Thermal Physiology of Warm-Spring Colonists: Variation among Lake Chub (Cyprinidae: *Couesius plumbeus*) Populations*

Charles-A. Darveau[1,†]
Eric B. Taylor[1,2]
Patricia M. Schulte[1]
[1]Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, British Columbia V6T 1Z4, Canada; [2]Biodiversity Research Centre and Beaty Biodiversity Museum, University of British Columbia, 6270 University Boulevard, Vancouver, British Columbia V6T 1Z4, Canada

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ABSTRACT

In northern Canada, lake chub (Cyprinidae: *Couesius plumbeus*) have colonized a variety of thermal springs that differ substantially from the ancestral environment in both mean temperature and thermal variation. To examine whether this environmental change is associated with differences in physiological traits, we compared the thermal breadth, capacity for acclimation of thermal tolerance, and metabolic enzymes in populations of lake chub from three habitats: a warm but variable hot spring, a thermally constant warm spring, and a seasonally variable temperate lake. Thermal breadth was generally lowest in fish from the constant environment, and this difference was statistically significant in fish acclimated at 10° and 25°C. Critical thermal maximum (CT_{max}) increased with increasing acclimation temperature in all populations. CT_{max} was similar among populations when acclimated at high temperatures but greater in the variable-spring population acclimated to low temperature (10°C). Critical thermal minimum was also dependent on acclimation temperature in all populations but differed among populations such that fish from the stable-spring habitat were not as tolerant to cold temperature when acclimated to 25°C. Temperate- and variable-spring populations showed an increase in mitochondrial enzyme activities (citrate synthase and cytochrome c oxidase) with decreasing acclimation temperature, but this response was absent in the stable-temperature population. Protein content did not change with acclimation temperature in the stable-temperature population, while it increased with decreasing acclimation temperature in both variable thermal habitat populations. Our study suggests that interpopulation variation in thermal physiology is associated with habitat thermal variability.

Introduction

Temperature has pervasive effects on biological systems and thus may be important in establishing the distribution and abundance patterns of ectotherms (Bullock 1955; Hochachka and Somero 2002). The thermal range that a species can inhabit may vary from narrow (in stenotherms) to wide (in eurytherms; reviewed in Somero et al. 1996), and the position and breadth of that range can be modified by acclimation (Cossins and Bowler 1987). The capacity to make the biochemical and physiological adjustments associated with acclimation, however, varies substantially among species (Cossins and Bowler 1987). Although there has been substantial debate in the evolutionary physiology literature with respect to the adaptive value of acclimation (e.g., Wilson and Franklin 2002; Woods and Harrison 2002), it is clear that the capacity for beneficial acclimation, a form of adaptive phenotypic plasticity, would be expected to evolve only under certain circumstances (Padilla and Adolph 1996; for a review, see Piersma and van Gils 2010). These theoretical considerations suggest that plasticity will evolve only when the environment varies in a predictable way and on an appropriate timescale with respect to the life span of the organism and the speed of the plastic responses and is likely to be lost in constant environments when positive selection for plasticity is relaxed—a process that may occur rapidly if the costs of maintaining plasticity are high (Lahti et al. 2009).

Acclimatory responses are common in ectotherms inhabiting temperate environments that are characterized by predictable seasonal variation in temperature. At the whole-animal level, compensatory changes in locomotor performance and thermal tolerance are often observed (Beitinger et al. 2000; Johnston and Temple 2002). For example, Beitinger et al. (2000) reviewed variation in upper and lower limits among fish species (measured as critical thermal maxima and minima, the temperature at which fish are no longer able to maintain equilibrium) and demonstrated that this trait shows a high degree of plasticity and that the extent of the plasticity tends to be greatest in temperate-zone fishes. At the suborganismal level, ectotherms often respond to thermal acclimation by modifying aspects of cellular metabolism, including mitochondrial and membrane properties (reviewed in Guderley 2004). For example, a typical compensatory response to cold acclimation consists of an in-

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† Corresponding author. Present address: Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, Ontario K1N 6N5, Canada; e-mail: cdarveau@uottawa.ca.

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crease in mitochondrial abundance, as indicated by the activity of mitochondrial enzymes citrate synthase and cytochrome c oxidase, and has been documented in fish (reviewed in Guderley 2004), marine invertebrates (Lurman et al. 2010), amphibians (Berner and Puckett 2010), and reptiles (Seebacher 2005). Thus, metabolic compensations appear to be a stereotypical response of eurythermic animals that remain active in low-temperature conditions.

The colonization of thermal springs by temperate-zone fishes provides an interesting model system in which to explore the effects of relaxation of selection on the capacity for thermal acclimation. The lake chub (*Couesius plumbeus*) is a small-bodied member of the Cyprinidae (carps and minnows) that is native to a vast area of North America. The lake chub is native to lakes and streams from the Atlantic basin in Canada and the United States, west to the Rocky Mountains in the United States, north and west to the Pacific basin in British Columbia (BC), and north and east to the Arctic basin in northwestern Canada and Alaska and to Labrador (Lee et al. 1980). The lake chub achieved this large range from postglacial range expansion following retreat of the Wisconsinan glaciers beginning about 10,000 years ago and in the process of deglaciation have colonized a small number of thermal springs in northwestern BC (McPhail 2007). The vast majority of lake chub populations, however, live in lakes and streams that experience an environment with strong seasonal variation in temperature, with water temperatures ranging from approximately 4°C in the winter to the mid-20s (°C) in the summer. In contrast, thermal-spring colonists experience much more stable thermal conditions. For instance, Atlin Warm Spring (northwestern BC) maintains a relatively constant water temperature of 23°–25°C year-round despite a yearly average daily air temperature of 0.5°C, average daily temperatures that range between −15° and −5°C from December to March, and daily air temperatures that range between 6° and 13°C from May to September (Environment Canada 2011). We hypothesized that thermal-spring populations, which experience relatively constant temperatures, would encounter relaxed selection for thermal plasticity and thermal breadth. Based on this, we predicted that thermal-spring populations would have narrower thermal breadth (a smaller difference between the maximum and minimum thermal tolerance) and that the extent of phenotypic plasticity would be reduced, both at the whole-organism level and at the level of metabolic enzymes. To test these predictions, here we sampled warm-water springs and temperate-lake habitat populations and performed an acclimation experiment to assess population-level differences in maximum and minimum critical temperature tolerance and their capacity to acclimate, as well as muscle metabolic enzyme acclimation response.

Material and Methods

Fish Collection

Lake chub (*Couesius plumbeus*) were collected from three locations in BC (BC fish collection permit SU/WL/SM05-13320, BC park use permit GP0510326, Canada Fisheries and Oceans transfer licence 10409 a-e). Populations living in contrasting thermal habitats were sampled, including two thermal-spring populations: Liard Hot Spring (59°25′42.69″, −126°5′18.32″) and Atlin Warm Spring (59°24′13.98″, −133°34′31.21″) populations. Various habitats (creeks, rivers, ponds, and lakes) neighboring the springs were sampled using a seine net and minnow traps, but no lake chub were captured during our sampling effort. As a result, we utilized fish from Green Lake (51°22′34.60″, −121°15′46.97″), which (although distant from the two spring populations) is likely representative of the putative ancestral thermal habitat. In fact, all study populations belong to a common "western" mitochondrial DNA lineage (E. B. Taylor, unpublished data), and the limited geographic distribution of contemporary lake chub populations in the Columbia River (part of the Pacific Refuge) and the Yukon River (the heart of the Bering Refuge) suggests that all lake chub in western North America probably had a common origin from a Great Plains refuge (cf. McPhail and Lindsey 1970). All populations were sampled during the months of July and August 2005, using minnow traps or a dip net. Live fish (80 per population) were transported in aerated plastic bags filled with the respective habitat water at a density of approximately 1 fish/L and maintained on ice in a Styrofoam box placed in a cardboard box for transport. Water temperature ranged from 3° to 7°C during transport, which ranged from 8 h for the Green Lake population to 24 h for the spring populations. In addition, for spring populations, 10 individuals were frozen immediately in liquid nitrogen after capture and transported in a cryogenic shipper. Water temperatures in each habitat at the time of sampling were recorded using a digital temperature probe (Quartz Digi-Thermo, Fisher Scientific).

Fish Holding Conditions and Temperature Acclimation Experiment

Upon arrival at the University of British Columbia (UBC), fish were transferred to four 100-L aquaria where the initial water temperature was set to 17°C. Each aquarium was divided into three compartments with plastic mesh to accommodate 11 individuals from each population. Temperatures within each aquarium were changed by 1°C/d until they reached the four acclimation temperatures of 5°, 10°, 20°, and 25°C (achieved average temperatures were actually 6°, 9.5°, 19.5°, and 24.5°C). Once experimental temperatures were reached, fish were acclimated to those conditions for 2 mo before measurements were initiated, to ensure sufficient time for physiological acclimation responses to take place (Johnston and Dunn 1987). Fish were maintained at a 12L : 12D photoperiod and fed every other day with bloodworms. Water quality was monitored periodically, and water changes were initiated as required, such that half of the tank volume was changed every 3 wk. When these holding conditions were used, low mortality occurred over a 3-mo period (one to three fish per group), except for the Atlin Warm Spring population acclimated to 20° and 25°C, where 11 and six individuals died, respectively, during the period in between critical temperature measurements. All ma-

nipulations were carried out under approved UBC animal care protocol A01-0180.

Critical Temperature Tolerance Measurements

After the temperature acclimation period, critical temperature minimum (CT_{min}) and maximum (CT_{max}) tolerance measurements were performed 2 wk apart. Fish were transferred from their acclimation tank to 1-L containers that were aerated and submerged in a common reservoir serviced with a recirculating water bath. Initial temperature matched the acclimation temperature of the experimental group, and fish were allowed to adjust to the experimental apparatus for 30 min before the beginning of the experiment. Temperature was increased or decreased by approximately 0.3°C/min. Individual fish were monitored until they lost equilibrium, preventing normal upright swimming; we used this end point as our measure of CT_{max} or CT_{min}, respectively. The temperature of each 1-L container was measured to account for heterogeneous temperature distribution within the common reservoir. For CT_{min} measurements, the common reservoir's water was substituted with ethylene glycol, and dry ice pellets were added to the common reservoir as needed to reach the lowest temperatures at the appropriate rate. Upon completion of the measurements, individuals were transferred back to their acclimation tank for a recovery period of 2 wk. Among the fish tested, all recovered from the CT_{min} trials, and three individuals (two from Green Lake and one from Atlin Warm Spring) did not recover from the CT_{max} trials. For each individual, the thermal breadth (ΔCT) was calculated as the difference between CT_{max} and CT_{min}.

Enzyme and Protein Assays

Two weeks following CT_{max} measurements, fish were anesthetized using MS222 (100 mg/L), body mass and body length were measured, and an axial muscle sample was dissected and frozen immediately in liquid nitrogen and stored at −80°C until enzyme assays. The location of the axial muscle sample was standardized for all fish directly anterior to the caudal peduncle.

Fish axial muscle samples were homogenized (Polytron) in 9 vol of buffer composed of Tris-HCl 75 mM pH 7.5 at 25°C, $MgCl_2$ 3 mM, EDTA 5 mM, DTT 1 mM, and Triton X-100 0.5% (vol/vol). Crude homogenates were centrifuged at 500 *g* for 5 min at 4°C, and supernatant was kept for enzyme assays and protein measurements. We measured the maximal activities, V_{max}, of citrate synthase (CS), cytochrome oxidase (COX), pyruvate kinase (PK), and lactate dehydrogenase (LDH) in axial muscle. All enzyme assays were conducted in triplicate at 25°C, and kinetic assays were monitored for 5 min with a Molecular Devices SpectraMax 190 using the following conditions: CS: Tris-HCl 100 mM pH 8.0 at 25°C, DTNB 0.1 mM, Acetyl CoA 0.3 mM, and oxaloacetate 1 mM (omitted from control); COX: potassium phosphate 50 mM pH 7.5 at 25°C, cytochrome c 0.05 mM (reduced using sodium hydrosulfite 2 mg/mL); PK: imidazole-HCl 50 mM pH 7.5 at 25°C, $MgCl_2$ 10 mM, KCl 100 mM, ADP 5 mM, NADH 0.15 mM, phosphoenolpyruvate 3.5 mM (omitted from control), lactate dehydrogenase in excess; and LDH: imidazole-HCl 50 mM pH 7.5 at 25°C, NADH 0.15 mM, pyruvate 0.5 mM (omitted from control). All chemicals were purchased from Sigma Chemicals.

Protein assays were performed using the bicinchoninic acid method (Sigma Chemicals). Assays were performed in duplicates and using bovine serum albumin as protein standard.

Statistical Analyses

All statistical analyses were performed using SYSTAT 12 software. Dependent variables (CT_{max}, CT_{min}, ΔCT, muscle enzyme activities, and protein content) were analyzed with analyses of covariance (ANCOVA), using population and acclimation temperature as factors and body mass as covariate. We also used condition factor as covariate, and results were qualitatively the same and are therefore not reported. Assumptions of the ANCOVA were tested, and in a few cases logarithmic or square-root transformation of the data was required. Due to missing data for one group (20°C-acclimated Atlin Warm Spring population), an ANCOVA was performed without the 20°C-acclimated groups and using all populations. Another analysis was performed without the Atlin Warm Spring population and using all acclimation temperature groups. Finally, field-collected samples were included in an ANCOVA without the Green Lake population and 20°C acclimation group. Multiple comparisons with Bonferroni adjustments were performed following ANCOVAs. Enzyme activities were analyzed when expressed both per gram of tissue and per milligram protein.

Results

Field Observations

The thermal habitats of the three populations differed greatly during the sampling period. Green Lake is a temperate lake typical of the BC interior; it is approximately 14 km long and 1.5 km wide (surface area, ~23 km^2), where the average depth is 10.3 m (maximum depth, 36 m). Winter and bottom temperatures during summer stratification are homogeneous, at around 4°C, and the highest average surface temperature reported varied from 18.5° to 21°C in July 2001–2004 (BC Lake Stewardship Society, Kelowna, BC, 2004). Green Lake fish were captured at a depth ranging from 1 to 2 m in littoral areas, where water temperature was a constant 17°C in early July. The Liard Hot Spring habitat consists of a hot springs complex where lake chub are found in two distinct areas, the Alpha and Delta-Epsilon complexes. We sampled the Alpha complex only because the other areas were closed, owing to the presence of black bears. In the Alpha complex, lake chub were found in the warm-water swamp, where warm water from the Alpha pool (~53°C at the source) flows into the Alpha swamp, forming a moderate-size shallow swamp (between 100 and 200 m wide and several hundred meters long). In the Alpha swamp, temperature was spatially and temporally variable; a single location ranged from 15.6° to 26.5°C, depending on the time of

day and solar radiation, and fish were observed in water ranging from 14.1° to 27.9°C, according to the location in the swamp at the same time period. Atlin Warm Spring forms a small round-shaped pool (~20 m in diameter; maximum depth, ~1 m), where water temperature at the source located in the center of the pool was 28°C. The pool drains into Atlin Lake several hundred meters downstream, via a small stream (~1 m wide). Water temperature was 26.5°–28°C in the main warm-water pool and 25°–26°C approximately 100 m downstream (air temperature was ~25°C midday). During the sampling period, Atlin Warm Spring fish were located exclusively in small streams, where temperature ranged from 25° to 26°C. Field observations taken during the month of February (M. Connor, Taku River–Tlingit First Nations Fisheries Department, Atlin, BC, personal communication), when the air temperature was −21°C midday, suggest that most fish are found in the warm-water pool, where temperature ranged from 24.6° to 26.2°C on the periphery; some fish were found in the small stream ranging from 22.8° to 24.5°C approximately 100 m downstream.

Transport and Mortality

Fish from both Green Lake and Liard Hot Spring coped with transport without any signs of disturbance. During shipping, fish were held for 8–24 h in well-aerated bags of water on ice, resulting in a gradual decrease in water temperature to approximately 3°–7°C through most of the shipping period. Atlin Warm Spring fish were treated the same way, but ~60% of the fish were cold-stunned (ventral side up in the shipping boxes) on arrival at UBC after 16 h in transit. The majority of Atlin Warm Spring fish recovered when placed in an aquarium at 17°C following shipping, although approximately 10% mortality occurred.

Morphological Measurements

Body mass of fish captured differed among populations, where Green Lake fish weighed 2.870 ± 0.262 g, Atlin Warm Spring fish weighed 2.109 ± 0.294 g, and Liard Hot Spring fish weighed 2.052 ± 0.243 g (population: $F_{2,71} = 3.06$, $P = 0.053$; temperature: $F_{2,71} = 0.97$, $P = 0.383$; temperature × acclimation: $F_{4,71} = 0.282$, $P = 0.889$). The calculated condition factor also differed among populations and also between acclimation temperature groups (population: $F_{2,71} = 26.86$, $P < 0.001$; temperature: $F_{2,71} = 5.87$, $P = 0.004$; temperature × acclimation: $F_{4,71} = 1.296$, $P = 0.280$). Atlin Warm Spring fish and Liard Hot Spring fish had similar condition factors (1.232 ± 0.033 and 1.173 ± 0.027, respectively), and Green Lake fish showed lower values (0.938 ± 0.029) at all acclimation temperatures. Condition factor was lower in fish acclimated to 5°C, but pairwise comparisons showed no differences among acclimation groups within populations.

Critical Temperature Tolerance

An ANCOVA showed a significant effect of population and acclimation temperature on CT_{max} values, with no significant interaction (fig. 1*A*; population: $F_{2,71} = 11.52$, $P < 0.001$; temperature: $F_{2,71} = 1{,}247.66$, $P < 0.001$; population × temperature: $F_{4,71} = 1.17$, $P = 0.33$; body mass: $F_{1,71} = 1.43$, $P = 0.24$). Pairwise comparisons showed that Liard Hot Spring fish have a higher CT_{max} than do the Atlin Warm Spring ($P = 0.021$) and Green Lake ($P = 0.005$) populations when acclimated to 10°C. CT_{max} significantly increased with increasing acclimation temperature in all populations, except between the 5° and 10°C acclimation groups for which no significant increase was observed.

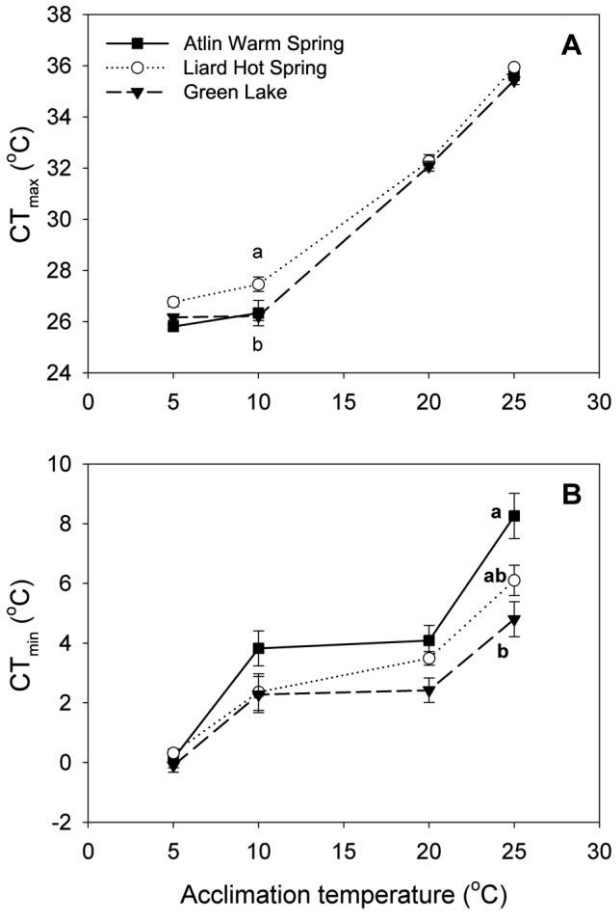

Figure 1. Mean (±SEM) critical thermal maximum (CT_{max}; *A*) and critical thermal minimum (CT_{min}; *B*) in three populations of lake chub (*Couesius plumbeus*) acclimated to four different temperatures. CT_{max} and CT_{min} differed significantly among populations and acclimation temperatures. Different letters indicate significant differences between populations at an acclimation temperature. For clarity, the statistical significance of the effects of acclimation temperature are not shown, but for all populations, CT_{max} estimations were 5° = 10° < 20° < 25°C ($P < 0.05$). Estimates of CT_{min} followed the same pattern, where 5° < 10° = 20° < 25°C ($P < 0.05$), except between 5° and 10°C for the Liard Hot Spring population ($P = 0.076$) and between 20° and 25°C for the Green Lake population ($P = 0.066$).

For CT_{min}, an ANCOVA showed a significant effect of temperature and population and a near-significant interaction term between these two factors (fig. 1*B*; population: $F_{2,96} = 10.81$, $P < 0.001$; temperature: $F_{3,96} = 83.61$, $P < 0.001$; population × temperature: $F_{6,96} = 2.13$, $P = 0.057$; body mass: $F_{1,96} = 1.13$, $P = 0.29$). The CT_{min} of the Atlin Warm Spring population increased more with increasing acclimation temperature than did that of the other two populations. Differences between populations were observed at 25°C, where Atlin Warm Spring fish had higher CT_{min} values than did Green lake fish ($P = 0.004$). Estimates of CT_{min} increased significantly with acclimation temperature, except from 5° to 10°C for the Liard Hot Spring population ($P = 0.076$), from 20° to 25°C for the Green Lake population ($P = 0.066$), and from 10° to 20°C for all populations.

There were differences among populations in ΔCT at the four acclimation temperatures (table 1; population: $F_{2,71} = 11.09$, $P < 0.001$; temperature: $F_{2,71} = 60.60$, $P < 0.001$; population × temperature: $F_{4,71} = 2.27$, $P = 0.055$; body mass: $F_{1,71} = 2.32$, $P = 0.132$). Atlin Warm Spring fish show a thermal range smaller than that of the Liard Hot Spring population when acclimated to 10°C ($P = 0.012$) and smaller than that of Green Lake fish when acclimated to 25°C ($P = 0.027$). For the Green Lake population, the thermal range differed among acclimation temperatures where 5° = 10° < 20° = 25°C ($P < 0.05$). For Liard Hot Spring fish, groups differed as follows: 10° < 20° = 25°C and 5° < 25°C ($P < 0.01$). Atlin Warm Spring fish showed a smaller thermal range when acclimated to 10°C ($P < 0.001$).

Muscle Enzyme Activity and Protein Content

The activity of the mitochondrial enzyme citrate synthase measured in the axial muscle showed a significant effect of population and temperature as well as a significant interaction between acclimation temperature and population (fig. 2*A*; population: $F_{2,71} = 12.35$, $P < 0.001$; temperature: $F_{2,71} = 42.86$, $P < 0.001$; population × temperature: $F_{4,71} = 3.84$, $P = 0.007$; body mass: $F_{1,71} = 3.67$, $P = 0.059$). In Atlin Warm Spring fish, no significant increase in activity with decreasing acclimation temperature was detected, while for the Green Lake and Liard Hot Spring fish, CS activity significantly increased by 71% and 141%, respectively, in low-temperature-acclimation groups (5° = 10° > 20° = 25°C; $P < 0.01$). No differences in enzyme activity were observed among populations when acclimated to warm temperatures (20° and 25°C), but at the lower 5° and 10°C acclimation temperatures, Liard Hot Spring fish had activity higher than that of Atlin Warm Spring fish ($P < 0.001$) and higher than that of Green lake population fish when acclimated to 5°C ($P = 0.014$). Finally, CS activity of fish collected in the field did not differ from that of fish acclimated to 25°C in the laboratory, nor did it differ between populations.

The enzyme COX followed a similar pattern, where its activity in the axial muscle changed with acclimation temperature to a different extent among populations (fig. 2*B*; population: $F_{2,71} = 12.80$, $P < 0.001$; temperature: $F_{2,71} = 42.20$, $P < 0.001$; population × temperature: $F_{4,71} = 6.39$, $P < 0.001$; body mass: $F_{1,71} = 0.201$, $P = 0.659$). Again, the Atlin Warm Spring population did not show an increase in activity associated with decreasing acclimation temperature. For the Green Lake fish, COX activity significantly increased by 86% in the low-temperature-acclimation groups (5° = 10° > 20° = 25°C; $P < 0.005$), while for Liard Hot Spring fish the activity increased by 133% and linearly with acclimation temperature (5° > 10° > 20° > 25°C; $P < 0.05$). There were no significant differences in activity among populations when fish were acclimated to warm temperatures (20° and 25°C), but when acclimated to 5°C the Atlin Warm Spring fish had activity lower than that of the other two populations ($P < 0.001$) and lower than that of the Green Lake population at 10°C ($P = 0.027$). Fish collected in the field did not differ from fish acclimated to 25°C in the laboratory or between populations.

The activity of the anaerobic glycolytic enzyme LDH differed among populations (fig. 2*C*; population: $F_{2,71} = 14.06$, $P < 0.001$; temperature: $F_{2,71} = 1.84$, $P = 0.167$; population × temperature: $F_{4,71} = 0.57$, $P = 0.685$; body mass: $F_{1,71} = 2.70$,

Table 1: Thermal breadth ($\Delta CT = CT_{max} - CT_{min}$) of lake chub (*Couesius plumbeus*) populations acclimated to four temperatures

	ΔCT (°C)		
Acclimation temperature (°C)	Atlin Warm Spring	Liard Hot Spring	Green Lake
5	25.68 ± .29	26.44 ± .19	26.27 ± .24
10	22.51 ± .69^{A}	25.10 ± .69^{B}	24.17 ± .64^{AB}
20	...	29.53 ± .48	28.76 ± .29
25	27.36 ± .70^{A}	29.84 ± .46^{AB}	30.63 ± .63^{B}

Note. An ANCOVA showed a significant effect of both factors (population: $F_{2,71} = 11.09$, $P < 0.001$; temperature: $F_{2,71} = 60.60$, $P < 0.001$; population × temperature: $F_{4,71} = 2.27$, $P = 0.055$; body mass: $F_{1,71} = 2.32$, $P = 0.132$). For each acclimation temperature, populations identified with different letters showed significant differences ($P < 0.05$ after Bonferroni adjustments). For clarity, the statistical significance of the effects of acclimation temperature are not indicated in the table, but for Green Lake, 5° = 10° < 20° = 25°C ($P < 0.05$); Liard Hot Spring, 10° < 20° = 25°C and 5° < 25°C ($P < 0.01$); and Atlin Warm Spring, 10° < 5° = 25°C ($P < 0.001$).

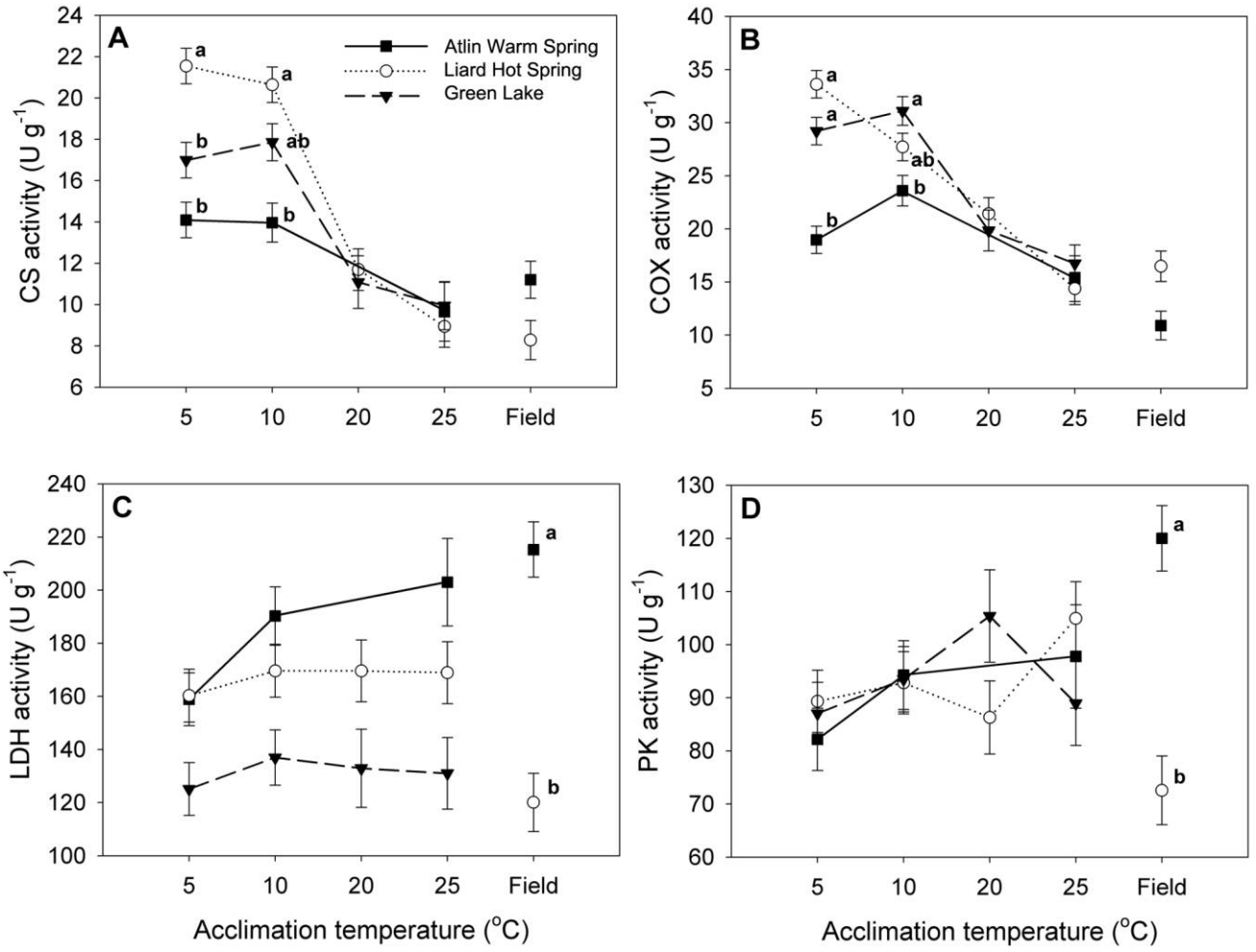

Figure 2. Activity (U g tissue^{-1}) of four energy metabolism enzymes (mean ± SEM) in the axial muscle of three populations of lake chub (*Couesius plumbeus*) acclimated to four temperatures. Two mitochondrial enzymes, citrate synthase (CS; *A*) and cytochrome c oxidase (COX; *B*), and two glycolytic enzymes, lactate dehydrogenase (LDH; *C*) and pyruvate kinase (PK; *D*), were measured. The activities of enzymes of fish collected in the field are reported for two populations. Different letters indicate significant differences between populations at an acclimation temperature. For clarity, the statistical significance of the effects of acclimation temperature are not shown, but for citrate synthase (*A*): Atlin Warm Spring, $P > 0.50$; Green Lake and Liard Hot Spring, 5° = 10° > 20° = 25°C ($P < 0.01$); and for cytochrome c oxidase (*B*): Atlin Warm Spring, $P > 0.50$; Green Lake, 5° = 10° > 20° = 25°C ($P < 0.01$); Liard Hot Spring, 5° > 10° > 20° > 25°C ($P < 0.05$). For lactate dehydrogenase (*C*) and pyruvate kinase (*D*), $P > 0.15$.

$P = 0.105$). Multiple comparisons indicated that Green Lake fish acclimated to 10° and 25°C tended to have lower LDH activity compared with Atlin Warm Spring fish acclimated to the same temperature ($P = 0.055$ and 0.071, respectively), but no other differences between populations acclimated to the same temperature were detected. The LDH activity of fish collected in the field did not differ from that of fish acclimated to 25°C but was nearly twofold higher in the Atlin Warm Spring population ($P < 0.001$).

For PK, activity was not affected by acclimation temperature or population (fig. 2*D*; population: $F_{2,71} = 1.54$, $P = 0.221$; temperature: $F_{2,71} = 1.47$, $P = 0.237$; population × temperature: $F_{4,71} = 0.48$, $P = 0.747$; body mass: $F_{1,71} = 6.23$, $P = 0.015$). By contrast, field-collected Atlin Warm Spring fish had a nearly twofold higher activity ($P = 0.002$), and field-collected fish did not differ from fish acclimated to 25°C.

Axial muscle homogenate protein content showed a significant effect of both acclimation temperature and population (fig. 3; population: $F_{2,71} = 8.29$, $P = 0.001$; temperature: $F_{2,71} = 12.20$, $P < 0.001$; population × temperature: $F_{4,71} = 1.15$, $P = 0.341$; body mass: $F_{1,71} = 9.87$, $P = 0.002$), although pairwise comparisons did not identify any differences between populations. The Atlin Warm Spring population showed no significant change in protein content with acclimation temperature, and the protein content of field-caught animals was not different from that of lab-maintained fish ($P > 0.138$). In Green Lake fish, protein content differed between the 10°- and 25°C-acclimated fish ($P < 0.05$), and for Liard Hot Spring fish,

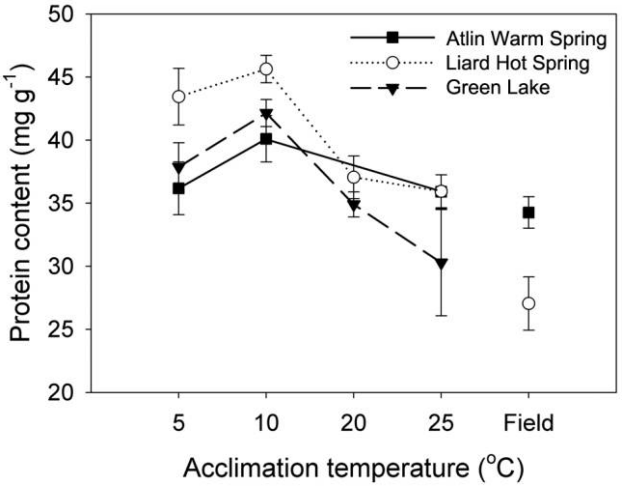

Figure 3. Protein content of lake chub (*Couesius plumbeus*) axial muscle of three populations acclimated to four temperatures. The protein content of fish collected in the field is also reported for two populations. There were no significant differences in protein content between populations at any acclimation temperature. Differences in protein content with acclimation temperature were Atlin Warm Spring, $P > 0.50$; Green Lake, $10° > 25°C$ ($P < 0.05$); Liard Hot Spring, $10° > 20° = 25°C$ ($P < 0.01$).

$10° > 20° = 25°C$ ($P < 0.01$). Liard Hot Spring fish captured in the field did not differ from fish acclimated to 25°C, and no differences among populations were detected.

To further assess differences in enzyme activity among populations and independent of protein content, we performed statistical analyses using activity expressed per milligram protein. The activity of CS expressed per milligram protein differed significantly among populations and acclimation temperatures (population: $F_{2,71} = 3.99$, $P = 0.023$; temperature: $F_{2,71} = 10.03$, $P < 0.001$; population × temperature: $F_{4,71} = 1.77$, $P = 0.145$; body mass: $F_{1,71} = 0.09$, $P = 0.757$). Pairwise comparisons showed that for Liard Hot Spring fish, the activity increased in cold-acclimated groups ($5° > 20° = 25°C$ and $10° > 25°C$; $P < 0.05$). The activity of COX was also significantly different among populations and acclimation temperatures with a significant interaction term (population: $F_{2,71} = 11.85$, $P < 0.001$; temperature: $F_{2,71} = 7.51$, $P = 0.001$; population × temperature: $F_{4,71} = 2.57$, $P = 0.045$; body mass: $F_{1,71} = 4.42$, $P = 0.039$). When acclimated to 5°C, Atlin Warm Spring fish showed a lower activity than did the other two populations ($P < 0.05$), and Liard Hot Spring fish had higher activity at 5° than at 25°C ($P < 0.001$).

Discussion

We examined the thermal physiology of three populations of lake chub living in contrasting thermal habitats: Green Lake, a temperate lake with predictable seasonal variation in temperature; Liard Hot Spring, where temperature varies substantially on an acute timescale; and Atlin Warm Spring, which has a stable thermal environment. We predicted that populations living in stable conditions would have a narrower zone of thermal tolerance (difference between CT_{max} and CT_{min}), but this prediction was only partially supported. Fish from Atlin Warm Spring tended to have a narrower zone of thermal tolerance than did the other populations (table 1), but this difference was not statistically significant for all comparisons at all acclimation temperatures. The Atlin Warm Spring fish showed a reduced zone of thermal tolerance when acclimated to warm temperature (25°C), due to a higher CT_{min}. The population from the thermally variable Liard Hot Spring had a larger zone of thermal tolerance when acclimated to 10°C, as a result of an increased CT_{max}. Similar trends were observed at the other acclimation temperatures, particularly with respect to the constant-temperature population in Atlin Warm Spring, and thus our data were generally consistent with our prediction that populations from more variable environments would have a larger zone of thermal tolerance (i.e., greater thermal breadth).

We also predicted that populations living in stable conditions would have reduced phenotypic plasticity (in terms of ability to acclimate) compared with populations from more variable environments. This prediction was supported at the biochemical level; the extent of plasticity of the Atlin Warm Spring population was reduced to the point that we could not detect any increases in mitochondrial enzyme activity with cold acclimation, suggesting a loss of phenotypic plasticity in this population (fig. 2). In contrast, the hypothesis of reduced phenotypic plasticity in stable environments was not supported for either CT_{max} or CT_{min} (fig. 1); if anything, plasticity in CT_{min} was greatest in the population from the stable thermal environment (fig. 1*B*). These differences in the plasticity of thermal tolerance resulted in greater reductions in the breadth of thermal tolerance for the Atlin Warm Spring population at high acclimation temperatures. These results are potentially consistent with a model in which the maintenance of cold tolerance is costly. In this scenario, populations from variable habitats would be selected to maintain cold tolerance even when acclimated to warm temperatures, while populations from the constant warm conditions could accrue cost savings by reducing cold tolerance except under conditions of cold acclimation. Our results highlight the loss of plasticity at the biochemical level in a population now experiencing a stable thermal habitat and an apparent change in shape of the relationship between thermal tolerance and acclimation temperature, reducing the breadth of tolerance in the stable-spring population when acclimated to higher temperatures.

Populations Studied

The interpretation of differences observed between springs and temperate-lake populations assumes that spring populations are unique and isolated. Observations we made in both spring habitats agree with those presented by McPhail (2001). In the Liard Hot Spring complex we sampled the Alpha swamp, where fish were restricted to a small area. McPhail (2001) extensively characterized the habitat and identified one area where some seepage from the thermal complex to a small stream might

occur; no lake chub were captured in the surrounding area, and the author concluded that at present Liard Hot Spring complex lake chubs are completely isolated. The Atlin Warm Spring population also appears to be isolated. We observed fish in small streams restricted near the main warm-water pool, and observations in the winter showed that fish remain in the same area and some migrated to the main warm-water pool (see "Results"). McPhail (2001) made similar observations and also sampled the stream for about 100 m upstream of Warm Bay, where the spring enters Atlin Lake, and no fish were observed. We also sampled Warm Bay using a seine net, which was effective, as we captured many least cisco (*Coregonus sardinella*) and Arctic grayling (*Thymallus arcticus*), but no lake chub were observed in the lake. The spring populations sampled appear isolated and are unlikely to be reinvaded by neighboring populations.

When trying to distinguish populations based on morphological features, we noted differences in body mass and condition factor between the temperate lake and the two spring populations. The Green Lake fish were slightly larger, which agrees with the observations of McPhail (2001) that warm-water spring populations show lower frequency of larger individuals. Condition factor differences indicate that the Green Lake population fish are more slender. The condition factor obtained for the Green Lake population (0.94) is in line with another temperate-lake habitat from a distant population (0.96; Phibbs et al. 2011). The analysis performed by McPhail (2001), and using multiple morphological traits, suggests that population-level variation in morphology is common in lake chub, and warm-water spring populations fall within the variation of all populations sampled in his study. In other words, populations of lake chub vary in morphology, and this variation is not strictly associated with habitat temperature. Although we cannot assess the origin of morphological differences observed, we have accounted for this difference in our analyses, and the physiological differences we reported are independent of morphological variation.

Whole-Animal Critical Temperature Tolerance

At the whole-animal level, populations of lake chub differed significantly in critical temperature maximum and minimum, as well as their thermal breadth, indicated by the difference in critical temperature tolerances (fig. 1; table 1). Population-level variation in whole-animal critical thermal tolerance has been documented in a variety of species (for a review, see Angilletta 2009), including fishes (e.g., Fields et al. 1987). For example, similar to the results presented here, Amargosa pupfish (*Cyprinodon nevadensis*) inhabiting a relatively thermally constant spring had a narrower thermal breadth than did fish from the nearby and more thermally variable river habitats (Hirschfield et al. 1980). The differences in thermal tolerance that we observed here could be the result of acclimation, developmental plasticity, or evolutionary change. Acclimation to variable environments has been shown to increase thermal breadth in fish (Feldmeth et al. 1974), but we acclimated all our fish to constant temperatures prior to testing to reduce these effects. Alternatively, the observed differences in thermal tolerance between lake chub populations could be due to developmental plasticity, as the environment experienced during early development has been shown to alter adult thermal tolerance in fishes such as zebrafish *Danio rerio* (Schaefer and Ryan 2006). The possibility of relatively rapid evolution in thermal tolerance is supported by studies on fish exposed to the thermal effluent of nuclear power plants (Meffe et al. 1995) and experimental evolution studies in threespine stickleback (*Gasterosteus aculeatus*) in which CT_{min} declined by several degrees Celsius within three generations of selection in a novel thermal environment (Barrett et al. 2010).

The shape of the relationship between acclimation temperature and thermal tolerance estimates also tended to vary among populations, as suggested by differences among populations only at certain temperatures and near-significant interaction terms in our analyses of CT_{min} and thermal breadth. Contrary to our prediction, the Atlin Warm Spring population did not show reduced phenotypic plasticity in the form of a reduced capacity to undergo acclimation of CT_{max} or CT_{min}. In fact, in the case of CT_{min}, this population exhibited somewhat greater plasticity (fig. 1*B*). Although we did not detect a significant interaction between population and acclimation temperature in the ANCOVA ($P = 0.057$), CT_{min} differed significantly between populations at warm acclimation temperatures but not at cold acclimation temperatures (with the Atlin Warm Spring fish being the least tolerant at warm acclimation temperatures and thus demonstrating the largest change in CT_{min} with acclimation and the greatest plasticity). Demonstrations of variation in plasticity are relatively rare in fishes. For example, populations of the coastal Atlantic killifish (*Fundulus heteroclitus*) differ in thermal tolerance such that populations living at the northern and southern extremes of the species range differ in CT_{max} and CT_{min} by ~1.5°C, regardless of acclimation temperature (Fangue et al. 2006), such that there are no differences between populations in the plasticity of thermal tolerance in this species (Healy and Schulte 2012). It is likely that for a complex phenotype such as whole-animal thermal tolerance, where the physiology behind the measured phenotype likely involves several components of neuromotor control affected by temperature, multiple trade-offs could be involved, resulting in complex relationships between plasticity and environmental variability. Angilletta et al. (2003) commented on the diversity of shapes in thermal reaction norms and how they can vary as a function of the trade-offs involved (specialist-generalist, acquisition, and allocation). A more systematic description of the relationship with fine acclimation temperature intervals would be necessary to assess the shape of these curves and differences among populations, to further comment on these potential trade-offs.

Muscle Thermal Acclimation Response

The increase in aerobic potential of muscle tissue, partly by increasing mitochondrial capacity, is a stereotypical response exhibited by many fish species acclimated to low temperature (Johnston and Maitland 1980; Eggington and Sidell 1989; Guderley and St. Pierre 2002; Pörtner 2002; Guderley 2004). The temperate-habitat representative population from this study, the Green Lake population, shows a clear acclimation response of both mitochondrial enzymes CS and COX (1.7- and 1.8-fold increase, respectively; fig. 2*A*, 2*B*), within the range reported for other species (see Grim et al. 2010). The absence of acclimation response found for the stable Atlin Warm Spring population points toward a relaxed selection for plasticity in muscle metabolic phenotype. In contrast, the greater-fold change of CS and COX enzyme activity in the variable Liard Hot Spring population (2.4- and 2.3-fold increase, respectively), although not significantly different from the Green Lake population, agrees with the idea that the degree of thermal habitat variation drives the extent of physiological plasticity.

Variation in the cold-temperature acclimation response among populations of a species has been documented in a few species of fish. Variation in cold-acclimation response of mitochondrial enzymes has been studied in *F. heteroclitus*, where the enzyme CS increased in activity in fish acclimated to 5°C when compared with individuals acclimated to 15° and 25°C but only in a population originating from the northern end of the species distribution (Fangue et al. 2009). Surprisingly, a similar pattern was not observed for COX activity in their study, but a complementary study confirmed that, indeed, northern populations of *F. heteroclitus* increased mitochondrial content when acclimated to cold temperatures, while southern populations did not (Dhillon and Schulte 2011). Another series of studies on Atlantic cod (*Gadus morhua*) showed population-level variation in mitochondrial cold-acclimation response. When populations living at various latitudes in Atlantic Europe were sampled, the activity of the mitochondrial enzymes CS and COX of the white muscle increased with decreasing acclimation temperature but to a lesser extent in populations living in more southern latitude (Luccassen et al. 2006). In addition, another study showed that those populations varied in acclimation response for another aspect of physiology associated with aerobic locomotion, the cardiac myoglobin level (Lurman et al. 2007). Phenotypic plasticity of cellular responses associated with temperature acclimation thus varies as a function of environmental temperature regime. The origin of such differences remains to be established, and future work assessing the role of thermal environment during development in shaping adult metabolic acclimation response will clarify mechanisms involved in generating population-level diversity.

Not only did acclimation to cold temperatures induce changes in mitochondrial capacity but also our results show a clear increase in the protein content of our homogenates, which are composed at least of all soluble proteins (fig. 3). Multiple cellular phenotypes acclimate with temperature, and protein content changes likely represent these global cellular changes. Quantitative changes as reported in this study where the content of specific proteins is increased at low temperatures and qualitative changes where isoforms of proteins are differentially expressed seasonally (Crockford and Johnston 1990) likely account for the observed change in protein content we measured. In addition, protein synthesis rates are also influenced by acclimation temperature, being higher during cold acclimation (Loughna and Goldspink 1985; Watt et al. 1988). The increase in protein content of the axial muscle homogenates observed in the two variable thermal habitats is therefore indicative of an overall reorganization, while the absence of protein content difference with acclimation temperature in the stable Atlin Warm Spring population indicates an absence of this global cellular change.

Despite parallel changes in both mitochondrial enzyme activities and protein content, differences in acclimation response among populations remain when enzyme activities were expressed per unit protein. For both CS and COX, Atlin Warm Spring fish did not increase their activity when acclimated to low temperature. Functionally, mitochondrial enzyme activity expressed per unit muscle mass represents the metabolic potential of fish musculature, while the activity of enzymes expressed per unit protein indicates additional changes associated with muscle composition differences. The absence of changes in glycolytic enzymes (expressed per unit mass), while both mitochondrial and protein content of the tissue increased with decreasing acclimation temperature, indicates that changes in cellular machinery are targeted as opposed to simple changes in muscle composition such as water and lipid content. Finally, the possibility that differences among populations represent differences in abundance of muscle fiber types is likely, but similar proportions of glycolytic and mitochondrial enzymes in fish acclimated to warm temperatures do not suggest that this is the case. The mechanisms generating such differential acclimation response remain to be resolved.

Conclusions

We showed that spring colonists exhibit diverse physiological phenotypes associated with acclimation to environmental temperature. At the whole-animal level, critical thermal tolerance varied as a function of the habitat thermal regime, where the stable-spring population had a reduced tolerance and the variable-spring habitat population showed a slightly improved tolerance. These differences in tolerance, however, were not reflected at all environmental temperatures, indicating that simple predictions from specialist-generalist trade-offs cannot account for the variation observed. The mechanism driving this diversity is still unresolved but could be the product of local adaptation or irreversible developmental plasticity. Whichever mechanisms explain these differences among populations, ecological isolation based on thermal tolerance is almost certainly an outcome; Atlin Warm Spring fish acclimated to their habitat temperature (~25°C) cannot tolerate temperatures below 8°C, unless they acclimate beforehand. At the cellular level, the phenotypic plasticity of aerobic energy metabolism in response to acclimation

temperature varied according to our predictions, where the extent of the response is a function of the extent of environmental variability. Moreover, we showed that this stereotypical response can be lost in populations experiencing stable conditions, suggesting substantial costs of this response and implying allocation or acquisition trade-offs (Angilletta et al. 2003). The evolutionary origin of these population-level differences remains to be confirmed, but, clearly, habitat thermal regime across a stability-variability continuum can have a profound impact on physiological phenotypes.

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