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Positive Effects of Acclimation Temperature on the Critical Thermal Maxima of *Ambystoma mexicanum* and *Xenopus laevis*

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ABSTRACT.—**An organism's thermal limits provide valuable insights into species distributions and potential climate change impacts. The critical thermal maximum (CT_{max}) in particular has been widely used for among-species analyses of thermal tolerances. However, CT_{max} measurements can depend on important context-dependencies, including acclimation temperature and experimental procedure. We investigated acclimation effects on CT_{max} in two amphibian species commonly used as laboratory models, *Ambystoma mexicanum* (Axolotls) and *Xenopus laevis* (African Clawed Frogs), using the standard dynamic method. Both species exhibited an increased CT_{max} following acclimation to a warm temperature (24°C) relative to cold acclimation (8°C). We also found that warm-temperature acclimation resulted in significant mass losses in both species, possibly reflecting metabolic costs of living at higher temperatures. Our CT_{max} measurements for *A. mexicanum* were consistent with CT_{max} measurements for closely related salamander species, but CT_{max} measurements for cold-acclimated *X. laevis* were inconsistent with a prior study of this species. This might have been because of the earlier study employing a less common curve-fitting method to estimate CT_{max}. These results highlight the importance of accounting for recent acclimation temperatures when estimating amphibian thermal limits and provide further support for the generalization that most amphibian species exhibit increased CT_{max} following warm-temperature acclimation.**

Global climate change is threatening organisms and ecosystems with more-frequent extreme temperature events (Easterling, 2000; Fischer and Schär, 2008). Amphibians are particularly sensitive to temperature variation because of their ectothermic physiology and ongoing threats from temperature-dependent diseases (Raffel et al., 2013; Price et al., 2019). To predict climate change impacts on amphibian populations, it is important to understand the thermal tolerances of potentially threatened species (Ehrlén and Morris, 2015; Gunderson and Leal, 2015). Perhaps the most widely measured and best-standardized index of thermal tolerance is an organism's critical thermal maximum (CT_{max}), usually defined in practice as the upper temperature at which an organism is no longer capable of a coordinated locomotor response to external stimuli (Lutterschmidt and Hutchison, 1997; Kingsolver and Umbanhowar, 2018). The CT_{max} measurements are available from the literature for many amphibian species (e.g., Hutchinson, 1961; Brattstrom and Lawrence, 1962), but we still lack standardized CT_{max} measurements for many species that are potentially threatened by climate change.

Although CT_{max} is sometimes assumed to be a fixed characteristic of a given species, this assumption is often false because of thermal acclimation responses that result in altered thermal tolerances following prolonged exposure to higher or lower temperatures (Wilson and Franklin, 2002). The "beneficial acclimation" hypothesis postulates that organisms will exhibit adaptive plastic responses following exposure to a high or a low acclimation temperature, increasing their performance at this temperature relative to unacclimated organisms (Wilson and Franklin, 2002). This is a common pattern in the primary literature, with amphibian species nearly always exhibiting increased CT_{max} following warm-temperature acclimation, despite other aspects of thermal performance frequently exhibiting other types of acclimation effects (reviewed by Rome et al., 1992; but see Riquelme et al., 2016). Based on these prior results, we predicted that the two focal amphibian species in this study would exhibit increased CT_{max} values following warm-temperature acclimation.

A challenge with using literature values for CT_{max} measurements, particularly when examining among-species patterns, is that CT_{max} measurements have been obtained using different methods that might not always yield comparable results (Lutterschmidt and Hutchison, 1997; Terblanche et al., 2007). Most CT_{max} measurements have been acquired using the "dynamic method," which involves increasing the temperature of an organism's controlled environment at a constant rate until the organism exhibits a loss of righting response, disorganized locomotor activity, or the onset of muscle spasms (Lutterschmidt and Hutchison, 1997; Kingsolver and Umbanhowar, 2018). However, other methods for obtaining CT_{max} estimates have been used, such as the "static method" in which researchers measure time to some endpoint at a constant stressful temperature (Lutterschmidt and Hutchison, 1997), or curve-fitting methods in which researchers obtain thermal performance data at lower ($<CT_{max}$) temperatures and use a statistical model (e.g., polynomial fit) to estimate the temperature at which performance equals zero (Cortes et al., 2016). To continue building a database of CT_{max} measurements with comparable values, we agree with recommendations by Lutterschmidt and Hutchison (1997) to focus on the more widely used dynamic method.

The present study focused on measuring thermal acclimation effects on the CT_{max} of two model amphibian species: African Clawed Frogs (*Xenopus laevis*) and Axolotls (*Ambystoma mexicanum*). These are the two most widely used model frog and salamander species (respectively) in cellular, developmental, and genetic research (Burggren and Warburton, 2007; Haas and Whited, 2017). Climate change is thought to have contributed to a recent *A. mexicanum* population decline, making investigations into its thermal biology potentially informative for conservation efforts (Contreras et al., 2009).

Perhaps surprisingly given the scientific importance of these two species, we could determine no prior study that used the standard dynamic method to measure thermal acclimation effects on the CT_{max} for adult *A. mexicanum* or *X. laevis*. We were

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unable to find any CT_{max} estimates for *A. mexicanum* in the primary literature, though CT_{max} measurements of closely related species are available (Delson and Whitford, 1973; Nietfeldt et al., 1980). The only study we found that investigated thermal limits of adult *X. laevis* was that of Cortes et al. (2016), who used a nonstandard curve fitting method based on a second-order polynomial fit to thermal performance data (righting response-time measurements). Given the limited availability of CT_{max} data for these two species and their importance as model organisms, it is valuable to obtain new CT_{max} measurements using the standard dynamic method.

Here we present measurements of thermal acclimation effects on the CT_{max} of adult *X. laevis* and *A. mexicanum* using the standard dynamic method. We hypothesized that *X. laevis* and *A. mexicanum* would have thermal responses similar to other amphibian species and, based on this hypothesis, we predicted that both species will exhibit a positive effect of warm temperature acclimation on CT_{max}.

Materials and Methods

Animal Sources and Maintenance.—Wild type *A. mexicanum* larvae were acquired as 1–2 cm larvae between December 2017 and June 2018 from the Ambystoma Genetic Stock Center (Lexington, KY; NIH: P40-OD019794) and raised to the juvenile stage (3–4 cm with limbs). Groups of 5–6 individuals were maintained in 22 × 30 × 6-cm trays filled with 2 L of 20°C Kordon Amquel®-treated water. They were fed a standard diet of five live blackworms (*Lumbriculus variegatus*) per animal twice weekly. Wild type *Xenopus laevis* tadpoles (NF 48–55) were obtained in September 2017 from a commercial research supplier (Nasco) and raised to adults. Groups of 15–30 tadpoles were housed in opaque 27 × 33 × 14-cm tanks filled with 6 L of 20°C Kordon Amquel®-treated water. Adults were housed in groups of 3–4 individuals, which were fed three times weekly with a post-metamorphic "frog brittle" provided by the supplier. Tadpoles were fed a crushed mix of frog brittle, also three times weekly. We provided polyvinyl chloride (PVC) pipes for both species during social housing as a refuge to hide from aggressive conspecifics. All animals were maintained on a 12 : 12; light : dark cycle and checked daily for overall health and water quality. Water changes were performed at every feeding.

Thermal Acclimation Experiment.—*Xenopus laevis* adults ($n = 19$) and *A. mexicanum* juveniles ($n = 19$) were randomly assigned to acclimation treatments (8°C = cold acclimation and 24°C = warm acclimation), which were chosen to reflect ecologically relevant seasonal temperature variation for these species (Major and Wassersug, 1998; García et al., 2009). We determined sex of *X. laevis* according to the protruding cloaca of females ($n = 10$) or its absence in males ($n = 9$). *Ambystoma mexicanum* were too young to determine sex through external observation; therefore, sex differences were not considered for this species. Animals ($n = 38$) were further assigned to four temporal blocks, constraining the randomization to ensure a similar number of each species in each acclimation temperature by block combination. Animals were then maintained at their acclimation temperature for 3 wk, during which time all animals were maintained individually in perforated 1.89-L GladWare plastic containers filled with 1 L water. Incubators ($n = 19$) were constructed as described by Raffel et al. (2013) using Styrofoam coolers, heat tape, and adjustable thermostats, except that heat tape was placed along the sides of each incubator instead of along the floor (i.e., convective instead of conductive heat transfer to animal containers). To monitor actual temperatures experienced by animals during the acclimation period, one HOBO data logger, set to record hourly measurements, was floated in the container of a randomly selected animal within each incubator. These temperatures remained close to their target temperatures throughout the acclimation period (warm incubators: 24.23 ± 1.57°C standard deviation (SD); cold incubators: 8.34 ± 0.81°C SD). To maximize the number of true incubator-level replicates for within-species acclimation effects, each acclimation incubator contained just one *X. laevis* and one *A. mexicanum*. The positions of the two animals' containers were rotated daily to control for the possibility of within-incubator temperature variation. Twice weekly, *X. laevis* and *A. mexicanum* were fed blackworms ad libitum, and soiled water was replaced with water preheated to the appropriate temperature. Feeding and cleaning occurred on the same 2 days of the week regardless of temporal block, resulting in a variable time interval of 2 days (Blocks 2 and 3) or 3 days (Blocks 1 and 4) between the last feeding and initiation of the CT_{max} measurement. To assess possible effects of the acclimation period on organisms' mass, the mass of each animal was measured immediately before and after its acclimation period. All animals were maintained at a 12 : 12 photoperiod.

Thermal Tolerance Measurements.—We measured CT_{max} using the dynamic (or "ramping") method, i.e., exposing each animal to a constant gradual increase in temperature until they reach a critical endpoint (Lutterschmidt and Hutchison, 1997). Each animal was weighed and then placed in a temperature-controlled water bath filled to a 12-cm depth (4.7 L) of 17°C water, which was heated at a constant rate of 0.745°C min^{-1} until CT_{max} was reached, as indicated by a loss of righting response (LRR). For both species, we defined LRR as the failure of an animal to turn itself back over within 10 sec. We measured LRR at 1-min intervals during the ramping assay by using a glass rod to turn each animal onto its back. Once an animal exhibited LRR, we ended the trial by moving it back to its starting temperature (17°C). High activity levels (e.g., attempts to escape their container or bite the glass rod, or rapid swimming in response to the glass rod) were consistent indicators of righting capacity in preliminary trials, so we assumed these behaviors were accurate indicators of righting capacity during experimental trials rather than inducing unnecessary stress by forcing high-activity animals onto their backs. Animals typically regained righting capacity and resumed normal swimming behavior within 30 sec of being returned to 17°C, and all animals regained righting capacity within 3 min.

All CT_{max} measurements were completed within 30 min of an animal being removed from its acclimation temperature. We performed the CT_{max} assay on two individual animals concurrently, one from each species randomly selected from different acclimation temperatures, with three exceptions in which different species from the same acclimation temperature were assayed together because of an odd number of animal pairings in three temporal blocks. The two animals were separated by placing each into one side of a two-celled cage constructed from plastic mesh. To measure the rate of heating, a HOBO data logger was placed in the water bath and set to record temperature at 1-min intervals. Once an organism exhibited LRR or inverted swimming, we recorded the length of assay and time of day to verify the precision of the heating rate collected by the HOBO data logger, and the final temperature was recorded using a digital aquarium thermometer.

TABLE 1. Summary statistics for effects of thermal acclimation temperature (8°C or 24°C) on the CT_{max} and body masses of *X. laevis* and *A. mexicanum*. SE in parentheses.

	X. laevis		*A. mexicanum*	
Variable	8°C	24°C	8°C	24°C
Sample size	10	9	10	9
CT_{max} mean (°C)	33.4 (0.32)	37.4 (0.14)	33.1 (0.20)	37.1 (0.19)
CT_{max} range (°C)	31.8–35.2	36.8–38.6	33.2–34.5	36.4–38.0
Mass before (g)	72.81 (11.96)	56.1 (12.20)	5.02 (0.29)	3.73 (0.34)
Mass after (g)	69.95 (11.66)	48.85 (10.77)	5.45 (0.25)	3.50 (0.39)
ΔMass (g)	−2.86 (0.59)	−7.28 (1.57)	0.42 (0.09)	−0.23 (0.14)

Statistical Analysis.—Descriptive statistics were obtained using Microsoft Excel (Version 2007, location: Redmond, WA), and all analyses were performed using R version 3.5.1 statistical software (R Core Team, 2018). Linear regression models (function "lm") were used to assess effects of acclimation temperature on CT_{max} and mass changes for both *X. laevis* and *A. mexicanum*. Main and interactive effects of sex and acclimation temperature on CT_{max} were assessed for *X. laevis* by adding "Sex" as an additional predictor in the primary CT_{max} model; predictor significance was assessed using *F*-tests with Type II sum of squares ("Anova" command in package "car"; Fox and Weisberg, 2011). Temporal block was removed from all final models because it did not significantly improve any of them (all $P > 0.1$).

RESULTS

Xenopus laevis exhibited significantly higher CT_{max} values when acclimated to 24°C ($CT_{max} = 37.4 \pm 0.14$°C standard error [SE]) than when acclimated to 8°C ($CT_{max} = 33.4 \pm 0.32$°C SE; Tables 1,2). *Ambystoma mexicanum* also exhibited significantly higher CT_{max} values when acclimated to 24°C ($CT_{max} = 37.1 \pm 0.19$°C SE) than when acclimated to 8°C ($CT_{max} = 33.1 \pm 0.20$°C SE; Tables 1,2). No significant main or interactive effects of sex on CT_{max} were detected for *X. laevis* (both $P > 0.1$). Both species also experienced significantly greater mass losses during 24°C acclimation relative to animals acclimated to 8°C (Tables 1,2). Additional summary statistics, including ranges of observed CT_{max} values and mean values for masses before and after acclimation, are reported in Table 1.

DISCUSSION

Consistent with our prediction, both *A. mexicanum* and *X. laevis* exhibited evidence for positive effects of warm-temperature acclimation on CT_{max}. These results are consistent with previous findings of positive correlations between CT_{max} and acclimation temperature in other amphibian species (Rome et al., 1992; Davies et al., 2015), likely because of increased expression of heat shock proteins (Heikkila et al., 1985).

Our CT_{max} measurements for warm-acclimated *A. mexicanum* were comparable to published measurements for warm-acclimated larvae, juveniles, and neotenic adults of its sister species, *Ambystoma tigrinum* (Tiger Salamander) (36.0–39.0°C for 20 or 28°C acclimation; Delson and Whitford, 1973; Nietfeldt et al., 1980). Our cold-acclimated *A. mexicanum* had lower CT_{max} values than did 10°C-acclimated larval or adult *A. trigrinum* observed by Delson and Whitford (all >35°C; 1973), but they were comparable to CT_{max} measurements of cold-acclimated larval *A. tigrinum* obtained by Nietfeldt et al. (33–35°C for 2 or 10°C acclimation; 1980). Both prior studies found positive effects of warm acclimation on *A. tigrinum* CT_{max} values, and one study revealed substantial within-species differences in CT_{max} values among populations and life stages (Delson and Whitford, 1973; Nietfeldt et al., 1980). These comparisons further emphasize the importance of accounting for differences in experimental procedures (e.g., acclimation temperatures, heating rates, and life stages investigated) when comparing results across studies.

Our CT_{max} measurement for warm-acclimated *X. laevis* was comparable to that estimated by a prior study of this species (36.2 ± 0.7°C SE) but lower than their CT_{max} estimate for *X. laevis* acclimated to 10°C (41.8 ± 2.2°C; Cortes et al., 2016). Cortes et al (2016) also did not detect a significant effect of acclimation temperature on their CT_{max} estimates. This discrepancy might partly be because of our use of the dynamic method to measure CT_{max} in contrast to the curve-fitting methodology employed by Cortes et al. (2016). It is generally acknowledged that different methods for obtaining CT_{max} estimates can generate conflicting results (Lutterschmidt and Hutchison, 1997). The dynamic method is widely preferred because it is simpler to use, requires fewer animals, can be performed in less time, and provides a direct measurement of the temperature at which a locomotor performance drops to zero (Lutterschmidt and Hutchinson, 1997).

During the 3-wk acclimation period, both species experienced losses in body mass at the warm acclimation temperature. *Xenopus laevis* lost body mass at both acclimation temperatures, with a greater loss at the warm temperature, whereas *A. mexicanum* only lost mass at the warm-acclimation temperature. This pattern might have been driven by positive effects of temperature on animals' metabolic rates, leading to higher energy expenditure at warmer temperatures (Irwin et al., 1998). This pattern is consistent with past observations showing that *A. mexicanum* have higher metabolic rates when measured at warmer temperatures or following acclimation to cooler temperatures (i.e., a "cooler is better" pattern; Irwin et al., 1998). Mass losses during warm-temperature acclimation likely indicate that animals were unable to compensate for increased metabolic rates by increasing food consumption, either because of limited food availability in this specific experiment or fundamental differences in the temperature dependence of food assimilation (i.e., digestion) vs. metabolism, as has been observed in studies of bony fish (Lemoine and Burkepile, 2012). Regardless of the cause of this pattern, these results indicate the importance of avoiding long-term exposure to excessively warm temperatures for these two laboratory model species.

Our results provide further support for the idea that temperatures experienced by animals in the recent past (i.e., past couple weeks) are useful in predicting changes in thermal tolerances and emphasize the need to account for acclimation temperature when conducting among-species comparisons of

Table 2. Regression results for effects of acclimation temperature on the critical thermal maximum (CT$_{\text{max}}$) or the change in mass (ΔMass) in each species. The single explanatory variable in each model was acclimation temperature in °C.

Response variable	Species	Coefficient ± SE	r^2	F	df	P
CT$_{\text{max}}$	*X. laevis*	0.250 ± 0.0017	0.86	105.8	(1,17)	<0.001
	A. mexicanum	0.254 ± 0.0024	0.93	215.8	(1,17)	<0.001
ΔMass	*X. laevis*	−0.276 ± 0.0010	0.47	7.58	(1,17)	0.014
	A. mexicanum	−0.041 ± 0.1000	0.48	15.8	(1,17)	<0.001

CT$_{\text{max}}$ (e.g., Rohr et al., 2018). This study adds to our knowledge of amphibian thermal tolerances by providing standardized measurements of CT$_{\text{max}}$ values for two scientifically important model species, and our finding of negative effects of warm temperature exposure on body mass provides potentially useful information for improving animal welfare in laboratory conditions. Our focus on lab-bred strains of both species means that we should be cautious about extrapolating these results to wild populations. For the sake of ecological relevance, future studies should seek to replicate these results using wild-caught animals and seek to account for potential context-dependencies such as geographic differences, behavioral responses, or effects of diurnal temperature fluctuations (Hutchinson and Ferrance, 1970; Hutchinson and Maness, 1979).

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