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

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ABSTRACT.—In this study we report mortality rates of adults of Rana pипiens to mild acid exposure and
pH preferences for R. pипiens and R. clamitans. We exposed adult R. pипiens 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. pипiens (N = 24) and R.
camitans (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. pипiens are sensitive to mildly acidic conditions, especially when emerging from
hibernation, and that adults of both R. pипiens 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 re-
stance factors (spleenic and peripheral white blood cell numbers and the phagocytic efficien-
cy of these cells) of ranids. In these experiments we found that adults of Rana pипiens, R. clami-
ts, and R. catesbeiana have different sensitivities to low pH with R. pипiens being the most
sensitive (unpubl. data). Immune function de-
clines during hibernation (Cooper et al., 1992)
and frogs emerging from hibernation may be
particularly vulnerable to disease until their im-
une function is restored (Maniero and Carey,
1997). We tested the hypothesis that acid sensi-
tivity is also different for adults of R. pипiens 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. pипiens ("mortality study"). We conducted a
second experiment to compare pH preferences
of R. pипiens to those of the less acid-sensitive R.
camitans ("preference study"). The ability to de-
tect the acidity of the environment may be par-
ticularly important for R. pипiens because of their
susceptibility to mild acidic conditions at all
life stages. Female frogs should choose carefully
the site for oviposition because R. pипiens em-
byros 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-Lescaut, 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. pипiens 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 breed-
ing season, and were held unfed in cold storage
by the supplier before shipment. The third ship-
ment 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 pre-
ference study 36 frogs, 24 R. pипiens and 12 R.
camitans (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. picipiens. The minimum voluntary temperature of R. picipiens 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. picipiens 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 airflow.

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 (Schirier-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
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 76.1 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) (χ² = 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 (χ² = 7.85, P = 0.005).

**Preference Study.—* There was no significant difference in the number of moves during the test and control runs made by either *Rana pипiens* (t = 1.480, P > 0.1) or *R. clamitans* (t = 0.375, P > 0.5). There was no significant difference in the number of moves during the test and control runs made by either *R. pипiens* (t = 1.480, P > 0.1) or *R. clamitans* (t = 0.375, P > 0.5). There was also no species effect on the number of moves made in either of these conditions. *R. pипiens* moved 92 ± 13 times in control runs versus 69 ± 28 times for *R. clamitans* (F = 0.74, P = 0.39). In experimental runs *R. pипiens* 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. pипiens* 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. pипiens* 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, χ² = 3.84, H = 4.12, P > 0.05) and an interaction of pH and species on time spent in buffers (χ² = 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. pипiens*, F = 38.8, P < 0.0001; *R. clamitans*, F = 7.47, P = 0.002). For *R. pипiens*, 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 7.0 treatment (Fig. 2).
FIG. 2. The average percent of time (± SE) R. pipsiens and R. clamitans spent in each pH (N = 24 R. pipsiens 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. pipsiens. Our data indicate that R. pipsiens 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. pipsiens 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 de-
tect 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 (Brod-kin et al., unpubl. data). Therefore, the different pH preferences are consistent with different pH tolerances for the two species.

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LITERATURE CITED


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