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Publication Date

2023-06-01

DOI

10.1016/j.cbpb.2023.110836

Peer reviewed

REVIEW

Title Page

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Loss of mitochondrial performance at high temperatures is correlated with upper thermal tolerance among populations of an intertidal copepod

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Abstract

Environmental temperatures have pervasive effects on the performance and tolerance of ectothermic organisms, and thermal tolerance limits likely play key roles underlying biogeographic ranges and responses to environmental change. Mitochondria are central to metabolic processes in eukaryotic cells, and these metabolic functions are thermally sensitive; however, potential relationships between mitochondrial function, thermal tolerance limits and local thermal adaptation in general remain unresolved. Loss of ATP synthesis capacity at high temperatures has recently been suggested as a mechanistic link between mitochondrial function and upper thermal tolerance limits. Here we use a common-garden experiment with seven locally adapted populations of intertidal copepods (*Tigriopus californicus*), spanning approximately 21.5° latitude, to assess genetically based variation in the thermal performance curves of maximal ATP synthesis rates in isolated mitochondria. These thermal performance curves displayed substantial variation among populations with higher ATP synthesis rates at lower temperatures (20-25 °C) in northern populations than in southern populations. In contrast, mitochondria from southern populations maintained ATP synthesis rates at higher temperatures than the temperatures that caused loss of ATP synthesis capacity in mitochondria from northern populations. Additionally, there was a tight correlation between the thermal limits of ATP synthesis and previously determined variation in upper thermal tolerance limits among populations. This suggests that mitochondria may play an important role in latitudinal thermal adaptation in *T. californicus*, and supports the hypothesis that loss of mitochondrial performance at high temperatures is linked to whole-organism thermal tolerance limits in this ectotherm.

Keywords: *Tigriopus californicus*, critical thermal maxima, local adaptation, ATP synthesis, latitudinal, thermal performance curve

Main text

Temperature has pervasive effects on the performance and survival of ectothermic organisms (Somero et al., 2017), and in aquatic ectotherms thermal tolerance limits likely influence both latitudinal ranges and shifts in range limits as a result of increasing environmental temperatures (Sunday et al., 2012). Consequently, resolving the biochemical and physiological mechanisms underlying thermal tolerance limits is key to understanding not only current biogeographic distributions, but also impacts of climate change. Several possible mechanisms have been linked to variation in upper thermal tolerance in aquatic species including molecular chaperone expression (Tomanek, 2008; Gleason & Burton, 2015), neural function (Miller & Stillman, 2012), whole-animal oxygen consumption (Pörtner, 2002; Eliason et al., 2011) and mitochondrial function (Christen et al., 2018; Iftikar & Hickey, 2013; Iftikar et al., 2014; Michaelsen et al., 2021).

Loss of mitochondrial performance was previously discounted as a possible mechanism underlying upper thermal tolerance because capacities for oxidative phosphorylation were maintained at temperatures beyond whole-organism tolerance limits (e.g., state III respiration, Somero et al., 1996; Somero, 2002; Pörtner, 2002). Yet, latitudinal variation in mitochondrial genotype may be affected by natural selection (Camus et al., 2017), and many aspects of mitochondrial function are thermally sensitive (Chung & Schulte, 2020), including traits that are often plastic when organisms are exposed to different environmental temperatures (e.g., Chung & Schulte, 2015; Chung et al., 2017a, b, 2018; Bryant et al., 2018). The synthesis of ATP, a key function of mitochondria in eukaryotic cells, may be impaired at temperatures that are similar to whole-organism thermal tolerance limits (Iftikar & Hickey, 2013; Iftikar et al., 2014; Harada et

al., 2019; Healy et al., 2019). Therefore, it is possible that loss of the capacity to generate ATP contributes to setting acute thermal tolerance limits at higher levels of biological organization.

The intertidal copepod *Tigriopus californicus* inhabits supralittoral tidepools along the Pacific coast of North America from Baja California, Mexico to Alaska, USA, and there is essentially no migration between distinct rocky outcrops (Burton & Feldman, 1981). This species has short generation times (~1 month) and is easily cultured in a laboratory, creating an ideal system for the study of local thermal adaptation. Even after many generations of laboratory rearing, previously published work has consistently resolved latitudinal variation in upper thermal tolerance among *T. californicus* populations with a significant correlation between tolerance and variation in maximum habitat air temperatures (Willett, 2010; Kelly et al., 2012; Pereira et al., 2017; Leong et al., 2018; Willett & Son, 2018; Healy et al., 2019). Population differences in tolerance have been most clearly linked with differences in the expression of molecular chaperones, such as heat-shock proteins, during and following heat stress (Schoville et al., 2012; Kelly et al., 2017; Graham & Barreto, 2019; Tangwancharoen et al., 2018, 2020; Healy et al., 2019; Harada & Burton, 2019), and knockdown of heat-shock protein beta 1 reduces the maximum temperature that these copepods can tolerate (Barreto et al., 2015). In addition to the important role of molecular chaperones, recent studies have proposed that loss of mitochondrial ATP synthesis capacity at high temperatures may also be associated with tolerance limits in *T. californicus* (Harada et al., 2019; Healy et al., 2019). However, these studies examined copepods from at most three populations spanning only a small portion of the species range (~4.2° latitude), which limits both the predictive power of this association and the potential relevance to latitudinal thermal adaptation overall. Thus, our current study examines the relationships between mitochondrial ATP synthesis, thermal tolerance limits and latitudinal adaptation in

seven populations of *T. californicus* spanning ~21.5° latitude. We assess thermal performance curves (TPCs) for maximal ATP synthesis rate (i.e., change in synthesis rate across temperatures) in mitochondria isolated from these copepods after several generations of common-garden laboratory rearing.

Adult *T. californicus* were collected from supralittoral tidepools from San Roque, Mexico (SR; 27° 10′ 48″ N, 114° 23′ 52″ W), La Bufadora, Mexico (BF; 31° 43′ 25″ N, 116° 43′ 19″ W), San Diego, USA (SD; 32° 44′ 41″ N, 117° 15′ 19″ W), Bird Rock, USA (BR; 32° 48′ 51″ N, 117° 16′ 24″ W), Santa Cruz, USA (SC; 36° 56′ 58″ N, 122° 02′ 47″ W), Pescadero Beach, USA (PE; 37° 15′ 35″ N, 122° 24′ 51″ W) and Pacific Crest, Canada (PC; 48° 49′ 48″ N, 125° 09′ 06″ W). Copepods were maintained in several population-specific 250 mL laboratory cultures at Scripps Institution of Oceanography (La Jolla, CA, USA) made up with filtered seawater (35 psu; 0.44 μm pore size), and held at 20 °C and a 12 h:12 h light:dark photoperiod. These salinity and temperature conditions are ecologically relevant across the majority of *T. californicus*' latitudinal range (e.g., Leong et al., 2018), and are commonly used in studies addressing latitudinal thermal adaptation in this species (e.g., Pereira et al., 2017; Harada et al., 2019; Healy et al., 2019). Powdered spirulina (Salt Creek, Inc., South Salt Lake City, UT, USA) and ground TetraMin Tropical Flakes (Spectrum Brands Pet LLC, Blacksburg, VA, USA) were added to the cultures weekly as a food source, and copepods also consumed natural algal growth in the cultures. These conditions were maintained for at least seven months (~7 generations) prior to the start of experiments.

TPCs for *in vitro* maximal ATP synthesis rates were determined from 20 to 36 °C for mitochondria isolated from copepods of each population using protocols similar to Harada et al. (2019) and Healy et al. (2019). Based on preliminary tests, 11 haphazardly selected adults from

each population were held without food overnight. In the morning, the individuals were pooled by population, and then homogenized by hand with a Teflon-on-glass homogenizer in 800 μL of ice-cold buffer (400 mmol L^{-1} sucrose, 100 mmol L^{-1} KCl, 70 mmol L^{-1} HEPES, 6 mmol L^{-1} EGTA, 3 mmol L^{-1} EDTA, 1% w/v BSA, pH 7.6). Homogenates were centrifuged at 1,000 g and 4 °C for 5 min. The supernatants were collected, and were centrifuged again at 11,000 g and 4 °C for 10 min. The second set of supernatants were removed, and the pelleted mitochondria were resuspended in 275 μL of buffer (560 mmol L^{-1} sucrose, 100 mmol L^{-1} KCl, 70 mmol L^{-1} HEPES, 10 mmol L^{-1} KH_2PO_4, pH 7.6) for the ATP synthesis assays. Thus, each ATP synthesis trial included a pool of mitochondria isolated from 11 copepods from each of the seven populations, and six trials were conducted in total (i.e., n = 6 per population).

Using fluorometry to quantify ATP, ATP synthesis rates were determined at 20, 25, 30, 32, 34 and 36 °C with saturating concentrations of electron transport system (ETS) substrates for complex I (5 mmol L^{-1} pyruvate, 2 mmol L^{-1} malate), complex II (10 mmol L^{-1} succinate) and complex V (1 mmol L^{-1} ADP). Note these conditions are equivalent to those used to measure state-III oxygen consumption in mitochondrial respirometry studies (e.g., Chung et al., 2017a). 25 μL of each mitochondrial isolation were added to 0.2 mL polymerase chain reaction tubes for each assay temperature. Assays were initiated by the addition of substrates, and were conducted for 10 min at the desired temperatures. ATP synthesis was stopped at the end of the assays by the addition 25 μL of CellTiter-Glo (Promega, Madison, WI, USA), which also enables ATP concentration to be measured. Separate 25-μL aliquots of the mitochondrial isolations had 25 μL of CellTiter-Glo added immediately prior to ATP quantification to determine the initial concentrations of ATP in the assays. These preparations and assay solutions were incubated with CellTiter-Glo in the dark for 10 min. After incubation, luminescence was quantified with a

Fluroskan Ascent® FL (Thermo Fisher Scientific, Waltham, MA, USA), and ATP concentrations were assessed by comparison to a standard curve (5 to 10,000 nmol L^{-1} ATP). Synthesis rates were calculated by subtraction of the initial ATP concentrations from the final concentrations followed by division by 10 (min).

Variation in log-transformed ATP synthesis rates was tested with a mixed-effect linear model implemented with the *lmerTest* package v3.1.3 (Kuznetsova et al., 2017) in *R* v4.2.0 (R Core Team, 2022) with population and temperature as fixed factors, and replicate as a random factor ($\alpha = 0.05$). Separate models within each population and each assay temperature were fit to examine effects of these factors further, as well as potential overall differences between two latitudinal groupings of the populations (southern, warm-adapted: SR, BF, SD and BR; northern, cold-adapted: SC, PE and PC; e.g., Tangwancharoen et al., 2018). To compare loss of ATP synthesis capacity at high temperatures to variation in upper thermal tolerance among populations, critical thermal maximum (CT_{max}) data for the seven populations in the current study were obtained from a previously published study investigating effects of developmental plasticity on thermal tolerance (Healy et al., 2019).

Across the temperature range in the current study, there was clearly variation in ATP synthesis rate among populations ($df = 6$, $F = 3.50$, $p = 0.0081$) and temperatures ($df = 5$, $F = 121.03$, $p < 2.2 \times 10^{-16}$; Fig. 1); there was also a significant population-by-temperature interaction ($df = 30$, $F = 4.47$, $p = 1.4 \times 10^{-10}$). The thermal sensitivities for ATP synthesis were generally low ($Q_{10} = 1.47 \pm 0.34$, $\mu \pm \sigma$, from 20 to 30 °C), which may be a consequence of the portion of the TPC examined (i.e., no assay temperatures below 20 °C). However, temperature significantly affected ATP synthesis within every population ($df = 5$, $F \geq 7.58$, $p \leq 1.9 \times 10^{-4}$), and there was variation among populations at all temperatures except for 36 °C ($df = 6$, $F \geq 3.02$, $p \leq 0.017$).

Furthermore, there were overall differences between populations from southern or northern latitudes at 20 and 25 °C (df = 1 for both, F = 8.29 and 8.26 respectively, $p = 0.035$ for both) with populations from northern latitudes generally tending to have higher ATP synthesis rates than populations from southern latitudes.

Since the highest ATP synthesis rate achieved across all temperatures varied among *T. californicus* populations, Healy et al. (2019) suggested that the proportional loss of synthesis capacity at high temperatures may be a key factor linking mitochondrial performance and upper thermal tolerance in this species. For most of the populations in the current study, the highest ATP synthesis rate across temperatures was observed at 30 or 32 °C (Fig. 1), and the variation among populations did not group by latitude (30 °C: BR, PE and PC, and 32 °C: BF, SD and SC). The southernmost population (SR), which has recently been proposed to potentially represent a different species of the *Tigriopus* genus (*T. bajaensis*; Barreto et al., 2018; Phillips, 2020), displayed its highest ATP synthesis rate at 25 °C; however, synthesis rates were relatively insensitive to temperature from 25 to 30 °C in this population (≤ 3.5% variation, on average). To compare the proportional loss of ATP synthesis capacity across populations, we normalized the synthesis rates within each population by dividing by the highest rate detected for the population. This normalization, resulting in a highest rate of 1 in all populations (Fig. 2A), revealed that the proportional synthesis rates for the different populations separated by latitude at high temperatures (latitudinal group df = 1, $F \geq 5.68$, $p \leq 0.022$ at 34 and 36 °C, and population df = 6, F = 4.37, $p = 0.0021$ at 36 °C) with southern populations maintaining ATP synthesis at proportionally higher rates than northern populations, whereas at lower temperatures (≤ 32 °C) there was no variation among the populations (latitudinal group df = 1, $F \leq 0.68$, $p \geq 0.42$, and population df = 6, $F \leq 1.02$, $p \geq 0.43$).

To index loss of ATP synthesis capacity at high temperatures, we used linear approximations between 34 and 36 °C to determine the temperature at which ATP synthesis rate was half of the maximal rate for each population. We then compared these temperatures to previously published CT_{max} values for adults from the same laboratory cultures and holding conditions as those in the current study (Healy et al., 2019). Average temperatures resulting in a 50% loss of maximal ATP synthesis rate were highly correlated with upper thermal tolerance limits among the seven *Tigriopus* populations ($r^2 = 0.93$, df = 5, t = 8.39, $p = 3.9 \times 10^{-4}$; Fig. 2B).

In general, mitochondrial performance is highly sensitive to temperature (e.g., Chung & Schulte, 2020), and variation in mitochondrial function across temperatures may contribute to metabolic capacity limitations at temperature extremes (Pörtner, 2002). Previous studies have indicated that loss of the ability to synthesize ATP at high temperatures may occur at similar temperatures to upper thermal limits in some species (Iftikar & Hickey, 2013; Iftikar et al., 2014), including *T. californicus* (Harada et al., 2019; Healy et al., 2019). In the current study, we confirm the hypothesis proposed by Harada et al. (2019), and demonstrate that there is a tight correlation between genetically based variation in the upper thermal limits of organismal tolerance and ATP synthesis capacity across seven populations spanning the majority of *T. californicus*' latitudinal range. It is possible this pattern is the result of parallel selection on the two traits as a result of temperature differences among populations, but the strength of relationship observed suggests a mechanistic link between the two traits may be more likely.

The correlation between losses of ATP synthesis capacity at high temperatures and upper thermal limits in *T. californicus* adds an additional mechanism of thermal adaptation to the substantial evidence linking heat-shock protein (hsp) expression and function with upper thermal tolerance in this species. Expression levels of many hsps increase as a result of acute heat stress

in laboratory-reared *T. californicus* from both within- and between-population crosses (Schoville et al., 2012; Kelly et al., 2017), and variation in the extent of hsp induction during heat stress has been positively associated with upper thermal limits among populations (Schoville et al., 2012; Graham & Barreto, 2019; Tangwancharoen et al., 2018, 2020). Similarly, variation in upper thermal tolerance due to differences in rates of warming during acute temperature exposures or in developmental temperatures are also positively associated with variation in hsp expression (Harada & Burton, 2019; Healy et al., 2019). Moreover, RNAi knockdown of hsp beta 1 (*hspb1*) results in decreased tolerance of high temperatures (Barreto et al., 2015), and Hspb1 proteins from a warm-adapted population (SD) perform better than Hspb1 proteins from a cold-adapted population (SC) in thermal protection assays (Tangwancharoen et al., 2020). Variation in hsp expression among populations is primarily observed after exposure to thermal stress (Schoville et al., 2012), so it is unlikely that these differences impact the performance of isolated mitochondria in the current study. Thus, ATP synthesis capacity and hsp expression may independently contribute to mechanisms underlying variation in tolerance in *T. californicus*, and the correlation between mitochondrial function and CT_{max} observed here may partially relate to the locomotory end point of CT_{max} measurements in this species. However, CT_{max} and lethal metrics of upper thermal tolerance (e.g., the temperature that results in 50% mortality after a 1-h thermal exposure [LD_{50}])" typically resolve similar patterns of variation among *T. californicus* populations (Pereira et al., 2017; Harada et al., 2019; Healy et al., 2019), suggesting the correlation between losses of ATP synthesis capacity and tolerance limits would hold regardless of methodology.

An alternative possibility is that hsps and mitochondrial function may interact to determine organismal thermal tolerance in *T. californicus*. For instance, hsps have the potential to buffer mitochondrial proteins against denaturation at high temperatures (Martin et al., 1992),

and maintenance of ATP supply during heat stress may be necessary to support effective heat-shock responses (Zhang & Dong, 2021). The greater induction of hsps in warm-adapted populations compared to cold-adapted populations of *T. californicus* (Schoville et al., 2012; Tangwancharoen et al., 2018), and the larger changes in CT_{max} than in the thermal limits of ATP synthesis capacity among populations (Fig. 2B) are consistent with the expectations of these potential interactions. However, although increased expression of hsp mRNA can occur rapidly in *T. californicus* (Harada & Burton, 2019), the total time of a CT_{max} trial (~1.5 h) may be too short to produce substantial increases in the hsp protein pool (e.g., Tomanek, 2008), particularly compared to the longer time periods (~3 d) that are used to assess lethal metrics of thermal tolerance (e.g., LD_{50}) following abrupt heat stresses in this species (Willett, 2010; Kelly et al., 2012; Leong et al., 2018). Regardless of these different possibilities for a mechanistic link between ATP synthesis capacity and upper thermal tolerance, our results clearly show that the two traits are correlated among populations. Given that the latitudinal variation in upper thermal tolerance in this species is thought to be adaptive (e.g., Pereira et al., 2017), our data also suggest that local thermal adaptation of mitochondrial function is pervasive in *T. californicus*.

Although ATP synthesis rates were not assessed at temperatures below 20 °C in the current study, the shapes of the TPCs for ATP synthesis among populations were consistent with potential tradeoffs between mitochondrial performance at cold and warm temperatures. In general, maximal ATP synthesis rates were higher in northern populations than in southern populations at low temperatures (20 and 25 °C). This countergradient pattern may compensate for the slowing thermodynamic effect of cold on biological reaction rates (Conover & Schultz, 1995). Thus, it is possible that selection to maintain the structural flexibility required for function at low temperatures (Somero et al., 2017) reduces the resilience of ATP synthesis capacity at

high temperatures in northern populations. Harada et al. (2019) found that ATP synthesis supported by electron donation to ETS complex II was less resilient to high temperatures than synthesis supported by electron donation to complex I. Therefore, variation in the effects of high temperatures on the interactions between these complexes and the other proteins of the ETS or the phospholipids of the inner mitochondrial membrane merits further investigation.

Taken together, our findings demonstrate that mitochondrial performance has likely been shaped by local thermal adaptation across latitudes in *T. californicus*. The genetic basis of this adaptation is evident from the common-garden approach used in this study; despite multiple generations of culture under laboratory conditions, isolated mitochondria retained population-specific TPCs that reflect patterns consistent with the known variation in air temperatures among habitats. Loss of ATP synthesis capacity occurs at similar temperatures to whole-organism thermal tolerance limits with a strong association between the two traits among populations, which is consistent with a possible mechanistic role for loss of mitochondrial performance in determining maximum tolerated temperatures in this ectothermic species.

Acknowledgements

Funding: this work was supported by National Science Foundation (DEB1556466 and IOS1754347).

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Figure legends

Fig. 1. Thermal performance curves for maximal ATP synthesis rates supported by electron donation to complex I and II (CI&II) in mitochondria isolated from four warm-adapted southern populations (A – San Rogue [SR]: dark red, diamonds, dotted-dashed line; La Bufadora [BF]: pink, squares, dotted line; San Diego [SD]: red, triangles, dashed line; Bird Rock [BR]: orange, circles, solid line) and three cold-adapted northern populations (B – Santa Cruz [SC]: blue, squares, dotted line; Pescadero Beach [PE]: light blue, triangles, dashed line; Pacific Crest [PC]: navy, circles, solid line) of *T. californicus*. Filled symbols show population means, and smaller empty background symbols display individual data points.

Fig. 2. Proportional maximum ATP synthesis rates supported by electron donation to complex I and II (CI&II) in seven populations of *T. californicus* after normalization by dividing by the highest rate measured across 20 to 36 °C (A), and the relationship between critical thermal maxima (CT_{max}) from Healy et al. (2019) and the high temperatures producing 50% maximal ATP synthesis rates among populations (B). Populations: San Rogue (SR: dark red, diamonds, dotted-dashed line), La Bufadora (BF: pink, squares, dotted line), San Diego (SD: red, triangles, dashed line), Bird Rock (BR: orange, circles, solid line), Santa Cruz (SC: blue, squares, dotted line), Pescadero Beach (PE: light blue, triangles, dashed line) and Pacific Crest (PC: navy, circles, solid line). A: asterisks – significant difference between southern and northern populations (SR, BF, SD and BR vs SC, PE and PC), dagger – significant difference among populations, and dotted light grey line – 50% threshold for ATP synthesis rate. B: dashed dark grey line – line of best fit for significant correlation between CT_{max} and the temperature of 50% maximal ATP synthesis rate.

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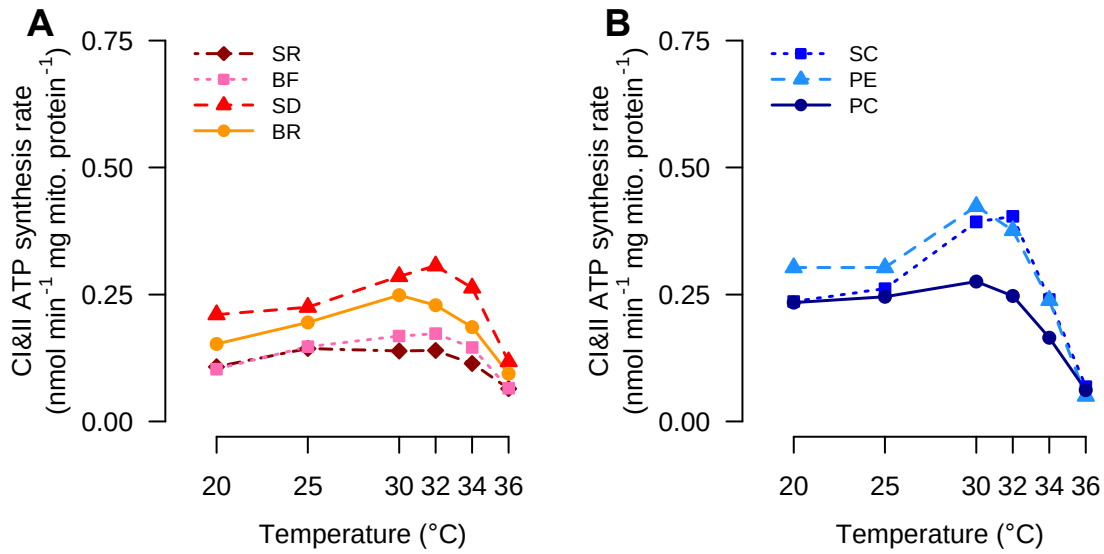
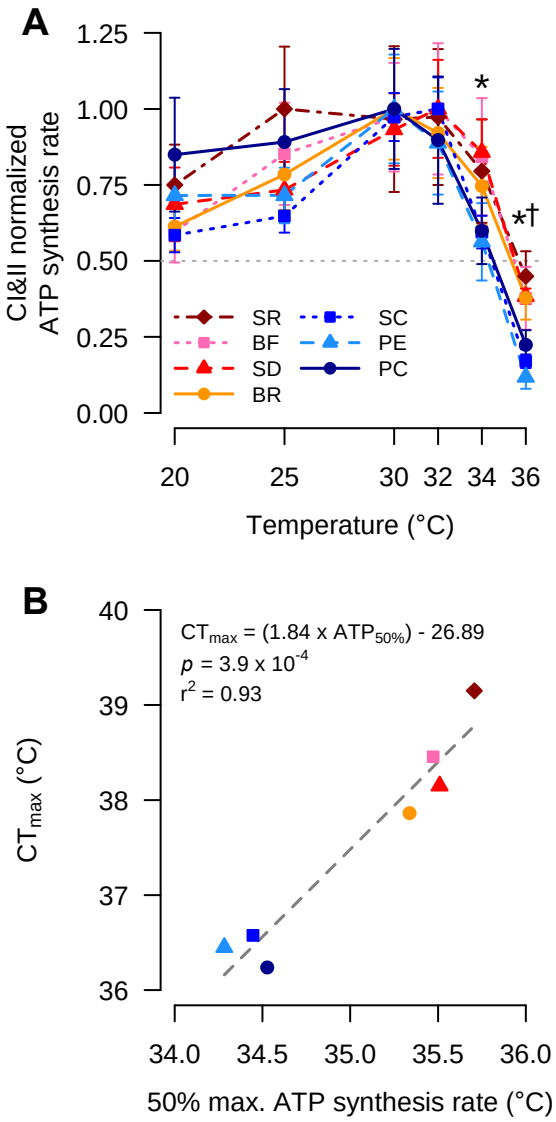


447
448
449

Figure 2.


A
1.25
1.00
0.75
0.50
0.25
0.00
CI&II normalized
ATP synthesis rate
*
*†
SR
BF
SD
BR
SC
PE
PC
20
25
30
32
34
36
Temperature (°C)
B
40
39
38
37
36
CTmax (°C)
CTmax = (1.84 x ATP50%) - 26.89
p = 3.9 x 10-4
r2 = 0.93
34.0
34.5
35.0
35.5
36.0
50% max. ATP synthesis rate (°C)

459 Graphical abstract
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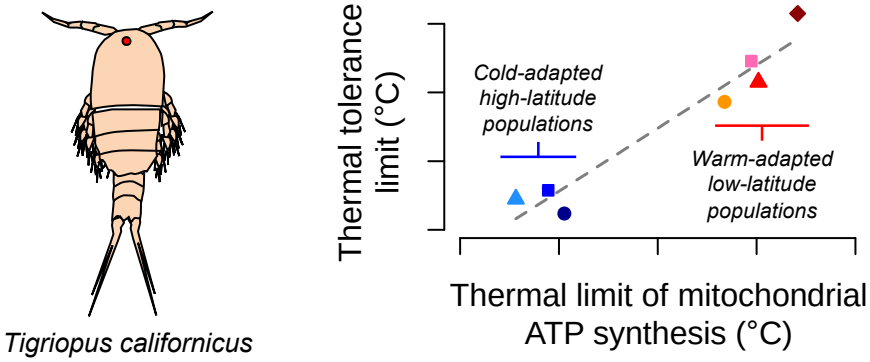

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