RESEARCH ARTICLE

Temperature during embryonic development has persistent effects on metabolic enzymes in the muscle of zebrafish

Meghan E. Schnurr, Yi Yin and Graham R. Scott*

ABSTRACT

Global warming is intensifying interest in the physiological consequences of temperature change in ectotherms, but we still have a relatively poor understanding of the effects of temperature on early life stages. This study determined how embryonic temperature (T_E) affects development and the activity of metabolic enzymes in the swimming muscle of zebrafish. Embryos developed successfully to hatching (survival ≥88%) from 22 to 32°C, but suffered sharp increases in mortality outside of this range. Embryos that were incubated until hatching at a control T_E (27°C) or near the extremes for successful development (22 or 32°C) were next raised to adulthood under control conditions at 27°C. Growth trajectories after hatching were altered in the 22°C and 32°C T_E groups compared with 27°C T_E controls, but growth slowed after 3 months of age in all groups. Maximal enzyme activities of cytochrome c oxidase (COX), citrate synthase (CS), hydroxyacyl-coa dehydrogenase (HOAD), pyruvate kinase (PK) and lactate dehydrogenase (LDH) were measured across a range of assay temperatures (22, 27, 32 and 36°C) in adults from each T_E group that were acclimated to 27 or 32°C. Substrate affinities (K_m) were also determined for COX and LDH. In adult fish acclimated to 27°C, COX and PK activities were higher in 22°C and 32°C T_E groups than in 27°C T_E controls, and the temperature optimum for COX activity was higher in the 32°C T_E group than in the 22°C T_E group. Warm acclimation reduced COX, CS and/or PK activities in the 22 and 32°C T_E groups, possibly to compensate for thermal effects on molecular activity. This response did not occur in the 27°C T_E controls, which instead increased LDH and HOAD activities. Warm acclimation also increased thermal sensitivity (Q_{10}) of HOAD to cool temperatures across all T_E groups. We conclude that the temperature experienced during early development can have a persistent impact on energy metabolism pathways and acclimation capacity in later life.

KEY WORDS: Developmental plasticity, Phenotypic plasticity, Thermal performance curve, Acclimatization, Muscle phenotype

INTRODUCTION

Environmental temperature has a profound influence on the biological function of ectothermic organisms. The survival and performance of populations are limited to a finite range of temperatures, the breadth of which is a crucial determinant of distribution and abundance (Angilleta, 2009; Schulte et al., 2011). Thermal optima and performance breadth can be altered by temperature acclimatization, and although the underlying mechanisms have been studied for decades (Fry and Hart, 1948), research interest has intensified of late

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in an effort to understand the potential ecological impacts of global climate change (Pörtner and Farrell, 2008; Somero, 2011). However, much of what we know about the effects of temperature on ectothermic vertebrates comes from studies of juveniles and adults. There has been relatively little emphasis on how temperature affects early life stages and how temperature can interact with the developmental programme to alter phenotypic plasticity in later life (Beldade et al., 2011; Overgaard et al., 2011).

Adjustments in the metabolic pathways that dictate energy production are important for temperature acclimation in adult ectotherms. Cold acclimation increases mitochondrial abundance and/or oxidative capacity in muscle and other tissues in a variety of fish species (e.g. Johnston and Maitland, 1980; Egginton and Sidell, 1989; McClelland et al., 2006; Orczewska et al., 2010). This response is thought to improve cellular function by counteracting the kinetic effects of temperature on the catalytic rates of mitochondrial enzymes or on the rates of oxygen/metabolite diffusion (Hubley et al., 1997; Guderley, 2004; Kinsey et al., 2011; O'Brien, 2011). However, the presence and magnitude of metabolic temperature acclimation in adult fish depends on the magnitude of temperature change and can vary between populations and species (Bremer and Moyes, 2011; Dhillon and Schulte, 2011).

Our recent work suggests that the thermal environment experienced in early life can have a profound influence on exercise performance (Scott and Johnston, 2012). We found that embryonic temperature (T_E) affected prolonged swimming performance (critical swimming speed, U_{crit}) in adult zebrafish in response to a short-term thermal challenge, such that fish raised at a particular T_E had reduced thermal sensitivity at its respective T_E. Similarly, U_{crit} after a prolonged period of warm acclimation was higher in fish raised at warmer T_E than in those raised at a cold T_E. The overall effects of developmental temperature on performance at warm temperatures therefore appear to be consistent with the beneficial acclimation hypothesis (Huey et al., 1999). In contrast, U_{crit} after cold acclimation was highest in fish raised at low and high T_E, and lowest in fish raised at intermediate T_E. The effect of T_E on cold acclimation was based on substantial variation in the expression of several metabolic genes in the swimming muscle (detected using high-throughput mRNA sequencing, RNA-Seq), but the mechanisms of the observed effects of T_E on short-term thermal plasticity and warm acclimation capacity was unclear.

The objectives of this study were to determine: (1) the thermal breadth of embryonic development in zebrafish [*Danio rerio* (Hamilton 1822)] and (2) how developing at the extremes of this range affects the capacity of energy metabolism pathways and the temperature acclimation response in the swimming muscle of adult fish. Zebrafish are well-suited to this study for a number of reasons. Zebrafish are native to slow-flowing rivers and streams in the Ganges and Brahmaputra River basins in southern Asia, where daily temperatures typically fluctuate by ~5°C and can range from as low as 6°C in winter to as high as ~38°C in summer (Spence et al., 2008;

List of symbols and abbreviations

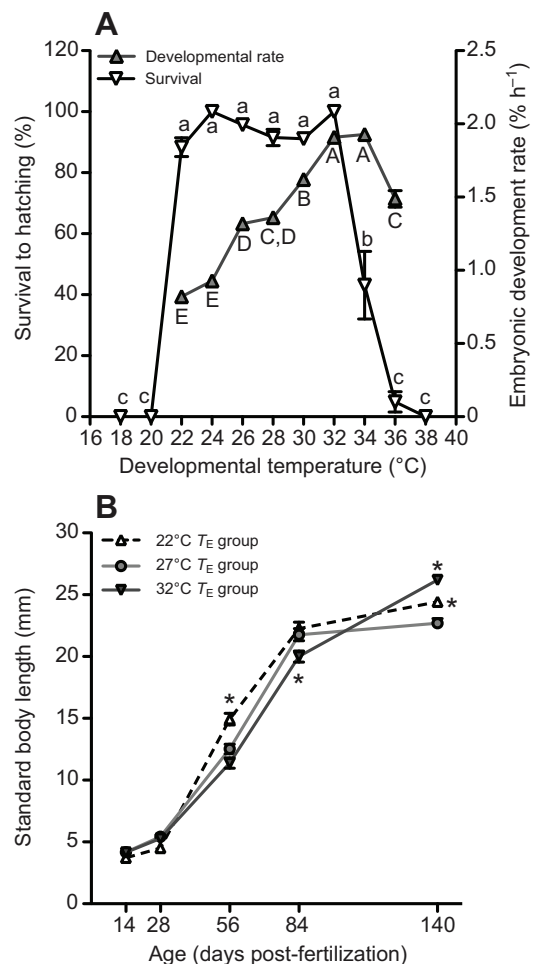|	
COX	cytochrome c oxidase
CS	citrate synthase
Cytc[Fe^{2+}]	reduced cytochrome c
HOAD	3-hydroxyacyl-coA dehydrogenase
K_m	substrate affinity
LDH	lactate dehydrogenase
PK	pyruvate kinase
Q_{10}	temperature coefficient
T_A	acclimation temperature
T_a	assay temperature
T_E	embryonic temperature
T_{opt}	temperature optimum
U_{crit}	critical swimming speed
V_{max}	maximal enzyme activity

Fig. 1. The effects of embryonic temperature on development and growth in zebrafish. (A) Temperature had a significant overall effect on the proportional survival to hatching ($F_{10,48}$=83.76, P<0.001) (assessed using one-factor ANOVA), and there was high survival from 22 to 32°C. Temperature also had a significant overall effect on the rate of embryonic development, expressed as a percentage of development to hatching ($F_{10,48}$=638.51, P<0.001). Data are means ± s.e.m. for replicate culture dishes (n=3–7 dishes; see Materials and methods). Groups with different letters were significantly different in Bonferroni pairwise comparisons (uppercase letters refer to comparisons of developmental rate data; lowercase letters refer to comparisons of survival data). (B) Embryonic temperature (T_E) had a persistent effect on growth after hatching, as reflected by a significant T_E effect ($F_{2,568}$=6.42, P=0.0018) and T_E × age interaction ($F_{8,568}$=6.42, P<0.0001). Asterisks represent a significant difference from the 27°C T_E group in Bonferroni pairwise comparisons.

López-Olmeda and Sánchez-Vázquez, 2011). Natural populations typically reproduce during the monsoon season, when food is readily available and temperatures range from ~23 to 31°C (Spence et al., 2008). We tested two hypotheses that could explain our previous observations on the effects of embryonic temperature on swimming performance (Scott and Johnston, 2012) based on differences in muscle enzyme function: (1) that embryonic temperature would shift the thermal optima of metabolic enzyme activity towards the T_E, and (2) that development at a low embryonic temperature would blunt the warm acclimation response of metabolic enzymes. We focused our efforts on enzymes that reflect the flux capacity of oxidative phosphorylation [cytochrome c oxidase (COX)], the citric acid cycle [citrate synthase (CS)], glycolysis [pyruvate kinase (PK) and lactate dehydrogenase (LDH)] and fatty acid oxidation [hydroxyacyl-coA dehydrogenase (HOAD)].

RESULTS

Thermal breadth of embryonic development

Embryonic development was successful from 22 to 32°C, as reflected by high proportional survival to hatching (≥88%) (Fig. 1A). Survival declined to ~43% at 34°C, and was negligible above 34°C and below 22°C. Temperature had a strong effect on the rate of embryonic development (Fig. 1A), which increased 2.33-fold from 22 to 32°C (i.e. Q_{10}=2.33). Developmental rate was highest from 32 to 34°C, beyond which the rate dropped.

Effects of T_E on growth persisted into later life, even though larvae were transferred to a common 27°C at hatching. Compared with fish raised at 27°C as embryos, fish raised at 22°C tended to be larger (as reflected by standard body length) and fish raised at 32°C tended to be smaller at 8 and 12 weeks after hatching (Fig. 1B). There were comparable differences in standard length and body mass in the separate group of fish that were sampled for enzyme measurements at 3 months after fertilization (Table 1) (see below). Standard length changed very little after 12 weeks of age in the 22°C and 27°C T_E groups, but the 32°C T_E group still exhibited significant growth beyond this age.

Effects of T_E on the activity of metabolic enzymes

We examined the effects of T_E values that represent the cold (22°C) and warm (32°C) extremes for normal development, relative to near-optimal thermal conditions (27°C). T_E had persistent effects on the capacity of energy metabolism pathways in the muscle of adult zebrafish that were raised at a common 27°C after hatching, as reflected by differences in maximal enzyme activities assayed at 27°C (Fig. 2A). The maximal activities of COX and PK were significantly higher in both the 22°C and 32°C T_E groups than in the 27°C T_E

controls. The activities of CS and HOAD were higher in the 22°C T_E group than in the 27°C and 32°C T_E groups. However, there were differences in body mass and length between embryonic temperature groups (Fig. 1B, Table 1), and we observed a significant positive effect of body mass on the activities of all enzymes except for PK (Fig. 2B, Table 2). Therefore, the residuals from these regressions were also used to compare T_E groups, the results of which suggested that the differences in COX and PK activity between T_E groups were independent of variation in body mass (Table 3). In contrast, there was no significant variation in body mass residuals for CS and HOAD, suggesting that the differences between T_E groups for these enzymes were caused largely by variation in body mass.

T_E also had persistent effects on the temperature optimum (T_{opt}) for COX activity in fish that were raised at 27°C and acclimated to

Table 1. Body size of fish sampled for enzyme measurements

	T_A (°C)	22°C T_E group	27°C T_E group	32°C T_E group
Body mass (mg)	27	210±13	168±13	141±17*
	32	187±21	173±11	132±6
Standard length (mm)	27	21.6±0.5	20.3±0.8	17.6±0.8*,**
	32	20.9±0.8	20.1±0.5	18.0±0.3*

T_A, acclimation temperature; T_E, embryonic temperature.
$N=10$ per treatment group. Degrees of freedom: T_A, 1; T_E, 2; $T_A \times T_a$, 2; error, 54. Two-factor ANOVA statistics for body mass were as follows: T_A, $F=0.618$, $P=0.435$; T_E, $F=9.257$, $P=0.0003$; $T_A \times T_a$, $F=0.479$, $P=0.622$. Two-factor ANOVA statistics for standard length were as follows: T_A, $F=0.0935$, $P=0.761$; T_E, $F=14.03$, $P<0.0001$; $T_A \times T_a$, $F=0.340$, $P=0.713$.
*,**Significant difference from 22°C or 27°C T_E group, respectively, in pairwise comparison ($P<0.05$).
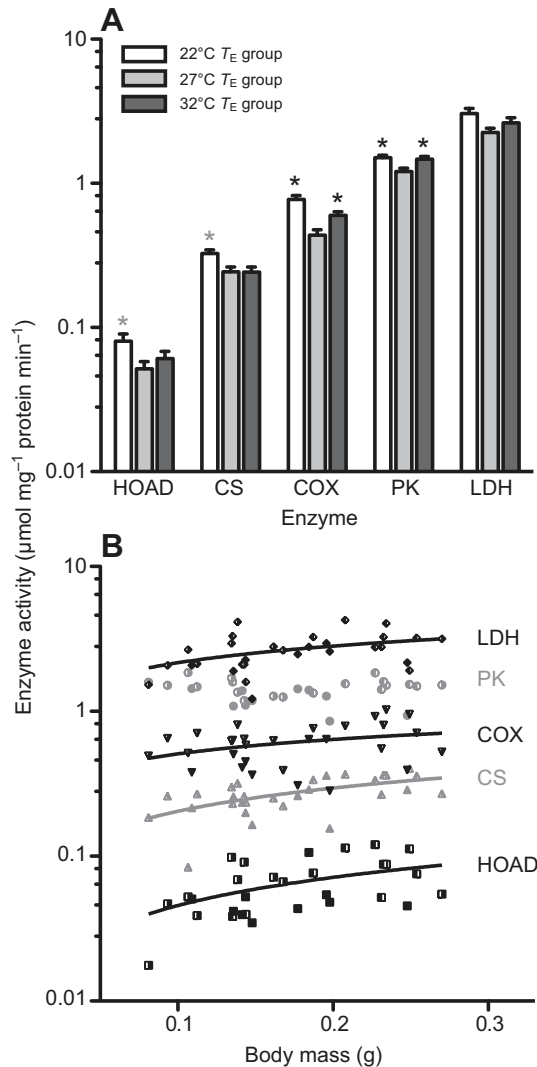

Fig. 2. Embryonic temperature (T_E) had a persistent effect on enzyme activities in the axial swimming muscle of adult zebrafish that were raised at 27°C after hatching. (A) There was a significant effect of T_E on the activities of hydroxyacyl-coA dehydrogenase (HOAD; $F_{2,27}=3.48$, $P=0.045$), citrate synthase (CS; $F_{2,27}=5.86$, $P=0.008$), cytochrome c oxidase (COX; $F_{2,27}=15.40$, $P<0.001$) and pyruvate kinase (PK; $F_{2,27}=6.62$, $P=0.005$), but not lactate dehydrogenase (LDH; $F_{2,27}=3.19$, $P=0.057$; assessed using one-factor ANOVA). Asterisks represent a significant difference from the 27°C T_E group in Bonferroni pairwise comparisons (only black asterisks were still significant after correcting for body mass; see Table 3). (B) With the exception of PK, there was a positive relationship between enzyme activity and body mass (see Table 2). The 22°C T_E group has only the left side of each symbol filled, the 27°C T_E group is completely filled, and the 32°C T_E group has only the right side of each symbol filled.

that temperature as adults. T_{opt} for maximal COX activity was 27°C in the 22°C T_E group but was 32°C in the 32°C T_E group, whereas activity in the 27°C T_E controls was similar across all assay temperatures (Fig. 3). In contrast, there were no distinct T_{opt} detected for the other enzymes assayed, as activity generally increased progressively with warming across the full range of assay temperatures (22, 27, 32 and 36°C). T_E also affected the substrate affinity (K_m) of COX activity, as the 22°C and 32°C T_E groups had lower K_m for reduced cytochrome c when assayed at 22°C than the 27°C T_E controls (Fig. 4).

Effects of T_E on thermal acclimation

T_E had persistent effects on the thermal acclimation response for all enzymes assayed. We did not use a residual approach to correct for body mass in the following comparisons with T_E groups, because there were no significant effects of acclimation temperature on body mass (Table 1). Warm acclimation at 32°C reduced maximal COX activity in the 22°C and 32°C T_E groups, but did not have a significant effect in the 27°C T_E controls (Fig. 3). COX K_m was not significantly affected by acclimation temperature, but it generally declined with increasing assay temperature (Table 4). Similar effects of T_E were observed for the acclimation response of maximal PK activity, as acclimation to 32°C reduced PK activity in the 22°C and 32°C T_E groups, but had no effect (trending towards an opposite effect) in the 27°C T_E controls (Fig. 5). Maximal CS activity was also reduced by acclimation to 32°C in the 22°C T_E group, but CS activity was not significantly affected by acclimation in the 27°C and 32°C T_E groups (Fig. 6).

In contrast to the above enzymes, for which the predominant effect of 32°C acclimation was a greater response in the 22°C and/or 32°C T_E groups, LDH activity was only affected by warm acclimation in the 27°C T_E controls. Maximal LDH activity increased substantially after acclimation to 32°C in the 27°C T_E controls, particularly at warmer assay temperatures, but was unaffected by acclimation in the 22°C and 32°C T_E groups (Fig. 7). This was not associated with any effects of T_E or acclimation temperature on the substrate affinity of LDH for pyruvate (Table 5). Maximal HOAD activity also increased in the 27°C T_E controls after acclimation to 32°C, but was not affected by acclimation in the 22°C and 32°C T_E groups (Fig. 8).

Thermal sensitivities of maximal enzyme activity, as reflected by temperature coefficients (Q_{10}), were not generally affected by T_E (Fig. 9). The exceptions were significant effects of T_E on Q_{10} for COX and PK in zebrafish acclimated to 27°C. In this regard, COX activity was less sensitive to cooler temperatures in the 27°C T_E controls than in the 22°C and 32°C T_E groups, concurrent with the lack of a definitive T_{opt} in the former group (Fig. 3). Interestingly, acclimation to 32°C increased the Q_{10} for HOAD activity between assay temperatures of 22 and 27°C, suggesting that warm

Table 2. Allometric scaling of metabolic enzyme activities in the muscle of zebrafish

	COX	CS	HOAD	PK	LDH
a (±s.e.m.)	1.09±0.36	0.70±0.16	0.20±0.08	1.27±0.23	5.23±1.45
b (±s.e.m.)	0.33±0.18	0.53±0.13	0.65±0.23	−0.05±0.10	0.38±0.16
R^2	0.102	0.375	0.242	0.010	0.185
SS	0.957	0.096	0.016	1.59	13.29

Maximal enzyme activities (V_{max} in µmol mg^{-1} min^{-1}) of fish acclimated to 27°C and assayed at 27°C were regressed to body mass (M_b) using an allometric equation ($V_{max}=aM_b{}^b$; 28 degrees of freedom).
COX, cytochrome c oxidase; CS, citrate synthase; HOAD, hydroxyacyl-coA dehydrogenase; PK, pyruvate kinase; LDH, lactate dehydrogenase; SS, sum of squares.

acclimation increased the sensitivity of the HOAD enzyme to cooler temperatures (bottom panels of Fig. 9).

Variation in muscle protein content (which was 39.0±0.5 mg protein per gram tissue overall) did not contribute to any of the results discussed above. There were no significant effects of T_E ($F_{2,54}=0.99$, $P=0.38$), acclimation temperature ($F_{1,54}=3.60$, $P=0.06$) or their interaction ($F_{2,54}=0.89$, $P=0.42$) in two-factor ANOVA. There was a slight tendency for muscle protein to be lower in 32°C-acclimated fish (38.2±0.6 mg g^{-1} overall) than in 27°C-acclimated fish (39.9±0.7 mg g^{-1} overall), but the magnitude of this non-significant effect was much lower than the significant effects of acclimation on the activities of many enzymes.

DISCUSSION
Our present findings suggest that the thermal conditions experienced during embryonic development have effects that persist into adulthood on the metabolic capacities of the muscle, the thermal optima of enzymes, and the thermal acclimation response. Zebrafish raised near the cold or warm extremes for successful development had higher COX and PK activities than fish raised at an intermediate ('optimal') temperature. T_E also had a positive association with the T_{opt} of COX. The acclimation response of mitochondrial oxidative capacity also appeared to be enhanced by development near the cold and/or warm extremes of embryonic survival, as reflected by stronger inverse relationships between acclimation temperature and the activities of mitochondrial (COX, CS) and glycolytic (PK) enzymes. The thermal environment experienced during early life may therefore have a strong influence on the physiological response to warming temperatures in later life.

Developmental plasticity of enzyme activities and thermal optima
Our finding that T_E had a persistent effect on the thermal optimum of COX activity, even after all fish were raised for several months

after hatching at 27°C, supports our first hypothesis. Enzyme activity is generally expected to be exponentially related to assay temperature (according to the Arrhenius equation) if the effects of temperature on enzyme activity are strictly due to thermodynamic effects on molecular movement (Schulte et al., 2011). This is somewhat reflected in the present study, insofar as the activity of most enzymes increased with increasing assay temperature. However, this was not the case for COX. The activity of this enzyme varied little across temperatures, differing by at most 30% across a 14°C range of assay temperatures and generally peaking between 27 and 32°C (Figs 3, 9). Similar patterns have been observed in some fish, amphibian and reptile species (Godiksen and Jessen, 2001; Glanville and Seebacher, 2006; Niehaus et al., 2011), but not in others (Caldwell, 1969; Blier and Lemieux, 2001). Furthermore, there were clear differences in thermal optima between the 22°C T_E group (T_{opt} of 27°C) and the 32°C T_E group (T_{opt} of 32°C). These findings provide some insight into the underlying mechanism of our previous observation that T_E influences the short-term thermal sensitivity of swimming performance (Scott and Johnston, 2012). For example, zebrafish raised at 22°C as embryos maintain higher swim speeds after transfer to 22°C than fish raised at 32°C, possibly in part because their COX enzyme was better suited to function at cooler temperatures.

T_E also had a persistent effect on the maximal activities of multiple enzymes in the muscle, even after fish were raised for several months after hatching at a common 27°C temperature. After correcting for the effects of variation in body mass, both the 22°C and 32°C T_E groups had higher activities of COX and PK than the 27°C T_E controls (Fig. 1, Table 3). These results were probably not caused by differences in fibre type composition of the muscle, because there are no differences between T_E groups in the total transverse area of either slow oxidative (red) or fast oxidative (pink, intermediate) fibres in the axial musculature at 27°C (Scott and Johnston, 2012). However, embryonic temperature does affect the

Table 3. Body mass residuals of muscle enzyme activities

	T_E (°C)			Results from one-factor ANOVA	
	22	27	32	F	P
COX	0.13±0.04*	−0.16±0.04	0.04±0.05*	11.49	<0.001
CS	0.024±0.012	−0.025±0.018	0.001±0.021	1.93	0.165
HOAD	0.007±0.007	−0.012±0.007	0.004±0.007	2.03	0.151
PK	0.13±0.06*	−0.19±0.06	0.06±0.06*	7.09	0.003
LDH	0.18±0.26	−0.37±0.14	0.19±0.20	2.46	0.105

COX, cytochrome c oxidase; CS, citrate synthase; HOAD, hydroxyacyl-coA dehydrogenase; PK, pyruvate kinase; LDH, lactate dehydrogenase; T_E, embryonic temperature.
Residuals (means ± s.e.m. are shown, $n=10$ per treatment group) were calculated from the allometric regressions of enzyme activity (V_{max} in µmol mg protein^{-1} min^{-1}) to body mass that are shown in Fig. 2B and Table 2.
*Significant difference from 27°C T_E group ($P<0.05$).
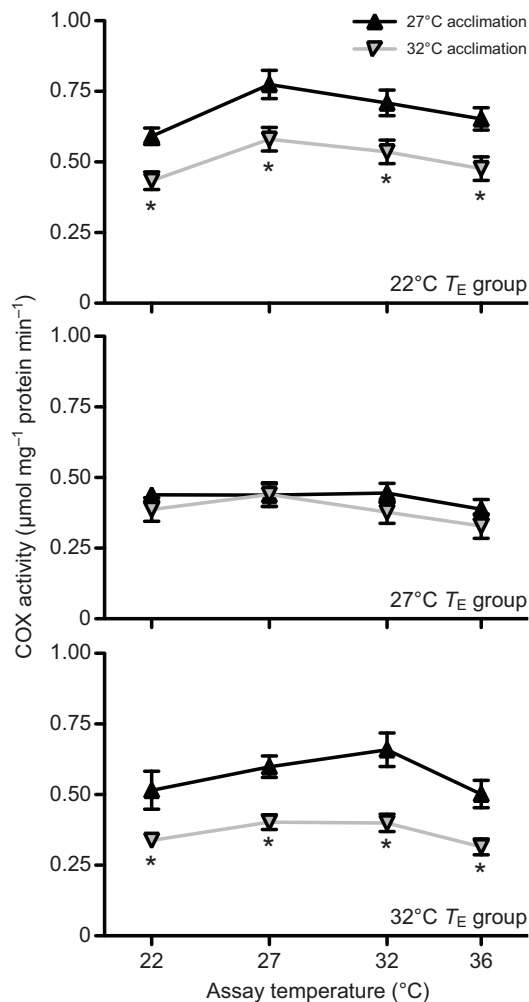Degrees of freedom in ANOVA: T_E, 2; error, 27.

Fig. 3. Embryonic temperature (T_E) affected the acclimation response of cytochrome c oxidase (COX) in the muscle, as well as the temperature dependence of its activity. Acclimation to a warmer temperature reduced COX activity in the 22°C T_E group ($F_{1,72}=37.10$, $P<0.001$) and the 32°C T_E group ($F_{1,72}=45.42$, $P<0.001$), but did not affect activity in the 27°C T_E group ($F_{1,72}=2.74$, $P=0.103$; assessed using two-factor ANOVA). The optimal temperature for high COX activity was generally lower in the 22°C T_E group than in the 32°C T_E group. Asterisks represent a significant difference between acclimation temperatures, within an assay temperature, in Bonferroni pairwise comparisons ($n=10$ per treatment group).

Fig. 4. Embryonic temperature (T_E) affected the kinetics of cytochrome c oxidase (COX) in the muscle for its substrate, reduced cytochrome c. Kinetics are represented by substrate affinity (K_m), the concentration of reduced cytochrome c that supports 50% of maximal activity. There was a significant interaction between T_E and assay temperature (T_a) on K_m in fish acclimated to 27°C ($F_{6,108}=2.36$, $P=0.035$), which are shown in the figure. There was also a significant main effect of T_E on K_m in fish acclimated to 32°C ($F_{2,108}=4.91$, $P=0.009$; data shown in Table 4). The asterisk represents a significant difference between the 27°C T_E group and both other T_E groups in Bonferroni pairwise comparisons ($n=10$ per treatment group).

sampled at 4 months of age, when there were small differences in body size between T_E groups (Table 1) and there was allometric variation in activity for most enzymes studied (Fig. 2). Accounting for this variation using a residual approach (Table 3) suggested that differences in size per se (but not necessarily instantaneous growth rate) did not cause the differences in COX and PK activities between T_E groups.

The persistent effects of T_E on muscle enzyme activities could affect the overall metabolism of the animal. The axial muscle powers swimming and makes up a large proportion of body mass, so the metabolic processes of this tissue will have a strong influence on whole-body metabolic rates. Previous work has shown that developmental temperature affects resting oxygen consumption in larval zebrafish (Barrionuevo and Burggren, 1999) as well as resting and maximal oxygen consumption in larval cyprinids (Wieser and Forstner, 1986; Kaufmann and Wieser, 1992). These observations should be partly due to the well-known kinetic effects of temperature on reaction rates, but could also result from effects on the various physiological factors that control metabolism. Metabolic rate and oxygen consumption are system-level processes that are influenced by all steps in the pathway of oxygen and fuel delivery and oxidation, including the activity of metabolic enzymes and the abundance of mitochondria (Suarez and Moyes, 2012). In this regard, T_E has been shown to alter mitochondrial abundance in the muscle of larvae from several fish species (Vieira and Johnston, 1992; Brooks and Johnston, 1993). Although we did not measure mitochondrial abundance, it is often inferred from COX activity (Duggan et al., 2011). T_E may therefore have persistent effects on whole-animal energy metabolism by altering COX activity and mitochondrial abundance in the muscle.

Developmental plasticity of metabolic temperature acclimation
Our observation that the warm acclimation response of metabolic enzymes was enhanced by development near the cold and/or warm extremes of embryonic survival did not support our second hypothesis. We found in previous work that both 27°C and 32°C T_E groups swam better after acclimation to 34°C than the 22°C T_E

size of both red fibres (Scott and Johnston, 2012) and fast glycolytic (white) fibres (Johnston et al., 2009) in zebrafish: fibres tend to be larger but fewer in number in fish raised at 22°C or 31–32°C as embryos compared with fish raised at 26–27°C. The observation that muscle COX and PK activities are higher in concert with an increase in muscle fibre size (but not total area) in the 22°C and 32°C T_E groups provides correlative evidence that there may be metabolic consequences for the often observed effect of T_E on muscle cellularity (Vieira and Johnston, 1992; Rowlerson and Veggetti, 2001; Stoiber et al., 2002; McClelland and Scott, 2013).

The extent to which T_E affected enzyme activities through long-term alterations in muscle growth phenotype is unclear. The T_E treatments occurred at a time of rapid and significant development and growth of the muscle (Hinterleitner et al., 1989; Johnston, 2006; McClelland and Scott, 2013), but impacts persisted into adulthood when growth rate had slowed (Fig. 1). Nevertheless, zebrafish were
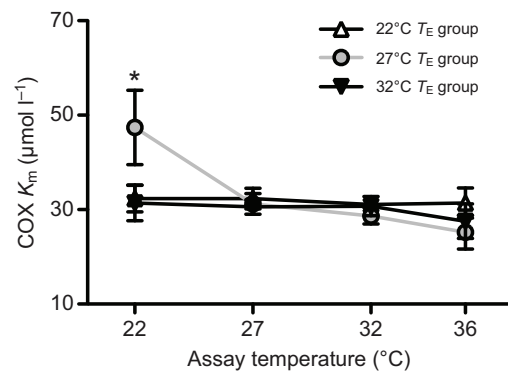
Table 4. Substrate affinity (K_m) for cytochrome c oxidase

T_A (°C)	T_a (°C)	22°C T_E group	27°C T_E group	32°C T_E group
27	22	32.4±2.8	47.4±7.9	31.4±3.7
	27	32.4±2.2	31.2±2.2	30.6±1.5
	32	31.1±1.4	28.7±1.7	30.7±2.0
	36	31.4±3.2	25.2±3.6	27.6±3.7
32	22	40.1±5.3	52.9±7.5	39.2±6.3
	27	34.0±1.6	39.9±1.9	31.9±2.0
	32	29.1±1.6	32.0±2.3	27.8±2.5
	36	24.6±3.3	26.3±4.0	18.3±2.5
Results from two-factor ANOVA within each T_E group (F, P)				
	T_A	0.006, 0.941	2.09, 0.153	0.103, 0.749
	T_a	2.98, 0.037	10.95, <0.001	4.75, 0.005
	$T_A \times T_a$	2.16, 0.100	0.257, 0.856	2.29, 0.086

K_m is the concentration of reduced cytochrome c that supports 50% of maximal activity (in units µmol l^{-1}).
T_E, embryonic temperature; T_A, acclimation temperature; T_a, assay temperature.
Degrees of freedom in ANOVA: T_A, 1; T_a, 3; $T_A \times T_a$, 3; error, 72. N=10 per treatment group.
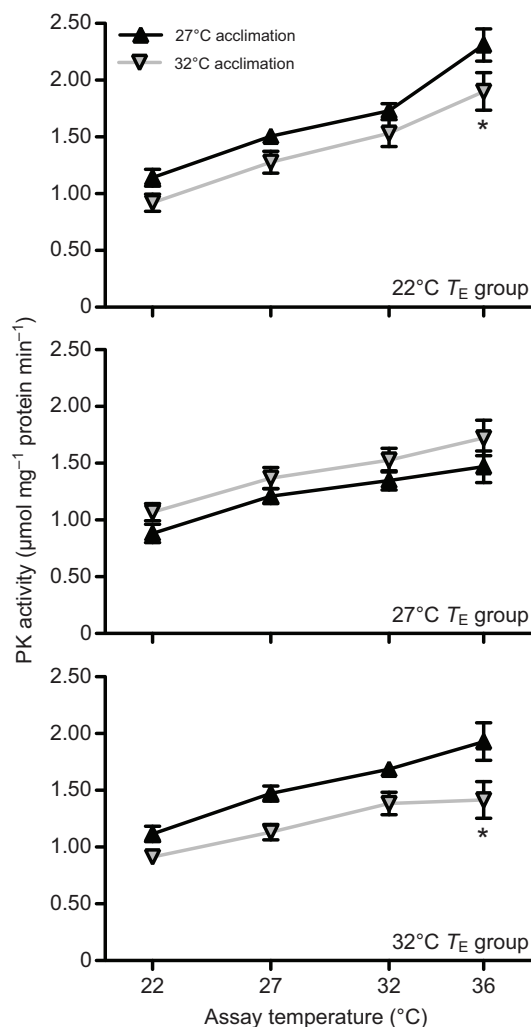

Fig. 5. Embryonic temperature (T_E) affected the acclimation response of pyruvate kinase (PK) activity in the muscle. Acclimation to a warmer temperature reduced PK activity in the 22°C T_E group ($F_{1,72}$=12.60, P<0.001) and the 32°C T_E group ($F_{1,72}$=22.02, P<0.001), but increased activity in the 27°C T_E group ($F_{[1,72]}$=6.91, P=0.011; assessed using two-factor ANOVA). Activity increased with assay temperature in all T_E groups (P<0.001). Asterisks represent a significant difference between acclimation temperatures, within an assay temperature, in Bonferroni pairwise comparisons (n=10 per treatment group).

group (Scott and Johnston, 2012), and had hypothesized here that this would be explained by an enhanced metabolic warm acclimation response in the 27°C and 32°C T_E groups. The discrepancy could reflect the effects of the small differences in acclimation temperature used between studies (32°C here versus 34°C previously). However, it is also possible that the 27°C T_E group uses an alternative but still effective strategy for acclimating to warm temperatures, such as increasing the capacity for anaerobic glycolysis (Fig. 7). Consistent with this idea, carbohydrate content of the muscle increases in response to 34°C acclimation in zebrafish raised under control conditions, but has no effect on total muscle lipid stores (Vergauwen et al., 2010). Differences between T_E groups in other processes that are important for warm acclimation, such as the induction of heat shock proteins (Vergauwen et al., 2010), may also contribute to the observed differences in performance.

Even though our second hypothesis was not supported, our current findings are generally consistent with our previous observation that T_E has a persistent effect on thermal acclimation capacity (Scott and Johnston, 2012). For example, we showed that the cold acclimation response for U_{crit}, slow oxidative fibre density and/or mRNA expression of metabolic genes were enhanced in both 22°C and 32°C T_E groups compared with the 27°C control T_E group. This observation is mirrored in the present study in that many of the metabolic responses to warm acclimation in the 22°C and 32°C T_E groups were similar and enhanced compared with the 27°C control T_E group.

The cellular mechanisms underlying developmental thermal plasticity are not well understood. It has been suggested that T_E alters the abundance and/or the balance between proliferation and differentiation of myogenic progenitor cells in the muscle (Johnston et al., 2003; Steinbacher et al., 2011). Having more undifferentiated myoblasts in the muscle should influence the potential for muscle plasticity during thermal acclimation, because it would affect the ability to recruit new muscle fibres or add nuclei to existing muscle fibres (Johnston, 2006). This could help explain the results of the present study, because the nuclear content of muscle is an important determinant of metabolic enzyme expression (Dalziel et al., 2005). Fibre-type composition of the muscle can change in response to thermal acclimation (Johnston and Lucking, 1978), and this could have differed between T_E groups, possibly related to the presence of myogenic progenitor cells. T_E also changes the developmental expression of muscle transcription factors (e.g. myogenic regulatory factors) (Macqueen et al., 2007), and could regulate genes important for muscle phenotype and plasticity through epigenetic mechanisms (Johnson and Tricker, 2010).
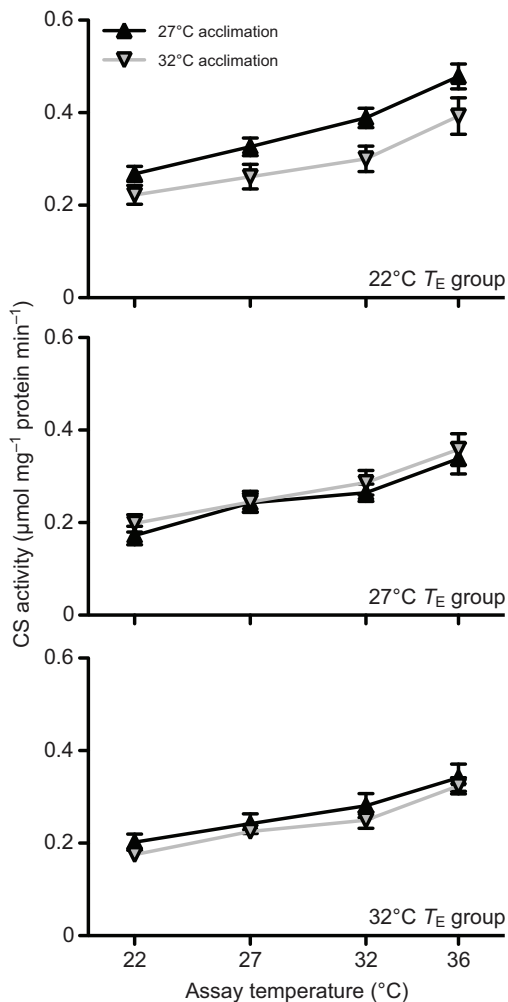
Fig. 6. Embryonic temperature (T_E) affected the acclimation response of citrate synthase (CS) activity in the muscle. Acclimation to a warmer temperature reduced CS activity in the 22°C T_E group ($F_{1,72}$=15.50, P<0.001), but had no significant effect in the 27°C T_E group ($F_{1,72}$=0.96, P=0.329) or the 32°C T_E group ($F_{1,72}$=2.711, P=0.104; assessed using two-factor ANOVA). Activity increased with assay temperature in all T_E groups (P<0.001; n=10 per treatment group).

Fig. 7. Embryonic temperature (T_E) affected the acclimation response of lactate dehydrogenase (LDH) activity in the muscle. Acclimation to a warmer temperature increased LDH activity in the 27°C T_E group ($F_{1,72}$=32.52, P<0.001), but had no significant effect in the 22°C T_E group ($F_{1,72}$=0.002, P=0.962) or the 32°C T_E group ($F_{1,72}$=0.008, P=0.927; assessed using two-factor ANOVA). There was an overall increase in activity with assay temperature in all T_E groups (P<0.001). Asterisks represent a significant difference between acclimation temperatures, within an assay temperature, in Bonferroni pairwise comparisons (n=10 per treatment group).

Changes in the lipid composition of mitochondrial membranes could act in concert with the changes observed here to mediate the effects of thermal acclimation and developmental plasticity on metabolic capacity in the muscle. Temperature acclimation has a well-known effect on the lipid composition of membranes (Hazel, 1995), and can thus affect the activity of membrane-bound enzymes (Seebacher et al., 2010). The effect of T_E on the changes in membrane composition during thermal acclimation is not explicitly known, but it is intriguing that the most prominent differences between T_E groups were observed for COX (an enzyme normally located in the inner mitochondrial membrane). Detergents are used in the COX assay to minimize the direct effects of membrane lipids on the measured activity, but it is possible that some of the variation observed was caused by differences in the impact of residual lipids that were not removed. Alternatively, COX activity may be altered in response to acclimation temperature and/or T_E in response to changes in the membrane-lipid microenvironment (Frick et al., 2010; Martin et al., 2013).
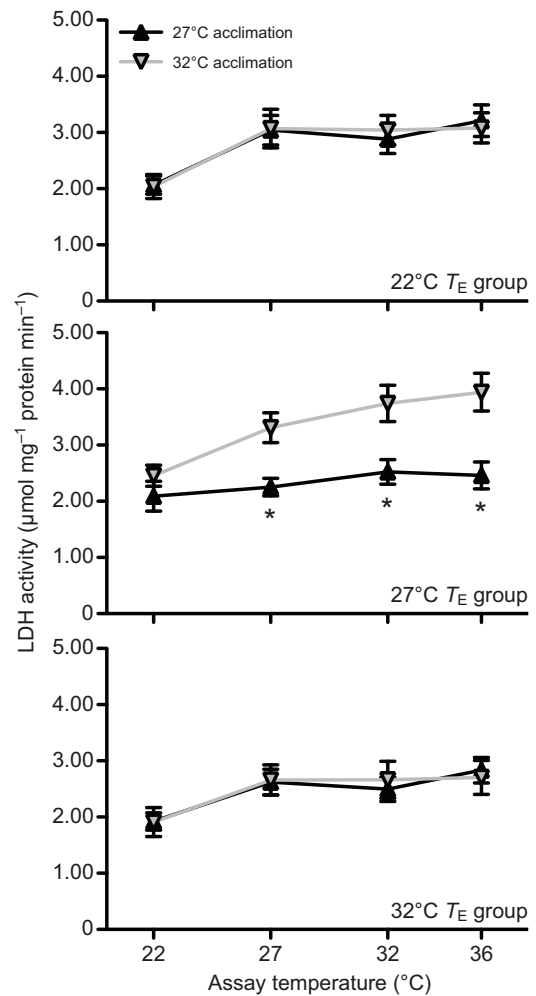
Although there were few effects of T_E on the temperature sensitivity of enzymes (with the exception of COX, see above), warm acclimation changed the temperature sensitivity of HOAD in all T_E groups. The Q_{10} of HOAD activity between assay temperatures of 22 and 27°C increased from ~2.7 in 27°C-acclimated fish to ~3.6 in 32°C-acclimated fish (Fig. 9). The cause of this increase in the sensitivity of HOAD to cold is unclear, but it could result from a change in the predominant isoform that is expressed in the muscle.

Thermal breadth of embryonic development

The high embryonic survival we observed between 22 and 32°C is consistent with previous reports that cumulative hatching success was lower in zebrafish after embryonic development at 21°C (~60%) compared with 26 and 33°C (~80%) (Hallare et al., 2005). Our results agree with the general expectation that there are sharp transitions in survival at the upper and lower thermal limits for

Table 5. Substrate affinity (K_m) for lactate dehydrogenase

T_A (°C)	T_a (°C)	22°C T_E group	27°C T_E group	32°C T_E group
27	22	41.4±3.7	51.1±2.9	58.1±7.4
	27	126.0±15.1	104.1±13.4	116.7±10.7
	32	86.8±12.7	88.6±7.9	76.9±5.7
	36	116.7±16.5	91.5±7.5	119.5±8.9
32	22	45.7±5.4	45.0±3.7	54.4±9.8
	27	119.2±13.3	113.9±12.3	126.7±24.4
	32	89.6±7.9	91.8±7.9	88.5±10.2
	36	109.0±14.5	109.5±7.6	104.2±6.0
Results from two-factor ANOVA within each T_E group (F, P)				
	T_A	0.048, 0.827	1.04, 0.311	0.007, 0.935
	T_a	17.31, <0.001	19.76, <0.001	12.66, <0.001
	$T_A \times T_a$	0.137, 0.938	0.702, 0.554	0.580, 0.630

K_m is the concentration of pyruvate that supports 50% of maximal activity (in units µmol l^{-1}).
T_E, embryonic temperature; T_A, acclimation temperature; T_a, assay temperature.
Degrees of freedom in ANOVA: T_A, 1; T_a, 3; $T_A \times T_a$, 3; error, 72. N=10 per treatment group.
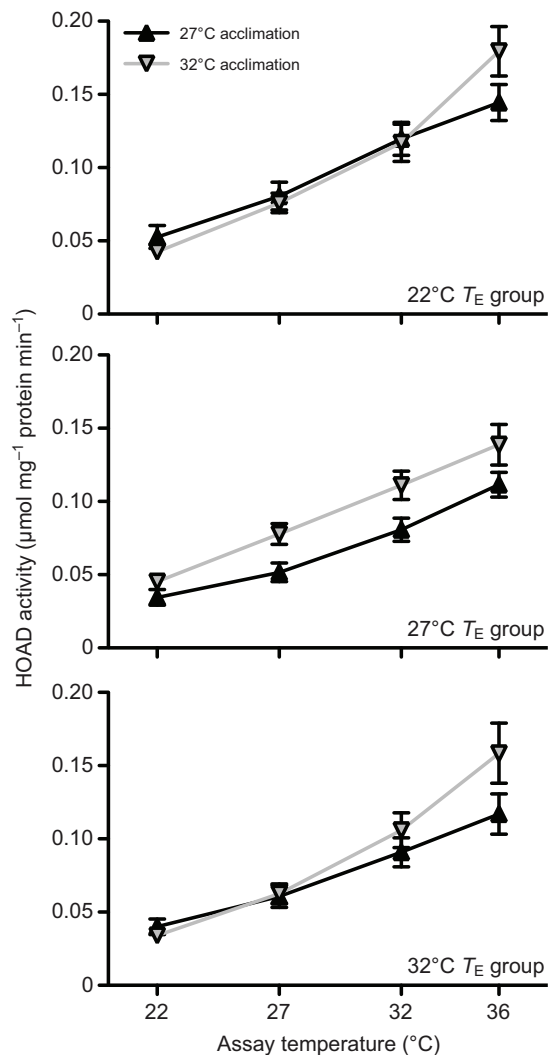

Fig. 8. Embryonic temperature (T_E) affected the acclimation response of hydroxyacyl-coA dehydrogenase (HOAD) activity in the muscle. Acclimation to a warmer temperature increased HOAD activity in the 27°C T_E group ($F_{1,72}$=15.94, P<0.001), but had no significant effect in the 22°C T_E group ($F_{1,72}$=0.329, P=0.568) or the 32°C T_E group ($F_{1,72}$=2.775, P=0.100; assessed using two-factor ANOVA). Activity increased with assay temperature in all T_E groups (P<0.001; n=10 per treatment group).

development, and that these thermal limits lie well within the tolerance range of adults (Angilleta, 2009). Abrupt thermal limits for development have been suggested to occur at the temperatures when cell division is no longer possible, caused by reversible effects of temperature (rather than irreversible effects, such as denaturation) on the activity of transcriptional enzymes and cell cycle proteins (van der Have, 2002), but empirical evidence for this suggestion is lacking. Temperatures within the survivable range did not lead to any noticeable increases in the proportion of developmental abnormalities, indicating that there is some robustness in the developmental programme to moderate temperature challenges (Braendle and Félix, 2008; Frankel et al., 2010). Temperature had a typical Q_{10} effect on full embryonic development between 22 and 32°C, consistent with previous observations on the rate of somitogenesis (a process that occurs during the segmentation period between roughly 15 and 30% of development to hatching) (Schröter et al., 2008).

Conclusions

Developmental plasticity in response to temperature can have striking and persistent effects on metabolic pathways, muscle phenotype and exercise performance in ectothermic organisms. We have shown here that T_E influences the capacity for metabolic temperature acclimation and the thermal sensitivity of oxidative enzymes in zebrafish. However, we still know relatively little about the underlying mechanisms of developmental thermal plasticity, and what influence it might have on the success of different species as climate change progresses (Beldade et al., 2011). Understanding the capacity of different organisms for both developmental and adult phenotypic plasticity will be essential to appreciate which species and life stages are most sensitive to thermal changes in the environment.

MATERIALS AND METHODS
Study animals

The zebrafish (*D. rerio*) used in this study were an outbred stock purchased from a tropical fish wholesaler (Aquality; Mississauga, ON, Canada). Two independent breeding groups, each containing eight males and eight females, were established for use in all experiments. These fish were maintained in a semi-recirculating system at 27–28°C (12 h:12 h light:dark photoperiod) and were fed three times per day with an alternating diet of tropical flake food and fresh brine shrimp. Three series of experiments were performed to determine (1) the thermal breadth of embryonic development, (2) the effects of embryonic temperature on post-hatching growth and (3) the effects of T_E
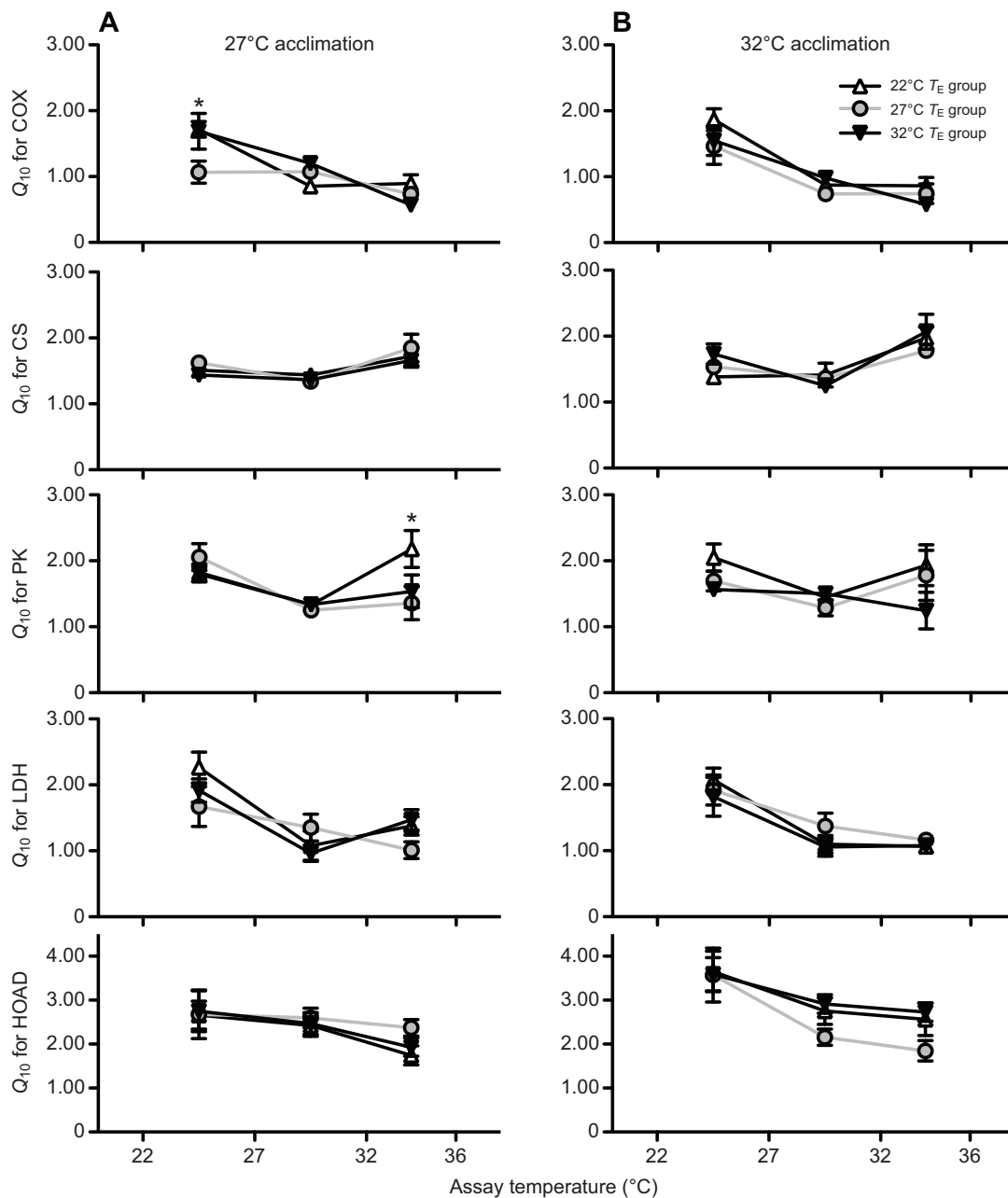
Fig. 9. The effects of embryonic temperature (T_E) on the temperature sensitivity of metabolic enzymes in the muscle. Temperature coefficients (Q_{10}; the fold-change in activity for a 10°C increase) were calculated between assay temperatures (T_a; see Materials and methods). T_E affected Q_{10} in zebrafish acclimated to 27°C (but not in those acclimated to 32°C) for cytochrome c oxidase (COX) and pyruvate kinase (PK), but there was no effect of T_E on Q_{10} for citrate synthase (CS), hydroxyacyl-coA dehydrogenase (HOAD) or lactate dehydrogenase (LDH). Acclimation to a warmer temperature increased Q_{10} for HOAD between assay temperatures of 22 and 27°C, reflecting an increased sensitivity to cooler temperatures (n=10 per treatment group).

on metabolic enzymes. Embryos from a minimum of two spawnings from each family were collected at the 1–8 cell stage and raised in Petri plates (50 embryos in 50 ml per plate) using standard protocols (Westerfield, 2007).

Experiment 1: thermal breadth of embryonic development
Embryos were raised at each of a range of temperatures (every 2°C from 18 to 38°C). The average developmental stage of each plate and the proportion of dead or abnormally developing embryos were recorded every 12 h (starting at 12 h post-fertilization) until all surviving embryos had hatched. The stage of embryonic development was scored from 0 (i.e. fertilization) to 100% using the expected temporal progression of zebrafish development at 28.5°C as a reference (Kimmel et al., 1995).

Experiment 2: effects of T_E on post-hatch growth
Embryos were raised at each of three temperatures (±0.5°C) that represent near-optimal (27°C), cold-extreme (22°C) and warm-extreme (32°C) temperatures for normal development. After hatching, larvae were maintained at a common temperature of 27±0.5°C in small tanks. Larvae were fed several times a day a mix of commercial larva food, spirulina and brine shrimp larvae (as appropriate). Fish were then transferred from static conditions to the semi-recirculating system at roughly 1 month of age, and were then gradually transitioned onto the same feeding routine as the parental groups. Standard body length was measured for a random sample of 26 to 64 fish from each embryonic temperature treatment group at 2, 4, 8, 12 and 20 weeks post-fertilization. This was accomplished by lightly

anaesthetizing the fish in buffered MS-222 ($0.08\,\mathrm{g\,l^{-1}}$) and then imaging each individual under a stereomicroscope.

Experiment 3: effects of T_E on enzyme activities

Embryos were raised as in Experiment 2, except that they were not anaesthetized and measured during development. Once fish reached 3 months of age they were acclimated to either 27 or 32°C for 30 days. Temperature acclimation treatments were conducted in static 50 l glass tanks. Tank water was filtered and aerated, and optimal temperatures (±0.5°C) were maintained using aquarium heaters. Approximately one-third of the tank volume was replaced with fresh dechlorinated tap water at the appropriate temperature at least twice a week to maintain water quality (nitrate, nitrite, ammonia and pH). Fish were euthanized (immersion in buffered MS-222; $0.8\,\mathrm{g\,l^{-1}}$), weighed and measured, and then sampled at the end of the 30 day acclimation period. The majority of both the red and white axial muscle was isolated altogether by removing the head, skin, fins and organs. Muscle samples were quickly immersed in liquid nitrogen and then stored at −80°C.

Enzyme assays

The frozen muscle samples were weighed and homogenized on ice in 20 volumes of homogenization buffer (concentrations in $\mathrm{mmol\,l^{-1}}$: 20 Hepes, 1 EDTA and 0.1% Triton-X-100) at pH 7.0 (this and all subsequent pH values refer to measurement at 20°C), and were re-frozen and stored at −80°C until assayed. The maximal activities ($V_{\max}$) in muscle homogenates of CS, HOAD and PK were assayed at each of four assay temperatures (22, 27, 32 and 36°C) by measuring absorbance at 340 nm (HOAD and PK) or 412 nm (CS) for 5 min. Assays were performed under the following conditions (in $\mathrm{mmol\,l^{-1}}$): CS, 50 Tris, 0.5 oxaloacetate, 0.3 acetyl-coa, 0.1 5,5-dithiobis-2-nitrobenzoic acid, pH 8.0; HOAD, 50 imidazole, 0.1 acetoacetyl-coA, 0.3 NADH, pH 7.2; PK, 50 MOPS, 5 phosphoenolpyruvate, 0.15 NADH, 5 ADP, 0.01 fructose-1,6-bisphosphate, 100 KCl, 10 $MgCl_2$, excess coupling enzyme (lactate dehydrogenase), pH 7.4. Preliminary experiments verified that substrate concentrations were saturating for all of the above assays.

The activity of COX and LDH were measured for several different starting concentrations of substrate at each of the four assay temperatures. COX activity was assayed with 100, 50, 25 or $10\,\mathrm{\mu mol\,l^{-1}}$ of reduced cytochrome c ($\mathrm{Cytc[Fe^{2+}]}$) in $50\,\mathrm{mmol\,l^{-1}}$ Tris (containing the desired cytochrome c concentration and 0.5% Tween-20, pH 8.0) by measuring absorbance at 550 nm for 5 min. Cytochrome c was reduced in the assay buffer using sodium dithionite (Spinazzi et al., 2012), and the latter was then oxidized by bubbling the buffer with air for at least 2 h on ice. The background oxidation rate of reduced cytochrome c was negligible. LDH activity was assayed in buffer containing 1000, 500, 250, 100, 50 or $10\,\mathrm{\mu mol\,l^{-1}}$ of pyruvate, $0.15\,\mathrm{mmol\,l^{-1}}$ NADH and $50\,\mathrm{mmol\,l^{-1}}$ Hepes (pH 7.0) by measuring absorbance at 340 nm for 5 min. The reported $V_{\max}$ is the highest empirical measurement of activity at each assay temperature. Substrate affinity (K_m) was determined by interpolating the substrate concentration at 50% of the empirically determined $V_{\max}$ value.

Enzyme activities of each individual were measured in triplicate on a SpectraMax Plus 384 microplate reader with temperature control (Molecular Devices, Sunnyvale, CA, USA). COX was assayed after a single freeze–thaw cycle, and the remaining enzymes were assayed after two or more freeze–thaw cycles. Background reaction rates were measured in control reactions that did not contain one of each enzyme's substrates, as follows: COX, $\mathrm{Cytc[Fe^{2+}]}$; CS, oxaloacetate; HOAD, acetoacetyl-CoA; LDH, pyruvate; PK, phosphoenolpyruvate. Enzyme activities were determined by subtracting the background rate from the rates measured in the presence of substrate. For HOAD, LDH, PK and COX assays, we calculated extinction coefficients (ε) empirically by constructing standard curves of absorbance versus NADH or $\mathrm{Cytc[Fe^{2+}]}$ concentration in the buffers appropriate for each assay. We used an ε of 13.6 optical density ($\mathrm{mmol\,l^{-1}}$) $\mathrm{cm^{-1}}$ for CS assays. Enzyme activities are expressed in international units relative to muscle protein content ($\mathrm{\mu mol\,mg\,protein^{-1}\,min^{-1}}$). Protein contents of samples were measured in triplicate using the bicinchoninic acid method (Sigma-Aldrich, Oakville, ON, Canada), following the manufacturer's instructions.

Calculations and statistics

Temperature coefficients (Q_{10}) between upper (T_2) and lower (T_1) temperatures of interest were calculated for developmental rate and the $V_{\max}$ for each enzyme as:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2 - T_1)}, \tag{1}$$

where R_2 and R_1 are rates at T_2 and T_1, respectively.

Data are shown as means ± s.e.m. error (except when data points from individual samples are shown). The enzyme activity value for each individual was the average of its triplicate measurements (minus background; see above), and the mean and standard error of these values are reported. One- and two-factor ANOVA and Bonferroni multiple comparisons tests were used as appropriate to assess the effects of T_E, acclimation temperature and/or assay temperature. A significance level of $P<0.05$ was used throughout.

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