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# Evolutionary implications of acidification: a frog's eye view

BY

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## ABSTRACT

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Understanding the diversity of life is one of the main aims of evolutionary biology, and requires knowledge of the occurrence and causes of adaptive genetic differentiation among geographically distinct populations. Environmental stress caused by acidity may cause strong directional selection in natural populations, but is little explored from an evolutionary perspective. In this thesis, a series of laboratory experiments and field data was used to study evolutionary and ecological responses of amphibians to environmental acidity.

Local adaptation to acid stress was studied in the moor frog (*Rana arvalis*). The results show that acid origin populations have higher acid stress tolerance during the embryonic stages than neutral origin populations, and that acid and neutral origin populations have diverged in embryonic and larval life-histories. The mechanisms underlying adaptive differentiation are partially mediated by maternal effects related to extra-embryonic membranes and egg size. Acid origin females invest in larger eggs and have a stronger egg size-fecundity trade-off than females from neutral areas, likely reflecting adaptive differentiation in maternal investment patterns.

Potential carry-over effects of low pH, and the effects of UV-b/pH interaction were investigated in the common frog (*R. temporaria*). The results suggest that amphibian larvae are able to compensate for the negative effects of acidity experienced early in life, if conditions later turn beneficial. *R. temporaria* populations differed in their sensitivity to synergistic effects of low pH/UV-B, indicating variation in population responses to environmental stress.

In conclusion, these results suggest rapid evolution in response to human induced environmental change, much of which may be mediated via adaptive maternal effects. Acidification may be a powerful selective force shaping life-history evolution.

*Key words:* acid stress, amphibians, local adaptation, maternal effects, maternal investment

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  - IV** Räsänen, K., Söderman, F., Laurila, A. and Merilä, J. Acidification shapes maternal investment in the moor frog: variation in egg size and reproductive trade-offs. *Manuscript*.
  - V** Räsänen, K., Laurila, A. and Merilä, J. 2002. Carry-over effects of embryonic acid conditions on development and growth of *Rana temporaria* tadpoles. *Freshw. Biol.* 47: 19-30.
  - VI** Pahkala, M., Räsänen, K., Laurila, A., Johansson, U., Björn, L.O. and Merilä, J. 2002. Lethal and sublethal effects of UV-B/pH synergism on common frog embryos. *Conserv. Biol.* 16:1063-1073.
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## INTRODUCTION

Understanding the diversity of life and the role that natural selection plays in evolution are some of the major goals of evolutionary biology. As such, much of evolutionary biology aims at understanding the occurrence and causes of adaptive variation among geographically distinct populations (Endler 1986). The evolution of local adaptations requires variation in local conditions causing selection, heritable genetic variation, and limited gene flow (Endler 1986). One important selective factor creating local adaptations is environmental stress (Hoffmann and Parsons 1991, 1997; Linhart and Grant 1996). Anthropogenic environmental changes expose many organisms to novel stressors and are often the underlying cause of rapid evolution (e.g. Reznick and Ghalambor 2001), and have even been suggested to be the major selective force in contemporary evolution (Palumbi 2001). Acidification derived from both natural and anthropogenic sources, exposes various organisms to stressful conditions. This is demonstrated by the negative effects of low pH on survival, growth and development both in terrestrial and aquatic organisms (e.g. Haines 1981; Pierce 1985; Wyman and Hawksley-Lescault 1987; Bradford et al. 1998; Rusek and Marshall 2000; Rowe and Freda 2000). Low pH may cause strong directional selection and because of large spatial variation in pH, local adaptations to acidity could be expected. However, the evolutionary implications of acidification have been little explored (but see: e.g. Pierce 1985; Fischer et al. 2001).

### *Adaptive maternal effects*

Maternal effects may provide an efficient mechanism of adaptation to environmental variation (Mousseau and Fox 1998). They are broadly defined as phenotypic variation in offspring that is a consequence of the mother's phenotype rather than the genetic constitution of the offspring (Roff 1998). Genetic maternal effects may provide a mean for rapid local differentiation due to selection among families and due to epistatic interactions between the maternal and offspring genotypes (Wade 1998). However, also environmental maternal effects may have adaptive value (Lacey 1998). Maternal effects are important, for example, in the determination of offspring size (Mousseau and Fox 1998) and may be modified by environmental conditions (e.g. Gliwicz and Guisande 1992; Parichy and Kaplan 1992; Einum and Fleming 1999; Agrawal et al. 1999; reviewed in Rossiter 1998). It is generally assumed that maternal effects are the main contributors to variation in early life-history traits (Bernardo 1996a,b; Mousseau and Fox 1998). However, the relative importance of additive, non-additive genetic and maternal effects as determinants of geographic variation in early life-history traits has rarely been investigated (but see e.g. Haugen and Vøllestad 2000; Kennington et al. 2001).

Low pH has often very strong negative effects on performance of early life-stages (e.g. see Haines 1981, Pierce 1985, Rowe and Freda 2000 for reviews), and selection acting either through maternal or genetic effects at these stages could be strong in acid

environments. However, although maternal effects have been implicated in embryonic acid tolerance (amphibians: Pierce and Sikand; Pierce and Harvey 1987; fish: Edwards and Gjedrem 1979), next to nothing is known about maternal effects and their adaptive value in acid stress tolerance. One well-known example of maternal effects is the positive correlation between egg size and offspring size in a variety of species (see e.g. Bernardo 1996a; Kaplan 1998 for reviews). The effect of initial size on offspring performance may vary between growth environments (e.g. Gliwicz and Guisande 1992, Parichy and Kaplan 1992, Einum and Fleming 1999; Moran and Emler 2001), but little is known about how initial size effects vary among populations. The effects of initial size are often especially pronounced in stressful environments (e.g. Fox and Mousseau 1996; Moran and Emler 2001), and thereby initial size could also be important for offspring performance in acid environments. However, whether this is the case, and whether the effects of egg size vary between acid and neutral origin populations, has not been investigated.

### *Life-history evolution*

The evolution of life-histories is a central theme in evolutionary biology, and diverse abiotic and biotic environmental factors have been identified as selective factors (Roff 1992, Stearns 1992), some of the most prominent being the effects of climatic variation and predation risk. Trade-offs, arising from e.g. mechanical and physiological constraints, are an important concept in life-history theory (Roff 1992; Stearns 1992), because they set the limits for the optimization of individual fitness. Because of trade-offs between, for instance, size and age at maturity (Roff 1992, 2000) and growth and differentiation rates (Arendt 1997), individuals may need to optimise their performance in environments that are temporarily limited and provide poor conditions for growth and development. Since growth and development rates slow down under acid conditions, spatial variation in pH could cause selection on life-histories of organisms. This could be especially the case in time limited (e.g. seasonal and temporal) environments.

Another major set of trade-offs influencing the evolution of life-histories are those concerning reproduction (Roff 1992, Stearns 1992). To maximise their life-time fitness, females need to decide upon how to allocate resources between current and future reproduction (e.g. when to start reproducing, how much resources to allocate to reproduction versus maintenance and growth) on one hand, and between individual offspring, on the other (Roff 1992). Variation in environmental acidity could contribute to variation in maternal investment patterns through at least two different, but not mutually exclusive, pathways. First, since acid environments are potentially stressful, acidity may affect a female's allocation of resources between growth/maintenance and reproduction. Second, if egg size influences offspring performance differently in acid and neutral environments, and if females are subject to the trade-off between egg size and fecundity (e.g. Smith and Fretwell 1974; Roff 1992), selection acting on egg size and number may have different outcomes in acid and neutral habitats.

*Carry-over effects*

Conditions experienced during early development may have a profound effect on performance during later life (Goater, 1994; Pechenik et al. 1998; Lindström, 1999, Phillips 2002). In addition to maternal effects, also environmental factors experienced by the individual itself at an early stage may carry over to later life stages (Pechenik *et al.*, 1998; Lindström, 1999; Phillips 2002). Through carry-over effects, even sub-lethal, short-term increases in stress could have long-term consequences for the performance and fitness of individuals and may eventually become evident at the population level (Pechenik *et al.*, 1998; Lindström, 1999; Maltby, 1999; Beckerman et al. 2002). However, carry-over effects have been relatively little explored in ecological studies. Because of the temporal acid pulses experienced by many organisms during spring snow melt and rainfall (Haines 1981; Morris et al 1989) carry-over effects of even short-term acidity could influence performance of many organisms even in environments that normally are circumneutral.

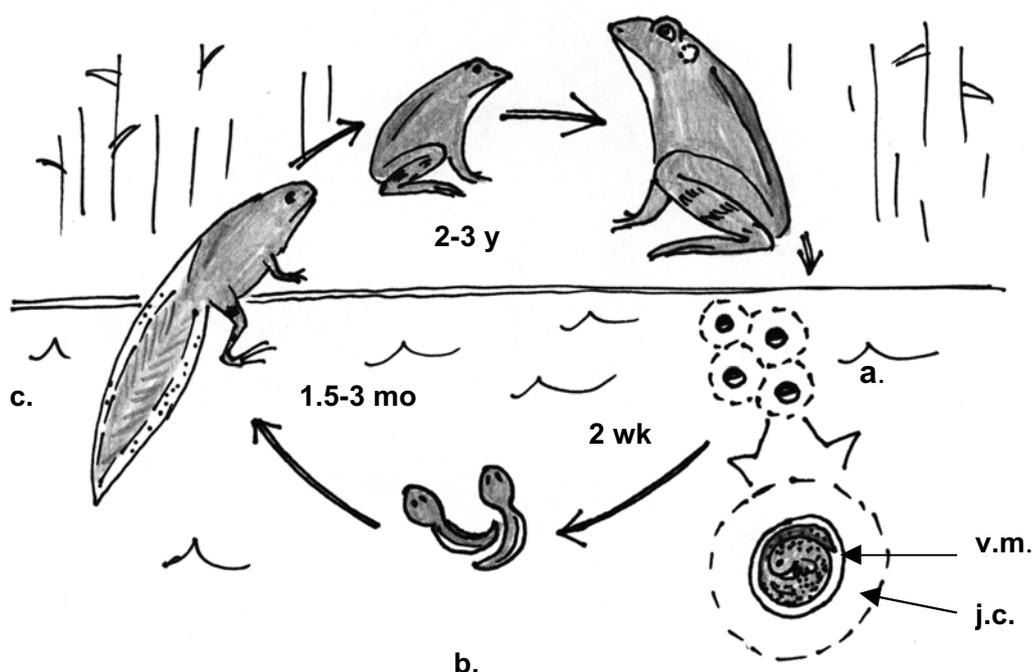
*Interactive effects*

Any statement about a species tolerance to a single stressor is subject to an array of assumptions. For instance, that different populations of the same species do not vary in their sensitivity, and that there are no synergistic effects with other stressors that result in reduced tolerance. However, the levels of stressors and their combinations differ among populations of the same species. Hence, interactive effects could often be important determinants of stress tolerance, and populations subject to different environmental conditions may differ in their tolerance. Several studies show that responses of organisms to acidity depend on a range of biotic (e.g. predation, Jung and Jagoe 1995; intra- and interspecific competition, e.g. Warner et al. 1993, Pehek 1995) and abiotic factors (e.g. aluminium concentration, Freda and McDonald 1990; calcium concentration, Dale et al. 1985; temperature: Dale et al. 1985; Horne and Dunson 1994). One stressor which may covary with acidification, is ultraviolet-B radiation (UV-B; Schindler et al. 1996, Lean 1998). Synergistic effects of UV-B and low pH could be a particular concern in Scandinavia and northern America where large areas have suffered from acidification (Brodin 1993; AMAP 1998). Consequently, studies of combined effects of low pH and UV-B radiation may be important for understanding responses of populations to either of these factors (Long *et al.* 1995; Hatch and Blaustein 2000).

*Amphibian life-cycle and mechanisms of acid toxicity*

Amphibians provide a good model for studies of local adaptation as they experience a wide range of environments and are relatively limited in their movements (Ward et al. 1992; Beebee 1996). The latter implies restricted gene flow and facilitates evolution through adaptive genetic differentiation among populations (Hendry et al. 2001). Furthermore, amphibians are very sensitive to various environmental perturbations, and

likely experience strong selection as indicated by population declines and range reductions in a large number of species world wide (Alford and Richards 1999; Houlihan *et al.* 2000). Spatial variation in pH may be important for local genetic differentiation in amphibian populations as suggested by the large variation in acid tolerance both among (e.g. Gosner and Black 1957, Leuven *et al.* 1986, Andrén *et al.* 1988) and within amphibian species (e.g. Pierce and Harvey 1987; Tyler-Jones *et al.* 1989, Karns 1992). However, most studies of amphibian acid tolerance do not control for environmental variation arising from previous acid exposure and maternal effects, and critical tests of local adaptation to acidity in amphibians have been lacking (but see Pierce and Harvey 1987).



**Figure 1.** Amphibian life-cycle with approximate development time between stages indicated. V.m.=vitelline membrane, j.c.=jelly coat.

Amphibians are a typical example of organisms with complex life-cycles (Wilbur 1980, Moran 1994; Fig. 1) and are thereby confronted with acid stress at different stages and through different mechanisms. In this thesis, two Swedish frog species, the moor frog *Rana arvalis* and the common frog *R. temporaria* were investigated. These species lay their eggs directly in water early in spring, thereby potentially exposing embryos to peak acidity (Pierce 1985; Rowe and Freda 2000). The embryos are surrounded by gelatinous

egg capsules and a vitelline membrane (Fig. 1a), which provide protection from various environmental hazards (Duellmann and Trueb 1994). Under acid conditions, however, these structures may confer to greater mortality as a result of the “curling effect” (Dunson and Connell 1992), whereby embryos may develop normally but fail to hatch. This effect may be due to changes in the physical/chemical composition of the extraembryonic membranes, failure of the hatching enzyme and/or the reduced ability of embryos to move (Dunson and Connell 1992). The embryo is considered to be the most sensitive stage (Pierce 1985, Rowe and Freda 2000) and there is large inter-specific variation in embryonic acid tolerance. Depending on the species, 50 % mortality may occur in the pH range of 3.5 to 5.0 (e.g. Gosner and Black 1957; Leuven et al. 1987; Böhmer and Rahmann 1990).

Following hatching, larvae become free-swimming (Fig. 1b) and are directly exposed to the ionic composition of the surrounding media. At the larval stage the main contributor to acid stress is apparently disrupted sodium balance (Freda and Dunson 1984), which mainly affects growth and development rates, but at extreme levels and long exposure may lead to death. Following metamorphosis (Fig. 1c), *R. arvalis* and *R. temporaria* become largely terrestrial (Fig. 1). Metamorphic size and age are important fitness components in animals with complex life cycles (Wilbur 1980; Moran 1994), since large size at metamorphosis improves juvenile survival and adult fecundity (e.g. Berven 1990; Tejedo 1992; Scott 1994; Taylor et al. 1998). Accordingly, the negative effects of acidity on metamorphic traits may have important ecological ramifications in amphibian populations, and selection could be expected to counteract these by influencing patterns of metamorphosis. There are only few studies investigating acid tolerance of terrestrial stages of amphibians, but these indicate that amphibians may be negatively affected by acidity also at these stages (e.g. Wyman et al. 1995; Vatnick et al. 1999).

## AIMS OF THE THESIS

In this thesis, I studied evolutionary and ecological responses of amphibian populations to acidity in attempt to investigate the following main questions:

1. *Have amphibians adapted to locally prevailing acidity?* I compared the performance of embryos (**I, V**) and larvae (**III, V**) from populations originating from acid and neutral pH environments.
2. *What are the mechanisms underlying local adaptation to acidity?* I investigated the influence of egg size (**I, II**) and extra-embryonic membranes on embryonic survival (**II**), and conducted reciprocal crosses between an acid and a neutral origin population, to study the relative importance of maternal, non-additive genetic and genetic effects in embryonic performance (**II**). In paper **III**, I studied the effects of hatching size on tadpole performance in acid and neutral environments.

3. *How does variation in environmental acidity influence life-histories?* I investigated variation in embryonic (**I, II, V**) and larval (**III**) growth and development rates in acid and neutral origin populations of *R. arvalis* (**I, II, III**) and *R. temporaria* (**V**). I also studied among population variation and covariation in maternal investment (egg size, egg number and total reproductive output) and other maternal traits (growth, age and size) in *R. arvalis* (**IV**).

4. *Do acid conditions experienced early in life influence later performance?* I investigated potential carry-over effects of embryonic acid exposure on larval performance in two *R. temporaria* populations (**V**).

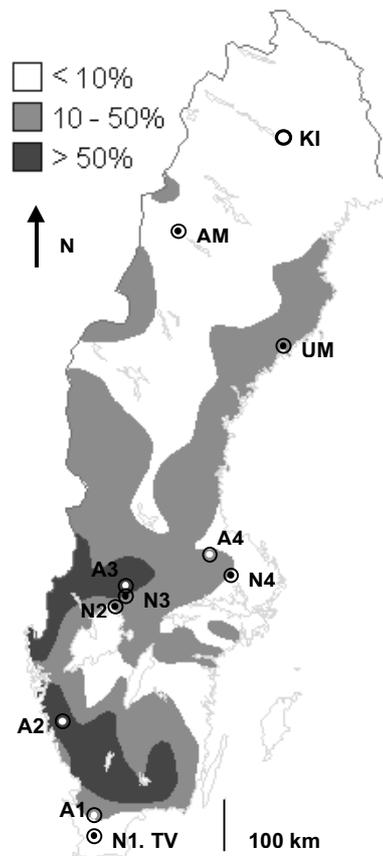
5. *How does acidity interact with other environmental stressors?* I investigated geographic variation in interactive effects of low pH and UV-B radiation on embryos in a southern and a northern *R. temporaria* population (**VI**).

Apart from paper **IV**, which is based on field collected material, all investigations are based on common garden experiments conducted in the laboratory.

## STUDY SPECIES AND POPULATIONS

*R. arvalis* (**I, II, III, IV**) and *R. temporaria* (**V, VI**) belong to the brown frogs and are widely distributed in the western Palearctic (Gasc *et al.* 1997). Both species occur in a wide range of habitats and a relatively broad range of pH levels. *R. temporaria*, however, is more acid sensitive (Leuven *et al.* 1986, Andrén *et al.* 1988), avoids extremely acid sites (e.g. Leuven *et al.* 1986, Aston *et al.* 1987), and breeds at higher altitudes and latitudes (Gasc *et al.* 1997). In both species, females produce one clutch of eggs (*R. arvalis* ca. 500-1500 eggs; **IV**, *R. temporaria* ca. 500-3000 eggs, Fog. *et al.* 1997, A. Laurila, pers. comm) per year.

In Sweden, anthropogenic acidification has resulted in low pH in the terrestrial and freshwater habitats in large parts of the country since the onset of industrialisation, and especially since the 1960s' (Fig. 2, Brodin 1993, Renberg *et al.* 1993). The acid (A) and neutral (N) populations of *R. arvalis* (A1: Järnhatten, A2: Tottatjärn, A3: Lilla Brödhällartjärn, A4: Södefors; N1: Tvedöra, N2: Lindrågen, N3: Norenberg and N4: Häggedal) used in this study originated from southern and central Sweden from a mean pH range of 4.2 to 7.5 (**I-IV**, Fig. 2). The study populations of *R. temporaria* (TV: Tvedöra, UM: Umeå, AM: Ammarnäs, and KI: Kiruna) originated from southern and northern Sweden (**V, VI**, Fig. 2) and from a pH range of 4.8 to 7.0.



**Figure 2.** Locations of study populations and acidification situation (proportion of acidified water bodies) in Sweden 1990. No major changes have occurred since this period Source: Swedish Environmental Protection Agency.

## METHODS

Males and females were collected at the onset of breeding at each site, transported to Uppsala, and artificially mated in the laboratory to produce full-sib families. Laboratory fertilisations ensured that all larvae from a given mating were full-sibs and that eggs were not pre-exposed to differential acidity.

### *Rearing of embryos and tadpoles*

The laboratory experiments investigated the periods from fertilisation to hatching (gill absorption completed; Gosner stage 25, Fig.1, **I, II, V, VI**), and from hatching to metamorphosis (emergence of at least one forelimb; Gosner stage 42, Fig. 1, **III, V**; Gosner 1960). Embryos and larvae were reared at two (**III, V, VI**) or three (**I, II, III**) pH levels. Reconstituted soft water (RSW, APHA 1985) was used in all experiments to allow the comparison of populations without confounding effects of unwanted variation in

water chemistry. RSW has a nominal pH of 7.2-7.6 and in the low pH treatments, pH was adjusted to the nominal level with H<sub>2</sub>SO<sub>4</sub>.

The experiments were carried out in climate rooms (**I-III, V, VI**) or in flow-through aquaria (**VI**). Embryos were raised in groups of 30-50 and tadpoles individually in plastic vials containing treatment water. Tadpoles were fed *ad libitum* with finely chopped spinach. Water and food in the experimental vials were renewed every second (**III**) or third (**V**) day.

The effect of extra-embryonic membranes on embryonic survival was tested by enzymatically removing the gelatinous egg capsules from a part of the eggs (**II**). Carry-over effects were investigated by raising embryos from fertilisation to hatching in one of two (low and neutral) pH treatments, and at hatching larvae were either swapped between the treatments or kept in the same treatment until metamorphosis (**V**). In the UV-B treatments, embryos were exposed to differential UV-B levels by altering the time of UV-B exposure in the aquaria (**VI**).

#### *Response Variables*

Survival, size and development rate were the main response variables (**I-III, V, VI**). Hatchling size was measured as total length and embryonic development time (age at hatching) estimated as the time elapsed from fertilisation until Gosner stage 25. To get an estimate of initial size and tadpole growth, wet weight was measured on hatchlings (**III, V**), three weeks and six weeks old tadpoles (**III**), as well as on metamorphs (**III, V**). At metamorphosis, body (**III, V**) and total length (**V**) of each individual was measured. Larval development rate (age at metamorphosis) was estimated as number of days elapsed from initiation of the experiment to metamorphosis.

Egg size was measured on 20-40 eggs per female from photographic images with NIH image analysis programme. Snout-vent length was measured and the number of eggs counted for females in paper **IV**. Age of females was estimated with standard skeletochronological methods (Hemelaar 1985; Castanet et al. 1993). The potential rates of divergence in embryonic acid tolerance among acid and neutral populations was estimated in haldanes (*h*; Lynch 1990; Gingerich 1993; Hendry and Kinnison 1999, **I**).

#### *Experimental design and statistical analyses*

In paper **II**, reciprocal crosses among the A1 and N1 populations were used to investigate the relative importance of additive genetic, non-additive genetic and maternal effects in geographic variation in embryonic acid tolerance and embryonic growth and development. These consisted of full-sib families in four parental origin types (AA: both parents of acid origin, AN: female acid – male neutral, NA: female neutral – male acid, NN: both parents of neutral origin). The interpretation here is that if the differences are due to additive genetic effects, the hybrid crosses should show intermediate values to

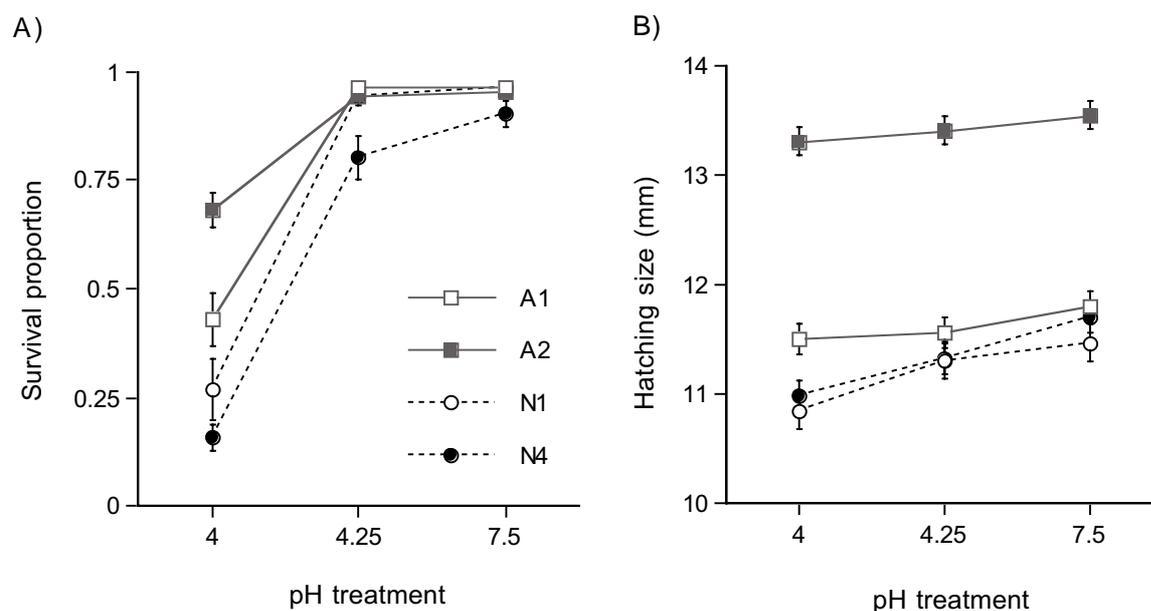
those of pure crosses and deviations from this pattern indicate non-additive genetic and maternal effects (Lynch and Walsh 1998). The experiments were performed as randomized blocks and analysed with factorial univariate and multivariate generalised linear models (GLM) and generalised mixed models (GLMM). All analyses were performed with the SAS statistical package (SAS 1996, Inc.).

## RESULTS AND DISCUSSION

### *Local adaptation*

I found that acidity reduced embryonic survival in *R. arvalis* by 30-80 % at pH 4.0 (Fig. 3a, **I**). However, survival was two to three folds higher in the acid origin populations than in the neutral origin populations (Fig. 3a). The negative effects of acidity on growth (Fig. 3b) and development were also more pronounced in neutral origin hatchlings. In terms of hatchling age, however, only one of the two acid origin populations (A2) had higher acid tolerance than the neutral origin populations (**I**). Furthermore, A2 population showed higher acid tolerance both in embryonic survival and development than A1. A2 population originates from lower pH and a geographic area that has suffered more strongly from anthropogenic acidification and situates centrally within a large acidified region (Fig. 2). Therefore, differences in acid tolerance between the A1 and A2 embryos could be explained by differences in the strength of selection or gene flow from neighbouring, less well adapted populations. The estimated divergence rates (0.007-0.102 *h*) for embryonic tolerance in *R. arvalis* were in many cases higher than the median value of 0.035 (for < 80 generations) reported by Kinnison and Hendry (2001). These results suggest that the strong selection caused by acidity on survival and growth of *R. arvalis* embryos, has resulted in local adaptation, which, furthermore, may have been rapid (see also Andr en et al. 1989). However, since the exact time for when selection for acid tolerance started is not known, some caution is warranted for the rate of divergence.

I did not find direct evidence for higher physiological acid tolerance in the acid origin tadpoles, when I compared larval performance of the *R. arvalis* populations N4 and A2 (the least and best adapted according to embryonic data and with large difference in egg size (**III**)). However, under acid conditions large hatching size translated to faster development and metamorphosis at similar size than that of smaller hatchlings in the acid population. This indicates that large hatching (egg) size may counteract negative effects of acidity on larval growth and development and therefore be adaptive in acid environments. The implications of larger size are discussed further below in relation to life-history variation.

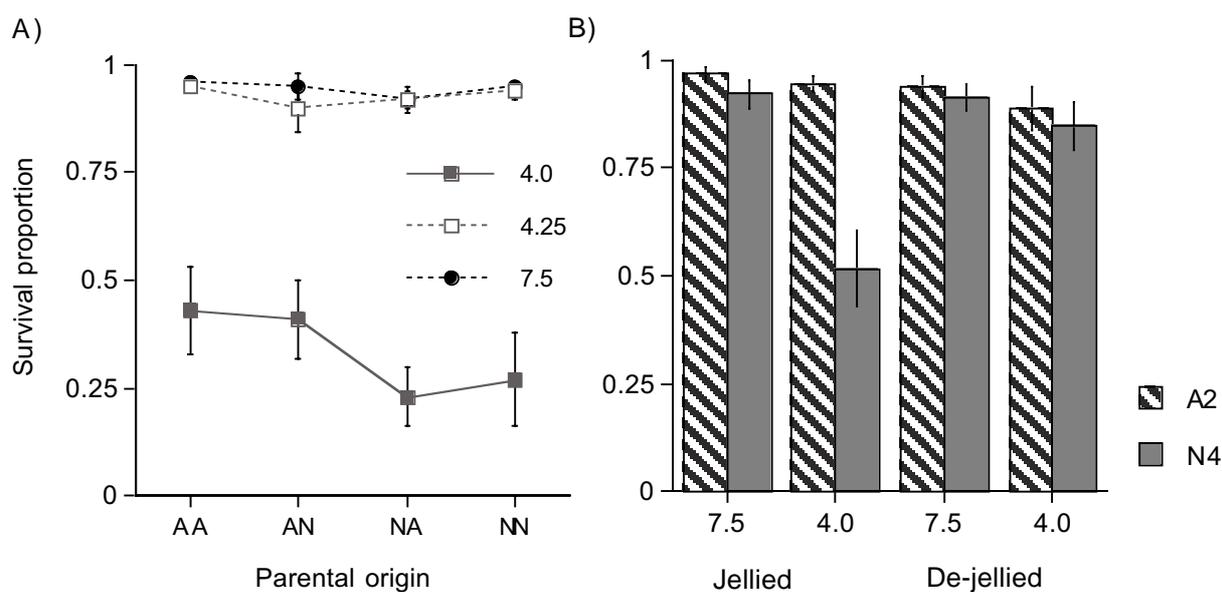


**Figure 3.** Mean ( $\pm$  S.E.) for embryonic survival (a) and least square means ( $\pm$ S.E) for hatchling size (b) in two acid origin and two neutral origin *R. arvalis* populations in three pH treatments.

I found no evidence for local adaptation to acidity in *R. temporaria* either during the embryonic or larval stages (V). In general, differences among acid tolerance studies may partially be explained by differential experimental conditions and by inherent differences in species tolerance levels. However, they are also likely to reflect differences among selective environments. The *R. arvalis* populations that show increased acid tolerance, originate from areas which have a very low pH ( $\text{pH} \geq 3.8$ ) as compared to acid ( $\text{pH} \geq 4.8$ ), but potentially still relatively benign environments experienced by the *R. temporaria* populations.

#### *Adaptive maternal effects*

The reciprocal crosses between A1 and N1 populations showed that among population variation in embryonic acid tolerance (survival) is maternally determined, indicating adaptive maternal effects (II, Fig. 4a). Maternal effects in embryonic tolerance have been hypothesised to depend on egg size, but I found only weak and inconsistent effects of egg size on embryonic survival in *R. arvalis* (I, II). Maternal effects have been implicated as determinants of embryonic acid tolerance also in *R. sylvatica* (Pierce and Sikand 1985, Pierce and Harvey 1987), but there has previously been no evidence of adaptive maternal effects. Likewise, there is generally little evidence for egg size effects in embryonic acid tolerance (Clark and LaZerte 1987, Pierce et al. 1987, Andrén et al. 1989; Merilä et al. 2002a).

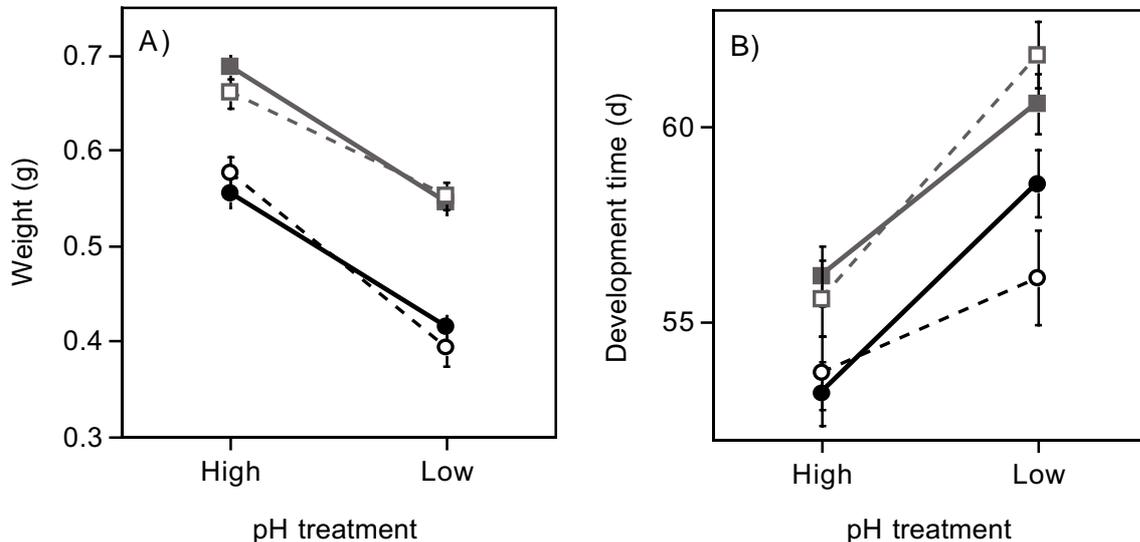


**Figure 4.** Embryonic survival in reciprocal crosses (A) between an acid (A1) and neutral (N1) origin population (first letter indicates maternal origin) in three pH treatments and B) in two populations (A2, N4) when jelly layers were retained (jellied) or removed (de-jellied) in two pH treatments. The values are means  $\pm$  S.E.

Removal of the gelatinous membranes (jelly) from eggs in the A2 and N4 populations (Fig. 4b) increased survival of N4 embryos but did not affect survival of A2 embryos, indicating that extraembryonic may determine intra-specific variation in embryonic survival under acid conditions, and thereby suggest a potential mechanism for adaptive maternal effects (II). Likewise, inter-specific differences in embryonic acid tolerance were related to the extra-embryonic membranes in two *Xenopus* species (Picker et al. 1993). However, whether variation in these structures has a genetic basis or merely is an environmental maternal effect needs to be investigated.

Egg size is strongly positively correlated with hatching size in amphibians (Kaplan 1998), and hatching size, thus, reflects maternal investment. The comparison of the A2 and N4 populations showed that hatching size affects larval performance in *R. arvalis* (Fig. 5, III). The effects were moderate to strong in the acid origin population, and weak in the neutral origin population (III). In the acid origin population, the effect of initial size depended on the growth environment; under acid conditions, initially large larvae were able to develop faster (hatchling weight-development time:  $r = -0.34$ ,  $P < 0.01$ ) and reach similar size at metamorphosis than initially smaller larvae, whereas under neutral conditions large hatching size translated to large metamorphic size (hatchling weight – metamorphic weight:  $r = 0.38$ ,  $P < 0.001$ ), but not influence development time. These results support the view that initial size may be a strong determinant of larval performance but that the effects of initial size may vary with growth environments and be population specific. Furthermore, the results suggest that although egg size has little

effects on embryonic acid tolerance, large egg size may be adaptive in acid environments due to its positive effects on larval performance.

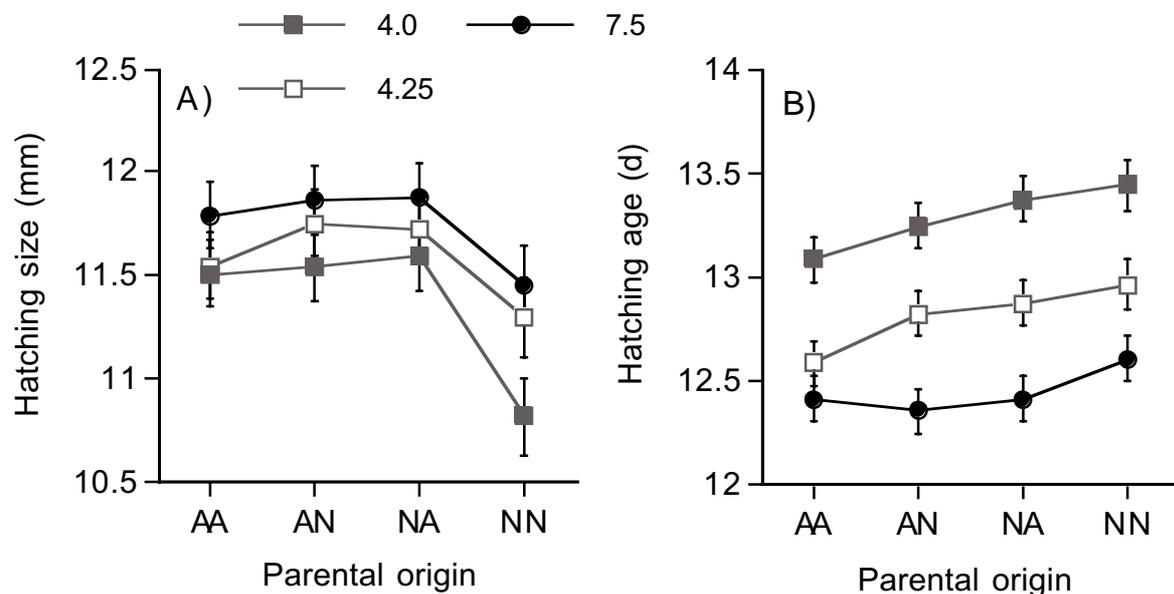


**Figure 5.** Metamorphic performance of *R. arvalis* tadpoles from and acid (A2) and a neutral (N4) population without (solid lines) and with (broken lines) correcting for initial size effects. Values are LSmeans  $\pm$  S.E. from models in paper III.

Embryonic traits have usually a strong maternal component (Mousseau and Fox 1998). However, the reciprocal crosses between A1 and N1 populations showed that geographic variation in hatching size of *R. arvalis* is partly determined by non-additive genetic and/or maternal-genetic effect interactions (Fig. 6a, **II**), whereas hatching age variation is mainly determined by additive genetic effects (Fig. 6b, **II**). These results show that although maternal effects often are strong determinants of early life-history variation, geographic divergence in early life-history traits may also be genetically determined. I found no statistically significant evidence for either maternal or genetic effects in embryonic acid tolerance when measured in terms of sub-lethal effects (i.e. change in hatching size and age between the neutral and acid treatments, **II**). The underlying factors contributing to among population variation in sub-lethal acid tolerance traits need further investigation.

#### *Life-history variation*

My results suggest that *R. arvalis* populations originating from low and neutral pH habitats differ in growth and development rates at embryonic (**I**, **II**) and larval (**III**) stages, indicating that spatial variation in environmental acidity may have a profound impact on organism life-histories. Firstly, acid origin embryos were larger at hatching (Fig. 3) and/or developed faster than their conspecifics from neutral populations (**I**). Second, acid origin larvae maintained their initial size advantage and grew faster throughout their aquatic development period, but developed slower than neutral origin larvae (Fig. 5, **III**).



**Figure 6.** Hatching size (A) and age (B) in the four types of reciprocal crosses in three different pH treatments. Codes on x-axis refer to the different crossing combinations A = acid origin, N = neutral origin, female origin indicated by the first of the two letters. The values represent LSmeans  $\pm$  S.E. from models in paper II.

Although the reasons for life-history variation among acid and neutral origin populations are largely unclear, several lines of reasoning suggest that selection may favour larger and/or faster developing genotypes in acid environments. First, large initial size may be beneficial if maternal investment in large eggs (e.g. more nutrients) compensates for increased energetic demands of larvae caused by acid stress. Large individuals may also render more tolerant of physiological acid stress due to a smaller surface-to-volume ratio (Verma and Pierce, 1994; Edwards and Gjedrem 1979; Shuter and Ihssen 1991) or a better osmoregulatory capacity (e.g. McCormick and Saunders 1987). In addition, large size and fast growth rates may diminish risk of predation from large insect predators (Travis et al. 1985, Jung and Jagoe 1995), that become top predators in acidified lakes (Henrikson 1989).

Second, the combination of fast growth and slow development in acid origin larvae could be explained by several different, but not mutually exclusive, factors. Slow development of A2 larvae may be a result of maternal investment in large but slow developing propagules (e.g. Bradford 1990). Selection may also have favoured an increase in genetic growth rates and a decrease in genetic development rates, because both have a positive effect on size (Roff 1992, 2000). Furthermore, if fast growth and large size are the target of selection in acid origin populations, slow developmental rates may also be caused by a trade-off between growth and developmental rate (Arendt 1997).

Third, faster growing genotypes could be selected for under conditions where phenotypic growth rates tend to slow down and when time available for larval development is limited (Conover and Schultz 1995; Arendt 1997), as represented by acid habitats in seasonal environments. Since the N2 population is situated roughly 350 km north than the A2 population, a latitudinal cline in genetic development rates explaining the faster development of the neutral population can not be excluded. However, the faster growth rates of A2 larvae are contradictory to this prediction.

Clearly, the ultimate reasons for among population variation in larval life-histories of *R. arvalis* originating from acid and neutral environments remain elusive and warrant further study. Furthermore, although these results are indicative of adaptive maternal effects mediated via egg size, detailed studies are needed to confirm this since, hatchling traits may also have a genetic basis (II).

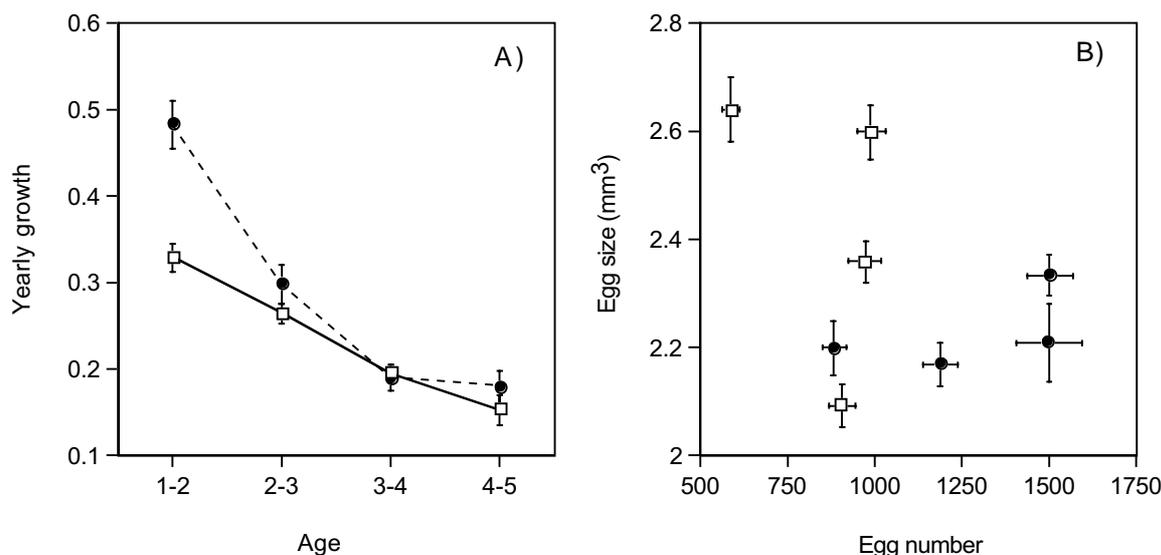
#### *Maternal investment*

I found that *R. arvalis* females from acid areas invest in large eggs than females from neutral areas (IV), providing further evidence that large egg size is selected for in acid environments. The total reproductive output and growth rate (Fig. 7, IV) was lower in acid origin females than in neutral origin females, indicating limited resource availability (e.g. Dunham 1978) or physiological limitations in a harsher environment. The difference between acid and neutral origin females in total reproductive output may also reflect a relationship between the evolution of egg size and the evolution of total reproductive output (Winkler and Wallin 1987, Caley et al. 2001). Nevertheless, large egg size is unlikely a result of higher levels of resources available for acid origin females.

Furthermore, I found a negative relationship between egg number and egg size in acid origin females but not in neutral origin females (IV). This suggests that at the phenotypic level selection on large egg size may strengthen the trade-off between egg size and number, as previously found in a selection experiment in *Drosophila* (Schwarzkopf et al. 1999). Phenotypic trade-offs between egg number and size have previously been found in a variety of organisms (reviewed in Roff 1992; Stearns 1992), but evidence for difference in strength of a trade-off among wild populations is limited.

In acid origin females, egg size increased more with female size, whereas in neutral origin females egg size increased with female age egg size, suggesting that egg size in acid and neutral origin populations may partly be determined by different mechanisms (IV). One possible explanation is that in acid environments, where large egg size may be adaptive, females may maximise their fitness by increasing egg size (rather than egg number) with increasing body size, whereas the opposite may be true for females inhabiting neutral environments. Because of the apparent trade-off between egg number and egg size in the acid origin females, another possible reason for the difference among acid and neutral origin females may lay in constraints to reproduction (Roff 1992). These could arise, for

instance, as a result of strength of mechanical limitations as has been found in other ectotherms (e.g. Kaplan and Salthe 1979; Sinervo and Licht 1991). In neutral origin females, egg size may be more influenced by e.g. maternal physiology (Sinervo and Licht 1991), which is further supported by the observation that egg number in neutral origin females decreased with age. In neutral origin females an increase in egg size with age may have become at the cost of reduced egg number – possibly indicating a trade-off arising from limitations of maternal quality.



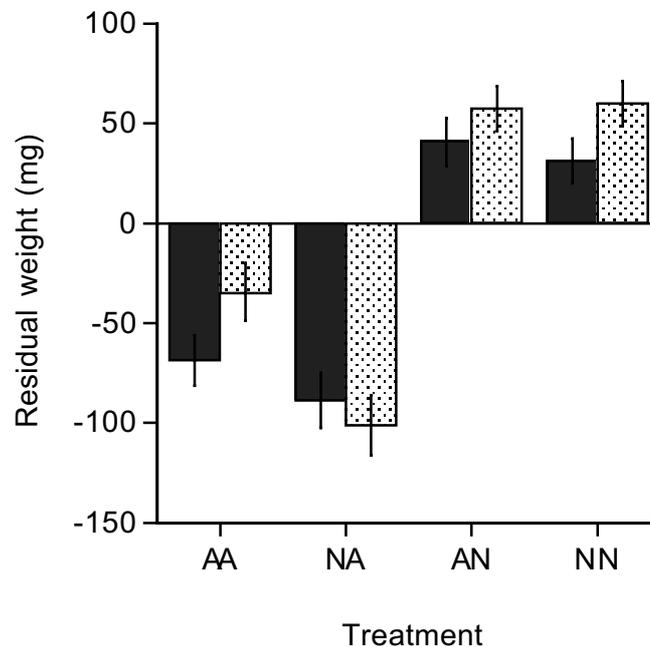
**Figure 7.** Mean ( $\pm$  S.E.) of yearly growth (a) rates for acid and neutral origin females and for egg size and egg number (b) in four acid (solid) and four neutral *R. arvalis* populations.

#### Carry-over effects

I found that *R. temporaria* tadpoles in the neutral embryonic – acid larval treatment were more adversely affected by acidity than tadpoles in the acid embryonic – acid larval treatment, apparent through their impaired growth performance until the age of three weeks (Fig. 8, V). This suggests that exposure to acidity during the embryonic stage may have increased the ability of larvae to tolerate continued acid exposure, i.e. they may have benefitted from an acclimation effect. The evidence for acclimation to low pH in fish is mainly negative (Wood, 1989) and has rarely been investigated in amphibians (but see: Freda and Dunson 1984, McDonald et al. 1994). However, the mechanisms behind this remain currently unclear and need further study.

Developmental time or size at metamorphosis in *R. temporaria* tadpoles raised initially under acid conditions, did not differ from those raised in neutral conditions (V), indicating a compensatory increase in growth once moved to neutral water. This suggests that if environmental conditions turn beneficial, amphibians may be able to counteract the earlier negative effects of acidity. However, more extreme acidity than used in my

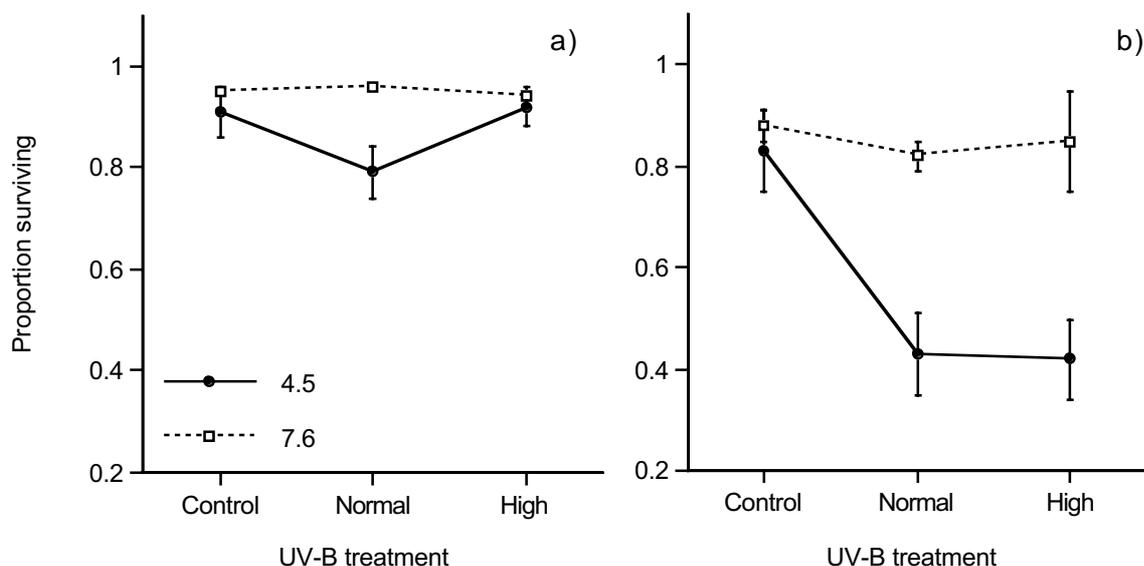
experiment may occur during spring snow-melt and heavy rain, and the effects of these events could be stronger than indicated here.



**Figure 8.** Mean residual body weight in four treatment groups (AA = acid embryonic- acid larval, NA = neutral embryonic – acid larval, AN = acid embryonic – neutral larval, NN = neutral embryonic – neutral larval) and two populations (dark bars = Ammarnäs, pale bars = Kiruna) of *R. temporaria*. The bars present least square means ( $\pm$  S.E.).

#### *UV-b/pH interaction*

Exposure of *R. temporaria* embryos simultaneously to stress from UV-b radiation and low pH increased the negative effects of both stressors (Fig. 9, VI), indicating that they acted in a synergistic fashion. Furthermore, the strength of the synergistic effect was stronger on the northern population than in the southern population (Fig. 8, V). These results suggest that conclusions about a species tolerance to a certain stressor should be treated with caution when based on single populations and in the absence of other relevant stress factors (see also Long et al 1995, Kiesecker and Blaustein 1995). For instance, *R. temporaria* is generally considered to be UV-b tolerant (Cummins *et al.* 1999; Langhelle *et al.* 1999; Merilä *et al.* 2000b; Pahkala *et al.* 2000, but see Pahkala et al. 2001), but its sensitivity to low pH may make it vulnerable to UV-B radiation. These results indicate that the effects of a stressor and its interactions with other stressors may be highly population specific.



**Figure 9.** Survival of two populations (a. southern, b. northern) of *R. temporaria* embryos in three UV-B and two pH treatments. Values are least square means ( $\pm$  S.E.) from models in paper VI.

### GENERAL CONCLUSIONS

My results suggest that environmental acidity may be a powerful selective force initiating genetic differentiation among populations at different life stages and that, when selection is sufficiently strong, this adaptation may occur fast. This provides further support for human caused environmental stress driving contemporary evolution (e.g. Reznick and Ghalambor 2001; Palumbi 2001). However, extinction is always an alternative to adaptation (e.g. Henriksen et al. 1989), and it is important to keep in mind that both the pattern and the rate of an environmental change, as well as available genetic variation, affect the ability of a population to adapt to a changing environment (Lande and Shannon 1996; Hoffmann and Parsons 1997; Kinnison and Hendry 2001). *R. arvalis* presents a relatively acid tolerant species and more sensitive species may fail to adapt to similar acidity.

My results further suggest that much of the underlying mechanisms of adaptation to acid environments in amphibians involves maternal effects and that in acid environments selection acts upon increasing egg size at the cost of egg number. This supports the view that maternal effects are often an important pathway for adaptation (Mousseau and Fox 1998), and that environmental stress may be one underlying factor in shaping evolution of reproductive life-histories (e.g. Forbes and Calow 1997). However, despite the often strong maternal contribution to variation in early life-stages, genetic effects may also contribute to geographic variation in early life histories. Furthermore, acid stress may

have strong negative effects also on other fitness components, such as metamorphic traits in amphibians. Selection to counteract these negative effects may result in geographic variation in genetic growth and development rates and thereby have a profound effect on individual life-histories.

Short-term acid events may have no lasting effects on performance in amphibians, at least under conditions where resources are not limited, suggesting that the negative effects of moderate stress during one stage of an organism's development may be compensated for later in life if conditions become favorable. The interactive effects with other environmental variables need to be considered when investigating the susceptibility of organisms to stressful environmental conditions and when interpreting geographic variation in stress tolerance.

#### *Future directions*

From the results of my thesis it seems clear that environmental stress caused by acidity may be a strong selective factor in natural populations. However, as in any interesting subject in biology, more questions are raised than answered. These involve the quantitative genetic basis of acid tolerance and life-history variation, the effects of acidity in natural conditions, and the trade-offs between acid tolerance and other fitness traits. Acid tolerance studies may also prove a good candidate for the recently emerged concept of evolutionary physiology (Feder et al. 2000). Large scale population comparisons, reciprocal transplant studies and investigations of the genetic basis of maternal effects are logical next steps to confirm some of the hypothesis raised here. Although my thesis has taken an amphibian perspective, all these questions are relevant for other organisms that experience spatial variation in environmental pH. Future studies will reveal further upon the general evolutionary effects of environmental stress caused by acidity.

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