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Maintenance of the alcohol dehydrogenase polymorphism in Tiger Salamanders, II. Differences in biochemical function among allozymes

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Summary

1. Previous studies of Tiger Salamanders demonstrated that variation in alcohol dehydrogenase (Adh) contributed significantly to associations between multilocus heterozygosity and oxygen consumption traits, and that Adh variation was associated with levels of pond-oxygen and metamorphic ability in extreme oxygen environments. Here Adh allozymes are characterized kinetically, and relationships between Adh and oxygen-related physiological traits (ATP/Hb, 2,3-DPG/Hb) are measured.

2. Kinetic differences were measured among Adh allozymes in the acetaldehyde-to-ethanol direction: k_{cat}/K_m ratios (the catalytic constant divided by the Michaelis–Menton constant) were significantly higher in Adh-SF than the other two genotypes, and in Adh-SS compared with Adh-FF. No significant differences were measured in the ethanol to acetaldehyde direction.

3. Adh-SS had a significantly higher ATP/Hb than Adh-FF, with the Adh-SF intermediate. In addition, a significant interaction between Hb and body mass was measured, such that Adh-FF showed a negative relationship between Hb concentration and body mass while the other two genotypes showed a positive relationship.

4. These results are consistent with the hypothesis that variation at the Adh locus has adaptive and physiological significance, and that functional differences among Adh allozymes partly explain significant associations between multilocus genotype and organismal traits.

Key-words: ATP, enzyme kinetics, haemoglobin, k_{cat}/K_m

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Introduction

The evolutionary and physiological consequences of allelic variation at enzyme loci (allozymes) have been debated for 30 years (reviewed in Mitton 1997). A large number of studies have identified the potential adaptive significance of both single-locus and multilocus allozyme variation through measurement of correlations between allozyme genotype and components of Darwinian fitness. Although such correlations demonstrate selection of the allozyme variant, they do not by themselves answer whether this selection is for the locus of study or for a linked locus. (Following the definitions of Sober (1984) and Endler (1986), selection for a trait denotes a cause–effect relationship between that trait and selection, whereas selection of a trait is an observation of selection and does not assign causal significance.) Several studies have attempted to resolve this issue for single loci by examining functional differences among allozymes

in an appropriate ecological context (Feder & Watt 1992). This approach has been successful in demonstrating both the functional and adaptive significance of variation of phosphoglucose isomerase (Pgi) in *Colias* butterflies, lactate dehydrogenase (Ldh) in the teleost fish *Fundulus heteroclitus*, and leucine amino peptidase (Lap) in the Blue Mussel, among others (reviewed in Mitton 1997). Attempts to uncover the physiological and adaptive significance of multilocus allozyme variation have been more difficult. Clark & Koehn (1992) have argued that metabolic efficiency, measured as pathway flux and protein maintenance, underlie this significance; however, their ideas have yet to be tested empirically. Hawkins & Day (1999) have demonstrated that protein turnover in mussels decreases with increasing allozyme heterozygosity, and have argued that differential turnover in more heterozygous protein pools produces positive correlations between heterozygosity and components of fitness. We suggest herein that functional differences among

allozymes at an individual locus may in part explain correlations between multilocus heterozygosity and components of fitness.

Multilocus heterozygosity was correlated with resting and active oxygen consumption in Tiger Salamanders (Mitton, Carey & Kocher 1986). Regression analysis of the data identified two of eight loci, Pgi and alcohol dehydrogenase (Adh), as contributing significantly to this relationship. In the same study, body size was significantly different among Adh genotypes in Tiger Salamanders collected from two natural populations. In a later study, Carter (1997) demonstrated significant relationships between Adh genotype frequencies and pond oxygen levels, with one homozygote being less frequent in hypoxic ponds, the other homozygote being less frequently in ponds supersaturated with oxygen, and the heterozygote showing no relationship. The same study also demonstrated differences in ability to metamorphose among Adh genotypes in extreme oxygen conditions, but not in normal oxygen condition, in both field and laboratory experiments. Under normal oxygen conditions, individuals of the three genotypes metamorphosed in the same frequency in which they occurred in the population. In both hypoxic and hyperoxic (supersaturated) conditions, Adh heterozygotes metamorphosed in the numbers expected, but fewer Adh homozygotes metamorphosed than expected, giving rise to differences in relative frequencies among genotypes in metamorphosed salamanders. These results demonstrated selection of Adh genotypes, but did not address whether selection was actually for Adh. Demonstrating selection for a locus is difficult and time consuming; witness, for example, the more than 20 years of study of Pgi in *Colias* butterflies (reviewed in Watt 1991). The best first step in such a process is to examine allozymes for differences in kinetic function (Feder & Watt 1992). Our primary objective in this study is to determine whether catalytic efficiencies differ among Adh allozymes; if not, then the selection measured in Mitton *et al.* (1986) and Carter (1997) cannot be for the Adh protein, but must have some other cause. Furthermore, identification of differences in catalytic efficiency among Adh allozymes would be evidence that the multilocus results presented in Mitton *et al.* (1986) were caused at least in part by functional differences at a single locus. We will use the ratio of k_{cat} to K_m (the catalytic constant divided by the Michaelis–Menton constant) as an index of catalytic efficiency because it 'provides a more realistic estimate of catalytic activity' (Hochachka & Somero 1984, p. 378) and because it has been used in previous studies of allozyme function (e.g. Powers *et al.* 1993).

Our second objective is to identify how variation in Adh might affect the organismal phenotype. The two prior studies of Adh in Tiger Salamanders identified oxygen consumption and environmental levels of oxygen as factors related to Adh variation; in addition, functional connections have been measured between

Ldh and ATP and haemoglobin in *Fundulus* (Powers *et al.* 1993), and Adh might potentially affect redox balance, and hence ATP production, as described by Burton & Place (1986). Therefore we focused on relationships between Adh and physiological traits that affect oxygen consumption, specifically concentrations of ATP, 2,3-DPG and haemoglobin (Hb). ATP and 2,3-DPG are allosteric effectors of Hb-oxygen binding in Tiger Salamanders (Wood, Hoyt & Burggren 1982). Increased levels of ATP and 2,3-DPG cause a 'right shift' in the Hb-oxygen binding curve, and long-term exposure to hypoxia in Tiger Salamanders results in a decline in ATP and 2,3-DPG and a subsequent 'left-shift' in the Hb-oxygen binding curve (Wood *et al.* 1982). Studies of Ldh-B variation in *Fundulus heteroclitus* at 10 °C demonstrated that relative to the Ldh-B_{aa} homozygote, the Ldh-B_{bb} homozygote had a higher swimming performance associated with increased levels of ATP/Hb and a right shift in the Hb-oxygen dissociation curve (Powers *et al.* 1993), which suggests possible explanations for previous results (Mitton *et al.* 1986; Carter 1997) and potential future research directions in salamanders. Here we tested the hypothesis that the ATP/Hb and 2,3-DPG/Hb differ among Adh genotypes in Tiger Salamanders.

Materials and methods

Gilled Tiger Salamanders *Ambystoma tigrinum* (Green) were collected by seining ponds near Leader and Gunnison, Colorado. Collected individuals were killed by cervical dislocation, the liver dissected from the carcass, and the gall bladder carefully removed from the liver. All but ≈20 mg of liver from each individual was placed in a labelled 1.5 ml Eppendorf tube (Eppendorf Scientific Inc., Westburg, NY) and stored at –70 °C. The remaining 20 mg of liver from each individual was homogenized in 2 volumes of ice-cold 0.11-M Tris (tris(hydroxymethyl)-aminomethane), 0.005-M EDTA (ethylenediaminetetraacetic acid) buffer adjusted to pH 7.5 with HCl. The homogenate was centrifuged for 10 min at top speed in an Eppendorf desktop microfuge kept at 4 °C. Each homogenate was genotyped for Adh using starch gel electrophoresis in a 22.9-mm Tris, 5.2-mm citrate monohydrate, pH 8.0 gel buffer, and a 0.687-M Tris, 0.157-M citrate, pH 8.0 electrode buffer. Gels were electrophoresed for 5 h at 4 °C at 130 V and 50 mA, then sliced and stained for Adh in 20 ml of a 0.1-M Tris, 1% agar overlay buffer at pH 7.0 in the presence of 1.5 ml 95% ethanol, 10 mg NAD (nicotinamide–adenine dinucleotide), 4 mg 3-[4,5-Dimethylthiazol-2-yl]-2.5-diphyltetrazolium bromide (MTT) and 2 mg PMS (phenazine methosulphate).

The enzyme from each Adh genotype was purified in a separate procedure, using a modification of the methods of Julia, Farres & Pares (1987). Approximately 10 g of liver that had been stored at –70 °C, from 10 to 15 different individuals of the same genotype, were used for each purification; unless otherwise

Table 1. Results for a typical purification of Adh from Tiger Salamanders. The total protein and total activity listed for each stage are what remained after completion of that stage

Purification stage	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)
Raw homogenate	2705	143	0.053
Second filtration	373	81	0.217
DEAE column	91	71	0.780
AMP column	1.85	8.36	4.519
Heat treatment	1.29	8.36	6.480

stated, all purification procedures were conducted at 4 °C. The pooled livers were weighed to the nearest 0.01 g and homogenized in 3 volumes of ice-cold 0.011-M Tris, 0.0005-M EDTA buffer, adjusted to pH 7.5 with HCl, in a glass on glass homogenization tube. The crude homogenate was centrifuged at 25 000 g for 30 min. After centrifugation the pellet was discarded and the supernatant filtered through glass wool to remove fat. The filtrate was then centrifuged at 100 000 g for 1 h; the pellet was discarded, and the supernatant was again filtered through glass wool to remove any remaining fat. The filtrate was applied to a 50-ml column of diethylaminoethyl (DEAE) Sephadex equilibrated in the homogenization buffer at a flow rate of $\approx 1 \text{ ml min}^{-1}$. Approximately 75% to 90% of the Adh activity came off the column in the straw-coloured fraction that eluted immediately. The fractions containing the heavily coloured liver pigments were slowed considerably on the column, and were later washed off in a 0.11-M Tris, 2-M NaCl solution and discarded. The column was stored in the cleansing buffer to which 0.05% NaN_3 had been added. The straw-coloured fraction was then applied to a 4-ml AMP (adenosine 5'-monophosphate)-Sephacose column, which was equilibrated in a 0.11-M Tris, 0.005 M EDTA buffer, adjusted to pH 7.5 with HCl, at a flow rate of $\approx 0.5 \text{ ml min}^{-1}$. Approximately 75–80% of the Adh activity bound to the column; the column was then washed with the buffer for 20 h to remove non-Adh proteins. The column was washed with a solution of 66 μM NADH dissolved in the equilibration buffer to elute the Adh. Most of the Adh activity was eluted in the first 20 ml. This eluent was then reduced in volume to 2 ml by centrifugation in Centricells (Polysciences Inc., Fisher Scientific, Pittsburgh, PA). The supernatant from this volume reduction process was heat treated at 62 °C for 45 min, immediately placed on ice for 10 min, and finally centrifuged at 3500 g at 4 °C to remove the precipitated protein. This supernatant was used for kinetic assays. Total protein determinations for each step of the purification were done following the procedures of Peterson (1977).

SDS (sodium dodecyl sulphate) acrylamide tube gels were run to determine the purity of individual enzyme preparations. Each gel was composed of 1 part of a 1.5-M Tris, 0.001-M EDTA, 0.01-M N,N,N',N' -tetramethylethylenediamine (TEMED), 0.02% NaN_3 solution adjusted to pH 8.9 with HCl; 2.34 parts of a

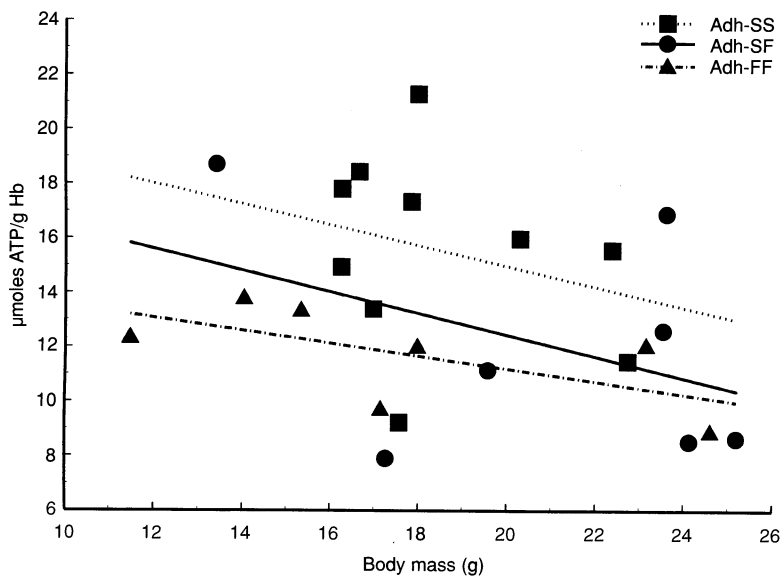
3.86-M acrylamide, 0.036-M bis, 0.02% NaN_3 solution; and 2 parts of a 0.006-M ammonium persulfate solution. The final concentration of SDS in each tube gel was 0.1%. Samples were mixed 9 to 1 in a 10% SDS, 10% beta-mercaptoethanol solution, and were placed in a boiling water bath for 2 min. After boiling, one drop of glycerol was added to each sample, and one sample was loaded on the top of each tube gel. The gels were run in an electrode buffer of a 1 to 20 dilution of a 0.05-M Tris, 0.38-M glycine, 0.1% SDS, 0.02% NaN_3 electrode buffer stock, which was adjusted to pH 8.3 with HCl. Concentrated bromophenol blue was added to the upper electrode buffer tray to mark the progress of the front. Gels were run for 2–3 h at 3 mA/gel. Gels were stained with Fast Green RR for 18 h, destained for 48 h in a 45% methanol, 9% acetic acid solution, and stored in a 7% acetic acid solution. Purity of each preparation was quantified using a gel scanner on a Gilford spectrophotometer at 533 nm.

Enzyme assays were run in both reaction directions for Adh in a Varian spectrophotometer (Varian Instruments, Walnut Creek, CA) at 340 nm at 25 °C (Cornell & Veech 1983). Standard assays in the acetaldehyde-to-ethanol reaction direction were run in a 0.11-M Tris, 0.005-M EDTA buffer adjusted to pH 7.5 with HCl; final substrate concentrations in the cuvette were 100 mM acetaldehyde and 0.1 mM NADH. Standard assays in the ethanol-to-acetaldehyde direction were run in the same buffer described above, with 50 mM ethanol and 2.6 mM NAD per cuvette. The kinetic studies in each reaction direction were conducted in the conditions just described, with the concentrations of the acetaldehyde and ethanol systematically varied from 100 to 0.03 mM to yield typical Michaelis–Menton plots.

For the measurements of haemoglobin, ATP (adenosine 5'-triphosphate) and 2,3-DPG (diphosphoglycerate), additional animals were collected from La Veta Pass, Colorado, and acclimated to laboratory conditions for several weeks before being killed by cervical dislocation. Blood was collected into physiological saline. Haemoglobin was measured in blood using the cyanmethaemoglobin method following Sigma kit no. 525 (Sigma Chemical Company, St Louis, MO). ATP was measured in blood with the quantitative, enzymatic determination of Bucher (1947) as modified by Adams (1963) using Sigma kit no. 366-UV. 2,3-DPG was measured quantitatively by the enzymatic determination of Lowry *et al.* (1964);

Table 2. Means and (standard errors) for kinetic parameters of the three Adh allozymes, measured at pH 7.5 and 25 °C

Allozyme	K_m (mM)	V_{max} (OD min ⁻¹)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)
Acetaldehyde to ethanol				
Adh-SS	0.096 (0.004)	0.0348 (0.0007)	4.77 (0.020)	49.49 (2.08)
Adh-SF	0.074 (0.003)	0.0360 (0.0006)	6.02 (0.095)	81.35 (3.94)
Adh-FF	0.102 (0.008)	0.0380 (0.001)	3.30 (0.113)	32.35 (2.94)
Ethanol to acetaldehyde				
Adh-SS	0.190 (0.0221)	0.0160 (0.0006)	0.438 (0.016)	2.30 (0.280)
Adh-SF	0.182 (0.0192)	0.0285 (0.0008)	0.504 (0.014)	2.77 (0.303)
Adh-FF	0.233 (0.0165)	0.0268 (0.0007)	0.322 (0.008)	1.44 (0.133)

**Fig. 1.** ATP/Hb as a function of body mass for each of the three Adh genotypes. Refer to Table 3 for statistical significance of main effects and covariate.

modified by Keitt (1966) and Rose & Liebowitz (1970), using Sigma kit no. 35-UV.

Results

The Adh purification results are presented in Table 1. The methods and results for salamander Adh purification are very similar to those for rats conducted by Julia *et al.* (1987), with the addition of a heat treatment to remove a single remaining contaminating

protein. Using these methods, purity levels ranging from 70% to 90% were obtained; in all cases, a single protein appeared to be the only contaminant left in the Adh preparation. Because this contaminant protein was of similar molecular mass to Adh, attempts to remove it with a molecular sieve column failed.

The means and standard errors of K_m , k_{cat} , V_{max} and the k_{cat}/K_m ratios in both reaction directions are presented in Table 2. In both reaction directions, the order of the magnitude of the k_{cat}/K_m ratios is Adh-SF > Adh-SS > Adh-FF. In the ethanol-to-acetaldehyde reaction, none of these differences was statistically significant. In the acetaldehyde-to-ethanol reaction direction, k_{cat}/K_m was significantly higher for Adh-SF than Adh-SS ($t = 12.92$, $df = 4$, $P < 0.001$), and k_{cat}/K_m was significantly higher in Adh-SS than in Adh-FF ($t = 7.74$, $df = 4$, $P < 0.01$), indicating significant differences in catalytic efficiencies among allozymes. Differences among K_m and V_{max} values in both reaction directions were not statistically significant, nor were differences significant for k_{cat} in the ethanol to acetaldehyde directions. In the acetaldehyde-to-ethanol reaction direction, k_{cat} was significant higher for Adh-SF than Adh-SS ($t = 3.69$, $df = 4$, $P < 0.05$), and k_{cat} was significantly higher in Adh-SS than in Adh-FF ($t = 4.02$, $df = 4$, $P < 0.05$).

The amount of ATP/Hb among Adh genotypes is plotted with body mass as a covariate in Fig. 1; the P -values from the analysis of covariance, conducted using the SAS general linear models (GLM) procedure (SAS Institute Inc., Cary, NC), are presented in Table 3. No significant interactions existed between

Table 3. Effects of Adh genotype and individual body mass on blood chemistry variables. Tabled are the P -values for each variable in each analysis of covariance (ANCOVA; Rohlf & Sokal 1981; Sokal & Rohlf 1981). ANCOVA was conducted using the GLM procedure in SAS. In analyses with non-significant interaction terms between mass and Adh, the interaction term was dropped and the model rerun. In such cases, Xs replace the value of P

	ATP/HB	ATP	Hb	DPG/HB	DPG
ADH	0.0499	0.4480	0.0245	0.6664	0.7979
Mass	0.1006	0.9880	0.0065	0.9846	0.4956
Mass × ADH	XXXXXX	XXXXXX	0.0231	XXXXXX	XXXXXX
Contrasts					
ADH-SS vs ADH-FF	0.0196	0.2107	0.0214	0.8479	0.5988
Heterozygotes vs homozygotes	0.6769	0.8667	0.4015	0.3837	0.6771

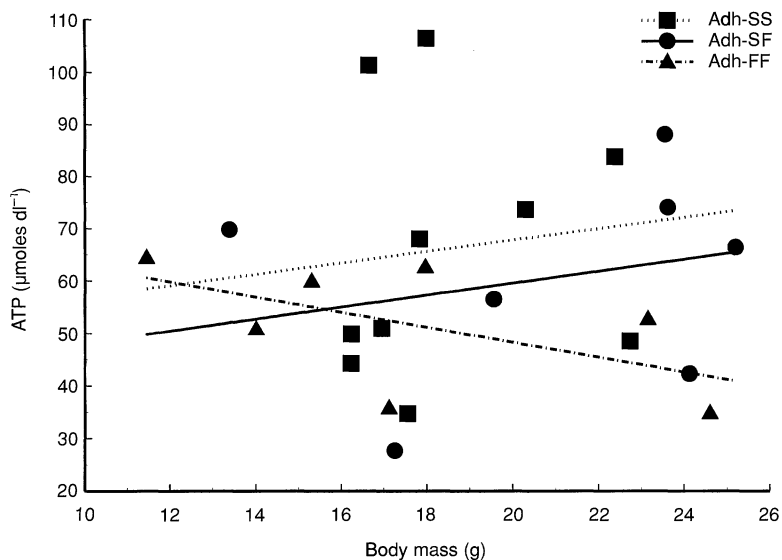


Fig. 2. ATP as a function of body mass for each of the three Adh genotypes. Refer to Table 3 for statistical significance of main effects and covariate.

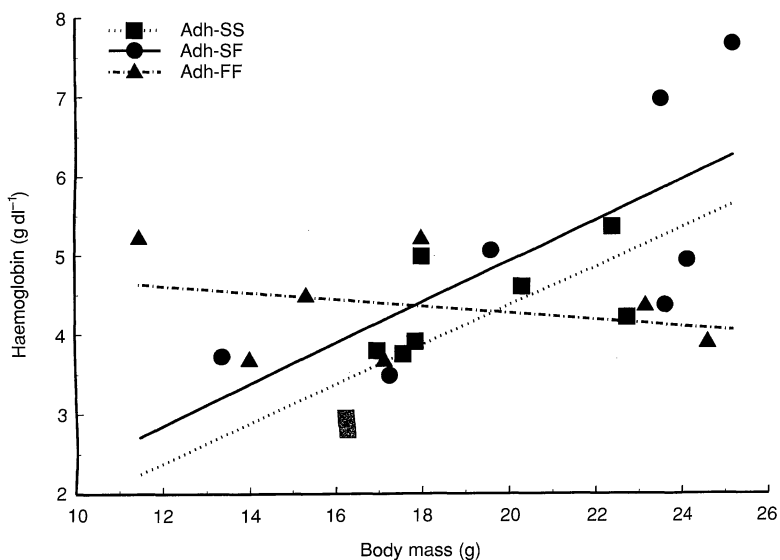


Fig. 3. Hemoglobin as a function of body mass for each of the three Adh genotypes. Refer to Table 3 for statistical significance of main effects, covariate and the interaction.

Adh and body mass, so that term was dropped from the statistical analysis; the effect of body mass was marginally non-significant ($P = 0.10$) and was retained in the final model. Adh had a marginally significant effect on ATP/Hb ($P = 0.0499$; Table 3), with the heterozygote being intermediate between the two homozygotes. The *a priori* comparison of the heterozygotes *vs* both homozygotes revealed no significant differences, as would be expected by the intermediate position of the heterozygote. However, the difference between the two homozygotes was statistically significant ($P = 0.019$; Table 3). The total amount of ATP and the total amount of Hb were also analysed separately. No statistically significant differences existed

for any variables in the analysis of ATP (Table 3 and Fig. 2); however, Hb showed a significant interaction between Adh and body mass, significant main effects of Adh and body mass, and a significant difference between homozygotes (Table 3 and Fig. 3). Hb in the Adh-FF homozygote declined with increasing body mass, while the other two genotypes showed a positive relationship between body mass and Hb. Finally, no statistically significant differences were measured in the analysis of 2,3-DPG/Hb or in the analysis of 2,3-DPG alone (Table 3).

Discussion

The measurement of k_{cat}/K_m ratios identified significant differences in catalytic efficiency among Adh allozymes in the acetaldehyde-to-ethanol reaction direction but not in the ethanol-to-acetaldehyde direction. The fact that the Adh heterozygote showed the highest k_{cat}/K_m ratio suggests that overdominance (i.e. direct heterozygote superiority) is occurring at this locus. Overdominance is rare for enzyme kinetic variables, but has been reported for Pgi in *Colias* butterflies (Watt 1983) and for Pgm in oysters (Pogson 1991). Biochemical overdominance in this case is consistent with the results of Carter (1997) which showed patterns of overdominance in relation to Darwinian fitness in that Adh-SF individuals metamorphosed equally well in all environments, irrespective of the oxygen conditions of the water. The significantly higher k_{cat}/K_m for the Adh-SS allozyme *vs* the Adh-FF is more difficult to interpret in the context of the results of Carter (1997), although in that study Adh-FF individuals metamorphosed poorly in both hypoxic and hyperoxic water, whereas Adh-SS metamorphosed poorly only in hypoxic water. Regardless, the kinetic results presented herein are consistent with selection occurring for variation at the Adh locus.

These results also suggest that associations between multilocus heterozygosity and components of fitness may be caused, at least in part, by functional differences among the allozymes of the individual loci used in multilocus studies. In the original study by Mitton *et al.* (1986) multilocus heterozygosity was positively associated with active oxygen consumption and negatively associated with resting oxygen consumption, and Adh and Pgi were the two loci shown to significantly contribute to those relationships. Carter (1997) provided more information on the relationships between Adh and oxygen consumption, but did not clarify the role of Adh in the results of Mitton *et al.* (1986). The enzyme kinetic data presented herein shows Adh-SF individuals having the highest k_{cat}/K_m ratio, which is consistent with Adh having a significant impact on the multilocus results of Mitton *et al.* (1986). Hence, these data support the idea that the multilocus results of Mitton *et al.* (1986) may be explained in part by functional differences at individual loci. Such a result does not exclude explanations developed by Clark &

Koehn (1992) and Hawkins & Day (1999), but does argue that the biochemical function of the individual loci be considered when interpreting results of multi-locus studies.

Precisely how the biochemical differences measured among Adh allozymes might influence Darwinian fitness is still unclear. The ATP/Hb was significantly associated with Adh, with it being lowest in Adh-FF individuals, suggesting that those individuals may have a left-shifted Hb-oxygen dissociation curve relative to Adh-SS individuals. Such a left-shift is frequently associated with acclimation to hypoxia (Wood *et al.* 1982); Carter (1997) demonstrated that frequencies of Adh-SS individuals were low, and frequencies of Adh-FF individuals were high, in natural populations of salamanders living in hypoxic ponds. However, Carter (1997) also measured lower than expected metamorphosis of Adh-FF individuals in hypoxic water. Hence Adh-FF individuals may be better acclimated to hypoxia, which would account for their higher frequency in hypoxic ponds, although such acclimation would not seem to assist them during metamorphosis. Additional work focusing on ATP/Hb and other factors which influence oxygen delivery to tissues is required to clarify how kinetic differences among Adh allozymes might affect organismal traits, such as acclimation to hypoxia and the ability to metamorphose. In addition, the effect of environmental conditions (e.g. temperature, pH) on Adh kinetics and ATP/Hb levels need to be investigated.

The analysis of Hb revealed a significant interaction between body mass and Adh genotype, with the relationship between body mass and Hb slightly negative in Adh-FF, and strongly positive in the other two genotypes. This suggests that Adh-FF individuals have relatively higher Hb when smaller (and hence younger) and relatively less when larger (and older); however, the importance of this difference, and whether it is related to metamorphosis, is not clear.

Although the results presented herein are consistent with Adh variation having physiological and adaptive consequences, they raise many questions about the function of Adh in these animals. First, what is the functional role of this enzyme in Tiger Salamanders? The fact that Adh variation is associated with oxygen tension and metamorphosis (Carter 1997) and that significant differences were measured in k_{cat}/K_m in the acetaldehyde-to-ethanol direction (Table 2), suggests that these animals may produce ethanol under hypoxic conditions, as some teleost fish do under anoxia (Shoubridge & Hochachka 1980, 1981, 1983; Van den Thillart & Van Waarde 1985, 1991; Nilsson 1988; Van den Thillart & Verbeek 1991). In those fish, production of ethanol as the end-product of anaerobic metabolism prevents the accumulation of toxic metabolic products because the ethanol probably escapes by diffusion across the gills into the surrounding water. Although this process inflicts a cost via a loss of carbohydrate,

it does seem to help survival of anoxia. In addition, eliminating ethanol in this fashion may affect the energetic status of the animal by helping maintain a proper redox balance (Burton & Place 1986).

Second, how is Adh function related to ATP/Hb, and to Hb itself? Once the organismal function of Adh and ethanol is understood, hypotheses concerning connections between Adh function and ATP/Hb can be constructed based on potential effects of Adh on redox balance, and hence ATP production, as described by Burton & Place (1986). Work on *Fundulus* has revealed numerous functional connections between Ldh and ATP/Hb (Powers *et al.* 1993), and similar lines of research could be developed for Adh in Tiger Salamanders. However, what the effect of Adh on Hb itself may be is currently not clear.

Finally, how might varying water oxygenation affect the kinetics of Adh? Although Adh genotype frequency is correlated with pond oxygen levels, no such relationship exists for temperature (Carter 1997), which is an environmental variable that frequently impacts enzyme function in ectotherms (e.g. Powers *et al.* 1993; Watt 1991). We currently favour the hypothesis that intracellular pH changes as a salamander experiences differing water oxygen levels, with pH falling under hypoxic conditions, and perhaps rising under hyperoxic conditions. Such a hypothesis could be tested in laboratory studies of Tiger Salamanders; once the range of internal pH environments that the animals experience has been established, additional kinetic studies on Adh allozymes at varying pHs could be conducted.

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References

- Adams, H. (1963) 5' Triphosphate determination with phosphoglycerate kinase. *Methods of Enzymatic Analysis* (ed. H. U. Bergmeyer), pp. 539–543. Academic Press, New York.
- Bucher, T. (1947) Über ein phosphatübertragendes garungsferment. *Biochimica Biophysica Acta* **1**, 292–299.
- Burton, R.S. & Place, A.R. (1986) Evolution of selective neutrality: further considerations. *Genetics* **114**, 1033–1036.
- Carter, P.A. (1997) Maintenance of the Adh polymorphism in tiger salamanders I. Genotypic differences in time to metamorphosis in extreme oxygen environments. *Heredity* **78**, 101–109.
- Clark, A.G. & Koehn, R.K. (1992) Enzymes and adaptation. *Genes in Ecology* (eds R. J. Berry, T. J. Crawford &

- G. M. Hewitt), pp. 193–228. Blackwell Scientific Publications, Oxford.
- Cornell, N.W. & Veech, R.L. (1983) Enzymatic measurement of ethanol or NAD in acid extracts of biological samples. *Analytical Biochemistry* **132**, 418–423.
- Endler, J.A. (1986) *Natural Selection in the Wild*. Princeton University Press, Princeton, NJ.
- Feder, M.E. & Watt, W.B. (1992) Functional biology of adaptation. *Genes in Ecology* (eds R. J. Berry, T. J. Crawford & G. M. Hewitt), pp. 365–392. Blackwell Scientific Publications, Oxford.
- Hawkins, A.J.S. & Day, A.J. (1999) Metabolic interrelations underlying the physiological and evolutionary advantages of genetic diversity. *American Zoologist* **39**, 401–411.
- Hochachka, P.W. & Somero, G.N. (1984) *Biochemical Adaptation*. Princeton University Press, Princeton, NJ.
- Julia, P., Farres, J. & Pares, N. (1987) Characterization of three isozymes of rat alcohol dehydrogenase. *European Journal of Biochemistry* **162**, 179–189.
- Keitt, A.S. (1966) Pyruvate kinase deficiency and related disorders of red cell glycolysis. *American Journal of Medicine* **41**, 762.
- Lowry, O.H., Passonneau, J.V., Hasselberger, F.X. & Schulz, D.W. (1964) Effect of ischemia on known substrates and cofactors of the glycolytic pathway in the brain. *Journal of Biological Chemistry* **239**, 18.
- Mitton, J.B. (1997) *Selection in Natural Populations*. Oxford University Press, New York.
- Mitton, J.B., Carey, C. & Kocher, T.D. (1986) The relation of enzyme heterozygosity to standard and active oxygen consumption and body size of tiger salamanders, *Ambystoma tigrinum*. *Physiological Zoology* **59**, 574–582.
- Nilsson, G.E. (1988) A comparative study of aldehyde dehydrogenase and alcohol dehydrogenase activities in crucian carp and three other vertebrates: apparent adaptations to ethanol production. *Journal of Comparative Physiology B* **158**, 479–485.
- Peterson, G.L. (1977) A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Analytical Biochemistry* **83**, 346–356.
- Pogson, G.H. (1991) Expression for overdominance for specific activity at the phosphoglucosmutase-2 locus in the Pacific oyster, *Crassostrea gigas*. *Genetics* **128**, 133–141.
- Powers, D.A., Smith, M., Gonazles-Villasenor, I., DiMichele, L., Crawford, D., Bernardi, G. & Lauerman, T. (1993) A multidisciplinary approach to the selectionist/neutralist controversy using the model teleost, *Fundulus heteroclitus*. *Oxford Surveys in Evolutionary Biology* **9**, 43–108.
- Rohlf, F.J. & Sokal, R.K. (1981) *Statistical Tables*. Freeman, New York.
- Rose, Z.B. & Liebowitz, J. (1970) Direct determination of 2,3-diphosphoglycerate. *Analytical Biochemistry* **35**, 177–183.
- Shoubridge, E.A. & Hochachka, P.W. (1980) Ethanol: novel end product of vertebrate anaerobic metabolism. *Science* **209**, 308–309.
- Shoubridge, E.A. & Hochachka, P.W. (1981) The origin and significance of metabolic carbon dioxide production in the anoxic goldfish. *Journal of Molecular Physiology* **1**, 315–338.
- Shoubridge, E.A. & Hochachka, P.W. (1983) The integration and control of metabolism in the anoxic goldfish. *Journal of Molecular Physiology* **4**, 165–195.
- Sober, E. (1984) *The Nature of Selection: A Philosophical Inquiry*. Bradford/MIT Press, Cambridge, MA.
- Sokal, R.K. & Rohlf, F.J. (1981) *Biometry*. Freeman, New York.
- Van den Thillart, G. & Van Waarde, A. (1985) Teleosts in hypoxia: aspects of anaerobic metabolism. *Molecular Physiology* **8**, 393–409.
- Van den Thillart, G. & Van Waarde, A. (1991) pH changes in fish during environmental anoxia and recovery: the advantages of the ethanol pathway. *Physiological Strategies for Gas Exchange and Metabolism* (eds A. J. Woakes, M. K. Grieshaber & C. R. Bridges), pp. 173–190. Cambridge University Press, Cambridge.
- Van den Thillart, G. & Verbeek, R. (1991) Anoxia induced oxygen debt of goldfish (*Carassius auratus* L.). *Physiological Zoology* **64**, 525–540.
- Watt, W.B. (1983) Adaptation at specific loci. II. Demographic and biochemical elements in the maintenance of the *Colias* PGI polymorphism. *Genetics* **103**, 691–724.
- Watt, W.B. (1991) Biochemistry, physiological ecology, and population genetics – the mechanistic tools of evolutionary biology. *Functional Ecology* **5**, 145–154.
- Wood, S.C., Hoyt, R.W. & Burggren, W.W. (1982) Control of hemoglobin function in the salamander, *Ambystoma tigrinum*. *Molecular Physiology* **2**, 263–272.

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