

## Thermal constraints on glycolytic metabolism in the New Zealand abalone, *Haliotis iris*: the role of tauropine dehydrogenase

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**Abstract** Black-foot abalone, *Haliotis iris*, were sampled from two populations in warm northern waters, and from two in colder southern waters. Abalone muscle is characterised by high activity of the glycolytic pyruvate reductase enzyme, tauropine dehydrogenase (TDH). Adductor muscle TDH was profiled for thermostability and activity to test the hypothesis that the enzyme may show adaptation in titre or kinetic characteristics reflecting thermal habitat. Temperature dependency of the apparent Michaelis-Menten constant of TDH for pyruvate ( $_{app}K_{m_{pyr}}$ ) suggested eurythermal enzyme behaviour below 20°C, and compromised function at the higher temperatures of northern populations occurring in the summer months. Thermostability profiles and enzyme activities suggest TDH expression does not differ significantly among populations ( $P > 0.05$ ), indicating that this locus shows no compensation for temperature. The optimal temperature for efficient TDH function, estimated from  $V_{max}/_{app}K_{m_{pyr}}$ , is close to 20°C. The possible thermal constraints on glycolytic metabolism in *H. iris* are discussed.

**Keywords** abalone; temperature; metabolism; tauropine dehydrogenase; glycolysis; *Haliotis iris*

### INTRODUCTION

Ectotherms frequently display rapid growth rates when living close to their upper thermal limits, although this is often to the detriment of terminal size (Atkinson 1996; Clarke 1996; Partridge & French 1996; Van Voorhies 1996). Temperature has been suggested as a constraint that results in size clines of several species of abalone living in temperate waters (Lindberg 1992). The decline in size and abundance of black-foot abalone, *Haliotis iris* Martyn, in the warmer northern waters of New Zealand (Poore 1972; McShane & Naylor 1995) may be another example of this phenomenon.

Theoretical mechanisms explaining size-clinal distributions have focused primarily upon impacts of temperature on developmental processes (Atkinson 1996; Van Voorhies 1996). However, we consider that temperatures approaching thermal limits of ectotherms may also compromise metabolism. At the cellular level, compensations for changes in habitat temperature may be met by allosteric modulation of enzymes, changes in enzyme titre, or induction of specific isoenzymes (Hochachka & Somero 1984). Thermal adjustments of metabolic rate allow for the conservation of substrate fuel reserves (Hochachka & Somero 2002), and failure to acclimatise might divert and compromise metabolic reserves that would otherwise be available for growth.

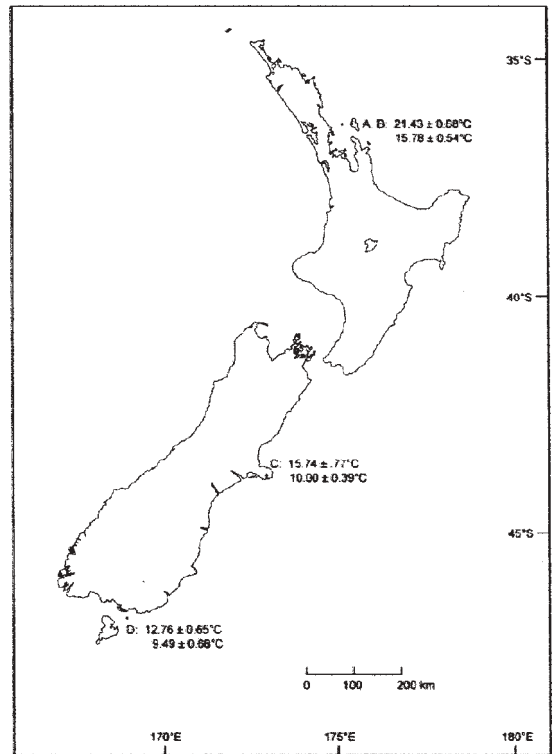
Variations in environmental temperature are associated with adaptive shifts in the kinetic parameters of numerous metabolic enzymes (Place & Powers 1979; Somero 1995; Somero et al. 1996; Powers & Schulte 1998). In particular, selective pressures on the terminal glycolytic enzyme, lactate dehydrogenase (LDH), appear to occur where habitat temperatures differ by as little as 3°C (Graves & Somero 1982). Black-foot abalone occur over the entire latitudinal range of New Zealand, and temperature may also be expected to influence the properties of tauropine dehydrogenase (TDH) at the terminal glycolytic step. TDH occurs in only a few animal groups, but is the predominant pyruvate

reductase enzyme in haliotids, and highlights the importance of anaerobiosis in the adductor muscle (Gäde 1988). Although functionally analogous, TDH is often co-expressed with LDH (Livingstone 1991), but appears to maintain lower cytosolic redox potential during extended anaerobic episodes (Fields 1983), and accounts for 90–95% of the glycolytic redox maintenance in the adductor muscle of *H. iris* (Wells & Baldwin 1995; Wells et al. 1998).

About 45% of the total mass of the abalone is adductor-foot muscle complex, used primarily for locomotion and clamping to rocks in turbulent water. With poor muscle perfusion, there is a strong dependence on anaerobic glycolysis to fuel working muscles (Wells & Baldwin 1995; Wells et al. 1998; Donovan et al. 1999). Accordingly, we have tested the hypothesis that *H. iris* from warm water populations in the North Island of New Zealand and abalone from cold water populations in the South Island differ in the functional characteristics of TDH at the terminal step of glycolysis. If anaerobic metabolism is compromised in warm, northern waters, then it may, in part, explain the size and distribution of species. Thus, abalone from two separate northern populations were compared with two southern populations, and TDH characterised from the maximal activity ( $V_{max}$ ) and the apparent Michaelis-Menten constant for pyruvate ( $app K_m_{pyr}$ ) as a function of temperature. Catalytic efficiency ( $V_{max}/K_m$ ) and Arrhenius activation energy were also determined and related to a pH-temperature coefficient determined from adductor tissue.

## METHODS AND MATERIALS

Abalone were collected from North Island populations at Little Barrier (36°12'S, 174°48'E) and Leigh (36°19'S, 174°48'E), and from South Island populations at Banks Peninsula (43°40'S, 172°55'E) and Stewart Island (46°55'S, 168°11'E) during the summer at sea temperatures of 21, 20, 16, and 14°C respectively. Corresponding mean shell lengths  $\pm$  SD and range (mm) were 102.14  $\pm$  9.91 (90–121), 87.29  $\pm$  7.13 (79–97), 90.75  $\pm$  10.47 (69–108), and 130.92  $\pm$  15.63 (110–157). Sea surface temperature (SST) data were provided by the National Climatic Data Center, Ashville, North Carolina, United States. Temperature was calculated for 50 km<sup>2</sup> grids encompassing the site co-ordinates. Data from April 1983 to April 2003 were taken from the optimum interpolation sea surface temperature (OISST) data set (Reynolds & Smith 1994), and the 20-year means



**Fig. 1** Location of collection sites, A–D, for *Haliotis iris*, showing 20-year mean maximum and minimum monthly sea surface temperatures  $\pm$  SD.

calculated for the maximum and minimum mean monthly temperatures (Fig. 1).

Portions of adductor muscle from the central core were excised after shucking, rapidly frozen in liquid nitrogen, and later stored at  $-80^{\circ}\text{C}$ .

## Tauropine dehydrogenase activity

Tissue was rapidly thawed and slices shaved from a portion of the adductor in a dorso-ventral plane. An Ultra-Turrax T-25 homogeniser with an 8-mm diam. shaft (Janke & Kunkel, Germany) was used to prepare homogenates in 1:10 w/v extraction buffer at pH 7.4 (50 mM imidazole-HCl, 1 mM EDTA, 2 mM  $\text{MgCl}_2$ ) and 50% glycerol. Samples were centrifuged at 13 000 g for 20 min at 4°C, and the supernatant retained for assay. Total activity was determined from 20- $\mu\text{l}$  aliquots of supernatant in a final volume of 1 ml buffer at pH 7.0 (80 mM imidazole-HCl containing 0.15 mM NADH, 80 mM taurine, 1mM EDTA, 1mM  $\text{MgCl}_2$ ) and with 2.5 mM pyruvate added to start the reaction. Oxidation of

NADH was followed at 340 nm, using a Shimadzu UV-1601 recording spectrophotometer fitted with a constant temperature cell-holder ( $\pm 0.02^\circ\text{C}$ ). LDH and non-specific activity were subtracted from a blank containing no taurine, and activity presented as  $\text{mmol min}^{-1} \text{g}^{-1}$  tissue weight. Comparison of fresh and frozen tissues confirmed that activity was not decreased by freezing tissues.

### Characterisation of TDH

Maximal velocity ( $V_{\text{max}}$ ) and apparent Michaelis-Menten constants ( $K_{\text{app}}$ ) were estimated in duplicate between 5 and  $45^\circ\text{C}$ . Temperatures in the reaction cuvette were verified with an Eirelec MT130-TC thermocouple probe ( $\pm 0.05^\circ\text{C}$ ). General assay conditions are as above. The  $K_{\text{app}}$  was measured at several concentrations of both taurine and pyruvate to evaluate co-substrate preference and saturation. The  $K_{\text{app}}$  for taurine was determined at pyruvate concentrations of 0.25, 0.5, and 2.5 mM; the  $K_{\text{app}}$  for pyruvate at taurine concentrations of 5, 20, 50, and 80 mM.

In view of the temperature-dependence of tissue pH (Yancey & Somero 1978),  $\Delta\text{pH}/\Delta\text{T}^\circ\text{C}$  was determined for tissue homogenates from 12 animals in 0.54M NaCl, and found to be c.  $-0.014$  pH units  $^\circ\text{C}^{-1}$ . For this reason, and to maintain constant chloride concentration when pH was changed in the reaction buffers, 50 mM HEPES buffers ( $\Delta\text{pH}/\Delta\text{T}^\circ\text{C} = -0.014$ ) were substituted for imidazole-HCl. The effect of pH on TDH activity at  $20^\circ\text{C}$  was made with buffers adjusted for pH with the aid of a Radiometer PHM-40 pH meter and PHC-2406 electrode using certified buffer standards. Optimal pH was determined by fitting a second order polynomial curve.

Temperature effects on the activity of TDH were evaluated under assay conditions as above, and the activation energy,  $\mu$ , of TDH, was derived from the

slope of an Arrhenius plot from the following equation:

$$K_{T_2} = K_{T_1} e^{\mu/R[(T_2-T_1)/T_2T_1]}$$

where R is the universal gas constant ( $8.3143 \text{ J mol}^{-1} \text{ K}^{-1}$ ), e is the base of the natural logarithm, and K, the rate at temperature T (K). Values of  $\mu$  were determined from the slopes derived from 19 individuals over the temperature ranges.

### Thermostability

Population differences in the thermal stability of TDH were compared using a method based on that of Dahlhoff & Somero (1993a). Homogenates were prepared as described, and incubated at  $35^\circ\text{C}$  for time periods of 0 (control), 10, 20, 30, and 60 min. Aliquots of 100  $\mu\text{l}$  were removed in 200- $\mu\text{l}$  tubes which were placed in ice-cold water to arrest further denaturation, then assayed for activity as outlined above. Five animals from each location were tested in duplicate.

### Statistics and data analysis

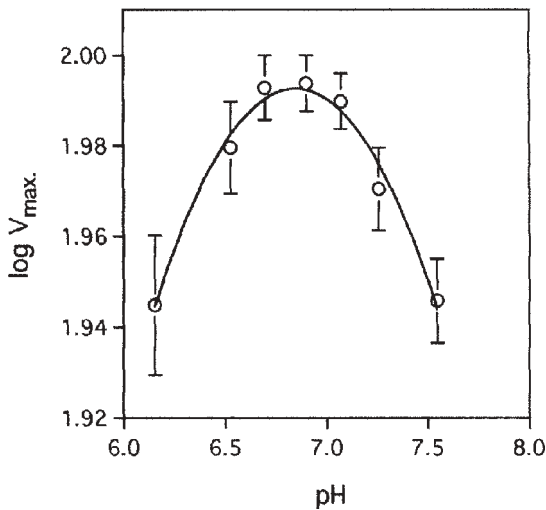
All statistical tests were performed using SAS v6.03. Significant differences were established at  $P < 0.05$  following ANOVA and Tukey's (HSD) test. Estimates of the kinetic parameters  $K_{\text{app}}$  and  $V_{\text{max}}$  were made using the Marquardt-Levenberg algorithm, and the non-linear regression component of the Sigma Plot<sup>®</sup> software package.

## RESULTS

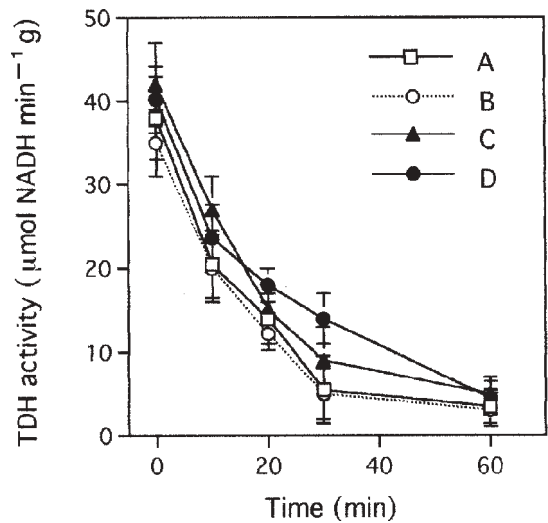
No significant differences in TDH activity were found between the two northern populations (thus excluding site effects), and there were no latitudinal differences ( $P > 0.05$ ; Table 1). For the size range of abalone used in this study, there was no evident

**Table 1** Activities of adductor muscle tauroxine dehydrogenase ( $V_{\text{max}}$ ,  $\mu\text{mol min}^{-1} \text{g}^{-1}$ ) in abalone (*Haliotis iris*) from four collection sites and two measurement temperatures. Data are means  $\pm$  SD.

Population	$20^\circ\text{C}$	$10^\circ\text{C}$
A, Little Barrier ( $n = 7$ )	$65.7 \pm 3.9$	$28.7 \pm 2.0$
B, Leigh ( $n = 7$ )	$67.8 \pm 7.2$	$28.6 \pm 2.9$
C, Banks Peninsula ( $n = 19$ )	$67.4 \pm 6.0$	$30.7 \pm 4.4$
D, Stewart Island ( $n = 12$ )	$63.7 \pm 3.9$	$25.2 \pm 3.2$
Mean ( $n = 45$ )	$65.3 \pm 0.6$	$28.4 \pm 0.2$



**Fig. 2** Effect of pH on tauropine dehydrogenase (TDH) activity at 20°C. Data are means  $\pm$  SEM ( $n = 5$ ).



**Fig. 3** Profiles of residual tauropine dehydrogenase activity as a measure of denaturation after incubation at 35°C. Data are means  $\pm$  SEM from populations at sites A–D, ( $n = 5$ ).

**Table 2** Effects of pyruvate and taurine concentrations on  $V_{\max}$  and  $appK_m$  for tauropine dehydrogenase at 20°C. Data are means  $\pm$  SD ( $n = 7$ ).

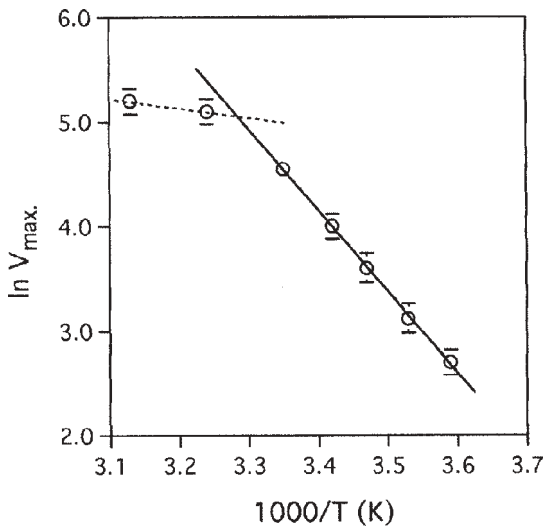
	$V_{\max}$	Co-substrate $appK_m$
0.25 mM pyruvate	22.6 $\pm$ 2	40.2 $\pm$ 9.5
0.5 mM pyruvate	39.4 $\pm$ 4.3	32.5 $\pm$ 4.5
2.5 mM pyruvate	65.3 $\pm$ 0.6	24.7 $\pm$ 2.9
5 mM taurine	14.6 $\pm$ 0.4	0.48 $\pm$ 0.05
20 mM taurine	31.3 $\pm$ 2	0.48 $\pm$ 0.09
50 mM taurine	54.5 $\pm$ 3.7	0.40 $\pm$ 0.01
80 mM taurine	67.8 $\pm$ 7.2	0.50 $\pm$ 0.06

allometric relationship for TDH activity ( $r^2 = 0.073$ ). The pH optimum of TDH is c. 6.9 (Fig. 2) for site B, and was similar for samples from sites A, C, and D ( $P > 0.05$ ). Residual activity profiles of heat-shocked TDH assayed at 20°C did not suggest latitudinal differences in thermostability of the enzyme ( $P > 0.05$ ; Fig. 3).

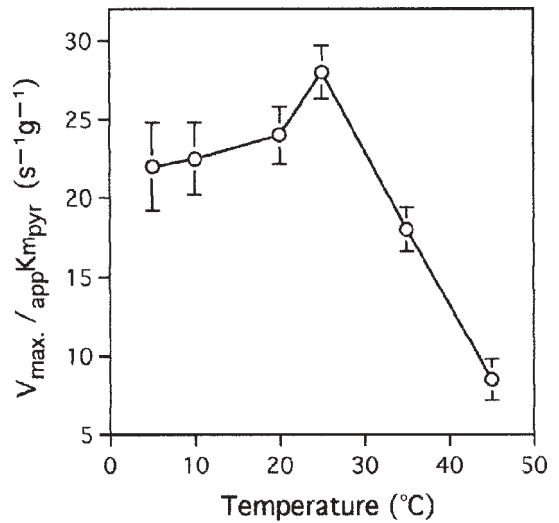
The  $appK_{m_{pyr}}$  did not change significantly with reductions of taurine concentration ( $P > 0.5$ ), but  $appK_{m_{taur}}$  increased significantly with reducing pyruvate ( $P < 0.05$ , Table 2), suggesting that TDH modulation is dependent on pyruvate concentration. Saturating concentrations of taurine appeared to be substantially lower than *in vivo* concentrations reported for *H. iris* (Bewick et al. 1997).

The activation enthalpy of 57 kJ mol<sup>-1</sup> (=13.6 kcal mol<sup>-1</sup>) derives from the slope of the Arrhenius plot (Fig. 4) and reflects elevated  $V_{\max}$  with increasing temperature. An apparent break temperature, defined as the break in slope of the Arrhenius plot at the upper temperature range (Somero et al. 1996), occurred at c. 26.5°C for site B and was similar for sites A, C, and D.

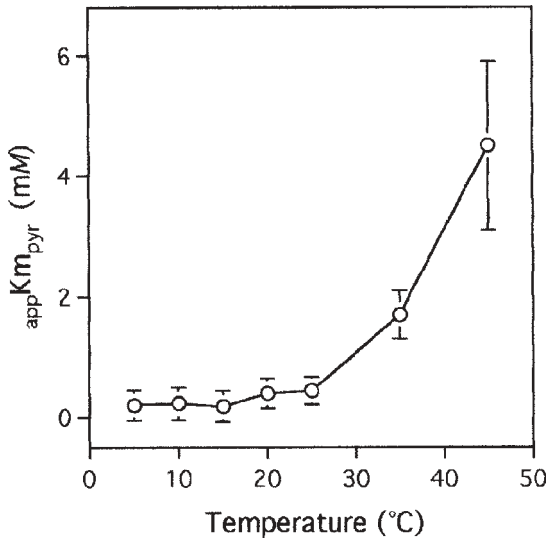
The effect of temperature on the apparent Michaelis-Menten constant for pyruvate ( $appK_{m_{pyr}}$ ) is shown for samples from site B in Fig. 5. The rapid increase in  $appK_{m_{pyr}}$  above the conserved range of  $appK_{m_{pyr}}$  values (to c. 25°C) is typical of LDH enzymes (Somero 1995), and was similar for samples from other sites ( $P > 0.05$ ).



**Fig. 4** Effect of measurement temperature on tauropine dehydrogenase activity from abalone, *Haliotis iris*, at 20°C. Arrhenius break-temperature is depicted by the temperature at which the lines intersect. Data are means  $\pm$  SEM ( $n = 19$ ).



**Fig. 6**  $V_{max} / \text{app}K_{m\text{pyr}}$  ratios as a measure of the relative efficiency of an enzyme at various temperatures. Data are means  $\pm$  SEM ( $n = 19$ ).



**Fig. 5** Effect of temperature on the apparent Michaelis-Menten constant for pyruvate ( $\text{app}K_{m\text{pyr}}$ ). Data are means  $\pm$  SEM ( $n = 19$ ).

**DISCUSSION**

Although there are several examples of temperature-dependent expression of LDH variants among ectotherms (Place & Powers 1979; Holland et al. 1997; Hochachka & Somero 2002), there are few invertebrate examples illustrating apparent selection at the terminal step of anaerobic glycolysis. In several marine invertebrates modification of the terminal step has given rise to some unusual pyruvate reductases, including octopine dehydrogenase (ODH), and TDH in haliotids (Fields 1983; Gäde & Grieshaber 1986; Gäde 1988; Livingstone et al. 1990). Given the biogeographical distribution of black-foot abalone and exposure to significant seasonal temperature cycles (see Fig. 1), qualitative or quantitative compensation at the TDH locus might be expected. Yet, despite functional differences suggesting thermal adaptation in the isoforms of ODH from the sea anemone, *Metridium senile* (Walsh & Somero 1981), several scallops, *Euvola ziczac* (Perez et al. 2000), *Placopecten magellanicus* (Volckaert & Zouros 1989), *Argopecten purpuratus* (Martinez et al. 2000), and the mussel, *Mytilus edulis* (Beaumont & Toro 1996), TDH is likely to be monomorphic in *H. iris*.

The ratio of  $V_{max} / \text{app}K_{m\text{pyr}}$  is a relative measure of the efficiency of TDH and decreased above 25°C (Fig. 6).

Comparisons of protein stability and the effects of temperature on enzymatic properties such as the apparent Michaelis-Menten constant ( $\text{app}K_m$ ) reflect the trade-off between thermostability and reactivity,

and may reveal both the temperatures and the range of temperatures to which species are adapted (Somero 1995). Measurements in excess of physiological temperatures are generally needed to confirm differences in thermal stability of isoforms (Somero et al. 1996), yet in Fig. 3 we are unable to see any population differences for *H. iris* that might indicate differences in thermal plasticity of the enzyme. Disruption of protein function however, occurs at temperatures well below those causing total denaturation. At temperatures close to the upper and lower limits of thermal distribution for the species (Table 1) there appear to be no population differences in  $V_{\max}$ .

The temperature sensitivity of  $_{\text{app}}K_{\text{m,pyr}}$  for TDH (Fig. 5) showed that values are conserved over the approximate biogeographical temperature range for *H. iris*. These data suggest that black-foot abalone are competent eurytherms below c. 20°C. Eurythermy of TDH function is further enhanced by the pH dependence of the muscle homogenates from *H. iris* ( $\Delta\text{pH}/\Delta\text{T}^{\circ}\text{C} = -0.014$ ). Temperature-dependent  $K_{\text{m,pyr}}$  values for LDH from taxa ranging from bacteria to vertebrates show remarkable conservation that may reflect an evolutionary constraint on pyruvate binding dynamics (Somero et al. 1996). Temperature-substrate interactions of TDH and LDH are therefore broadly similar. Furthermore, temperature-driven changes in the ionisation of histidyl groups within active sites of LDH appear responsible for changes in  $_{\text{app}}K_{\text{m,pyr}}$  and pH optima (Yancey & Somero 1978; Somero 1995). Shifts above or below physiological substrate ranges appear to shift the  $_{\text{app}}K_{\text{m,pyr}}$  values outside universally conserved substrate concentrations and may compromise catalytic function (Somero et al. 1996). Values for  $_{\text{app}}K_{\text{m,pyr}}$  in other invertebrate TDHs are similar to those for LDH at the same temperature (Gäde 1988; Sato et al. 1991). The Japanese echinoderm, *Asterina pectinifera*, shows a high TDH value of  $_{\text{app}}K_{\text{m,pyr}}$  (0.46 mM) at its extreme upper thermal limit of 25°C (Kanno et al. 1996), a value similar to that obtained for *H. iris*. Thus, the thermal limit of *H. iris* TDH is approached, or exceeded above 20°C.

Comparison of maximal TDH activities of *H. iris* with the tropical abalone *H. asinina* suggests a conservation of catalytic rates relative to environmental temperature. TDH activity of *H. asinina* measured at a typical habitat temperature of 25°C, was 48.3  $\mu\text{mol min}^{-1} \text{g}$  (Baldwin & Wells unpubl. data) compared with *H. iris* activities of 46.2 and 105.4  $\mu\text{mol min}^{-1} \text{g}$  at 15 and 25°C.

The Arrhenius break-temperature for mitochondrial respiration correlates with acclimation temperature for the stenothermal abalone *H. kamtschatkana* (Dahlhoff & Somero 1993b). We were unable to find differences in the break temperature (c. 26.5°C) between populations of *H. iris*, suggesting poor acclimation ability of the glycolytic pathway at the upper thermal limit. We should caution however, that aerobic pathways of an organism may show thermal adaptation, while anaerobic pathways might not (Crockett & Sidell 1990).

Large individuals and dense populations of *H. iris* inhabit waters that average close to 15°C, and suggest conservation of maximal TDH activities relative to thermal habitat. These observations parallel those for LDH in barracuda species (Graves & Somero 1992), killifish (Crawford et al. 1990; Powers & Schulte 1998), and for malate dehydrogenase in North American abalones (Dahlhoff & Somero 1993b) which all show correlations with habitat temperatures. In addition, Arrhenius break-points of enzymes generally exceed the thermal limits of the whole organism (Gutfreund 1995; Hochachka & Somero 2002). Interestingly, cultured *H. iris* expire at temperatures over 25°C, close to the Arrhenius break-temperature (Fig. 4). The temperature for optimum growth rates of *H. rufescens* is well below its thermal maximum (Diaz et al. 2000).

The ratio of  $k_{\text{cat}}$  (the catalytic rate constant), or  $V_{\max}$ , relative to  $K_{\text{m}}$  provides an index of enzymatic efficiency (Somero & Seibenaller 1979) and may have evolved to match environmental conditions, such as pH and temperature, where higher values are more efficient (Gutfreund 1995; Powers 1998). Examples of thermodynamic tuning of this ratio have been reported in the LDHs of Antarctic fishes, deep-sea fishes, and temperate fishes (Somero 1978; Somero & Seibenaller 1979; Pogson 1988). The best example of optimisation of this ratio is found in allelic distributions of LDH in the killifish heart. These alleles show a clinal distribution in values that appear optimal in their respective thermal ranges (Powers & Schulte 1998). Since  $k_{\text{cat}}$  is derived in purified enzymes, the maximal  $V_{\max}/_{\text{app}}K_{\text{m}}$  ratio for *H. iris* homogenates provides an analogous efficiency ratio which occurred close to 20°C, coinciding with approximate maximal habitat temperatures for the species. It may be argued however, that at thermal extremes, thermodynamic efficiency is modulated by different events. At warmer temperatures, demands on limited substrate increase, but in cooler temperatures reduced catalytic

rates do not incur a metabolic cost. Several species of temperate abalone have narrow thermal ranges for optimal growth (Leighton 1974; Gilroy & Edwards 1998; Diaz et al. 2000). Furthermore, thermally stressed abalones have been observed to lift shells to increase convection (Hecht 1994). Perhaps shell elevation is a failure of muscle adduction rather than an active response.

Interspecific differences in stability of enzymes and  $K_{app}$  values in response to temperature change are closely linked to the biogeographical range of a species (Somero et al. 1996). For example, temperature effects on malate dehydrogenase from five species of Eastern Pacific abalones correspond with their latitudinal distribution (Dahlhoff & Somero 1993a). Other examples are LDH properties in phylogenetically related fish species (Graves & Somero 1982; Fields 1995). It is less clear whether such relationships apply to populations within a species.

It is reductive to suggest that the TDH locus alone instigates the physical demise of *H. iris* in warmer waters. Locomotion in haliotids is predominantly anaerobic and energetically expensive (Donovan et al. 1999). Hemocyanin, the respiratory protein of *H. iris*, also displays compromised function at 20°C (Wells et al. 1998), although high rates of oxygen delivery to the adductor and foot muscles may not be a priority with hemocyanin providing an oxygen reserve supporting a predominantly anaerobic energy production (Behrens et al. 2002). A theme of thermally constrained protein function becomes apparent, and the lack of TDH heterozygosity and impaired function above 20°C may compromise energy flux through anaerobic metabolism, and consequently limit terminal size in warmer waters.

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