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Heat hardening as a function of developmental stage in larval and juvenile *Bufo americanus* and *Xenopus laevis*

Elizabeth Sherman*, Daniel Levitis

Natural Sciences, Bennington College, Bennington, VT 05201, USA

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Abstract

1. We studied heat hardening in tadpoles and juveniles of the temperate *Bufo americanus* and the subtropical *Xenopus laevis* by comparing the critical thermal maxima (CTMax) of heat-pretreated animals with the CTMax of control (non-pretreated) animals of the same developmental stages.
2. While premetamorphic tadpoles (Gosner stages 26–43) of both species exhibited heat hardening, metamorphosing *B. americanus* tadpoles (stages 44–45) were unable to heat harden and, in fact, exhibited a decrease in CTMax following pretreatment while metamorphosing *X. laevis* tadpoles still exhibited heat hardening.
3. This difference may reflect the more stressful nature of the more extensive metamorphosis of *Bufo* compared to that of *Xenopus*.

Keywords: Tadpoles; Heat hardening; CTMax; Anuran development; *Bufo*; *Xenopus*; Thermal tolerance

1. Introduction

Vertebrate ectotherms exhibit elevated levels of thermal tolerance following a brief, sublethal thermal stress. This phenomenon, heat hardening, is an acute short-lived response that permits animals to cope with transient fluctuations in environmental temperature and appears to be adaptive. Among amphibians, heat hardening is particularly significant given the fluctuations in daily temperature to which they may be exposed (Spotila et al., 1989; Rome et al., 1992). The thermal environment of anuran amphibians changes during their lifetime as most begin life as aquatic organisms which later metamorphose into terrestrial or semi-terrestrial animals (Sherman, 1980; Ultsch et al., 1999). Moreover, both the thermal tolerance of tadpoles (Cupp, 1980; Sherman, 1980; Floyd, 1983) and their selected temperatures (Dupre and Petranka, 1985; Wollmuth and Crawshaw, 1988) change during their ontogeny. Therefore, we predicted that the heat hardening capacity of tadpoles might also change during their development.

In this study, we examined the heat hardening capacity of tadpoles and juvenile toads of *Bufo americanus* and *Xenopus laevis*. Heat hardening was measured as an increase in the critical thermal maximum (CTMax) of animals that had been pretreated with a thermal stress compared to the CTMax of non-pretreated control animals.

The thermal physiology of anurans is related to their geographic distribution (Ultsch et al., 1999) and therefore we might expect differences in heat hardening between the temperate *B. americanus* and the subtropical *X. laevis*. Amphibians from temperate climates typically experience more extreme temperature fluctuations than tropical and subtropical forms (Ultsch et al., 1999). Moreover, *Bufo* tadpoles can be found in

*Corresponding author. Tel.: +1-802-440-4466; fax: +1-802-440-4461.

E-mail address: sherman@bennington.edu (E. Sherman).

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temporary ponds, often exploiting high temperatures which encourages metamorphosis (Sherman, 1980), while *Xenopus* typically inhabits permanent ponds and has a more protracted metamorphosis (Wassersug and Hoff, 1982).

Wassersug (1996) has argued that pipids, such as *X. laevis*, which remain aquatic following transformation exhibit a less abrupt and extensive metamorphosis than bufonids which transform rapidly into terrestrial toadlets. A number of studies have reported a decrease in thermal tolerance of anuran larvae undergoing metamorphosis (Cupp, 1980; Sherman, 1980; Floyd, 1983). Sherman (1980) has suggested that such a decrease is due to the stressful nature of metamorphosis which requires extensive differentiation. If *Bufo* has a more extensive, abrupt, and therefore stressful metamorphosis than *Xenopus*, perhaps the capacity of *Bufo* tadpoles to increase their thermal tolerance or heat harden would be compromised during metamorphosis compared to *Xenopus* tadpoles.

2. Materials and methods

2.1. Animals

Larval and postmetamorphic (juvenile) *B. americanus* were collected in and around Krause Pond, Bennington, Vermont, US, in June and July of 1991 and 1992. *X. laevis* tadpoles were obtained from Nasco Biological Supply from August 1998 to May 1999. All animals were maintained at room temperature (20–22°C) with a 12-h photoperiod centered at noon (EST). Previously untreated *X. laevis* tadpoles that transformed in the lab were tested as juveniles. All tadpoles were maintained in aquaria with aged tap water. *B. americanus* tadpoles were fed boiled lettuce and *X. laevis* tadpoles were fed commercial *Xenopus* tadpole nutrient. Juvenile *B. americanus* were maintained in watch glasses on moist paper towel and were fed flightless fruit flies. Transformed *X. laevis* were kept in aquaria and were fed commercial food pellets. Most animals were tested between 8 and 14 days of capture or receipt. Three *Bufo* tadpoles of the earliest stage used (see below) were tested after only 3 days in order to ensure that they had not developed into a later stage. However, Lutterschmidt and Hutchison (1997) note that acclimation is rapid in ectotherms and occurs within a few days. All experiments were performed between 1000 and 1500 h EST.

2.2. Determination of thermal tolerance

We used the CTMax as the index of thermal tolerance, defined as the temperature at which locomotor activity becomes so disorganized that animals are unable to escape from lethal conditions (Cowles and Bogert, 1944). Test animals typically exhibited lower levels of heat incapacitation such as listing and loss of righting ability. Lutterschmidt and Hutchison (1997) argue that onset of spasms (OS) is a preferred CTMax endpoint. However, they were reporting on adult ectotherms. As in fish, OS is difficult to observe reliably in tadpoles (see Cupp, 1980; Sherman, 1980; Menke and Claussen, 1982; Floyd, 1983; Skelly and Freidenburg, 2000). We found that the most consistent endpoint of thermal incapacitation for all stages of tadpoles was an abrupt cessation of movement, even when prodded, and the temperature at which that occurred was designated as the CTMax. Other studies of thermal tolerance in tadpoles also use abrupt cessation of movement as the endpoint, permitting appropriate comparisons with our data (Cupp, 1980; Sherman, 1980; Menke and Claussen, 1982; Floyd, 1983; Skelly and Freidenburg, 2000).

CTMax determinations followed the methods of Sherman (1980). One or two individuals (depending on size) were placed in a beaker of 500 ml of aged tap water at room temperature (20–22°C) and permitted to adjust for 15–20 min prior to heating. Then the water was heated on a hot plate at $1\,^{\circ}\mathrm{C\,min^{-1}}$, a rate at which there would be no lag between ambient temperature and deep body temperature (Hutchison, 1961). The water was aerated continuously and stirred with a Fisher Scientific Digital Thermometer (calibrated to nearest 0.1°C) which insured complete mixing of the water. As heating progressed, tadpoles were prodded gently with the thermometer to see if they responded. The temperature at which all movement suddenly ceased was recorded as the CTMax and the tadpoles were promptly removed to room temperature water. If an animal did not revive in the cool water, the CTMax had been exceeded and the datum was discarded. Following the CTMax determination, tadpoles were staged according to Gosner (1960). The snout-vent length of postmetamorphic individuals was recorded.

2.3. Heat hardening experiments

A variety of treatments has been used in previous studies to induce heat hardening in amphibians. The temperature to which the animals are exposed, the duration of exposure, and the interval between elevated temperature exposure and heat hardening determination all have been manipulated. In several studies, animals were heated to within 2–5°C of CTMax (Easton et al., 1987; Spotila et al., 1989; Yu et al., 1998). The CTMax of tadpoles, however, changes throughout development and a temperature of 2°C below the CTMax of premetamorphic tadpoles might exceed the CTMax of metamorphosing individuals (Sherman, 1980). Moreover, the CTMax of different species is different (Cupp, 1980), so we could not use one temperature for both

species. Conversely, we wanted to use one pretreatment temperature for all animals of the same species rather than, say 2°C below CTMax for each stage. It would be more ecologically significant to compare the responses of animals of different stages to the same heat stress (Lutterschmidt and Hutchison, 1997).

Our pilot studies indicated that a pretreatment of 10 min at 2–3°C below the CTMax of metamorphosing tadpoles (Gosner stages 44–45) followed by a 2-h interval at room temperature (20–22°C) would reliably induce heat hardening in most stages. In the pretreatment of *B. americanus*, two animals were placed in 500 ml of aerated aged tap water at room temperature and then heated at $1°C\,min^{-1}$. They were held at 37 (± 0.5)°C for 10 min and then placed back in room temperature water. The pretreatment of *X. laevis* was the same except that animals were held at 32 (± 0.5)°C. Following a 2-h interval at room temperature, the CTMax of the animals was determined as above. The stage (tadpoles) or snout-vent length (juveniles) of pretreated animals was recorded following the experiments. Heat hardening was measured as an increase in the mean CTMax of pretreated animals of a particular stage or stage group compared to the mean CTMax of non-pretreated control animals of the same stage or stage group.

We tested *B. americanus* tadpoles from Gosner stages 32–46 and *X. laevis* tadpoles from stages 26–46. *X. laevis* tadpoles metamorphosed at a much larger size than *B. americanus* tadpoles. The *Bufo* juveniles tested were 1.0–1.4 cm long while *Xenopus* juveniles were 2.5–4.0 cm long.

2.4. Statistics

In order to assess the effect of heat pretreatment on tadpoles of different stages, we applied an ANOVA model for each species with treatments nested within stages using the General Linear Model of SYSTAT 9 (SPSS, Inc.). In some cases, we further teased out the interaction between treatment and stage by doing pairwise Student's-*t* tests comparing the mean CTMax of control and pretreated animals within selected developmental stage groupings.

3. Results

The CTMax of untreated (control) animals and heat-pretreated animals of various stages are displayed in Figs. 1 and 2. Treatment effects were highly significant in both species (*Bufo*: $p<0.001$; *Xenopus*: $p<0.001$). The development of anuran larvae is a continuous process and thus the stage groupings are somewhat arbitrary but based on descriptions of Gosner (1960), Sherman (1980), and McDiarmid and Altig (1999). Tadpoles of stages up to 35 are characterized by growth of the trunk and tail. The hind limbs experience rapid growth and differentiation from stage 36 and continue to grow through stage 41. The front limbs erupt at stage 42 marking the beginning of metamorphosis which is completed with the resorption of the tail at stage 46.

B. americanus and *X. laevis* exhibited several similar responses in this study. In both species, the CTMax of control animals decreased during metamorphosis

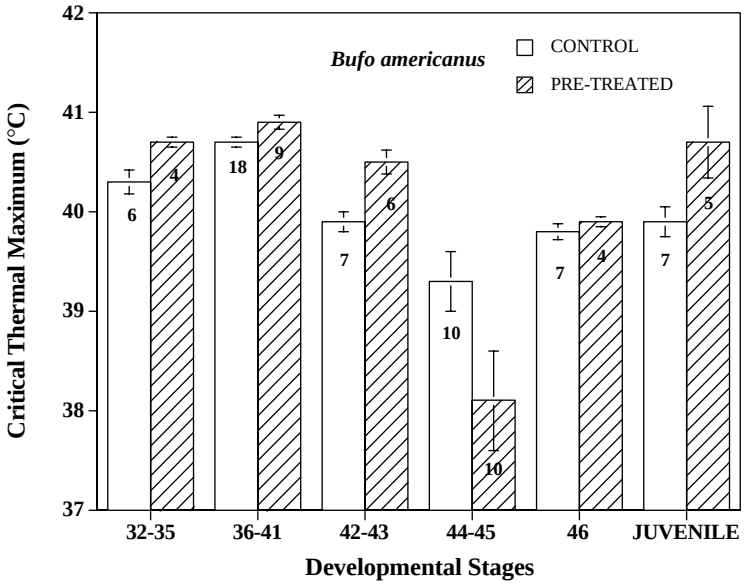

Fig. 1. Mean CTMax of control (open bars) and pretreated (shaded bars) *B. americanus* of different developmental stages (± 1 SEM). Sample size is indicated within each bar.

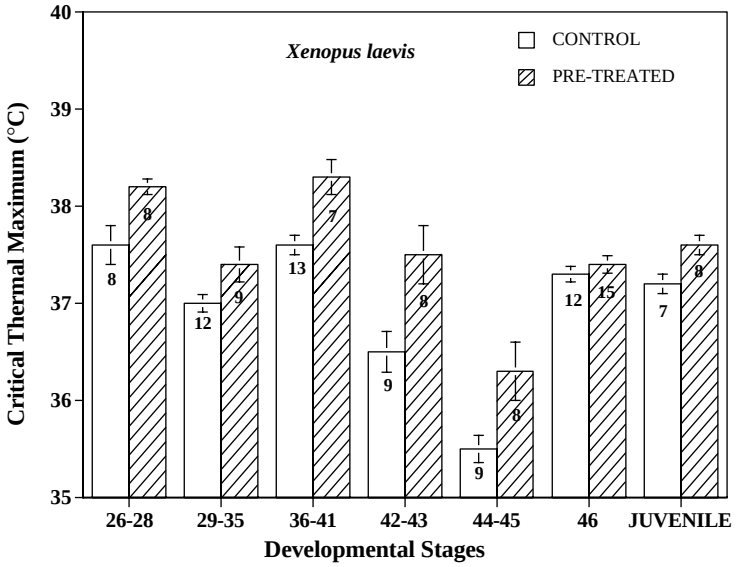

Fig. 2. Mean CTMax of control (open bars) and pretreated (shaded bars) *X. laevis* of different developmental stages (±1 SEM). Sample size is indicated within each bar.

(beginning with stages 42–43, culminating in stages 44–45) compared to earlier and postmetamorphic stages (Figs. 1 and 2). The drop in CTMax from stages 36–41 to stages 44–45 was 1.4°C in *Bufo* (Fig. 1) and 2.1°C in *Xenopus* (Fig. 2). In both species, tadpoles through stage 43 exhibited heat hardening (Figs. 1 and 2). Among the larvae of both species, tadpoles of stages 42–43 exhibited the greatest magnitude of heat hardening, 0.6°C in *B. americanus* ($p = 0.0012$) and 1.0°C in *X. laevis* ($p = 0.007$). At the completion of metamorphosis, stage 46, neither species displayed heat hardening (Figs. 1 and 2) though the CTMax of control animals had increased compared to metamorphic individuals (stages 44–45). Juveniles of both species exhibited significant heat hardening, 0.8°C in *B. americanus* (Fig. 1; $p = 0.002$) and 0.4°C in *X. laevis* (Fig. 2; $p = 0.007$).

Some important differences distinguish the responses of these two species. At all stages, the CTMax values of *B. americanus* were higher than those of *X. laevis*. The mean CTMax of control premetamorphic *B. americanus* tadpoles ranged from 40.3°C to–40.7°C (Fig. 1), while those of premetamorphic *X. laevis* tadpoles ranged from 37.0°C to 37.6°C (Fig. 2). Similarly, juvenile American toadlets had a mean CTMax of 39.9°C while that of *X. laevis* was 37.2°C. However, the most profound difference between these two species occurred during the throes of metamorphosis (stages 44–45): *X. laevis* tadpoles were still able to heat harden by 0.8°C (Fig. 2; $p = 0.013$), while the CTMax of pretreated *B. americanus* actually decreased by 1.2°C (Fig. 1; $p = 0.028$).

Typically, animals of both species recovered from CTMax determinations within a minute of being placed in room temperature water. During CTMax tests using tadpoles with distinct tails, hind limbs, and forelimbs exposed (stages 42–44), the limbs of the tadpoles (i.e. "adult parts") stopped moving at lower temperatures than the tails (i.e. "larval parts") in both species. However, metamorphosing *Bufo* tadpoles sometimes required up to 4 h in cool water to recover, while metamorphosing *Xenopus* tadpoles recovered immediately.

4. Discussion

Comparisons of ontogenetic changes in thermal tolerance of anurans among different studies are somewhat problematic given the different treatment regimes in different studies. Nevertheless, the pattern of ontogenetic change in thermal tolerance exhibited by *B. americanus* and *X. laevis* in our study is similar to that reported in other studies. Thermal tolerance decreases during metamorphosis compared to the thermal tolerance of premetamorphic larvae (Table 1), and this pattern is likely common for anurans. In studies in which tadpole groupings distinguish among premetamorphic tadpoles (through stage 41), tadpoles just entering metamorphosis (following the eruption of forelimbs, stages 42–43) and tadpoles in metamorphic climax (stages 44–45), the decrease in CTMax begins during stage 42 and continues through stage 45. The reported decrease in CTMax from premetamorphic to metamorphosing tadpoles ranges from over 4°C in *B. w. fowleri* and *B. marinus* to roughly 0.5–2°C in *B. americanus*, *X. laevis*, *B. terrestris* and *R. pipiens*,

Table 1
Change in mean CTMax (°C) of anurans from premetamorphic stages to metamorphic stages. The various acclimation temperatures and stage comparisons are presented from different studies

Species	Acclimation temperature (°C)	Premetamorphic stages	Metamorphic stages	Change in mean CTMax (°C)	Study
Bufonidae					
B. terrestris	22	35–39	40–44	−0.5	Noland and Ultsch (1981)
	27	35–39	40–44	−0.7	Noland and Ultsch (1981)
B. americanus	20	42	44	−1.93	Cupp (1980)
	20–22	36–41	44–45	−1.4	Present study
	20–22	42–43	44–45	−0.7	Present study
	30	42	43–44	−0.73	Cupp (1980)
B. w. fowleri	21.8–24	42–43	44	−4.1	Sherman (1980)
	20	42	43–44	−0.67	Cupp (1980)
	30	42	43–44	−1.15	Cupp (1980)
B. marinus	25	40	44	−3.9	Floyd (1983)
	30	40	44	−5.3	Floyd (1983)
Ranidae					
R. pipiens	22	35–39	40–44	−0.5	Noland and Ultsch (1981)
	27	35–39	40–44	0	Noland and Ultsch (1981)
R. sylvatica	20	42	43–44	−0.73	Cupp (1980)
	30	42	43–44	−0.57	Cupp (1980)
R. catesbeiana	25	28–35	43–44	−1.51	Menke and Claussen (1982)
Hylidae					
Pseudacris triseriata	20	42	43–44	−0.19	Cupp (1980)
	30	42	43–44	−1.09	Cupp (1980)
Microhylidae					
Gastrophryne carolinensis	20	42	43–44	−1.53	Cupp (1980)
	30	42	43–44	−2.89	Cupp (1980)
Pipidae					
X. laevis	20–22	36–41	44–45	−2.1	Present study
	20–22	42–43	44–45	−0.9	Present study

and *R. sylvatica* (Table 1). While the various species exhibited decreases of different magnitudes, these reported differences may be related to the varying treatment regimes and groupings of developmental stages in the different studies.

There appear to be several interrelated predictors of tadpole CTMax including latitude, time of year at which larvae appear, and relative permanence of larval ponds, all of which affect the environmental temperatures to which tadpoles are exposed. Bufonids, which can live in temporary ponds, typically exhibit higher CTMax than sympatric ranids that reproduce in more permanent ponds (Noland and Ultsch, 1981; Ultsch et al., 1999). Predictably, bufonids are found at temperatures closer to their CTMax than are ranids, which may facilitate rapid metamorphosis from transient ponds (Sherman, 1980; Noland and Ultsch, 1981). Ponds in the tropics and subtropics exhibit smaller temperature variations than temperate ponds (Ultsch et al., 1999) and indeed the values of CTMax of *B. americanus* which we report,are roughly 3°C higher than *X. laevis* at all stages of development. Bufonid tadpoles have been reported in water of temperatures as high as 42°C (Sherman, 1980) but data on larval ecophysiology of *X. laevis* are lacking.

Cupp (1980) hypothesized that the decrease in CTMax during metamorphosis represents an adaptive shift downward in the thermal physiology of anurans in anticipation of the lower temperatures to which they will be exposed as adults. However, the increase in CTMax following metamorphosis reported here in *X. laevis* and *B. americanus* juveniles and in *B. w. fowleri* juveniles and adults (Sherman, 1980) supports Sherman's notion that metamorphosis is a stressful condition due to extensive differentiation and metamorphosing animals are simply unable to mobilize mechanisms to cope with high temperatures. Moreover, the thermal environment of adult and larval *X. laevis* is likely to be the same as they are found in the same ponds (Tinsley et al., 1996), although there are no data describing the different microenvironments that these animals might select.

The effect of heat pretreatment on thermal tolerance of animals of different stages is summarized in Fig. 3. Premetamorphic tadpoles of both *X. laevis* and *B. americanus* exhibited a significant capacity for heat hardening (Fig. 3). This capacity would be adaptive for

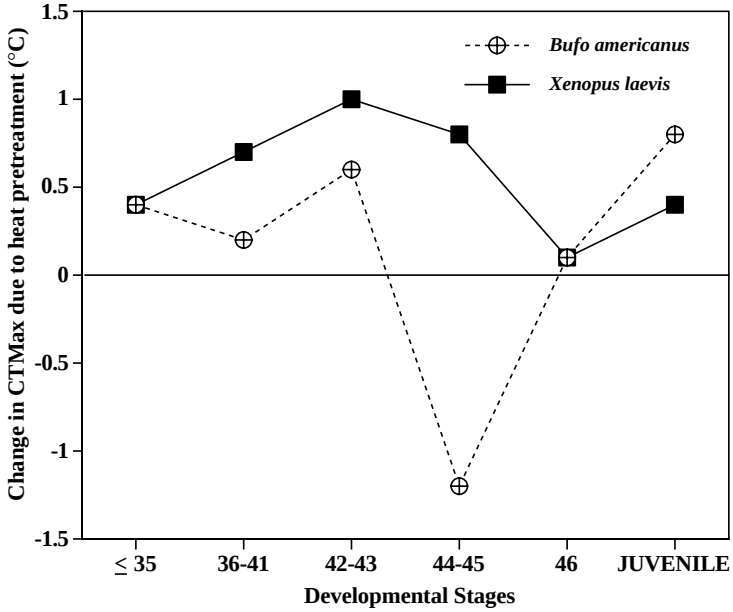

Fig. 3. Effect of heat pretreatment on thermal tolerance of *B. americanus* and *X. laevis* of different stages. Points represent differences between mean CTMax of heat-pretreated animals and control animals of a given developmental stage (data from Figs. 1 and 2). Points above the 0 line reflect heat hardening except for stage 46, in which the difference between the mean CTMax of control and pretreated animals was not significant (*Bufo*: $p = 0.19$; *Xenopus*: $p = 0.20$). Heat pretreatment of *Bufo* stages 44–45 resulted in a significant decrease in thermal tolerance ($p = 0.028$; see text). Earliest stages studied were 32 for *Bufo* and 26 for *Xenopus*.

tadpoles facing daily fluctuations in pond temperatures. Typically, amphibians experiencing heat hardening manifest an increase in thermal tolerance of 1°C or less (Rome et al., 1992). In the only other study of heat hardening in tadpoles of which we are aware, Menke and Claussen (1982) reported an increase in thermal tolerance of 0.4°C in premetamorphic (stages 28–35) *Rana catesbeiana*, though their methods differed from ours considerably. Nevertheless, in our study premetamorphic tadpoles of *B. americanus* of stages 32–35 and premetamorphic tadpoles of *X. laevis* stages 29–35 exhibited heat hardening of 0.4°C as well (Fig. 3).

Within each species, the tadpoles that exhibited the greatest degree of heat hardening were individuals of stages 42–43 (Fig. 3; 0.6°C in *B. americanus* and 1.0°C in *X. laevis*). Similarly, in their study of *R. catesbeiana* tadpoles, Wollmuth and Crawshaw (1988) noted that early metamorphic tadpoles tended to select the highest temperatures of any stages. In order to minimize the amount of time spent in metamorphosis, their most vulnerable period (Wassersug and Sperry, 1977; Ultsch et al., 1999), it might be advantageous for tadpoles to select warmer temperatures (Wollmuth and Crawshaw, 1988) and be capable of enduring those temperatures by heat hardening. However, Wollmuth and Crawshaw (1988) also noted a decrease in selected temperature during late metamorphosis and suggested that this might be characteristic of anuran development.

In our study, late metamorphic *X. laevis* tadpoles (stages 44–45) though having a lower thermal tolerance than premetamorphic animals, still were able to heat harden (Fig. 3). However, in what may be the most striking result of our study, the thermal tolerance of late metamorphic *B. americanus* tadpoles actually decreased by 1.2°C following heat pretreatment (Fig. 3). The decrease in thermal tolerance of both species during metamorphic climax suggests that metamorphosis is indeed stressful. The fact that metamorphosing *B. americanus* tadpoles fail to heat harden and, in fact, exhibit a lower thermal tolerance following heat pretreatment, suggests that metamorphosis might be more stressful for *B. americanus* than *X. laevis*. Moreover, metamorphosing American toad tadpoles required up to 4 h to recover following CTMax determinations, while comparable stage *Xenopus* tadpoles recovered immediately.

Wassersug (1996) has argued that *Xenopus* has a less extensive and less abrupt metamorphosis compared to neobatrachians such as bufonids. *Xenopus* remains aquatic as an adult. It has functional lungs through most of its larval life (Wassersug and Feder, 1983), a comparatively long larval period, and a protracted metamorphosis of roughly 14 d (Wassersug and Sperry, 1977). By contrast, *B. americanus* undergoes more extensive differentiation at metamorphosis (Wassersug and Hoff, 1982). It acquires, for example, functional

lungs only upon metamorphosis (Wassersug and Feder, 1983). Yet the radical shift in its morphology and physiology necessary to accommodate the change from an aquatic to terrestrial habitat occurs during a metamorphic climax as brief as 5 or 6 d (Sherman, personal observation). The more extensive and abrupt differentiation of *B. americanus* during metamorphosis is reflected not simply in its inability to heat harden but, perhaps more significantly, in its decrease in thermal tolerance following a heat stress (Fig. 3).

Individuals of both species that had just completed metamorphosis (stage 46) did not exhibit heat hardening (Figs. 1 and 2). Compared to metamorphosing animals, this represents a recovery of thermal resistance in *B. americanus* as the thermal tolerance of pretreated animals remained the same rather than decreased. However, the significance of the inability of stage 46 *X. laevis* to heat harden is unclear given that animals in metamorphic climax were able to heat harden (Fig. 2).

Among *B. americanus*, the developmental stage exhibiting the greatest degree of heat hardening is the juvenile (Fig. 3). Bufonid toadlets are heliothermic (Sherman, 1980) and the capacity to heat harden is likely to be beneficial given that such behavior enhances their growth rate and shortens their time to adult size (Lillywhite et al., 1973).

Comparisons of the thermal physiology of *B. americanus* and *X. laevis* reported here must be made cautiously as we used individuals from a wild population of *B. americanus* and laboratory reared (for many generations) *X. laevis*. Moreover, a great deal is known about the physiological ecology of bufonid larvae, while there is little comparable literature for wild *Xenopus* larvae.

4.1. Future questions

We hypothesize that the more abrupt and extensive the differentiation of a metamorphosing anuran, the more compromised will be its ability to heat harden during that transformation. Of course, it is difficult to draw phylogenetic conclusions based only on two species and further studies of thermal tolerance during metamorphosis of different species are warranted. Since the 1980s, little attention has been paid to the significance of ontogenetic change in thermal tolerance of anurans. But studies of thermo-physiological correlates of metamorphosis among different amphibian taxa may shed light on fitness consequences of such transformations (see Angilletta et al., 2002).

The mechanisms underlying heat hardening in tadpoles are not known. Increased cellular thermal tolerance following a brief heat stress has been correlated with increased synthesis of heat shock proteins (hsps) (Feder, 1999). However, a causal relationship between hsp synthesis and heat hardening has not been established in several species of salamanders in which it has been studied (Easton et al., 1987; Spotila et al., 1989; Yu et al., 1998). Nevertheless, an investigation of levels of constitutive hsps and hsp synthesis among tadpoles of different stages and species might shed light on the mechanisms underlying the ontogenetic changes in their thermal physiology. Moreover, our observation that the tail seems to have a higher thermal tolerance than the legs of the same metamorphosing individual suggests that there might be differences in constitutive hsps between "adult parts" and "tadpole parts" within the same individual. While hsp synthesis in adult and embryonic amphibians has been reported, studies of hsp synthesis in anurans undergoing metamorphosis are entirely lacking. Finally, Denver (1997) reported that circulating levels of corticosteroid stress hormones increase at metamorphosis in anurans. The relationship among corticosteroids, hsps, and thermal tolerance of metamorphosing tadpoles is unknown and deserves further investigation.

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