

The work from which this
copy was made
included the following
copyright notice:

Copyright 19⁹²

The Zoological Society
of London

OKS Document Delivery

Journal Title: Journal of zoology (1987)

Volume: 228

Issue:

Month/Year:

1992

Pages: 557-570

Article Author: BEATTIE, RC

Article Title: THE EFFECTS OF PH, ALUMINUM
CONCENTRATION AND TEMPERATURE ON
THE EMBRYONIC-DEVELOPMENT OF THE
EUROPEAN COMMON FROG, RANA-
TEMPORARIA

circulating)

Call #: 590.6 Z863p

Location: Main Library Periodicals (Nor

**CUSTOMER HAS REQUESTED
E-Mail**

Angie Reisch
24251 CR 260
Morrison, OK 73061

ILLiad TN: 731395



The effects of pH, aluminium concentration and temperature on the embryonic development of the European common frog, *Rana temporaria*

R. C. BEATTIE¹, R. TYLER-JONES^{1*} AND M. J. BAXTER²

¹Department of Life Sciences, Nottingham Polytechnic, Clifton Lane, Nottingham NG11 8NS
and ²Department of Mathematics, Statistics and Operational Research, Nottingham Polytechnic,
Clifton Lane, Nottingham NG11 8NS

(Accepted 12 November 1991)

(With 1 figure in the text)

The individual and combined effects of pH, aluminium concentration and temperature, on the development of common frog (*Rana temporaria*) embryos from an upland area of northern England, were investigated in a controlled laboratory study. There was strong evidence to suggest that embryonic survival was lower at pH 4.5 compared with pH 6.0. At pH 4.5, embryonic survival was reduced at the highest aluminium concentrations. There was no strong evidence for a reduction in embryonic survival at lower aluminium concentrations.

Gastrulation and hatching appeared to be the most sensitive stages to both pH and aluminium concentration.

There was strong evidence to suggest that temperature-shocked embryos had reduced survival when compared with those kept at a constant temperature, particularly at pH 4.5.

There was substantial variation in the survival of embryos from different clutches in the same pond with respect to low pH, aluminium concentration and temperatures. Thus *R. temporaria* has a wide genetic base from which to tolerate environmental changes. It is suggested, however, that the lethal and sublethal effects of low pH, high aluminium concentration and low temperature, could lead to a decrease in recruitment to the adult populations of *R. temporaria* in upland, northern England.

Contents

| | Page |
|-------------------------------|------|
| Introduction | 557 |
| Materials and methods | 558 |
| Results | 562 |
| Discussion | 565 |
| References | 567 |
| Appendices | 569 |

Introduction

As a result of their aquatic embryonic and larval development, many species of amphibians are potentially affected by acid deposition. Although substantial interspecific variation in acid tolerance exists, the embryos of most amphibian species appear to be fairly acid tolerant with severe reductions in survival occurring only below pH 4.5 (Gosner & Black, 1957; Pough & Wilson, 1977; Punzo, 1983; Clark & LaZerte, 1985, 1987). However, acidified waters are often

* Present address: Cripps Computing Centre, University of Nottingham, Nottingham, NG7 2RD

associated with elevated aluminium concentrations and even moderate concentrations of aluminium can reduce embryo survival and hatching success (Clark & Hall, 1985; Clark & LaZerte, 1985, 1987).

In the last few decades *Rana temporaria* populations in the UK appear to have diminished (Simms, 1969; Cooke, 1972; Prestt, Cooke & Corbett, 1974; Beebee, 1983) and it has recently been suggested that the decline in numbers in some upland areas may be due, in part, to the effects of acidification (Fry & Cooke, 1984). *Rana temporaria* in upland areas of the UK breed in acidic ponds with high levels of dissolved aluminium (Cummins, 1986; Aston, Beattie & Milner, 1987). The pH and aluminium concentrations found in these ponds have been shown, under laboratory conditions, to increase embryo mortality and to depress the growth of both embryos and larvae (Cummins, 1986; Tyler-Jones, Beattie & Aston, 1989).

In addition to being more susceptible to acidification, upland areas also tend to be colder and subject to greater climatic variation than regions of lower altitude. Although frog populations in upland areas breed later in the year than lowland populations, mean pond temperatures tend to be lower during the period of embryonic development (Beattie, 1987). The temperature in upland ponds can be near freezing for long periods (Beattie, 1987; Beattie, Aston & Milner, 1991) and these low temperatures often result in increased egg mortality (Beattie, 1987).

Thus the survival of *R. temporaria* embryos in upland areas may be adversely affected by both low temperatures and the effects of acidification. A study in an upland area of northern England found that embryonic mortality and the incidence of embryonic abnormalities were correlated with low pond temperatures and high inorganic monomeric aluminium concentrations, respectively (Beattie, Aston & Milner, 1991).

In this study, the effects of water pH, aluminium concentration and temperature on the development of *Rana temporaria* embryos were investigated under controlled conditions, in an attempt to resolve their individual and combined effects. As severely low temperatures are unlikely to occur throughout the entire period of embryonic development in upland ponds (Beattie, 1987), the effect of low temperature was assessed by lowering temperature only during gastrulation, the most sensitive stage of development (Grainger, 1959).

Materials and methods

Apparatus

The constant flow apparatus used was essentially that described by Sadler & Lynam (1986) but included some minor modifications (Fig. 1). Deionized water from the header tank and salt solutions from reservoirs were pumped to seven 15 litre plastic containers (experimental chambers) each suspended in a tank and each containing water of different pH and aluminium concentration. Water pH in each chamber was maintained at a preset value by titration with acid or alkali as appropriate. The water in the chambers was mixed by aeration and overflowed into the tanks. From the tanks the water drained into a waste collection tank and was returned, via a filter and deionizer, to the header tank. The flow rate of water into each chamber was approximately 100 ml min^{-1} giving a replacement time of about 15 h (Sprague, 1969).

The water temperature in the tanks was maintained at 10°C (range $9\text{--}11^\circ\text{C}$). Lighting was provided by artificial lights on a 12 h light; 12 h dark regime.

Water quality

Water pH in the experimental chambers was maintained at either pH 6.0 or pH 4.5 by the controlled addition of $0.05 \text{ mol}\cdot\text{l}^{-1}$ KOH or $0.05 \text{ mol}\cdot\text{l}^{-1}$ H_2SO_4 , respectively.

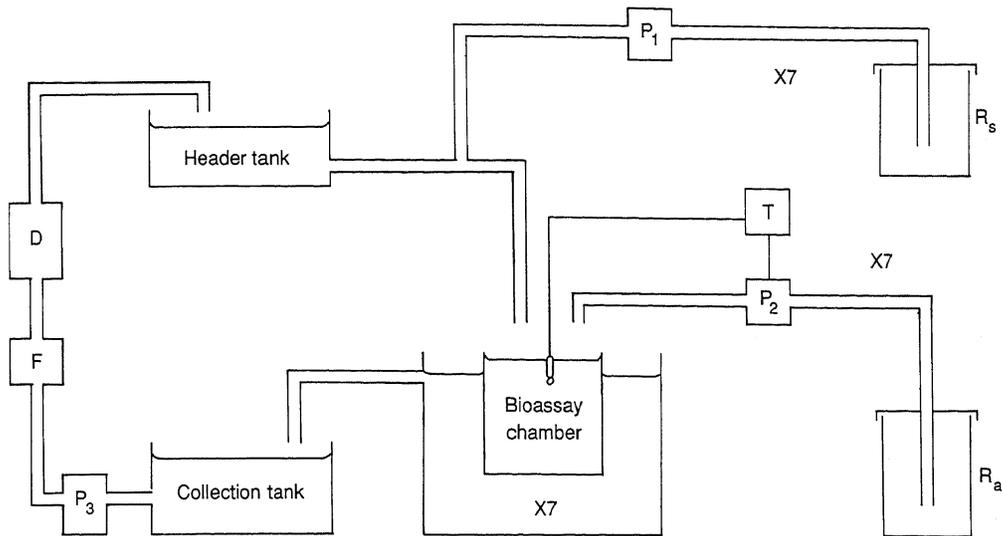


FIG. 1. Diagram of apparatus used for bioassays in this study. For further details see text and Sadler & Lynam (1986). Labelled items are: P_1 and P_2 , peristaltic pumps, P_3 , main circulation pump, T, titrator unit and pH meter, F, filter, D, twin bed deionizer, R_s , salt solution reservoir and R_a , acid solution reservoir. As indicated, but not shown, the apparatus included seven bioassay chambers, each with associated peristaltic pumps and acid and salt solution reservoirs.

The nominal concentrations of the cations, Na^+ , K^+ , Mg^{2+} and Ca^{2+} in the chambers were the same as those used by Tyler-Jones, Beattie & Aston (1989). Nominal aluminium chloride (added as $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$) concentrations were 0 and $3.71 \mu\text{mol} \cdot \text{l}^{-1}$ at both pH 6.0 and pH 4.5 and, at pH 4.5 only, 7.41, 11.12 and $14.83 \mu\text{mol} \cdot \text{l}^{-1}$ (0, 100, 200, 300 and $400 \mu\text{g} \cdot \text{l}^{-1}$ Al, respectively).

The concentrations of total acid-soluble and inorganic monomeric aluminium in each chamber were measured regularly following fractionation and analysis by the catechol violet method of Dougan & Wilson (1974) as modified by Lynam & Sadler (1987). The concentrations of Na, K, Mg and Ca were determined with an inductivity couple plasma atomic emission spectrometer (Bausch & Lomb, series no. 34000).

Biological material

Newly laid clutches of *Rana temporaria* eggs were collected from a pond (longitude $2^\circ 18' 11''$ W, latitude $54^\circ 48' 2''$ N, altitude 556 m) in an upland area of northern England. Water pH in different parts of the pond, measured with a Radiometer pHM80 portable pH meter, varied from pH 4.25 to pH 4.88. Water temperature and conductivity near the spawn were 8°C and $50 \mu\text{S} \cdot \text{cm}^{-1}$, respectively. A water sample was taken for the measurement of the major cations and total acid-soluble and inorganic monomeric aluminium. The concentrations were determined as $3.97 \mu\text{mol} \cdot \text{l}^{-1}$ total acid-soluble Al, $2.15 \mu\text{mol} \cdot \text{l}^{-1}$ inorganic monomeric Al, $0.148 \text{mmol} \cdot \text{l}^{-1}$ Na, $0.011 \text{mmol} \cdot \text{l}^{-1}$ K, $0.021 \text{mmol} \cdot \text{l}^{-1}$ Mg and $0.035 \text{mmol} \cdot \text{l}^{-1}$ Ca. Newly laid egg clumps were selected so that, at the time of collection, eggs were between stages 1 and 3 (1–2 cell stages) (Gosner, 1960). The eggs were transported to Nottingham in pond water held in an insulated box to minimize increases in temperature.

Experimental protocol

The eggs in each clutch were examined and 5 clutches were chosen in which the eggs were still at the 2–4 cell

stages (stages 3–4, Gosner, 1960). From each clutch, 280 fertilized eggs were separated out and divided into 14 groups of 20 eggs. Each group of 20 eggs was placed into a plastic pot (approximately 166 cm³), the sides of which were perforated and which had open ends covered by nylon mesh to allow the exchange of water. Two pots containing eggs from each clutch were placed into each of the 7 experimental chambers. Thus each chamber held 10 separate groups of eggs, 2 groups from each of the 5 clutches.

Initially, the eggs were examined regularly without removing them from their pots. This ensured a minimum of disturbance to the eggs to avoid the possibility of mechanical damage. Eggs were staged according to Gosner (1960). When all the eggs had reached stages 8–9 (mid-to-late cleavage), 5 pots, one with eggs from each clutch, were removed from each tank and transferred to 5 litre plastic containers holding water of a quality similar to that in which they had previously been held. These were then transferred to an incubator at approximately 2°C (range 0–5°C). When the eggs had reached stages 11–12 (mid-to-late gastrula), the containers were removed from the incubator and the water allowed to warm to 10°C before the pots were returned to the experimental chambers.

The experiment was terminated when all surviving embryos had reached stage 20 (gill circulation) and when all those which were judged likely to hatch had hatched. Therefore, at the termination of the experiment, those embryos which had been kept at a constant 10°C had reached a much later stage of development than those embryos which had been subjected to a lower temperature during gastrulation. The eggs and hatched larvae were all preserved in 70% ethanol for later examination. The number of hatched, morphologically normal larvae were noted and were classified as surviving embryos. Those which had not hatched to normal, free-swimming larvae were placed into one of 3 categories: larvae which had hatched but had deformities of the spine or tail resulting from a failure to retract the yolk plug completely; hatched larvae with other deformities (e.g. vesicles or growths, bent tails); and embryos which had either died within the egg or had failed to hatch and remained constricted in the perivitelline membrane.

Survival of deformed larvae

To investigate the ability of larvae with deformities resulting from incomplete yolk plug retraction to survive, surplus eggs from the clumps used in the previous experiment were divided into 2 groups and were kept in either circumneutral (pH 6.0) water without aluminium or water at pH 4.5 with 14.83 $\mu\text{mol} \cdot \text{l}^{-1}$ total acid-soluble aluminium. After hatching, 40 normal larvae from the controls and 20 larvae with deformities of the spine and tail from the low pH, high [Al] water were selected. Twenty normal larvae and 2 groups, each of 10 normal and 10 deformed larvae, were placed into separate tanks (22 cm \times 30 cm \times 21 cm) containing aerated, dechlorinated tap water at 10°C. The larvae were fed on fish pellets every 2–3 days and the water was changed once a week. The number of dead and surviving larvae in each category (normal and deformed) was noted regularly in each tank. All dead larvae were removed soon after death was confirmed.

Statistical analyses

The effect of different variables on the survival rate of frog embryos was investigated by fitting logistic regression models using maximum likelihood estimation via the statistical package GLIM. The relevant statistical theory is given in Dobson (1990) and Cox & Snell (1989) with additional material on the application of GLIM in Healy (1988) and Aitkin *et al.* (1989).

In the terminology of Cox & Snell (1989) the groups of 20 eggs were different levels of a factor corresponding to different experimental units; aluminium concentration, temperature and pH correspond to treatments. The prime focus of research interest was on treatment effects and their interactions.

It was assumed that, for a fixed combination of factor levels, the observed proportion (p) of embryos to survive within a group of 20 eggs could be modelled using the binomial distribution with θ , the constant probability of the survival of an embryo. This requires the assumption that the survival of an embryo in a group of 20 eggs is independent of the survival of the other embryos within the group. This assumption is

questionable for at least some of the experiments and is discussed in more detail after the formal description of the methodology that follows.

For a fixed pH level, the experimental designs were in the form of unreplicated factorial designs ($5 \times 5 \times 2$ for pH=4.5 and $5 \times 2 \times 2$ for pH=6.0) (Cox & Snell, 1989). A logistic regression model, with main effects only, then has the form:

$$\ln [\theta/(1-\theta)] = \mu + \alpha_i + \beta_j + \gamma_k \quad (1)$$

where α_i , β_j and γ_k model the effects of levels i , j and k of the 'animal', 'aluminium' and 'temperature' factors. The right-hand side of equation (1) is analogous to models used in linear modelling and, in a similar way, interaction terms of the form $(\alpha\beta)_{ij}$ can be added.

To investigate the effects of pH, all experiments at the lowest 2 aluminium concentrations were used. This constituted a ($5 \times 2 \times 2 \times 2$) factorial design and required an extra term, such as δ_i , in the main effects model (1).

In fitting the models, terms were added sequentially with main effects first; then first-order interactions, second-order interactions, and so on. 'Analysis of deviance' tables (analogous to the 'analysis of variance table' in linear models) were then constructed in which the difference in successive fits allowed the significance of the added terms to be assessed via a log-likelihood ratio statistic, having (asymptotically) a χ^2 distribution, under the null hypothesis of no significant effect.

If the first-order interactions were found to be significant, then the contribution of the different components $(\alpha\beta)_{ij}$, etc. could be similarly assessed. Unlike linear models, the contribution to the analysis of deviance depends on the order of fitting, so it should be checked that conclusions are robust with respect to this order.

Models that require high-order interaction terms to 'explain' the data can be difficult to interpret. It is often more useful to seek a parsimonious model that provides a good description of (most of) the data and which can be readily interpreted. In the present context, this involved fitting main effect models only, followed by the use of standard procedures (Cox & Snell, 1989; Aitkin *et al.*, 1989) to identify unusual observations that were outlying or influential in some sense. The effect of omitting such observations from the analysis was then investigated. Inspection of Table II makes it clear that there are quite strong patterns in the data disrupted by some rather unusual results. These include the results at aluminium concentration $7.41 \mu\text{mol}\cdot\text{l}^{-1}$ and the 3 results (Al concentration = $0 \mu\text{mol}\cdot\text{l}^{-1}$; pH = 4.5; temperature = constant; animals 2 and 3) and (Al concentration $3.71 \mu\text{mol}\cdot\text{l}^{-1}$; pH 6.0; temperature = lowered; animal 3). These obviously unusual results were also identified within the statistical analysis using methods for the identification of outliers and influential data. The survival rate was clearly much lower than patterns elsewhere in the table led us to expect. In this case, several possibilities arose:

- these 3 values (and Al concentration $7.41 \mu\text{mol}\cdot\text{l}^{-1}$) were those primarily responsible for the departures from a good parsimonious model; if 'genuine' they implied the existence of complex interactions for which a biological explanation was needed;
- these results violated the model assumptions; in particular it could have been the case that survival within these clutches was not independent (because of infection spreading, etc.);
- the observations were genuinely unusual and we were 'unlucky' in their occurrence in the data; they reflect natural variability rather than interaction.

It seemed sensible to analyse the results with and without the offending observations in order to see how conclusions were affected by particular values. Also, because of the clear doubt about the validity of the independence assumption employed, not too much emphasis is placed on the exact levels of significance derived from it. Instead, we have sought parsimonious models that explain a large proportion of the variation in the data; in many of the analyses formally 'significant' interactions are substantively unimportant compared to the main effects. Interpretation has also concentrated on the identification of important treatment effects and interactions with variation between the experimental units or blocks of secondary interest.

Results

Water quality

Data for the nominal and measured water concentrations of total acid-soluble and inorganic monomeric aluminium and Na, K, Mg and Ca for each chamber are given in Table I. The high levels of potassium in chambers with pH 6.0 water is due to the addition of KOH. At pH 6.0 inorganic monomeric aluminium accounted for 30.3% of total aluminium whereas at pH 4.5 inorganic monomeric aluminium was the predominant (87.5% to 91.3%) form. Also included in Table I are data for water pH, and the concentrations of the above cations for water from the containers in which embryos had been kept whilst in the incubator. These samples were taken just prior to the point when the eggs were returned to the experimental chambers.

Development rate

Embryos which had been held at a constant 10°C in water at pH 6.0 with 0 $\mu\text{mol}\cdot\text{l}^{-1}$ Al reached stages 17-18 (tail bud) after 7 days and, except for a small number of mortalities, all had gill circulation and had hatched after 13 days. The embryos which had been held in similar water but

TABLE I

Nominal and measured water quality values in the bioassay chambers. The top row of figures for each chamber gives the nominal values for pH and cation concentrations. The next row gives the measured values for water chemistry expressed as mean \pm S.E. with the number of observations in parentheses. The third row of figures are measured values for water pH and total and monomeric aluminium concentrations from chambers holding spawn after 162 h of cold treatment in an incubator. All concentrations are given in $\mu\text{mol}\cdot\text{l}^{-1}$

| Chamber | pH | Total aluminium | Inorganic monomeric Al | Na | K | Ca | Mg |
|---------|------|----------------------|------------------------|---------|----------|----------|----------|
| 1 | 6.0 | 0 | — | 190 | 23.0 | 38.0 | 25.0 |
| | | 0 (4) | 0 (4) | 213 (1) | 30.2 (1) | 41.4 (1) | 27.6 (1) |
| 2 | 5.90 | 0 | 0 | | | | |
| | 6.0 | 3.71 | — | 190 | 23.0 | 38.0 | 25.0 |
| 3 | 6.66 | 4.78 \pm 0.81 (4) | 0.91 \pm 0.34 (4) | 211 (1) | 66.5 (1) | 39.9 (1) | 26.8 (1) |
| | | 1.85 | 0.56 | | | | |
| 4 | 4.5 | 0 | — | 190 | 23.0 | 38.0 | 25.0 |
| | 4.65 | 0 (3) | 0 (4) | 193 (1) | 19.9 (1) | 37.7 (1) | 25.1 (1) |
| 5 | 4.5 | 0 | 0 | | | | |
| | 4.42 | 3.71 | — | 190 | 23.0 | 38.0 | 25.0 |
| 6 | 4.5 | 3.53 \pm 0.03 (3) | 3.09 \pm 0.15 (4) | 186 (1) | 19.2 (1) | 36.7 (1) | 24.3 (1) |
| | | 2.08 | 1.37 | | | | |
| 7 | 4.5 | 7.41 | — | 190 | 23.0 | 38.0 | 25.0 |
| | 4.40 | 7.77 \pm 0.06 (3) | 7.08 \pm 0.11 (4) | 201 (1) | 22.5 (1) | 54.1 (1) | 9.4 (1) |
| 8 | 4.5 | 4.78 | 4.15 | | | | |
| | 4.15 | 11.12 | — | 190 | 23.0 | 38.0 | 25.0 |
| 9 | 4.5 | 11.37 \pm 0.13 (3) | 10.38 \pm 0.14 (4) | 199 (1) | 22.3 (1) | 37.9 (1) | 26.0 (1) |
| | | 8.23 | 7.86 | | | | |
| 10 | 4.5 | 14.83 | — | 190 | 23.0 | 38.0 | 25.0 |
| | 4.40 | 15.97 \pm 0.31 (5) | 14.34 \pm 0.51 (5) | 205 (1) | 24.3 (1) | 39.7 (1) | 26.7 (1) |
| | | 9.86 | 9.34 | | | | |

TABLE II

Number of *R. temporaria* embryos surviving (out of 20 eggs in all cases except * where $n=19$ and ** where $n=21$) from egg clutches produced by five different females (A-E), when kept in water at either pH 6.0 or pH 4.5 with 0-14.83 $\mu\text{mol}\cdot\text{l}^{-1}$ Al and subjected to two temperature regimes. The two temperature regimes used were a constant 10°C throughout embryonic development and a normal temperature of 10°C but with a reduction to between 0 and 5°C during gastrulation

| Nominal total acid-soluble aluminium ($\mu\text{mol}\cdot\text{l}^{-1}$) | pH level | Clutches | | | | | | | | | |
|--|----------|---------------------------|--------------------------|----|----|-----|-----|-----|----|----|------|
| | | A | | B | | C | | D | | E | |
| | | Constant temperature (CT) | Reduced temperature (RT) | CT | RT | CT | RT | CT | RT | CT | RT |
| 0 | 4.5 | 16 | 15 | 0 | 13 | 4 | 20 | 16 | 10 | 18 | 16** |
| | 6.0 | 20 | 18* | 20 | 20 | 19* | 20 | 19 | 17 | 19 | 17 |
| 3.71 | 4.5 | 20 | 20 | 16 | 6 | 16 | 16 | 18 | 20 | 18 | 11 |
| | 6.0 | 20 | 20 | 20 | 19 | 19* | 10* | 19* | 17 | 16 | 17 |
| 7.41 | 4.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 0 | 0 |
| 11.12 | 4.5 | 18 | 0 | 6 | 0 | 10 | 0 | 6 | 0 | 1 | 0 |
| 14.83 | 4.5 | 5 | 1 | 1 | 0 | 1 | 0 | 5 | 0 | 0 | 0 |

were subjected to reduced (0-5°C) temperature during gastrulation had reached only stage 11 (mid gastrula) by day 7 and stages 17 and 18 after 13 days.

Survival of embryos

The raw data are presented in Table II. Several features of these data that affect, and are identified by, the statistical analysis are noted. These are:

- the absence of survivors for Al=7.41 $\mu\text{mol}\cdot\text{l}^{-1}$, particularly at a constant temperature, which was unexpected given values in the surrounding cells;
- the low survival under control conditions at pH=4.5 for animals B and C was in sharp contrast to all other values at the two lowest aluminium concentrations;
- the unusual value for survival at Al=3.71 $\mu\text{mol}\cdot\text{l}^{-1}$ and lowered temperature for animal C, relative to other values at pH=6.

To incorporate these effects into a statistical model is likely to involve high-order interaction terms; alternatively, as discussed, we may seek a simple model for the data other than these results which require separate discussion.

The poor survival at Al=7.41 $\mu\text{mol}\cdot\text{l}^{-1}$, pH 4.5 at both constant (10°C) and lowered temperatures (0-5°C) was unexpected. In a comparable study using the same species of frog from the same geographical area, the average survival at Al=7.41 $\mu\text{mol}\cdot\text{l}^{-1}$, pH 4.5 and constant temperature (10°C) was 68% (Tyler-Jones, Beattie & Aston, 1989). This suggests that a catastrophic event occurred in the tank holding these 200 eggs at Al concentration 7.41 $\mu\text{mol}\cdot\text{l}^{-1}$,

as there is no obvious biological explanation for this result. The atypical results highlighted in (b) and (c) cannot be explained in terms of known biological theory other than they may well reflect the wide variation in the survival of frog eggs from different parents.

Analysis of deviance tables for a variety of analyses are given in the tables in **Appendix 1**. Tables A1, B1 and C1 are the analyses for the saturated models for the data at pH = 4.5, pH = 6.0; and the two lowest aluminium concentrations, respectively. All the data were used in each case. For A1 and C1 the second-order terms were formally significant at high levels but of limited importance relative to the main effects and first-order terms. For B1 the first-order terms were relatively important in the sense that they accounted for 39% of the total deviance. Here and elsewhere references to statistical 'significance' should be read in the light of the caveat at the end of the previous section.

For pH = 4.5, on omitting the Al = 7.41 $\mu\text{mol}\cdot\text{l}^{-1}$ data, no significant simplification was achieved (Table A2). When a main effects model was fitted to these data and residual analysis applied, the observations identified under (b) were (predictably) clear outliers. Omitting them gives rise to Table A3, for which the highest-order interactions were not significant.

In Table A3, and notwithstanding the statistically significant first-order terms, the main effects accounted for 81% of the total deviance. Full details of the analyses of the interactions are not given here but may be summarized by stating that the animal/aluminium interaction accounted for about 11–12% of the deviance and the aluminium/temperature interaction for about 6%.

For pH = 6.0 the main effects model accounted for 58% of the deviance. The use of diagnostic statistics reveals, as expected, that the result identified in (c) above was highly influential. Its omission resulted in a model in which none of the interactions and none of the main effects was significant at the 5% level. In other words, any attribution of significance was entirely dependent on this single observation. In the model using all the data, none of the treatment interactions was significant.

The analyses to investigate the effects of pH were the least satisfactory from a statistical point of view. Using all the data, only 36% of the deviance was accounted for by a main effects model. The three observations identified in (b) and (c) also emerged from an analysis of residuals/influence. Tables C2 and C3, obtained after omitting the first two and then all three of these results, gave main effects models that accounted for 38% and 49% of the total deviance which is quite low.

Contrasting Table C1 with C2 and C3 showed that the significance of second-order terms in C1 was dependent on the unusual values identified. In the final two models, the treatment interactions (as opposed to interactions involving the experimental units) make almost no contribution to the deviance.

Parameter estimates for selected main effects models are shown in the tables in **Appendix 2** where analyses are identified as in **Appendix 1**. The parameterizations:

$$\alpha_1 = \beta_1 = \gamma_1 = \delta_1 = 0$$

were adopted so that other estimates measured departures from these bases, a negative sign suggesting a worsening of survival chances (and assuming that interactions were relatively unimportant).

For the aluminium concentrations, a natural 'null hypothesis' was that $\beta_i = 0$ for $i = 1, \dots, 5$ with the alternative that $\beta_1 \geq \beta_2 \geq \beta_3 \geq \beta_4 \geq \beta_5$ (assuming that increased Al was detrimental to survival). While the null hypothesis in analysis A1 must clearly be rejected, the estimates were unsatisfactory in the sense that $\hat{\beta}_2 > \hat{\beta}_1$ and $\hat{\beta}_3 > \hat{\beta}_4 > \hat{\beta}_5$. Omitting Al = 7.41 $\mu\text{mol}\cdot\text{l}^{-1}$ did not otherwise markedly

affect the results; omitting additionally the two outliers gave results consistent with $\beta_1 = \beta_2 > \beta_3 > \beta_4$ and resulted in a sharp increase in the estimated temperature effect.

All three models showed a significant temperature effect and were consistent with the higher aluminium concentrations having a markedly adverse effect on survival probabilities. Our preferred model A3 was additionally consistent with there being no significant differences in survival at the lower aluminium concentrations.

The analyses for pH = 4.5 and pH = 6 gave broadly similar estimates, although in the latter case, the significance of all but the animal effects depended on a single observation.

For the analyses C1 and C2 (which used subsets of the A and B analyses and were thus not independent of them) a mixed picture emerges. The differences between the two aluminium concentrations were either marginally significant (as in B1) or non-significant (as in A3) depending on whether or not the two outliers were used. Temperature was significant in the second but not the first of the analyses shown. The estimates for C3 (not shown) were broadly similar to those for C2.

These latter analyses must be surrounded by the caveat that the interaction terms were not negligible. Perhaps the most important point to note is that in all the analyses undertaken, pH was found to be significant, with lower survival at pH = 4.5. (This remained true on allowing for animal/pH interactions that are not reported here.)

The main conclusions from the statistical analysis of these results can be summarized as follows:

- (a) There was strong evidence that a pH of 4.5, as opposed to pH 6.0, reduced the prospect of embryonic survival, regardless of the data treatment.
- (b) Evidence from experiments at pH = 4.5 showed clearly the deleterious effects of aluminium at the highest concentrations. From all the analyses undertaken, there was no strong evidence for a major difference in effect at the lower aluminium concentrations.
- (c) There was strong evidence for a temperature effect, with temperature-shocked embryos having a reduced chance of survival, if we omitted two outliers at pH = 4.5 and included an influential observation at pH = 6. If only pH = 4.5 data were used, some effect was present regardless of the data treatment. While this conclusion makes scientific sense, it did depend on how the available data were used.
- (d) Although there was statistical evidence of interactions in the model, such interactions were either: (1) largely dependent on three unusual values; (2) were numerically unimportant even if statistically significant; or (3) involved experimental unit/treatment interactions. In particular, there was little evidence of any treatment/treatment interaction.

Survival of deformed larvae

Increased aluminium concentrations resulted mainly in grossly deformed embryos, the majority of which failed to hatch. At pH 4.5 with $14.83 \mu\text{mol} \cdot \text{l}^{-1}$ Al, many of the embryos still had yolk plugs at stage 17.

The survival of normal and abnormal (deformities of the spine and tail) larvae was monitored for three weeks past hatching. At the end of that period, of the 40 normal larvae used, 39 were still alive whereas none of the 20 deformed larvae had survived.

Discussion

There was strong evidence to suggest that embryonic survival in *R. temporaria* was lower at pH 4.5, compared with survival at pH 6.0. In a previous study using embryos from the same pond,

kept in similar conditions, no pH effect was found (Tyler-Jones, Beattie & Aston, 1989). This reflects the substantial inter-clump variation which exists within a pond. Clearly, pH 4.5 is a critical level for the survival of *R. temporaria* embryos. Long-term exposure to pH < 4.5 can reduce embryonic survival (Andr n *et al.*, 1988), as can short-term exposure to pH < 4 (Olsson *et al.*, 1987; Cummins, 1989). The survival of embryos of most other amphibian species is also affected by exposure to pH < 4.5 (Gosner & Black, 1957; Pierce, 1985; Freda & Dunson, 1985, 1986). Gastrulation and hatching appear to be particularly sensitive to low pH.

At pH 4.5, embryonic survival decreased at the higher aluminium concentrations. This is in agreement with the results from an earlier study (Tyler-Jones, Beattie & Aston, 1989). Aluminium is also toxic to the embryos of other amphibian species at low pH (Clark & LaZerte, 1985). At a nominal aluminium concentration of $3.71 \mu\text{mol}\cdot\text{l}^{-1}$, only 19% of the aluminium was in the inorganic monomeric form at pH 6.0, but at pH 4.5 the inorganic monomeric form accounted for 88% of total acid-soluble aluminium. Thus the pH-dependency of aluminium toxicity is probably due to changes in aluminium speciation, with inorganic monomeric aluminium, predominant at low pH, presenting the greatest toxicity. As with low pH, gastrulation and hatching seem to be the stages most sensitive to aluminium.

Olsson *et al.* (1987) found no effect of increasing aluminium concentration on the survival of *R. temporaria* embryos, despite using concentrations up to $1600 \mu\text{g}\cdot\text{l}^{-1}$ ($59.3 \mu\text{mol}\cdot\text{l}^{-1}$). Similarly, Andr n *et al.* (1988) were also unable to detect any effect of aluminium on embryonic survival, but did find an increase in the number of larval deformities with both a decrease in pH and increasing aluminium concentration. These deformed larvae included those in which the yolk plug had failed to retract completely during gastrulation. In the present study, larvae with this deformity were included as embryo mortalities as survival after hatching is severely limited compared to normal larvae.

The discrepancies between survival at high aluminium levels in this study and the studies of Olsson *et al.* (1987) and Andr n *et al.* (1988) may also be attributable to differences in water hardness. Calcium levels in the water used by Olsson *et al.* (1987) and Andr n *et al.* (1988) were 8–10 times those used in the present study. High calcium concentrations tend to moderate the toxic effects of low pH in amphibian embryos (Freda & Dunson, 1985; Cummins, 1989) and also of aluminium in amphibian larvae (Gascon, Planas & Moreau, 1987).

There was strong evidence to suggest that temperature-shocked embryos had a reduced survival compared with those kept at a constant temperature, particularly at pH 4.5. Minimum pond temperature has been shown to be a significant factor in the survival of *R. temporaria* embryos in field-based experiments (Beattie, Aston & Milner, 1991).

There was little evidence of interactions between the effects of the three environmental variables. Previously, no interaction was found between the effects of pH and aluminium on the survival of *R. temporaria* embryos kept under comparable conditions (Tyler-Jones, Beattie & Aston, 1989). Similarly, no interaction was found between temperature and pH in *Ambystoma maculatum* (Pough & Wilson, 1977). However, a pH-temperature interaction has been demonstrated for hatching success in one species of salamander, *Ambystoma jeffersonianum* (Pough & Wilson, 1977).

The levels of pH and aluminium used in this study encompass the range of values measured in upland ponds in the northern Pennines (Beattie, 1980; Aston, Beattie & Milner, 1987; Beattie, Aston & Milner, 1991) and Scotland (Cummins, 1986). Water temperatures in upland ponds are invariably low during the frog breeding season; water temperature can be below 5°C for much of the time and temperatures of 0°C are not infrequent (Beattie, 1987; Beattie, Aston & Milner,

1991). Embryonic mortality may be increased in ponds with acid water and high aluminium concentrations. If the water temperature is low during development, particularly during either gastrulation or hatching, embryonic survival may be further decreased.

It is clear from the present study and previous work (Tyler-Jones, Beattie & Aston, 1989; Beattie, Aston & Milner, 1991), that there is substantial variation in the survival of *R. temporaria* embryos from different clutches of eggs in the same pond, with respect to pH, aluminium concentration and temperature. Regional ecotypes also exist in *R. temporaria* (Kozłowska, 1971; Beattie, 1987; Tyler-Jones, Beattie & Aston, 1989). Similarly, intraspecific variation in tolerance to acidity has been shown in other amphibian species (Pierce & Harvey, 1987). Thus *R. temporaria* may well have the capacity to adapt to new environmental conditions.

In conclusion, the direct lethal effects of these three variables, combined with sublethal effects, such as reductions in embryonic and larval growth rates (Cummins, 1986; Tyler-Jones, Beattie & Aston, 1989), and indirect ecological effects, such as changes in habitat structure and food resources (Clark, 1986), could lead to a decrease in recruitment to the adult population. (It is difficult to assess the impact of these effects given our poor understanding of the factors limiting the size of frog populations.) However, the fact that *R. temporaria* embryos appear to have a wide tolerance to environmental change may ameliorate the effects of acidification to some extent.

Financial support for this study was provided by the Central Electricity Generating Board. The authors would also like to thank Mrs S. Lynam (Ratcliffe Technology Centre, Powergen) for the analyses of water Na, K, Ca and Mg concentrations. Dr R. J. Aston (Ratcliffe Technology Centre, Powergen) gave invaluable help and encouragement.

REFERENCES

- Aitkin, M., Anderson, D., Francis, B. & Hinde, J. (1989). *Statistical modelling in GLIM*. Oxford Statistical Science Series No. 4. Oxford Science Publications.
- Andrén, C., Henrikson, L., Olsson, M. & Nilson, G. (1988). Effects of pH and aluminium on embryonic and early larval stages of Swedish brown frogs *Rana arvalis*, *R. temporaria* and *R. dalmatina*. *Holarctic Ecol.* **11**: 127-135.
- Aston, R. J., Beattie, R. C. & Milner, A. G. P. (1987). Characteristics of spawning sites of the common frog (*Rana temporaria*) with particular reference to acidity. *J. Zool., Lond.* **213**: 233-242.
- Beattie, R. C. (1980). A physico-chemical investigation of the jelly capsules surrounding eggs of the Common frog (*Rana temporaria temporaria*). *J. Zool., Lond.* **190**: 1-25.
- Beattie, R. C. (1987). The reproductive biology of Common frog (*Rana temporaria*) populations from different altitudes in northern England. *J. Zool., Lond.* **211**: 387-398.
- Beattie, R. C., Aston, R. J. & Milner, A. G. P. (1991). A field study of fertilization and embryonic development in the common frog, *Rana temporaria*, with particular reference to acidity and temperature. *J. appl. Ecol.* **28**: 346-357.
- Beebee, T. J. C. (1983). Habitat selection by amphibians across an agricultural land-heathland transect in Britain. *Biol. Conserv.* **27**: 111-124.
- Clark, K. L. (1986). Distributions of anuran populations in central Ontario relative to habitat acidity. *Wat. Air Soil Pollut.* **30**: 727-734.
- Clark, K. L. & Hall, R. J. (1985). Effects of elevated hydrogen ion and aluminium concentrations on the survival of amphibian embryos and larvae. *Can. J. Zool.* **63**: 116-123.
- Clark, K. L. & LaZerte, B. D. (1985). A laboratory study of the effects of aluminium and pH on amphibian eggs and tadpoles. *Can. J. Fish. aquat. Sci.* **42**: 1544-1551.
- Clark, K. L. & LaZerte, B. D. (1987). Intraspecific variation in hydrogen ion and aluminium toxicity in *Bufo americanus* and *Ambystoma maculatum*. *Can. J. Fish. aquat. Sci.* **44**: 1622-1628.
- Cooke, A. S. (1972). Indications of recent changes in status in the British Isles of the frog (*Rana temporaria*) and the toad (*Bufo bufo*). *J. Zool., Lond.* **167**: 161-178.
- Cox, D. R. & Snell, E. J. (1989). *Analysis of binary data*. Monographs on Statistics and Applied Probability No. 32. Chapman & Hall.

- Cummins, C. P. (1986). Effects of aluminium and low pH on growth and development in *Rana temporaria* tadpoles. *Oecologia* **69**: 248-252.
- Cummins, C. P. (1989). Effect of calcium on survival times of *Rana temporaria* embryos at low pH. *Funct. Ecol.* **2**: 297-302.
- Dobson, A. J. (1990). *An introduction to generalized linear models*. London: Chapman & Hall.
- Dougan, W. K. & Wilson, A. L. (1974). The absorptiometric determination of aluminium in water. A comparison of some chromogenic reagents and the development of an improved method. *Analyst, Camb.* **99**: 413-430.
- Freda, J. & Dunson, W. A. (1985). The influence of external cation concentration on the hatching of amphibian embryos in water of low pH. *Can. J. Zool.* **63**: 2649-2656.
- Freda, J. & Dunson, W. A. (1986). Effects of low pH and other chemical variables on the local distribution of amphibians. *Copeia* **1986**: 454-466.
- Fry, G. L. A. & Cooke, A. S. (1984). Acid deposition and its implications for nature conservation in Britain. *NCC, Focus on Nature Conservation* No. 7: 1-64.
- Gascon, C., Planas, D. & Moreau, G. (1987). The interaction of pH, calcium and aluminium concentrations on the survival and development of wood frog (*Rana sylvatica*) eggs and tadpoles.
- Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**: 183-190.
- Gosner, K. L. & Black, I. H. (1957). The effects of acidity on the development and hatching of New Jersey frogs. *Ecology* **38**: 256-262.
- Grainger, J. N. R. (1959). The effect of constant and varying temperatures on the developing eggs of *Rana temporaria* L. *Zool. Anz.* **163**: 267-277.
- Healy, M. J. R. (1988). *GLIM, an introduction*. Oxford: Clarendon Press.
- Kozłowska, M. (1971). Differences in the reproductive biology of mountain and lowland Common frogs, *Rana temporaria* L. *Acta biol. cracov.* **14**: 17-32.
- Lynam, S. & Sadler, K. (1987). *Evaluation of a method for the measurement of aluminium in freshwaters permitting the fractionation into monomeric and polymeric forms and organic complexes*. CERL publ. TPRD/L/3082/R86.
- Olsson, M., Hogstrand, C., Dahlberg, A. & Berglind, S.-Å. (1987). Acid-shock, aluminium and presence of *Sphagnum aurantiacum*: effects on embryological development in the common frog, *Rana temporaria* and the moor frog, *Rana arvalis*. *Bull. Envir. Contam. Toxicol.* **39**: 37-44.
- Pierce, B. A. (1985). Acid tolerance in amphibians. *Bioscience* **35**: 239-243.
- Pierce, B. A. & Harvey, J. M. (1987). Geographic variation in acid tolerance of Connecticut wood frogs. *Copeia* **1987**: 94-103.
- Pough, F. H. & Wilson, R. E. (1977). Acid precipitation and reproductive success of *Ambystoma* salamanders. *Wat. Air Soil Pollut.* **7**: 307-316.
- Prestt, I., Cooke, A. S. & Corbett, K. F. (1974). British amphibians and reptiles. In *The changing flora and fauna of Britain* **6**: 229-254. Hawksworth, D. L. (Ed.). London: Academic Press.
- Punzo, F. (1983). Effects of environmental pH and temperature on embryonic survival capacity and metabolic rates in smallmouth salamander, *Ambystoma texanum*. *Bull. envir. Contam. Toxicol.* **31**: 467-473.
- Sadler, K. & Lynam, S. (1986). Some effects of low pH and calcium on the growth and tissue mineral content of yearling brown trout, *Salmo trutta*. *J. Fish Biol.* **29**: 313-324.
- Simms, C. (1969). Indications of the decline of breeding amphibians at an isolated pond in marginal land, 1954-1967. *Br. J. Herpet.* **4**: 93-96.
- Sprague, J. B. (1969). Measurement of pollutant toxicity to fish. I. Bioassay methods for acute toxicity. *Wat. Res.* **3**: 793-821.
- Tyler-Jones, R., Beattie, R. C. & Aston, R. J. (1989). The effects of acid water and aluminium on the embryonic development of the common frog, *Rana temporaria*. *J. Zool., Lond.* **219**: 355-372.

Appendix 1: Analysis of deviance tables

*Summary of the results of the statistical analyses for the survival of **R. temporaria** embryos kept in water at either pH 6.0 or pH 4.5 and with 0–14.83 $\mu\text{mol}\cdot\text{l}^{-1}$ total acid-soluble aluminium. In addition to these water quality treatments, embryos were subjected to either constant temperature (10°C) throughout development until hatching or a similar temperature for most of the development period but with a reduction in temperature during gastrulation*

| | Deviance | Degrees of freedom |
|--|----------|--------------------|
| A1 pH 4.5 data only | | |
| Main effects | 544.6 | 9 |
| 1st-order terms | 205.3 | 24 |
| 2nd-order terms | 36.8 | 16 |
| Total | 786.8 | 49 |
| B1 pH 6.0 data only | | |
| Main effects | 35.7 | 6 |
| 1st-order terms | 23.8 | 9 |
| 2nd-order terms | 2.2 | 4 |
| Total | 61.7 | 19 |
| C1 pH 4.5 and pH 6.0 data | | |
| Main effects | 102.4 | 7 |
| 1st-order terms | 112.6 | 15 |
| 2nd-order terms | 65.7 | 13 |
| 3rd-order terms | 6.9 | 4 |
| Total | 287.7 | 39 |
| A2 pH 4.5 (omitting Al=7.41 $\mu\text{mol}\cdot\text{l}^{-1}$) | | |
| Main effects | 412.0 | 8 |
| 1st-order terms | 161.1 | 19 |
| 2nd-order terms | 36.8 | 12 |
| Total | 609.9 | 39 |
| C2 pH 4.5 and pH 6.0 data (omitting 2 outliers) | | |
| Main effects | 65.7 | 7 |
| 1st-order terms | 85.4 | 15 |
| 2nd-order terms } 3rd-order terms } | 22.3 | 15 |
| Total | 173.4 | 37 |
| A3 pH 4.5 (omitting Al=7.41 $\mu\text{mol}\cdot\text{l}^{-1}$ and 2 outliers) | | |
| Main effects | 469.2 | 8 |
| 1st-order terms | 106.8 | 19 |
| 2nd-order terms | 5.8 | 10 |
| Total | 581.8 | 37 |
| C3 pH 4.5 and pH 6.0 (omitting Al=7.41 $\mu\text{mol}\cdot\text{l}^{-1}$ and 2 outliers) | | |
| Main effects | 78.7 | 7 |
| 1st-order terms | 67.2 | 15 |
| 2nd-order terms } 3rd-order terms } | 14.9 | 14 |
| Total | 160.7 | 36 |

Appendix 2: Parameter estimates for models with main effects only

| | pH=4.5 | | | pH=6.0 | | pH 4.5 & pH 6.0 |
|------------|--------------|--------------|--------------|--------------|--------------|-----------------|
| | A1 | A2 | A3 | B1 | C1 | C2 |
| α_1 | 0 | 0 | 0 | 0 | 0 | 0 |
| α_2 | -2.26 (0.31) | -2.42 (0.33) | -1.94 (0.35) | 0.01 (1.42) | -1.94 (0.38) | -1.27 (0.40) |
| α_3 | -1.17 (0.30) | -1.29 (0.31) | -0.72 (0.34) | -2.40 (1.08) | -1.45 (0.39) | -0.74 (0.43) |
| α_4 | -0.45 (0.29) | -0.88 (0.31) | -1.04 (0.35) | -2.07 (1.09) | -0.97 (0.41) | -0.97 (0.41) |
| α_5 | -1.31 (0.30) | -1.44 (0.31) | -1.70 (0.34) | -2.61 (1.07) | -1.25 (0.39) | -1.25 (0.40) |
| β_1 | 0 | 0 | 0 | 0 | 0 | 0 |
| β_2 | 0.97 (0.25) | 0.98 (0.24) | 0.06 (0.28) | -0.95 (0.43) | -0.45 (0.20) | -0.19 (0.22) |
| β_3 | -4.13 (0.40) | — | — | — | — | — |
| β_4 | -2.20 (0.25) | -2.23 (0.25) | -3.37 (0.32) | — | — | — |
| β_5 | -3.52 (0.33) | -3.58 (0.34) | -4.76 (0.40) | — | — | — |
| γ_1 | 0 | 0 | 0 | 0 | 0 | 0 |
| γ_2 | -0.65 (0.19) | -0.85 (0.20) | -1.70 (0.25) | -1.50 (0.48) | -0.25 (0.20) | -1.02 (0.24) |
| δ_1 | — | — | — | — | 0 | 0 |
| δ_2 | — | — | — | — | 1.67 (0.23) | 1.21 (0.24) |
| μ | 2.01 (0.28) | 2.30 (0.30) | 3.61 (0.39) | 5.94 (1.13) | 2.08 (0.36) | 2.97 (0.40) |

A1 B1 C1 = All data used

A2 = Omitting A1 = 7.41 $\mu\text{mol}\cdot\text{l}^{-1}$ A3 C2 = Omitting A1 = 7.41 $\mu\text{mol}\cdot\text{l}^{-1}$ and 2 outliers