels. The LowNa, LowCa treatment at pH 3.5 was 33% below pre-treatment levels, and was significantly less than all other treatments as well as pre-treatment ($P<0.05$ for all). The LowNa, HighCa and HighNa, LowCa treatments were intermediate, declining 18% from pre-treatment levels ($P<0.05$).

The above results showed that increased sodium and calcium concentrations could reduce the effects of pH 3.5 on body sodium concentration when potassium was constant. A further test of this effect was made by holding potassium and sodium constant and varying calcium and pH (Table 4). Varying water calcium concentration had no effect on body sodium concentration at pH 5.8. At pH 3.5 with no external calcium, body sodium concentration was 48% lower than controls. An increase in calcium from 0 to 25 μ*M* was correlated with increased body sodium concentration; above 25 μ*M* there was no consistent effect on body sodium concentration. Plasma sodium concentrations of fish in the extreme calcium concentrations (0 and 1250 μ*M*) at pH 5.8 and in the high calcium concentration at pH 3.5 were not different from those of controls kept in 25 μ*M* calcium (Table 4).

Table 4. Effects of exposure of *E. obesus* for two weeks to varying calcium concentrations at pH's 5.8 and 3.5 on body sodium, potassium, and water concentration and plasma sodium and hematocrit

[CA] μ*M*	Wet body mass g(n)	Body [Na] μmol/g WBM	Body [K] μmol/g WBM	Body water % WBM	Plasma [Na] m*M*	Hematocrit %
pH 5.8						
0	1.7±0.2 (10)	57.2±0.8	77.3±0.8	76.5±0.4	135.6±1.9	53.5±2.5
15	2.1±0.3 (10)	57.2±1.7	72.5±1.5	76.8±0.5	–	–
25 (control)	2.4±0.3 (8)	55.3±1.0	70.1±3.3	75.8±0.4	135.0±3.3	45.6±3.2
125	1.7±0.2 (9)	57.2±1.3	75.1±0.8	76.3±0.5	–	–
625	2.0±0.2 (10)	55.4±0.6	74.9±0.8	75.9±0.3	–	–
1250	2.0±0.4 (10)	56.0±1.3	72.9±2.1	75.9±0.3	130.7±1.7	51.5±5.5
pH 3.5						
0	1.7±0.2 (7)	27.2±2.1*	51.7±2.1*	75.9±0.7	83.1±7.7*	75.8±4.0*
15	1.5±0.3 (8)	36.4±2.4*	58.8±2.6*	75.1±0.4	–	–
25	1.6±0.2 (10)	45.2±2.6*	57.1±2.2*	75.6±0.5	–	–
125	1.5±0.2 (7)	49.1±1.7	65.4±1.0	75.3±0.8	–	–
625	1.9±0.3 (9)	43.1±1.8*	65.8±0.8	74.3±0.3	–	–
1250	1.2±0.1 (9)	48.4±0.8	67.2±1.1	75.3±0.3	124.3±2.6	54.4±3.8

All values are means±SE. Asterisk indicates significant difference from control ($P<0.05$). WBM = wet body mass; $n=4$ for all plasma samples

Table 5. The relationship between body water (*W*) and body sodium concentration (*Na*) for *E. obesus* exposed to varying sodium and calcium concentrations at pH 5.8 and 3.5, and *L. gibbosus* exposed to ASW of varying pH

Treatment	*n*	Equation	Minimum [Na] μmol/g DBM	Minimum body water % WBM	r^2	*P*
Varying [Ca]						
pH 3.5	49	W=72.5+0.016Na	90.7	72.8	0.18	<0.01
pH 5.8	57	W=66.2+0.042Na	190.4	73.5	0.83	<0.01
Varying [Na] and [Ca]						
pH 3.5	26	W=72.6+0.015Na	137.4	73.2	0.12	>0.05
pH 5.8	23	W=68.9+0.039Na	202.4	76.0	0.74	<0.01
L. gibbosus	68	W=77.8−0.015Na	120.0	72.4	0.09	<0.025

All sodium and calcium concentrations were combined by pH for *E. obesus*. *DBM* = dry body mass

Plasma sodium was significantly lower than controls ($P<0.05$) in fish in pH 3.5 with no calcium, and all the fish died before the end of the exposure period. Body potassium concentration at pH 3.5 was significantly lower ($P<0.05$) than controls (pH 5.8, 25 μ*M* calcium) at 0, 15, and 25 μ*M* Ca (Table 3). Body potassium concentration was linearly related to body sodium concentration (K = 36.7+0.64 Na, $r^2=0.49$, $P<0.01$; units of μmol/g WBM) for all treatments combined. Hematocrit of fish exposed to pH 3.5 with no calcium was significantly greater ($P<0.05$) than that of controls (Table 4).

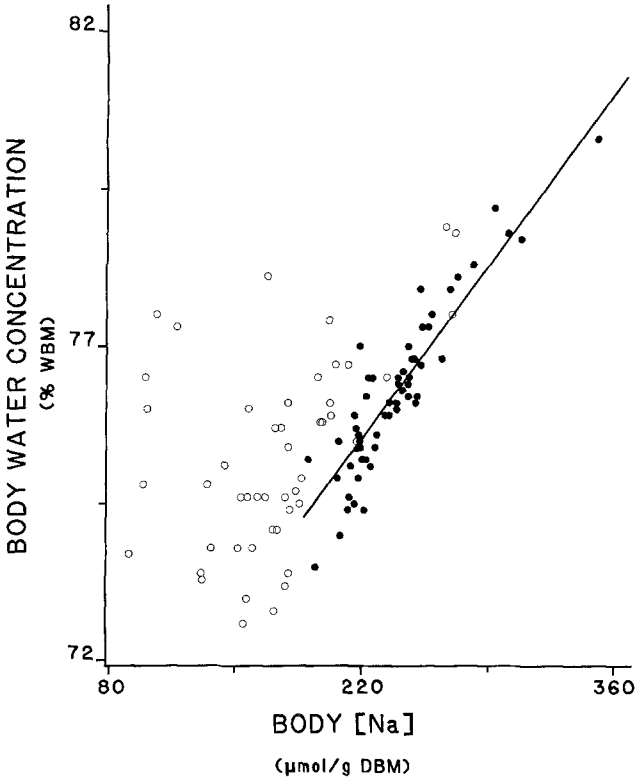

Fig. 5. Scatter plot of body water concentration versus body sodium concentration for *E. obesus* exposed to various calcium concentrations at pH's 5.8 (closed circles) and 3.5 (open circles; calcium treatments were combined by pH), with the regression line for pH 5.8 (W = 66.2 + 0.04 Na, $r^2=0.83$, $P<0.05$)

Body water

Body water, as % wet body mass (WBM), of *E. obesus* exposed to pH 3.5 with varying calcium or sodium tended to be slightly lower or about the same as those of fish exposed to pH 5.8 (Tables 3 and 4). In contrast, body water of *L. gibbosus* exposed to pH's 4.0 and below were significantly higher than controls (Table 2). Regression of body water concentration against body sodium concentration by pH treatment for *E. obesus* revealed a pattern (Table 5). Body water (W) was directly and linearly related to body sodium concentration (Na) in the pH 5.8 treatments of Tables 3 and 4. Only one group at pH 3.5 (varying Ca) showed such a significant relationship, and the r^2 value was very low (0.18). Although the minimum body sodium concentrations were much lower in the pH 3.5 treatments, the minimum body water values were not different. A plot of body water against body sodium concentration for the varying calcium test (Table 4) is presented to illustrate this pattern (Fig. 5). A similar regression for *L. gibbosus* revealed a poor, but significantly negative relationship between body water and body sodium concentration. In contrast to *E. obesus*, body water increased as body sodium declined (Table 5). Body sodium concentration was expressed as μmol/g dry body mass (DBM) for these analyses, instead of the usual μmol/g WBM, to remove the effects of possible changes in mass due to changes in water

content. A regression of body sodium concentration (μmol/g DBM) against dry mass showed no relation, indicating that the size range of the fish used in these experiments was not great enough to have any effect on body sodium concentration.

Discussion

E. obesus exhibited only a small, transient disturbance in sodium balance upon exposure to pH 3.5 arising from inhibition of J_{in} and moderate stimulation of J_{out}. During continued exposure to pH 3.5 J_{out} appeared to decline. In comparison, *L. gibbosus* exhibited a large increase in J_{out} upon exposure to pH 3.5 with little sign of recovery; death resulted in less than 12 h. Even at pH 4.0, while J_{out} of *E. obesus* was very slightly stimulated during initial exposure, J_{out} of *L. gibbosus* was 5 times greater. Clearly, *E. obesus* demonstrates a strikingly lower sodium permeability of the gills than *L. gibbosus* at pH 4.0 and 3.5.

It seems that *E. obesus* and *L. gibbosus* do not differ in their inability to actively absorb sodium at pH 4.0 and below. Similar results have been achieved in studies of J_{in} in several species of trout (Packer and Dunson 1970; McWilliams and Potts 1978; McDonald et al. 1983). pH 4.0 is acutely lethal to most trout, but is well above lower lethal limits for *E. obesus*. Thus, it might seem reasonable to expect *E. obesus* to be able to actively absorb sodium at pH's below 4.0. However, despite the exceptional acid tolerance of this sunfish, J_{in} shows an inhibition by low pH similar to that of the less acid tolerant species.

E. obesus showed no increase in J_{in} during 24 h exposure to pH's 4.0 and 3.5. No compensation in J_{in} at these pH's occurred, although longer term recovery can not be ruled out. Other fresh water animals show a range of ability to recover J_{in} under acidic conditions. Brown trout recovered J_{in} in a matter of days, depending on the exposure pH and the water calcium levels (McWilliams 1980). Tadpoles of *Rana pipiens* exposed to pH 4.5 increased J_{in} ten-fold after 7 days (Freda and Dunson 1984). Rainbow trout exposed to pH 4.0 soft water, however, showed no recovery of J_{in} (McDonald et al. 1983).

Plasma sodium concentration increased above pre-treatment levels at pH 4.5 and greater (Table 1). The size of the increase was linearly related to the exposure pH; there was no change at pH 3.5 and the greatest increase was at pH 7.5. This may reflect a linear relationship between J_{in} and pH. If J_{out} is constant and near zero, then the increase in plasma sodium concentration as pH increases might be due to increasing J_{in}.

The inability of *E. obesus* to actively take up sodium at pH's below 4.0 emphasizes the critical importance of control of J_{out} at these low pH's. *E. obesus* exhibited mild to moderate acceleration of J_{out} upon exposure to pH's 4.0 and 3.5, but it gradually declined during continued exposure. Thus *E. obesus* not only resists increases in J_{out} at low pH's, it also seems to be able to restrict permeability to sodium even further during continued exposure. Krout and Dunson (1985) found that extremely low pH's were required to stimulate J_{out} in two species of air-breathing fish (bowfin and eastern mudminnow) native to acidic waters. *Rana pipiens* larvae showed some decreases in J_{out}, after initial increases, during prolonged exposure to low pH (Freda and Dunson 1984). Rainbow trout also reduced J_{out}, after initial increases, during 40 h exposure to pH 4.0 (McDonald et al. 1983). Studies on trout have shown that survival times at low pH were closely related to rate of net sodium loss (Robinson et al. 1976; Swarts et al. 1978). *E. obesus* shows an unusual ability to limit increases in J_{out} at low pH and to recover from acid stimulated efflux over a very short period of time (a few hours) during exposure to low pH. These adaptations probably play a primary role in the ability of *E. obesus* to prevent excessive sodium losses and to inhabit acidic environments.

Body sodium concentration of *E. obesus* exposed to pH 3.5 for five weeks declined by 30% after two weeks, but was stable in the following three weeks. Reduction of J_{out} under these conditions probably plays an important role in the prevention of further sodium losses. Body sodium concentration was not disturbed at pH 4.0, again probably largely due to reduction of J_{out}.

pH appears to be a very important factor controlling body sodium in *E. obesus* only below pH 4.0, where sodium levels were depressed. In contrast, body sodium concentrations of *L. gibbosus* at pH's below 4.5 were depressed. Recent studies have shown that sodium balance of several other species of fish is greatly disrupted at pH 4.0. Rainbow trout showed depression of plasma sodium levels and no recovery during 5 days of exposure to pH 4.0–4.5 (McDonald et al. 1980). Brown trout lost 10% of their blood sodium after 50 h at pH 4.0 (Leivestad and Muniz 1976). Body sodium concentration of brook trout exposed to pH's less than 4.5 decreased markedly (Packer and Dunson 1970).

Variation in external calcium and sodium concentrations has little effect on body sodium con-

centration at high pH. Only one treatment at pH 5.8 (HighNa, LowCa) out of 9 (Tables 3 and 4) was different from pre-treatment or control groups, and it was increased relative to its pre-treatment. It is only at pH 3.5 that increased calcium or sodium concentrations act to mitigate the effects of low pH on body sodium concentration. The influence of calcium at pH 3.5 is substantial, but only over a narrow concentration range. Body sodium concentrations of fish exposed to pH 3.5 with no external calcium were depressed by 48%, but as external calcium concentration increased to 25 μM, body sodium concentration increased. Above 25 μM, calcium concentration had no additional effect on body sodium concentration. It has been proposed that the key mechanism for control of sodium efflux at low pH is increased calcium affinity of the gill epithelia. Studies indicate that sodium efflux increases when hydrogen ions leach calcium from the gill, allowing sodium to diffuse out (McWilliams 1983; McDonald and Rogano 1986). The fact that external calcium concentration above 25 μM had no additional effect on body sodium concentration at pH 3.5 indicates that calcium affinity of the gills in such a low pH tolerant fish is great.

E. obesus exposed to pH 3.5 lost from 10 to 50% of their body sodium depending on the experimental conditions. Packer and Dunson (1970) showed that brook trout die when about 50% of their body sodium is lost. Milligan and Wood (1982) proposed a sequence of events triggered by sodium loss that culminates in death. The immediate result of sodium loss is plasma dilution and creation of an osmotic gradient across cell membranes. This in turn causes shrinkage of the extracellular fluid space as water moves into cells along the osmotic gradient. This leads to further disturbances and ultimately death. While *E. obesus* at pH 3.5 clearly suffered massive sodium losses at pH 3.5, the result was not necessarily death. Although two separate groups of fish were used to measure plasma and body sodium levels it is interesting to note that a 15% drop in body sodium concentration during the first week of acid exposure did not coincide with a drop in plasma sodium concentration (Figs. 3 and 4). Further, plasma sodium concentration did decline during weeks 2 and 3, while body sodium concentration stopped declining after 2 weeks. In another experiment (Table 1), plasma sodium concentrations increased above controls after 2 weeks at pH 4.5 and above, but body sodium concentrations did not change. Further study of the partitioning of sodium stores is in order to determine whether plasma sodium levels can be regulated independently of total sodium. It is possible that sodium sequestered in bone may be utilized to maintain plasma levels in the face of dropping body sodium levels.

Another possibility, for which there is some evidence, is the excretion of water proportionate to sodium losses to maintain plasma sodium levels. Body water concentration appears to be closely and positively tied to body sodium concentration for *E. obesus* (Table 5). In contrast, body water is negatively related to body sodium concentration in *L. gibbosus*. If *E. obesus* were to excrete water proportionately to sodium loss, then plasma sodium levels would be maintained. Water excretion to maintain plasma sodium concentrations must have limits, and indeed plasma sodium concentration did decline during the second and third weeks of exposure to pH 3.5 (Fig. 4). If water were excreted renally, some sodium must also be lost. This is not a problem at high body sodium concentrations, but it would magnify the problem of sodium loss at low body levels. As body sodium or water levels decline below a certain point, it seems that control of body water may become uncoupled from that of body sodium (Fig. 5). As body sodium continues to decline, water levels remain unchanged or even increase. This could explain the poor or non-significant relationships between body water and body sodium concentration at pH 3.5 (Table 5), and the lack of significant differences between body water content of fish in various treatments at pH 3.5 and pre-treatment or control fish (Tables 3 and 4).

If water loss were proportionately less than that of sodium, plasma sodium will decline. Hematocrits of *E. obesus* exposed to pH 3.5 increased to 76% after two weeks (Table 3) as plasma sodium levels declined, implicating a shrinkage in plasma volume. During the five week test at pH 3.5 (Fig. 4) hematocrit increased to 72% after two weeks. Plasma sodium concentration continued to fall during the third week, but hematocrit declined, indicating that the plasma volume might be expanding. Fish exposed to pH 3.5 in varying calcium concentrations exhibited significantly lower body potassium levels than those at pH 5.8, and body potassium concentration was linearly related to body sodium concentration. *L. gibbosus* exposed to pH 4.0 for one week also had depressed body potassium concentrations, but plasma potassium concentration was elevated. Potassium is the primary intracellular cation balancing sodium in the extracellular fluid. Perhaps it is allowed to diffuse out of the cells down its electrochemical gradient as sodium is lost and is excreted by the kidney.

The net effect would be to reduce the osmotic imbalance created by the loss of sodium, but it would require that *E. obesus* and *L. gibbosus* be able to tolerate reduced intracellular potassium levels. Intracellular potassium concentration did decline with plasma chloride concentration in brown trout exposed to pH 4.0–4.6 (Fugelli and Vislie 1982). Rainbow trout exposed to low pH exhibited decreased intracellular potassium concentration, increased plasma potassium concentration, and increased branchial efflux and renal excretion (McDonald and Wood 1981), all apparent symptoms of severe ionic disturbance leading to death. Yet the long term survival of *E. obesus* at pH 3.5 and *L. gibbosus* at pH 4.0 argues that potassium decline may be an adaptive response not necessarily associated with mortality.

There is evidence to suggest that *E. obesus* accumulates sodium when conditions are favorable. Fish sampled from holding tanks in June soon after collection as part of experiments described above had a mean body sodium concentration of 47 μmol/g WBM (Table 3, pH 5.8 pre-treatment). This had risen to 58 μmol/g WBM in August (Table 3, pH 3.5 pre-treatment), a level typical of most teleosts (Evans 1979). Similar results were achieved for fish collected over the range of collecting dates (May to October). Plasma sodium concentration of fish exposed to ASW of varying pH's (Table 3) rose from an initial level of 120 m*M* to as much as 137 m*M* in just two weeks. Typical plasma sodium concentrations for teleosts is 150–160 m*M* (Evans 1979). Sodium may be accumulated in concert with absorption of water to expand the extracellular fluid volume. This ability to take up sodium during favorable conditions may play an important role during exposure to pH's below 4.0. If death occurs when some minimum body or plasma sodium concentration is reached, then sodium accumulated and stored under favorable conditions may act as a buffer against losses at unfavorably low pH's.

E. obesus thrives in softwater environments low in pH, while few other fish can survive for long in such a rigorous environment. *E. obesus* has several specific adaptations related to sodium balance that confer tolerance to low pH. Control of J_{out} plays a primary role in adapatation to acidic environments, but this species can also tolerate large sodium losses (up to 48%) at low pH's. Rainbow trout, conversely, defend a constant ionic state in the face of low pH (McDonald and Rogano 1986), which is believed to be typical of most teleosts. It is possible that a resistance strategy alone is not energetically feasible in extremely low pH environments, resulting in the multifaceted adaptations of: (a) resistance to sodium loss and water gain at low pH due to increased gill permeability, (b) tolerance of low body sodium levels (which involves excretion of water and potassium as sodium is lost), and (c) rapid replenishment of sodium when conditions ameliorate.

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