No evidence for cold-adapted life-history traits in cool-climate populations of invasive cane toads (*Rhinella marina*)

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Abstract

As an invasive organism spreads into a novel environment, it may encounter strong selective pressures to adapt to abiotic and biotic challenges. We examined the effect of water temperature during larval life on rates of survival and growth of the early life-history stages of cane toads (*Rhinella marina*) from two geographic regions (tropical vs. temperate) in the species' invaded range in eastern Australia. If local adaptation at the southern (cool-climate) invasion front has extended the cold-tolerance of early life-stages, we would expect to see higher viability of southern-population toads under cooler conditions. Our comparisons revealed no such divergence: the effects of water temperature on rates of larval survival and growth, time to metamorphosis, size at metamorphosis and locomotor performance of metamorphs were similar in both sets of populations. In two cases where tropical and temperate-zone populations diverged in responses to temperature, the tropical animals performed better at low to medium temperatures than did conspecifics from cooler regions. Adaptation to low temperatures in the south might be constrained by behavioural shifts (e.g., in reproductive seasonality, spawning-site selection) that allow toads to breed in warmer water even in cool climates, by gene flow from warmer-climate populations, or by phylogenetic conservatism in these traits.

Introduction

In ectotherms, environmental temperature controls developmental rates and a wide range of physiological processes [1]. Traits such as embryonic developmental trajectory, time to metamorphosis, body size at maturity, locomotor performance and growth rate are sensitive to developmental temperature [2–6]. As a result, geographical variation in thermal environments can limit the distribution of a species. At such a range-edge, a species may be under intense selection to adjust its tolerances to the thermal challenges it encounters, either by physiologically-based adaptation or behavioural flexibility [7, 8]. Phenotypically plastic responses commonly adjust organismal traits to the local environment [9]. Many studies on amphibians have documented geographic variation in traits that plausibly enhance the ability of range-edge
individuals to survive under the relatively extreme environmental conditions that they encounter. Those traits include the critical thermal minimum [9–11] and maxima [12], morphology [13], embryonic developmental rates [14] and body sizes [15].

How rapidly can such adjustments occur? Invasive species provide a robust model system with which to examine the time course of initial responses to encountering novel challenges [16–18]. A growing literature on invasive species provides many examples of adaptations that enhance dispersal rates, reduce predation vulnerability, and facilitate competitive superiority against native taxa [19–23]. Cane toads in Australia have been intensively studied in this respect, but most attention has focused on the toad’s invasion of tropical habitats [24, 25]. At their southeastern (cool-climate) range-edge, cane toads are moving slowly through coastal habitats [26–29]. Low temperatures in this region may be limiting range-expansion [29, 30].

Studies in other parts of the toads’ Australian range have revealed rapid evolutionary changes in traits that affect dispersal rate [31, 32], life-history traits [33–35], hydric balance [36, 37] and thermal biology [10, 11, 38]. Given these rapid changes, toads may also have evolved an ability to deal with (or at least, exhibit sufficient plasticity to tolerate) low temperatures at their southern range-edge. To evaluate this possibility, we conducted laboratory studies to measure responses of early life-stages of tropical versus temperate-zone cane toads to a range of water temperatures.

Results

Hatching success of eggs

The eggs of tropical toads had higher hatching success than did those of temperate-zone toads at all temperatures tested (main effect of location $F_{1,87} = 12.76, P < 0.001$; interaction temperature*location $F_{2,87} = 0.19, P = 0.82$; see Fig 1A). Mean hatching success was higher at 25˚C than at warmer or cooler temperatures, but this effect did not attain statistical significance ($F_{2,87} = 2.80, P = 0.07$).

Rates of survival and growth of tadpoles

Tadpole survival was not significantly affected by the location of origin ($F_{1,94} = 0.007, P = 0.93$) or by temperature ($F_{2,94} = 0.13, P = 0.87$; interaction location*temperature $F_{2,94} = 0.91, P = 0.40$; Fig 1B). Rearing temperature influenced larval growth rate ($F_{2,100} = 8.56, P < 0.001$) in similar ways in the two populations (location $F_{1,100} = 0.009, P = 0.92$; interaction location*temperature $F_{2,100} = 1.07, P = 0.34$). Tadpoles grew more slowly at 20˚C than at the other two temperatures (Tukey posthoc 20˚C < 25˚C or 30˚C; see Fig 1C).

Duration of larval period

Metamorphosis was delayed if tadpoles were kept at lower temperature ($F_{2,2594} = 661.91, P < 0.0001$; posthoc tests show that all three treatments differ significantly from each other), and was delayed in temperate-zone toads compared to tropical conspecifics (main effect location $F_{1,2594} = 86.32, P < 0.0001$; interaction location*temperature $F_{2,2594} = 0.37, P = 0.69$; Fig 1D).

Mass at metamorphosis

Metamorphs from 20˚C were larger than were those from warmer temperatures ($F_{2,2594} = 127.59, P < 0.0001$), with a significant interaction between temperature and location ($F_{24,2594} = 12.76, P < 0.0001$); a rearing temperature of 25˚C resulted in smaller metamorphs in
Locomotor performance of metamorphs

Tadpoles raised in cooler water produced metamorphs that took shorter hops (i.e., needed more hops to cover the same distance; $F_{2,1112} = 39.28$, $P < 0.001$; posthoc tests show that all three treatments differ significantly from each other) and temperate-zone toads took shorter hops than did tropical conspecifics (location effect $F_{1,1112} = 47.56$, $P < 0.0001$; interaction temperature*location $F_{2,1112} = 0.60$, $P = 0.55$; see Fig 2B). The time required for a metamorph to
Fig 2. The effects of temperature at which eggs and larvae of cane toads *Rhinella marina* were raised on body size at metamorphosis, locomotor behaviour, and locomotor speed of metamorphs. Graphs show effect of rearing temperature on (a) mass at metamorphosis, (b) number of hops required for a metamorph to travel 1 m in laboratory raceway trials, and (c) the time taken for a metamorph to travel 1 m in laboratory raceway trials. The panels show data from toads whose parents were collected in two locations: tropical (red) and temperate-zone (blue) Australia. The
travel all the way along a 1-m raceway was greater for animals that had been raised in cooler water during larval life (temperature effect $F_{2,1112} = 166.24$, $P < 0.0001$), and the increase in time taken after low-temperature rearing was greater for temperate-zone (NSW) metamorphs than for tropical (Qld) metamorphs (interaction temperature x location $F_{2,1112} = 18.82$, $P < 0.0001$; see Fig 2C).

Discussion

Organisms that are expanding their range (like the cane toad in Australia) may encounter novel environmental conditions. They may cope with such challenges either physiologically or behaviourally. Although previous work has reported rapid adaptation and behavioural adjustments of cane toads in response to selective forces within tropical and arid environments [31, 32, 36, 39] and shifts in thermal minimum tolerances in adult toads encountering cool conditions [10, 11], we saw no such shifts in life-history traits of the early life-stages of toads in response to water temperature. Although toads in southern Australia are exposed to water-bodies that are substantially cooler than those used for spawning in tropical Australia [6, 40] or the toads’ native range [41], the thermal responses of eggs and tadpoles were very similar in tropical versus temperate-zone populations. In an analogous result, Volpe [42] found no difference in response to experimental temperature of *Incilius (Bufo) valliceps* embryos from thermally diverse breeding environments (early and late breeding seasons).

Overall, our results highlight the broad thermal tolerance of *Rhinella marina* eggs and tadpoles. Substantial numbers of eggs hatched, and tadpoles survived and grew, even at 20°C—cooler than in potential spawning ponds at the southern edge of the toad’s current Australian range [6]. Such eurythermy may enable the cane toad to breed successfully in a wide range of conditions, and selection to enhance viability at low temperatures accordingly may be weak. Rearing temperature significantly affected many life-history traits, as previously reported by Wijethunga et al. [6], but we lack field data to assess the fitness consequences of variation in traits such as larval duration and size at metamorphosis.

The only traits for which we detected significant differences between populations in their thermal responses were mass and hopping speeds post-metamorphosis. Temperate-zone toads had smaller metamorphs at an intermediate rearing temperature (25°C) than did their tropical counterparts (Fig 2A). For locomotor speeds, cooler conditions during larval life reduced performance more in temperate-zone toads than in their tropical counterparts (Fig 2B, 2C). Neither of these effects is easily reconciled with the idea of adaptive changes to cool environmental conditions. The durations of larval life were broadly similar in tropical and temperate-zone toads (Fig 1D), so that smaller size at metamorphosis in southern (temperate-zone) animals cannot be interpreted as a tradeoff for faster development [43]. All else being equal, a larger size at metamorphosis is likely to enhance fitness, because smaller metamorphs are more vulnerable to threats such as desiccation, predation, and parasite attack [44]. For locomotor speed, likewise, faster speeds are likely to enhance rather than reduce metamorph fitness; and thus, the slower locomotion of temperate-zone (NSW) metamorphs raised at low temperatures seems unlikely to be adaptive.

The overall similarity in thermal dependency of survival, growth and performance between widely-separated (tropical vs. temperate) populations might reflect a lack of selection for cold-tolerance in eggs and larvae at the southern range edge. For example, the southern limit of the
toad’s distribution may be determined primarily by traits such as weather-limited foraging opportunities for adult toads [30] with weak or no selection on larval thermal biology. Selection for cold-tolerance would be further reduced if females delay spawning until summer weather brings pond temperatures close to those experienced year-round in tropical Australia. Local adaptation also may be opposed by gene flow from surrounding populations exposed to higher temperatures [45] or frequent human-assisted translocation of toads to the southern range-edge from warmer climates [46]. Alternatively, toads may not have been exposed to these cool-climate conditions for long enough to adapt [47]. Selection for cold-tolerance would be further reduced if females delay spawning until summer weather brings pond temperatures close to those experienced year-round in tropical Australia. Local adaptation also may be opposed by gene flow from surrounding populations exposed to higher temperatures [45] or frequent human-assisted translocation of toads to the southern range-edge from warmer climates [46]. Alternatively, toads may not have been exposed to these cool-climate conditions for long enough to adapt [47].

Even if natural selection favours cold tolerance at the expanding range-edge, however, that selection may be ineffective because of tradeoffs with other traits under selection (such as increased dispersal rates at expanding range edges [30–33]). In summary, our results do not provide any support for the prediction that cane toads are evolving cold-tolerant eggs, larvae and metamorphs as the species penetrates southwards into cooler parts of Australia.

Materials and methods
Study area and species
Since they were introduced to Queensland (Qld) in 1935, cane toads have spread west across tropical Australia and south along the New South Wales (NSW) coast, sandwiched between mountains to the west and the Pacific Ocean to the east [29, 50]. Cane toads are large bufonids whose powerful toxins kill many native Australian vertebrates [24]. Female cane toads lay up to 30 000 eggs in a string in shallow ephemeral ponds [51]. Previous field studies have reported that in the Australian tropics, cane toads spawn in water averaging 32˚C [40]; but conditions are cooler (~28.5˚C in summer, 20˚C in spring, 24˚C in autumn) in their southern temperate-zone breeding ponds [6, 48].

To investigate whether toads from the cool southern (temperate-zone) versus warm northern (tropical) parts of their Australian distribution differ in cold tolerance, we collected adult male and female toads from three populations in tropical Australia (Queensland; centered on 18˚00’19.1"S, 146˚00’31.1”E, where they have been established for 80 years) and from three populations from the southernmost range-edge in temperate-zone Australia (northeastern New South Wales; centered on 33˚56’38.69"S, 147˚56’57.41”E) where toads have been present for less than 20 years [29]. Climatic conditions are much warmer at the tropical site than at the temperate-zone site (see Fig 3 for data on mean monthly air temperatures).

Methods
Adult toads were housed at the University of Sydney for one month, in tubs (390 x 280 x 140 mm; two toads per tub) with ad lib access to water, and food provided three times a week. Room temperature was 22–23˚C. The adult toads were then injected with hormone (0.5 mL/L Lucrin, Abbott Australasia, Botany, NSW; see Kelehear et al. [52] for details) in an attempt to obtain two clutches of eggs from each population (i.e., six clutches per site). In practice, we obtained five viable clutches from tropical Queensland toads (two each from Townsville and Tully, one from Cairns) and three from temperate-zone areas in New South Wales (two from Bungawalbin, one from Brooms Head). Strands of 100 eggs from each clutch were then raised at 20, 25, or 30˚C, bracketing the range of temperatures available at the tropical (Qld) and temperate-zone (NSW) breeding ponds in Australia. Each temperature treatment contained four
replicates, and was maintained using aquarium heaters (AquaWorld, Model HT 2150, Boyu Group, Guangdong, China), with temperature recorded daily (EC-PCST Testr35 multiparameter, model PTTEST35, Eutech, Singapore; accuracy ±0.01˚C). The water was aerated for 15 min each day with a 220–240-V aerator (Resun LP-100, Longgang, Shenzhen, China). Tadpoles were fed with frozen lettuce every day, with weekly water changes. Water within each tub (28 x 38 x 20 cm) was conditioned using tap water conditioner (API Tap Water Conditioner, Chalfont, Pasadena; 5 mL per 20 L). We recorded egg hatching success (% hatched), and % survival and body lengths of tadpoles after four weeks.

When tadpoles metamorphosed (completely resorbed their tails), we recorded the date and weighed them. Metamorphs were raised in tubs (390 x 280 x 140 mm; half water, half sand), with each tub containing 20 metamorphs from the same thermal treatment. Food (day-old crickets) was provided three times per week. Room temperature was 22–23˚C. The young toads were fed with one-day-old crickets twice weekly. Locomotor performance of metamorphs was tested at 4 weeks of age by placing each animal at the start of a 1-m-long raceway and prompting it to run by gently tapping the urostyle with a soft brush. Room temperature was held constant (23–24˚C) and we recorded the total time taken to travel 1 m, and the number of hops required.

Ethics statement

All experimental protocols were approved by the University of Sydney Animal Care and Ethics Committee (Animal Care and Ethics Protocol Number L04/08-2012/3/5807) and were conducted in accordance with all relevant guidelines and regulations, including the ARRIVE guidelines. Euthanasia was performed humanely by immersion in MS-222. Husbandry methods were designed to assure that animals were maintained in good health and without stress.

Statistical analysis

Analysis of Variance (ANOVA) was performed to compare northern tropical (Qld) versus southern temperate-zone (NSW) populations of toads in terms of % of eggs hatching, % tadpole survival, length of the tadpoles after 4 weeks, time to metamorphosis, mass at metamorphosis, and locomotor ability (time and number of hops required to travel 1 m). To improve
normality of distributions, we arcsin-transformed data on the proportion of eggs hatching and proportion of tadpoles surviving, and ln-transformed data on metamorph mass and locomotor traits (the number of hops and times taken required to cover 1 m on the raceway). The independent variables were location (tropical/temperate-zone) and temperature of rearing treatment. We also included clutch # as a random variable, and replicates within clutch as a nested effect (JMP version 15: SAS Institute Inc., Cary, NC). Preliminary analyses revealed no significant link between performance and body size, so we did not include mass as a covariate in the analyses of locomotor traits.

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