Effects of temperature acclimation and diet on energetics and behavior of the Mexican axolotl (*Ambystoma mexicanum*)

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Abstract:

Many organisms exhibit physiological responses to monthly or seasonal temperature shifts, but mechanisms driving these “thermal acclimation” effects remain unclear. Prior work on Mexican axolotls (*Ambystoma mexicanum*) revealed that warm-temperature exposure led to reduced metabolic performance at a new test temperature. We postulated that this pattern was driven by a thermal mismatch between metabolic energy expenditures and energy assimilation (i.e., digestion), resulting in energetic stress at warm temperatures. The metabolic theory of ecology (MTE) predicts higher metabolic rates at warmer temperatures, whereas the dynamic energy budget (DEB) theory predicts that animals with a reduced energy reserve should have reduced metabolic performance. Linking these frameworks together might explain the observed thermal acclimation effects seen in axolotl metabolism. To test the proposed linkages between temperature, metabolism, energetics, and physiological stress, I exposed axolotls to two acclimation temperatures (7°C and 25°C) and three levels of food availability. I then measured their effects on metabolic rates, behavior, and blood lipids. I used linear regression models to test for main and interactive effects of acclimation temperature, performance temperature, and food availability on axolotl metabolic rate, activity levels, and blood lipids. Results generally showed support for the prediction of higher metabolic rates with warmer temperatures and higher levels of reserve energy, with the exception of warm-acclimated fasted animals that exhibited higher than expected metabolic rates at the cooler performance temperature. This result was driven by increased activity levels in this treatment combination, possibly due to increased foraging behavior in animals with the lowest levels of reserve energy. These results support the hypothesis that axolotl thermal responses are driven by thermal mismatches between energy assimilation and expenditure, as predicted by MTE and DEB.

Current Research:

Climate change negatively impacts ecosystems around the world, making it important to better understand organism responses to temperature change (Pounds et al. 1999). When there are changes in temperature, organisms' net energy gain may be severely impacted by food availability and metabolic expenses (Huey and Kingsolver, 2019). The metabolic theory of ecology (MTE) provides a powerful framework for predicting thermal responses, based on the assumption that physiological processes are constrained by organism metabolic rates, which scale predictably with body mass and temperature (Brown et al. 2004). Although MTE has been widely applied to describe organism responses to current temperature (i.e., thermal performance curves), less attention has been given to potential applications of MTE for explaining physiological changes following extended exposure to warm or cool temperatures (i.e., thermal acclimation responses).

Thermal acclimation is typically defined as a change in an organism's thermal performance curve, because of past exposure to high or low temperatures (Paull et al. 2015). A "beneficial acclimation" response is often assumed when discussing thermal acclimation. The "beneficial acclimation hypothesis" postulates that organisms will have highest performance at those temperatures at which they have been acclimated (e.g., those acclimated at warm temperatures will perform best at warm temperatures) (Wilson & Franklin, 2002). However, many organisms have been found to exhibit a "cooler-is-better" pattern, including the Mexican axolotl, a model organism commonly used in developmental biology research (Irwin et al. 1998). A potential explanation of the "cooler-is-better" pattern is the "thermal stress hypothesis", which states that warm-temperature exposure is stressful, leading to reduced performance as exposure to that temperature continues (Paull et al. 2015). One possible reason for energetic stress at warm

temperatures has been proposed by Lemoine and Burkepile (2012), who postulated a mismatch in the thermal performance curves between energy assimilation (i.e., digestion) and energy expenditures (i.e., metabolic rates).

For this "thermal energetics mismatch hypothesis" to explain the observed "cooler-is-better" acclimation effect on axolotl metabolic rates, we must make three core assumptions. First, this hypothesis makes the implicit assumption that an organism's metabolic rate is directly related to the size of an organism's energy reserve. This assumption is based on another important theoretical framework in ecology, the dynamic energy budget (DEB) theory, which predicts that an animal's reduced energy reserve will lead to reduced physiological performance (Freitas et al. 2010). Animals with larger energy reserves (i.e., animals with higher amounts of fatty acids in the blood) are therefore predicted to have higher respiration rates than animals with depleted energy reserves (i.e., animals with lower amounts of fatty acids in the blood). A second assumption is that warm temperature exposure will increase an axolotl's energy expenditures due to MTE-predicted higher metabolic rates at warmer temperatures. A third assumption is that axolotls will be limited in their ability to compensate for increased metabolic expenditures at warmer temperatures, due to constraints on their ability to consume and assimilate additional food. A prior study found support for the latter two assumptions in axolotls, showing little change in food assimilation rates at warmer temperatures despite a marked increase in axolotl metabolic rates (Brady, 2019). Linking MTE and DEB theory through the relationships between metabolic rate, temperature, physiological stress, and energetics helps provide an explanation toward the observed "cooler-is-better" pattern seen in previous studies.

The primary purpose of this study was to test the first assumption of the "thermal energetics mismatch hypothesis", that axolotl resting metabolic rates increase in response to both

temperature and the size of the axolotl energy reserve. I predicted that increased expenditure of energy at high acclimation temperatures or with restricted diets would generate lower energy reserves in these treatments, resulting in lower levels of blood lipids and lower temperature-dependent metabolic rates in these treatment combinations. To test this prediction, I conducted an experiment crossing temperature and thermal acclimation treatments with three levels of food availability.

Methodology:

Acclimation:

Fifty-four axolotls were obtained from the Ambystoma Genetic Stock Center at the University of Kentucky: twenty-four as larvae in October 2017 and thirty as juveniles in April 2019. The fifty-four juvenile axolotls were randomly assigned to one of four temporal blocks (A, B, C, and D) in which they were moved to a refrigerated room with an ambient temperature of 8°C. Mass of each animal was measured before being placed into the experiment. The axolotls were then placed in incubators made from Styrofoam coolers and heat tape, set at either 25°C or 7 °C to begin acclimation. In each incubator, three axolotls were housed individually in clear 1-liter deli cups with 800 mL of Amquel®-treated tap water. The axolotls in each incubator received one of three diets: no food (fasted), 5-6 blackworms (regular) or 30-40 blackworms (excess). The acclimation period lasted three weeks during which the axolotls were fed twice per week (except for those in the fasted treatment) and their water changed three times per week. The deli cups with axolotls were rotated daily to control for temperature variation within the incubator. HOBO loggers were also placed inside each incubator to record temperature readings and to ensure the consistency of temperatures.

Respirometry:

After the three-week acclimation period, the axolotls were weighed, moved to a new "performance" incubator set to their performance temperature and then their respiration rates were measured. Prior to temperature shifts, 400 mL of water was removed from each deli cup, decreasing the amount of time needed to change the water temperature. Half of the axolotls remained at their acclimation temperature, making this temperature their performance temperature as well. The other half was moved from 7°C to 25°C or vice versa. In order to prevent temperature shock, these axolotls were placed in a "step" incubator set at 16 °C for 30 minutes. The axolotls were held in their "performance" incubator for at least 30 minutes before transitioning over to mason jars to take dissolved oxygen (DO) measurements. The mason jars were filled with aerated Amquel®-treated water heated to each animal's assigned temperature as well as a mesh disk to protect the animal from the DO probe. The lid of the jar had a hole in the center, so a DO probe could be inserted to measure the dissolved oxygen content while the axolotl was still in the mason jar. After the initial measurement was taken, water was poured to fill the top of the jar and an airtight cap was screwed on. This was to validate that the changes in DO was based solely on the axolotl's respiration and not due to air leaking into the mason jar. The axolotls in the mason jars were then moved to "respiration" incubators set at their performance temperatures and remained there for one hour, after which a final DO measurement was taken. Then, the axolotls were moved back to their "performance" incubators for the remainder of the experiment.

Behavior:

Behavior responses were recorded within an hour of completing the respirometry portion of the experiment. Each axolotl was observed for 5 minutes, and a stopwatch was used to

measure the length of time the axolotl was seen to be moving. The stopwatch was started each time the axolotl began to move and paused once the axolotl stopped moving. This value was used as the proportion time moving for each individual axolotl. After the timed observation, I noted the type and extent of movement the axolotl exhibited. I created and used an ethogram to measure the activity index seen by each axolotl. A range of 0-5 was used, ranging from no movement to aggressive thrashing. This data was used to determine if changes in activity level play a role in metabolic response to temperature shift.

Necropsy:

The day following respiration and behavior measurements, axolotls were anaesthetized in 0.03% Benzocaine for roughly 10 minutes or until the axolotl was noticeably immobile, and subsequently euthanized by partial decapitation. Blood from the axolotl was collected via 70-µL heparinized capillary tubes and then transferred to plasma separator tubes where the samples were placed on ice. The tubes were centrifuged at 2200 rpm for 5 minutes at room temperature. Plasma was then removed from the centrifuged tubes and those samples were stored at -20°C. The axolotls, after dissection, were preserved in 10% formalin.

Serum Triglyceride Assays:

A serum triglyceride determination kit (Sigma Aldrich #TR0100) was used to find the concentrations of “total” and “true” triglycerides in the blood plasma samples taken from the axolotls. The “total” triglycerides included both glycerol and the other “true” triglycerides in the concentration. Two 96 well plates were used for testing samples and were read by the Epoch Biotek plate reader at a wavelength of 540nm. On each plate, there were standards of 5 different concentrations made from a 1:2 serial dilution (130 ng/uL, 65 ng/uL, 32.5 ng/uL, 16.25 ng/uL, 8.125 ng/uL). Each plate included a single technical replicate for each plasma sample, four

blanks, four negative wells, and four wells per standard. The standards and plasma samples were distributed across the plate to reduce spatial confoundment. The standard curves that were used had a concentration of up to 32.5 ng/uL because this is the range we expected our results to fall into. Concentration of glycerol and "total" triglycerides were found by fitting regression lines to the standard curves as opposed to the original protocol, which suggested using a single standard to do the calculations. To calculate the "true" triglyceride concentration, I made a new standard curve by recalculating the absorbance values: average final absorbance – (blank*0.8) = "true" triglyceride absorbance. These corrected values were then plotted against the concentration of the standards and the resulting curve were used to determine "true" triglyceride concentration.

Statistics:

Results from the experiment were analyzed using R statistical software v.4.0.3 (R Developmental Core Team, 2020) and R studio. I used linear mixed effects models to test for interactive effects of my primary predictor variables (acclimation temperature, performance temperature, and food provisioning) on my primary response variables (respiration rates, activity levels, behavior, and lipids). F-statistics were calculated using Type II sums of squares and Kenward-Rogers degrees of freedom. Table 1 lists the statistics for natural log transformed respiration rate and behavior where acclimation incubator and performance incubator were used as random grouping factors. Table 2 lists the statistics for the linear regression model of the interaction effects on respiration rate per treatment group where no random grouping factors were used due to one animal from each treatment group being placed in an incubator. Finally, Table 3 lists the statistics for true and total triglyceride concentrations where acclimation incubator was used as a random grouping factor. The data of the experiment were analyzed and compiled into graphs made in Excel using pivot tables. Figure 1 illustrates the results of natural

log transformed mass scaled respiration rate with a mass scaling coefficient of 0.75 (A), proportion time moving (B), and activity index (C). Figure 2 depicts the concentrations of true triglycerides (A) and total triglycerides (B).

Results:

Respirometry:

Mass, performance temperature, and food treatment had a significant effect on respiration rate ($F_{1, 33.1} = 17.8$, $p < 0.001$, $F_{1,10.9} = 25.9$, $p < 0.001$, and $F_{2, 26.7} = 7.70$, $p = 0.02$ respectively). We found a significant interaction effect between food treatment and performance temperature on respiration rate ($F_{2, 26.5} = 9.59$, $p = 0.01$), displayed in Figure 1A. Additionally, we found a significant three-way interaction between acclimation temperature, food treatment, and performance temperature on respiration rate ($F_{2, 27.4} = 3.56$, $p = 0.042$). Exploring interaction effects among the three food treatment groups, acclimation temperature and performance temperature had a significant effect on respiration rate for the regular ($F_{1, 10} = 26.05$, $p < 0.001$ and $F_{1, 10} = 74.92$, $p < 0.001$ respectively) and excess ($F_{1, 8} = 6.95$, $p = 0.030$ and $F_{1, 8} = 30.99$, $p < 0.001$ respectively) food treatment groups (Fig. 1A). We found block to have a significant effect on respiration rate ($F_{3, 8} = 4.21$, $p = 0.046$) as well for the excess food treatment group. Within the fasted food treatment group, the interaction between acclimation temperature and performance temperature had a significant effect on respiration rate ($F_{1, 11} = 6.08$, $p = 0.031$).

Behavior:

We found a significant three-way interaction between acclimation temperature, food treatment, and performance temperature for both the activity index ($F_{2, 26.9} = 3.92$, $p = 0.032$) and proportion time moving ($F_{2, 27.8} = 3.52$, $p = 0.043$), driven by an interactive effect of acclimation

and performance temperature within the fasted food treatment group ($F_{1,11} = 6.08$, $p < 0.031$). Unlike in the other food treatments, fasted animals had higher activity levels at the colder performance temperature if they were warm-acclimated (Figs. 1B and 1C). We also found a significant negative main effect of acclimation temperature on the activity index ($F_{1, 12.5} = 9.02$, $p = 0.011$) and proportion time moving ($F_{1, 13.2} = 14.1$, $p = 0.002$), as illustrated in Figures 1B and 1C. Mass and food treatments also had significant main effects on proportion time moving ($F_{1, 34.9} = 6.04$, $p = 0.019$ and $F_{2, 27.1} = 3.56$, $p = 0.042$ respectively).

Serum Triglyceride Assays:

The food treatment has significant effects on true triglyceride concentration ($F_{2, 27.7} = 9.45$, $p < 0.001$) and total triglyceride concentration ($F_{2, 27.5} = 8.19$, $p = 0.002$). Animals in treatments with higher food availability generally had higher blood lipid concentrations (Fig. 3).

Discussion:

Our results generally supported our initial predictions of temperature acclimation and diet effects on the energetics and behavior of Mexican axolotls. Consistent with the predictions of DEB theory, increased energy reserves led to higher respiration rates. The results we found from our serum triglyceride assay analysis showed that food treatment had a significant effect on blood lipids found in the axolotl's blood (Table 3). Increasing the food availability resulted in higher blood lipid concentrations, suggesting that giving animals more food did indeed result in increased energy reserves (Fig. 2). As was found in a similar study conducted by Irwin et al. (1998), fasted animals displayed lower blood lipid concentrations (Fig. 2), suggesting that starving the axolotls leads to depleted energy reserves. Additionally, since the excess food

treatment group had the highest triglyceride concentrations, we also found the group to have a general trend of higher respiration rates, further supporting DEB theory.

As predicted by MTE, warmer performance temperatures generally resulted in a higher respiration rate. For two of the three food treatment groups (Fig. 1A), we found an observed pattern of respiration rate being driven by the interaction of performance temperature and food treatment (Table 1). Specifically, in both the regular and excess food treatment groups, we saw significant effects of acclimation temperature and performance temperature on respiration rate (Table 2), further indicating how changing the environment an axolotl is placed in can affect its metabolic rate. However, we found a deviation from this observed pattern in the fasted treatment group (Fig. 1A), where the warm acclimated axolotls had a lower respiration rate when moving to a warmer performance temperature. This could be explained by the significant three-way interaction found between acclimation temperature, food treatment, and performance temperature on respiration rate (Table 1). After looking more into this deviated pattern, analyzing the effects on respiration rate in the fasted food treatment group led to the result of a significant interaction effect between acclimation temperature and performance temperature (Table 2). The metabolic rate of these warm acclimated axolotls was higher at the cooler performance temperature, apparently contradicting MTE predictions.

It is important to keep in mind that what we measured was total metabolic rate, and MTE's predictions apply primarily to temperature effects on resting metabolic rates. Animals can also have increased metabolic rates due to elevated activity levels (Hervant et al., 2001) as seems to have been the case in the specific treatment combination of fasting, warm acclimation, and a cooler performance temperature (Figs. 1B and 1C). Elevated activities in the warm-acclimated and fasted axolotls could be a result of increased foraging behavior for these animals,

which had lower energy reserves than other treatment combinations based on their blood lipid levels. This result is consistent with a prior study that found a curvilinear effect of fasting on salamander activity, in which animals had greatly increased activity in response to the first 60 days of the starvation period presumably to increase the chances of finding food (Hervant et al., 2001). Additionally, Irwin et al. (1998) found a similar result where the warm-acclimated fasted axolotls had higher respiration rates at the cooler performance temperature, but they also found the cold-acclimated fasted axolotls to have higher respiration rates as well when compared to the fed acclimated axolotls.

Despite this deviation for the fasted food treatment group, warm-acclimated axolotls generally had lower metabolic rates for both regular and excess levels of food (Fig. 1A), which was consistent with the findings from Brady (2019). As postulated by Paull et al. (2015), acclimating to warmer temperatures compared to colder temperatures would result in an energy deficit and subsequently decrease the axolotl's metabolic rate, which we found to be the case based on our results (Figs. 1A and 2). Combining the initial predictions of MTE and DEB theory discussed previously, our findings support this "thermal energetics mismatch hypothesis" where the axolotl's resting metabolic rate will increase as a result of temperature acclimation effects and the size of its energy reserve. Being placed in warmer performance temperatures and increasing energy reserves will generally lead to an elevated resting metabolic rate for a Mexican axolotl.

Conclusion:

By studying the effects of thermal acclimation and nutrition on axolotls, we can use these data to improve the husbandry of these animals. Prior studies, including the current one, have

found a “cooler is better” pattern, which correlates to warmer temperatures being more energetically stressful to axolotls. With this in mind, controlling the temperature changes can limit the amount of stress placed on axolotls and correct the damage these changing conditions can have on the resulting environment. Testing both temperature acclimation and food availability effects also furthers knowledge in thermal biology of ectotherms as a whole, and helps improve axolotl husbandry by studying the indices of energetic stress in these organisms. Further studies can be conducted to examine the relationship of temperature on axolotl growth in natural environments.

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Table 1. Effects of acclimation temperature, performance temperature, and food treatment on respiration rate and behavior. Measurements of mass were taken immediately after the acclimation period of three weeks before dissolved oxygen measurements were taken. Respiration rate was natural log-transformed to improve normality. Variables that significantly improved each model are highlighted in bold font. The three models included acclimation incubator and performance incubator as a random grouping factor. F-statistics were calculated using Type II sums of squares and Kenward-Rogers degrees of freedom.

Response	Predictors	F	d.f.	P
Respiration rate (mg O_2 × $M^{-3/4}$ × hr^{-1})	Block	1.98	3, 10.7	0.177
	ln (Mass)	**17.8**	**1, 33.1**	**<0.001**
	Acclimation temperature (AcclTemp)	4.27	1, 13.2	0.059
	Food treatment (Food)	**7.70**	**2, 26.7**	**0.002**
	Performance temperature (PerfTemp)	**25.9**	**1, 10.9**	**<0.001**
	AcclTemp × Food	1.77	2, 26.9	0.190
	AcclTemp × PerfTemp	2.22	1, 10.7	0.165
	Food × PerfTemp	**9.59**	**2, 26.5**	**0.001**
	AcclTemp × Food × PerfTemp	**3.56**	**2, 27.4**	**0.042**
Activity index	Block	0.92	3, 10.9	0.462
	Mass	2.49	1, 30.9	0.125
	AcclTemp	**9.02**	**1, 12.5**	**0.011**
	Food treatment	0.76	2, 26.5	0.477
	PerfTemp	0.15	1, 10.9	0.711
	AcclTemp × Food	1.43	2, 26.6	0.256
	AcclTemp × PerfTemp	0.07	1, 10.9	0.791
	Food × PerfTemp	1.34	2, 26.3	0.279
	AcclTemp × Food × PerfTemp	**3.92**	**2, 26.9**	**0.032**
Proportion time moving	Block	1.30	3, 10.6	0.325
	Mass	**6.04**	**1, 34.9**	**0.019**
	AcclTemp	**14.1**	**1, 13.2**	**0.002**
	Food	**3.56**	**2, 27.1**	**0.042**
	PerfTemp	1.92	1, 10.7	0.194
	AcclTemp × Food	2.89	2, 27.1	0.073
	AcclTemp × PerfTemp	3.09	1, 10.7	0.107
	Food × PerfTemp	0.49	2, 26.7	0.616
	AcclTemp × Food × PerfTemp	**3.52**	**2, 27.8**	**0.043**

Table 2. Effects of acclimation temperature and performance temperature on respiration rate per specified food treatment. Respiration rate was natural log-transformed to improve normality. Variables that significantly improved each model are highlighted in bold font. The model did not include incubator as a random grouping factor due to one animal per food treatment group being placed in each incubator. F-statistics were calculated using Type II sums of squares and Kenward-Rogers degrees of freedom.

Response	Food treatment	Predictors	F	d.f.	P
Respiration rate (mg O_2 × $M^{-3/4}$ × hr^{-1})	Fasted	Block	0.71	3, 11	0.567
		ln (Mass)	3.81	1, 11	0.077
		Acclimation temperature (AcclTemp)	0.12	1, 11	0.734
		Performance temperature (PerfTemp)	1.66	1, 11	0.225
		AcclTemp × PerfTemp	**6.08**	**1, 11**	**0.031**
	Regular	Block	2.90	3, 10	0.088
		ln (Mass)	3.43	1, 10	0.094
		AcclTemp	**26.05**	**1, 10**	**<0.001**
		PerfTemp	**74.92**	**1, 10**	**<0.001**
		AcclTemp × PerfTemp	1.30	1, 10	0.282
	Excess	**Block**	**4.21**	**3, 8**	**0.046**
		ln (Mass)	1.75	1, 8	0.222
		AcclTemp	**6.95**	**1, 8**	**0.030**
		PerfTemp	**30.99**	**1, 8**	**<0.001**
		AcclTemp × PerfTemp	0.081	1, 8	0.783

Table 3. Effects of acclimation temperature and food treatment on blood lipid levels. Both response variables were natural log-transformed to improve normality. Variables that significantly improved each model are highlighted in bold font. Both models included acclimation incubator as a random grouping factor. F-statistics were calculated using Type II sums of squares and Kenward-Rogers degrees of freedom.

Response	Predictors	F	d.f.	P
True triglycerides	Block	1.23	3, 11.9	0.343
	Acclimation temperature (AcclTemp)	2.67	1, 12.6	0.127
	Food treatment (Food)	**9.45**	**2, 27.7**	**< 0.001**
	AcclTemp × Food	0.40	2, 28.8	0.671
Total triglycerides	Block	0.68	3, 12.0	0.580
	AcclTemp	2.03	1, 12.6	0.178
	Food	**8.19**	**2, 27.5**	**0.002**
	AcclTemp × Food	0.55	2, 28.6	0.584

Table 4. Activity index ethogram ranging from 0 to 5 based on behavior patterns seen from juvenile Mexican axolotls after respiration measurements were taken and proportion time moving was observed.

Behavior	Behavior Description
No movement (0)	*A. mexicanum* would remain still (no voluntary movements) remaining on the bottom of their container.
Isolated movement (1)	During periods of inactivity, *A. mexicanum* would occasionally reposition or slowly move a single limb, both front limbs, both back legs, or its tail.
Walking (2)	*A. mexicanum* would walk along the bottom of its container, relatively slowly, which would include a few strides per bout, but varied in speed and number of strides. Animals would occasionally attempt to climb up the side of their containers in this way.
Random swimming (3)	To swim, *A. mexicanum* would quickly move its tail from side to side and keep its limbs by its sides. Animals would typically shift which direction they were facing at least once during random swimming bouts due to the small size of their containers.
Nosing behavior (4)	*A. mexicanum* would swim downwards toward the edge where the bottom and side of its container met, bumping and/or rubbing its nose there.
Aggressive thrashing (5)	Occasionally, *A. mexicanum* would quickly contract one side of their body and then the other. resulting in very intense, aggressive thrashing.

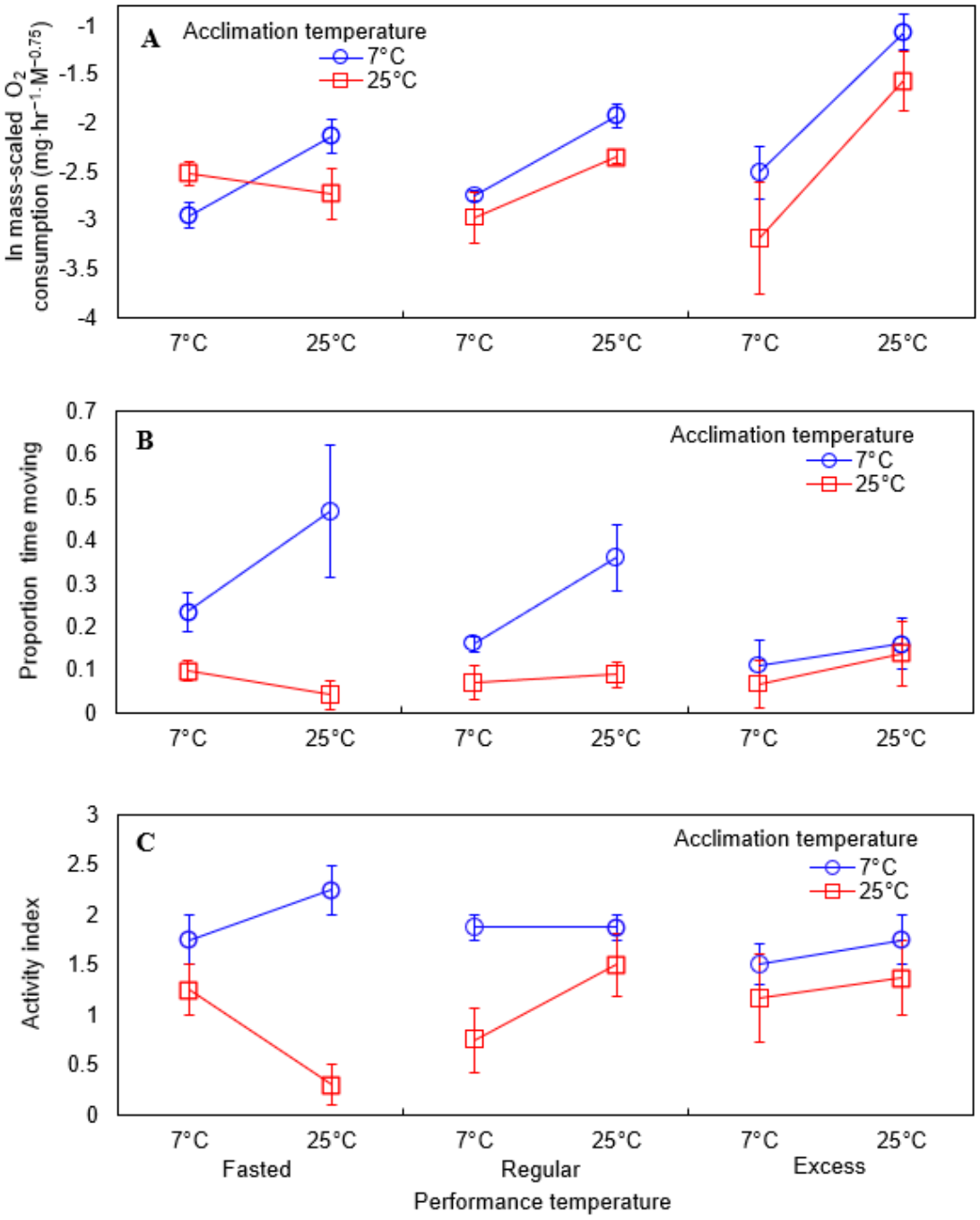

Figure 1. Respiration and behavior measurements. Temperature and food treatment effects on (A) natural log transformed mass-scaled O_2 consumption with a scaling coefficient of 0.75, (B) proportion time moving, and (C) activity index of juvenile Mexican axolotls at their respective performance temperatures after being exposed to their acclimation temperatures for three weeks. Error bars are ± SE.

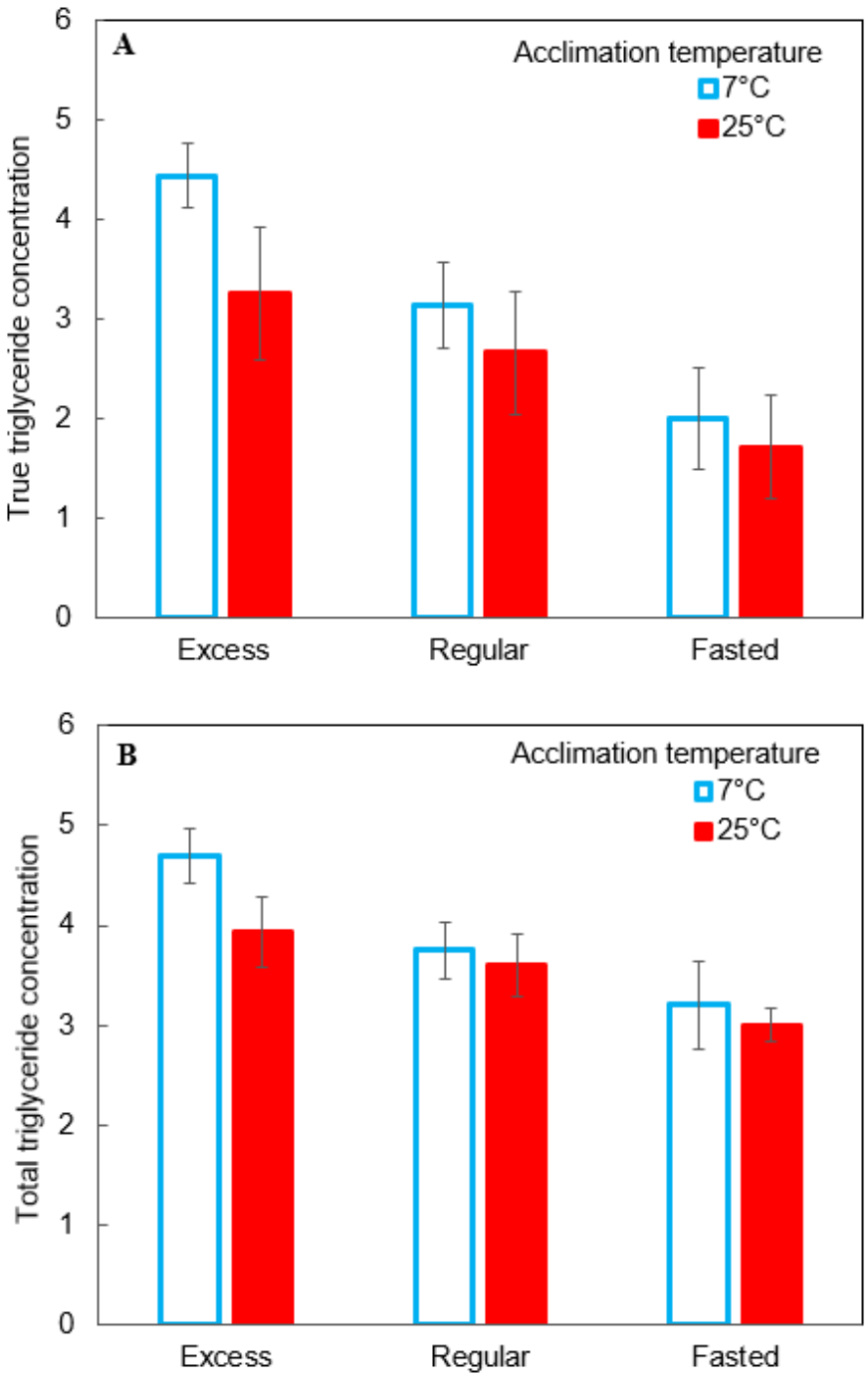

Figure 2. Triglyceride concentrations. (A) True triglyceride concentrations and (B) total triglyceride concentrations from serum triglyceride assays done on juvenile Mexican axolotls after necropsy after being exposed to their acclimation temperatures for three weeks. Error bars are ± SE.