# The Acid Test: pH Tolerance of the Eggs and Larvae of the Invasive Cane Toad (*Rhinella marina*) in Southeastern Australia

**Uditha Wijethunga**
**Matthew Greenlees**
**Richard Shine***
School of Biological Sciences A08, University of Sydney, New South Wales 2006, Australia

Accepted 2/28/2015; Electronically Published 4/16/2015

## ABSTRACT

Invasive cane toads are colonizing southeastern Australia via a narrow coastal strip sandwiched between unsuitable areas (Pacific Ocean to the east, mountains to the west). Many of the available spawning sites exhibit abiotic conditions (e.g., temperature, salinity, and pH) more extreme than those encountered elsewhere in the toad's native or already invaded range. Will that challenge impede toad expansion? To answer that question, we measured pH in 35 ponds in northeastern New South Wales and 8 ponds in the Sydney region, in both areas where toads occur (and breed) and adjacent areas where toads are likely to invade, and conducted laboratory experiments to quantify effects of pH on the survival and development of toad eggs and larvae. Our field surveys revealed wide variation in pH (3.9–9.8) among natural water bodies. In the laboratory, the hatching success of eggs was increased at low pH (down to pH 4), whereas the survival, growth, and developmental rates of tadpoles were enhanced by higher pH levels. We found that pH influenced metamorph size and shape (relative head width, relative leg length) but not locomotor performance. The broad tolerance range of these early life-history stages suggests that pH conditions in ponds will not significantly slow the toad's expansion southward. Indeed, toads may benefit from transiently low pH conditions, and habitat where pH in wetlands is consistently low (such as coastal heath) may enhance rather than reduce toad reproductive success. A broad physiological tolerance during embryonic and larval life has contributed significantly to the cane toad's success as a widespread colonizer.

*Keywords:* acidity, pH, *Bufo marinus*, development, invasive species, physiological tolerance, amphibian.

*Corresponding author; e-mail: rick.shine@sydney.edu.au.

*Physiological and Biochemical Zoology* 88(4):433–443. 2015.  1522-2152/2015/8804-4143$15.00. DOI: 10.1086/681263

## Introduction

Introduced species are an increasing problem for conservation worldwide, often exerting severe impacts on native taxa (Kupferberg 1997; Lodge et al. 2000; Johnson et al. 2007). Wildlife managers thus need to predict the likelihood of invasion into specific habitat types in order to identify conservation priorities and focus management efforts on areas likely to be imperiled (Sakai et al. 2001). Several approaches have been developed for this purpose, mostly centered on bioclimatic models that identify areas that provide abiotic (i.e., thermal, hydric) conditions similar to those within the invader's native range and/or the areas already invaded (Kearney et al. 2008). Other models integrate information on organismal functioning to create individual-based predictions on the conditions necessary for the invasive species to thrive (Kearney et al. 2008). Although these models are useful, they are difficult to extrapolate to conditions that do not occur within the invader's current range. As a consequence of escaping from competitors and predators, many invasive taxa can spread into areas outside the climatic envelope occupied within their native range (Sakai et al. 2001; Broennimann et al. 2007; Tingley et al. 2014). To assess the probability of such extensive spread, we need to identify the abiotic challenges presented by these extralimital regions and the invader's ability to tolerate those conditions (e.g., McCann et al. 2014).

Many successful invasive species exhibit broad physiological tolerances (Lee 2002), but generalities are elusive. For example, a species that is highly tolerant in one niche dimension (e.g., thermal) may have narrow tolerances on another (e.g., hydric; Tingley et al. 2012). Thus, it is not enough to simply rank invasive species as tolerant or sensitive to abiotic extremes; we need to explore each parameter (and the interactions of parameters) in order to predict distributional limitations and the potential for niche occupancy. Thus, managers need to quantify the nature of challenges in an environment that has yet to be invaded and assess whether the invader will be able to deal with those challenges.

We have conducted a study of this type at the southern extreme of the invaded range of cane toads (*Rhinella marina*; Linnaeus 1758) in Australia. Native to Central America and South America, this large bufonid anuran has been translocated to more than 40 countries worldwide in misguided attempts to control insect pests (Lever 2001). Despite its origins in well-watered tropical areas, the cane toad has spread suc-

cessfully in many Australian habitats that superficially appear to be climatically unsuitable, including arid, semidesert regions (Florance et al. 2011; Tingley and Shine 2011) and high mountains (Newell 2011; McCann et al. 2014). That success reflects broad thermal tolerances (Floyd 1984) and a flexible adjustment of activity patterns to moisture availability in the landscape (Brown et al. 2011). For example, cane toads have colonized arid sites by modifying their times and places of activity and their desiccation thresholds for movement (Tingley and Shine 2011; Tingley et al. 2012; Webb et al. 2014). They have colonized high, cold montane areas by rapidly adjusting their critical thermal minima (McCann et al. 2014).

Thermal and hydric extremes are not, however, the only challenges faced by cane toads at the expanding edges of their invaded range in Australia. At the southern invasion front in northeastern New South Wales (NSW), the toad faces novel challenges. The Great Dividing Range runs parallel to the east coast for 3,500 km, mostly less than 100 km from the ocean (McGlashan and Hughes 2001). Sandwiched between the ocean and the mountains, a narrow coastal corridor makes up the major route for farther-southward expansion of the toad invasion. The front is progressing less rapidly in this area than in tropical Australia (Urban et al. 2007), plausibly reflecting thermal constraints (Sutherst et al. 1996). Nonetheless, other factors may constrain toad invasion as well. In these near-coastal areas, few ponds may provide the conditions available at spawning sites used by toads in other parts of their range (Hagman and Shine 2006). Salinity and pH, as well as temperature, in these south-coastal ponds may well differ from conditions seen in toad-spawning ponds over the rest of the anuran's extensive range.

Pond acidity may constrain the ability of toads to breed along Australia's east coast. Wetlands in coastal heath and wallum, widespread in this region, exhibit high concentrations of tannins and low pH, generally ranging from 3.5 to 4.1 (Barth and Wilson 2010). Early developmental stages of many amphibians are sensitive to acidity (Pierce 1985, 1993; Freda 1986). Deposition of spawn into water that is highly acidic or basic can cause embryonic mortality by preventing expansion of perivitelline membranes (Gosner and Black 1957) and inhibiting hatching enzymes (Dunson and Connell 1982). Such conditions often delay hatching and disrupt development (Gosner and Black 1957). Although amphibian larvae are more tolerant of acidity than are embryos (Freda 1986), tadpoles in low-pH conditions display elevated sodium efflux (Freda and Dunson 1985*b*), depressed growth rate (Freda and Dunson 1985*b*), delayed metamorphosis, smaller size at metamorphosis, and poor reproductive success (Wilbur and Collins 1973; Smith-Gill and Berven 1979). However, tolerance to acidity varies interspecifically (Pierce 1993), and some bufonids can develop successfully under acidic conditions (e.g., pH 4–4.5: *Bufo calamita* eggs [Beebee 1986]; pH 4.1–6.3: *Bufo americanus* eggs [Clark and Lazerte 1985; Dale et al. 1985; Glooschenko et al. 1992]; pH 4.8: *Bufo bufo* larvae [Dolmen and Skei 2006]).

To assess whether local environmental conditions may inhibit the ability of this invasive species to successfully reproduce, we measured pH in water bodies in northeastern NSW, near the southern front of the main toad invasion. We repeated the work in Sydney (500 km farther south), where an extralimital population of toads occurs. We also conducted a laboratory experiment, raising eggs and tadpoles at pH levels recorded in natural water bodies to clarify how pH affects hatching success, development, and performance of both tadpoles and metamorph toads.

## Material and Methods

### *Study Species and Area*

Introduced to northeastern Australia in 1935, cane toads have now spread across much of the Australian tropics and subtropics, from northwestern Australia to the southeastern coast (Urban et al. 2007; Phillips et al. 2010*a*). In Australia (and in their native range; Evans et al. 1996), female toads have been reported to lay their eggs in shallow lentic water bodies with a pH of 5–7, temperature of 27°–32°C, salinity of 0.01–0.02 ppt, and dissolved oxygen content of 67%–107% in freshwater (based on measurements in 23 water bodies; Hagman and Shine 2006; Semeniuk et al. 2007). Female cane toads lay up to 30,000 eggs in a single clutch (Lever 2001), with the eggs hatching within 1–2 d and developing for 2–6 wk before metamorphosing (Alford et al. 1995; Lever 2001).

We conducted our fieldwork close to the toad invasion front in northeastern NSW. Toads first appeared in this area between 1964 and 1966, at Byron Bay, and had spread throughout most of the study area by 1977 (van Beurden and Grigg 1980). In this region, they occupy (and thus have the opportunity to breed in) coastal bogs, swamps, shallow coastal lagoons, roadside culverts, and ponds among dry sclerophyll forest and farmland (van Beurden and Grigg 1980). Rainfall and temperature across this region are mildly seasonal; mean annual rainfall is 568 mm, with most of the rain occurring from May to November (Bureau of Meteorology 2013).

### *Surveys of Water Bodies*

We measured the water quality (salinity and temperature, including pH) of 43 ponds—35 ponds from areas within and adjacent to the toad's invaded range in northeastern NSW (centered on 33°56′38.69″S, 147°56′57.41″E) and an additional 8 ponds from Sydney, where an isolated population of toads has recently become established (33°56′38.69″S, 147°56′57.41″E). Pond pH was measured 1–4 times per pond. These water bodies ranged from small roadside culverts to sediment-retention basins, natural wetlands, and farm dams and included several sites where toads are known to have spawned. Measurements were taken between November 2012 and September 2013, between 0900 and 2000 h. We measured pH using a digital meter (EC-PCST Testr35, Eutech, Singapore; accuracy $\pm 0.01$ pH). We measured pH between 1–2 m from the edge of the pond by immersing the probe of the digital pH meter to a depth of 5 cm. The meter was gently agitated for 2–3 min in the water, and the reading was taken immediately

after withdrawing and gently shaking excess water off the probe. In nine ponds, we recorded pH daily over three consecutive days. At each pond, pH was measured at two separate locations, at least 2 m apart (two measurements per site each day when measurements were taken). The average pH for each pond was calculated based on the pH readings from September–October 2012 and March–September 2013.

### *pH Tolerance of Toad Eggs and Larvae*

Laboratory experiments were conducted in 20-L plastic containers, filled to 50 mm below the top. Water in each container was preconditioned with aquarium water ager (API Tapwater Conditioner, Chalfont, PA; 1 mL per 76 L) and aerated with a 220–240-V aerator (Resun LP-100, Longgang, Shenzhen, China) pumping through a 28.4-mm cylindrical air stone (A960 Marina Air Stone, Hagen, Baie d'Urfé, Quebec). Eggs were assigned to one of five treatments reflecting the range of acidity levels (pH 4–10; see below) found in our field surveys; rooms in which experimental containers were kept were temperature controlled, allowing water temperature to remain at a level typical of natural ponds (21°–22°C). Lighting was provided artificially, on a 12L : 12D photoperiod. During the experiments, we measured pH and temperature daily with a pH meter (EC-PCST Testr35, model PTTEST35, Eutech, Singapore) and a digital thermometer (KM28, Comark Instruments, Beaverton, OR; accuracy $\pm 0.1$°C). We maintained pH in the experimental containers at 4, 5.5, 7 (neutral), 8.5, or 10 by controlled addition of 1 M $H_2SO_4$ (for acid treatments) or 1 M NaOH (for alkaline treatments).

To stimulate oviposition and fertilization, we injected adult cane toads (from the northeastern NSW study area) with 0.4 mL (males) or 0.8 mL (females) of Leuprorelin acetate (0.5 mg/mL; Lucrin, Abbott Australasia, Botany, NSW) diluted at 1 : 20 with saline (see Kelehear et al. 2009 for details). Following deposition of eggs into water of pH 7, viable eggs containing early embryos (Gosner stage 10–13, 100 per container; Gosner 1960) were placed into 15-L solutions of the five pH treatments, with six replicates per treatment (randomly arranged in the experimental area). We monitored the development of the embryos daily until hatching. Dead embryos (decomposing or covered in fungus; Pough 1976) were identified and removed to prevent fouling.

To measure the impact of pH on larval life, we maintained the tadpoles in the same pH conditions under which they had been kept as eggs. Differential survival in the egg stage (see below) thus translated into unequal sample sizes for the larval study. The number of surviving larvae was counted weekly. To minimize any effects of variation in density as a consequence of differential mortality, we consolidated tadpoles within treatment groups weekly after the third week posthatching. We fed the tadpoles with boiled lettuce ad lib. twice per week, also renewing the water at these times. We photographed five individuals per treatment each week to determine their Gosner stage and snout-vent length (SVL), the latter using the image analysis software ImageJ (Abràmoff et al. 2004). We calculated metamorphic age for each tadpole as the number of days elapsed between Gosner stages 25 (a week after hatching) and 42 (when the forelimbs were developed). We estimated the daily rate of body growth of tadpoles in each treatment as the increment in body length between Gosner stages 25 and 42, divided by the metamorphic age. Metamorphosing tadpoles (those at stage 42) were reared in separate containers (under the same pH as previously) for 3 wk, until they reabsorbed their tail (Gosner stage 46). We then measured their locomotor performance (speed and endurance on a racetrack; see Llewelyn et al. 2010 for details of methods), SVL, intraorbital distance (IOD), and tibia length (using digital calipers; accuracy $\pm 0.01$ mm). All animal experiments were conducted according to the guidelines for care and use of animals approved by the ethics committee of the University of Sydney (ethics protocol approval L04/08-2012/3/5807).

### *Statistical Analyses*

We used ANOVA in SPSS, version 21 (IBM, Armonk, NY), and an $\alpha$ level of $P = 0.05$ for statistical analyses of egg survival (hatching %) and metamorph traits. When ANOVA gave significant results, we used pairwise comparisons to identify significant differences among treatments. Analyses for survival of tadpoles and growth over time involved repeated measurements on the same treatment over time and were based on average results for each container to avoid pseudoreplication. We used generalized estimation equations (GEE) to determine whether pH affects the growth and survival of tadpoles and the variation of pH in ponds over time (Zeger et al. 1988; Lee et al. 2007).

*Model 1.* We used GEE with a gamma error distribution (log link function) and an autoregressive (AR1) working correlation matrix to determine the effect of rearing pH on the growth rates of tadpoles; this was done separately for the first 4 wk and the last 4 wk. The two periods were examined separately because of differences in density among treatments in the first 4 wk due to differential mortality. Approximately equal densities were maintained among replicates from the fourth until the eighth week, after which differential rates of metamorphosis again generated density divergences among replicates. To explore how pH influences the growth rates of tadpoles over time, we added an interaction term (pH × week). We used GEE with a gamma distribution (log link function) and an AR1 working correlation matrix to assess the relationship between this interaction and two dependent variables (tadpole body lengths and growth rates).

*Model 2.* To examine whether pH affected tadpole survival, we used the same GEE design as above (including the pH × week interaction) on data for the first 4 wk of the experiment (because the density differed among treatments over the first 4 wk, as above).

*Model 3.* Because tadpole density varied among treatments during the first 4 wk and because growth rate and survival are likely to depend on competitor density, we used GEE with a gamma error distribution (log link function) and an AR1

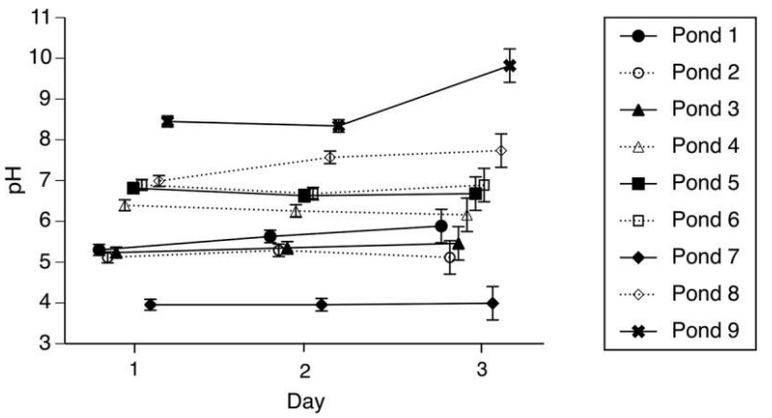


Figure 1. Variation of pH in a sample of nine ponds in northern New South Wales, Australia, over three consecutive days. These data were collected using a digital pH meter. The figure shows mean values and associated standard errors.

working correlation matrix to explore the effect of the interaction term (pH × tadpole density) on the growth rates and survival of tadpoles, based on data from the first 4 wk. Additionally, we ran the model with a third-order interaction (pH × density × week). Corrected quasi-likelihood under the independent model criterion verified that the best model included only main effects.

*Model 4.* To test the variation of pond pH over time, we used GEE with a gamma error distribution (log link function) and an AR1 working correlation matrix, as above.

## Results

### *Surveys of Water Bodies*

Overall, we recorded considerable variation in the pH of water bodies (from pH 4 to pH 10). Among ponds where pH was measured over consecutive days, variation over time was marginally significant ($P = 0.032$) and much lower than variation among ponds ($P < 0.0001$; fig. 1).

### *pH Tolerance of Toad Eggs*

Hatching success varied with pH (ANOVA: $F_{4,25} = 10.220$, $P < 0.0001$; see fig. 2) and was greatest in highly acidic water. At pH 4, an average of 63% of eggs developed to stage 30–32 (hatching). The proportion of hatching declined with increasing pH, with only 7% surviving to hatching from the most alkaline treatment (post hoc Tukey's honestly significant difference between treatments: pH $4 > 5.5 = 7 > 8.5 > 10$).

### *pH Tolerance of Toad Larvae*

The pH to which a toad tadpole was exposed affected the animal's growth rate (total length of the tadpoles, $P < 0.05$). At higher pH, tadpoles were larger (positive regression coefficients; table 1). The impact of pH on growth rates varied through time (interaction pH × week, $P < 0.0001$). Tadpoles in alkaline water (pH 8.5 and 10) grew quickly after hatching, whereas growth was slow in their siblings raised in acidic (pH 4) water (fig. 3). The impact of pH on tadpole growth continued into the last 4 wk of the experiment ($P < 0.0001$; fig. 3; table 1). The positive regression coefficients at pH 5.5, 7, and 8.5 show increasing growth rates of the tadpoles through time. In highly acidic conditions (pH 4), however, growth rates decreased with time (negative regression coefficient).

The survival rates of tadpoles were also affected by pH (model 2; $P < 0.0001$; fig. 4), with the effect changing through time (interaction pH × time, $P < 0.0001$; table 2). An additional analysis that incorporated tadpole density showed that survival rates were lower in more crowded containers and more so at some times than others (thus, interaction pH × tadpole density × week, $P < 0.0001$ in model 3) over the first 4 wk. Growth rates, in

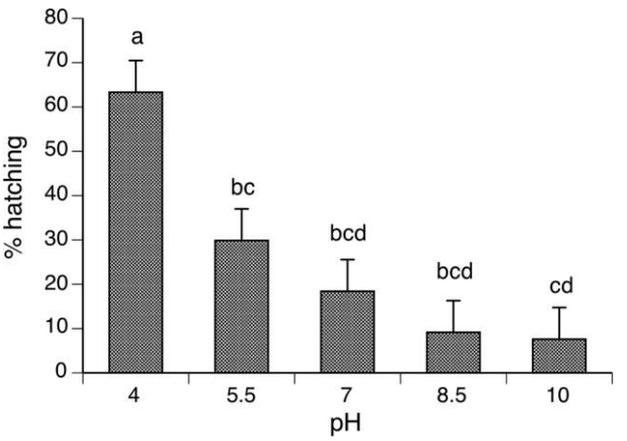


Figure 2. Hatching success (%) of cane toad eggs in different pH treatments. These data were obtained by counting the number of hatchlings in each treatment after exposing 100 egg strands in each treatment. The pH treatments to which the eggs were exposed were pH 4, 5.5, 7, 8.5, and 10. The figure shows mean values and associated standard errors. Means not sharing the same letters indicate statistically significant differences between the treatments as determined by one-way ANOVA, followed by Tukey's pairwise comparison.

Table 1: Analyses of the generalized estimation equation (GEE) parameter estimates of tadpole length during weeks 5–8 at a range of pH treatments

| | | | 95% Wald confidence interval | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| Parameter | $\beta$ | SE | Lower | Upper | Wald $\chi^2$ | df | $P$ |
| Intercept | 2.582 | .0419 | 2.500 | 2.664 | 3,801.955 | 1 | <.0001 |
| pH 8.5 | .404 | .0423 | .321 | .487 | 90.869 | 1 | <.0001 |
| pH 7 | .296 | .0684 | .161 | .430 | 18.668 | 1 | <.0001 |
| pH 5.5 | .306 | .0466 | .215 | .398 | 43.306 | 1 | <.0001 |
| pH 4 | 0[a] | … | … | … | … | … | … |
| Week | .032 | .0037 | .025 | .039 | 76.548 | 1 | <.0001 |
| pH 8.5 × week | .034 | .0050 | .024 | .044 | 45.564 | 1 | <.0001 |
| pH 7 × week | .059 | .0161 | .028 | .091 | 13.621 | 1 | <.0001 |
| pH 5.5 × week | .047 | .0131 | .022 | .073 | 13.171 | 1 | <.0001 |
| pH 4 × week | 0[a] | … | … | … | … | … | … |
| Scale | .005 | … | … | … | … | … | … |

Note. Analysis of model 1 GEE parameter estimates based on the robust variance estimates, using an autoregressive working correlation matrix, with total length of tadpoles during weeks 5–8 as the outcome variable and pH and pH × week as explanatory variables.

[a]Set to 0 because this parameter is redundant.

contrast, were relatively unaffected by tadpole densities (e.g., similar growth rates in pH treatments 5.5, 7, and 8.5 despite disparate densities; fig. 3; table 3).

The duration of larval life was broadly similar in most pH treatments, but those that were raised in alkaline water (pH = 8.5) emerged earlier than did their siblings raised at pH 5.5 or 7 ($F_{2,102} = 3.605$, $P = 0.031$; table 4). The pH treatment also influenced a metamorph's SVL ($F_{2,66} = 3.997$, $P = 0.023$) and its tibia length relative to SVL ($F_{2,66} = 11.986$, $P < 0.0001$). Metamorphs that emerged from alkaline water (pH = 8.5; table 4) were larger (fig. 5) and longer legged than those from other pH treatments (table 4). The IOD (relative to SVL) of metamorphs from pH 5 was smaller than that of metamorphs from pH 7 and 8.5 ($F_{2,66} = 10.367$, $P < 0.0001$; table 4). Notably, metamorph morphometrics (tibia, SVL, and IOD) were nonindependent of larval duration (Pearson's correlation, $P < 0.01$). However, neither locomotor speed (body lengths per second; $F_{2,66} = 0.527$, $P = 0.593$; table 4) nor endurance (total body lengths traveled before exhaustion; $F_{2,66} = 0.146$, $P = 0.864$) and absolute speed ($F_{2,66} = 1.038$, $P = 0.36$) of metamorphs was significantly affected by prior pH exposure.

## Discussion

As cane toads extend southward along the coast of NSW, these invasive anurans are encountering potential breeding sites that differ from those in the toad's native range (Evans et al. 1996) and in already colonized parts of Australia (Hagman and Shine 2006; Semeniuk et al. 2007). Temperatures are lower than in most areas already occupied by cane toads, below the tadpoles' thermal optima, but still within the tolerance limits of toad embryos and larvae (Floyd 1983). In our water body surveys, spatial heterogeneity in pond temperatures (both within and among ponds) was low. In contrast, pH conditions were variable through space and (to a lesser degree) time. Our experimental studies show that major aspects of the cane toad's early life history (hatching success, larval growth rate, development rate and survival, metamorph morphology) are significantly affected by pH variation over the range that we measured in natural water bodies. Importantly, however, we recorded at least some survivors over a wide range of pH treatments (pH 4–8). Thus, variable (and sometimes extreme) pH conditions in water bodies in southeastern Australia likely will affect toad viability (and thus colonization and estab-

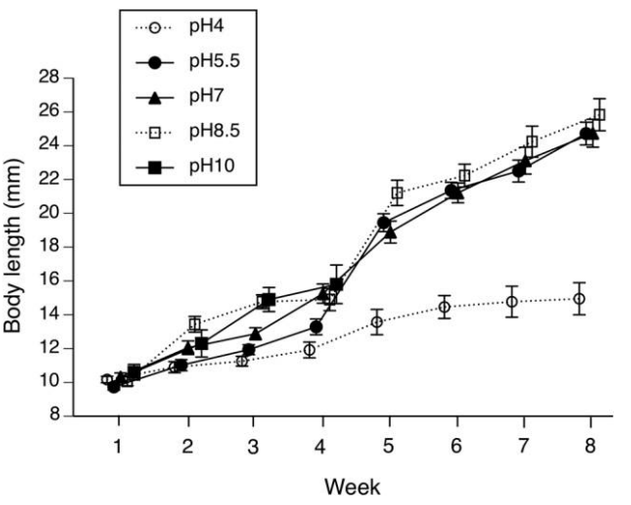


Figure 3. Effect of pH treatment on total body length (mm) of cane toad tadpoles through time. Growth data was obtained by photographing 30 tadpoles from each treatment every week and measuring the total body length using ImageJ software. Tadpole density was not equal among treatments during the first 4 wk of the experiment but was equal during the last 4 wk (weeks 5–8) of the exposure. The figure shows mean values and associated standard errors.

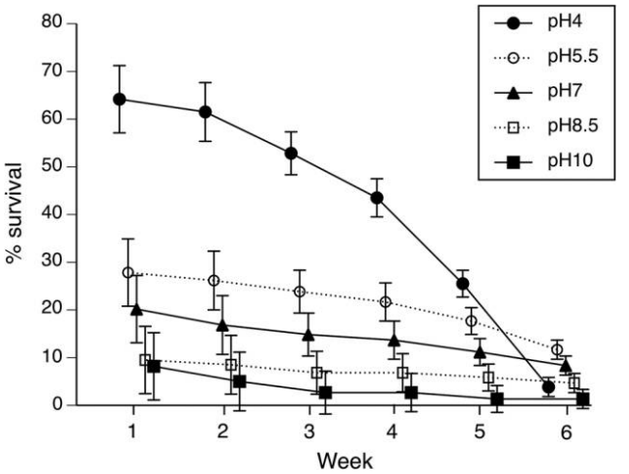


Figure 4. Effect of pH treatment on survival rates (%) of successfully hatched cane toad tadpoles. The number of tadpoles were counted weekly in each pH treatment until they began to metamorphose. The figure shows mean values plus associated standard errors.

lishment success) but are unlikely to constrain the eventual limit of toad distribution. As in many successful colonizing species, the cane toad has a broad physiological tolerance of pH variation, as it does (at least as adults) for temperature (McCann et al. 2014).

In several respects, our results differ from those of studies on other anuran taxa. For example, many anurans show high egg mortality at low pH (Dunson and Connell 1982; Freda 1986; Freda and Dunson 1986), apparently due to disruption of superficial tissues (Tome and Pough 1982). In contrast, we found the highest hatching success of cane toad eggs at the lowest pH we studied (pH 4), perhaps because the thick, gelatinous capsule around the eggs buffers early embryos from prevailing environmental conditions. The underlying cause for higher embryonic survival at pH 4 remains unclear but might also involve suppression of microorganisms and might be affected by water body chemistry, such that lab results do not reliably mimic field effects (Persson et al. 2007). Future experimental work could usefully examine this topic.

Despite enhancing hatching success, low pH reduced larval survival. The same may be true in other anurans, based on reports of successful hatching but no surviving larvae in ponds with low pH (Freda and Dunson 1986). Survival of tadpoles in acidic water may be reduced by disruption of ionic regulation, especially the loss of sodium (Freda and Dunson 1984, 1985*b*). Acute exposure to low pH inhibits active uptake of $Na^+$ and $Cl^-$ by the gills and causes massive loss of these ions. Larval death ensues when about half of the body's $Na^+$ content is lost (Freda and Dunson 1984).

Toad tadpoles raised in lower pH showed slower rates of growth, as did the tadpoles of *Rana pipiens* (albeit over a far more narrow range of pH 4.4 vs. pH 5.8; Freda and Dunson 1985*a*, 1985*b*; Freda 1986). Conversely, more alkaline water (pH 8.5) enabled earlier metamorphosis. The energy costs of ion-exchange challenges at low pH (above) might explain that effect, and alternatively (or additionally), low pH may elicit a reduction in swimming and feeding activity (as it does in other aquatic vertebrates such as fishes; Tembo 2009). In turn, lower growth rates may reduce tadpole viability by rendering the larvae more susceptible to ingestion by gape-limited predators and by extending the duration of larval life. Prolongation of development may result in continued exposure to stressful conditions (Denver 2009; Bowerman and Christopher 2010) or even result in tadpoles failing to metamorphose before their ephemeral pond dries out (Wilbur 1984; Freda 1986). In our own study, the effects of pH on growth were confounded with effects of density due to differential mortality rates. Amphibian larval density affects per capita growth rates, survivorship,

Table 2: Analysis of the generalized estimation equation (GEE) parameter estimates of effect of treatment on survival of the tadpoles at a range of pH treatments

| | | | 95% Wald confidence interval | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| Parameter | $\beta$ | SE | Lower | Upper | Wald $\chi^2$ | df | $P$ |
| Intercept | 4.722 | .1580 | 4.413 | 5.032 | 893.566 | 1 | <.0001 |
| pH 8.5 | −1.877 | .7542 | −3.355 | −.398 | 6.192 | 1 | .013 |
| pH 7 | −1.946 | .3708 | −2.672 | −1.219 | 27.536 | 1 | <.0001 |
| pH 5.5 | −1.422 | .3410 | −2.091 | −.754 | 17.402 | 1 | <.0001 |
| pH 4 | −1.195 | .2315 | −1.648 | −.741 | 26.620 | 1 | <.0001 |
| Week | 0[a] | ... | ... | ... | ... | ... | ... |
| pH 8.5 × week | −.368 | .0419 | −.450 | −.285 | 76.928 | 1 | <.0001 |
| pH 7 × week | .209 | .1596 | −.103 | .522 | 1.720 | 1 | .190 |
| pH 5.5 × week | .249 | .0596 | .132 | .366 | 17.471 | 1 | <.0001 |
| pH 4 × week | .224 | .0671 | .092 | .355 | 11.107 | 1 | .001 |
| Scale | .210 | ... | ... | ... | ... | ... | ... |

Note. Analysis of model 2 GEE parameter estimates based on the robust variance estimates, using an autoregressive working correlation matrix, with survival of tadpoles (within the first 4 wk) as the outcome variable and time, pH, and pH × week as explanatory variables.

[a] Set to 0 because this parameter is redundant.

Table 3: Analysis of generalized estimation equation (GEE) parameter estimates of the length of tadpoles at weeks 1–4 at a range of pH treatments

| | | | 95% Wald confidence interval | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| Parameter | $\beta$ | SE | Lower | Upper | Wald $\chi^2$ | df | $P$ |
| Intercept | −45.377 | … | … | … | … | 1 | <.0001 |
| pH 4 | 41.698 | .7345 | 40.258 | 43.138 | 3,222.532 | 1 | <.0001 |
| pH 5.5 | 40.520 | .3449 | 39.844 | 41.196 | 13,802.322 | 1 | <.0001 |
| pH 7 | 42.260 | .4605 | 41.357 | 43.162 | 8,420.762 | 1 | <.0001 |
| pH 8.5 | 39.541 | 1.4407 | 36.717 | 42.364 | 753.259 | 1 | <.0001 |
| pH 10 | 0[a] | … | … | … | … | … | … |
| Week | 8.581 | … | … | … | … | 1 | <.0001 |
| Density | .741 | … | … | … | … | 1 | <.0001 |
| pH 4 × week × density | .009 | .0039 | .001 | .017 | 5.190 | 1 | .023 |
| pH 5.5 × week × density | .006 | .0040 | −.002 | .014 | 2.463 | 1 | .117 |
| pH 7 × week × density | .012 | .0089 | −.005 | .030 | 1.941 | 1 | .164 |
| pH 8.5 × week × density | −.056 | .0271 | −.109 | −.003 | 4.278 | 1 | .039 |
| pH 10 × week × density | −.051 | … | … | … | … | 1 | <.0001 |
| pH 4 × density | −.770 | .0104 | −.790 | −.749 | 5,503.561 | 1 | <.0001 |
| pH 5.5 × density | −.741 | .0112 | −.763 | −.719 | 4,356.906 | 1 | <.0001 |
| pH 7 × density | −.779 | .0242 | −.826 | −.731 | 1,034.859 | 1 | <.0001 |
| pH 8.5 × density | −.603 | .0762 | −.752 | −.453 | 62.476 | 1 | <.0001 |
| pH 10 × density | 0[a] | … | … | … | … | … | … |
| pH 4 × week | −8.678 | .2619 | −9.191 | −8.164 | 1,097.898 | 1 | <.0001 |
| pH 5.5 × week | −8.302 | .1215 | −8.541 | −8.064 | 4,666.559 | 1 | <.0001 |
| pH 7 × week | −8.719 | .1808 | −9.073 | −8.364 | 2,324.473 | 1 | <.0001 |
| pH 8.5 × week | −7.759 | .4697 | −8.680 | −6.839 | 272.844 | 1 | <.0001 |
| pH 10 × week | 0[a] | … | … | … | … | … | … |
| Scale | .634 | … | … | … | … | … | … |

Note. Model 3 GEE parameter estimates based on the robust variance estimates, using an autoregressive working correlation matrix, with total length of tadpoles during weeks 1–4 as the outcome variable and pH, week, density, pH × week × density, pH × density, and pH × week as explanatory variables.

[a]Set to 0 because this parameter is redundant.

and time to metamorphosis (Wilbur 1977; Dash and Hota 1980; Crump 1981; Semlitsch and Caldwell 1982; Cameron 1994). Nonetheless, differences in tadpole body sizes among our treatments cannot be attributed entirely to density effects, because growth rates differed substantially even in pH treatments with very similar densities (fig. 3). We note also that our experimental design mimicked the actual conditions in the pond; for example, tadpoles growing up in water that caused high egg survival (such as pH 4 in our experiment) would subsequently encounter higher conspecific densities than would tadpoles growing up in a pond where pH conditions resulted in low egg survival.

Interestingly, pH treatments affected developmental trajectories as well as rates of growth, development, and survival. Similar plasticity is widespread in anurans (Newman 1992; Skelly 1997). Toad metamorphs emerging from alkaline treatments (pH 8.5) had larger bodies and relatively longer limbs, and the metamorphs from slightly acidic water (pH 5.5) had narrower heads (smaller intraorbital distance) relative to body length than did metamorphs from other treatments. In our study, these treatment-induced differentials in metamorph size and shape did not translate into performance differentials. Neither the absolute speed nor the distance jumped differed significantly among toadlets from the four treatments.

Table 4: Mean values of morphometric characteristics and locomotor performance of the toadlets at different pH treatments

| Treatment (pH) | SVL (mm) | IOD[a] | Tibia length[a] | Metamorphic age (wk) | Absolute speed (cm/s) | Endurance (body lengths/trial) | Locomotor speed (body lengths/s) |
|---|---|---|---|---|---|---|---|
| 5.5 | 9.256 | .327 | .419 | 9.00 | .549 | 417.1 | .587 |
| 7 | 9.327 | .351 | .400 | 8.80 | .581 | 455.1 | .613 |
| 8.5 | 9.983 | .331 | .426 | 7.70 | .690 | 395.7 | .684 |

Note. These values are calculated from analysis of variance. SVL = snout-vent length; IOD = intraorbital distance.

[a]As a proportion of SVL.

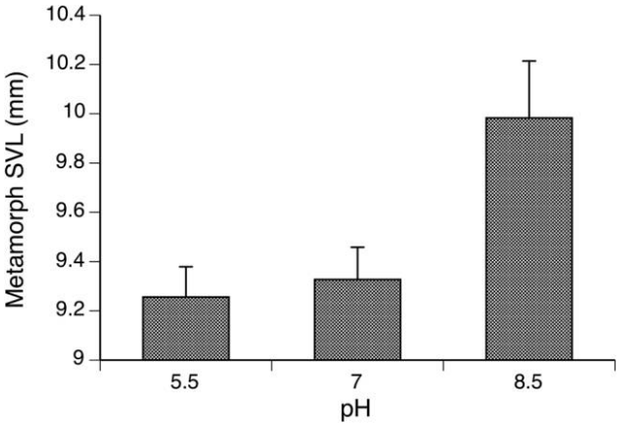


Figure 5. Effect of pH treatment on snout-vent length (SVL; mm) of cane toad metamorphs. The SVL of each metamorph was measured using vernier calipers after 3 wk of metamorphing in each pH treatment (pH 5.5, $n = 32$; pH 7, $n = 28$; pH 8.5, $n = 9$). The figure shows mean values and associated standard errors.

Cane toads in their native range (in Venezuela) reportedly breed in clear water with pH only slightly higher than neutral (7.1–7.6), with low water turbidity, and at a water temperature of 29°–30°C (Evans et al. 1996). Similar pH results have been obtained from studies of toad-spawning ponds in northern NSW (slightly north of our own study sites), with water close to neutral (pH 6.99–7.22; Semeniuk et al. 2007). In tropical Australia, however, toads often spawn in more acidic water bodies (pH 5.28–5.33; Hagman and Shine 2006). In combination, these studies suggest that toads often spawn in relatively neutral acidities (both in the native range and in the invaded range) but sometimes breed in ponds that offer more extreme pH conditions. Our laboratory study shows that in both the embryonic and larval stages, cane toads can tolerate a wide range of pH as extreme as any that we have recorded in nature. Takano and Iijima (1937) also reported that the tadpoles of *Rhinella marina* can successfully develop at pH 4–9.

It is difficult to translate these experimentally induced shifts in survival, growth, larval duration, and metamorph traits into any simple evaluation of viability. From a management perspective, we want to know how the recruitment of cane toads is affected by pond pH conditions, but to do so, we need to follow individuals through their long lives (Lever 2001) to measure lifetime consequences of variation in these traits. Plausibly, many of the traits that are affected by pH impact toad viability. This is certainly true for survival measures, with pH affecting both egg survival and tadpole survival—but in a complex fashion, such that the optimal pH for egg survival was catastrophic for tadpole survival. Similarly, tadpole growth rates and larval durations often may have major impacts on fitness, but the nature of those relationships varies strongly through time and space (Travis 1984; Indermaur et al. 2010; Llewelyn et al. 2010; Phillips et al. 2010*b*). Finally, metamorph body size, shape, and locomotor ability may also affect subsequent fitness (Berven and Gill 1983; Smith 1987; Altwegg and Reyer 2003).

Other critical gaps in our knowledge involve the spatial and temporal dynamics of pH in natural water bodies, the ability of reproductive adult toads to assess sites before spawning, and the potential for tadpoles to detect and avoid suboptimal levels of acidity within water bodies. For example, heavy rainfall may create transiently low pH conditions (Cook 1983), thereby enhancing egg survival for the brief (approximately 2-d) embryonic period, but then over the next few days, pH may rise to conditions that are optimal for tadpole growth and survival. Also, tadpoles may be able to track changes in pH and move to places (based on water depth, temperature, freshwater inflow, shading, etc.) that minimize their exposure to harmful conditions. Behavioral plasticity of this type might enable tadpoles to buffer ambient variation in pH levels and exploit different parts of their pond environment at different developmental stages.

Finally, what do our results mean for the continued southward expansion of the cane toad invasion in southern Australia? At first sight, the highly acidic conditions in near-coastal regions (notably in wallum habitat) seem as if they might impede the invader's progress. Our study is discouraging in this respect. First, cane toads can survive and metamorphose after developing under a wide range of pH conditions. Second, extremely acidic water can enhance rather than reduce toad survival in the egg stage, although continued exposure to pH 4 throughout the larval period was invariably fatal. Given the enormous fecundity of cane toads, with tens of thousands of eggs per clutch, even a low-percentage survival rate translates into a large number of metamorphs. Thus, we doubt that pH challenges will substantially curtail the toad's southern expansion. Factors such as low temperature (Kolbe et al. 2010) or interactions with native biota (Cabrera-Guzmán et al. 2011, 2012) may well be more important in this respect.

## Acknowledgments

We thank Stewart Harris (Sutherland Shire Council) for logistical support and assistance with fieldwork; Jason Bishop and Jeff Thomas (NSW NPWS), Pamela Gray (Tweed Shire Council), Glenn Murray, Peter Geise, Lesley Reid, Chris Shannon, Sharon Lehman, and Russell Jago for assistance with access to field sites; Josh Amiel for assistance in statistical analysis; Melanie Elphick for assistance with figure preparation; and Chalene Bezzina, Catherine Michel, Jai Thomas, Sam McCann, and George Madani for assistance with fieldwork and husbandry. The work was funded by the Australian Research Council.

## Literature Cited

Abràmoff M.D., P.J. Magalhães, and S.J. Ram. 2004. Image processing with ImageJ. Biophotonics Int 11:36–43.

Alford R., M. Cohen, M. Crossland, M. Hearnden, and L. Schwarzkopf. 1995. Population biology of *Bufo marinus* in northern Australia. Pp. 173–181 in C.M. Finlayson, ed.

Wetland research in the wet-dry tropics of Australia. Office of the Supervising Scientist, Jabiru, Northern Territory, Australia.

Altwegg R. and H.U. Reyer. 2003. Patterns of natural selection on size at metamorphosis in water frogs. Evolution 57:872–882.

Barth B.J. and R.S. Wilson. 2010. Life in acid: interactive effects of pH and natural organic acids on growth, development and locomotor performance of larval striped marsh frogs (*Limnodynastes peronii*). J Exp Biol 213:1293–1300.

Beebee T.J.C. 1986. Acid tolerance of natterjack toad (*Bufo calamita*) development. Herpetol J 1:78–81.

Berven K.A. and D.E. Gill. 1983. Interpreting geographic variation in life-history traits. Am Zool 23:85–97.

Bowerman J. and A.P. Christopher. 2010. Ability of Oregon spotted frog (*Rana pretiosa*) embryos from central Oregon to tolerate low temperatures. Northwest Nat 91:198–202.

Broennimann O., U.A. Treier, H. Müller-Schärer, W. Thuiller, A. Peterson, and A. Guisan. 2007. Evidence of climatic niche shift during biological invasion. Ecol Lett 10:701–709.

Brown G.P., C. Kelehear, and R. Shine. 2011. Effects of seasonal aridity on the ecology and behaviour of invasive cane toads in the Australian wet-dry tropics. Funct Ecol 25:1339–1347.

Bureau of Meteorology. 2013. Climate data. http://www.bom.gov.au. Accessed October 2012.

Cabrera-Guzmán E., M. Crossland, and R. Shine. 2011. Can we use the tadpoles of Australian frogs to reduce recruitment of invasive cane toads? J Appl Ecol 48:462–470.

Cabrera-Guzmán E., M.R. Crossland, and R. Shine. 2012. Predation on the eggs and larvae of invasive cane toads (*Rhinella marina*) by native aquatic invertebrates in tropical Australia. Biol Conserv 153:1–9.

Cameron P.G. 1994. Growth and survival of postmetamorphic toads: interactions among larval history, density, and parasitism. Ecology 75:2264–2274.

Clark K.L. and B.D. Lazerte. 1985. A laboratory study of the effects of aluminum and pH on amphibian eggs and tadpoles. Can J Fish Aquat Sci 42:1544–1551.

Cook R.P. 1983. Effects of acid precipitation on embryonic mortality of *Ambystoma* salamanders in the Connecticut Valley of Massachusetts. Biol Conserv 27:77–88.

Crump M.L. 1981. Energy accumulation and amphibian metamorphosis. Oecologia 49:167–169.

Dale J.M., B. Freedman, and J. Kerekes. 1985. Acidity and associated water chemistry of amphibian habitats in Nova Scotia. Can J Zool 63:97–105.

Dash M.C. and A.K. Hota. 1980. Density effects on the survival, growth rate, and metamorphosis of *Rana tigrina* tadpoles. Ecology 61:1025–1028.

Denver R.J. 2009. Stress hormones mediate environment-genotype interactions during amphibian development. Gen Comp Endocrinol 164:20–31.

Dolmen D. and J.K. Skei. 2006. Effects of pH, aluminium, and soft water on larvae of the amphibians *Bufo bufo* and *Triturus vulgaris*. Can J Zool 84:1668–1677.

Dunson W.A. and J. Connell. 1982. Specific inhibition of hatching in amphibian embryos by low pH. J Herpetol 16:314–316.

Evans M., C. Yáber, and J.-M. Hero. 1996. Factors influencing choice of breeding site by *Bufo marinus* in its natural habitat. Copeia 1996:904–912.

Florance D., J.K. Webb, T. Dempster, M.R. Kearney, A. Worthing, and M. Letnic. 2011. Excluding access to invasion hubs can contain the spread of an invasive vertebrate. Proc R Soc B 278:2900–2908.

Floyd R.B. 1983. Ontogenetic change in the temperature tolerance of larval *Bufo marinus* (Anura: Bufonidae). Comp Biochem Physiol A 75:267–271.

———. 1984. Variation in temperature preference with stage of development of *Bufo marinus* larvae. J Herpetol 18:153–158.

Freda J. 1986. The influence of acidic pond water on amphibians: a review. Water Air Soil Pollut 30:439–450.

Freda J. and W.A. Dunson. 1984. Sodium balance of amphibian larvae exposed to low environmental pH. Physiol Zool 57:435–443.

———. 1985*a*. Field and laboratory studies of ion balance and growth rates of ranid tadpoles chronically exposed to low pH. Copeia 1985:415–423.

———. 1985*b*. The influence of external cation concentration on the hatching of amphibian embryos in water of low pH. Can J Zool 63:2649–2656.

———. 1986. Effects of low pH and other chemical variables on the local distribution of amphibians. Copeia 1986:454–466.

Glooschenko V., W.F. Weller, P.G.R. Smith, R. Alvo, and J.H.G. Archbold. 1992. Amphibian distribution with respect to pond water chemistry near Sudbury, Ontario. Can J Fish Aquat Sci 49:114–121.

Gosner K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190.

Gosner K.L. and I.H. Black. 1957. The effects of acidity on the development and hatching of New Jersey frogs. Ecology 38:256–262.

Hagman M. and R. Shine. 2006. Spawning site selection by feral cane toads (*Bufo marinus*) at an invasion front in tropical Australia. Austral Ecol 31:551–558.

Indermaur L., B.R. Schmidt, K. Tockner, and M. Schaub. 2010. Spatial variation in abiotic and biotic factors in a floodplain determine anuran body size and growth rate at metamorphosis. Oecologia 163:637–649.

Johnson C.N., J.L. Isaac, and D.O. Fisher. 2007. Rarity of a top predator triggers continent-wide collapse of mammal prey: dingoes and marsupials in Australia. Proc R Soc B 274:341–346.

Kearney M., B.L. Phillips, C.R. Tracy, K.A. Christian, G. Betts, and W.P. Porter. 2008. Modelling species distributions without using species distributions: the cane toad in Australia under current and future climates. Ecography 31:423–434.

Kelehear C., J. Webb, and R. Shine. 2009. *Rhabdias pseudosphaerocephala* infection in *Bufo marinus*: lung nematodes reduce viability of metamorph cane toads. Parasitology 136: 919–927.

Kolbe J.J., M. Kearney, and R. Shine. 2010. Modeling the consequences of thermal trait variation for the cane toad invasion of Australia. Ecol Appl 20:2273–2285.

Kupferberg S.J. 1997. Bullfrog (*Rana catesbeiana*) invasion of a California river: the role of larval competition. Ecology 78: 1736–1751.

Lee C.E. 2002. Evolutionary genetics of invasive species. Trends Ecol Evol 17:386–391.

Lee J.-H., T.A. Herzog, C.D. Meade, M.S. Webb, and T.H. Brandon. 2007. The use of GEE for analyzing longitudinal binomial data: a primer using data from a tobacco intervention. Addict Behav 32:187–193.

Lever C. 2001. The cane toad: the history and ecology of a successful colonist. Westbury Academic and Scientific, Otley, West Yorkshire.

Llewelyn J., B.L. Phillips, R.A. Alford, L. Schwarzkopf, and R. Shine. 2010. Locomotor performance in an invasive species: cane toads from the invasion front have greater endurance, but not speed, compared to conspecifics from a long-colonised area. Oecologia 162:343–348.

Lodge D.M., C.A. Taylor, D.M. Holdich, and J. Skurdal. 2000. Nonindigenous crayfishes threaten North American freshwater biodiversity: lessons from Europe. Fisheries 25: 7–20.

McCann S., M. Greenlees, D. Newell, and R. Shine. 2014. Rapid acclimation to cold allows the cane toad to invade montane areas within its Australian range. Funct Ecol 28: 1166–1174.

McGlashan D. and J. Hughes. 2001. Genetic evidence for historical continuity between populations of the Australian freshwater fish *Craterocephalus stercusmuscarum* (Atherinidae) east and west of the Great Dividing Range. J Fish Biol 59: 55–67.

Newell D. 2011. Recent invasions of World Heritage rainforests in north-east New South Wales by the cane toad *Bufo marinus*. Aust Zool 35:876–883.

Newman R.A. 1992. Adaptive plasticity in amphibian metamorphosis. BioScience 42:671–678.

Persson M., K. Räsänen, A. Laurila, and J. Merilä. 2007. Maternally determined adaptation to acidity in *Rana arvalis*: are laboratory and field estimates of embryonic stress tolerance congruent? Can J Zool 85:832–838.

Phillips B.L., G.P. Brown, and R. Shine. 2010*a*. Evolutionarily accelerated invasions: the rate of dispersal evolves upwards during the range advance of cane toads. J Evol Biol 23:2595–2601.

———. 2010*b*. Life-history evolution in range-shifting populations. Ecology 91:1617–1627.

Pierce B.A. 1985. Acid tolerance in amphibians. BioScience 35: 239–243.

———. 1993. The effects of acid precipitation on amphibians. Ecotoxicology 2:65–77.

Pough F.H. 1976. Acid precipitation and embryonic mortality of spotted salamanders, *Ambystoma maculatum*. Science 92:68–70.

Sakai A.K., F.W. Allendorf, J.S. Holt, D.M. Lodge, J. Molofsky, K.A. With, S. Baughman, et al. 2001. The population biology of invasive species. Annu Rev Ecol Syst 32:305–332.

Semeniuk M., F. Lemckert, and R. Shine. 2007. Breeding-site selection by cane toads (*Bufo marinus*) and native frogs in northern New South Wales, Australia. Wildl Res 34:59–66.

Semlitsch R.D. and J.P. Caldwell. 1982. Effects of density of growth, metamorphosis, and survivorship in tadpoles of *Scaphiopus holbrooki*. Ecology 63:905–911.

Skelly D.K. 1997. Tadpole communities: pond permanence and predation are powerful forces shaping the structure of tadpole communities. Am Sci 85:36–45.

Smith D.C. 1987. Adult recruitment in chorus frogs: effects of size and date at metamorphosis. Ecology 68:344–350.

Smith-Gill S.J. and K.A. Berven. 1979. Predicting amphibian metamorphosis. Am Nat 113:563–585.

Sutherst R.W., R.B. Floyd, and G.F. Maywald. 1996. The potential geographical distribution of the cane toad, *Bufo marinus* L. in Australia. Conserv Biol 10:294–299.

Takano S. and K. Iijima. 1937. Studies on the life history and habitat of *Bufo marinus* L. in Formosa. Pt. 1. The relation between the growth of the tadpole and the quality of the breeding water. Report of the Government Sugar Experiment Station, Tainan, Formosa, Japan 4:195–213.

Tembo R.N. 2009. The sublethal effects of low-pH exposure on the chemoreception of *Poecilia sphenops*. Arch Environ Contam Toxicol 57:157–163.

Tingley R., M.J. Greenlees, and R. Shine. 2012. Hydric balance and locomotor performance of an anuran (*Rhinella marina*) invading the Australian arid zone. Oikos 121:1959–1965.

Tingley R. and R. Shine. 2011. Desiccation risk drives the spatial ecology of an invasive anuran (*Rhinella marina*) in the Australian semi-desert. PLoS ONE 6:e25979.

Tingley R., M. Vallinoto, F. Sequeira, and M.R. Kearney. 2014. Realized niche shift during a global biological invasion. Proc Natl Acad Sci USA 111:10233–10238.

Tome M.A. and F.H. Pough. 1982. Responses of amphibians to acid precipitation. Pp. 245–254 in R.E. Johnson, ed. Acid rain/fisheries. American Fish Society, Bethesda, MD.

Travis J. 1984. Anuran size at metamorphosis: experimental test of a model based on intraspecific competition. Ecology 65:1155–1160.

Urban M.C., B.L. Phillips, D.K. Skelly, and R. Shine. 2007. The cane toad's (*Chaunus* [*Bufo*] *marinus*) increasing ability to invade Australia is revealed by a dynamically updated range model. Proc R Soc B 274:1413–1419.

van Beurden E.K. and G.C. Grigg. 1980. An isolated and expanding population of the introduced toad *Bufo marinus* in New South Wales. Aust Wildl Res 7:305–310.

Webb J.K., M. Letnic, T.S. Jessop, and T. Dempster. 2014. Behavioural flexibility allows an invasive vertebrate to survive in a semi-arid environment. Biol Lett 10:20131014.

Wilbur H.M. 1977. Density-dependent aspects of growth and metamorphosis in *Bufo americanus*. Ecology 58:196–200.

———. 1984. Complex life cycles and community organization in amphibians. Pp. 195–224 in P.W. Price, C.N. Slobodchikoff, and W.S. Gaud, eds. A new ecology: novel approaches to interactive systems. Wiley, New York.

Wilbur H.M. and J.P. Collins. 1973. Ecological aspects of amphibian metamorphosis: nonnormal distributions of competitive ability reflect selection for facultative metamorphosis. Science 182:1305–1314.

Zeger S.L., K.-Y. Liang, and P.S. Albert. 1988. Models for longitudinal data: a generalized estimating equation approach. Biometrics 44:1049–1060.