

Effects of Acid Stress in Adult *Rana pipiens*

MARC BRODKIN^{1*}, ITZICK VATNICK¹, MARTIN SIMON²,
 HOLLYANN HOPEY¹, KRISTINE BUTLER-HOLSTON¹,
 AND MICHELLE LEONARD¹

¹*Department of Biology, Science Division, Widener University, Chester, Pennsylvania 19013*

²*Biology Department, Benedictine College, Atchison, Kansas 66002*

ABSTRACT The decline in frog populations is a well-recognized worldwide phenomenon and infectious disease has been implicated as a major cause in the global decline of amphibian populations. *Rana pipiens* are disappearing from many habitats where they used to flourish, and environmental acidification has been considered as a possible contributor to this disappearance. We present a model that integrates the results of several experiments on the effects of acid exposure on natural resistance and mortality of adult *Rana pipiens*. These studies suggest that different components of the natural defense mechanisms of these frogs have different acid sensitivities. We have shown previously that exposure to pH 5.5 leads to a reduction in splenic white blood cell number, viability, and to colonization of the spleen with both Gram positive and Gram negative bacteria. In this paper we show that exposure to pH 6.0 did not affect the number or viability of splenic white blood cells but did result in colonization of the spleen by bacteria. We also show that cold exposure by itself does not cause a systemic bacterial infection in adult *Rana pipiens*, but acid stress following cold exposure does. The data presented in this paper provide empirical evidence to support the hypothesis that acid stress may be a contributor to the decline of *Rana pipiens* in the northeastern region of the United States. *J. Exp. Zool.* 298A:16–22, 2003. © 2003 Wiley-Liss, Inc.

INTRODUCTION

The coordinated effort to elucidate the causes for global amphibian decline is now entering its second decade. Many biotic and abiotic factors have been identified as possible causes for this decline (Freda and Dunson '85; Bradford '91; Freda, '91; Bradford et al. '92; Brodtkin et al. '92; Blaustein et al. '94; Howdshell and Smalley '96; Lannoo '96) and both are likely contributors to the host-pathogen interactions that may lead to mortality in amphibian populations. Infectious disease has been hypothesized as one factor that may be responsible for amphibian decline (Carey et al., '99; Vatnick et al., '99). Infection by chytrid fungi has been suggested as the cause of major die-offs of amphibians in both Central America and Australia (Berger et al., '98). This observation provides further support for the role of pathogens in the global decline of frog populations. It is unclear why pathogens emerged as a threat to amphibian populations in the past 20 years. One cause may be environmental stress that has been suggested as a possible cause for the weakening of

the natural defense mechanisms of amphibians (Carey et al. '99).

Acid is a prevalent environmental stressor in the northeast United States (Driscoll et al., 2001). We have examined the effects of acid stress on the natural defense mechanisms and bacterial colonization of the spleen of *R. pipiens* (Simon et al., 2002, see Table 1). Exposure of *R. pipiens* to pH 5.5 resulted in 70% mortality within 10 days (Vatnick et al., '99; Fig. 1). Their natural defenses are compromised to a much greater degree than those of other ranid frogs (*R. catesbeiana* and *R. clamitans*) exposed to the same acid condition (Brodtkin et al., unpublished observation).

Cold exposure also has deleterious effects on the structure and function of frogs' immune system (Cooper et al., '92). Carey ('93) and Maniero and Carey ('97) suggested that

*Correspondence to: Marc Brodtkin, Department of Biology, Science Division, Widener University, One University Place, Chester, Pennsylvania 19013. E-mail: Brodtkin@pop1.science.widener.edu

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TABLE 1. Comparison of bacterial colonization of the spleen in all 4 experiments (Colony Forming Units per spleen \pm SE)

	Control	Experimental
Acid pH 5.5 (Simon <i>et al.</i> 2002)	19 \pm 5	3029 \pm 1045*
Acid pH 6.0 (Experiment 1)	16.7 \pm 5.8	353.1 \pm 125.9*
Cold (Experiment 2)	1.3 \pm 1.5	12.7 \pm 10.6
Acid pH 5.5 after Cold (Experiment 3)	26.3 \pm 51.2	241.7 \pm 346.2*

Values were compared in each experiment (across rows). An asterisk * signifies statistically significant difference ($p < 0.05$) using the appropriate test (see text for details).
 Treatments for Experiment 1 were pH 6.0 for experimental group and pH 7.0 for control.
 Treatments for Simon *et al.*, 2002 and Experiment 3 were pH 5.5 for experimental group and pH 7.0 for control group.
 Treatments for Experiment 2 were 5°C for experimental group and 25°C for control group.

environmental stress (e.g. cold and/or acid exposure) is the initiating factor in a cascade of physiological events that may start with immunosuppression followed by systemic distribution of opportunistic and virulent bacteria, ultimately leading to the death of adult frogs. Our data in this paper and a recent report (Simon *et al.*, 2002) support this hypothesis.

Cold-exposure prior to acid-exposure caused an increase in adult *R. pipiens*' mortality (Vatnick *et al.*, '99). This treatment mimicked the natural emergence of frogs from hibernation. The immune system of frogs is compromised due to cold-exposure during winter hibernation (Cooper *et al.*, '92). During the post-hibernation

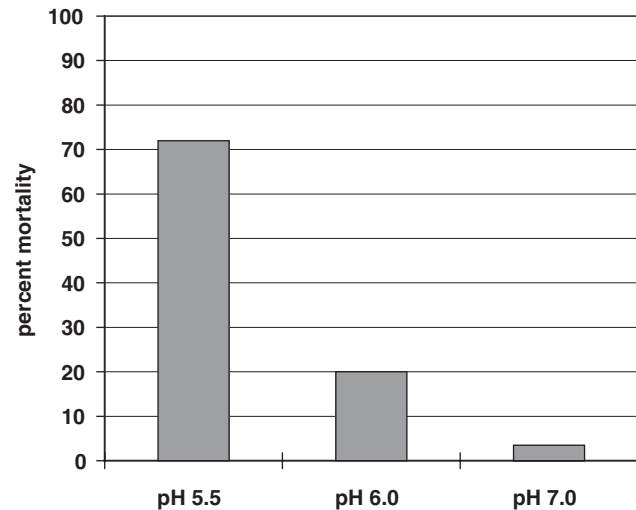


Fig. 1 Mortality of *Rana pipiens* after 10-day exposure to pH 5.5, pH 6.0, or pH 7.0.

period *R. pipiens* are likely to spend significant time in ponds and lakes in order to breed. During this post-hibernation period streams, ponds, and lakes in the Northeast United States suffer from episodic acidification due to snowmelt and rain events (Driscoll *et al.*, 2001). Therefore, the period following emergence from hibernation is probably the part of the year when adult *R. pipiens* in the Northeast United States are most vulnerable to acid stress.

Both Gram positive and Gram negative bacteria have been associated with systemic infection in frogs (Glorioso *et al.*, '74; Carr *et al.*, '76). In addition, potential pathogenic species of *Aeromonas*, *Pseudomonas*, *Escherichia*, *Klebsiella*, and *Proteus* have been isolated from the intestinal contents of healthy *R. pipiens* (Van Der Waaij *et al.*, '74, Banas *et al.*, '88). These potential pathogens can be a source of systemic infections and death in immunocompromised animals (Blecha and Kelley, '81; Regnier and Kelley '81; Blecha *et al.*, '82; Marnila *et al.*, '95).

In this paper we present a hypothetical model (Fig. 2) that provides the link between host-pathogen interactions, acid exposure, and mortality in adult *R. pipiens*. We also present data from our previous work (Vatnick *et al.*, '99; Simon *et al.*, 2002) as well as data from three new experiments that support a model we propose in this paper on the mechanisms by which acid exposure affects the

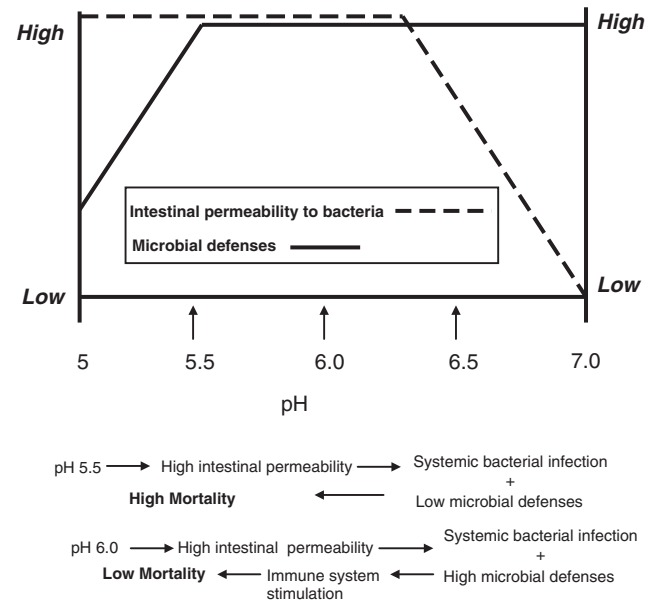


Fig. 2 A hypothetical model of the effects of acid exposure on intestinal permeability and microbial defenses of adult *Rana pipiens*.

innate immune function of adult *R. pipiens*. Our model proposes that the physiological processes leading to mortality include changes in gut permeability and compromised natural defense mechanisms.

MATERIALS AND METHODS

Animals

Rana pipiens of both sexes (10–15 cm in length) used for the experiments reported in this paper were purchased from Amphibians of North America, Nashville, TN and shipped via airmail to our laboratory. The Widener University Institutional Animal Care and Use Committee approved all protocols for the experiments described in this paper.

Buffers

Buffers were prepared in 20 L carboys, using tap water, and each buffer was autoclaved for 1 hour. The pH 5.5 buffer was prepared with 4.8 mM citric acid anhydride, 9.5 mM sodium hydroxide, and 0.6 mM sodium citrate. The pH 6.0 buffer was prepared with 3.5 mM citric acid anhydride, 6.7 mM sodium hydroxide, 0.5 mM sodium citrate. The pH 7.0 buffer was prepared with 2.4 mM citric acid anhydride, 4.8 mM sodium hydroxide, and 0.3 mM sodium citrate. Buffer was changed daily with autoclaved fresh sterile citrate buffer. As a result frogs were not exposed to any source of exogenous bacteria.

Experiment 1 acid exposure

Frogs were acclimated to the laboratory in aquaria filled with 5 cm of aged tap water (conductance=0.366 mohs, Na^+ =0.14 mM, K^+ =0.3 mM, Mg^{++} =0.30 mM, and Ca^{++} =.24 mM) prior to the 14-day experiment. Each frog was fed three four-week old crickets and the water changed every other day with aged tap water. Frogs were randomly assigned to the acid group (pH 6.0, n=28) or control group (pH 7.0, n=20). During the fourteen days of the experiment frogs were placed in individual, autoclaved Tupperware containers containing the appropriate sterile buffered solutions that were changed daily. At the end of the experiment the surviving frogs were sacrificed within 72 hours. Animals were not fed during the entire duration of the experiment.

Experiment 2: cold exposure

Twenty-five frogs were acclimated to the laboratory in aquaria filled with aged tap water (5 cm) as described above. On day 0 of cold exposure, five frogs were randomly selected as controls for the cold exposed groups. These frogs were sacrificed and the white blood cells from the spleen and bacterial number from the spleen and intestine were assayed in the manner described below under bacterial colony counts. The remaining 20 frogs were maintained in aquaria lined with paper towels soaked in sterile aged tap water and placed in an environmental chamber (Lab-Line Instruments Inc., Melrose Park, IL) set at 20°C. The paper towels were changed every other day for the duration of the experiment. The temperature in the environmental chamber was gradually lowered 5°C per day to a final temperature of 5°C. The spleen and distal portion of the intestine from each of five frogs was sampled at 15, 22, 29, and 35 days of exposure to 5°C.

Experiment 3: acid exposure after cold exposure

Frogs were treated similarly to the frogs in Experiment 2 and exposed to 5°C for thirty-five days in an environmental chamber. At the end of the thirty-five day cold-exposure period, the temperature was gradually raised to 20°C at 5°C per day. The twenty-five surviving frogs were randomly assigned to either the acid exposed or control group. Fifteen frogs were assigned to the acid exposed group. These frogs were placed in individual, autoclaved Tupperware containers with a pH 5.5 buffer solution. The frogs in the control group were also placed in individual, autoclaved containers with a pH 7.0 buffer solution. These buffered solutions were changed daily.

During days five through fourteen of acid exposure, spleen and intestine samples were collected from a total of 12 frogs as described below. On days nine to fifteen spleen and intestine samples were collected from a total of ten control frogs.

We also calculated pooled mortality rate from several experiments including those described above in which frogs were exposed to pH 5.5, 6.0, and 7.0 for 14 days.

White blood cells counting and viability (Experiment 1)

A single cell suspension of splenic tissue was prepared using aseptic technique in sterile am-

intestine was found to be $4.54 \times 10^8 \pm 7.12 \times 10^8$ in the acid-exposed group, and $2.14 \times 10^8 \pm 2.78 \times 10^8$ in the control frogs (t test, $P > 0.05$).

Gram positive and Gram negative bacteria were isolated from both the spleen and the distal region of the intestine of nearly all acid-exposed frogs. The Gram positive species *Bacillus cereus* was isolated from both spleen and the distal region of the intestine in each of two acid-exposed frogs. The Gram negative species *Acinetobacter calcoaceticus* was isolated from both the spleen and intestine in the same acid-exposed frog.

DISCUSSION

We report in this paper pooled mortality data from several experiments conducted under the same conditions in the past few years for frogs exposed to pH 5.5, 6.0, and 7.0. These data were previously reported (Vatnick et al., '99). In Figure 1 we add new pooled mortality data for frogs exposed to pH 6.0. *Rana pipiens* exposed to pH 5.5 experienced 72% mortality (n=46) throughout a 10-day experimental period (Vatnick et al., '99). Frogs exposed to pH 6.0 experienced a much-reduced mortality rate of 20% (n=31). Frogs exposed to pH 7.0 experienced less than 2% mortality (n=52) during the experimental periods.

Acid-exposed (Experiment 1) frogs and frogs that were exposed to acid after cold-exposure (Experiment 3) exhibited a significant increase in bacterial colonization of the spleen. No such colonization was observed in frogs exposed to cold only (Table 1). Microbial defense mechanisms of vertebrates include the integrity of epithelial barriers, and non-specific humoral and cellular mechanisms, as well as their specific immune responses. Acid exposure weakens the natural defenses of frogs; the mechanisms for this occurrence are not completely understood. However, the effect of pH on adult frogs has been studied *in vitro*. These studies examined the effects of acidification on ion exchanges in isolated frog skin (e.g. Ferreira and Hill, '82). A wealth of information about paracellular as well as transcellular ion transport has been gathered (Ferreira and Hill, '82; Lyall et al., '92; Feder et al., '93). These studies are significant in that they demonstrate that acidic conditions disrupt the transport of ions in isolated skin *in vitro*.

Our model (Fig. 2) proposes that exposure to acidic conditions adversely affects *R. pipiens*' natural bacterial defenses by two separate me-

chanisms: 1) disrupting the proper functioning of the intestinal epithelium as a barrier to bacterial translocation; and 2) decreasing WBC number and viability. Our data show that a relatively narrow range of pH can elicit drastically different physiological processes that may determine the frogs' survival. In addition the model provides a framework to test the sensitivity of other adult frogs to an acidic environment. It may be that the intra-genera differences we found in ranids are found among other genera of frogs. It is logical to postulate that acid may affect other frogs in the same manner that it affects *R. pipiens*.

Frogs exposed to either pH 5.5 (Simon et al., 2002) or 6.0 (Experiment 1) exhibited colonization of the spleen with Gram positive and Gram negative bacteria. We have reported previously that the spleen of frogs exposed to pH 7.0 was either sterile or exhibited very low levels of bacterial colonization (Simon et al., 2003, Table 1). *Rana pipiens* exposed to pH 5.5 for 14 days exhibited colonization of the spleen with Gram negative bacteria that reside in their intestine under normal physiological conditions. Bacterial colonization of the spleen also occurs after exposure to pH 6.0 but does not result in frogs' mortality, and the total number of splenic WBC and the percent viable WBC does not decrease. Therefore, pH 6.0 may allow bacteria to transit across the intestinal epithelial barrier but does not impair significantly the natural defense mechanisms associated with white blood cells. The high survival of *R. pipiens* exposed to pH 6.0 (Fig. 1) may be attributed to the normal functioning of white blood cell-mediated non-specific defense systems as assessed by number and viability of splenic white blood cells. We have already reported (Simon et al., 2002) that frogs exposed to pH 5.5 exhibited a drastic reduction in the number and viability of splenic WBCs.

At pH 5.5 splenic WBC function and perhaps the intestinal epithelial barrier are compromised, therefore rendering the frogs vulnerable to systemic infections by their own intestinal bacteria. Several altered physiological and pathophysiological states lead to bacterial translocation across the intestinal epithelia (Ziegler et al., '88; Spaeth et al., '90; Wells, '90; Wells et al., '90; Deitch et al., '91). Therefore, it is quite possible that this may also be the case when frogs are exposed to acid. Our data show that exposure to pH 5.5 caused a decrease in white blood cell number, viability (Simon et al., 2003), and phagocytic efficiency (unpublished observations).

Both Gram positive and Gram negative bacteria have been associated with systemic infection in frogs. Potential pathogenic species of *Aeromonas*, *Pseudomonas*, *Escherichia*, *Klebsiella*, and *Proteus* have been isolated from the intestinal contents of healthy *R. pipiens* (Van Der Waaij et al., '74). We propose that in acid stressed frogs (frogs exposed to pH 5.5 or lower), these bacteria move across the intestine into the blood stream, encounter compromised natural defense mechanisms, and, as a result, can be found in the spleen. In a recent paper (Vatnick et al., '99) we demonstrated that *R. pipiens* exposed to pH 5.5 for 10 days exhibited 72% (n=46) mortality compared to only 3.5% mortality in frogs held in the same buffer at pH 7.0 (n=29). Further analysis of these data revealed that sensitivity to pH 5.5 varied with the physiological state of the frogs. Frogs collected early in the spring, having just emerged from hibernation, but prior to the breeding season, exhibited 100% (n=15) mortality within the first four days of exposure to pH 5.5. Our results provided empirical support to the suggestion by Maniero and Carey ('97) that frogs recently emerging from hibernation are more susceptible to microbial disease. This increased susceptibility may be due to the cold-induced suppression of the immune system and is compounded by exposure to environmental stressors such as acid (Harte and Hoffman, '89). Our model of the effect of acid stress on the natural defense mechanisms of *R. pipiens* also suggests that different parts of the natural defense systems of *R. pipiens* exhibit differential sensitivities to acid exposure.

Cold has deleterious effects on the structure and function of lymphoid organs and the natural defense mechanisms of frogs. Recovery of lymphoid tissue structure and natural defense function from cold exposure takes approximately 30 days (Cooper, '92). Therefore, frogs emerging from hibernation are vulnerable to bacterial disease particularly if they emerge into an acidic environment (Vatnick et al., '99).

Frogs' normal intestinal flora includes potential pathogenic bacteria. The effect of cold-exposure on the intestinal flora of frogs has been well documented (Van Der Waaij et al., '74; Carr et al. '76; Banas et al.'88). Among the species of aerobic bacteria that survive cold exposure are *Acintobacter sp.*, *Aeromons hydrophila*, *Bacillus sp.*, and *Pseudomonas sp.* (Carr et al., '76). We also isolated *Acintobacter sp.*, and *Bacillus sp.* in *R. pipiens* exposed to both 25°C and 5°C giving further

support to the selective action of cold-exposure on the intestinal flora.

We demonstrated that intestinal bacteria do not colonize the spleen in frogs exposed to pH 7.0 after a five-week cold-exposure. However, the same species of bacteria that normally reside in the distal portion of the intestine were found in the spleen in those frogs exposed to pH 5.5 following cold-exposure. These data suggest that bacteria move across the intestinal epithelial barrier, into the bloodstream, and as a result can be found in the spleen. Furthermore, it appears that acid-exposure and not cold-exposure allows colonization of the spleen by intestinal-bacteria. The resulting systemic bacterial infection coupled with the reduced function of the immune system (due to the length of time it takes to restore immune structure and function after cold exposure, Cooper et al., '92) may contribute to the high mortality of these frogs. These findings fit well with the frogs' annual cycle. Upon emergence from hibernation, *R. pipiens* enter bodies of water for breeding purposes. During the spring in the northeastern region of the United States these aquatic habitats are susceptible to episodic acidification due to mobilization of acid in the soil during snowmelt and a rise in the water table (Driscoll et al., 2001). The cold-induced compromised natural defenses, coupled with acid exposure may explain in part the recent decrease of *R. pipiens* populations in this region.

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