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ULTRASTRUCTURAL DESIGN OF ANURAN MUSCLES USED FOR CALL PRODUCTION IN RELATION TO THE THERMAL ENVIRONMENT OF A SPECIES

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Summary

I examined the aerobic trunk muscles, which are used for call production, of male frogs from species that breed in different thermal environments to test the hypothesis that cold-adapted frogs should have fewer capillaries per unit mitochondrial volume in oxidative muscles than warm-adapted frogs because of reduced mitochondrial function at low temperatures. The species of interest were the cold-temperate *Pseudacris crucifer* and the warm-tropical *Hyla microcephala* in the family Hylidae, and the cold-temperate *Rana sylvatica* and the warm-temperate *Rana clamitans* in the family Ranidae. Trunk-muscle mitochondrial volume, V_V(mt,f), was proportionally higher in species with higher mean calling rates (number of notes per hour), irrespective of the familial affinity of a species and the thermal environment in which it vocalized. Trunk-muscle capillary length density, J_V(c,f), was significantly lower in *P. crucifer* than in *H. microcephala* because of significantly higher mean fiber area, $\bar{a}$(f). Conversely, trunk-muscle J_V(c,f) was similar in the two ranid species. Using total capillary length, *J*(c), and total mitochondrial volume, *V*(mt,m), as a measure of maximal oxygen supply and demand, respectively, in trunk muscles, *J*(c)-to-*V*(mt,m) ratios were significantly lower in cold-adapted *P. crucifer* (4.3 km cm^{-3}) and *R. sylvatica* (4.8 km cm^{-3}) than in warm-adapted *H. microcephala* (7.1 km cm^{-3}) and *R. clamitans* (6.4 km cm^{-3}). In contrast, *J*(c)-to-*V*(mt,m) ratios in the more anaerobic gastrocnemius muscle of these species was not related to the thermal environment of a species, which may reflect capillaries conforming to microcirculatory functions, e.g. lactate removal, that take precedence over oxygen delivery. Mitochondrial cristae surface area, S_V(im,mt), in *P. crucifer* trunk and gastrocnemius muscles (37.7±1.6 and 35.9±1.5 m^2 cm^{-3} respectively) was, on average, similar to mammalian values, suggesting equivalent structural capacities of muscle mitochondria in these two taxa. Taken together, the present data suggest that trunk-muscle respiratory design may reflect a capillary supply commensurate with maximal levels of oxygen delivery set by mitochondria operating at different environmental temperatures. *P. crucifer* and *H. microcephala* trunk muscles were also characterized by a high lipid content, which contrasted with a near absence of trunk-muscle lipids in *R. sylvatica* and *R. clamitans*. The extraordinarily high lipid content of *P. crucifer* trunk muscles (26 % of muscle volume) may serve as an auxiliary oxygen pathway to mitochondria and thus compensate in part for this tissue's reduced capillary/fiber interface. The effect of potentially high depletion rates of trunk-muscle lipid stores on metabolic rates of male frogs while calling is discussed.

Key words: mitochondria, muscle capillarity, skeletal muscle, anuran, vocalization, thermal adaptation, ultrastructure, Hylidae, Ranidae, frog.

Introduction

Comparative studies of teleost fishes from different thermal environments offer compelling evidence that cold temperature is a strong determinant of ultrastructural respiratory design in the oxidative muscle of this vertebrate taxon. Most notably, cold-water fish possess muscle with more abundant mitochondria and, depending on the species, a more expansive microvasculature or higher lipid content than that observed in homologous muscle of warm-water fish (Londraville and Sidell, 1990; Sidell, 1991; Clarke and Johnston, 1996). Higher quantities of these subcellular structures in the musculature of cold-water fish are thought to conserve aerobic muscle activity at low tissue temperatures by compensating for lower activity levels of mitochondrial enzymes and/or the reduced diffusive movement of oxygen and metabolites to mitochondria (Tyler and Sidell, 1984; Sidell and Hazel, 1987; Egginton and Sidell, 1989).

Despite claims in the literature that the effects of temperature on muscle ultrastructure observed in fish may be widespread among ectothermic vertebrates (e.g. Sidell, 1988), the relationships between environmental temperature and ultrastructural design of amphibian and reptilian muscle are poorly understood. Frogs, however, are ideal ectotherms in

which to explore the interrelationships between aerobic muscle performance, subcellular respiratory design and the thermal environment of species for the same reasons that are often cited for fish. First, the activity temperature of different frog species varies widely, on both a temporal and spatial scale (Rome et al., 1992). Second, frogs possess a limited capacity for physiological thermoregulation and no capacity for regional endothermy occurring in any muscle tissue. Thus, both the metabolic physiology and muscle function of amphibians have a high thermal dependence (Hutchison and Dupré, 1992; Rome et al., 1992). Finally, anuran skeletal muscle is distinguished by either one fiber type or different fiber types segregated into discrete zones (Gans and De Gueldre, 1992), which greatly facilitates interspecific comparisons of muscle tissue with homogeneous fiber types.

The internal and external oblique muscles of male frogs, which are used for call production and are collectively referred to as trunk muscles, form one such set of muscles. These two muscles are composed of 100 % fast oxidative fibers and possess biochemical and contractile properties indicative of a tissue with a high capacity for aerobic metabolism (Pough et al., 1992; Prestwich, 1994; Bevier, 1995). Indeed, studies have demonstrated that sustained calling by male frogs is an aerobically supported natural behavior that engenders increases in rates of oxygen consumption above resting levels that, for some hylid treefrogs, are unprecedented among ectothermic vertebrates (e.g. Taigen and Wells, 1985; Taigen et al., 1985; Prestwich et al., 1989; Wells and Taigen, 1989).

Few studies have examined the ultrastructural properties of male trunk muscles in frogs, but the limited data that are available suggest that trunk-muscle respiratory design is influenced by environmental temperature in a manner different from that observed in and predicted from fish muscle. For example, Ressel (Ressel, 1996) examined tropical hylid and leptodactylid frogs that call at 26 °C and found that, while capillary length, J_V(c,f), and mitochondrial volume, V_V(mt,f), increased proportionately with increasing levels of calling activity, J_V(c,f)-to-V_V(mt,f) ratios across species were 42–60 % lower than mammalian values. These data have been used to support a hypothesis advanced by Marsh and Taigen (Marsh and Taigen, 1987) that trunk-muscle capillary length may reflect maximal oxygen flux rates set by mitochondria operating at environmental temperatures that are significantly lower than the internal temperature of eutherian mammals.

Implicit in this hypothesis are the underlying assumptions that changes in tissue temperature affect mitochondrial respiration rates to a greater degree than oxygen diffusion rates in trunk muscles and that the trunk-muscle mitochondrial content of cold-bodied frogs does not compensate fully for reduced levels of catalytic capacity at low temperatures. This hypothesis therefore predicts that species that call at cold temperatures will have lower J_V(c,f)-to-V_V(mt,f) ratios than do either tropical species or temperate-zone species that breed at warm temperatures.

The first objective of the present study was to test the above hypothesis by comparing the trunk-muscle respiratory design of two temperate-zone frogs that breed at very cold temperatures, the spring peeper *Pseudacris crucifer* and the wood frog *Rana sylvatica*, with that of two warm-adapted frogs, a tropical hylid, *Hyla microcephala*, and the temperate-zone green frog *Rana clamitans*. In an attempt to minimize potential effects of phylogeny on anuran muscle ultrastructure, I performed intrafamilial comparisons only, i.e. within Hylidae (*P. crucifer versus H. microcephala*) and within Ranidae (*R. sylvatica versus R. clamitans*). These intrafamilial comparisons also accounted for differences in trunk-muscle ultrastructure due to markedly different levels of calling activity exhibited by hylid and ranid frogs (Pough et al., 1992). I included lipid volume together with capillary length and mitochondrial volume in my assessment of trunk-muscle respiratory design because of the strong arguments put forth previously (e.g. Sidell, 1991; Dutta and Popel, 1995; Desaulniers et al., 1996) that intramuscular lipids may represent an auxiliary oxygen pathway in ectothermic skeletal muscle. Finally, I performed the same ultrastructural analysis on glycolytic leg muscle from the four species to determine whether interspecific differences in muscle respiratory design are restricted solely to oxidative muscle, as inferred by the hypothesis of Marsh and Taigen (Marsh and Taigen, 1987).

A reduced microvasculature in anuran trunk muscles may also be indicative of mitochondria with an intrinsically lower capacity for aerobic ATP production than that of mitochondria in mammalian muscle (Moyes et al., 1998). Consequently, a second objective of the present study was to quantify mitochondrial cristae surface area in the two muscles of interest to test the alternative hypothesis that a reduced microvasculature may be congruent with rates of oxygen consumption set by mitochondria with less cristae surface area than their mammalian homologues.

Materials and methods

Estimate of calling effort

The vocal behavior of *Pseudacris crucifer* Wied and *Hyla microcephala* Cope is well documented (Rosen and Lemon, 1974; Wells and Taigen, 1989; Bevier, 1995; Wells et al., 1996; Zimmitti, 1999). Both species have high capacities for sustained calling on a nightly basis and produce calls of similar intensity (approximately 105 dB SPL at 50 cm). *H. microcephala* produces a complex multi-note call, with each note being the product of a separate muscle contraction, whereas the call of male *P. crucifer* is a simpler single frequency-modulated ‘peep’ that represents a single muscle contraction. Accordingly, I used mean rate of note production as a behavioral correlate of aerobic trunk muscle activity for male *H. microcephala* calling at a fairly constant temperature of 26±1 °C (see Table 1). In contrast, *P. crucifer* have a prolonged breeding season that encompasses a wide temperature range, and their calling rate is highly temperature-dependent (Wells et al., 1996). Nonetheless, Zimmitti

(Zimmitti, 1999) reported that a subset of males is capable of much higher calling rates than other males on any given night during the breeding season. Thus, I used the calling rates reported for early season, high-calling males to obtain a mean value that is representative of the upper limit of trunk-muscle aerobic workload for *P. crucifer* active at temperatures of 7 °C or below (see Table 1).

For *Rana sylvatica* Leconte and *Rana clamitans* Latreille, I recorded calling males in natural choruses found in the vicinity of Storrs, Connecticut, USA. Thirty minute recordings were made of 20 different males for each species using a Realistic Directional Microphone (model no. 33-1062) and Marantz PMD 360 stereo cassette tape recorder. Males of both species were easily approachable, and my presence did not appear to alter their vocal behavior. Both species produce calls with several distinct notes delivered as a group (Wells, 1978; Wells and Bevier, 1997). Therefore, I used the number of notes per unit time to calculate mean calling rate because this value probably reflects the number of muscle contractions underlying vocal activity in these two species.

Tissue preparation

I collected five calling males of each species by hand from breeding populations in eastern Connecticut, USA, and Gamboa, Panama. I recorded the activity temperature of each species at the time of capture, being careful to note the location of calling males relative to the air/water interface. For both *P. crucifer* and *R. clamitans*, males were calling near shore or on floating vegetation with their pelvic region and hindlegs submerged in water. Thus, I recorded both air and water temperatures. For *R. sylvatica*, I measured water temperature only because males vocalized while actively searching for females in the water. Frogs were kept overnight in an environmental chamber at the University of Connecticut, Storrs, Connecticut, USA, at a temperature that was equivalent to their respective breeding temperatures. Tissue preparation was performed the following morning (<10 h after capture). The study site, breeding behavior and protocol for collecting male *H. microcephala* have been described previously (Wells and Taigen, 1989; Ressel, 1996).

Frogs were killed by decapitation followed by pithing of the spinal cord. I quickly excised the internal and external oblique muscles from each frog and analyzed them collectively as trunk muscles. I then removed the gastrocnemius muscle from each male, alternating between the right and left hindleg among individuals. I immediately weighed the muscle tissue on a Sartorius balance (±0.001 g), and then fixed, stained and embedded the tissue as described previously (Ressel, 1996).

Tissue sectioning

I selected two tissue blocks per muscle at random from the set of embedded tissue for each animal. Thus, 10 blocks per muscle were subsequently sectioned for each species. I then cut and stained semi-thin (1 μm) and ultrathin (50–60 nm) sections, as described in detail by Ressel (Ressel, 1996).

Morphometry

I obtained morphometric estimates of capillary-to-fiber ratio [N_N(c,f); number of capillaries/number of fibers] and capillary numerical density, N_A(c,f)0°, in transversely oriented fibers for each muscle sample by projecting images of semi-thin sections onto a digitizing tablet coupled to a video monitor and microcomputer (Ressel, 1996). I used muscle fiber area as the reference space in my estimates of capillary density, and restricted capillary counts to vessel profiles consisting of endothelium and pericytes only (Casley-Smith et al., 1975; Mathieu-Costello, 1993). On average, I examined 3–5 randomly selected fields per section, which yielded capillary counts for 150–250 fiber profiles for each muscle sample. This sampling protocol stabilized standard errors at ⩽15 % of the mean capillary density estimate, an acceptable margin of error for morphometric studies (Cruz-Orive and Weibel, 1990).

To estimate the degree of capillary bending and branching in the trunk muscles and gastrocnemius, I followed a widely used protocol (Mathieu et al., 1983) to calculate a capillary tortuosity coefficient, c(K,0), in the muscles of *P. crucifer*. Here, I assumed that c(K,0) values for *P. crucifer* muscle were representative of homologous muscle in the other species included in this study, being careful to apply this assumption only if each muscle sample experienced similar levels of fiber contraction during immersion fixation (Mathieu-Costello, 1993). Thus, I quantified and compared estimates of mean sarcomere length, l_o, in the trunk and leg muscles of the four species, employing a protocol described previously (Ressel, 1996).

Semi-thin sections of trunk muscles contained both transverse and longitudinal sections of fibers because the internal and external oblique fibers are oriented perpendicular to each other. Thus, I was able to estimate capillary numerical density in longitudinally oriented fibers, N_A(c,f)90°, with the same semi-thin sections used to estimate N_A(c,f)0°. For *P. crucifer* gastrocnemius, I estimated N_A(c,f)90° in separate longitudinal sections (angle parallel to the fiber axes, α=90 °) from randomly chosen tissue blocks. I then calculated J_V(c,f) and total capillary length, J(c), according to Conley et al. (Conley et al., 1987).

For morphometric estimates of mitochondrial volume, V_V(mt,f), intracellular lipid volume, V_V(li,f), and myofibril volume, V_V(mf,f), I recorded electron micrographs of ultrathin sections on 35 mm film (Kodak direct positive microfilm 2468) with a Philips EM 300. Ten micrographs per section were systematically recorded over the entire grid section area. Thus, I projected 100 micrographs per muscle for each species onto a screen equipped with a C 64 grid (see Appendix 3 in Weibel, 1979) to derive volume estimates using standard stereological calculations (StepOne Software, Department of Anatomy, University of Berne, Switzerland). No correction for section thickness or compression was made. Total mitochondrial volume, V(mt,m), and mean capillary diameter, $\bar{d}$(c), were calculated for each muscle sample as described previously (Hoppeler et al., 1987; Ressel, 1996, respectively). For *H. microcephala* trunk

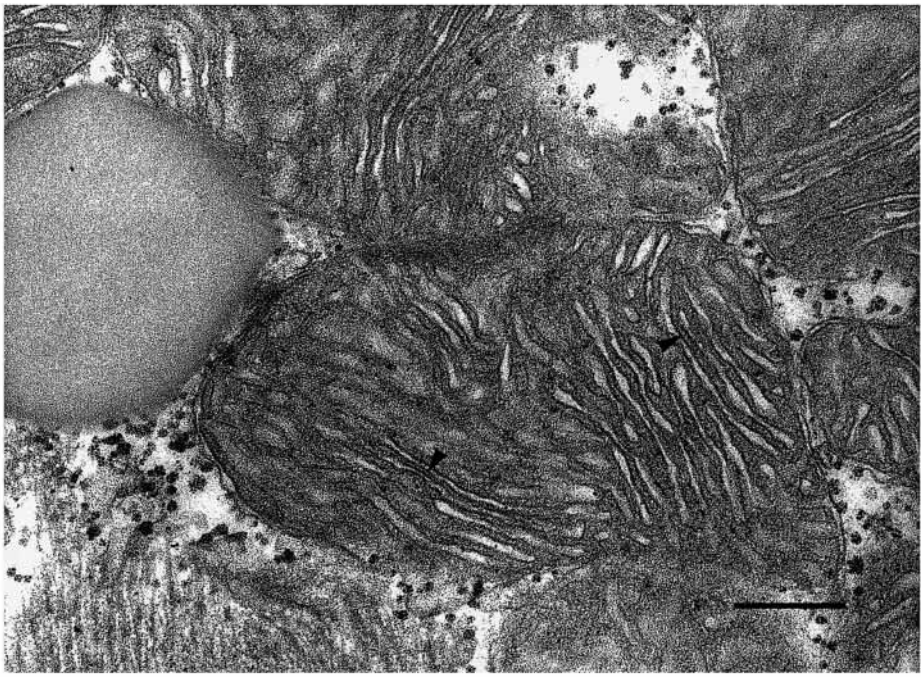

Fig. 1. Electron micrograph of isolated mitochondria from *Pseudacris crucifer* trunk muscles showing the preservation of outer and inner membranes. Arrowheads denote membrane profiles exhibiting a double-leaflet structure. Scale bar, 0.2 μm.

muscles, I used previously published values for some of the morphometric estimates considered in the present comparative analysis (Ressel, 1996).

I estimated the cristae surface area of mitochondria, S_V(im,mt), in *P. crucifer* trunk and gastrocnemius muscles after a preliminary examination of my muscle samples at high magnification (200 000×) revealed that these two tissue preparations were the only ones that had a large number of mitochondria in which some portion of inner membrane profiles clearly exhibited a double-leaflet structure (i.e. membranes oriented perpendicular to the plane of section; Schwerzmann et al., 1989) (Fig. 1). Thus, I selected at random one tissue block per muscle tissue for each male and cut ultrathin sections (≤40 nm) longitudinal to the fiber axis. Sections were mounted on 400 mesh copper grids and stained with 5 % uranyl acetate and 2.5 % lead citrate. Sixty micrographs were scored using a staggered cycloid arc test system (adapted from Baddeley et al., 1986). I was careful to project a randomly selected area of each mitochondrion so that the vertical axis of the test system aligned with the longitudinal orientation of the muscle axis (Cruz-Orive and Weibel, 1990).

I then obtained cristae surface area per unit volume of mitochondria, S_V(im,mt), using the following equation from Weibel (1979):

$$S_V(\text{im,mt}) = 2 \times (k/d) \times \text{magnification} \times \left[\sum_{i=1}^{n} I \Big/ \sum_{i=1}^{n} P \right],$$

where k and d refer to the total number of points and combined length of all cycloid arcs on the test system, respectively. Electron micrographs were scored at a magnification of 200 000×, and the number of intersections, I, between cycloid arcs and cristae membranes and the number of test points, P, that fell within the outer membrane boundary of the mitochondrion being examined were summed over all micrographs, n. No corrections were made for differences in section thickness or compression.

Statistical analyses

Values are expressed as means ± one standard error of the mean (S.E.M.). The standard error of volume estimates was calculated according to Hoppeler et al. (Hoppeler et al., 1981). The standard error of the different structural variables of capillary supply, fiber size and sarcomere length indicates variation among the five males per species. Standard errors of cristae surface area were approximated according to Weibel (Weibel, 1979).

Mann–Whitney *U*-tests of mean estimates and a two-way Kruskal-Wallis test of J(c)-to-V(mt,m) ratios in trunk muscles and gastrocnemius across species with family affinity (Hylidae or Ranidae) and activity temperature (low and high temperature) as between-subject factors were performed using Systat (Version 5.2). Differences were considered significant if $P<0.05$.

Results

Activity temperatures and level of vocal activity

Male *Pseudacris crucifer* and *Rana sylvatica* were active at ambient temperatures that did not exceed 5 °C and 7 °C, respectively (Table 1). Table 1 also shows that the higher activity temperature of male *Rana clamitans* was close to that reported previously for male *Hyla microcephala*. Male *R. sylvatica* had a mean calling rate that was 3.2 times that of male *R. clamitans* (Table 1).

Table 1. *Activity temperature, level of calling activity and muscle mass of hylid and ranid frogs*

	Pseudacris crucifer	*Hyla microcephala*	*Rana sylvatica*	*Rana clamitans*
Air temperature (°C)	1±1	26±1[a]	ND	22±1
water temperature (°C)	5	ND	7	23
Calling rate (notes h^{-1})	3075±70[b]	3800[a]	557±56	174±28
Trunk-muscle mass (g)	0.187±0.007*	0.056±0.004	0.597±0.043	2.50±0.231*
Gastrocnemius mass (g)	0.014±0.001	0.025±0.003*	0.189±0.02	0.982±0.054*

Values are expressed as mean ± 1 S.E.M.
*Significantly different between species (P<0.05).
Behavioral data, N=20 for each species; tissue mass data, N=5 for each species.
[a]Data from Wells and Taigen (1989).
[b]Data from Zimmitti (1999).
ND, not determined.

Intrafamilial differences in trunk-muscle and gastrocnemius ultrastructure

Trunk-muscle V_V(mt,f) was nearly equivalent in the two hylid treefrogs, whereas trunk-muscle V(mt,m) was significantly higher in *P. crucifer* than in *H. microcephala* because of a 3.3-fold difference in trunk-muscle mass (Tables 1, 2). The trunk muscles of male *P. crucifer* had a significantly higher V_V(li,f) and a significantly lower V_V(mf,f) than those previously described in homologous muscle of male *H. microcephala*. For *P. crucifer*, intracellular lipids accounted for over 26 % of trunk-muscle volume (Table 2; Fig. 2A).

Trunk-muscle V_V(mt,f) was significantly higher in *R. sylvatica* than in *R. clamitans* (Table 3). Further scrutiny of these data revealed that the trunk-muscle V_V(mt,f) and mean calling rate of the three temperate-zone species in this study were related in a manner similar to that observed in seven tropical species of hylid and leptodactylid frogs (Fig. 3). Trunk-muscle V(mt,m) was significantly higher in *R. clamitans* because muscle mass was significantly higher than that of *R. sylvatica* trunk muscles (Tables 1, 3). The trunk muscles of *R. sylvatica* and *R. clamitans* contained intracellular lipids that occupied less than 0.1 % of total muscle volume (Table 3; Fig. 2B,C).

Estimates of V_V(mt,f) in gastrocnemius were considerably lower than in the trunk muscles of all four species (Tables 2, 3; Fig. 2D). Within both families, the cold-adapted species had a significantly higher V_V(mt,f) than the warm-adapted species. However, *H. microcephala* and *R. clamitans* possessed gastrocnemius muscle with a significantly higher mass (Table 1), which resulted in equivalent values of V(mt,m) in the gastrocnemius muscles of the two hylid treefrogs and a significantly higher V(mt,m) in *R. clamitans* than in *R. sylvatica*. Intracellular lipids were found at very low or undetectable concentrations in the gastrocnemius of all four species (Tables 2, 3; Fig. 2D).

Intrafamilial differences in trunk-muscle and gastrocnemius capillary supply

Morphometric estimates of capillary supply in the two hylid and two ranid species are given in Tables 4 and 5, respectively. Mean sarcomere length varied by no more than 8 % for each intrafamilial comparison of trunk muscles and gastrocnemius.

Table 2. *Ultrastructural variables of muscle tissue from two species of hylid frog*

	Trunk muscles		Gastrocnemius muscle	
Variable	*Pseudacris crucifer*	*Hyla microcephala*	*Pseudacris crucifer*	*Hyla microcephala*
V_V(mt,f) (%)	24.5±0.9	23.8±0.7[a]	5.1±0.4*	3.1±0.1
V_V(li,f) (%)	26.4±1.6*	17.3±1.2[a]	0.09±0.05	0
V_V (mf,f) (%)	48.0±1.1*	57.1±1.1[a]	92.6±0.5	94.7±0.4
V(mt,m) (cm^3)	0.043±0.004*	0.013±0.001	0.001±0.0001	0.001±0.0001

Values are means ± 1 S.E.M.; N=5 for each species.
*Significantly different between species (P<0.05).
V_V(mt,f), volume of mitochondria per fiber volume; V_V(li,f), volume of intracellular lipids per fiber volume; V_V (mf,f), volume of myofibrils per fiber volume; V(mt,m), total volume of mitochondria.
[a]Data from Ressel (1996).

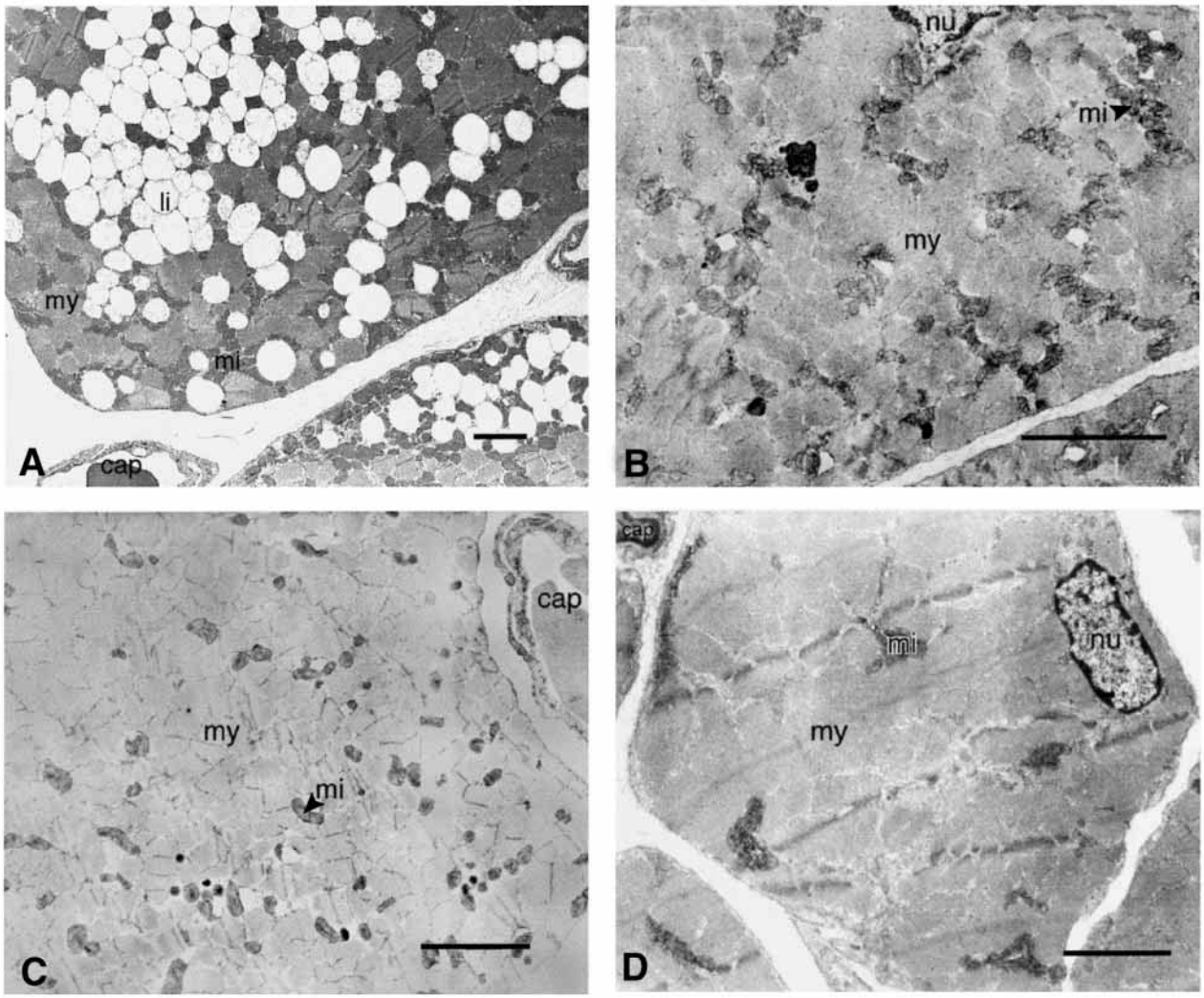

Fig. 2. Transverse sections of trunk muscles from (A) *Pseudacris crucifer*, (B) *Rana sylvatica* and (C) *Rana clamitans*. The transverse section of gastrocnemius from *R. sylvatica* (D) is representative of the four species in this study. Scale bars: 2 µm in A; 1 µm in B–D. my, myofibrils; mi, mitochondria; li, intracellular lipids; nu, nucleus; cap, capillary.

Thus, I used the c(K,0) values derived for *P. crucifer* muscles to calculate J_V(c,f) and J(c) for homologous muscle of the other species.

Capillary-to-fiber ratio, N_N(c,f), and $\bar{d}$(c) were, on average, not significantly different between the two hylid species in the trunk muscles, although N_N(c,f) was approximately 18 % higher in *P. crucifer* than in *H. microcephala* (Table 4). The mean fiber area of the trunk muscles was 1.9 times larger in *P. crucifer* than in *H. microcephala*. Consequently, trunk-muscle J_V(c,f) was 1.7 times higher in *H. microcephala* than in *P.*

Table 3. *Ultrastructural variables of muscle tissue from two species of ranid frog*

	Trunk muscles		Gastrocnemius muscle	
Variable	*Rana sylvatica*	*Rana clamitans*	*Rana sylvatica*	*Rana clamitans*
V_V(mt,f) (%)	13.0±0.9*	8.7±0.6	6.3±0.6*	4.4±0.3
V_V(li,f) (%)	0.05±0.04	0.09±0.05	0	0
V_V (mf,f) (%)	82.3±0.8	89.6±0.8	91.8±0.6	94.6±0.4
V(mt,m) (cm^3)	0.075±0.012	0.20±0.022*	0.011±0.001	0.039±0.001*

Values are means ± 1 S.E.M.; N=5 for each species.

*Significantly different between species (P<0.05).

V_V(mt,f), volume of mitochondria per fiber volume; V_V(li,f), volume of intracellular lipids per fiber volume; V_V (mf,f), volume of myofibrils per fiber volume; V(mt,m), total volume of mitochondria.

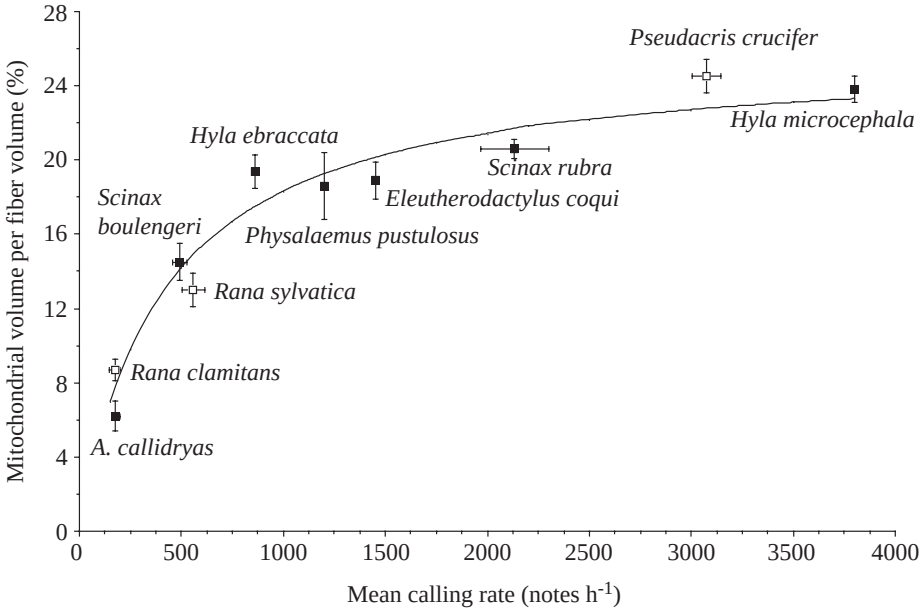

Fig. 3. Relationship between mean calling rate (N=20) and the trunk-muscle volume of mitochondria per fiber volume, V_V(mt,f) (N=5) among three temperate-zone frogs (open squares) compared with that of seven neotropical frogs, including *Hyla microcephala* (filled squares), from Ressel (1996). Values are means ± 1 S.E.M.

crucifer. Total mitochondrial volume (Table 2), however, was significantly higher in *P. crucifer* trunk muscles because of the larger muscle mass of this tissue (Table 1).

N_N(c,f) was significantly higher in the trunk muscles of *R. sylvatica* than in *R. clamitans*, while no significant difference in $\bar{a}$(f) was detected between the two ranid species (Table 5). However, total mitochondrial volume in the trunk muscles was significantly higher in *R. clamitans* than in *R. sylvatica* (Table 2) because of a larger trunk-muscle mass in this species (Table 1). Mean capillary diameter in *R. sylvatica* trunk muscles was 1.3 times larger than that found in the homologous muscle of *R. clamitans*.

In both intrafamilial comparisons of gastrocnemius muscle, estimates of J_V(c,f) were not significantly different between cold- and warm-adapted species. In contrast, J(c) was significantly higher in *H. microcephala* and *R. clamitans* than in *P. crucifer* and *R. sylvatica*, respectively. These differences can also be accounted for by the large differences in the gastrocnemius muscle mass between the hylid and ranid species. Mean capillary diameter in gastrocnemius was significantly larger in *R. sylvatica* than in *R. clamitans*, although not to the extent observed in the trunk muscles of these two species (1.0 μm larger *versus* 2.5 μm larger, respectively).

Effects of temperature and family affinity on capillary-to-mitochondria ratios

A two-way Kruskal–Wallis test revealed that activity temperature had a significant effect on J(c)-to-V(mt,m) ratios

Table 4. *Morphometric estimates of capillary supply, fiber size and sarcomere length of trunk muscles and gastrocnemius in two hylid frogs*

	Trunk muscles		Gastrocnemius muscle	
Variable	*Pseudacris crucifer*	*Hyla microcephala*	*Pseudacris crucifer*	*Hyla microcephala*
N_N(c,f)	1.86±0.17	1.53±0.10	0.80±0.04*	0.60±0.04
N_A(c,f)0 ° (mm^{-2})	718.6±72.1*	1222.4±101.0[a]	338.5±24.8	357.4±26.2
N_A(c,f)90 ° (mm^{-2})	425.6±63.9	ND	86.5±7.4	ND
c(K,0)	1.46±0.13	ND	1.11±0.03	ND
J_V(c,f) (mm^{-2})	1052.8±140.1*	1794.5±215.7[a]	374.4±28.8	396.8±29.06
J(c) (m)	187.8±26.4*	92.3±5.6	5.0±0.6	9.2±1.0*
$\bar{d}$(c) (μm)	7.51±0.22	7.8±0.2[a]	7.56±0.3	7.8±0.3
$\bar{a}$(f) (μm^2)	2565.4±125.5*	1312.75±52.4	2386.9±75.2*	1708.15±72.6
l_o (μm)	1.62±0.02	1.66±0.02[a]	1.84±0.02	2.0±.02

Values are means ± 1 S.E.M.; N=5 for each species.

*Significantly different between species (P<0.05).

N_N(c,f), capillary-to-fiber ratio; N_A(c,f)0 °, capillary numerical density in transversely oriented fibers; N_A(c,f)90 °, capillary numerical density in longitudinally oriented fibers; c(K,0), capillary tortuosity coefficient; J_V(c,f), capillary length per fiber volume; J(c), total capillary length; $\bar{d}$(c), mean capillary diameter; $\bar{a}$(f), mean cross-sectional fiber area; l_o, mean sarcomere length.

ND, not determined.

[a]From Ressel (1996).

Table 5. *Morphometric estimates of capillary supply, fiber size and sarcomere length of trunk muscles and gastrocnemius in two species of ranid frogs*

	Trunk muscles		Gastrocnemius muscle	
Variable	*Rana sylvatica*	*Rana clamitans*	*Rana sylvatica*	*Rana clamitans*
N_N(c,f)	1.29±0.08*	1.04±0.06	0.85±0.09*	0.60±0.04
N_A(c,f)0 ° (mm^{-2})	437.1±35.1	395.3±29.0	205.2±21.3	120.8±5.6
J_V(c,f)[a] (mm^{-2})	641.7±76.1	580.3±66.2	229.4±24.4	135.1±7.0
J(c)[a] (m)	357.8±20.6	1278.0±84.2*	40.3±2.2	124.8±8.1*
$\bar{d}$(c) (μm)	11.2±0.3*	8.7±0.4	10.3±0.3*	9.3±0.2
$\bar{a}$(f) (μm^2)	3179.0±164.8	2846.3±174.1	4264.5±255.0	4919.9±301.9
l_o (μm)	1.71±0.02	1.70±0.02	1.81±0.01	1.87±0.02

Values are means ± 1 S.E.M.; N=5 for each species.

*Significantly different between species (P<0.05).

N_N(c,f), capillary-to-fiber ratio; N_A(c,f)0 °, capillary numerical density in transversely oriented fibers; J_V(c,f), capillary length per fiber volume; J(c), total capillary length; $\bar{d}$(c), mean capillary diameter; $\bar{a}$(f), mean cross-sectional fiber area; l_o, mean sarcomere length.

[a]See Results for calculation of J_V(c,f) and J(c).

in trunk muscles (H=8.69, P<0.005). For both intrafamilial comparisons, the cold-adapted species had a lower J(c)-to-V(mt,m) ratio in the trunk muscles (*P. crucifer* and *R. sylvatica*: 4.3 and 4.8 km cm^{-3}, respectively) than the warm-adapted species (*H. microcephala* and *R. clamitans*: 7.1 and 6.4 km cm^{-3}, respectively).

In contrast, family affinity had a highly significant effect on J(c)-to-V(mt,m) ratios in gastrocnemius (H=14.29, P<0.001), with the two hylid species having more capillaries per unit volume of mitochondria, on average, than the two ranid species (hylid mean 7.1 km cm^{-3}; ranid mean 3.4 km cm^{-3}). Between the two hylid species, J(c)-to-V(mt,m) ratios in gastrocnemius were highly variable (*P. crucifer* 5.0 km cm^{-3}; *H. microcephala* 9.2 km cm^{-3}), whereas the gastrocnemius of *R. sylvatica* and *R. clamitans* exhibited similar J(c)-to-V(mt,m) ratios of 3.6 and 3.2 km cm^{-3} respectively.

Morphometric estimates of mitochondrial cristae surface area

For *P. crucifer*, mean values of cristae surface area per unit volume of mitochondria, S_V(im,mt), were similar in the trunk muscles and gastrocnemius, 37.69±1.55 and 35.93± 1.48 m^2 cm^{-3}, respectively.

Discussion

In the only previous study that addressed temperature effects on anuran muscle ultrastructure, Ballantyne and George (Ballantyne and George, 1978) reported that mitochondrial populations increased 2.4-fold in the m. triceps brachii of *Rana pipiens* upon acclimation to cold temperatures. These data have been used to suggest that anurans may exhibit ultrastructural adaptations to both short- and long-term cold exposure similar to those observed in fish muscle (Egginton and Sidell, 1989). However, the present data demonstrate that male *Pseudacris crucifer* and *Rana sylvatica*, which often breed at near-freezing temperatures, possess trunk muscles with fractional volumes of mitochondria that do not exceed the values observed in and predicted from warm-adapted frogs with similar calling efforts (Fig. 3). For *P. crucifer*, trunk-muscle V_V(mt,f) in relation to mean calling rate is slightly higher than predicted, but the magnitude of the difference is far smaller (6 %) than that reported for comparisons of oxidative muscle between cold- and warm-water fish (55–200 %; Sidell, 1988). In addition, my two intrafamilial comparisons of trunk-muscle V(mt,m) did not reveal consistently higher values in the two cold-adapted species. It is also noteworthy that the higher trunk-muscle V(mt,m) in *P. crucifer* compared with *Hyla microcephala* was the result of larger muscle mass and not a greater abundance of mitochondria, as seen in cold-water fish muscle.

My intrafamilial comparisons of gastrocnemius muscle did reveal an inverse relationship between V_V(mt,f) and environmental temperature between species, but when I factored in the mean muscle mass of this tissue, no consistent relationship was detected between V(mt,m) and the thermal environment of the species. In the case of the two ranid frogs, however, the fractional volume of mitochondria in the gastrocnemius may be related to interspecific differences in locomotor performance levels because previous studies have shown that exercising *R. sylvatica* have higher mass-independent rates of oxygen consumption than do exercising *R. clamitans* (Taigen et al., 1982; Pough and Kamel, 1984; Taigen and Beuchat, 1984). These combined data, in turn, are consistent with differences in the foraging and reproductive behavior of these two ranid species. For example, *R. sylvatica* actively search for food on land after breeding, whereas *R. clamitans* are sit-and-wait predators along the water's edge. Male *R. sylvatica* also actively swim towards females while calling during the breeding season, but male *R. clamitans* remain sedentary while calling (Wells, 1977; Howard, 1980).

My data also show that V_V(mt,f) was approximately two- to eightfold greater in trunk muscles than in gastrocnemius among the species in this study, which is consistent with the observation that male frogs generally exhibit significantly higher mass-specific metabolic rates during calling than during

locomotion (Pough et al., 1992). More abundant mitochondria in the trunk muscles relative to the gastrocnemius is also consistent with previous data that demonstrate markedly higher levels of sustained contractile activity and higher levels of citrate synthase (CS) activity in trunk muscles relative to leg muscles in a phylogenetically diverse group of male frogs (Marsh and Taigen, 1987; Pough et al., 1992; Bevier, 1995). On the basis of these analyses, ultrastructural indices of oxygen demand in anuran skeletal muscle appear to be more aligned with mammalian muscle than with fish muscle because mitochondrial content is causally linked to tissue oxidative capacity across different tissue types and across species, irrespective of their taxonomic position and thermal environment.

In assessing the match between oxygen delivery and demand in the trunk muscles of each species, the present results reveal that male *P. crucifer* and *R. sylvatica* exhibit J(c)-to-V(mt,m) ratios that are 40 % and 25 % lower, respectively, than those observed in *H. microcephala* and *R. clamitans*. These data, in turn, support the hypothesis (Marsh and Taigen, 1987) that the oxidative muscle of cold-adapted frogs should have a less dense capillary bed per unit volume of mitochondria than that of warm-adapted frogs. Yet, careful scrutiny of the present data reveal that a lower J(c)-to-V(mt,m) ratio in the trunk muscles of *P. crucifer* and *R. sylvatica* does not reflect fewer capillaries per trunk-muscle fiber. Indeed, my data indicate that the two cold-adapted anurans have higher capillary-to-fiber number ratios in their trunk muscles than do the warm-adapted anurans. Rather, the differences in trunk-muscle capillarity between the hylid and ranid frogs examined here are primarily a function of interspecific differences in mean fiber area and muscle mass.

Mathieu-Costello (Mathieu-Costello, 1993) has shown that morphometric estimates of mean fiber area and c(K,0) in mammalian and avian muscle are greatly influenced by the amount of fiber shortening that occurs during fixation. Although the effect of fiber shortening on fiber size has yet to be determined for amphibian muscle, it is unlikely that the interspecific differences in mean fiber area reported here are an artifact of differences in fixed fiber length because l_o varied by $\leqslant$5 % among trunk-muscle samples and by $\leqslant$8 % among gastrocnemius samples. On the basis of the predicted change in fiber cross-sectional area with sarcomere length in pigeon (*Columba livia*) pectoralis muscle (Mathieu-Costello, 1991) and dog hindlimb muscles (Mathieu-Costello et al., 1989), these interspecific differences in l_o would alter the mean fiber area of trunk muscles and gastrocnemius by only 6 % and 9 %, respectively. Similarly, my use of c(K,0) estimates from *P. crucifer* to derive J_V(c,f) values for all the species in this study appears to be justifiable because the error margin among species for this calculation is $\leqslant$10 %, assuming that changes in the fixed length of frog muscle fibers alter c(K,0) in a manner consistent with that reported for mammalian muscle (Mathieu-Costello, 1993). The error margins for mean fiber area and c(K,0), in turn, fall within the acceptable range established for morphometric estimates derived *via* stereology (Cruz-Orive and Weibel, 1990). At the same time, the absence of data on mean fiber area and capillary anisotropy in relation to sarcomere length in anuran muscle tissue limits comparisons of the present data with morphometric data from other vertebrate muscle that has been normalized to l_o=2.1 μm (Mathieu-Costello et al., 1989).

Two independent lines of evidence derived from the present and previous studies suggest that lower J(c)-to-V(mt,m) ratios in the trunk muscles of male *P. crucifer* and *R. sylvatica* may indeed reflect the microvasculature of anuran oxidative muscle conforming to reduced mitochondrial respiration rates at low tissue temperatures. First, mitochondria in anuran skeletal muscle do not appear to be inherently less oxidative than mitochondria in mammalian muscle on the basis of my morphometric assessment of S_V(im,mt) in *P. crucifer* muscle, which yielded values that are within the range of those measured in mammalian muscle (36–38 in frogs *versus* 20–40 $m^2\,cm^{-3}$ in mammals; Hoppeler et al., 1991). This result, however, should be viewed with some caution because comparisons of absolute values of S_V(im,mt) among studies are potentially impeded by volume changes in mitochondria associated with different protocols for tissue fixation and embedding (Schwerzmann et al., 1989).

Nonetheless, as a first approximation of S_V(im,mt) in anuran skeletal muscle, the present data suggest that the internal structure of mitochondria in *P. crucifer* muscle is consistent with that observed in one study of mammalian muscle in which S_V(im,mt) was derived in a similar manner (Schwerzmann et al., 1989). The methodology that I employed here ultimately precluded me from comparing S_V(im,mt) among the four anuran species in this study, but this result is still noteworthy from a comparative perspective because male *P. crucifer* exhibit a capillary-to-mitochondria ratio in trunk muscles that is approximately one-third that of mammalian muscle (4.3 *versus* 13 $km\,ml^{-1}$; Hoppeler and Billeter, 1991).

Second, if variable capillary-to-mitochondria ratios in the trunk muscles of different species of frogs reflect a structural and functional unit that is maximally designed for the thermal environment of a species, then one can predict that, at equivalent temperatures and at comparable levels of calling, male *H. microcephala* and *R. clamitans* should attain higher rates of oxygen consumption during calling, $\dot{V}_{O_2}$call, than *P. crucifer* and *R. sylvatica*, respectively. A direct test of this prediction in both families, although important to the functional significance of the results presented here, is problematic for the two ranid species because male *R. sylvatica* in natural choruses do not call at temperatures above 10 °C (Wells and Bevier, 1997; S. J. Ressel, personal observation).

P. crucifer, however, have a prolonged breeding season in which males are active at temperatures that can range from 4 to 23 °C (Wells et al., 1996; Zimmitti, 1999). Wells et al. (Wells et al., 1996) have shown that the calling rate of male *P. crucifer* is highly temperature-dependent. Because $\dot{V}_{O_2}$call is a simple linear function of mean calling rate, and the trunk muscles probably consume most of the oxygen utilized while males call (Pough et al., 1992), I can therefore use published metabolic and behavioral data for *P. crucifer* active at 23 °C

and for *H. microcephala* active at 26 °C to compare the relationship between $\dot{V}_{O_2}$call and mean calling rate in the two species at roughly similar temperatures. At selected mean calling rates of 1000, 3000 and 5000 notes h^{-1}, the predicted $\dot{V}_{O_2}$call values for *H. microcephala* are 0.58, 1.38 and 2.18 ml g^{-1} h^{-1} (Wells and Taigen, 1989), whereas predicted $\dot{V}_{O_2}$call values for *P. crucifer* are 0.45, 1.1 and 1.76 ml g^{-1} h^{-1} (Wells et al., 1996). These data indicate that *H. microcephala* exhibits higher mass-specific rates of oxygen consumption at a given level of calling than does *P. crucifer* and that this disparity in metabolic capacity increases with mean calling rate when oxygen flux rates within trunk-muscle fibers presumably approach maximal levels. Because V_V(mt,f) as well as maximal levels of citrate synthase (CS) activity are equivalent in the trunk muscles of the two species (Taigen et al., 1985; Bevier, 1995; this study), one can reasonably conclude that a less expansive capillary network may be limiting the metabolic performance during calling of *P. crucifer* at elevated temperatures.

This structure/function analysis is further supported by a comparison of actual measurements of peak $\dot{V}_{O_2}$call against potential peak $\dot{V}_{O_2}$call values over the natural thermal range in which male *P. crucifer* vocalize. At environmental temperatures of 7, 10, 15, 19 and 23 °C, Wells et al. (Wells et al., 1996) calculated peak $\dot{V}_{O_2}$call values of 0.74, 1.15, 1.51, 1.59 and 2.11 ml g^{-1} h^{-1}, respectively, from the calling activity of males in natural choruses. Using published data on the thermal sensitivity of CS activity in *P. crucifer* trunk muscles at assay temperatures of 5, 10, 15, 20 and 25 °C, I estimated potential peak $\dot{V}_{O_2}$call values to be 0.74, 1.18, 1.75, 2.27 and 2.75 ml g^{-1} h^{-1}, respectively (Zimmitti, 1999). Assuming that peak levels of CS activity reflect maximal levels of mitochondrial oxygen demand in *P. crucifer* trunk muscles, this comparison clearly shows that potential values are higher than actual values of $\dot{V}_{O_2}$call at temperatures above 10 °C, with the difference between the two metabolic estimates becoming progressively larger with increasing temperature. This analysis suggests, therefore, that *P. crucifer* may lack the capacity to deliver oxygen at a level commensurate with the oxygen demands of the mitochondrial respiratory enzymes in exercising trunk muscles at temperatures above 10 °C, which may reflect a trunk-muscle respiratory design that is maximally designed for cold temperatures.

Ultrastructural analysis of *P. crucifer* trunk muscles also revealed that endogenous lipid stores in this tissue far exceed those reported for any vertebrate skeletal muscle studied to date. Although unprecedented in the literature in terms of the proportion of total muscle fiber occupied by endogenous lipids (Fig. 4), this finding is consistent with the idea that male *P. crucifer* rely primarily on intramuscular lipids to fuel calling, especially for males that call early in the season when prey items are unavailable or indigestible (Wells and Bevier, 1997). Nonetheless, our understanding of a vertebrate's maximal capacity to accumulate endogenous substrate reserves without compromising the contractile properties of skeletal muscle needs to be reassessed due to the extraordinarily high lipid content of *P. crucifer* trunk muscles reported here (Weber, 1992).

Substantial accumulations of intracellular lipids in fish oxidative muscle are thought to enhance oxygen transfer in muscle tissue that has a lower than expected capillary-to-mitochondria ratio (e.g. Egginton and Sidell, 1989; Londraville and Sidell, 1990). Assuming that lipid bodies in anuran skeletal muscle consist primarily of triglycerides, and thus exhibit oxygen solubility and diffusivity properties similar to those proposed for endogenous fish oils (Sidell, 1988), then the extremely high lipid content in *P. crucifer* trunk muscles may compensate for a less extensive capillary network compared with that in *H. microcephala* trunk muscles. However, my preceding comparative analysis of temperature effects on

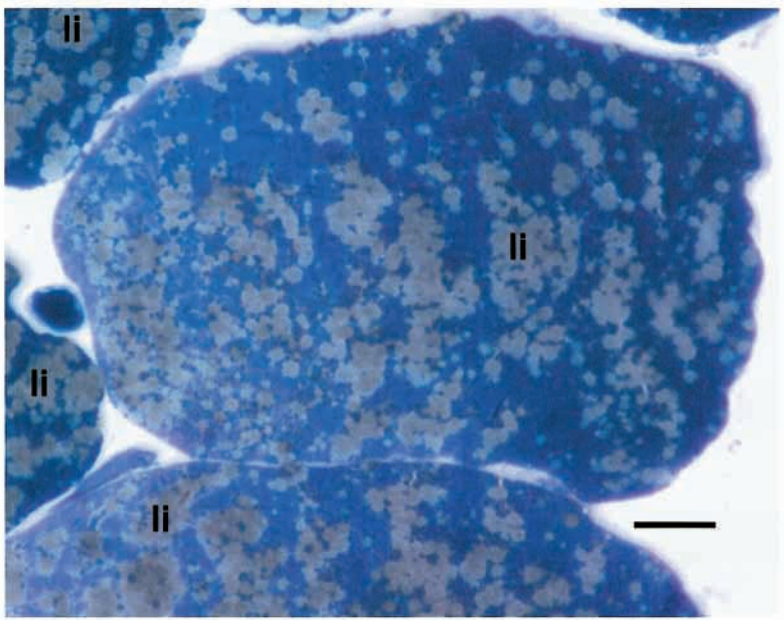

Fig. 4. Light micrograph of a transverse semi-thin section of *Pseudacris crucifer* trunk muscle showing extensive accumulation of intracellular lipids (li) throughout the muscle fibers. Scale bar, 10 μm.

oxygen consumption rates while calling suggests otherwise. While ample theoretical and empirical data support the idea that lipid inclusions may help facilitate transmuscular oxygen transport (Sidell, 1991), lower levels of CS activity in *P. crucifer* trunk muscles at cold temperatures may reduce the driving force of oxygen in individual fibers and thus obviate the need to supplement oxygen flux rates to mitochondria beyond the levels set by the total microvasculature. At warmer temperatures, however, trunk-muscle mitochondrial function is higher, and one should expect to see some measure of convergence between actual and potential values of $\dot{V}_{O_2}$call if lipids and capillaries function as a co-adapted oxygen delivery system in *P. crucifer* trunk muscles. One possible explanation for the lack of convergence observed here is that the higher energetic costs of calling at warmer temperatures may decrease trunk-muscle lipid stores of male *P. crucifer* to levels that create a mismatch between oxygen demand and oxygen supply *via* the capillaries and the remaining lipid compartment. Indeed, studies have shown that prolonged bouts of sustained calling by male frogs can deplete lipid stores in trunk muscles and laryngeal muscles, which are also used in call production in some anurans, by as much as 75 % (Eichelberg and Obert, 1976; Pough et al., 1992; Ressel, 1993).

A near absence of trunk-muscle lipids in *R. sylvatica* and *R. clamitan*s contrasts markedly with the high lipid content of trunk muscles in the two hylid species. Patterns of substrate use in calling frogs are thought to follow the general vertebrate scheme in which sustained aerobic muscle activity is supported by the oxidation of carbohydrates and lipids (Wells et al., 1995, Bevier, 1997). Thus, this finding suggests that structure/function analysis of trunk-muscle capillary supply in male *R. sylvatica* and *R. clamitans* requires a more integrative approach than that of hylid trunk muscles because J(c) in ranid trunk muscles may reflect the functional demands associated with maximal delivery rates of oxygen and free fatty acids from exogenous reserves to exercising trunk muscles (Hoppeler, 1998). The available evidence suggests, however, that male *R. sylvatica* lack large reserves of exogenous lipids in their abdomen and instead rely almost exclusively on trunk-muscle glycogen stores to fuel calling during a breeding season that usually lasts less than a week (Wells and Bevier, 1997). Although no data relevant to this analysis currently exist for male *R. clamitans*, there is a growing body of data indicating that male frogs of temperate-zone and tropical species in other families rely almost exclusively on endogenous stores of lipids and glycogen in the trunk muscles to fuel all levels of calling (Wells et al., 1995; Bevier, 1997; Grafe, 1997; Wells and Bevier, 1997). These data, in turn, imply that trunk-muscle respiratory design reflects a capillary supply commensurate with maximal levels of oxygen delivery to mitochondria in exercising muscles at different operating temperatures, with delivery rates of exogenous substrates during exercise having little, if any, influence on trunk-muscle J(c).

In contrast, my data suggest that the microvasculature of anuran gastrocnemius may follow different rules of assembly. J(c)-to-V(mt,m) ratios in anuran gastrocnemius are highly variable among three of the four species examined here and, in the case of *H. microcephala*, exceed those of the trunk muscles. A similar pattern has been observed in comparisons of mammalian muscle that vary in oxidative capacity, which may be indicative of a microvasculature in glycolytic vertebrate muscle conforming to lactate removal, to glycogen delivery or to a combination thereof (Hudlicka et al., 1987). Thus, oxygen delivery to mitochondria may not be the principal function of capillaries in anuran leg muscles with low oxidative capacity, which may account for the observation that gastrocnemius J(c)-to-V(mt,m) ratios are independent of the thermal environment of a species.

Although J_V(c,f) has long been viewed as a key structural index of the oxygen delivery system in vertebrate skeletal muscle (Hoppeler and Billeter, 1991), capillary volume, V_V(c,f), and capillary surface area, S_V(c,f), have been advanced as more accurate indices of blood-tissue exchange potential because they take capillary diameter into account (Mathieu-Costello, 1993). Ultrastructural analyses of trunk muscles from the two ranid species in this study indeed show that capillary diameter can vary markedly in homologous muscle of different species. However, large-bore capillaries may not represent an alternative strategy to enhance oxygen delivery to mitochondria in *R. sylvatica* trunk muscles because the apparent absence of subsarcolemmal mitochondria in anuran trunk muscles (Fig. 2A,C; Ressel, 1996) is inconsistent with prevailing models of muscle design that suggest that muscle mitochondria aggregate near capillaries to create a sufficient driving force for oxygen transfer across the capillary/fiber interface (Mathieu-Costello et al., 1991). Because mitochondria have a more homogeneous radial distribution in anuran oxidative muscle, levels of oxygen flux rates associated with larger but fewer capillaries are probably lower than oxygen transfer rates of a more dense capillary network on the basis of currently accepted theoretical models of determinants of Krogh's diffusion radius in muscle tissue (Weibel, 1984).

It has also been proposed that a more comprehensive assessment of subcellular oxygen supply in vertebrate oxidative muscle needs to integrate measurements of arterial blood hematocrit and/or hemoglobin content (e.g. Conley et al., 1987) and blood transit time (e.g. Kayar et al., 1994; Bicudo et al., 1996) with ultrastructural parameters of oxygen delivery. Unfortunately, the lack of available data prevent any attempt to compare the hematological properties of the anuran species examined here. However, future investigations of this nature will need to take into account the high thermal dependence of heart rate in anurans (Harri and Talo, 1975) and the wide fluctuations in blood hematocrit associated with the hydration state of individual frogs (Hutchison and Dupré, 1992) and with seasonal fluctuations in the oxygen-carrying capacity of anuran blood (Harris, 1972).

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