

Life in acid: interactive effects of pH and natural organic acids on growth, development and locomotor performance of larval striped marsh frogs (*Limnodynastes peronii*)

B. J. Barth and R. S. Wilson*

Integrative Ecology Lab, School of Biological Sciences, The University of Queensland, St Lucia, QLD 4072, Australia

*Author for correspondence (r.wilson@uq.edu.au)

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SUMMARY

The low pH of naturally acidic aquatic environments is the result of soft-water with low buffering capacity and high concentrations of natural organic acids. Our current understanding of the influence of pH on aquatic organisms is largely limited to laboratory studies conducted under controlled conditions with little incorporation of these organic acids. Recent studies suggest natural organic acids may influence the physiology of aquatic species independent of low pH. We examined the effects of pH and varying concentrations of natural wallum water, which is high in organic acids on the hatching success, growth and locomotor performance of larval striped marsh frogs (*Limnodynastes peronii*). Based on previous studies, we predicted that the detrimental effects of low pH would be further exacerbated by higher concentrations of naturally occurring organic acids (high concentrations of wallum water). In artificial soft-water, embryos experienced both reduced growth and reduced survival when exposed to low pH. However, greater concentrations of natural organic acids did not exacerbate these effects of low pH on growth and development. Instead, we found some evidence that the natural organic acids within wallum water improved growth and swimming performance across all pH treatments. Using path analyses to investigate the effects of pH and natural organic acid concentration on burst swimming performance, we found performance was directly affected by both body length and organic acid concentration. Our data further highlight our limited understanding of the importance of natural organic acids for aquatic organisms and the need to incorporate greater ecological relevance into these studies.

Key words: locomotion, organic acids, pH, performance, swimming, tadpoles.

INTRODUCTION

Understanding the range of abiotic factors that limit the abundance and distribution of organisms is an important goal within the field of physiological ecology (Garland and Carter, 1994). For aquatic organisms, environmental variables such as water flow, temperature, salinity and water chemistry are believed to be crucial in defining species boundaries and reproductive success (Menni et al., 1996; Brodman et al., 2003; Dunham et al., 2003; Mazerolle, 2005; Domingos et al., 2006; Lacoul and Freedman, 2006; Zivic et al., 2006). Stressful environments often contain unique assemblages of flora and fauna specialized for the specific conditions, typically to the exclusion of widespread and common species (Gosner and Black, 1957; Ingram and Corben, 1975; Meyer, 2004). For example, naturally acidic aquatic environments are commonly inhabited by distinct assemblages of species, including unique and endemic species of fish, crustaceans and amphibians (Gosner and Black, 1957; Bensink and Burton, 1975; Ingram and Corben, 1975; Saber and Dunson, 1978; Freda and Dunson, 1986; Picker et al., 1993). Amphibians are particularly interesting for studies of stressful aquatic environments, including those with a tolerance to low pH, because adults can selectively choose particular environments for breeding but eggs and larvae are constrained to aquatic habitats.

Exposure of freshwater aquatic organisms to low pH environments can result in increased mortality and decreased growth rate associated with the disruption of ion balance (McDonald et al., 1984; Freda and Dunson, 1984; Freda and Dunson, 1985; Pierce, 1985; Robinson, 1993; Moore and Klerks, 1998). For amphibians and fish the net loss of electrolytes (particularly Na^+) is largely

caused by damage of the gill epithelium preventing the maintenance of ionic homeostasis (Freda and Dunson, 1984; Frisbie and Wyman, 1992; Meyer et al., 2009). Low pH can cause decreased hatching success across a wide range of amphibian taxa (e.g. Gosner and Black, 1957; Dunson and Connell, 1982; Picker et al., 1993). Laboratory studies suggest a correlation between pH tolerance and inter-specific variation in distribution, with species inhabiting acidic environments exhibiting greater tolerance to low pH (Gosner and Black, 1957; Pierce, 1985; Freda and Dunson, 1986; Pierce and Wooten, 1992; Picker et al., 1993). The sub-lethal effects of low pH include decreased growth and increased developmental time for amphibians; however, the effects on whole-animal performance of amphibian larvae are not known (Freda and Dunson, 1985; Griffiths et al., 1993; Preest, 1993). This is surprising given that most micro-geographic changes in the distribution and abundance of species are the result of subtle influences of the abiotic and biotic environment, rather than direct mortality effects.

Our current understanding of the influence of pH on amphibian physiology is largely limited to laboratory studies conducted under artificial conditions with little use of natural organic acids. The low pH of naturally occurring acidic environments, including bogs, peat swamps and the wallum areas of eastern Australia, is the result of both soft-water with low buffering capacity and high concentrations ($>10\text{ mg l}^{-1}$) of natural organic acids (Bayly, 1963; Jackson, 1975; Hemmond, 1994). Natural organic acids (humic and fulvic acids) are a diverse, complex and poorly understood group of organic compounds that are often referred to generally as dissolved humic substances (McDonald et al., 2004). Natural organic acids derive

from the decomposition of biological material and have the potential to influence the physiology and ecology of freshwater organisms independent of pH (Jackson, 1975; Hemmond, 1994; Steinberg et al., 2006). The effect of humic substances on aquatic organisms remains poorly understood and generalizations are difficult (for review, see Steinberg et al., 2006). For example, humic substances are known to protect numerous aquatic organisms against heavy metal toxicity and ion loss in extremely soft-water (McGeer et al., 2002; Schwartz et al., 2004; Winter et al., 2005; Vigneault and Campbell, 2005; Kozlova et al., 2009). In contrast, humic substances can exert chemical stresses on a range of aquatic invertebrates (e.g. Timofeyev and Steinberg, 2006). However, for amphibian larvae, the detrimental effects of low pH on hatching success were found to be further exacerbated by naturally occurring bog water that contained high concentrations of natural organic acids (Dunson and Connell, 1982; Freda et al., 1990; Picker et al., 1993). Thus, the limited diversity and unique assemblage of species that inhabit natural low pH environments may be due to the combined direct effects of low pH and high concentrations of organic acids.

The freshwater bodies of the coastal sandy lowlands or 'wallum' environment of eastern Australia represent a typical naturally low-pH environment (Coaldrake, 1961). The wallum environment occurs between coastal northern New South Wales and southern Queensland and is characterized by sand dunes or flat, undulating country with acid soils and a high water table. Wallum water bodies typically have soft-water, with low buffering capacity and high levels of organic acids. This causes the water to be stained dark brown, similar to other low pH environments such as the 'blackwaters' of the Rio Negro in the Amazon. This represents a potentially stressful environment for aquatic organisms, with water bodies having a pH ranging between 3.0 and 6.5 (Bayly, 1964; Bensink and Burton, 1975; Meyer, 2004). The low pH water bodies of the wallum environments contain a unique assemblage of amphibians, including four vulnerable endemic frog species (*Litoria cooloolensis*, *L. freycineti*, *L. olongburensis* and *Crinia tinnula*), while most amphibian species that are common across eastern Australia are largely excluded from the low pH wallum environments. We examined the interactive effects of low pH and naturally occurring wallum water, which is high in natural organic acids, on the embryonic and larval development of a common amphibian species. We studied the striped marsh frog (*Limnodynastes peronii*), which has a widespread distribution along the eastern region of Australia. Although previous laboratory studies have revealed that larval *L. peronii* can develop to metamorphosis at a pH as low as 4.5 (Meyer, 2004) they are not often found within the low pH water bodies of the wallum environments. Given the limited distribution of *L. peronii* within these wallum environments, they represent an ideal species for examining the possible sub-lethal effects on growth and development that may limit the distribution of amphibian species into natural low pH environments.

We first investigated the effects of chronic exposure to artificial soft-water of different pH values on the embryonic and larval growth of *L. peronii* and their subsequent burst swimming performance. This initial experiment allowed us to determine a non-lethal but challenging pH environment to examine the interactive effects of pH and varying concentrations of natural organic acids on the hatching success, growth and locomotor performance of larval *L. peronii* using a simple factorial experiment. Measures of locomotor performance were used as a proxy for an individual's physical condition. Water collected from natural creeks and ephemeral water bodies within wallum habitats was used as a natural source of soft-water with high natural

organic acid concentrations. Based on previous studies (Dunson and Connell, 1982; Freda et al., 1990; Picker et al., 1993), we predicted that the detrimental effects of pH on *L. peronii* embryos and larvae would be further exacerbated in treatments having greater concentrations of wallum water. Using path analysis and Akaike's information criterion (AIC), we also investigated the relationship between pH and wallum water concentration on body size, condition and burst swimming performance by using competing models that incorporated both direct and indirect effects. Path analyses can be used by researchers to compete several *a priori* hypotheses against each other so as to determine which model best explains the available dataset.

MATERIALS AND METHODS

Striped marsh frog (*L. peronii* Duméril and Bibron 1841) spawn was collected from various sites in south-east Queensland, Australia, and immediately taken to the laboratory at The University of Queensland. Between six and 10 partial foam egg masses were collected for each experiment and individual eggs were evenly distributed among all treatments. Upon hatching, all larvae were fed finely chopped boiled spinach *ad libitum*, and complete water changes were made twice weekly. Embryos and larvae were maintained at 25°C (±1°C) in all experiments. Experiments were approved by The University of Queensland Animal Welfare and Ethics Committee and collection of egg masses and water was approved by the Queensland Parks and Wildlife Service.

The naturally occurring wallum water used in this study was collected from six undisturbed wallum habitats within Cooloola National Park and on North Stradbroke Island, south-east Queensland, Australia. All collected water was heavily stained dark brown and was between pH 3.5 and 4.1, as is typical of many wallum environments. Wallum water from the different sites was mixed for use in experiments and a detailed analysis of the chemical composition of this mixture, including humic and fulvic acid content, was conducted by the Analytical Service Unit at The University of Queensland (Table 1). Dilutions of the mixed wallum water were made for subsequent experiments (details below).

Experiment 1: effects of pH on embryonic and larval development

The embryonic and larval development of *L. peronii* was examined at pH levels of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.7. Egg masses were returned to the laboratory within 6–12 h of fertilization and immediately placed within their specific pH treatments. Five viable embryos were placed into each replicate tank ($N=20$ tanks per treatment) that contained 0.25 l of artificial soft-water (ASW). ASW consisted of 1.0 mg l⁻¹ MgSO₄·7H₂O, 0.7 mg l⁻¹ NaCl, 0.7 mg l⁻¹ CaCl₂·2H₂O, 0.1 mg l⁻¹ KCl and 0.21 mg l⁻¹ NaOH added to reverse osmosis water. The time taken for each individual embryo to hatch was recorded hourly, and animals that had not hatched 36 h after the first embryos had emerged were considered dead. For the quantification of total time to hatching, the time of fertilization (spawning) was estimated to be at 0.00 h on the night of collection. Based on development of the eggs at collection and the reproductive behaviour of this species, the estimated time of fertilization should be accurate to within 2 h. After three embryos had hatched within each replicate tank, one individual was selected at random and humanely killed using an overdose of the barbiturate MS222. The total length of this individual was measured using a calibrated optical micrometer under a dissecting microscope. A second individual was then selected at random from the remaining individuals and reared individually in 2.5 l of ASW until 12 days

Table 1. Chemical composition of water used for this study and water chemistry of natural wallum water collected from various locations in south-eastern Queensland

	H-acids	F-acids	TOC	Cl	Al	Ca	Cu	K	Mg	Na	S
Artificial soft-water (exp. 1)	0.00	0.00	0.00	8.1	0.0	1.9	0.00	0.5	0.90	3.96	1.30
Natural wallum water mixture (exp. 2)	9.1	19.3	18.5	27.3	0.16	2.0	0.01	3.8	1.64	10.9	1.17
0% wallum water (exp. 2)	0.00	0.00	0.00	25.4	0.00	2.0	0.00	3.8	1.64	11.8	2.16

Values are mg l^{-1} . See Materials and methods for details of experiments 1 and 2.

H-acids, humic acids; F-acids, fulvic acids; TOC, total organic carbon.

post-hatching under their specific pH treatment condition. After 12 days of development, maximum burst swimming performance was assessed at 25°C (described below). Following assessment of swimming performance, the head–body and tail lengths and body mass of each larva were recorded. Body mass was determined using a digital balance ($\pm 0.0001 \text{ g}$) after surface water had been removed from the tadpoles with absorbent paper towelling.

The burst swimming performance of each larva was assessed by recording three startle responses with a high speed digital camera (Redlake Motionscope, Cheshire, CT, USA) recording at 250 Hz. Only swimming responses that consisted of a C-start response from a resting position and with limited vertical displacement were analysed (Wilson and Franklin, 1999). Swimming sequences were elicited by gently touching the tip of the head of the larva within the swimming arena ($0.15 \text{ m} \times 0.15 \text{ m} \times 0.03 \text{ m}$ deep) at 25°C. Swimming sequences were filmed from a distance of 1.5 m by recording the image captured off a mirror angled at 45 deg. above the swimming arena. The accompanying Redlake software package was used to play back the swimming responses frame-by-frame to measure instantaneous changes in displacement. The first 140 ms of a startle response were analysed, beginning with the frame preceding the first detection of movement. Instantaneous displacement of the centre of the head–body region was calculated by digitizing the front and back of the head–body and calculating the mid-point of these co-ordinates. Instantaneous velocity was then determined by calculating the rate of change of displacement after a three-point moving average filter was applied to the data (Wilson, 2005). The maximum instantaneous speed for each individual was taken as the greatest absolute speed attained from all three recorded startle responses.

Experiment 2: effects of pH and natural organic acids on embryonic and larval development

The embryonic and larval development of *L. peronii* was examined at three pH levels (pH 4.5, 5.0 and 6.7) across three wallum water (ww) dilutions: 100% wallum water (100%ww), 50% wallum water (50%ww) and 0% wallum water (0%ww). The wallum water was diluted with artificial wallum water (AWW) which had a similar ionic composition to naturally occurring wallum water but did not contain the natural organic acids (i.e. humic or fulvic acids) (Table 1). Thus, apart from the humic and fulvic acids, all concentrations of other important elements in the AWW remained similar to that of 100% wallum water treatments. The AWW consisted of $16.5 \text{ mg l}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$, $30.0 \text{ mg l}^{-1} \text{ NaCl}$, $7.6 \text{ mg l}^{-1} \text{ CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $7.3 \text{ mg l}^{-1} \text{ KCl}$ mixed with reverse osmosis water. Wallum water ionic composition was determined by The University of Queensland's Analytical Service Unit.

From the first experiment it was evident that pH 3.5 and 4.0 were too stressful and resulted in 100% mortality after 12 days of development. Therefore, in this experiment, the pH levels of 4.5 and 5.0 were used as stressful but non-lethal conditions in order to examine the interactive effects of pH and naturally occurring wallum water. Egg masses were returned to the laboratory within 6–12 h of fertilization and immediately placed within their specific treatments. Two viable embryos were placed within each replicate tank ($N=25$ tanks per treatment) that contained 0.25 l of treatment water. After both embryos had hatched within each replicate tank, one individual was selected at random and humanely killed using an overdose of MS222. The time to hatching and total length of this individual were then measured. For the remaining individual, tail and head–body lengths and mass were recorded at day 6 and

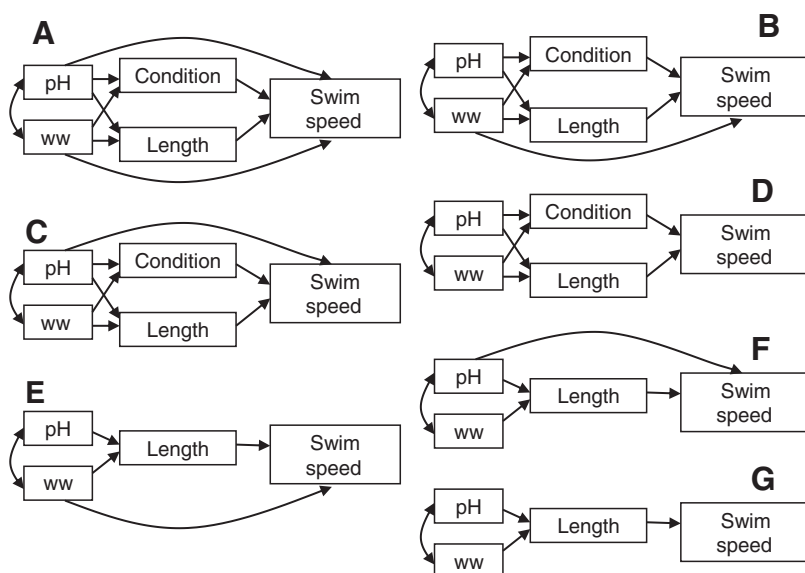


Fig. 1. Seven path models (A–G) predicting the relationships of pH and wallum water concentration to body size, condition and burst swimming performance. ww refers to the wallum water concentration used in each treatment. Length represents the total body length of each individual tadpole. Condition is the residuals of body mass regressed onto body length. Swim speed is the maximum burst swimming performance recorded for each individual.

day 12 of development. Burst swimming speed was also measured for each individual at day 12.

To describe the relationship between pH, wallum water, morphology and performance ability, we compared the fits of seven path models using an information-theoretic approach (Fig. 1). Each model was fitted to the data using AMOS 5.0 (SPSS Inc., Chicago, IL, USA). We used the residuals of body mass regressed onto body length as an index of body condition. Body condition data were log transformed to meet the assumptions of normality. Models were then ranked in order of likelihood by calculating the second-order Akaike information criterion (AIC):

$$AIC = \chi^2 + 2K + (2K(K + 1)) / (n - K - 1), \quad (1)$$

where χ^2 is the Chi-square goodness of fit, K is the number of estimated parameters and n is the sample size (Burnham and Anderson, 2002). Akaike weights were used to assess which model was the best descriptor of the data from the set of models.

Monitoring of pH

To ensure that specific treatments were within 0.2 units of the designated pH, each replicate container was monitored every 4–6 h throughout experiments. The pH of all containers was lowered when necessary using a dilute solution of acetic acid. All pH measurements were taken using either a Hanna HI9023 or Eutech Ecoscan pH5 pH meter with temperature-compensation probes. During the second experiment an acetate buffer was also used to help decrease any drift in pH. This buffer contained acetic acid and sodium acetate, both of which have been found to be non-toxic to amphibian larvae at these concentrations (Ireland, 1991; Dawson et al., 1996). Only small amounts of the buffer solutions were added so that the sodium concentrations were increased by approximately equal amounts in each treatment.

Statistical analyses

The influence of pH treatment on morphology and hatching success was analysed using a one-way ANOVA. Hatching success data were arc-sine transformed prior to analysis to meet the assumptions of normality. In experiment 1, the influence of pH treatment on maximum swimming speed was analysed using a general linear model with one continuous (body size) and one categorical predictor (pH). Body size was calculated from the first factor of a principal components analysis (PCA) of tail length, head–body length and body mass. The first factor described greater than 80% of the variation observed among individuals and was deemed a good

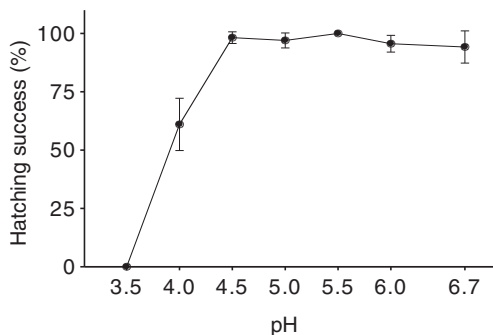


Fig. 2. The influence of pH on the hatching success of embryos of the striped marsh frog (*Limnodynastes peronii*) (experiment 1). A significant effect of pH on hatching success was detected ($F_{6,132}=117.6$; $P<0.0001$). Data represent means \pm 95% confidence limits.

predictor of overall body size. In experiment 2, the interactive effects of pH and wallum water on morphological measures were analysed using a general linear model with two categorical predictors (pH and wallum water treatment). Maximum swimming speed was compared among treatments using a general linear model with two categorical and one continuous predictor (body size). Body size was again taken as the first component from a PCA of tail and head–body length and body mass. *Post-hoc* tests of specific differences among treatments were performed using Tukey's comparisons. All statistical analyses were performed using Statistica 7.0. Significance was taken at the level of $P<0.05$. Data are presented as means \pm 95% confidence limits.

RESULTS

Experiment 1: effects of pH on embryonic and larval development

The hatching success of *L. peronii* embryos decreased significantly at lower pH ($F_{6,132}=117.6$; $P<0.0001$). Hatching success was greater than 94% across the pH range of 4.5 to 6.7, but *post-hoc* tests revealed that hatching success was significantly lower at both pH 4.0 (61.0 \pm 11.2%) (Tukey's; $P<0.0001$) and pH 3.5 (0%) (Tukey's; $P<0.0001$) than in other treatments (Fig. 2). However, the time to hatching ($F_{5,88}=1.45$; $P=0.21$) and total length at hatching ($F_{5,88}=2.03$; $P=0.08$) were unaffected by pH treatment.

After 12 days of development, 100% mortality had occurred within the pH 4.0 treatment. Survival across all other treatment levels was between 65 and 90%. At day 12, the body mass of surviving larvae was significantly affected by treatment pH ($F_{4,72}=4.72$; $P=0.002$), with the largest tadpoles at pH 6.0. However, total body length ($F_{4,73}=2.12$; $P=0.09$) and maximum swim speed ($F_{4,71}=0.86$; $P=0.50$) remained largely unaffected by pH treatment. Maximum swim speed was significantly influenced by total body size across all treatments, with larger tadpoles having a faster burst swimming performance ($F_{4,71}=7.26$; $P<0.001$).

Experiment 2: effects of pH and natural wallum water concentration on embryonic and larval development

Hatching success of *L. peronii* embryos was unaffected by pH ($F_{2,216}=2.74$; $P=0.07$) and wallum water concentration ($F_{2,216}=0.048$; $P=0.95$), with greater than 94% of individuals across all treatments successfully hatching. The time taken to hatch was significantly

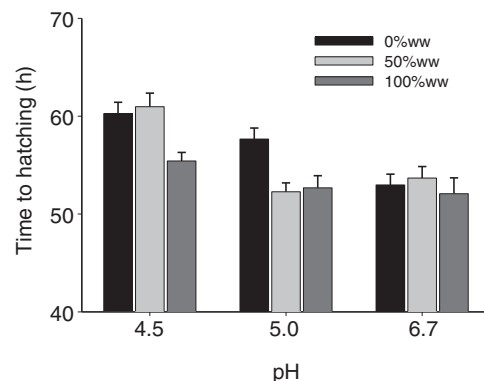


Fig. 3. The interactive effects of pH and wallum water concentration (ww) on the time to hatching of embryonic *L. peronii* (experiment 2). Significant effects of both pH ($F_{2,186}=71.1$; $P<0.0001$) and wallum water concentration ($F_{2,186}=25.6$; $P<0.0001$) were detected. Data represent means \pm 95% confidence limits.

affected by both pH ($F_{2,186}=71.1$; $P<0.0001$) and wallum water concentration ($F_{2,186}=25.6$; $P<0.0001$), with embryos at the lowest pH combined with 0%ww and 50%ww treatments taking the longest time to hatch (Fig. 3). In addition, the time to hatching at pH 5.0 was significantly faster in the 100%ww and 50%ww treatments than in the 0%ww treatment (Tukey's; $P<0.0001$). Total body length at hatching was not significantly affected by either pH ($F_{6,132}=0.34$; $P>0.05$) or wallum water concentration ($F_{6,132}=0.73$; $P>0.05$).

After 6 days of development, the body masses of larval *L. peronii* differed significantly among pH ($F_{4,193}=127.3$; $P<0.0001$) and wallum water treatments ($F_{4,193}=8.3$; $P<0.001$) (Fig. 4A). Total body length was also significantly influenced by pH ($F_{4,193}=148.8$; $P<0.001$) and wallum water conditions independently ($F_{4,193}=13.2$; $P<0.0001$) at day 6, with an increase in pH and wallum water concentration corresponding to increased mass and length (Fig. 4B).

After 12 days of development, the effects of pH and wallum water were still evident. The body masses of larvae were significantly influenced by treatment pH ($F_{4,190}=440.2$; $P<0.0001$) and wallum water treatment ($F_{4,190}=51.5$; $P<0.001$). Increasing pH and wallum water concentration resulted in an increased mass (Fig. 5A). In addition, the relationship between pH treatment and body mass was also affected by wallum water concentration ($F_{4,190}=38.6$; $P<0.001$), with the larger tadpoles coming from treatments with higher concentrations of wallum water (Fig. 5B). An increased wallum water concentration ($F_{4,190}=25.5$; $P<0.0001$) and pH ($F_{4,190}=487.2$; $P<0.001$) also resulted in an increased total body length.

Burst swimming performance at 12 days was significantly affected by both pH ($F_{2,189}=26.4$; $P<0.001$) and wallum water concentration ($F_{2,189}=9.10$; $P<0.001$) (Fig. 5C). Swimming speed was significantly slower for larvae from the pH 4.5 treatment than for both pH 5.0 and pH 6.7 (Tukey's *post-hoc*: $P<0.0001$), but no significant difference was detected between pH 5.0 and pH 6.7. When body size was used as a covariate in the analysis, swimming speed was still significantly influenced by both pH ($F_{2,188}=10.92$; $P<0.001$) and wallum water concentration ($F_{2,188}=4.1$; $P<0.001$). Larvae from more concentrated wallum water treatments had greater size-corrected swimming speeds than those from more dilute wallum water treatments. No significant interaction was detected between pH and wallum water treatment ($F_{2,188}=1.89$; $P<0.001$).

Of the seven path models that we evaluated, a model relating body length and wallum water concentration to swim speed (model E) was more than 64% likely to provide the best description of the data (Table 2; Fig. 6). Based on this model, total body length was directly affected by both pH and wallum water concentration. However, wallum water concentration and total body length also directly influenced swim speed. Because pH and wallum water

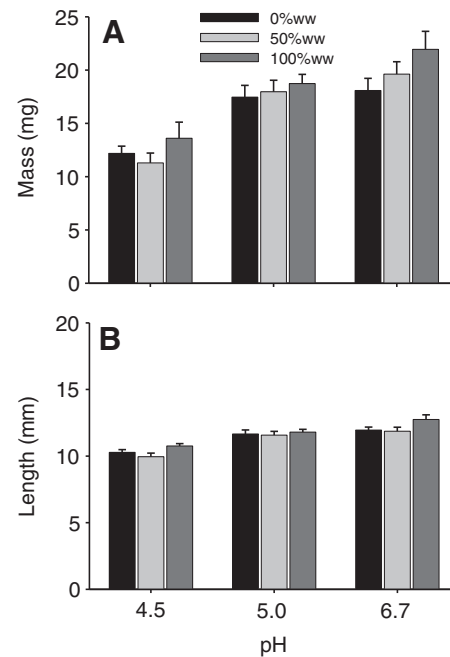


Fig. 4. The interactive effects of pH and wallum water concentration on the (A) mass and (B) length of larval *L. peronii* after 6 days of development. Significant effects of both pH and wallum water concentration were detected across treatments. Data represent means \pm 95% confidence limits.

concentration were uncorrelated, our interpretation of this model was unaffected by multi-collinearity.

DISCUSSION

We examined the effects of low pH on the survival, growth and development of *L. peronii* embryos and larvae, and the potentially important interactions with natural organic acids contained in wallum water. Embryos of *L. peronii* experienced both reduced growth and reduced survival when exposed to low pH and artificial soft-water. However, natural organic acids that are characteristic of natural wallum environments in south-east Queensland were not found to exacerbate these effects of low pH. Instead, we found some evidence that the natural wallum waters subtly improved growth and locomotor performance across all pH treatments.

In the absence of natural organic acids, the embryos and larvae of *L. peronii* suffered complete mortality at pH levels below 4.5 within 12 days of chronic exposure, and this mortality occurred

Table 2. A comparison of path analysis models describing the relationships between pH, wallum water treatment, total body length, body condition and burst swimming performance

Model	χ^2	d.f.	K	AIC	Δ_i	w_i
A: full model	2.629	1	19	103.96	55.49311	0
B: condition, length and wallum water	3.987	2	18	92.60	44.13316	0
C: condition, length and pH	11.195	2	18	99.81	51.34116	0
D: condition and length	16.181	3	17	93.89	45.42606	0
E: length and wallum water	2.247	1	13	48.47	0	0.6400
F: length and pH	5.067	1	13	51.29	2.82	0.1562
G: length	10.337	2	12	50.75	2.28883	0.2037

χ^2 , Chi-square goodness of fit; d.f., degrees of freedom; K, number of parameters; AIC, Akaike information criterion; Δ_i , differential of AIC; and w_i , Akaike weight. The Akaike weight of each model is the likelihood that a particular model best describes the data.

Model E was more than 60% likely to be the best predictor of the relationship between water treatment, morphology and performance.

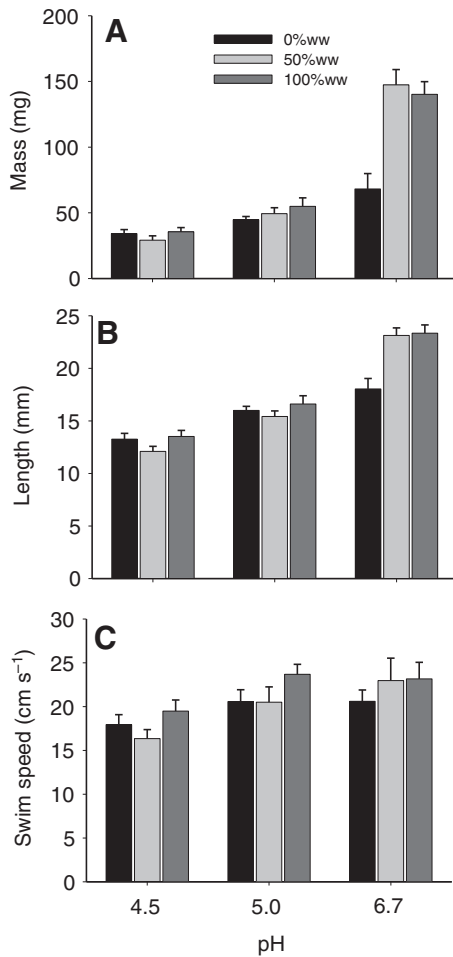


Fig. 5. The interactive effects of pH and wallum water concentration on the (A) mass, (B) length and (C) maximum swimming speed of larval *L. peronii* after 12 days of development (experiment 2). Significant effects of both pH and wallum water concentration were detected on mass and length (see Results). Data represent means \pm 95% confidence limits.

primarily before hatching. These values are similar to those previously reported for this species (Meyer, 2004) and other acid-sensitive amphibians (Gosner and Black, 1957; Pierce, 1985; Freda and Dunson, 1986; Whiteman et al., 1995). Most studies of amphibian larvae report that the embryonic life-stage is the most sensitive to low pH (Saber and Dunson, 1978; Pierce, 1985; Pierce and Wooten, 1992; Freda and Dunson, 1986). Although the mechanisms underlying the decreased hatching success are still largely unknown, it is likely to be due to chemical changes in the egg membrane that prevent the embryos from hatching rather than to any direct toxicity to the embryo (Gosner and Black, 1957; Freda and Dunson, 1986).

The decreased growth rate in the low pH treatments for *L. peronii* after 12 days of chronic exposure is probably due to damage of epithelium cells of the gill resulting in a loss of body sodium (Freda and Dunson, 1984; McDonald et al., 1984; Robinson, 1993; Meyer et al., 2009). Similarly, *Rana pipiens* tadpoles raised at the low pH of 4.5 were found to grow slower than those raised at pH 5.8 (Freda and Dunson, 1985). The growth rate and survival of the freshwater prawn *Macrobrachium rosenbergii* was also found to decrease at low pH (Chen and Chen, 2003). Neon tetras (*Paracheirodon innesi*), which are native to the acidic waters of the Amazon, possess

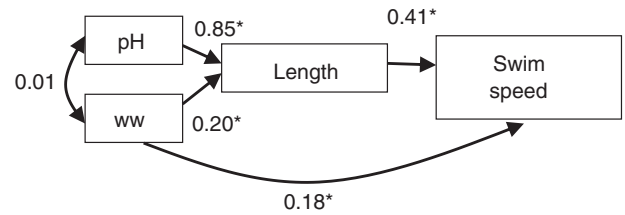


Fig. 6. The best path model (model E – see Fig. 1) that explains the relationships of pH and wallum water concentration to body size, condition and burst swimming performance. *Significant correlations among factors were detected at the level of $P < 0.05$.

a very high affinity Na^+ transporter that is completely insensitive to water pH and decreases the loss of sodium at low pH (Gonzalez et al., 1998). A similar mechanism may be important for acid-tolerant amphibian larvae under low pH conditions.

The lower swimming speeds observed for larvae raised under low pH conditions were associated with underlying differences in body size between the treatment groups. Burst swimming performance is frequently used as a correlate of fitness because of its perceived importance in predator avoidance. Although differences among treatments were due to body size, absolute rather than body-specific swimming speed is a more important determinant of predator escape. This suggests that the detrimental effects of low pH on swimming performance may reduce survival of larvae in the natural environment, because of decreased predator escape responses. Predation trials with larval pacific tree frogs *Hyla regilla* demonstrated that faster individuals were more likely to survive predation by garter snakes (*Thamnophis sirtalis*) than were slower tadpoles (Watkins, 1996).

Larvae of *L. peronii* raised in wallum waters with high natural organic acids were larger and exhibited faster swim speeds than those raised in water without natural organic acids at the same pH. In addition, embryos hatched sooner in water high in natural organic acids at low pH relative to artificial wallum water treatments, suggesting a positive effect of organic acids against the detrimental effects of low pH. This is interesting given that previous studies have suggested that humic substances may decrease hatching success at low pH (Dunson and Connell, 1982; Freda et al., 1990; Picker et al., 1993). Several previous studies of fish species from the naturally acidic blackwaters of the Amazon basin also suggest that the presence of natural humic substances in the water may have a positive influence on development, growth and ion fluxes (Gonzalez et al., 2002; Wood et al., 2003). For example, when Amazonian catfish (*Corydoras julii*) were exposed to low pH in Rio Negro water instead of deionized water (with the same concentrations of major ions), the detrimental effects of low pH on ion regulation were diminished. Like the wallum waters of south-east Queensland, the acidic blackwaters of the Rio Negro contain high concentrations of organic acids (i.e. dissolved humic substances). An increased growth rate at near neutral pH as seen in our study may also be due to the organic acids decreasing the diffusion of ions into the soft wallum water. Wood and colleagues suggested the mechanisms underlying the benefits of blackwaters at low pH are a protective function against the loss of sodium and chlorine ions at the gill surface (Wood et al., 2003). Interestingly, Gonzalez and colleagues suggest that the blackwater nature of the Amazon basin plays a key role in allowing the high degree of species diversity in the low pH environment (Gonzalez et al., 2002). Our path analysis revealed that the most likely model for predicting burst

swimming performance contained direct influences from total body length and wallum water concentration. Both wallum water concentration and pH also indirectly affected swimming performance by changes in total body length. This was not surprising given that total body length is often associated with an increased swimming speed for aquatic organisms, including larvae of *L. peronii* (Wilson and Franklin, 2000).

Because of the complex nature of humic substances, the exact mechanisms that either cause toxicity or provide a protective function are largely unknown. However, Campbell and colleagues found evidence that dissolved organic acids can bind to the cells of fish gills, which can change the permeability of the cell membrane and thereby affect the transport of molecules through the cell wall (Campbell et al., 1997). The organic acids may therefore have differing effects on the embryo and larvae of aquatic organisms depending on the molecules blocked. The primary protective effects of organic acids are often attributed to their ability to bind potentially toxic metal ions, rendering them biologically unavailable (Jackson, 1975; Frimmel, 1998; McGeer et al., 2002; Kozlova et al., 2009). The importance of dissolved humic substances in ameliorating or exacerbating the effects of low pH on homeostasis and their role in influencing the distribution and abundance of aquatic organisms are still poorly understood and warrant further study.

As with all studies on pH we had to alter the pH artificially. While it has been shown that acetic acid is a relatively non-toxic acid to amphibians (Ireland, 1991; Dawson et al., 1996) we cannot rule out the possibility that the results here are due to the use of acetic acid and may not be the same as those seen if we were to have used a different acid. Because of the complex nature of many of the natural organic acids it is difficult to know exactly how acetic acid alters their structure and therefore influences aquatic organisms. Our data also further highlight our limited understanding of the role pH and humic substances play in affecting the distribution and abundance of aquatic organisms, particularly amphibian populations. Although the ecological significance of humic substances remains unclear, this study shows that *L. peronii* embryos and larvae are not adversely affected by high concentrations of dissolved organic acids within the acidic water of wallum environments. This study also found that *L. peronii* embryos and larvae were intolerant of low pH; however, this does not entirely explain their absence from the lower pH environments of the stressful wallum habitats. It is unlikely that the combination of low pH and high dissolved organic acid content is what is exclusively limiting *L. peronii* reproduction in low pH environments. Rather, it is likely that the combined effect of stress caused by low pH and other potential stresses such as competition, predation, exposure to UV and a plethora of other influences are what is limiting the distribution of *L. peronii* in these stressful habitats.

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