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EXPERIMENTAL STUDIES OF THE EFFECTS OF ACIDITY AND ASSOCIATED WATER CHEMISTRY ON AMPHIBIANS

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Laboratory bioassays with 5 species of Nova Scotian amphibians (*Ambystoma maculatum, Hyla crucifer, Rana sylvatica, R. palustris,* and *Bufo americana*) and *Xenopus laevis* revealed pH 4.0 to 5.0 as the critical range within which developing embryos showed reduced hatching success, and below which hatching did not occur. Bioassays revealed differences in acid tolerance among various species of Nova Scotia amphibians. For some species, aluminum in combination with pH≤5, had a deleterious effect. Calcium ameliorated pH toxicity, but in some cases mortality was increased at very high Ca concentrations (≥50 ppm). Larvae of *Rana clamitans* and adult *Notophthalmus viridescens* survived exposure to pH as low as 3.3, but this tolerance was reduced at low temperatures.

Des bioessais portant sur 5 espèces d'amphibiens de la Nouvelle-Ecosse (*Ambystoma maculatum, Hyla crucifer, Rana sylvatica, R. palustris* and *Bufo americana*) et *Xenopus laevis* ont montré que le pH 4.0 à 5.0 constitue une zone critique à l'interieur de laquelle le taux d'éclosion était réduit. Au-dessous de cette zone l'eclosion ne se produisait pas. Des bioessais ont montré des différences spécifiques de tolérance à l'acide. Le calcium réduisant la toxicité associé au bas pH. Cependant, une trop forte concentration encalcium (>50 ppm) causait une augmentation de mortalité dans certains cas. Les tetards de *Rana* clamitaus ainsi. Que les adults de *Notophthalmus viridescens* ont résisté à un exposition à un pH aussi faible que 3.3; cette tolerance était réduite à basse tempèrature.

Introduction

The impacts of acidic deposition on amphibians, compared with fish and forests, have received relatively little attention. However, amphibiansm may be susceptible to acidification, and are ecologically important in some forest/pond ecosystems (Burton and Likens 1975a; Wassersug 1975; Seale 1980).

In the United States 90% of frog and toad species and 50% of salamander species lay their eggs in water; many of these breed in ephemeral pools filled with rainwater or snowmelt, as opposed to groundwater (Pough and Wilson 1977). In regions receiving acidic deposition, breeding ponds may have pH similar to precipitation, especially during spring (Pough and Wilson 1977). Since many amphibians enter temporary ponds to breed in spring, the most susceptible stages of their life histories (reproduction and early embryonic development) may coincide with the most acidic breeding site conditions.

Several field studies have suggested that habitat acidity can reduce population size or restrict distribution of amphibians (reviewed recently in Dale *et al.* 1985). Various laboratory studies support these field observations, and also suggest physical and chemical mechanisms of amphibian reproductive failure. Schlichter (1981) reported

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Table I Chemistry of the water types used in laboratory bioassays

	pH	Conduct. (μmho-cm)	Color (Hazen Units)	Alkalinity (mg/l)	Na (mg/l)
dechlorinated[1] tapwater	7.8	21.0	0	86	3.4
clearwater[2] oligotrophic	5.5	0.0	5	24	2.6
brownwater[3] oligotrophic	4.6	0.0	80	33	2.7

[1] From Dalhousie University.
[2] From Beaverskin Lake, Kejimkujik National Park, Nova Scotia.
[3] From Pebbeloggitch Lake, in the Park.

that pH≤6.5 decreased sperm motility in the leopard frog (*Rana pipiens*), and that the number of healthy embryos decreased at pH ≤6.3. Several authors have found that low pH caused deformities or arrest of embryonic development. In recent reviews of laboratory experiments, the sensitivity of embryonic development to acidic media was found to be rather similar among 14 amphibian species (Tome and Pough 1982; Pierce 1985). In most cases pHs of 3.7 to 3.9 during development produced mortality >85%, and prolonged exposure to pHs <4.0 caused mortality >50%. Gosner and Black (1957) found that embryos were more sensitive than larvae, with some embryos showing ≥85% mortality at pH 4.0. Pough (1976) reported that high egg mortality of yellow-spotted salamanders (*Ambystoma maculatum*) seen in New York ponds with pH<6, was also observed under laboratory conditions. Furthermore, the mortality occurred at the same developmental stages: neurulation, late gill stage, and hatching. Deformities observed in the field were also seen in the laboratory, with swelling near the heart, stunted gills, failure of yolk plug retraction, and deformation of the posterior trunk. Pough (1976) also noted that at pH 4 embryos were tightly coiled within their membrane. This coiling was attributed to failure of expansion of the perivitelline space, which caused a mechanical constriction of the embryo leading to death or deformation (Gosner and Black 1957; Pough 1976; Pough and Wilson 1977). When yellow-spotted salamander embryos which failed to hatch at pH 4.0 were removed from the egg membrane, they were unable to swim or straighten their bodies (Pough and Wilson 1977). Working with the African clawed frog (*Xenopus laevis*), Dunson and Connell (1982) reported that removal of the membrane from embryos at pH 4.3 (which otherwise would not have hatched), allowed normal development.

Objectives of the present study were: i) to conduct laboratory experiments to determine the effect of low pH on embryonic and early larval development of selected native amphibian species, and the African clawed frog; ii) to examine the effect of two frequently-occurring types of Nova Scotian freshwaters (i.e. acidic clear and brown oligotrophic water) on reproductive success of selected native amphibians and the African clawed frog; iii) to examine effects of calcium and aluminum additions, in combination with pH and water type, on reproductive success of selected native species and the African clawed frog; and iv) to examine the effect of low pH on the ability of frog larvae (*Rana clamitans*) and adult newts (*Notophthalmus viridescens*) to survive at different temperatures.

K →	Ca	Mg	SO_4	Cl	Al	Total Organ. Carbon
0.40	13.0	0.10	8.5	n.d.	0.10	n.d.
0.20	0.4	0.34	2.6	4.3	0.04	5.6
0.20	0.3	0.33	3.3	4.4	0.16	17.2

Materials and Methods

Egg collection

Eggs of native species were collected in the field or from wild amplexed adults brought into the laboratory. Eggs collected in the field were transported to the laboratory in their natural water and maintained in a controlled environment at 12°C until used.

Eggs used in the *X. laevis* assays were collected from adults induced to breed by injection of human chorionic gonadotropin (Carolina Biological Supply Co., injection method no. 4, male - 250 I.U., female - 500 I.U.). Breeding pairs were maintained in a 30 L aquarium fitted with a false bottom with a removable glass tray. With this apparatus undisturbed eggs could be removed prior to first cleavage and selected for viability prior to use in experiments.

Eggs were added to treatment waters either as intact masses (*Ambystoma maculatum, Rana sylvatica, R. palustris*) or as groups of single eggs (*Xenopus laevis, Hyla crucifer, Bufo americanus*). *R. sylvatica, A. maculatum,* and *X. laevis* were staged according to Rugh (1962). *Rana clamitans* was staged according to Gosner (1960).

Water types

Polyethylene containers were filled with dechlorinated tap water, clear oligotrophic water, or brown oligotrophic water. The latter two were collected from Beaverskin Lake and Pebbleloggitch Lake, respectively, in Kejimkujik National Park (Kerekes *et al.* 1982). Chemical constituents of the three water types are summarized in Table I. One experiment used 10% frog Ringers, consisting of 11.5 mM NaCl, 0.2 mM KCl, 0.14 mM $CaCl_2$, and 0.24 mM $NaHCO_3$ (Schlichter 1981).

Buffers

Three of the six *X. laevis* assays were buffered to control pH drift. Following Schlichter (1981), buffered solutions were 25 millimolar and consisted of either sodium acetate/acetic acid ($pK_a = 4.8$), MES = 2 [N-Morpholino] ethanesulfonic Acid ($pK_a = 6.1$), or HEPES = N-2-Hydroxyethylpiperazine-N' -2- ethanesulfonic Acid ($pK_a = 7.5$).

pH adjustment

pH was adjusted using H_2S0_4 and NaOH, and was measured at the experimental temperature. pH $\leq$4.25 were stable over three or four days, but at higher pH drift towards neutrality occurred, by as much as 0.5 pH units per day in preliminary, aerated experiments. We therefore adjusted pH once or twice daily. In this manner, pH was maintained within 0.2 pH units of the desired value for experiments reported here. All assays had a complete change of treatment water at least once weekly.

Water in several of our earlier experiments was aerated. Since this practice caused greater pH drift, it was discontinued for experiments reported here, with no apparent ill effect on developing embryos or larvae.

Aluminum and calcium

In assays using aluminum, solutions were made by adding 1000 ppm Al stock solution (made from $AlCl_3$ $6H_20$), and then adjusting to desired pH. Calcium solutions were made by adding 10,000 ppm Ca stock solution (made from $CaCl_2$ $2H_20$). Concentrations of Al and Ca were measured by atomic absorption spectro photometry. Concentrations reported are total Al and Ca, and include back ground concentration in assay waters (see Table I).

Monitoring

Assays ran until hatching was completed, and in several cases well into the larval phase. These times ranged from 1 to 7 weeks. Observations of developmental and hatching success were made during these periods.

Upon termination, each assay was scored (where applicable) for % hatched, % hatched but later died, and % hatched but deformed.

Bioassays

Over 1982-83, bioassays were performed on 7 native species and *X. laevis*. Methods used for each are summarized below, ordered by species.

Ambystoma maculatum

pH - tapwater 12°C. Intact egg masses were placed in 3 L of dechlorinated tapwater adjusted to pHs 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, or 7.0 (3 replicates/treatment), and incubated at 12°C for 41 days. Embryos were initially at, or prior to, the fourth cleavage division (stage 5, Rugh 1962).

pH - tapwater 21°C. This assay was identical to the previous, except the incubation temperature was 21°C, and the assay ran for 35 days.

pH - clear and brown oligotrophic water. Intact *A. maculatum* masses prior to the blastula stage (stage 6, Rugh 1962) were incubated at 12°C for 41 days in 4 L of either clear or brown oligotrophic water. These waters had been adjusted to pH 3.5, 4.0, 4.5, or 5.0, with 2 replicates/treatment.

Calcium - pH interactions. This assay was a 5 x 5 matrix of pH and calcium, with one replicate/treatment. Egg masses were divided into portions of 9-24 embryos, randomly assigned to treatment, and incubated at 21°C in 300 ml of dechlorinated tapwater adjusted to a particular pH and calcium concentration. pHs were 3.5, 4.0, 4.5, 5.0, and 6.0, and calcium concentrations were 10, 30, 50, 70, and 90 ppm. Embryos were initially at the late blastula stage (stage 9, Rugh 1962). The assay ran for 27 days.

Aluminum - pH interactions. This assay was a 4 x 5 matrix of aluminum and pH, with 2 replicates/treatment. pHs were 3.5, 4.0, 4.5, 5.0, and 6.0, and total aluminum concentrations were 0.1, 0.4, 0.7, and 1.1 ppm. Egg masses were divided

(range of 18-46 eggs/treatment), randomly assigned to treatment, and incubated at 21°C in 3 L of dechlorinated tapwater for 31 days. Embryos were initially at the late blastula stage (stage 9, Rugh 1962).

Hyla crucifer

pH - tapwater. Containers with 300 ml of dechlorinated tap water were adjusted to one of the following pHs: 3.0, 4.0, 5.0, 6.0, 7.0, or 8.0. Eggs were obtained in the laboratory from an amplexed pair of wild adults; There were 35 eggs (midblastula stage) per treatment. Assays were maintained at 20°C for 18 days.

pH - aluminum. This assay was a 5 x 4 matrix of pH and aluminum. pHs were 3.5, 4.0, 4.5, 5.0, and 6.0, and aluminum concentrations were 0.1, 0.4, 0.7 and 1.1 ppm. Two replicates per treatment (range 11 to 34 midblastula eggs) were incubated at 21°C in 100 ml of dechlorinated tapwater solution, for 16 days.

Rana sylvatica

pH - tapwater 12°C. Portions of egg masses were placed in containers with 3 L of dechlorinated tapwater adjusted to pH 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, or 7.0 (2 replicates/treatment), and incubated at 12°C for 18 days. Embryos were initially at, or prior to, the fourth cleavage division (stage 6, Rugh 1962).

pH - tapwater 21°C. This experiment was identical to the previous, except the incubation temperature was 21°C, and the assay ran for 11 days.

pH - clear and brown oligotrophic water. This bioassay involved intact egg masses at 12°C in clear or brown oligotrophic water. Four liter solutions were adjusted to pH 3.5, 4.0, 4.5, or 5.0, with 2 replicates/treatment. Embryos were initially at, or prior to, the fourth cleavage division (stage 6, Rugh 1962). The assay ran for 19 days.

Calcium - pH interactions. This bioassay was a 5 x 5 matrix of pH and calcium. Egg masses were divided into portions with 25-75 embryos, randomly assigned to treatment, and incubated at 21°C in 300 ml of dechlorinated tapwater adjusted to pH 3.5, 4.0, 4.5, 5.0, or 6.0, and/or calcium concentration of 10, 30, 50, 70, or 90 ppm. Embryos were initially at the gastrula stage (stage 12, Rugh 1962). The assay ran for 7 days.

Rana palustris

A single egg mass of *R: palustris* was divided into portions (37 to 62 eggs/treatment) and incubated for 26 days in 300 ml of 12°C dechlorinated tapwater adjusted to pH 3.0, 3.5, 3.8, 4.0, 4.3, 4.5, 5.0, 6.0, or 7.0. Embryos were at or before the 32 cell stage. There were 2 replicates/treatment, and the experiment ran for 26 days.

Bufo americanus

pH - tapwater - 21°C. This assay involved incubating groups of eggs at the early gastrula stage (46-51 eggs/treatment) in 3 L of 21°C dechlorinated tapwater adjusted to pH 3.0, 3.5, 3.75, 4.0, 4.25, 4.5, 5.0, or 6.0. The assay ran for 11 days, with 2 replicates per treatment.

Calcium - pH interactions. This assay was a 5 x 5 matrix of pH and calcium. pHs were 3.5, 4.0, 4.5, 5.0, or 6.0, and calcium concentrations were 10, 43, 76, 110, or 210 ppm. Groups of eggs at the gastrula stage (range 23-28 eggs per treatment) were incubated at 21°C in 300 ml of dechlorinated tapwater for 10 days, with 2 replicates per treatment.

Aluminum - pH interactions. This assay was a 5 x 6 matrix of pH and aluminum. pH was 3.5, 4.0, 4.5, 5.0, or 6.0, and aluminum concentration was 0.10, 0.60, 0.85, 1.10, 1.60, or 2.10 ppm. Groups of eggs at the early gastrula stage (47-52 eggs/treatment) were incubated at 21°C in 2 L of dechlorinated tapwater for 11 days, with 2 replicates per treatment.

Rana clamitans

Effect of temperature and pH on larvae. First-year larvae (stage 25 to 29, Gosner 1960) were assayed either at 5°C or 21°C. Larvae were collected in the field on 9 October, 1983 at a field pH of 6.4 and temperature of 13°C. Larvae were placed in 12°C aerated dechlorinated tapwater, and over the next month temperature was gradually reduced to 5°C. During this acclimation period larvae were fed boiled lettuce. For the 5°C assay, containers with 2 L of dechlorinated tap water were adjusted to the following pHs: 3.5, 3.8, 4.0, 4.3, 4.5, 5.0, or 6.0. There were 3 replicates per treatment, and 10 larvae per replicate. The experiment ran for 38 days.

For the 21°C assay larvae were gradually acclimated over 2 weeks to 21°C. The assay ran for 13 days, and was identical to the 5°C assay except for temperature, addition of pH 3.3 and 7.0 treatments, and deletion of pH 4.3.

Notophthalmus viridescens

Effect of temperature and pH on adults. Adult newts were assayed at 5°C and 21°C. Newts were collected in the field on 9 October, 1982 at a field pH of 6.2 and temperature of 12°C. They were kept in the laboratory in 12°C aerated dechlorinated tap water, and over the next month water temperature was gradually reduced to 5°C. During this acclimation period newts were fed raw beef liver. For the 5°C assay, containers with 2 L of dechlorinated tap water were adjusted to the following pHs: 3.5, 3.8, 4.0, 4.3, 4.5, or 5.0. There were 3 replicates per treatment, and 10 newts per replicate. The experiment ran for 38 days. For the 21°C assay newts were gradually acclimated over a 2-week period to 21°C. The assay ran for 13 days, and was identical to the 5°C assay except for temperature.

Xenopus laevis

Buffered/unbuffered - sodium acetate/acetic acid. Fourteen containers were prepared, each containing 300 ml of dechlorinated tap water. Seven of the containers were acidified using 25 mM sodium acetate/acetic acid as a buffer ($pK^a = 4.8$), following Schlichter (1981). The remaining containers did not have buffer added. The 7 pairs of acetate or non-acetate treatments were adjusted to the following pHs: 4.0, 4.5, 4.8, 5.0, 5.3, 5.5, or 6.0. Twelve eggs (prior to the eight cell stage, stage 5, Rugh 1962) were placed in each container. Assays were maintained at 21°C for 6 days.

A parallel assay was run to confirm observations made in the above experiment. Groups of 10 eggs (four cell stage, stage 4, Rugh 1962) were incubated at 21°C in 800 ml of 10% amphibian Ringers solution adjusted to pH 3.5, 4.0, 4.5, 5.0, 6.0, or 7.0. These solutions were either unbuffered or buffered with sodium acetate/acetic acid at a concentration of 25 mM. There were 2 replicates/treatment, and the experiment ran for 5 days.

Aluminum - pH interactions. Clear and brown oligotrophic waters were used in this assay. For each water type a 6 x 6 matrix of pH and aluminum concentration was established, with pH of 3.0, 3.5, 4.0, 4.5, 5.0, or 6.0, and aluminum concentration of 0.05 to 1.15 ppm. Microliter amounts of a 1000 ppm aluminum solution were pipetted into 100 mL of water to give desired aluminum concentrations.

Solutions were then adjusted to desired pH. Ten eggs (prior to the four cell stage, stage 3, Rugh 1962) were placed into each beaker. Assays were maintained at 21°C for 11 days.

Calcium - pH interactions. This assay was identical to the aluminum assay described above, except that: i) the matrix was 5 x 5 with pH 3.0, 3.5, 4.0, 4.5, or 5.0 and calcium concentration of 0, 20, 40, 60, or 80 ppm; ii) a 10,000 calcium solution was used; and iii) the assay ran for 10 days.

Table II *Ambystoma maculatum* bioassays in dechlorinated tapwater

pH	12°C		21°C	
	No. of eggs in mass	% hatched	No. of eggs in mass	% hatched
7.0	48	23	54	43
	52	52	47	51
6.0	86	6	62	40
	85	26	42	81
5.0	67	40	75	32
	57	11	76	46
4.5	56	2	118	2
	111	9	56	11
4.0	62	0	72	0
	42	0	83	1
3.5	>50	0	>50	0
	>50	0	>50	0
3.0	>50	0	>50	0
	>50	0	>50	0

Results

Ambystoma maculatum

pH effects at 12°C. No hatching or development of embryos were observed at pH 3.0 or 3.5 (Table II). At these pHs, the gelatinous mass that contained the eggs turned a milky-opaque colour within 2 hours of exposure, and the egg mass subsequently shrank in volume, becoming rubbery in texture.

At pH 4.0 egg masses turned slightly opaque; nevertheless embryos appeared to be developing normally. However, after 20 days of incubation all embryos were dead. At pH 4.5 embryos were tightly coiled within the perivitelline space. The 6% that did hatch were deformed with abnormal body curvatures (both scoliosis and kyphosis: effects on spinal development). Deformed larvae were observed using the double-staining technique of Hanken and Wassersug (1981), and indicated that fusion of some vertebrae had occurred; this may have caused the abnormal spinal curvatures.

At pH 4.5 only 6% of embryos hatched successfully, although unhatched developing embryos had appeared normal. Hatching success improved somewhat at pHs 5.0, 6.0, and 7.0 with 26%, 16%, and 38%, respectively. These latter percentages were less than obtained in preliminary experiments run one year previously at the same pHs but at 15°C (mean hatching success of 93%). This probably reflects some inherent inviability of the egg masses used in the 1983 experiments. Larger experiments with greater replication would have been desirable for documenting interspecific varia-

tion in viability; however experiments of such scope would have been difficult logistically, and were beyond our means.

pH effects at 21°C. No hatching or development were observed at pH 3.0 or 3.5 (Table II). At pH 4.0 one deformed embryo hatched, but it soon died. Unhatched embryos at pH 4.0 developed to the head/tail stage (stage 25, Rugh 1962). They were all tightly coiled within the vitelline membrane and died without hatching.

At pH 4.5 development was slow, compared to higher pHs, and many embryos died at an early stage. Seven percent of these embryos hatched. These were initially scoliotic, but at the end of the experiment they appeared normal and were able to swim. At higher pHs hatching success was improved.

Brown vs. clear oligotrophic water. This experiment, run at 12°C, did not indicate any differences in pH toxicity between the two water types. No hatching occurred at pH 3.5 or 4.0, while at pH 4.5 and 5.0 hatching averaged (n=2) 36% in the clear, and 30% in the brown oligotrophic water.

Calcium - pH interactions. Results of this experiment indicate that with calcium concentrations of at least 30 ppm at pH 4.0, hatching occurred (21% at 30 ppm calcium, 20% at 50 ppm, 8% at 70 ppm, 4% at 90 ppm), while none occurred at 10 ppm calcium. At pH >4.5, hatching success was high (overall mean of 15 treatments was 90%) at all calcium concentrations.

Aluminum - pH interactions. From pHs 4.5 to 6.0 aluminum had no effects on hatching success. However, at pH 4.0 mean (n=2) hatching success was decreased from 11% at 0.1 ppm total aluminum, to 3% at 0.4 ppm, and 2% at both 0.7 and 1.1 ppm. However, all larvae hatched at pH 4.0 died over the following few days.

Table III *Hyla crucifer* bioassay at 20°C in dechlorinated tapwater

pH	% hatched[1]	% hatched, but later died
8.0	83	0
7.0	80	0
6.0	80	0
5.0	77	11
4.0	54	100
3.0	0	-

[1] Of 35 individual eggs.

Hyla crucifer

pH effects. No hatching or development were observed at pH 3.0 (Table III). The gelatinous sheath around individual eggs (this species does not lay egg masses) was compressed in volume at pH 3.0 by a factor of *ca.* 1/2, and by a lesser degree at pH 4.0. However, the gelatin did not turn opaque.

At pH 4.0, hatching success was 54%, but all hatched larvae subsequently died. At this pH, developing embryos were tightly coiled within a compressed perivitelline space; head and tail of the embryos overlapped, for a total coiling of about 430 degrees of arc, compared with about 280 degrees at higher pH. All hatched larvae at pH 4.0 were deformed with gross body curvatures, were incapable of swimming, and all subsequently died.

At pH ≥5.0 hatching success averaged 80%, but there was higher mortality among hatched larvae at pH 5.0 (11% versus <1% over pH 6 to 8). Twenty percent of hatched larvae at pH 5.0 had body curvature deformities and impaired swimming.

Aluminum - pH interactions. There was no consistent effect of aluminum on hatching success of *H. crucifer* at any of the pHs where hatching occurred (i.e. pHs

Table IV *Rana sylvatica* bioassays in dechlorinated tapwater

pH	12°C		21°C	
	No. of eggs in mass	% hatched	No. of eggs in mass	% hatched
7.0	451	92	473	92
	409	95	213	98
6.0	333	51	378	77
	318	46	346	80
5.0	473	51	262	97
	357	39	319	90
4.5	340	38	337	95
	296	40	323	82
4.0	320	17	119	8
	313	6	276	<1
3.5	>200	0	>200	0
	>200	0	>200	0
3.0	>200	0	>200	0
	>200	0	>200	0

4.5, 5.0, and 6.0). No hatching occurred at pH 4.0, contrary to results presented in the previous section, which indicated a hatching success of 54%, although all of these hatched larvae soon died.

Rana sylvatica

pH effects at 12°C. No hatching or development were observed at pH 3.0 (Table IV). At this pH the gelatinous mass that contained the eggs turned a milky-opaque colour within 2 hours of first exposure. Overall volume of the egg mass subsequently shrank, and it became rubbery in texture over the next few days, ending up as a small, tight, spherical structure. At pH 3.5 some embryos developed to the neural plate stage (stage 14, Rugh 1962) before being aborted. Otherwise, these egg masses were similar to those at pH 3.0.

Over the pH range 4.0 to 7.0 hatching occurred. At pH 4.0 12% of the embryos hatched compared with an average of 44% over pH 4.5 to 6.0, and 94% at pH 7.0. pH 4.0 embryos were tightly coiled within the vitelline membrane, and the few that did hatch subsequently died.

pH effects at 21°C. No hatching or development occurred at pH 3.0 (Table IV). Four pH 3.5 embryos developed to the head/tail stage (stage 18, Rugh 1962), and muscular movement was detected; however, none of these hatched. An average of 50% of pH 4.5 to 7.0 embryos had hatched before any pH 4.0 embryos hatched, and when 90% of the former had hatched, only 4% of the pH 4.0 embryos had hatched. The latter hatched larvae were maintained at pH 4.0 for 30 days, after which they appeared normal in swimming and feeding behavior, compared with controls. Unhatched pH 4.0 embryos were all tighly coiled with >360° of arc.

Brown vs. clear oligotrophic water. This experiment did not indicate differences in pH toxicity between the two water types. No hatching occurred at pH 3.5, while at pH 4.0, 4.5, and 5.0 hatching averaged 52%, 84%, and 90% in the clear water, and 43%, 87%, and 93% in the brown water.

Table V *Rana palustris* bioassays at 12°C in dechlorinated tapwater

pH	No. of eggs[1]	% hatched	% hatched that later died
7.0	43	100	0
	37	100	0
6.0	41	100	0
	53	100	0
5.0	49	41	50
	22	36	50
4.5	40	5	50
	44	5	50
4.3	39	0	-
	48	0	-
4.0	62	0	-
	41	0	-
3.8	53	0	-
	52	0	-
3.5	55	0	-
	38	0	-
3.0	50	0	-
	43	0	-

[1] Excized from a single egg mass.

Table VI *Bufo americanus* bioassays at 21°C in dechlorinated tapwater

pH	No. of eggs[1]	% hatched
6.0	50	100
	51	100
5.0	50	100
	50	100
4.5	48	100
	46	100
4.3	50	100
	49	82
4.0	50	84
	50	96
3.8	50	0
	49	0
3.5	48	0
	49	0
3.0	47	0
	51	0

[1] Groups from a single egg strand.

Calcium - pH interactions. No embryos hatched at pH 3.5 or 4.0, though many developed to the stage where external gills were present (stage 19, Rugh 1962).

At pH 4.5 hatching success decreased with increasing calcium concentration (100% at 10 ppm calcium, 71% at 30 ppm, 41% at 50 ppm, 11% at 70 ppm, and 20% at 90 ppm). This effect did not occur at either pH 5.0 or 6.0, with hatching success uniformly >90% (mean of 10 observations = 94%).

Rana palustris

pH - tapwater. Hatching was reduced from 100% at pH 6.0 to 39% and 5% at pHs 5.0 and 4.5, respectively (Table V). At pH <4.5 no hatching occurred. Larvae hatched at pH 5.0 or 4.5 were deformed and could not swim properly; some died soon after hatching.

Bufo americanus

pH - tapwater. Hatching success ranged from 90% to 100% over pH 4.0 to 6.0 (Table VI). However, at pH <4.0 no hatching occurred. All embryos at pH <3.8 failed to develop, the gelatin coat surrounding each embryo became opaque, and embryos

Table VII *Rana clamitans* bioassays for larvae in dechlorinated tapwater

pH	% survival[1] after 38 days at 5°C	% survival after 14 days at 21°C
7.0	-	100
		100
		100
6.0	40	100
	20	100
	60	100
5.0	80	100
	80	100
	80	100
4.5	80	100
	90	100
	60	100
4.3	50	
	90	-
	80	
4.0	50	100
	90	100
	80	100
3.8	60	100
	90	100
	70	100
3.5	30	100
	60	100
	60	100
3.3	-	50
		0
		0

[1] Of 10 larvae in each replicate.

appeared to be touching each other, indicating failure of formation of the perivitelline space.

Calcium - pH interactions. This experiment demonstrated no effect of calcium on hatching success. Hatching success was >90% at all pHs and calcium concentrations from pH 4.0 to 6.0 (mean = 97%). No hatching occurred at pH 3.5.

Aluminum - pH interactions. As above, hatching was >90% at all pHs and total aluminum concentrations from pH 4.0 to 6.0 (mean = 96%), and no hatching occurred at pH 3.5.

Rana clamitans

Effect of temperature on pH tolerance. At 5°C, survival was <100% at all pHs, with lowest survival at pH 3.5 and 6.0 (Table VII). At 21°C, survival was lowest at pH 3.3 (averaging 17%), and was 100% at all higher pHs. Thus, cooler bioassay temperature negatively affected survival at all pHs. Nevertheless, many larvae survived relatively low pH (i.e, <4.0), in contrast to our observations of toxicity to embryonic and early larval development in other species.

Notophthalmus viridescens

Effect of temperature on pH tolerance. At 5°C, survival averaged 20% at pH 3.5, and 100% at all higher pHs (Table VIII). At 21°C, survival averaged 71% at pH 3.5, and was essentially 100% at all higher pHs. Thus, cooler temperatures predisposed adult newts to death at the lowest pH used in this bioassay.

Xenopus laevis

Buffered vs. unbuffered assay solutions. Effects of buffering with acetate on hatching success were quite clear. Embryos in unbuffered solutions hatched at pH ≥4.5 (Table IX). Embryos in acetate-buffered solutions did not hatch at pH <6. In the buffered solutions, embryonic development did not occur over pH 4.0 to 5.0; some development occurred at pH 5.3 and 5.5, but this was arrested at the 128-cell stage (stage 7, Rugh 1962). These observations were consistent in two separate experiments - one run in dechlorinated tapwater, and the other in amphibian Ringers solution.

Aluminum - pH - water type interactions. In neither brown nor clear water was hatching or development observed over the pH range 3.0 to 4.0 (Table X). In the brown water, hatching success was little affected by either pH from 4.5 to 6.0, or aluminum from 0.15 to 1.15 ppm. However, survival of hatched larvae was lower at the highest aluminum concentrations (especially at pHs 4.5 and 5.0; the effect was less at pH 6.0), although there were several anomalous results (e.g. at pH 4.5 and with 0.15 ppm aluminum).

Similar observations were made for hatching in the clear water from pH 4.5 to 6.0, apart from lower hatching at pH 5.0 with higher aluminum concentrations. Again, survival of hatched larvae was impaired at higher aluminum concentrations at pHs of 4.5 and 5.0, but not at pH 6.0. Differences in hatching or survival between the two water types were not consistent.

At 0.05 to 0.15 ppm aluminum, a high frequency of deformed larvae (body curvature) was found at pH 4.5, especially in the brown water. At more moderate pH (e.g. pH >5.0) but high aluminum concentration (e.g. >0.55), malformed larvae also occurred. Albinism and incomplete yolk sac absorption were frequent.

Calcium - pH - water type interactions.

No hatching occurred in either water type at pH 3.0 or 3.5 (Table XI). Hatching also did not occur at pH 4.0 with calcium concentrations of 0 to 40 ppm, but this effect was ameliorated at higher calcium concentrations. Similarly, pH 4.5 showed a toxic effect

Table VIII *Notophthalmus viridescens* bioassays for adult animals in dechlorinated tapwater

pH	% survival[1] after 38 days at 5°C	% survival after 14 days at 21°C
5.0	100	100
	100	100
	100	100
4.5	100	87
	100	100
	100	100
4.3	100	100
	100	100
	100	100
4.0	100	100
	100	100
	100	100
3.8	100	100
	100	100
	100	100
3.5	10	75
	20	87
	30	50

[1] Of 10 adults in each replicate.

Table IX *Xenopus laevis* bioassays in dechlorinated tapwater or amphibian Ringer's solution

pH	% hatched[1] in dechlorinated tapwater, 21°C		% hatched[2] in 10% amphibian Ringer's, 21°C	
	buffered[3]	unbuffered	buffered[3]	unbuffered
7.0	-	-	50	90
			0	50
6.0	17	58	0	100
			0	40
5.5	0	50	-	-
5.3	0	58	-	-
5.0	0	75	0	20
			0	90
4.8	0	42	-	-
4.5	0	67	0	40
			0	90
4.0	0	0	0	0
			0	0
3.5	-	-	0	0
			0	0

[1] Of 12 individual eggs per treatment.
[2] Of 10 eggs per replicate per treatment.
[3] Buffered with 25 millimolar sodium acetate/acetic acid.

Table X *Xenopus laevis* bioassays for interaction of pH and aluminum

	pH	Aluminum Concentration (ppm)					
		0.15	0.20	0.40	0.65	0.90	1.15
a) Brownwater oligotrophic	3.0	0(-)	0(-)	0(-)	0(-)	0(-)	0(-)
	3.5	0(-)	0(-)	0(-)	0(-)	0(-)	0(-)
	4.0	0(-)	0(-)	0(-)	0(-)	0(-)	0(-)
	4.5	88(100)[1]	100(38)	100(0)	100(0)	100(100)	100(100)
	5.0	100(0)	100(0)	100(0)	100(0)	90(100)	100(100)
	6.0	100(0)	100(0)	100(0)	100(0)	100(50)	100(40)
		0.05	0.10	0.30	0.55	0.80	1.05
b) clearwater oligotrophic	3.0	0(-)	0(-)	0(-)	0(-)	0(-)	0(-)
	3.5	0(-)	0(-)	0(-)	0(-)	0(-)	0(-)
	4.0	0(-)	0(-)	0(-)	0(-)	0(-)	0(-)
	4.5	100(0)	80(0)	86(0)	100(100)	100(100)	100(100)
	5.0	100(0)	100(0)	100(20)	80(100)	33(100)	20(100)
	6.0	100(0)	100(0)	100(14)	100(0)	100(0)	100(0)

[1] % hatching at 21°C (of 10 eggs, 70% proving to be fertilized and viable) followed by % dead of hatched larvae at end of experiment, in parentheses.

Table XI *Xenopus laevis* bioassay for interaction of pH and calcium

	pH	Calcium Concentration (ppm) 0	20	40	60	80
a) Brownwater oligotrophic	3.0	0(-)	0(-)	0(-)	0(-)	0(-)
	3.5	0(-)	0(-)	0(-)	0(-)	0(-)
	4.0	0(-)	0(-)	0(-)	9(100)	40(100)
	4.5	0(-)	40(100)	50(0)	91(0)	55(0)
	5.0	55(100)[1]	55(100)	80(0)	90(0)	60(0)
b) clearwater oligotrophic	3.0	0(-)	0(-)	0(-)	0(-)	0(-)
	3.5	0(-)	0(-)	0(-)	0(-)	0(-)
	4.0	0(-)	0(-)	0(-)	75(100)	78(100)
	4.5	10(0)	100(0)	90(0)	80(0)	90(0)
	5.0	80(0)	80(0)	70(0)	90(0)	90(0)

[1] Conditions as in footnote 1, Table X.

at 0 ppm calcium, but this was ameliorated at higher calcium concentrations. Thus, calcium clearly ameliorated pH toxicity in this species, but this only occurred at relatively high calcium concentrations.

Discussion

Acidity

All organisms have a physiological tolerance range for environmental variables, outside which they cannot survive. This is true for amphibians with regard to pH. Our bioassays were designed to determine the lower limit of pH tolerance for certain amphibian species, and they focussed mainly on the most susceptible life history stage. No hatching occurred below pH 4.0 (i.e. at pH 3.8), for any Nova Scotian amphibians tested. However, among the five native species assayed, some were more sensitive to moderate pH than others, as discussed below.

Ambystoma maculatum suffered a drastic reduction in hatching success at pH $\leq$4.5 (Table II). Similar results have been reported for both laboratory (Pough and Wilson 1977) and field studies (Pough 1976).

Hatching success of *Hyla crucifer* was not impaired at pH $\geq$4.5, although subsequent survival was reduced even at pH 5.0 (Table III), and some larvae were deformed. Gosner and Black (1957) reported the only comparable data for this species. They found that pH 4.2 allowed only 50% hatching, and that pH 3.8 was lethal.

Hatching of *Rana sylvatica* was relatively unaffected at pH $\geq$4.5 (Table IV). At pH 4.0 hatching success was greatly reduced, and developmental abnormalities occurred. At pH 3.5 embryonic development was arrested. Both Gosner and Black (1957) and Pierce *et al.* (1984) reported similar findings for this species; they found 50% hatching at pH 3.9 and 3.75, respectively, and no development at pH 3.5. In contrast, Tome and Pough (1982) reported 90% hatching at pH 4.0 and >50% at pH 3.5.

A drastic reduction in hatching success of *Rana palustris* occurred at pH <5.0 (Table V). The only other study on this species (Gosner and Black 1957) found pH 4.3 to be the "minimum limiting pH value", where >50% hatching occurred. Their data indicate a greater tolerance of low pH by this species, than do our observations. Gosner and Black (1957) worked with animals collected from or near acidic bog waters in New Jersey. Animals from that area may have evolved acid tolerance, but if not they at least represent a different population from animals collected in Nova Scotia. Other researchers have hypothesized that local populations of amphibians

may have evolved tolerance to low pH (Gosner and Black 1957; Tome and Pough 1982; Cook 1983), but to date no experimental evidence exists to support this.

Bufo americanus may be the most tolerant of the Nova Scotian species assayed. Hatching success was 100% at pH ≥4.0 (Table VI). However, at pH 3.8 there was no hatching. There are no comparitive data in the literature for this species.

Xenopus laevis had variable hatching success at pH ≥4.5, ranging from 0 to 100% at pH ≥4.5 and from 50 to 100% at pH 5.0, depending on the assay (Tables IX-XI). However, no hatching occurred at pH ≤4.0. These results agree with those of Saber and Dunson (1978) and Dunson and Connell (1982), where pH 3.9 was lethal. However, Tome and Pough (1982) found no significant differences in hatching success from pH 4.0 to 9.0 (60-70% hatching success), and even at pH 3.5 they had 30-40% hatching. Their results indicate greater tolerance of low pH, than shown by other studies.

In summary, amphibian species vary in susceptibility to acidity in laboratory assays. Some such as *B. americanus*, tolerate relatively extreme conditions. Others such as *R. palustris*, are less tolerant.

Aluminum

In no aluminum bioassays were there discernable effects of aluminum; hatching seemed to only be affected by acidity of the water. However, in the *X. laevis* assay, aluminum did have an effect on deformities and post-hatching mortality (Table X). One other study has investigated effects of aluminum on amphibian reproduction. Clark and Hall (unpublished) found that hatching successes in the field of *B. americanus, R. sylvatica,* and *A. maculatum* were highly negatively correlated with total Al, inorganic monomeric Al, total AlF complexes, $Al^{3}\varnothing$, and acidity.

Calcium

Addition of calcium to the assay medium, in an attempt to ameliorate effect of pH, had variable results, depending on the amphibian species being assayed. Acid tolerance of *B. americanus* was unaffected by calcium. *A. maculatum* exhibited a weak positive effect, while *R. sylvatica* suffered reduced hatching with the addition of calcium. *X. laevis* was the only species which clearly benefitted from calcium. The addition of 60 to 80 ppm calcium at pH 4.0 allowed hatching at this pH, which was otherwise lethal (Table XI). Freda and Dunson (1984) partially elucidated the reason for calcium's effect. Acute exposure of amphibian larvae to low pH (2.5 to 4.0) was found to depress sodium influx while markedly accelerating its efflux. The resulting net loss of 50% of body sodium was fatal. They found that an increase in external calcium concentration (from 0 to 200 ppm) extended survival time by slowing loss of sodium. However, since only four sites sampled in our parallel field studies (Dale *et al.* 1985) had calcium concentrations of >60 ppm (and these were all high-pH sites), the positive effect of calcium on survival of larvae in acidic situations is probably not an important factor for Nova Scotia amphibians.

Brownwater versus clearwater oligotrophic

In no bioassays were there clear differences between clear and brown oligotrophic water. In one assay that tested aluminum against the two water types using *X. laevis*, there was also no effect. Of interest in this latter experiment was the possibility that aluminum would be less toxic in the brown water due to chelation by organic compounds (Baker and Schofield 1982). A decreased toxicity was not observed.

Other researchers have investigated direct effects of brown water on amphibian reproductive success, in both the field and laboratory (Gosner and Black 1957; Saber and Dunson 1978; Hagstrom 1980). These studies have revealed that brown waters are toxic in themselves to developing amphibians. However, cause of the toxicity is not

clear; low pH of these waters, toxic organic compounds such as fulvic acids (Saber and Dunson 1978), or metals may be responsible.

Buffers

We initially used buffered assay solutions to eliminate pH drift during exposure periods, following the procedure of Schlichter (1981). However, it is obvious that the sodium acetate/acetic acid buffer system is toxic to *X. laevis* (Table IX), and it should not be used in pH bioassays with this or other amphibian species. This acetate-toxicity effect probably explains toxic effects observed by Schlichter (1981) at relatively high pH, in acetate-buffered solutions using *Rana pipiens*.

Deformities

For all assays starting with early stage embryos, deformities resulting from low pH were similar to those described elsewhere for similar species (Gosner and Black 1957; Pough 1976; Pough and Wilson 1977; Dunson and Connell 1982). The most obvious deformation was curvature of the spine, either scoliosis or kyphosis, possibly caused by mechanical constriction of the embryo within the vitelline membrane. The causal mechanism for this constriction has been discussed by other researchers; it appears to involve decreased ability to uptake water, effectively preventing formation of the perivitelline space. It is also possible that hatching is prevented by inactivation of hatching enzymes, which are pH sensitive (Gosner and Black 1957; Dunson and Connell 1982). In many cases curvature that we observed carried over to the larval stage, resulting in an inability to swim properly, which would lead to an obvious and drastic decrease in fitness. Ossification of vertebrae in such a situation, resulting in fusion of neighboring vertebrae, would permanently handicap the organism. Unfortunately, larvae with this defect were not followed through metamorphosis, and it is not known how (or if) this defect would manifest itself in adults.

Temperature

Although the effect of incubation temperature on pH tolerance of developing embryos (*A. maculatum* and *R. sylvatica*; Tables II and IV) was assayed, the results were not conclusive. Other researchers have addressed this question more directly (Pough and Wilson 1977; Tome and Pough 1982). It does appear that very low water temperature in combination with low water pH reduces survival of amphibians. Both *R. clamitans* larvae and *N. viridescens* adults experienced increased mortality at 5°C compared to 21°C (Tables VII and VIII). This increased sensitivity to pH at low temperature, may indicate that larvae or adults which overwinter in water or are present during spring thaw may be at greater risk than species which do not follow this strategy, although it is not known what temperature and pH range this latter group (i.e. terrestrial hibernators) experiences.

Implications of Acidification

In some regions receiving acidic deposition, the pH of small, ephemeral water bodies can be similar to that of local precipitation (Pough and Wilson 1977). Unlike larger, more permanent bodies of water, these small ponds or pools can be created quickly by accumulation of rain or snowmelt. Frequently, these pools have little or no capacity to buffer or dilute incoming acid: i) owing to their small catchments, runoff is not in contact with soil long or intimately enough to significantly raise pH; ii) in early spring, deciduous foliage has not yet been produced, and rainwater acidity is not decreased by contact with leaves or stems (Prager and Freedman 1984); and iii) the ground may still be frozen, preventing runoff from percolating through soils and contacting neutralizing agents. Also, snow packs which have accumulated through-

out the winter, tend to concentrate acidic substances near surfaces of snow crystals, so that initial meltwater is relatively concentrated with acid (Arland *et al.* 1980). Finally, it is early spring rains, or warm periods when spring thaw begins, that cue early spring breeders to enter ponds. Therefore, the most susceptible stages of many amphibian life histories (early embryonic and larval development), coincide with the most acidic breeding site conditions.

The implication of the preceeding discussion is that amphibians may suffer reproductive failure if breeding habitats become too acidic. The disappearance of amphibian populations could have detrimental effects on trophic relations in the ecosystem involved. Studies of hardwood forests at Hubbard Brook in New Hampshire revealed that salamanders were the largest and highest quality vertebrate food resource available to tertiary consumers such as birds, reptiles, or mammals (Burton and Likens 1975a, 1975b), some of which are known to prey heavily upon amphibians at least occasionally (Debenedictis 1974; Arnold and Wassersug 1978; Cecil and Just 1979; Racey and Euler 1983). In addition, amphibians appear at breeding sites in spring, coinciding with a period of low food availability for some vertebrate predators (Gerrell 1969). Amphibians are also important as top predators or primary consumers in fishless forest ponds (Dodson and Dodson 1971; Orser and Shure 1972; Seale 1980).

Relation to Field Observations

For all the native amphibian species that we assayed, the pH range of 4-5 is critical; within this range developing embryos suffer reduced hatching success, and below it hatching does not occur. It may be important then, that the mean annual pH of rain falling in Nova Scotia over 1977-1980 was 4.5-4.6 (Underwood 1982; Anonymous 1983). Under this loading, typical clearwater oligotrophic waterbodies in Nova Scotia currently have pH >5.0, while typical coloured humic waters have pH of *ca.* 4.5-5.0 due to the presence of naturally acidifying organic acids (Kerekes *et al.* 1982). Thus, for coloured waters particularly, the possibility exists that some disruption of amphibian breeding may occur, and this could increase if more widespread or more intense acidification of otherwise suitable habitats were to occur in the future.

We should note, however, that some of our field observations (Dale *et al.* 1984) indicate a somewhat greater tolerance of acidity by some species, than might be expected from some of our laboratory bioassays. For example, we observed successful hatching of *A. maculatum* and *R. sylvatica* in the field at a pH of 4.1, and *R. clamitans* larvae were collected in an extremely acidic (but eutrophic) lake with a mean pH of 4.0 (minimum pH of 3.8), and from an acidic ditch with a pH of 3.9 (Dale *et al.* 1984; Kerekes *et al.* 1984). These field observations indicate a rather high degree of acid tolerance in these species.

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