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The Effects of Exposure to Mild Acidic Conditions on Adult Frogs (*Rana pipiens* and *Rana clamitans*): Mortality Rates and pH Preferences

I. VATNICK,[1] M. A. BRODKIN,[1] M. P. SIMON,[2] B. W. GRANT,[1] C. R. CONTE,[1] M. GLEAVE,[1] R. MYERS,[1] and M. M. SADOFF[1]

[1]*Biology Department, Science Division, Widener University, One University Place, Chester, Pennsylvania, 19013, USA E-mail: Vatnick@pop1.science.widener.edu, and*
[2]*Biology Department, Benedictine College, Atchison, Kansas, 66002, USA*

ABSTRACT.—In this study we report mortality rates of adults of *Rana pipiens* to mild acid exposure and pH preferences for *R. pipiens* and *R. clamitans*. We exposed adult *R. pipiens* to mildly acidic conditions for a ten day period under controlled laboratory conditions. Frogs exposed to citrate buffer at pH 5.5 for 10 d exhibited 72% mortality as compared with 3.5% mortality in the control group held at pH 7.0. Furthermore, within the pH 5.5 group there was a difference in acid sensitivity based on season. All of the frogs that had recently emerged from hibernation died within the first four days of exposure to pH 5.5, whereas frogs that were post breeding suffered 58% mortality throughout the 10 d of the experiment.

This study also examined the pH preferences of adult frogs. Individuals of *R. pipiens* (N = 24) and *R. clamitans* (N = 12) were placed in a six-compartment experimental chamber filled with three different pH solutions buffered to pH 4.0, 5.5, and 7.0. Frogs of both species preferred the more neutral pHs. Our results suggest that adults of *R. pipiens* are sensitive to mildly acidic conditions, especially when emerging from hibernation, and that adults of both *R. pipiens* and *R. clamitans* can discriminate between acidic and neutral pH environments.

During the last six years we have studied the effect of environmental stressors on natural resistance factors (splenic and peripheral white blood cell numbers and the phagocytic efficiency of these cells) of ranids. In these experiments we found that adults of *Rana pipiens, R. clamitans,* and *R. catesbeiana* have different sensitivities to low pH with *R. pipiens* being the most sensitive (unpubl. data). Immune function declines during hibernation (Cooper et al., 1992) and frogs emerging from hibernation may be particularly vulnerable to disease until their immune function is restored (Maniero and Carey, 1997). We tested the hypothesis that acid sensitivity is also different for adults of *R. pipiens* that have recently emerged from hibernation versus post-breeding adults.

In this paper we report the effect of exposure to mildly acidic conditions (pH 5.5 for 10 d) on mortality of post-hibernation and post-breeding *R. pipiens* ("mortality study"). We conducted a second experiment to compare pH preferences of *R. pipiens* to those of the less acid-sensitive *R. clamitans* ("preference study"). The ability to detect the acidity of the environment may be particularly important for *R. pipiens* because of their susceptibility to mild acidicic conditions at all life stages. Female frogs should choose carefully the site for oviposition because *R. pipiens* embryos and larvae have a high mortality in pH of 5.5; many other species can tolerate pHs that are about 1 pH unit lower (Pierce, 1985). Although there have been a few studies of salamanders (Mushinsky and Brodie, 1975; Wyman and Hawksley-Lescault, 1987), there are no studies of the behavioral responses of adult frogs to acid conditions (Pierce, 1985) or the sensitivities of adults of different species to these conditions (Pierce, 1985; Carey, 1993).

MATERIALS AND METHODS

Animals.—In both experiments *R. pipiens* and *R. clamitans* were purchased from Amphibians of North America, Nashville, TN and shipped via airmail to our laboratory. In the mortality study frogs were received in three separate shipments. The first shipment of 30 post-breeding frogs was received in September 1997 and a second shipment of 28 frogs was received on January 1998. These frogs were captured prior to entering hibernation, had finished the breeding season, and were held unfed in cold storage by the supplier before shipment. The third shipment of 25 frogs, freshly caught from the wild in the northeastern United States, was received in the first week of March 1997, having recently emerged from hibernation. Each frog was fed 2–3 crickets (*Acata domestica*) on the day of arrival and every other day thereafter except during the runs of the preference experiment. In the preference study 36 frogs, 24 *R. pipiens* and 12 *R. clamitans* (22 males and two females for *R. pi-*

piens and 10 males and two females for *R. clamitans*) were used.

In both experiments, frogs were immediately placed in aged tap water (pH 7.4–8.0) upon arrival, and were housed in 40 l aquaria with no more than five frogs to an aquarium for 2–6 d before the start of an experiment.

Experimental Protocol: Mortality study.—Forty eight to seventy two hours prior to the experiment, frogs were placed individually in autoclaved Rubbermaid® polypropylene containers filled with 1 liter of sterilized, aged tap water. Holes were drilled in lids of the containers to allow maximal airflow. These containers were placed in an environmental chamber (Lab-Link Environette; Lab Line Instrument Inc.) set at 29 C ± 0.1 C with a 12:12 light:dark cycle. This temperature is well within the observed minimum and maximum voluntary temperatures of *R. pipiens.* The minimum voluntary temperature of *R. pipiens* is 17.8 C and the maximum is 34.7 C with a wide range of observed field temperatures (Brattstrom, 1963). Previous studies showed that *R. pipiens* held at an initial temperature of 5 C and tested at a final temperature of 29 C acclimate to this new temperature very fast (½ AT = 0.18 d; Duellman and Trueb, 1994). On the first day of the 10 d experiment, the sterile water was replaced with citrate buffer adjusted to a pH of either 5.5 (experimental) or 7.0 (control). The buffers were autoclaved prior to use and changed twice daily approximately at 0800 h and 2000 h on each day of the experiment. The pH of the buffers was checked at the beginning and end of each 12 h period. The 5.5 buffer maintained its pH within 0.2 pH units while the pH 7.0 buffer increased its pH to a value not higher than 8.2. The differences in the buffering capacity at each pH were due to the pKa of the citrate buffer and the base load of the frogs' urine.

Experimental protocol: Preference study.—Twenty-four hours prior to the experiment, frogs were individually placed in autoclaved Rubbermaid® polypropylene containers filled with 1 liter of sterilized, aged tap water. Holes were drilled in lids of the containers to allow maximal air flow.

The experimental chamber was made from a Rubbermaid® 120-l trash container composed of low-density polyethylene. The container was cut to a height of 10 cm. Five centimeter high dividers were sealed in place with GE Silicone II Tub and Tile Sealant creating six equal sized, water-proof, wedge-shaped compartments. A 10 cm length of a 2.4 cm diameter PVC tubing was notched and fitted over the dividers at the center of the chamber. During the experimental runs, a Plexiglas® cover rested on the center post and the outside rim of the chamber. This left a 5 cm clearance between the tops of the compartment dividers and the Plexiglas® cover. The compartments were identified by number, one through six. An infrared light was fixed in the outside wall of each compartment and provided illumination for a surveillance video camera. The camera was mounted approximately 0.7 m above the experimental chamber and was connected to a monitor and a VCR. The VCR, a Panasonic Time Lapse Video Recorder Model #6040, was set to compress 24 h into one hour of taping. A second identical chamber was constructed and used as a conditioning chamber. The purpose of the conditioning chamber was to allow the frogs to become accustomed to the novelty of the circular chamber prior to the actual the experimental runs.

An experimental run for each frog included three 24-h periods, a conditioning period, a test run, and a control run. The conditioning period always preceded the test and control runs. For the conditioning period a frog was placed in the conditioning chamber that was partially filled with approximately 450 ml of aged tap water per compartment. The 24 h conditioning run prior to the experimental and control runs was designed to accustom the frogs to the test arena. In preliminary runs without conditioning, frogs moved significantly more in the first 24 h of being placed in the chamber regardless of the pH (all pH 7.0 or 3 different pHs), indicating that the frogs explored their novel environment upon being placed in the experimental chamber.

The test run consisted of partially filling the compartments of the experimental chamber with the three different buffers, one buffer in each of two compartments. The assignment of buffer to compartment was made randomly. A frog was placed in a randomly chosen starting compartment, and the movements of the frog within the chamber were videotaped for 24 h. The control run was identical to the test run with the exception that all sections were filled with the pH 7.0 buffer. The order of test and control runs alternated with each frog. The total number of moves per run, the total number of visits to each compartment, and the total amount of time spent in each compartment and each buffer were quantified from the videotapes.

Statistical Analysis.—Mortality rates between pH treatments and between seasons were compared using two-way non-parametric ANOVAs (Schrier-Ray-Hare extension of KW test; Sokal and Rohlf, 1995). For the preference experiment the data were analyzed using a parametric ANOVA and a non-parametric Tukey test for multiple comparisons (Sokal and Rohlf, 1995).

Solutions: Mortality Study.—Citrate buffer was adjusted to a pH of 5.5 or 7.0 by addition of

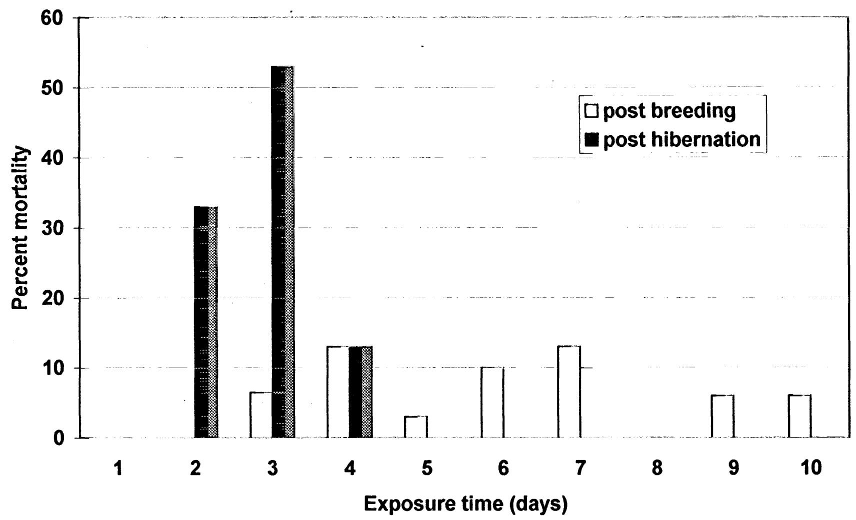

FIG. 1. Mortality of *Rana pipiens* exposed to pH 5.5 for 10 d (N = 64).

10N NaOH. The osmolarity of the buffers was determined to be 280 milliosmoles by a freezing point depression test. The freezing point test was conducted after the buffers were adjusted to their proper pH.

Solutions: Preference Study.—Citrate buffers adjusted to three different pHs, 4.0, 5.5, and 7.0, were placed in the various compartments of the experimental chamber. All buffers were autoclaved prior to use and their pH was measured at the beginning and end of each experimental run. Tap water (pH 7.4–8.0) aged in 76 l containers with an aerator for a minimum of one day was used in the conditioning chamber and individual containers.

RESULTS

Mortality Study.—Frogs exposed to citrate buffer of pH 5.5 for 10 d exhibited 72% (N = 46) mortality compared to only 3.5% mortality for frogs held in the same buffer at pH 7.0 (N = 29) (χ^2 = 33.47, $P < 0.005$). Mortality commenced after two d of exposure to pH 5.5, peaked at day 3 and 4 of treatment and continued throughout the 10 d of the experiment. The 3.5% mortality in the control group represents a single frog, which expired on day 4 of the experiment.

Season affected the sensitivity of frogs to pH 5.5. Frogs collected early in the spring, immediately following hibernation, but prior to the breeding season, exhibited 100% (N = 15) mortality within the first four d of exposure to pH 5.5. Frogs collected later in the season, post-breeding and prior to hibernation, exhibited 58% (N = 31) mortality over the 10 days of exposure (Fig. 1). The rates of mortality between the pre-breeding frogs and post-breeding frogs were significantly different from each other (χ^2 = 7.85, $0.01 > P < 0.005$).

Preference Study.—There was no significant difference in the number of moves during the test and control runs made by either *R. pipiens* (t = 1.480, $P > 0.1$) or *R. clamitans* (t = 0.375, $P > 0.5$). There also was no species effect on the number of moves made in either of these conditions. *R. pipiens* moved 92 ± 13 times in control runs versus 69 ± 28 times for *R. clamitans* (F = 0.74, P = 0.39). In experimental runs *R. pipiens* moved 64 ± 11 times versus 60 ± 9 times for *R. clamitans* (F = 0.9, P = 0.76). Therefore, treatment did not appear to influence activity levels and both species were equally active.

For control runs, the position of the compartment did not significantly affect the number of visits by frogs (*R. pipiens* F = 0.71, P = 0.62; *R. clamitans* F = 0.24, P = 0.94). Position also did not affect the time the frogs spent in each compartment (*R. pipiens* F = 1.82, P = 0.11; *R. clamitans* F = 0.77, P = 0.58). These data indicate that there was no location bias.

There was an effect of the species on the time spent in the buffers (two-way non-parametric ANOVA, χ^2 = 3.84, H = 4.12, $P < 0.05$) and an interaction of pH and species on time spent in buffers (χ^2 = 5.99, H = 7.4, $P < 0.05$). For the experimental runs, there was a significant difference in the time spent at the different pHs for both species (*R. pipiens*, F = 38.8, $P < 0.0001$; *R. clamitans*, F = 7.47, P = 0.002). For *R. pipiens*, Tukey multiple comparison tests indicated avoidance of both acidic treatments in comparison to the pH 7.0 treatment (Fig. 2). For *R. clamitans*, Tukey multiple comparison tests showed significant avoidance only to the lowest pH treatment (Fig. 2).

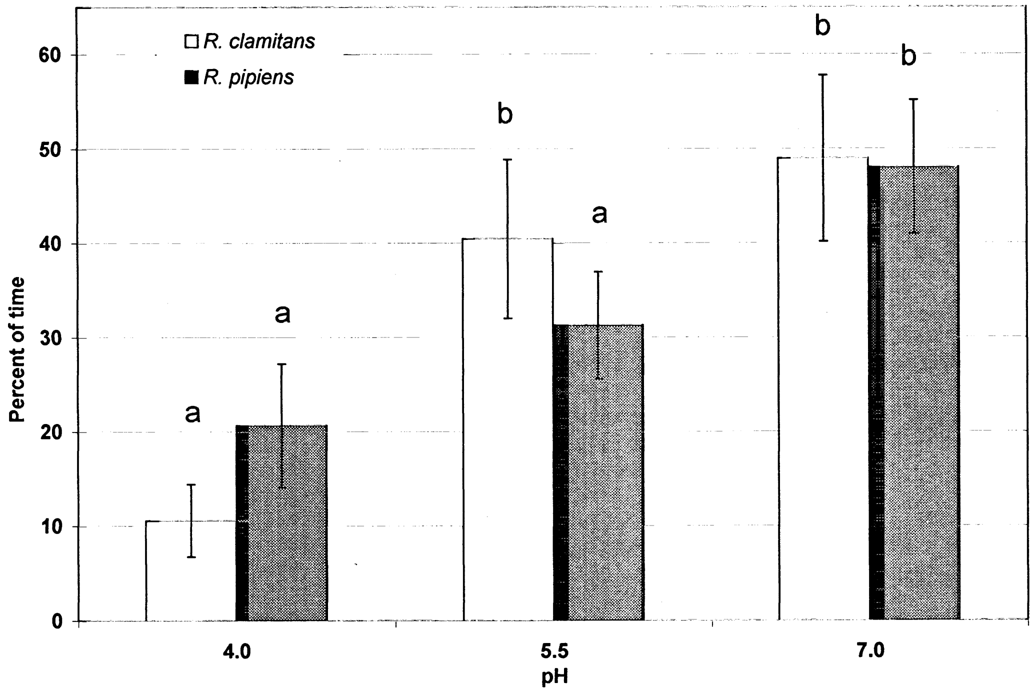

FIG. 2. The average percent of time (±SE) *R. pipiens* and *R. clamitans* spent in each pH (N = 24 *R. pipiens* and N = 12 for *R. clamitans*). Letters above error bars indicate significant differences among the three groups within a species revealed by a non-parametric Tukey test.

DISCUSSION

Acid is a prevalent environmental stressor for North American frogs. In the last few decades many aquatic environments have become increasingly acidic (Likens and Bormann, 1974; Freda, 1986; Bradford et al., 1992). Although a great deal of work has been done on the effects of pH on developing frogs (Gosner and Black, 1957; Schlichter, 1981; McDonald et al., 1984; Freda and Dunson, 1985; Pierce, 1985; Bradford et al., 1992; Griffiths and Beebee, 1992) there is a notable lack of studies on adult frogs. A few studies have demonstrated that acidic conditions disrupt the transport of ions in isolated skin in vitro (Ferreira and Hill, 1982; Lyall et al., 1992; Feder et al., 1993; Urbach et al., 1994).

Our mortality study is the first to demonstrate acid sensitivity of adult *R. pipiens*. Our data indicate that *R. pipiens* stressed by mildly acidic conditions (pH 5.5) experience a high level of mortality (58%) within a relatively short period of time (10 d). This observation fits well with hypotheses by other investigators (Glorioso et al., 1974; Carey, 1993; Maniero and Carey, 1997) that environmental stress is the initiating factor in a cascade of physiological events (immunosuppression and systemic distribution of opportunistic and virulent bacteria) which may ultimately contribute to the death of adult frogs.

Developing frogs exhibit interspecific and interpopulation differences in their sensitivity to acid conditions (Pierce, 1985; Griffiths and Beebee, 1992). It was therefore logical to hypothesize that these differences also occur in adult frogs. Our data indicate that adult *R. pipiens* exhibited individual differences in their sensitivity to acidic exposure. Sixty percent of those exposed to pH 5.5 died during a 10 d exposure. The remaining 40% that survived must differ in their ability to withstand such conditions. The physiological mechanisms that enable this group to survive are currently under investigation. Our results also provide empirical support for the suggestion by Maniero and Carey (1997) that the heightened vulnerability of frogs emerging from hibernation may be compounded by environmental stressors. For example, Cooper et al. (1992) demonstrated that exposure to cold during hibernation suppresses the immune system of frogs. In our mortality study, the group of frogs that just emerged from hibernation exhibited 100% mortality within four days of acid exposure compared to only 58% mortality over the 10 day exposure period in the post-breeding, pre-hibernation group. Therefore, frogs emerging from hibernation are vulnerable until their immune capacity is restored.

Our data also suggest that ranid frogs can detect the pH of the surrounding environment and preferentially choose a neutral pH. Comparison of experimental and control runs showed that frogs made equal numbers of moves in both runs, indicating that the acid conditions did not influence general levels of activity. The lack of differences in the average number of moves made by each species may suggest that the mechanism for sampling and assessing the environmental pH is similar for both species of frogs.

Adults of both *R. pipiens* and *R. clamitans* spent the greatest amount of time in pH 7.0 and the least amount of time in pH 4.0 (Fig. 2). However, the *R. pipiens* avoided both pHs 4 and 5.5 while *R. clamitans* spent almost equal amounts of time in pH 5.5 and 7.0 and avoided only pH 4. These data correspond to the acid sensitivity of these two species. Exposure to pH of 5.5 causes high rates of mortality in adult *R. pipiens*, but adults of *R. clamitans* exposed to similar acidic conditions experience no mortality (Brodkin et al., unpubl. data). Therefore, the different pH preferences are consistent with different pH tolerances for the two species.

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