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Anuran activity energetics

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AN ABSTRACT OF THE THESIS OF Scott R. Landrey for the Master of Science in Biology presented July 16, 1982.

Title: Anuran Activity Energetics

Subtitle I: The Correlation of LDH Michaelis Constant, Specific Activity and Isoenzyme Patterns in Nine Species of Anurans

Subtitle II: Physiological Differences in Exercised, Unexercised and Fresh Caught Frogs

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

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PART I Anuran Lactate Dehydrogenases

The gastrocnemius muscle of nine species of anurans were assayed for lactate dehydrogenase specific activity, apparent K_m and LDH isozyme ratio. There was no correlation found between LDH isoenzymes and either specific activity or K_m . A strong correlation existed

between specific activity and K_m ($r = .94$). The M subunit is responsible for the increase in specific activity with an increase in K_m in the anurans tested. This evidence suggests the M-LDH subunit maximized K_m to increase specific activity in different species of anurans. Studies involving environmental factors in respect to conservation of K_m in fish M4 LDH are possible explanations of the kinetic differences in different anurans.

PART II Reconditioning in *Rana catesbeiana*

Rana catesbeiana were exercised to fatigue three times a week from the time of capture. At the end of the sixth week the frogs were tested for resistance to fatigue and maximal oxygen consumption rate. To properly assess the effects of training, a group of frogs was tested immediately after capture and a group of frogs was tested six weeks after capture that was not exercised. The gastrocnemius muscle was homogenized and assayed for maximal lactate concentration, citrate synthase activity and lactate dehydrogenase isozyme activity. The blood was assayed for hemoglobin concentration. The unexercised frogs had significantly lower hemoglobin concentration and $\dot{V}O_{2\max}$ than both the exercised and immediately captured frogs. Therefore, cardiovascular deconditioning should be addressed in activity studies involving captive anurans.

ANURAN ACTIVITY ENERGETICS

by
Scott Landrey

A thesis submitted in partial fulfillment of the
requirements for the degree of

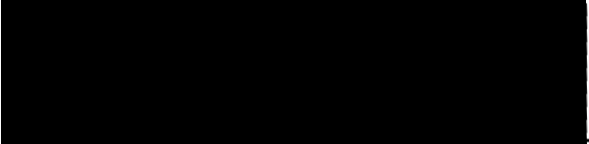
MASTER OF SCIENCE
in
BIOLOGY


Portland State University


1982

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:


The members of the Committee approve the thesis of Scott Landrey
presented July 30, 1982.

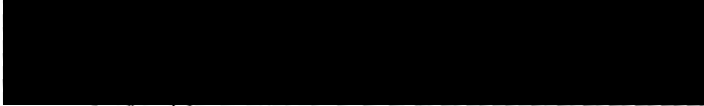

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TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vi
LIST OF FIGURES	vii
INTRODUCTION	1
PART I	
INTRODUCTION	6
MATERIALS AND METHODS	7
Experimental Animals	7
Experimental Procedure	7
RESULTS	9
LDH Isozyme Patterns	9
Species Differences in the Percent of LDH Subunits	9
Specific Activity vs Isozyme Ratio	10
Michaelis Constant vs Isozyme Ratio	10
DISCUSSION	10
PART II	
INTRODUCTION	23
MATERIALS AND METHODS	24

PAGE

Experimental Animals	24
Experimental Procedure	24
RESULTS	26
DISCUSSION	28
REFERENCES	35

LIST OF TABLES

PART I

TABLE

PAGE

I Lactate dehydrogenase maximal specific activity (S.A.) and apparent Km as determined by the Linweaver-Burke plot for nine species of anurans. Percent type LDH isozyme subunit is also given. Assay conditions explained in materials and methods. For all regressions $P < 0.05$ 12

PART II

I A chart of the results of the field (F), chronically exercised-captive (E), and unexercised-captive (Un) *Rana catesbiana*. The frogs were examined for weight (GW); for the aerobic parameters, maximal oxygen consumption ($\dot{V}O_2$ max), hemoglobin concentration (Hb), and citrate synthase activity (CS); and for the anaerobic parameters of fatigued muscle lactate concentration at 5 minutes into exercise (lactate) and muscle lactate dehydrogenase activity (LDH). An * denotes this group was significantly different from the other two groups. 29

LIST OF FIGURES

PART I

FIGURE	PAGE
1. LDH isozyme patterns of five anuran gastrocnemius muscles .	11
2. Graph of LDH specific activity (μ mole/min-gm tissue) versus Km (m molar) in nine species of anurans.	13 13
3. Graph of LDH specific activity versus percent type H-LDH in nine species of anurans.	14
4. Graph of Km versus percent type H-LDH in nine species of anurans.	15

PART II

1. Performance as time to fatigue in minutes during the exercise regime of 42 days in the chronically exercised frogs.	30
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INTRODUCTION

This thesis presents two separate experiments concerning aerobic and anaerobic amphibian energetics. The first study (Part I) investigates differential alterations in the kinetics of the anaerobic enzyme lactate dehydrogenase, in nine species of anurans with varying natural histories. The second study (Part II) studies changes in different anaerobic and aerobic physiological parameters in a single species (*Rana catesbeiana*) after six weeks of chronic exercised, unexercised-captive and for field frogs. The common theme of these two experiments involves the potential for physiological changes due to environmental stress. Part I examines different species to infer a possible evolutionary relationship to environmental stresses at the molecular level of lactate dehydrogenase. Part II comprises the effects on captivity on aerobic and anaerobic capacities of *R. catesbeiana*.

The pattern of activity metabolism varies between toads and frogs. Frogs exhibit a pronounced saltatory response during an activity bout, often to the point of total exhaustion (Bennett, 1974). In contrast, toads move slowly in response to threatening situations and do not appear to exhaust. Bennett and Licht (1974) suggest that burst activity in frogs and some other amphibians, is achieved with extensive glycolysis i.e. anaerobic metabolism and high lactate accumulation). Toads do not accumulate as high a concentration of lactate during an

activity bout as do frogs (Carey, 1979).

While Hutchison and Miller (1979) support the premise that lactate levels of terrestrial amphibians are strongly correlated with the animals' pattern of activity, their data on aquatic amphibians do not fit the activity pattern hypothesis. The aquatic frog, *Xenopus*, (Hutchison and Miller, 1979) is both active and produces very high quantities of lactate. Alternatively, the Urodele, *Necturus*, is less active and produces much less lactate than *Xenopus* yet still relies heavily on anaerobic lactate metabolism for energy above maintenance needs (Miller and Hutchison, 1979).

Taigen et al (1982) measured aerobic and anaerobic metabolism during maximum exercise in 7 families (17 species total) of anurans of varying ecological types and locomotor modes. Their study showed that no single factor (i.e. predator avoidance, predatory mode or locomotory pattern) alone could explain differences in the exercise physiology of each species. Rather, the exercise physiology is interrelated to many factors of their biology.

Bennett (1974) has examined the relative activity rates and apparent Michaelis constants (K_m) of the glycolytic enzymes phosphofructokinase (PFK, ATP: D-fructose 6-phosphate 1-phosphotransferase) and lactate dehydrogenase (LDH, NAD + : Lactate oxidoreductase) in an attempt to establish a physiological basis for the difference in patterns of energy generation between frogs and toads. The enzyme activity in frog PFK and LDH was twice that of the toad. The K_m for each enzyme in both species was the same. Bennett concludes: "The differential demands for activity in this group may be met solely by

differential enzymatic concentrations and not alterations of enzymatic composition of structure".

There are two ways an animal can increase enzyme specific activity (activity/gm tissue). First, by increasing the enzyme concentration, secondly, by increasing the catalytic properties of the enzyme. When the enzyme concentration is increased, a change in specific activity alone is evidenced. However, if the catalytic properties are changed, both the K_m and the specific activity may be altered. The tissue homogenate's catalytic properties could be altered either by changing the subunit composition (isozyme) or the structure of a single type of enzyme in the tissue.

In diploid vertebrate tissue two subunits of LDH are synthesized: the "aerobic" type H (heart) LDH subunit, and the "anaerobic" type M (muscle) LDH subunit. LDH is a tetramer with subunits that can randomly recombine. The H and M subunits can combine to form five LDH isozymes. In vertebrate muscle the M type kinetically favors pyruvate reduction to lactate during anaerobic glycolysis. The type H-LDH subunit predominates in the heart tissue and kinetically favors lactic acid oxidation to pyruvate during respiration (Kaplan et al, 1968; Cahn, 1962; Dawson et al, 1964). LDH hybrid isozymes follow closely with slight deviation predicted and experimental kinetic values for stoichiometric mixtures of H₄ and M₄ LDH (Braswell Data, 1975). Breast muscle LDH of birds shows a strong correlation between type of flight and proportion of H-LDH subunits (Wilson, 1963). Various species of amphibians also have differing amounts of hybrid LDH isozymes in their striated muscle (Salthe, 1965).

Alternatively, Vessel (1966, 1968) and Wuntch et al (1970) claim that the physiological significance of LDH isozymes might not be due to kinetic differences (pyruvate inhibition) or to be correlated to the aerobicity of the tissue investigated. If anuran muscle does not show a correlation between LDH isozyme subunit proportion and either the aerobicity, glycolytic potential or activity pattern, yet there is still a difference in LDH kinetics, it is possibly due to changes in the LDH between species. Atkinson (1977) has concluded it would be energetically advantageous for an organism to change an enzyme's catalytic ability rather than to increase the enzyme concentration.

Glycolytic enzymes such as LDH can increase substrate turnover (Fersht, 1977 and Hochachka, 1980). Fersht states it is catalytically advantageous to bind substrates weakly to maximize substrate turnover. An enzyme with strong binding (low K_m) to its substrate, yet with substrate concentrations much greater than the K_m would be rate limited by the enzyme-substrate and enzyme product binding. This is alleviated by strong binding of the transition state first proposed by Haldane (1930) and elaborated by Pauling (1948) and weak binding of the enzyme-substrate and enzyme-product.

Adaptations to sustained chronic exercise in amphibians has recently been studied in *Rana pipiens* (Cummings, 1980) and *Xenopus laevis* (Miller and Camelliere, 1981). In both studies, exercised anurans increased performance compared to their respective unexercised controls. This increase in performance was correlated to an increase in citrate synthase activity in exercised Ranads (Cummings, 1980). Cummings associated this with an increase in aerobic metabolism in Ranads since

an analogous increase in citrate synthase activity in mammals parallels an increase in $\dot{V}O_2$ (Holloszy et al, 1976; Winder et al, 1973). Exercised *Xenopus* showed no such correlation between increased performance and citrate synthase activity (Miller and Camelliere, 1981).

Whether chronic exercise improves performance, or whether it is only reconditioning, frogs is the major question addressed in this paper. In contrast to the previous two anuran chronic exercise papers, this paper will compare field animals, trained and untrained. Neither Cummings (1979) or Miller and Camelliere (1981) compared their exercised frogs to field frogs. Both previous studies purchased frogs from commercial suppliers. No mention was made about how long these animals were in confinement and captivity. In humans, bed confinement causes deconditioning of performance paralleled with muscular and cardiovascular deterioration (Stremmel et al, 1976; Salton et al, 1968). Therefore, if analogous deconditioning processes occur in frogs, the differences in performance as well as the physiological parameters between trained and untrained frogs might only be to maintain the trained animals at field frog levels of conditioning.

Neither Cummings (1979) or Miller and Camelliere (1981) measured maximal oxygen consumption rates ($\dot{V}O_{2max}$) directly in their training studies. In mammals, $\dot{V}O_{2max}$ and stamina (performance) correlate with blood oxygen carrying capacity (Wranne and Woodson, 1973; Woodson et al, 1978; Ekblom et al, 1972). A correlation between $\dot{V}O_{2max}$ and hemoglobin concentration with no correlation between stamina and $\dot{V}O_{2max}$ or hemoglobin concentration has been demonstrated in anurans (Hillman, 1980).

PART I

INTRODUCTION

It is believed that frogs have greater anaerobic (burst) metabolism than toads, due to the frogs' higher concentration of lactate dehydrogenase (LDH) in its striated muscle and not alteration of catalytic rate of the frogs' lactate dehydrogenase (Bennett, 1974).

These conclusions were correlated to the animals' locomotor and predator defence behavior. Where the more anaerobic frogs escape predation by rapid saltatory burst, the more aerobic toad uses static defense postures. Taigen (1982) has recently shown that the behavioral patterns of seventeen anurans are much more diverse than Bennett's frog-toad model.

Bennett (1974) did not consider altering the LDH subunit type as a possible method of increasing the specific activity of the frog muscle over the toad. A relationship was found between the proportion of H type LDH subunits in bird breast muscle and each species of birds manner of flight (Wilson, 1963). Different species of anurans have differing LDH isozyme patterns in their striated muscle. Even if isozymes are not significant in altering the LDH specific activity of different anurans, a recent theory on the maximization of catalytic rate by increasing K_m in non-regulatory glycolytic enzymes (Fehrst, 1978; Hochachka, 1980) would contradict Bennett's (1974) finding of no alteration in K_m with

increased specific activity from toads to frogs.

By using classical enzyme kinetic and electrophoretic assays with nine species of anurans, this paper examines the following interrelated questions: 1) Do anurans with greater anaerobic ability have a higher LDH activity? 2) If there is an increase in activity, is it due to an increase in enzyme concentration or alteration of the Michaelis constant (K_m)? 3) If there is an alteration of K_m , is it due to differential isozyme subunit ratios, or alteration of the enzyme substrate binding of a single type of LDH subunit between different species?

MATERIALS AND METHODS

Experimental Animals

Rana pipiens and *Xenopus laevis* were purchased from biological supply houses. *Rana aurora* and *Hyla regilla* were collected in the tricounty area, Portland, Oregon. *Scaphiopus intermountainus* were collected in Deschutes County, Oregon, *Bufo alvarius*, *Bufo cognatus* and *Scaphiopus couchi* were collected in Southern Arizona (Arizona Scientific Collecting Permit 067, 1981).

Experimental Procedure

Experimental animals were killed by a blow to the head. The heart and gastrocnemius muscle were each removed and weighed; then homogenized in 3-10 volumes of 0.1M phosphate buffer pH 7.0. The resulting homogenate was centrifuged for ten minutes at full speed in a clinical centrifuge at 3000 rpm. The supernatant was immediately assayed for LDH activity then frozen.

Lactate dehydrogenase isozyme patterns were examined using starch gel electrophoresis. The starch gel consisted of 10% starch and 0.1M histidine gel buffer, pH 7.0. The tray buffer consisted of 0.05M citric acid-NaOH buffer, pH 7.0. The samples were electrophoresed for 6-8 hours at 150 volts, 30 amps, at 5°C. The bands were stained using a solution containing 384 mg lithium lactate, 24 mg nicotinamide adenine dinucleotide, 20 mg nitro blue tetrazolium and 0.8 mg para methyl sulfate dissolved in 40 ml of 0.1M tris buffer pH 8.

The LDH patterns were quantified using a Joyce Loebel densitophotometer. Each peak (isozyme band) was integrated by weighing the paper trace. The percentage of type H or type M LDH subunits were calculated from the integrated densitophotometer readings as follows:

$$\%M = \left[\frac{\text{wt. of M4 peak}}{\text{wt. of total}} \right] + \left[\frac{\text{wt. of M3H}}{\text{wt. of total}} \right] 0.75 + \left[\frac{\text{wt. of M2H2}}{\text{wt. of total}} \right]$$

$$0.5 + \left[\frac{\text{wt. of MH3}}{\text{wt. of total}} \right] 0.25$$

$$\%H = 100 - \%M$$

Each specimen's supernatant was assayed at 0.2, 0.15, 0.1, 0.05, 0.033, and 0.02 mM pyruvate in an assay medium containing 0.15 mM NADH, 0.1M phosphate buffer (pH 7.0, 20°C) to determine the maximal velocity and Km. Three individuals of each species were plotted on a lineweaver-Burke double reciprocal plot. The correlation coefficient, x(1/Km) and y(1/Vm) intercepts were determined by least squares linear regression. The regression coefficients were tested for significance using the student t-test.

Specific activity (S.A.) of the type M and type H LDH isozyme subunits were calculated from the S.A. of the homogenate as:

$$\text{S.A. type M} = \text{S.A. total homogenate} \times \%M$$

$$\text{S.A. type H} = \text{S.A. total homogenate} \times \%H$$

RESULTS

LDH Isozyme Patterns

Electrophoresed gastrocnemius muscle and heart tissue homogenates stained for LDH for five species are shown in Figure 1. This zymogram clearly shows different species of anurans have LDH differing structurally and compositionally (isozymic). *Hyla regilla* gastrocnemius muscle homogenates express four LDH bands; form M4 on the origin to MH3 migrating most anodally. *Scaphiopus couchi* has three bands; the M4 1 cm from the origin with the M3H and the M2H2 migrating anodally. *Rana pipiens*, *Bufo cognatus*, and *Bufo alvarius* all have a large M4 band and a very small M3H band. *Bufo cognatus* M4 migrated 0.5 cm anodally from the origin, but *Rana pipiens* and *Bufo alvarius* had no M4 migration. The M4 electrophoretic differences between these species implies possible chemical differences in muscle type subunits. There are also clear qualitative and quantitative differences in the bands expressed between the different species.

Species Differences in the Percent of MLDH Subunits

Table I shows the quantified LDH isozyme patterns for each species in terms of percent of the total LDH in the gastrocnemius that are of the muscle type LDH subunits. Anurans have from 99% (*Bufo cognatus*)

down to 61% M-LDH subunits (*Scaphiopus intermountainous*).

Specific activity (S.A.) was related to the Km regression line coefficient of determination ($r = 0.94$, $P < 0.25$). Three clusters of animals are apparent: A group with both low Km's and low specific activities comprising *Bufo boreas*, *Rana aurora*, *Scaphiopus intermountainous*, *Bufo cognatus*, and *Hyla regilla*; a group with intermediate Km's and intermediate specific activities consists of *Bufo alvarius*, *Scaphiopus couchi*, and *Xenopus laevis*. Only *Rana pipiens* had both a high Km and high specific activity.

Specific Activity vs Isozyme Ratio

There was no significant correlation ($r^2 = 0.27$, $P = .5$) between increasing specific activity and isozyme ratio of LDH in these species of anurans (Figure 3). These data indicate that distinct compositional differences in the percent of type H subunit in various species of anurans has little or no bearing on the specific activity of the LDH muscle tissue homogenate.

Michaelis Constant vs Isozyme Ratio

No correlation between increasing Km and isozyme composition in anurans ($r^2 = 0.26$, $P = 0.5$) was found in these anurans (Figure 4).

DISCUSSION

The results of this study allow discussion of three interrelated questions, which for clarity will at first be considered separately:

1) Do anurans with greater anaerobic ability have a higher LDH

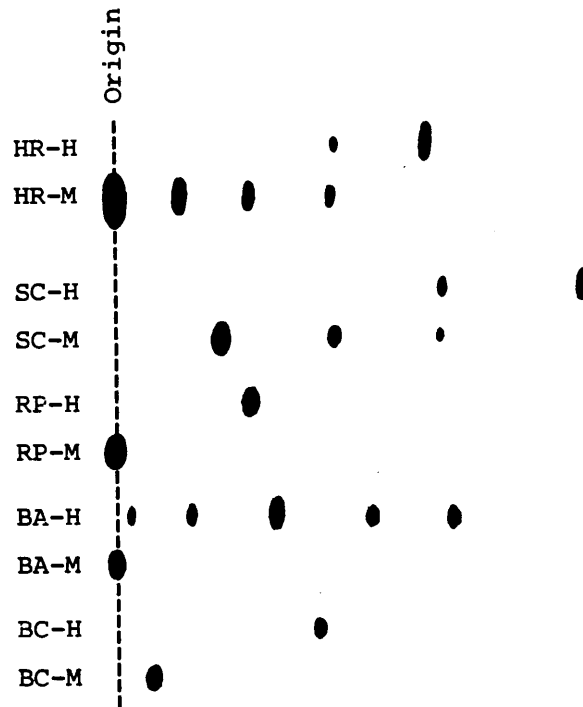


Figure 1. LDH isozyme patterns of five anuran gastrocnemius muscles

M and H = heart tissue homogenates

HR = *Hyla regilla*

SC = *Scapiopus couchi*

RP = *Rana pipiens*

BA = *Bufo avlarius*

BC = *Bufo cognatus*

TABLE I

LACTATE DEHYDROGENASE MAXIMAL SPECIFIC ACTIVITY (S.A.) AND APPARENT K_m AS DETERMINED BY THE LINWEAVER-BURKE PLOT FOR NINE SPECIES OF ANURANS. PERCENT TYPE LDH ISOZYME SUBUNIT IS ALSO GIVEN. ASSAY CONDITIONS EXPLAINED IN MATERIALS AND METHODS.
FOR ALL REGRESSIONS $P < 0.05$.

Species	S.A. IU/g-min x 10^3	K_m app. m molar	%M	%H
1. <i>Rana pipiens</i> $r = 0.98$	2.53	1.28	97	3
2. <i>Scaph. couchi</i> $r = 0.99$	1.57	0.54	86	14
3. <i>Bufo alvarius</i> $r = 0.99$	1.57	0.54	94	6
4. <i>Xen. laevis</i> $r = 0.98$	1.49	0.74	80	20
5. <i>Rana aurora</i> $r = 0.81$	0.568	0.21	93	7
6. <i>Scaph. intermount.</i> $r = 0.97$	0.369	0.19	61	39
7. <i>Bufo boreas</i> $r = 0.91$	0.268	0.36	85	15
8. <i>Hyla regilla</i> $r = 0.98$	0.258	0.07	84	16
9. <i>Bufo cognatus</i> $r = 0.99$	0.058	0.10	99	1

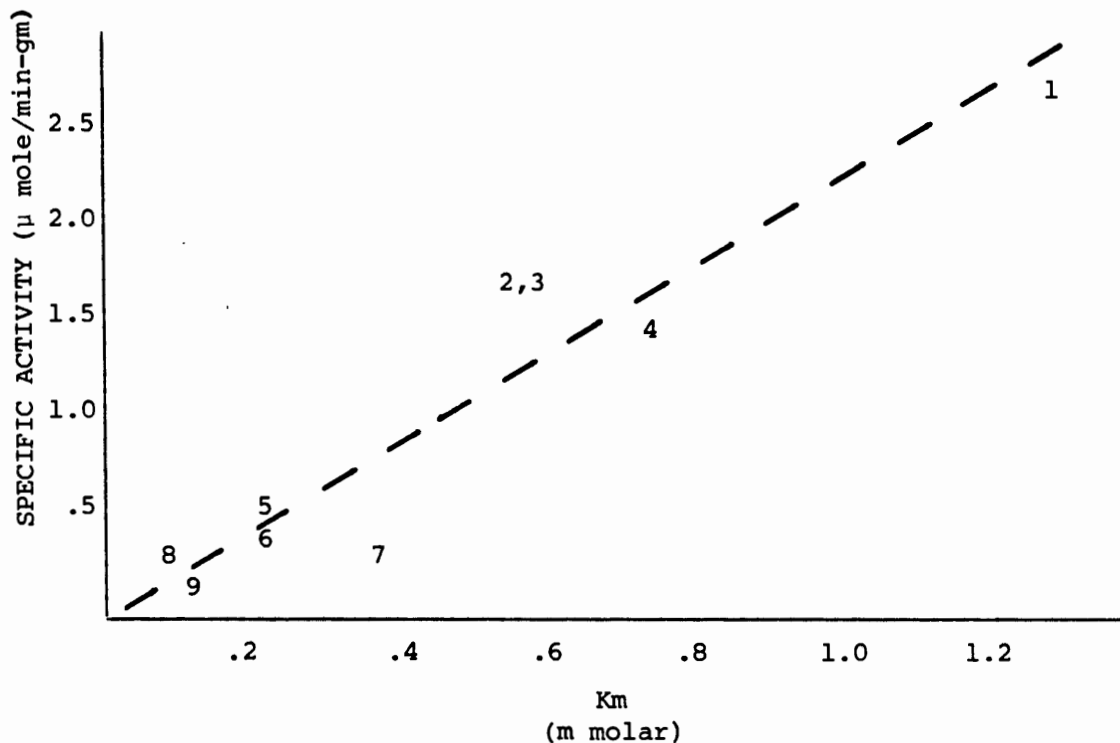


Figure 2. Graph of LDH specific activity (μ mole/min-gm tissue) versus Km (m molar) in nine species of anurans. Numbers on the following graph correspond to species below:

- 1 = *Rana pipiens*
- 2 = *Scaphiopus couchi*
- 3 = *Bufo alvarius*
- 4 = *Xenopus laevis*
- 5 = *Rana aurora*
- 6 = *Scaphiopus intermountainous*
- 7 = *Bufo boreas*
- 8 = *Hyla regilla*
- 9 = *Bufo cognatus*

Regression line S.A. = $2.07 \text{ Km} + 0.0038$.

Correlation coefficient $r = 0.94$ ($P < 0.025$)

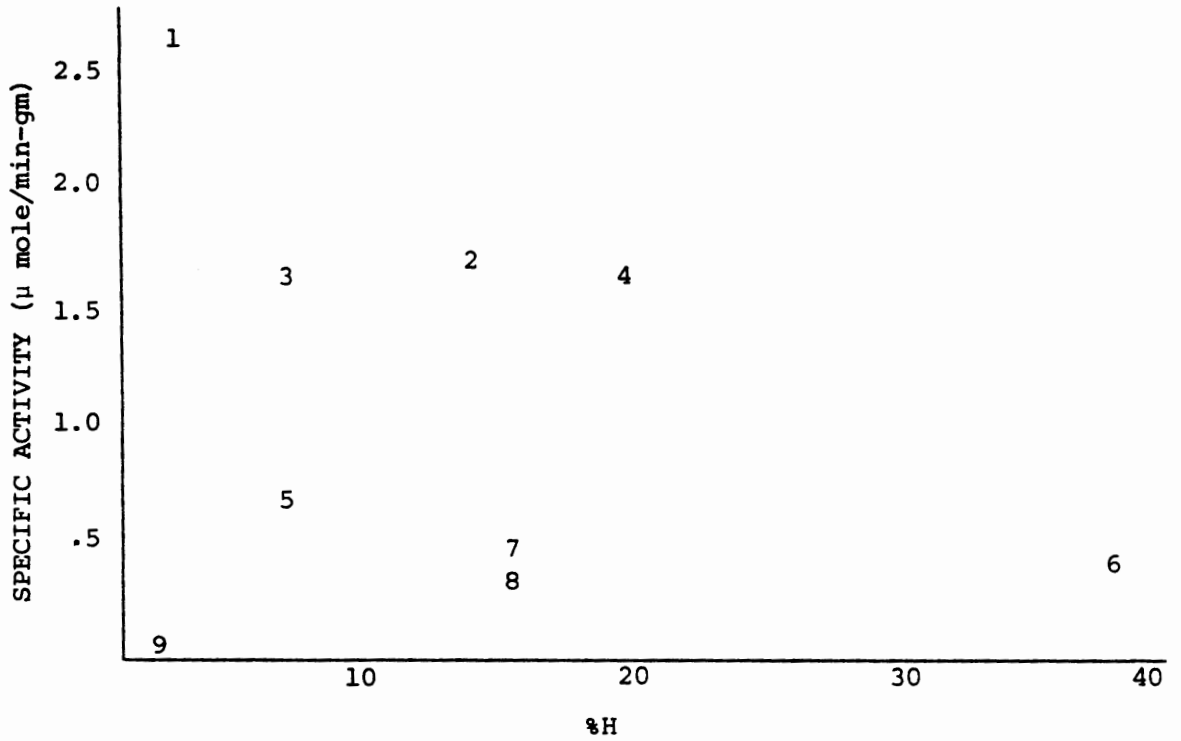


Figure 3. Graph of LDH specific activity versus percent type H-LDH in nine species of anurans. Numbers correspond to species as per Figure 2. Regression coefficient $r^2 = -0.27$ ($P=0.3$).

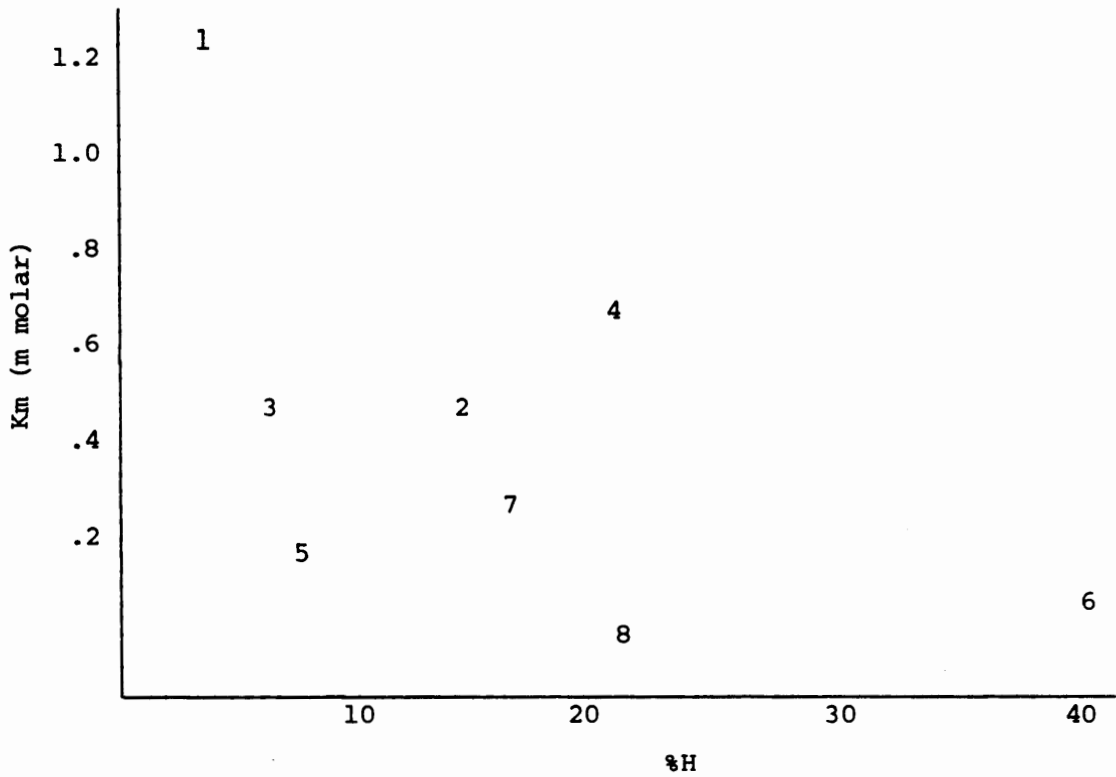


Figure 4. Graph of Km versus percent type H-LDH in nine species of anurans numbers correspond to species as per Figure 2. Regression coefficient $r^2 = -0.26$ ($P=0.9$).

activity? 2) Is this increase in activity due to an increase in enzyme concentration or an alteration in K_m ? 3) If there is an alteration in K_m is it due to differential isozyme ratios or alterations of a single LDH subunit between different species?

Both *Rana pipiens* and *Bufo boreas* activity patterns are correlated with their LDH specific activity (Bennett, 1974). Lactic acid levels are also correlated with these two anurans' activity patterns (Bennett, 1974; Putnam, 1979; Carey, 1979), as well as terrestrial amphibians in general (Hutchison and Miller, 1978). If only *Rana pipiens* and *Bufo boreas* are considered, the specific activity as well as isozyme patterns would fit the activity patterns of the two anurans. However, when data for all nine species of anurans in this study are analysed, this correlation is less clear. All Ranids are considered more anaerobic, with greater jumping ability than Bufonids. Yet, only *Rana pipiens*' LDH specific activity is significantly higher than several species of Bufonids. Salthe (1965) measured the pyruvate ratios of LDH in heart extracts of 85 amphibians. While there was a strong correlation between the LDH pyruvate ratio and environmental oxygen availability, a large overlap existed between 17 species of Ranids and three species of Bufonids. From Salthe's data (1965) and the findings of this paper, it is possible that the concentration of LDH in anuran tissue is not indicative of, or responsible for, the maximal lactate levels or the activity patterns of anurans.

In this study, LDH specific activity from nine species of anurans strongly correlate to an increase in the LDH apparent K_m . This is in contrast to Bennett's (1974) finding of no difference in K_m with LDH

Vmax in only two species. The LDH has decreased enzyme substrate binding affinity (higher K_m) in species with higher specific activity Figure 2. ($P < 0.025$). The correlation between increasing K_m and increasing specific activity is consistent with the theoretical treatment of Ferhst (1977). The concept that the enzyme binds the transition state of the substrate most strongly was first described by Haldane (1930) and elaborated by Pauling (1948). Furthermore, while the transition state of the substrate is bound strongly to the enzyme, Ferhst states that the substrate binding to the enzyme is minimized (higher K_m). In this study, high LDH specific activity (i.e. *Rana pipiens*) is achieved by decreasing the binding affinity of LDH for pyruvate as compared to anurans with lower specific activity (i.e. *Bufo boreas*).

Ferhst (1977) considered glycolytic enzymes as examples of enzymes which maximize rates to the loss of binding affinity. Lactate dehydrogenase fits well into the characteristic glycolytic enzyme with a highly evolved maximum rate. One criterion for a "perfectly evolved enzyme" that maximizes rate is that K_m is greater than the substrate concentration. This ratio (K_m/S) for LDH is 1.2 (Ferhst, 1977) and therefore LDH is an enzyme with reaction rate being diffusion limited. The results of this study show that LDH's from anurans have evolved a maximum rate for pyruvate reduction and this rate is increased between species by increasing K_m .

The change in LDH K_m with LDH rate is probably not isolated to anurans. A tentative correlation between LDH K_m and Vmax is apparent for three species of fish (Yancey and Somero, 1978).

Changing the V_{max} by changing K_m is one of the two ways which Atkinson (1977) believes an organism's enzyme can change enzyme activity relative to steady state substrate concentrations. The alternative; increasing the amount of protein committed to catalyze a given reaction is detrimental to the organism. Atkinson's reasoning for there being a limit to increasing a given protein is explained in this example: "Several enzymes catalyzing reactions in major pathways occur at concentrations near 10^{-5} M, but the levels of most enzymes must be considerably lower. One thousand enzymes at an average concentration of 10^{-5} M with an average molecular weight of 10^5 would sum to 1 kg/liter, or a density of 1 g/cm^3 . This would correspond approximately to the density of dry protein, and could be attained only if all water and other cell constituents were excluded."

A typical cell contains 50 mg/cm^3 protein (Atkinson, 1977). From this Atkinson (1977) states: "Long ago, at a time so remote that common ancestors of all present-day organisms were alive, organisms must have reached a point where an increase in the level of protein would have been injurious. Since that time, the evolution of further complexity (new sequences and new reactions, hence, new enzymes) has been possible only at the expense of either deletion or decrease in amount of preexisting enzymes. Decrease in amount of an enzyme, if its function is retained, requires an increase in catalytic effectiveness; thus there must have been rather strong selective pressure for improvement of enzymes in this regard."

The muscle cells of anurans are under the same constraints as described above by Atkinson. To increase LDH specific activity greatly

between species would require changes in catalytic activity instead of greatly increasing the concentration of LDH in muscle cells. This was observed in this work.

Skeletal muscle of amphibians expresses various combinations of the two LDH isozymes subunits (Salthe, 1965). These two subunits, H and M, have markedly different properties (Hochachka, 1980). The H type LDH is inhibited by lower concentrations of pyruvate than is the type M LDH. The H type LDH subunit is also the predominate subunit in aerobic organs such as the heart and favors lactate oxidation. M type LDH, the predominate LDH in muscle, favors pyruvate reduction during anaerobic glycolysis (Kaplan, et al, 1968; Markert, 1965). Differential combinations of H and M LDH subunits *in vitro* correlate to proportional changes in kinetic properties (Ainslie, 1971; Braswell, 1975). Vessel (1968) and Wuntch et al (1970) indicate that the physiological significance of LDH isozymes are not due to either pyruvate inhibition or correlated to the need for oxygen of the tissue investigated.

Although anurans in this study express between 1 to 40 percent H type LDH subunits in gastrocnemius muscle, there is no correlation between in vivo (homogenate) kinetic properties (K_m , $P=0.3$; specific activity, $P=0.9$) and isozyme ratio. Therefore, changes in LDH kinetic properties of species of anurans are not caused by in vivo variation of the isozyme ratios.

Furthermore, the type H LDH subunit is only a small percentage of the LDH in muscle. In eight of the nine anurans tested it was less than twenty percent. By including the fact that the H LDH subunit has both lower turnover number and K_m per unit enzyme than the type M LDH

(Hultin, 1975), it becomes clear that the type H subunit plays no significant part in the increase of K_m with anurans with increasing LDH specific activity. Therefore, an increase in K_m of anurans with increasing specific activity in crude muscle homogenates is probably due to alterations in M LDH subunit between the anurans.

Since the LDH specific activity and K_m of all nine species of anurans do not correlate with their respective maximum lactic acid accumulation and/or genus specific activity patterns, the eco-physiological significance of the differential kinetics of anuran M-LDH is open to question. Work done in the conservation of fish M4 LDH K_m in respect to the environmental temperature (Yancey and Somero, 1979), osmolality (Yancey and Somero, 1979), and pressure (Siebnaller and Somero, 1978) might give some clues, since amphibians also must respond evolutionarily to these same parameters. These parameters along with the anuran's activity patterns may explain most of the physiological significance of the differential kinetics of anuran LDH and would be of interest to study.

In fish M4 LDH, K_m 's follow closely the muscle pyruvate concentrations (Yancey and Somero, 1978). This agrees with Atkinson's (1976) idea that enzyme K_m 's are likely to have selective value for several interrelated reasons. A M4 LDH with too high a K_m (low affinity) may never use most of its catalytic ability. On the other hand, an M4 LDH with too low a K_m (high affinity) will be at its maximum capability at all times. During high glycolytic flux the LDH could not catalyze all the pyruvate, severely limiting production of ATP for burst metabolism by anaerobic metabolism.

An ideal M4 LDH will probably have a K_m somewhat greater than the pyruvate concentration of resting muscle; such an enzyme will not interfere with the oxidative metabolism during resting periods, react quickly to increasing pyruvate levels and yet have substantial catalytic capacity in reserve for periods of extreme anaerobiosis (Yancey and Somero, 1978). No comparative studies on anuran pyruvate concentration have been reported.

Environmental oxygen availability might play some role in elevating the K_m and LDH specific activity in some anurans. *Rana pipiens* remain inactive in the mud at the bottom of ponds up to five months during hibernation (Poczopko, 1959). The oxygen tension in the mud is very low (Hutchison et al, 1964; Poczopko, 1959). Both the resting oxygen consumption (Fromm and Johnson, 1955) and tissue metabolism (Barger and Johnson, 1941; Rose and Drotman, 1967) differ seasonally in frogs. Anoxic winter acclimated frogs submerged in 5°C water for greater than 120 hours increased lactate levels six times over that of resting levels (Christenson and Penny, 1973). *Rana pipiens* might use the low affinity M LDH to allow higher concentrations of lactate to accumulate in the tissue before mass action slows the enzyme to equilibrium (possibly prolonging ATP production during hibernation).

In summary, no significant correlation was found between the crude muscle homogenate LDH kinetics and maximum lactate concentrations or activity patterns from the respective species of anurans. The increase in LDH specific activity is strongly correlated to an increase in K_m . The H LDH subunit represents a small percentage of the total LDH in the muscle, and therefore contributes marginally to the activity of the

muscle homogenate. The increased K_m (lower affinity) of M LDH in anurans with higher M LDH specific activity agrees with Ferhst's (1977) theory for the evolution of maximum rate through maximizing K_m . Therefore, in anurans M LDH the rate of the pyruvate reduction is diffusion limited. The anurans with more LDH specific activity in their skeletal muscle do so by loosening their LDH-pyruvate binding and probably not by increasing enzyme concentration as proposed by Bennett (1974).

PART II

INTRODUCTION

Sustained chronic exercise increases performance in both *Rana pipiens* (Cummings, 1979) and *Xenopus laevis* (Miller and Camelliere, 1981). The frogs in both studies were bought from supply houses. It was not mentioned how long the animals were in captive confinement. In humans, bed confinement causes deconditioning of performance, muscles and the cardiovascular system (Stremmel, 1976; Salton et al, 1968). Both previous chronic exercise studies on frogs (Cummings, 1979; Miller and Camelliere, 1981) measured aerobic ability by citrate synthase activity and not by measuring maximal oxygen consumption directly.

This paper will examine the relationship of performance (stamina) of trained, untrained and field frogs to aerobic capacity directly ($\dot{V}O_2\text{max}$) and indirectly (citrate synthase activity). By examining aerobiosis at these different physiological levels this paper will discern: 1) if training and or deconditioning causes analogous changes in $\dot{V}O_2\text{max}$ and hemoglobin concentration as compared to Hillman's (1980) anemic frogs, 2) if aerobic capacity is affected by training and or deconditioning, at what level is it manifested, i.e. is $\dot{V}O_2\text{max}$ limited at the cardiovascular level or tissue level or both?

If training and or deconditioning does affect aerobic

capabilities, is anaerobic metabolism increased differentially to counteract the loss of aerobic ATP during an exercise bout? Hillman (1980) found an inverse correlation of muscle and blood lactate accumulation and aerobic capability in anemic frogs. Chronically exercised and unexercised *Rana pipiens* had the same resting and fatigue muscle lactate levels, but chronically exercised (trained) *Rana pipiens* had less lactate fifteen minutes after fatigue. Trained *Xenopus*, compared to untrained *Xenopus* had higher lactate levels both before and after an exercise bout. To assess the effects of training and or deconditioning on anaerobic metabolism, both muscle lactate concentrations and lactate dehydrogenase activity were assayed.

MATERIALS AND METHODS

Experimental Animals

Rana catesbeiana were collected in eastern Washington County, Oregon. The captured animals were returned to the lab and divided into three groups; trained, untrained, and field. The trained and untrained *R. catesbeiana* were kept in plastic containers in groups of three, at 18°C with a natural photoperiod. The containers were layered with small gravel and supplied with a dish of water. Animals were fed *Tenbrio* larvae ad libitum.

Experimental Procedure

The trained frogs were exercised three times a week for six weeks; by prodding the dorsal surface of the animal, thereby inducing the frog to hop. This practice was continued until the frogs became

refractory. The frog was then flipped over on its back. Failure of the animal to "right" itself after thirty seconds was considered fatigue. The fatigue point was the time at which the frog was last turned on its back.

The method for determining maximal oxygen consumption rates ($\dot{V}O_2\text{max}$) was that of Seymour (1973) and Hillman (1976). The frogs were rotated in a sealed metabolic chamber at 19°C. This has the effect of flipping the frog on its back and forcing it to expend energy to right itself. At five minutes an air sample was extracted from the chamber and analyzed with a Beckman OM-14 oxygen analyzer, to determine oxygen content. All values are expressed as $\text{ml O}_2 \text{ g}^{-1} \text{ h}^{-1}$ (STPD).

Bladder water was eliminated by cannulation, and the animals were weighed. The frogs were then stunned by a blow to the head. A small incision was then made into the apex of the heart ventricle. A 100 μl heparinized capillary was placed inside the ventricle to draw off blood for hemoglobin analysis. The heart and hindlimbs were then dissected from the carcass, weighed and used for analyses as described below.

Hemoglobin concentrations were determined by the cyanomethemoglobin method (Sigma bulletin #525) using human hemoglobin standards.

One weighed gastrocnemius was homogenized in 4 to 8 volumes of 50 mM Tris HCl (pH 8.2), 5 mM MgSO_4 , 1 mM EDTA. The homogenate was centrifuged at 1000 g for 10 minutes and stored at -20°C for both citrate synthase and lactate dehydrogenase activity.

The second weighed gastrocnemius was homogenized in 5 volumes of ice cold 8% perchloric acid. The homogenate was

centrifuged at 1000 x g for five minutes. The supernatant was frozen at -5°C for lactate analysis.

Lactate concentration was measured on a narrow bandwidth double beam Coleman spectrophotometer using the method described in Sigma bulletin #725 UV and modified proportionately from a three ml cuvette to a 1 ml cuvette.

The V_{max} of citrate synthase (E.C. No. 4.1.3.7) was determined according to a modified procedure of Srere (1969). The thawed supernatant was recentrifuged at 1000 x. The final reaction mixture (1 ml) contained 50 mM Tris HCl (pH 7.5), 0.1 mM 5,5'-dithiobis-(2-nitrobenzoate), 0.2 mM acetyl CoA, 0.5 mM oxaloacetate, 5 mM EDTA and 0.1 ml homogenate. The samples were then assayed at 25°C on a Coleman double beam spectrophotometer at 412 nm.

Lactate dehydrogenase activity was assayed in 0.2 M Tris HCl (6.9, 25°C), 1 mM pyruvate, 1 mM NADH in a Coleman double beam spectrophotometer at 340 nm.

RESULTS

Time to fatigue (performance) is plotted in Fig. 1 over the six weeks of exercise. The day after capture (day 0) the exercised frogs fatigued in $2.66 \pm .60$ minutes. Two days into training the exercised frogs' mean fatigue time was $2.00 \pm .25$ minutes. At five day the frogs oscillated back up to a $3.00 \pm .14$ minute mean fatigue time. The mean fatigue time gradually oscillated downward to 1.75 ± 0.18 minutes at 24 days into the exercise regimen. From this low point of 1.75 ± 0.18 minutes at 24 days, the mean fatigue time of 3.46 ± 0.60

minutes at 41 days. The mean fatigue time of the first day of training (2.66 ± 0.6 minutes) compared to the last day (day 41, 3.41 ± 0.46 minutes) of training are significantly different by the t test ($P < 0.05$).

No significant difference in total body weight (TBW, $P = 0.09$) was found between the exercised, unexercised and field frogs. No shift in body weight occurred due to either chronic exercise-confined, or nonexercised confined frogs in the hindlimb ($P = 0.07$) or the gastrocnemius ($P = 0.9$) compared to the field groups (Table 1).

No difference was found in heart weight between the exercised, unexercised and field groups ($P = 0.1$). Neither exercise nor laboratory confinement caused either hypertrophy or atrophy of heart tissue (Table 1).

The maximal oxygen consumption of unexercised frogs was significantly less than ($P < .025$) than either the exercised or field groups. The exercised and field frogs did not vary significantly from each other. Confinement (non-exercise) decreases the aerobic capacity while exercise sustains these frogs at field (fresh caught aerobic capacity (Table 1).

The hemoglobin concentration of unexercised frogs was significantly less than both the exercised and field frogs ($P < 0.025$). No difference was found between the exercised and field frogs (Table 1).

Citrate synthase activity of both the exercised and unexercised frogs was less than that of the field frogs ($P < 0.05$).

No significant differences exist between the three groups post

exercise lactate concentrations ($P=0.9$). Neither exercise nor confinement affect anaerobic metabolism as measured by the anaerobic end product, lactate.

No significant difference exists with lactate dehydrogenase activity among the three groups ($P=0.3$).

DISCUSSION

This discussion separately addresses the following questions as raised in the introduction: 1) Does chronic exercise actually improve a frog's performance, or does it only recondition, deconditioned frogs? 2) Does training and or deconditioning cause analogous changes in $\dot{V}O_2$ and hemoglobin concentrations as found in Hillman's (1980) anemic frogs? 3) If aerobic capacity is affected by training and or deconditioning, at what level is it manifested? 4) Does training and or deconditioning affect anaerobic metabolism?

The results of this paper show both training and deconditioning processes are possibly at work simultaneously. The trained frogs' performance improved significantly ($P<0.05$) from day 0 to day 41 of training. These frogs were one day out of the field on day 0, so training did increase performance above field performance. When the oscillatory nature of the performance over the training period is taken into account, it is possible at some time past day 41, the difference between field and trained frogs might diminish the significance of the increased performance. Miller and Camilliere's (1980) *Xenopus* also displayed oscillations in performance during their training period. Performance actually diminished after the peak of six

TABLE I

A CHART OF THE RESULTS OF THE FIELD (F), CHRONICALLY EXERCISED - CAPTIVE (E), AND UNEXERCISED - CAPTIVE (Un) *RANA CATESBIANA*. THE FROGS WERE EXAMINED FOR DIFFERENCES IN TOTAL BODY WEIGHT (TBW), HINDLIMB WEIGHT (HLW), GASTROCNEMIUS WEIGHT (GW); FOR THE AEROBIC PARAMETERS, MAXIMAL OXYGEN CONSUMPTION ($\dot{V}O_2$ MAX), HEMOGLOBIN CONCENTRATION (Hb), AND CITRATE SYNTHASE ACTIVITY (CS); AND FOR THE ANAEROBIC PARAMETERS OF FATIGUED MUSCLE LACTATE CONCENTRATION AT 5 MINUTES INTO EXERCISE (LACTATE) AND MUSCLE LACTATE DEHYDROGENASE ACTIVITY (LDH). AN * DENOTES THIS GROUP WAS SIGNIFICANTLY DIFFERENT FROM THE OTHER TWO GROUPS.

Parameter	T	Un	F	Significance
TBW (grams)	9.34 ± 2.51	9.33 ± 1.55	9.13 ± 1.33	P=0.9
HLW "	1.207 ± 0.354	1.23 ± 0.253	1.406 ± 0.175	P=0.7
GW "	0.0943 ± 0.0138	0.1083 ± 0.0299	0.1306 ± 0.0265	P=0.3
Heart "	0.0168 ± 0.0047	0.0203 ± 0.0055	0.0162 ± 0.0036	P=0.1
$\dot{V}O_2$ max ml/g-h	0.6179 ± 0.3060	0.4542 ± 0.1253*	0.6023 ± 0.0507	P<0.025
Hb g%	6.89 ± 1.05	5.26 ± 0.78*	6.88 ± 1.14	P<0.025
CS u-moles/g-m	145.6 ± 54.23	171.00 ± 65.82	317.22 ± 46.00*	P<0.05
lactate mg%	84.0 ± 18.1	82.9 ± 37.5	85.6 ± 41.4	P=0.9
LDH u-moles/g-m	71400 ± 35000	58433 ± 10378	66333 ± 12059	P=0.1

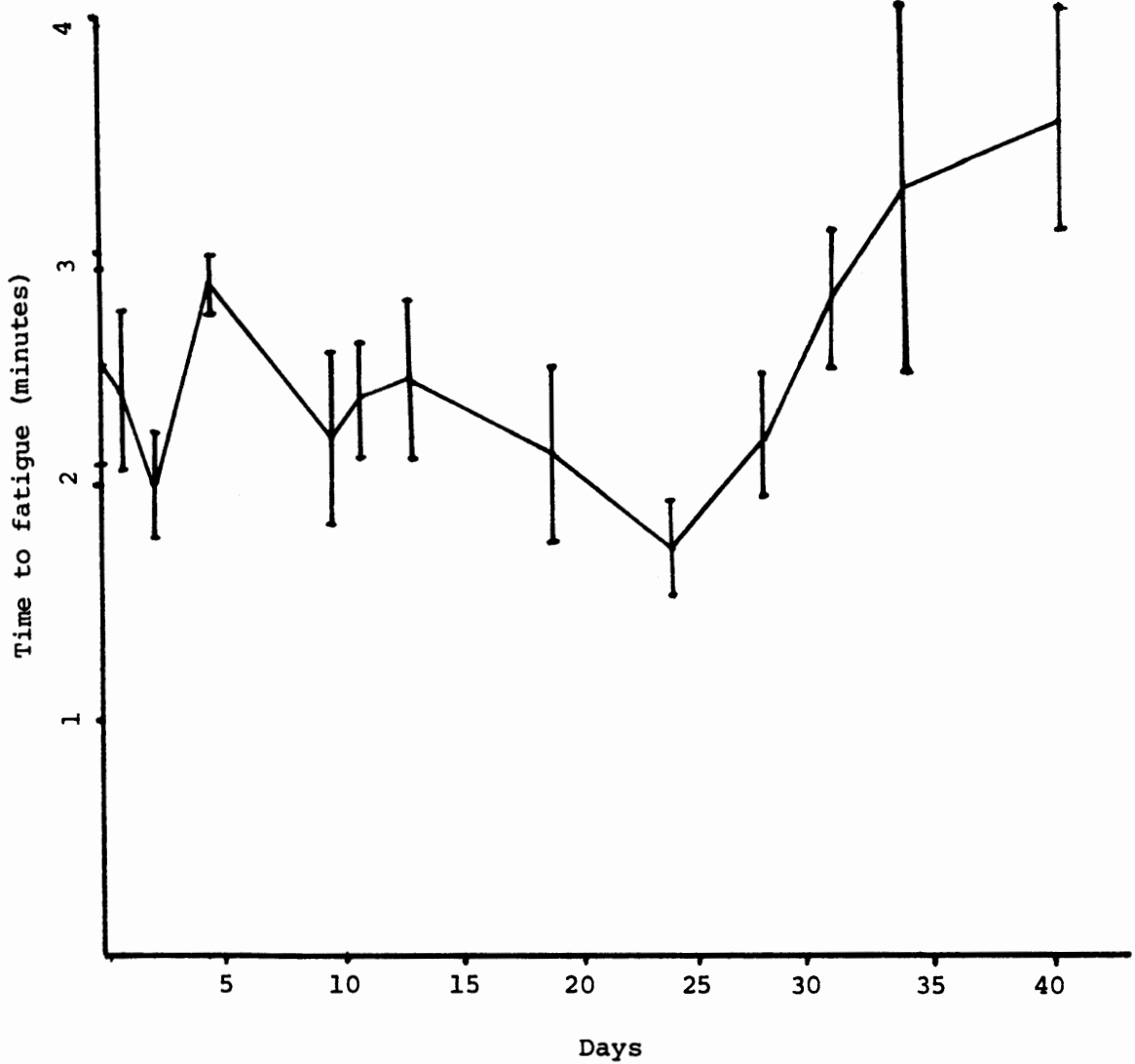


Figure 1. Performance as time to fatigue in minutes during the exercise regime of 42 days in the chronically exercised frogs. The final days mean performance of 3.41 ± 0.46 was significantly different when compared to the initial days (day 0) performance 2.66 ± 0.60 by the student t test ($P < 0.05$)

weeks into training in winter trained *Sceloporus* (Gleeson, 1979). Both previous anuran training papers (Cummings, 1979; Miller and Camilliere, 1980) show performance improvement comparing trained-captive frogs to untrained-captive frogs. In the lizard *Sceloporus occidentalis*, Gleeson (1979) had mixed results when comparing the performance of trained-captive to field lizards. Therefore, while performance did increase between field and trained *Rana catesbeiana* in this study, enough questions remain to warrant more study of performance through time.

Deconditioning is evidenced at the physiological level in unexercised-captive frogs. Both $\dot{V}O_2\text{max}$ and hemoglobin concentration are significantly lower ($P < 0.025$) in unexercised frogs compared to trained or field frogs. Hillman (1980) found a strong correlation between $\dot{V}O_2\text{max}$ and hemoglobin concentration in anemic frogs. In this work, the unexercised frogs lower $\dot{V}O_2\text{max}$ (0.5 ml/gm hr) and hemoglobin concentration (5.3 gm%) compared to the higher trained and field values of $\dot{V}O_2\text{max}$ (0.6 ml/gm hr) and hemoglobin concentration of (7 gm%) compare well with Hillman's (1980) data on an absolute basis of $\dot{V}O_2\text{max}$. Hillman found no correlation of stamina to either $\dot{V}O_2\text{max}$ or hemoglobin concentration. In this study the trained frogs performance did increase marginally over the field frogs. However, no significant differences are found between trained and field $\dot{V}O_2\text{max}$ and hemoglobin concentrations. The untrained frogs were not measured for performance.

Aerobic deconditioning in untrained compared to field and trained frogs is seen at both the whole organismal ($\dot{V}O_2\text{max}$) and cardiovascular (hemoglobin concentrations) levels. At the biochemical

level, aerobic deconditioning is seen in the trained-captive and untrained-captive frogs, as compared to the field frogs ($P < 0.025$). Performance of the trained frogs marginally increased or at least did not change over field frogs. Therefore, citrate synthase activity in this study is independent of performance and the maximal aerobic ability of these frogs.

Increased oxidative enzyme activity (citrate synthase, cytochrome oxidase) has been correlated to increased performance due to endurance training with mammals (Hollozy et al, 1976; Baldwin et al, 1976; Byland et al, 1977). Citrate synthase activity was correlated to (resting $\dot{V}O_2$) many vertebrates and invertebrates (Alp, 1976). Endurance trained *Rana pipiens* also have increased citrate synthase activity with increased performance (Cummings, 1979). Endurance trained *Xenopus laevis* citrate synthase activity (Miller and Camilliere, 1981) did not differ from untrained *Xenopus laevis*, even though endurance does increase in these frogs. In *Sceloporus occidentalis* (Gleeson, 1979) neither performance nor citrate synthase activity changed between trained, untrained, and field lizards. In this study the $\dot{V}O_{2\max}$ and hemoglobin concentration in the trained frogs remained at field levels, but, citrate synthase activity decreased from field levels. What factors are involved with the decreased citrate synthase activity were not studied.

Anaerobic metabolism at both the muscle and enzyme level (LDH activity) are not affected by either training or deconditioning from captivity. Muscle lactate concentrations in this study were taken after 5 minutes of exercise to fatigue. Cummings (1979) found trained

and untrained *Rana pipiens* muscle lactate concentrations did not differ after fatigue. However, the removal of lactate from muscle in 15 minutes by *Rana pipiens* was significantly greater in the trained than the untrained groups (Cummings, 1979). Trained *Xenopus laevis* has significantly greater resting and fatigue lactate levels when compared to untrained *Xenopus* (Miller and Camilliere, 1981). Hillman (1980) found lactate production during exercise is inversely correlated to maximal oxygen consumption in anemic *Rana pipiens*. In this present study, only static lactate levels were measured from fatigue. Since training affects $\dot{V}O_2$ max and hemoglobin concentrations (this study) and lactate production is inversely correlated to $\dot{V}O_2$ max (Hillman, 1980) it is possible that lactate production (a rate function) is affected by training while maximal lactate levels are regulated differently and therefore not affected by training or deconditioning.

In summary, this study has shown untrained-captive *Rana catesbeiana* do decondition at the whole organismal ($\dot{V}O_2$ max), cardiovascular (hemoglobin concentration) and biochemical (citrate synthase) levels compared to field animals. The trained-captive group maintained $\dot{V}O_2$ and cardiovascular hemoglobin concentrations near field levels, while citrate synthase activity decreased significantly from field frogs. Therefore, the aerobic capabilities of the trained-captive frogs is not affected by citrate synthase activities in these muscles. These results follow Hillman's (1980) theory of $\dot{V}O_2$ max being limited at the cardiovascular level in frogs, while performance is not affected by $\dot{V}O_2$ max. Anaerobically, neither maximal lactate levels (measured immediately after fatigue), nor lactate dehydrogenase are

affected either by captivity or training.

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