Physiological and Biochemical Adaptations to Training in *Rana pipiens*

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Summary. Fifteen *Rana pipiens* were trained on a treadmill thrice weekly for 6.5 weeks to assess the effects of training on an animal that supports activity primarily through anaerobiosis. Endurance for activity increased 35% in these frogs as a result of training ($P = 0.006$, Fig. 1). This increased performance was not due to enhanced anaerobiosis. Total lactate produced during exercise did not differ significantly for the trained or untrained animals in either gastrocnemius muscle (2.77 ± 0.21 and 2.82 ± 0.13 mg/g, respectively) or whole body (1.32 ± 0.10 and 1.47 ± 0.06 mg/g, respectively). Glycogen depletion also did not differ between the two groups (Fig. 2c). The primary response to training appeared to involve augmentation of aerobic metabolism, a response similar to that reported for mammals. Gastrocnemius muscles of trained frogs underwent a 38% increase over those of untrained individuals in the maximum activity of citrate synthase (14.5 ± 1.0 and 10.5 ± 0.9 μmoles/(g wet wt · min); $P = 0.008$). This enzyme was also positively correlated with the level of maximum performance for all animals tested ($r = 0.61$, $P < 0.01$) and with the degree of improvement in the trained animals ($r = 0.72$, $P < 0.05$). In addition to an increased aerobic capacity, the trained animals demonstrated a greater removal of lactate from the muscle 15 min after fatigue (Fig. 2b).

Introduction

Mammals characteristically respond to sustained, chronic activity with increased endurance for exercise. An amplification of the muscle's ability to derive energy from aerobic pathways underlies this change (Holloszy and Booth, 1976). Adjustments correlated with this augmented aerobic capacity include increased ability of the muscle to oxidize pyruvate (Baldwin et al., 1972), and increased activities of the enzymes of both the tricarboxylic acid cycle (Dohm et al., 1973) and the electron transport chain (Holloszy, 1967). These modifications are reflected in an increase in mitochondrial size and number (Gollnick and King, 1969).

The question arises as to how representative the mammalian pattern of response is for vertebrates in general. In this regard, it is of special significance to discover whether organisms in which locomotion is primarily dependent on anaerobic metabolism, e.g., amphibians and reptiles (Bennett and Licht, 1972, 1974), enhance their aerobic or their anaerobic capacities during training. The latter would entail augmentation of glycolysis, which has been demonstrated in rainbow trout forced to swim in fast currents (Hammond and Hickman, 1966).

Recent work by Gleeson (1979) indicates that the western fence lizard, *Sceloporus occidentalis*, augments neither aerobic nor anaerobic metabolism with chronic exercise. Indeed, this reptile, which relies to a great extent on anaerobic ATP production during activity (Bennett and Gleeson, 1976), shows no increase in its performance after training. Such refractoriness is surprising, for Baldwin et al. (1972) have demonstrated that changes in oxidative capacity are possible in all types of mammalian muscle fibers, including the highly anaerobic glycolytic fibers.

The present study was undertaken to ascertain whether the response of *Sceloporus occidentalis* to endurance training is characteristic of other reptiles and amphibians. The leopard frog, *Rana pipiens*, was chosen to assess training since its locomotory requirements are met predominantly through anaerobic metabolism (Bennett and Licht, 1974; Hutchison and Turney, 1975) and because it has low endurance capabilities and fatigues rapidly during stimulation to maximum activity (Bennett and Licht, 1974; Carey, 1976; Putnam, 1977).

Materials and Methods

Animals

Thirty adult, male *Rana pipiens* (30–45 g) were obtained from an animal supplier (Alburg, Vermont). All animals were prophylactically treated with tetracycline hydrochloride (5 mg/animal twice daily) for four days to assure uniformity in health. Five weeks were provided prior to undertaking experimentation to guarantee that the drug was completely metabolized. Animals were housed in a large plastic aquarium (4.5 × 0.56 × 0.31 m), through which water continually flowed at 15 °C. Elevated wooden stands allowed the animals free access into and out of the water. At one end of the aquarium, heat lamps were provided to permit behavioral thermoregulation. Animals were fed live crickets dusted with a vitamin supplement (Theralin, Lambert-Kay). Both trained and untrained groups were kept in the same tank and fed ad libitum three times a week on days in which no animals were exercised. Experiments were performed during March and April of 1978.

Experimental Design and Training Conditions

Animals were fatigued on a treadmill and arbitrarily assigned to either the trained or the untrained group, so as to represent animals of equal ability in both groups. The treadmill was enclosed on all sides with dimensions of 0.9 m in length and 0.13 m width and operated at 9.5 m/min. Animals commenced hopping when the treadmill was started. Animals hopped with no stimulus other than treadmill movement. As animals fatigued, contact with a soft bristle brush at the back end of the treadmill provided the necessary added stimulation to maintain movement. Fatigue was defined as the inability of the animal to right itself when placed on its back. Fatigue occurred shortly after the animals ceased hopping. Animal body temperatures during forced activity were 20–22 °C.

Training consisted of refatiguing animals after an initial bout of exhaustive activity. The training program lasted 6.5 weeks, during which animals were thrice weekly exercised to fatigue, given a 15-min rest period, and fatigued again. At the conclusion of the training program, one week without forced activity was provided before final activity and metabolic measurements were taken. No food was provided during the last four days. Animals from each group were assigned to one of three subgroups:

Rested: animals were at rest before sampling.

Fatigued: animals were exercised to exhaustion before sampling.

Recovered: animals were fatigued and allowed 15 min of rest before sampling.

Performance

The total distance traveled, the duration of activity, and the total number of hops until fatigue were recorded.

Enzymatic, Substrate, and Metabolic Measurements

Animals were decapitated and both gastrocnemius muscles were removed immediately. One muscle was homogenized (glass-glass homogenizer) in 8–12 volumes of cold 0.6 N perchloric acid and stored at 5 °C for analysis of glycogen and lactate. The contralateral muscle was homogenized (glass-glass) in 8–12 volumes of cold 50 mM Tris-HCl (pH 8.2), 5 mM $MgSO_4$, 1 mM EDTA and stored at −20 °C for citrate synthase analysis. Immediately after removal of the muscles, the remainder of the carcass was homogenized

in 5–8 volumes of cold 0.6 N perchloric acid with a Waring blender and stored at 5 °C for whole body lactate determinations.

The V_{max} of citrate synthase (E.C. No. 4.1.3.7) was determined according to a modified procedure of Srere (1969). The final reaction mixture (1.0 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 of homogenate. Samples were thawed overnight at 5 °C, then sonicated on ice for three 15-s periods with 30-s pauses. The samples were then assayed at 25 °C. Lactate analysis was performed with a lactate test kit (Sigma, 826-UV) by the method of Bennett and Licht (1972). Glycogen concentrations were assayed by the amyloglucosidase method of Keppler and Decker (1974). All three of these spectrophotometric tests were performed on a temperature regulated Zeiss model PMQ 3 spectrophotometer. All necessary reagents were obtained from the Sigma Chemical Company, St. Louis, MO.

Statistics

Differences between subgroups and within groups were tested using the Student's *t*-test when parametric requirements were satisfied and the Mann-Whitney U test when the non-parametric procedure was dictated. Lines of symmetry were drawn according to the method of Brace (1977). Results are expressed as means ± S.E.M.

Results

Training causes a significant increase in the ability of *Rana pipiens* to sustain activity (Fig. 1). The trained animals increased their maximum level of activity by a mean difference of 58.2 hops ($P = 0.006$), while the control frogs exhibit virtually no change ($P = 0.93$). The increased endurance of the trained animals is manifested by concurrent increases in the total distance traveled until exhaustion (54.2 to 67.9 m) and the absolute duration of the activity (5.7 to 7.2 min). These three activity parameters (hops, distance, time) are well correlated ($r = 0.91$, $P < 0.01$). Due to this correlation and a desire to simplify data presentation, only the total number of hops will be used in assessing the training effects.
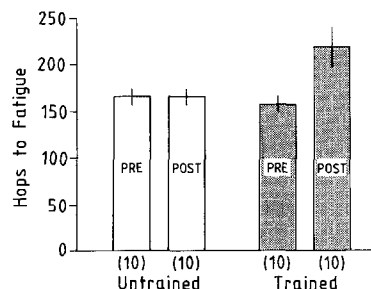

Fig. 1. Endurance of *Rana pipiens* expressed as the total number of hops to fatigue. Shaded bars are means of trained animals (pre- and post-training). Clear bars are means of untrained animals (over same time period). Sample sizes are given below each bar. Vertical lines represent ± S.E.M. Paired *t*-test is significant at $P = 0.006$
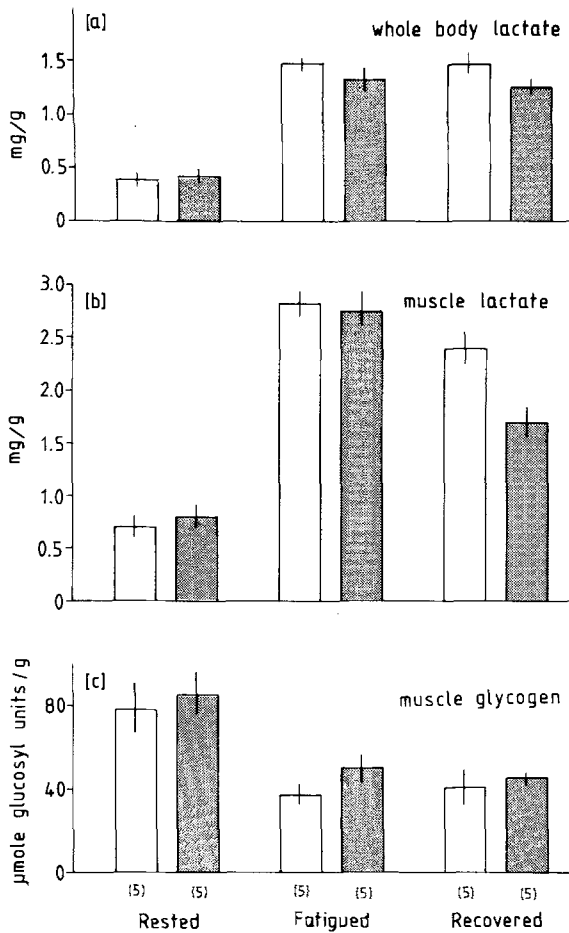
Fig. 2. a Whole body lactate. b Muscle lactate concentrations. c Muscle glycogen values. All values are on a body mass-specific basis. Bars are mean values in rested, fatigued, and recovered animals. Vertical lines represent ± S.E.M. Clear bars, untrained animals; shaded bars, trained animals. Sample sizes below each bar
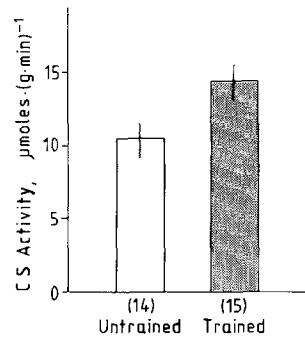

Fig. 3. Citrate synthase activity (V_{max}) on a body mass-specific basis. Sample sizes below each bar. Vertical lines represent ± S.E.M. Student's t-test significant at $P=0.008$
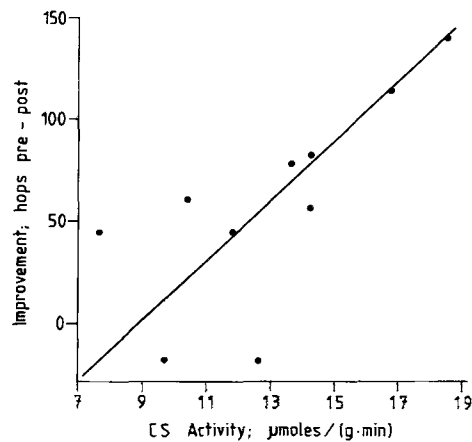

Fig. 4. Correlation of change in maximum activity of the trained animals pre- and post-training vs. citrate synthase (V_{max}), the last being expressed on a body mass-specific basis. Line of symmetry drawn according to Brace (1977), $r=0.72$, $P<0.05$

Lactate Production

Changes in whole body lactate are shown in Fig. 2a. Resting values do not differ significantly between trained and untrained animals (0.42 ± 0.04 and 0.39 ± 0.05 mg/g, respectively). After fatigue these values are elevated threefold (1.32 ± 0.10 and 1.47 ± 0.06 mg/g, respectively). Following the 15-min recovery period after fatigue, the whole body lactate values had not changed (1.25 ± 0.09 and 1.46 ± 0.10 mg/g, respectively). No significant difference was detected between trained and untrained animals at any time.

Muscle lactate concentrations (Fig. 2b) are similar for trained and untrained animals at rest and after fatigue. Values were elevated from 0.79 ± 0.10 to 2.77 ± 0.21 mg/g in the trained group and from 0.75 ± 0.13 to 2.82 ± 0.13 mg/g in the control animals.

The major difference between groups was found in animals which were allowed a 15-min rest following exercise. Trained animals reduced muscle lactate by 38% from peak values (2.77 ± 0.21 to 1.71 ± 0.15 mg/g) ($P=0.003$). A reduction of muscle lactate recorded in untrained animals (2.82 ± 0.13 to 2.39 ± 0.15 mg/g) was not significant ($P=0.058$). The removal of muscle lactate by the trained animals in the 15 min following exhaustion was significantly greater than that by control animals ($P=0.012$).

Glycogen Depletion

The trained and untrained animals had statistically similar levels of muscle glycogen at rest (85.2 ± 10.9 and 78.1 ± 12.2 μmole glucosyl units/g, respectively) and at fatigue (49.9 ± 5.8 and 37.4 ± 4.5 μmole glu-

cosyl units/g, respectively) (Fig. 2c). After the recovery period muscle glycogen had not changed significantly for either the trained or untrained frogs (45.5 ± 2.6 and 40.8 ± 8.5 μmole glucosyl units/g, respectively).

Citrate Synthase Activity

Citrate synthase activity (V_{max}) in trained frogs significantly exceeded that in controls. Values for the trained animals were 14.5 ± 1.0 μmoles (g wet wt·min)$^{-1}$ vs. 10.5 ± 0.9 μmoles (g wet wt·min)$^{-1}$ for the controls (Fig. 3). Citrate synthase activity is positively correlated with the absolute level of performance for both groups of animals ($r=0.61$, $P<0.01$) and with the degree of improvement for the trained animals ($r=0.72$, $P<0.05$) (Fig. 4).

Discussion

Training causes a significant increase in the ability of *Rana pipiens* to sustain activity (Fig. 1). This demonstrated increase in performance agrees favorably with data from training studies on mammals (Holloszy and Booth, 1976) and fish (Hammond and Hickman, 1966). In mammals, this adjustment is due in part to increased oxidative capacity of the muscle, whereas in the rainbow trout, the adaptation is due in part to increased anaerobiosis. Since *Rana pipiens* relies to a considerable degree on anaerobic metabolism for support of activity (Bennett and Licht, 1974), an adjustment increasing their anaerobic capacity might be anticipated. This increased anaerobiosis would, necessarily, be reflected by higher levels of lactate at fatigue and a greater depletion of muscle glycogen, the primary substrate for anaerobiosis. In fact, endurance trained *Rana pipiens* demonstrated neither of the above. Values for whole body and muscle lactate and muscle glycogen were similar for trained and untrained animals at fatigue (Fig. 2). Thus, expansion of the anaerobic systems was not the primary adjustment supporting increased exercise capacity.

Mammals reduce reliance on anaerobiosis during endurance training. Athletes exercising at maximal $\dot{V}_{O_2}$ have lower skeletal muscle and blood lactate concentrations when in the trained state (Hermansen, 1971). This effect is similar to the adjustment found in *Rana pipiens*, in that lactate production was lower in the trained animals when expressed as a function of the total number of hops performed (1.26 to 1.65 mg/g lactate per 100 hops, respectively). This result is due to the fact that the trained animals went further on the same total lactate production.

In mammals, the decreased reliance on anaerobiosis is counterbalanced by an increased utilization of the aerobic pathways (Holloszy and Booth, 1976). Increases in the levels of oxidative enzymes (Costill et al., 1976; Maxwell et al., 1977; Molé et al., 1971; Henriksson and Reitman, 1977; Fitts et al., 1975) and specifically citrate synthase (Holloszy et al., 1970; Winder et al., 1974) are correlated with the increased aerobic capacity. The maximum activity of citrate synthase provides a valid assessment of aerobic involvement in energy provision of representatives of many classes of vertebrate and invertebrate animals (Alp et al., 1976). The trained *Rana pipiens* demonstrated an adaptation similar to that of the mammals in that muscle citrate synthase activity increased 38% (Fig. 3). This change in enzyme activity indicates that the trained animals are supplementing their anaerobic ATP production with a pronounced increase in aerobic processes. The importance of citrate synthase as an indicator of the endurance is supported by the positive correlation of citrate synthase with the level of maximum performance among the animals tested ($r=0.61$, $P<0.01$). Since citrate synthase activity appears responsive to training, then a correlation of the degree of improvement with the increase in enzyme activity would further support the claim of an aerobic adaptation acquired through training. This correlation does exist and it provides the opportunity to view the aerobic nature of the training response independent of individual variation in response to training (Fig. 4).

Certainly, this evidence of increased aerobic capacity would be greatly supported by a direct measurement of oxygen consumption. However, the use of a treadmill of this size precluded effective measurement of this parameter. Despite this limitation, the treadmill was chosen because it stimulated natural hopping locomotion, inducing a realistic portrayal of muscle utilization during activity, and providing a standardized activity for all animals. The method of animal stimulation be it by electrical, manual, or rotational means is of considerable importance since different activity patterns will be elicited, thus affecting the proportion of anaerobic and aerobic contribution.

The trained frogs have modified the rate of recovery from fatigue as well as increasing aerobic capacity. Their more rapid removal of lactate from the muscle following an exhaustive bout of exercise (Fig. 2b) could involve three possible routes. One route could involve conversion of lactate to glycogen in the muscle. Recent research indicates that this process may be possible at slow rates (Hermansen and Vaage, 1977; Bendall and Taylor, 1970; Krebs and Woodford, 1965), despite low activities of the necessary

enzymes (Opie and Newsholme, 1967; Crabtree et al., 1972). However, if this mechanism were significant during recovery in these animals, higher muscle glycogen concentrations would be anticipated following the recovery period, contrary to observations (Fig. 2c). The other avenues of lactate removal involve circulatory transport and terminal oxidation. Post-fatigue monitoring of these systems was not done during these experiments, so assessment of their relative contributions is not feasible. Increases in capillary density have been observed in mammalian training studies (Andersen and Henriksson, 1977) and would not be inconsistent with the results presented here. Work specifically addressing these mechanisms of lactate removal could provide valuable information regarding recovery dynamics.

At the present time, considerable controversy exists in the field of amphibian energetics. Conclusions as to duration of activity and number of hops to fatigue are useful for comparisons only within the framework of these experiments. It is important to note, however, that lactate values found in this study are similar to results from investigations using other methods. Muscle lactate values in my animals after 5–7 min of activity are only slightly higher than those reported by Hutchison and Turney (1975) after 30 min of electrical stimulation. Whole body lactate concentrations are in agreement with those of Bennett and Licht (1974) obtained over a similar duration using prodding to elicit activity.

The adaptations to training presented in this study contrast sharply with the responses found in the lizard, *Sceloporus occidentalis,* in which no adaptation was found (Gleeson, 1979). The difference may be due to the different training methods utilized in each study. The possibility exists that the intensity of training in Gleeson's study was insufficient to elict adjustments in the lizards. Alternately, the distinction may lie in fundamental differences between the animals themselves, such as the different modes of locomotion utilized by the two animals.

Rana pipiens' ability to augment its metabolic capacity indicates that these animals do have some flexibility in responding to increased energetic demands. The training adjustments present in *Rana pipiens* parallel those in mammals. This study thus indicates that enhancement of aerobiosis during programs of chronic, sustained activity is not limited to animals with high aerobic capacities, but can also develop in animals more dependent on anaerobiosis.

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