

Effects of acid exposure on natural resistance and mortality of adult *Rana pipiens*

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Acid is a prevalent environmental stressor for North American frogs, and in the last few decades many aquatic environments have become increasingly acidic (Freda, 1986). Acidic pH may decrease sperm motility in *Rana pipiens* (Schlichter, 1981) and cause high mortality and developmental abnormalities in embryos and tadpoles (Bradford, et al., 1992; Freda and Dunson, 1985; McDonald et al., 1984; Pierce, 1985; Pierce et al., 1984; Schlichter, 1981). Lethal effects of low pH on adult frogs are poorly understood. A few studies demonstrated acidic conditions disrupt transport of ions in isolated skin *in vitro* (Feder et al., 1993; Ferreira and Hill, 1982; Lyall et al., 1992), which at pH below 4 -5 may cause death (Boutilier et al., 1992).

The relation of stress to immunosuppression is well documented in many vertebrates (Blecha and Kelley, 1981; Blecha et al., 1982; Marnila et. al 1995; Regnier and Kelley 1981). In addition, amphibians exposed to low pH may experience immunosuppression followed by microbial disease resulting in mortality (Carey, 1993). We previously demonstrated that adult *Rana pipiens* exposed to pH 5.5 for 10 d experienced high mortality rates (Vatnick et al., 1999). Here, we characterize the effects of acid exposure on defense systems of adult *Rana pipiens*.

Fifty frogs were purchased from Charles D. Sullivan Co. Inc. (Nashville, TN) and arrived via airmail. These were wild *Rana pipiens* from ponds in the northeastern United States captured by licensed collectors. In our lab, frogs were housed in individual plastic containers with 800-1,000 ml of aged tap water (conductance = 0.366 mohs, $\text{Na}^+ = 0.14 \text{ mM}$, $\text{K}^+ = 0.3 \text{ mM}$, $\text{Mg}^{++} = 0.30 \text{ mM}$, and $\text{Ca}^{++} = 0.24 \text{ mM}$) and were acclimated at room temperature for at least two days after arrival and prior to the start of any experiment. During experiments frogs were placed in individual autoclaved

containers containing sterilized citrate buffer. Buffers were prepared in 75 L carboys, using tap water, and each buffer was autoclaved for 1h. The pH 5.5 buffer was prepared by adding 4.8 mM citric acid anhydride, 9.5 mM sodium hydroxide, and 0.6 mM sodium citrate to 75 L of tap water. The pH 7.0 buffer was prepared by adding 2.4 mM citric acid anhydride, 4.8 mM sodium hydroxide, 0.3 mM sodium citrate. Buffer was changed every 2d with autoclaved fresh sterile citrate buffer, so frogs were not exposed to any source of exogenous bacteria. Animals were not fed during the 14d experimental period. Within 3 days after the experimental period, frogs were killed by diethyl ether asphyxiation, and their spleens were dissected out using aseptic technique. A single cell suspension of splenic tissue was prepared in sterile amphibian culture medium (Wolf et al., 1960). Two aliquots were prepared: one for counting total cell number and the other for a determination of bacterial number. The first aliquot was transferred to a hemocytometer and cells counted as either viable or nonviable based on exclusion of trypan blue. Viable cells exclude trypan blue (Mishell and Shiigi, 1980).

The second aliquot was used for enumeration of bacteria by the spread plate method. Serial two-fold dilutions were prepared in a laminar flow hood using sterile isotonic saline. Triplicate spread plates were prepared from each dilution on nutrient agar and incubated aerobically at 37°C. Twenty-four hours after initiation of incubation, bacterial colonies were counted and numbers recorded as colony forming units per spleen. Isolated bacterial colonies, obtained from the spleen squash spread plates, were stained by the Gram reaction method. Gram negative organisms were identified using the Enterotube II CCIS (Roche Diagnostic Systems, Nutley, New Jersey) method for identification of facultative anaerobes. Non-fermentative Gram

negative rods were identified using the Oxiferm identification system (Roche Diagnostic Systems, Nutley, New Jersey). Gram positive bacteria were identified using classical staining methods and substrate utilization tests. Total white blood cell numbers and viability were compared using a Student t test and reported as means \pm SE.

Mean number of WBCs \pm SE (84.5 ± 5.26 in a 1:10,000 dilution) from the spleen of frogs exposed to pH 5.5 was significantly lower ($t= 3.38$, $n= 50$, $p< .01$) than that present (157.5 ± 8.5 in a 1:10,000 dilution) in the spleen of the control group held at pH 7.0. Cell viability also was lower in frogs exposed to pH 5.5 ($52.4\% \pm 1.7$ SE) than those in the control group ($62.7\% \pm 1.6$ SE, $t=4.528$, $n=50$, $p<0.01$).

Frogs exposed to pH 5.5 had spleens colonized with both Gram positive and Gram negative bacteria. The Gram negative bacteria recovered from spleens of frogs exposed to pH 5.5 included *Hafnia alvei*, *Serratia marcescens*, *Klebsiella sp.*, *Pseudomonas sp.* and *Alcaligenes sp.*; Gram positive bacteria were from the genus *Bacillus*. Spleens of frogs exposed to pH 7.0 either were sterile or exhibited little bacterial colonization. Frogs exposed to pH 5.5 had significantly more bacterial colony forming units than frogs exposed to pH 7.0 (3029 ± 1045 versus 19 ± 5 SE, $t= 4.01$ $n=40$, $p<0.05$)

DISCUSSION

The results of the experiments reported herein support hypotheses suggested by other investigators (Carey, 1993; Carey et al., 1996; Glorioso et al., 1974; Maniero and Carey, 1997) that environmental stress is the initiating factor in a cascade of physiological events. These events may start with immunosuppression followed by systemic distribution of opportunistic and virulent bacteria, and may ultimately lead to

the death of adult frogs. Natural microbial defense mechanisms of vertebrates include the integrity of epithelial barriers, non-specific humoral and cellular mechanisms, and specific immune responses. Stress may affect all these components.

Exposure to pH 5.5 caused 72% mortality in adult *Rana pipiens* (Vatnick et al., 1999). In the present study, *Rana pipiens* exposed to pH 5.5 for 14 d had spleens colonized by Gram negative bacteria that normally reside in their gut. If disruption of epithelial ion transport during acid exposure (Ferreira and Hill, 1982) indicates disruption in the integrity of the epithelial barrier, bacteria may move from the intestine to blood and ultimately colonize the spleen. At pH 5.5 the gut epithelial barrier may be compromised, allowing endogenous bacteria to translocate into the blood stream. Our experimental evidence indicates that splenic WBC number and viability were both reduced due to low pH. Therefore the ability to respond to blood-borne bacteria may also be diminished, rendering frogs vulnerable to systemic infections by their own gut bacteria. Several altered physiological and pathophysiological states lead to bacterial translocation across the intestinal epithelia (Deitch et al., 1991; Spaeth et al., 1990; Wells, 1990; Wells et al., 1990; Ziegler et al., 1988).

Developing frogs exhibit interspecific and interpopulation differences in their sensitivity to acid conditions (Freda, 1986; Pierce 1985). It is logical to speculate that these differences also occur in adult frogs. However, the marine toad *Bufo marinus* did not exhibit reduced immune competence when exposed to pH 3.8 for 14 days (Carey et al., 1996). Therefore, the apparent difference between Carey's and our results may be explained by inter-specific variation in sensitivity to acid conditions. We noticed such variation even among ranids; *Rana clamitans* did not exhibit increased mortality when

exposed to pH 5.5 while *Rana pipiens* did (unpublished data). *Rana pipiens* also exhibited intraspecific variation in sensitivity to acid exposure. The 28% of *Rana pipiens* that survived exposure to pH 5.5 in our study must have differed in their ability to acutely withstand such conditions.

Our data shows that exposure to pH 5.5 caused a decrease in white blood cell number and viability. Furthermore, exposure to pH 5.5 caused colonization of the spleen by bacteria. Both Gram negative and Gram positive bacteria have been associated with systemic infection in frogs. Potential pathogenic species of *Aeromonas*, *Pseudomonas*, *Escherichia*, *Klebsiella*, and *Proteus* have been isolated from the gut contents of healthy *Rana pipiens* (Van Der Waaij et al., 1974). Our results suggest that in acid stressed frogs (exposed to pH 5.5 or lower), bacteria move across the gut into the blood stream, encounter compromised natural defense mechanisms, and as a result colonize the spleen. Resulting systemic infections combined with decreased natural defenses may in part cause increased mortality in *Rana pipiens*.

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