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José L. Bartheld, Paulina Artacho, Leonardo Bacigalupe



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Title: Thermal performance curves under daily thermal fluctuation: A study in helmeted water toad tadpoles.

Running head: TPCs under daily thermal fluctuation.

List of authors: José L. Bartheld¹, Paulina Artacho² & Leonardo Bacigalupe¹

1 Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de Chile, Campus Isla Teja, Valdivia, Chile.

2 Instituto Tecnológico del Salmón, Salmón Chile.

Corresponding author: José L. Bartheld

email: jlbartheld@gmail.com

Telephone number: +56964707864

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Abstract

Most research in physiological ecology has focused on the effects of mean changes in temperature under the classic “hot vs cold” acclimation treatment; however, current evidence suggests that an increment in both the mean and variance of temperature could act synergistically to amplify the negative effects of global temperature increase and how it would affect fitness and performance-related traits in ectothermic organisms. We assessed the effects of acclimation to daily variance of temperature on thermal performance curves of swimming speed in helmeted water toad tadpoles (*Calyptocephalella gayi*). Acclimation treatments were $20\text{ }^{\circ}\text{C} \pm 0.1\text{ SD}$ (constant) and $20\text{ }^{\circ}\text{C} \pm 1.5\text{ SD}$ (fluctuating). We draw two key findings: first, tadpoles exposed to daily temperature fluctuation had reduced maximal performance (Z_{max}), and flattened thermal performance curves, thus supporting the “vertical shift or faster-slower” hypothesis, and suggesting that overall swimming performance would be lower through an examination of temperatures under more realistic and ecologically-relevant fluctuating regimens; second, there was significant interindividual variation in performance traits by means of significant repeatability estimates.

Our present results suggest that the widespread use of constant acclimation temperatures in laboratory experiments to estimate thermal performance curves (TPCs) may lead to an overestimation of actual organismal performance. We encourage the use of temperature fluctuation acclimation treatments to better understand the variability of physiological traits, which predict ecological and evolutionary responses to global change.

1. Introduction

For ectotherms, temperature is perhaps the most important abiotic characteristic of the environment, as it affects virtually all organismal processes at different levels of organization (Angilletta et al., 2002; Hochachka and Somero, 2002). Temperature is known to have important effects on physiology, performance, and fitness (Huey and Berrigan, 2001; Huey and Kingsolver, 1989; Huey and Stevenson, 1979; Kingsolver and Gomulkiewicz, 2003; Kingsolver and Huey, 2008). In addition to increasing mean environmental temperatures, a higher frequency of extremely high temperatures and greater temporal variability have also been predicted to occur under climate change (Easterling et al., 2000; Rahmstorf and Coumou, 2011; Wang and Dillon, 2014). As stated, evidence suggests that an increment in both the mean and variance of temperature could act synergistically to amplify the negative effects that global warming will have on fitness and performance-related traits (Vasseur et al., 2014).

A practical approach to studying the physiological capabilities of ectotherms to given body temperatures is by estimating thermal performance curves (TPCs) (Huey and Stevenson, 1979). TPCs describe an organism's performance as a function of temperature, which generally have a concave shape. Performance increases with increasing body temperature from a critical minimum temperature (CT_{min}), reaching a maximum value (Z_{max}) at the optimal temperature (T_{opt}), and then rapidly decrease to a critical maximum temperature (CT_{max}) (Angilletta, 2006; Huey and Stevenson, 1979; Kingsolver and Gomulkiewicz, 2003). Two other important parameters derived from TPCs are thermal tolerance ($T_{tol} = CT_{max} - CT_{min}$) and thermal performance breadth (TBr). TBr is the range of body temperatures over which performance is greater than a given proportion of Z_{max} (e.g. $TBr_{80} = 80\%$ of Z_{max}) (Angilletta, 2006; Huey and Hertz, 1984; Izem and Kingsolver, 2005). TPC shape is similar across taxa, but high variation is

attributable to genetic, latitudinal, or acclimation effects (Clusella-Trullas et al., 2011; Izem and Kingsolver, 2005; Kingsolver, 2009; Ruiz-Aravena et al., 2014).

In general, studies of thermal ecology have focused on the effects of mean changes in temperature (Bozinovic et al., 2013), and TPCs have often been derived for certain organisms acclimating to a range of constant temperatures in controlled laboratory conditions (Gaitan-Espitia et al., 2014; Ruiz-Aravena et al., 2014; Scheiner, 2002). However, environmental temperatures are highly dynamic and tend to vary on annual, seasonal, and daily scales (Geerts, 2003; Paaijmans et al., 2010). In general, environmental temperature variability has been recognized as an important factor that affects several processes and fitness-related traits, such as gene expression (Podrabsky and Somero, 2004), development (Arrighi et al., 2013; Kaplan and Phillips, 2006; Kern et al., 2015; Paaijmans et al., 2013), performance (Bozinovic et al., 2013; Colinet et al., 2015; Kern et al., 2015; Meřaková and Gvoždík, 2009; Niehaus et al., 2011; Vasseur et al., 2014), and survival (Bozinovic et al., 2016; Estay et al., 2014; Lawson et al., 2015; Paaijmans et al., 2013). In particular, it has been observed that daily thermal fluctuations increase metabolic demands, compared with constant thermal conditions, inducing energetic trade-offs that can affect survival, body condition, growth, development, metabolic enzyme activity, and performance (Arrighi et al., 2013; Colinet et al., 2015; Kern et al., 2015; Niehaus et al., 2012). Despite the obvious need to understand the effects of acclimation for increased temperature means and variability, the number of studies addressing this is insufficient, especially in non-model vertebrate species.

Here, we investigated the effects of acclimation to daily thermal fluctuation using a simple experimental protocol to separate the effects of mean and daily temperature variation on swimming speed of helmeted water toad tadpoles (*Calyptocephalella gayi*; formerly *Caudiverbera caudiverbera*) (Donoso-Barros and Cej, 1962). We chose to focus on locomotor

performance, because it is a whole-animal phenotypic performance trait that has important ecological relevance and is also correlated with fitness (Burggren and Warburton, 2007; Husak, 2006; Jayne and Bennett, 1990; Le Galliard et al., 2004). In addition, *C. gayi* inhabit lentic ponds and streams with unstable temperatures throughout the year, with a larval development period lasting longer than 1.5 years. Anuran larvae are considered ideal organisms for studying thermal tolerance and thermal sensitivity in ectotherms (Burggren and Warburton, 2007) because their internal temperature is the same as that of their surrounding water environment, in comparison with semi-aquatic or fully terrestrial adults, in whom body temperature also depend on hydric interchanges with the environment (Spotila et al., 1992). They do not suffer dehydration when exposed to heat, so although tadpoles are capable of behavioral thermoregulation through habitat selection, physical restrictions of shallow ponds without thermal stratification limit their capacity to thermoregulate (Casterlin and Reynolds, 1978; Wu and Kam, 2005; Piasečná et al. 2015).

Since *C. gayi* inhabits relatively unstable environments, we hypothesized that should present a TPC sensitive to the thermal variation that it undergoes during acclimation, specifically how acclimation to daily temperature fluctuation should cause a reduction of maximal swimming performance (Z_{\max}) in comparison with individuals acclimatized at a constant temperature with the same mean temperature.

2. Materials and methods

2.1 Study animals and acclimation treatments

C. gayi is an endemic species of Chile and the single living species of *Calyptocephalella* genus, and is restricted to the temperate region (Aconcagua Province) to Puerto Montt (i.e., between 30° and 42°S) (Ceí, 1962). Current evidence indicates that populations of *C. gayi* are declining in their native range (Díaz-Páez and Ortiz, 2003). Fifty-four helmeted water toad tadpoles (*C. gayi*, Gosners 25-27) were obtained at a breeding farm in Santiago, Chile (33°S,

70°W). Tadpoles were transported to Universidad Austral de Chile laboratory facilities and were immediately placed in four aquariums with dechlorinated tap water, at a stocking density of 0.25 individuals per liter. Tadpoles were exposed to a 12L:12D photoperiod and fed *ad libitum* with commercial food for herbivorous fish.

After two weeks, tadpoles were randomly selected and transferred to six water baths (100 x 75 x 30 cm³), each with nine divisions (30 x 25 cm²) made from a plastic net (0.5 cm aperture) to individually hold each tadpole. The water baths were filled with dechlorinated tap water. Each water bath was equipped with a thermostatic heater (100 W; SERA, Heinsberg, Germany) and was connected to an air pump system (40 W, Sunsun HT-400, China) to enable equal heat and oxygen distribution throughout the bath. Three baths were randomly designated to the “constant temperature acclimation treatment.” These baths were placed on three shelves in a room where the air temperature was set to 13°C. The bath temperature was maintained at 20°C ± 0.1 SD. The three other baths were placed on the same three shelves in the same room and assigned to the “fluctuating temperature acclimation treatment.” For this treatment, the bath thermostat was set at 28°C and switched on automatically with a timer every 12 h (timer switch on/off time) to obtain a temperature ranging from 18°C to 22°C with a mean of 20°C ± 1.5 SD and a ramping of 0.25°C/h. Every five min, we recorded the water temperature of baths with the use of data loggers (HOBO Water Temp Pro v2 Data Logger, Onset Computer) (Fig. 1). The mean temperature of 20°C was chosen, as this represents the average water temperature experienced by *C. gayi* during the summer in Santiago (hydro meteorological database from the Chilean government agency DGA, Dirección General de Aguas; <http://snia.dga.cl/BNAConsultas/reportes>). In the two weeks of acclimation, tadpoles were exposed to a 12L:12D photoperiod and fed *ad libitum* with commercial food for herbivorous fish

(SERA, Heinsberg, Germany). Evaporated water and food remains were replaced and removed every three days.

2.2 Performance measures

As a measure of locomotor performance, we used swimming speed instead of instantaneous velocity or acceleration for two reasons: First, *C. gayi* tadpoles are relatively large (9 cm total body length), so the distance to estimate the locomotor performance would correspond only to three body lengths; second, in our field observations we saw that the escape behavior of this species is a vertical straight line in the direction of the bottom to hide in the sediment. Swimming speed was individually measured at six body temperatures in random order (5°C, 12°C, 20°C, 25°C, 30°C, and 35°C). Tadpoles (Gosner stages 25-27) were kept individually and brought to these target temperatures by heating or cooling them in a thermostat-controlled temperature water bath for at least one hour before measurements were obtained. Previous studies on amphibian tadpoles have indicated that this is an appropriate amount of time to reach desired temperatures (Richter-Boix et al., 2015). Measurements were made using a thermal bath provided with a swimming track. The track consisted of an open cross section of a Polyvinyl chloride (PVC) tube (1 m long x 10 cm wide x 7 cm deep) filled with water at a given temperature. The tube had marks at five cm intervals, and a digital camera (DSR Cannon D 70 set on 60 frames/s) was positioned above the tube. Individuals were randomly assigned to groups of 10 tadpoles, and the sequences of temperatures in each group were randomized. Additionally, tadpoles were always provided a minimum of 24 h of rest between trials. Each tadpole was placed at the end of the swimming track and was then chased by gently tapping the tail with a soft brush. We recorded three daily swimming trials (nonconsecutive) for each individual at each test temperature. The fastest velocity of the three trials, estimated over a 25 cm interval (that

represents approximately three body lengths) using the software Tracker 4.87 (<http://www.cabrillo.edu/~dbrown/tracker>), was considered the swimming speed ($\text{cm} \times \text{s}^{-1}$) for each test temperature and individual. Since locomotor performance may scale with body size (Gvozdik and Van Damme, 2006), and body size may confound the effect of swimming speed on escape success (Van Damme and Van Dooren, 1999), we used size-relative swimming speed (body lengths per seconds, $\text{BL} \times \text{s}^{-1}$) for constructing TPCs. The mean \pm SE total length of individuals in each treatment was 9.15 ± 0.10 cm (constant temperature) and 8.89 ± 0.10 cm (fluctuating temperature). After completing the swimming speed measurements, each individual was dorsally photographed from a standardized distance. The image processing software ImageJ (Abramoff et al., 2004) was used to measure the total length of each tadpole, i.e., the straight line distance from the tip of the snout to the tip of the tail.

2.3 Statistical analysis

Thermal performance curves were analyzed using a mixed modelling approach, as we have six repeated measures on the same individual, one at each test temperature. The response variable was swimming speed corrected by the body length ($\text{BL} \times \text{s}^{-1}$). The effect of acclimation treatment, test temperature, and their interactions were included as fixed effects and were evaluated through confidence intervals computed from the likelihood profile (Bates et al., 2013). Body temperature was included as a linear effect and a quadratic effect to account for the curvature of TPCs. Individuals as well as water baths were included as random effects and their statistical significance was assessed with likelihood ratio tests of nested models. In addition to the analysis described above, for each individual we numerically found the TPCs' parameters (CT_{min} , CT_{max} , TBr_{80} , Z_{max} , T_{opt} , T_{tol}) adjusting a second-order polynomial function with the software CurveExpert Professional v2.2 (Hyams, 2016), and then compared these parameters

among treatments with independent sample t-tests. If a tadpole reached a Gosner stage greater than 27, it was not considered in the analysis.

3. Results

3.1 Curvature and slope of swimming TPC

Fixed effects analysis indicated that the curvature of swimming TPCs was significantly affected by acclimation treatment (Table 1, Fig. 2). Fluctuating temperature ($20^{\circ}\text{C} \pm 1.5$ SD) acclimated tadpoles had lower performance ($t = -3.02$; $p < 0.01$; CI 95 for mean differences = 0.463 to 0.515) and the flatter curve ($t = 3.52$; $p < 0.01$; CI 95 for mean differences = -0.012 to -0.011) compared to constant temperature ($20^{\circ}\text{C} \pm 0.1$ SD) acclimated animals. A visual inspection of Fig. 2 indicates that performance differences between treatments are greater when performance reaches its maximum value (Z_{max}). The model that best describes the individual differences in thermal sensibilities (Fig. 3) was the one that accounted for individual differences in both the linear slope and curvature of the TPCs (table 2, model 4).

3.2 Thermal traits

The statistical comparison of numerically-derived parameters of tadpoles best fit model TPCs (table 3) showed significant differences between thermal treatments in terms of maximal performance (Z_{max}) ($t = 5.491$, $p < 0.01$). The other thermal traits were not affected by acclimation (Table 3).

4. Discussion

In this study, we experimentally evaluated the effects of daily thermal variability on swimming speed in helmeted water toad tadpoles. We compared TPCs of individuals acclimated to constant temperatures and to daily fluctuating temperatures. From this, we draw two key conclusions: first, tadpoles exposed to daily temperature variation had reduced maximal

swimming speeds (Z_{max}) and flattened TPCs, although other TPC parameters were not affected (CT_{min} , CT_{max} , T_{tol} , T_{opt} , T_{br80}); second, there was significant interindividual variation in the TPCs.

The observed reduction of maximal performance in the fluctuating treatment is difficult to explain, because the capacity for thermal acclimation of an organism is determined by several factors, such as phylogenetic history and past environmental conditions at multiples timescales (Seebacher et al., 2012, Gunderson and Stilman 2015, Sorensen et al, 2016). On the other hand, evolutionary theory gives us three theoretical models for TPC variation. First, the “vertical shift or faster-slower” hypothesis describes variation in overall performance across all temperatures. Alternatively, “horizontal shift or cooler-warmer,” describes variation in the location of maximum performance. Last, the “generalist-specialist trade-off” hypothesis involves variation in the width of TPCs, and identifies trade-offs between width and maximal performance (Angilletta et al., 2003; Frazier et al., 2006; Huey and Kingsolver, 1989; Izem and Kingsolver, 2005; Kingsolver, 2009). Because our results fit to a change in Z_{max} between constant and fluctuating regimens, we find enough evidence to support the “vertical shift or faster-slower” hypothesis, thus suggesting that overall swimming performance can be lower through examining temperatures under more realistic and ecologically-relevant fluctuating regimens.

The T_{opt} for both acclimation treatments was approximately 20°C, but fluctuating regimens exceeded this level to <18°C and > 22°C, so we speculate that the tadpoles were in chronically and thermally stressful conditions. Thus the observed reduction of maximal performance in the fluctuating treatment is consistent with Jensen’s inequality, a mathematical property of nonlinear functions (Ruel and Ayres, 1999). In relation to TPCs, the Jensen’s theorem states that temperature variability is predicted to consistently elevate or depress performance in relation to acquired performance at the same mean temperature in a constant regimen. The increases or decreases in performance only depend on whether the function is

accelerating or decelerating. Jensen's inequality predicts that in a thermally-fluctuating environment with a mean value above the TPC inflexion point, performance is predicted to be lower than in a constant environment with the same mean temperature (as observed in this experiment). Likewise, in a thermally variable environment, when the mean temperature is below the TPC inflection point, performance will be higher than it would be in a constant environment with the same mean temperature. Previous studies have reported contradictory results; some have shown that fluctuating thermal conditions tend to reduce performance relative to constant conditions (Arrighi et al., 2013; Bozinovic et al., 2013; Kingsolver et al., 2004; Kjaersgaard et al., 2012; Petavy et al., 2004), while others have reported increased performance in thermally-variable conditions (Bozinovic et al., 2011; Kingsolver et al., 2009). Despite such discrepancies, Jensen's inequality could explain the observed TPC phenomena, as differences in performance between constant and variable environments depend on where the mean temperature lies in relation to the inflection point. On the other hand, the *C. gayi* evolutionary history and physiological thermal capabilities is also an important factor to take into consideration, because the effects of environmental variation would ultimately be modulated by complex interrelationships between ecology and evolution. In this sense, the use of comparative phylogenetics could be used for testing adaptive hypotheses regarding history and performance in ectotherms (Nespolo et al., 2017).

The importance of studying locomotor performance is that this trait may determine if an organism is able to escape from predators, catch prey, or disperse (Arnold, 1983; Irschick and Garland, 2001). In addition, trait variation among individuals within populations is the 'raw material' upon which natural selection can act (Brodie and Russell, 1999); however, little is known about how thermal performance varies among individuals or whether it is an adaptive source of phenotypic variation (Careau et al., 2014). Moreover, individual variability in the

effect of thermal sensitivity on performance has been explicitly assessed in only a few studies in lizards (Artacho et al., 2013) and frogs (Careau et al., 2014; Preest and Pough, 1989). More field and laboratory studies should be conducted to understand the ecological and evolutionary significance of the observed interindividual variation in *C. gayi* tadpoles, and experiments should be conducted to determine the relationship between this phenotypic variation and fitness.

Our simple laboratory experiment demonstrates that minimal daily variation in temperature (18°C to 22°C with a mean of $20 \pm 1.5^\circ\text{C}$ SD), but spanning through the most thermally sensitive areas of the TPC can reduce performance; thus, constant acclimation temperatures in laboratory experiments may lead to an overestimation of performance, and many TPCs reported in the literature may overestimate what actually occurs in nature. We recommend caution when using maximum performance values (Z_{max}), as input in mechanistic predictive models that consider physiological sensitivity to increased temperature or other components of climate, and we must to consider the effects of temperature variability at different temporal scales.

Finally, we reiterate the recommendation of several studies (e.g. Bozinovic et al., 2016; Bozinovic and Poertner, 2015; Estay et al., 2014; Huey et al., 2012; Vasseur et al., 2014; Vázquez et al., 2015), it is imperative that we better understand the effects of increased thermal means and variability on physiological traits to predict ecological and evolutionary responses to ongoing global warming.

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6. Tables

Table 1. Significance tests of the simplest random effect model (model 1). Probabilities were calculated using the lmerTest package in R and Satterthwaite's approximation for estimating degrees of freedom. Bt, linear temperature component; Bt², quadratic temperature component; AT, acclimation treatment.

	β	<i>s.e.</i>	<i>d.f.</i>	<i>T</i>	<i>p value</i>
(intercept)	- 0.89	0.22	233	- 4.11	< 0.001***
Bt	0.48	0.02	205	21.33	< 0.001***
Bt ²	- 0.01	0.00	205	-21.27	< 0.001***
AT	0.13	0.29	232	0.44	0.660
AT : Bt	- 0.09	0.03	204	- 3.02	0.003**
AT : Bt ²	0.00	0.00	204	3.52	< 0.001***

*P < 0.05; **P < 0.01; ***P < 0.001

Table 2. Random effect models used and comparisons of them using REML likelihood ratio tests. Comparison between models (M) is shown; ID, individual tadpole; Bt, linear temperature component; Bt², quadratic temperature component; AT, acclimation treatment; AIC, Akaike Information Criterion; Loglike, log likelihood; ref M, reference model; a colon ':' indicates an interaction, and '|' indicates a conditional relationship. All models contain the full fixed effect structure alongside the random structure.

Model	Random terms	ref M	M d.f.	AIC	Loglike	χ^2	d.f.	<i>p</i> value
1	(1 ID)		8	489.24	-222.53			
2	(Bt ID)	1	9	461.85	-221.92	1.22	1	0.269
3	(Bt ² ID)	1	11	462.11	-220.06	4.95	3	0.175
4	((Bt+Bt ²) ID)	1	14	448.75	-210.38	24.32	6	< 0.001***
5	(AT ID)	1	11	466.77	-222.38	0.29	3	0.960

Models including water bath as a random effect always were less informative **P* < 0.05;

P* < 0.01; *P* < 0.001

Table 3. Parameters of thermal performance curves for each acclimation treatment were obtained from the quadratic model: Swimming speed = Body temperature + (Body temperature)². T-tests were made to compare the acclimation treatments.

<i>Parameter</i>	<i>Constant (n=19)</i>	<i>Fluctuating (n=21)</i>	<i>d.f.</i>	<i>T</i>	<i>p value</i>
Z_{\max}	4.03 ± 0.17	3.38 ± 0.12	40	3.19	0.003**
T_{opt}	20.43 ± 0.28	21.17 ± 0.25	40	- 1.98	0.054
CT_{\min}	1.81 ± 0.30	1.87 ± 0.33	40	- 0.13	0.896
CT_{\max}	39.08 ± 0.56	40.45 ± 0.46	40	- 1.91	0.063
T_{tol}	37.28 ± 0.70	38.59 ± 0.61	40	- 1.41	0.165
TBr_{80}	16.85 ± 0.40	17.26 ± 0.28	40	- 0.88	0.385

Parameters: Maximum performance (Z_{\max}), Thermal optimum (T_{opt}), Critical thermal minimum (CT_{\min}), Critical thermal maximum (CT_{\max}), Thermal tolerance (T_{tol}), Thermal performance breadth (TBr_{80}). Data include means \pm s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

7. Figures

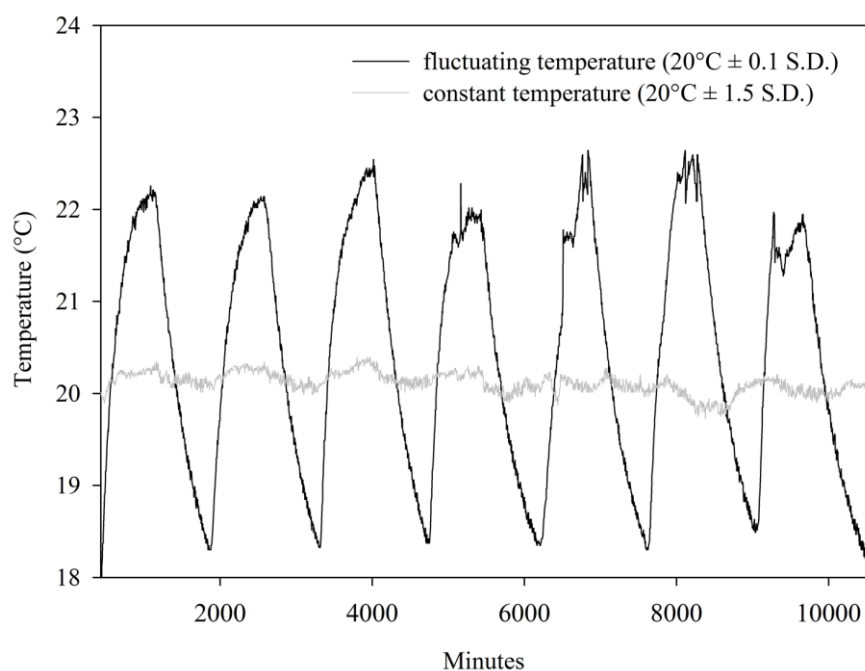


Figure 1. An example of the first week of temperature fluctuations in two acclimation treatments used to test the effects of daily temperature variation on swimming TPCs.

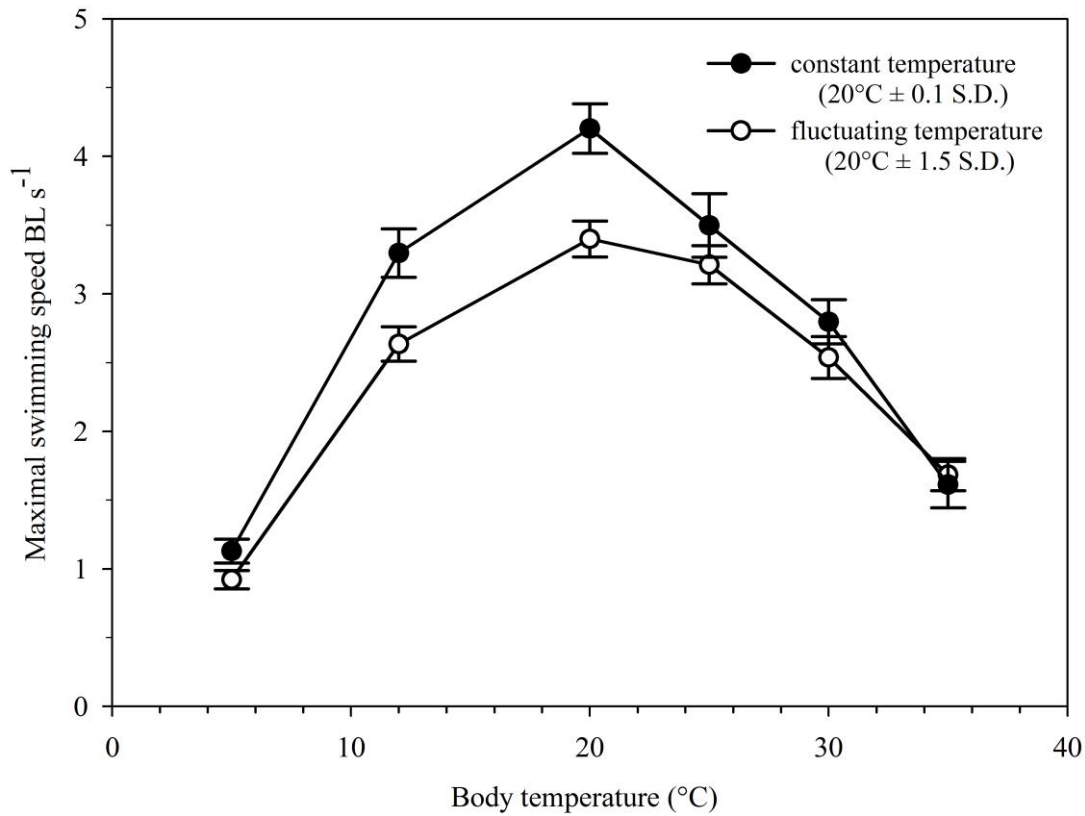


Figure 2. Maximal swimming performance curves of *Calyptocephalella gayi* tadpoles acclimated to constant ($20 \pm 0.1^\circ\text{C}$; $n=18$) and fluctuating (mean: $20 \pm 1.5^\circ\text{C}$; $n=25$) temperatures. Values are expressed in mean \pm s.e.m. Performance curves have the same optimum temperature (T_{opt}), but their maximum performance (Z_{max}) are significantly different (see tables 1 and 3).

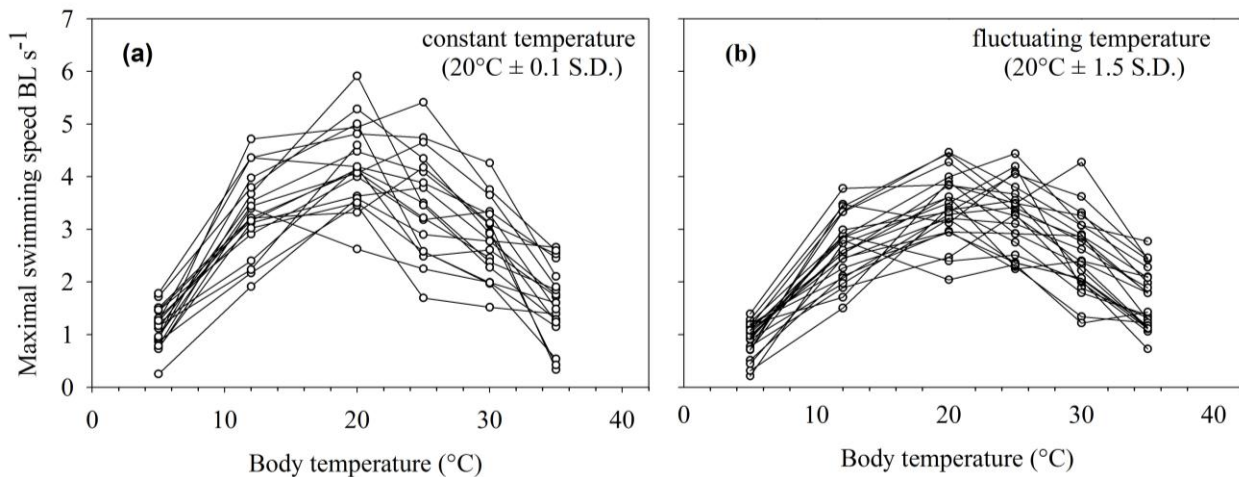


Figure 3. Individual variation in maximal swimming performance for 40 helmeted water toad tadpoles (*Calyptocephalella gayi*). Observed variation (open circles) and performance curves (lines) in **a)** tadpoles acclimated to constant temperature ($20^{\circ}\text{C} \pm 0.1 \text{ S.D.}$; $n=19$) and **b)** tadpoles acclimated to fluctuating temperature (mean: $21^{\circ}\text{C} \pm 1.5 \text{ S.D.}$; $n=21$).

Highlights

- We assessed acclimation effects to daily thermal fluctuation on thermal performance curves of swimming speed in helmeted water toad tadpoles (*Calyptocephalella gayi*).
- Tadpoles exposed to daily temperature fluctuation had reduced maximal performance and flattened thermal performance curves, although other thermal traits were not affected.
- We find evidence to support the “vertical shift or faster-slower” hypothesis,” thereby suggesting that overall swimming performance can be lower through examining temperatures under more realistic and ecologically-relevant fluctuating regimens.
- There was significant interindividual variation in performance.