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SUITABILITY OF MEASURED PARAMETERS AND MINIMUM SAMPLE SIZES REQUIRED TO QUANTIFY CAPILLARY SUPPLY TO FISH MUSCLE

Stuart Egginton and Ian A. Johnston

Department of Physiology, University of St. Andrews,
ST. ANDREWS, Fife, Scotland.

ABSTRACT

In the myotomal muscle of fish, metabolic fibre types are arranged in discrete layers. There is considerable variation in the capillary supply both to fast and slow muscle, and between homologous muscles of different species. Mitochondrial volume densities, $V_V(mt,f)$, in fish slow muscle (0.2 to 0.4) are similar to those found for mammalian heart and are positively correlated with the number of capillaries/mm^2 of muscle fibre. $V_V(mt,f)$ for fast fibres varies from 0.001 to 0.09. This paper examines the relative merits of different indices of capillary supply to fish muscle, and determines the minimum sample numbers required for reproducible estimates. A minimum sample size of up to 500 fibres was required to estimate capillary density, $N_A(c,f)$, for conger slow and fast muscle. These values may be substantially reduced in species with a higher capillary density. Estimation of capillary surface $S_V(c,f)$, and volume densities, $V_V(c,f)$, may provide a better estimate of the potential capillary gas exchange. However, these indices require a better understanding of the 3-Dimensional structure of the capillary bed.

INTRODUCTION

Oxygen supply to muscle fibres is dependent on many factors, including the density and surface area of capillaries, blood flow, perfusion distribution, myoglobin content, and other variables affecting the oxygen-haemo-

globin equilibrium and rate of mitochondrial respiration. Many different indices of capillary supply are in use, but none of these provide an adequate description of the physical dimensions of the capillary bed.

Mammalian limb muscles contain a mixture of different fibre types, with a higher capillary density found in red than white muscle (Hoppeler et al., 1981). Analysis of capillary supply is complicated since a single capillary may supply 3 or more different metabolic fibre types. The segmentally-arranged fish trunk musculature is known to be separated into distinct layers of different fibre types (Johnston, 1981), and would appear to offer a useful model for investigating the quantitative relationships between capillary supply and metabolism of specific fibre types.

Several theoretical, and practical limitations affect representative sampling of the capillary supply to fish muscles. Slow muscle is composed of a mixed size range of small diameter fibres, which receive 0 to 13 capillaries per fibre. The sample area is usually restricted to a narrow, superficial layer of muscle, in all but very large fish. In contrast, 90-95% of the myotome is composed of fast glycolytic fibres which present a very large area for sampling, but a heterogeneous and sparse distribution of capillaries makes the capillary supply difficult to quantify. The range of capillary densities found in fish varies from >6000 capillaries mm^{-2} in Anchovy slow muscle (Johnston, 1981) to 3-100 mm^{-2} for elasmobranch fast muscle (Totland et al., 1980). A further consideration which is often overlooked is regional variation in capillary supply within the myotome (Egginton and Johnston, 1982).

To our knowledge, there has been no previous attempt to assess minimum sample size required to overcome these limitations, or the usefulness of particular indices when applied to fish muscle.

MATERIALS AND METHODS

Conger eels (*Conger conger*, L), c.3kg body weight, 1m standard length) were obtained from St. Andrews Bay, Scotland. Fish were stunned and decapitated; small bundles (2-3 mm diameter) of fast and slow muscle fibres were rapidly excised from epaxial myotomes immediately posterior to the cloaca, and fixed at resting length in 3% gluteraldehyde, 0.15M phosphate buffer pH 7.4 at 20°C for 4 hours. Tissue was subsequently processed and

embedded in araldite CY212 (EM Scope, England). Ten blocks of transversely-orientated fibre bundles were prepared from each sample site, from each of 5 fish. Semi-thin sections (0.5 μm) were cut from 6 fast and 5 slow muscle blocks selected at random, but including at least 1 from each individual. Measurements of fibre cross sectional areas, $\bar{a}(f)$, and capillary counts, N(c), were made directly from toluidine blue-stained sections (magnification X400) using a microscope drawing arm in conjunction with a digital planimeter interfaced to a mini-computer. Each section contained an average of 250-300 slow or 200-250 fast muscle fibres. Measurements were made of all fibres in discrete bundles on each section, and analysis performed at regular increments of sample size, N(f), for both the cumulative and sub-sample means, using both individual fish and grouped data. Replication error was <5%. The index chosen for capillary density, $N_A(c,f)$, is likely to provide a better estimate than the index used in previous studies, which relates N(c) to section cross-sectional area, and includes a variable interstitial space component.

Similar analyses were made for slow myotomal fibres from 8 small Crucian carp (Carassius carassius, L.), 75g body weight. From a total of 64 blocks, single sections (0.5 μm thick) were taken from 10 blocks, and 20-30 fibres analysed. In addition, capillary cross sectional areas, $\bar{a}(c)$ and perimeters, $\bar{b}(c)$, were determined from low power electron micrographs (final magnification, X24000), and used to calculate capillary surface density, $S_V(c,f)$, and volume fraction, $V_V(c,f)$ (Hoppeler et al., 1981; see Table 2).

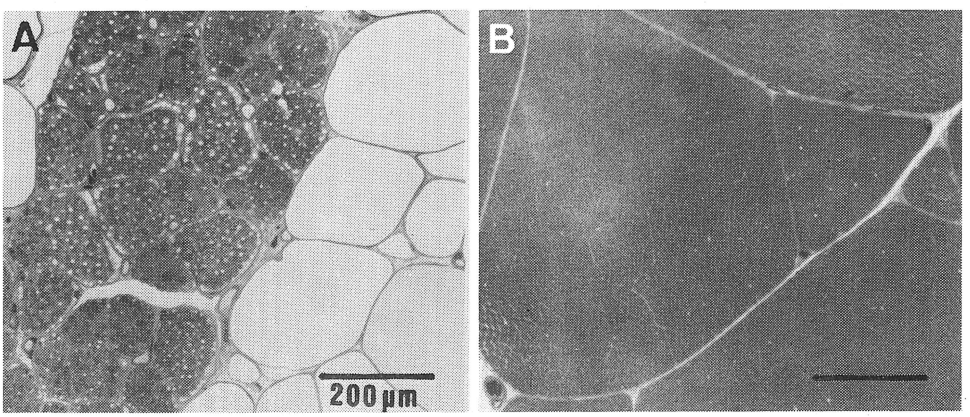

Fig. 1. 0.5 μm sections, toluidine blue stain. A) Conger slow muscle, showing the distribution of capillaries within the muscle layer and between the surrounding adipo-

cytes. B) Conger fast muscle. Note the range of fibre size and sparse capillary population.

RESULTS

The organisation of fast and slow fibres in conger myotomes is similar to that of other fish (Johnston, 1981) except that the slow muscle layer is bounded on both sides by adipocytes (Fig. 1). These regions have their own capillary network, which may contribute to the muscle oxygen supply.

Conger fast muscle contains fibres with a large range of cross sectional areas (Figs. 1,2). The variability of $\overline{a}(f)$, estimated as sample mean/extrapolated population mean, fell to below 10% after N(f) = 300 for the experimental group (Fig. 4). Despite a much narrower range of fibre cross sectional areas in conger slow muscle, a similar sample size was required to obtain a reproducible estimate for $N_A(c,f)$, (Fig. 4). Carp slow muscle had a higher capillary density, and consequently a smaller sample size (100-150 fibres) was required (Fig. 5). For all indices used, the average values for subsample estimates showed considerable variation, reflecting the heterogeneity of capillary supply, whereas the cumulative mean began to plateau after 100 fibres, for both individual fish and grouped sub-samples.

Sharing factor, $\overline{n}(f,c)$, varied little between either sub-samples or muscle types (Fig. 3) and is clearly of limited usefulness. Capillary density, $N_A(c,f)$, co-varied with capillary:fibre ratio, C:F, and fibre cross sectional area, $\overline{a}(f)$.

DISCUSSION

The average number of fibres around a capillary (sharing factor) does not reflect differences in capillary density. It is known to be an insensitive index in the more regular capillary network of mammals (Plyley, Groom, 1975) and, despite the larger range of values in fish muscle an average around 3 was found for all muscle types (Fig. 3). Such an index takes no account of $\overline{a}(f)$ or $\overline{a}(c)$, nor do the frequently used alternatives of C:F or $\overline{n}(c,f)$, the mean number of capillaries around a fibre. These indices would appear to be less useful for fish than mammalian muscle, due to the greater variance of $\overline{a}(f)$. However, some qualitative information can be obtained from these parameters as the magnitude of N(f) required for a

stable estimate of $N_A(c,f)$, varies as a function of $\overline{n}(c,f)$.

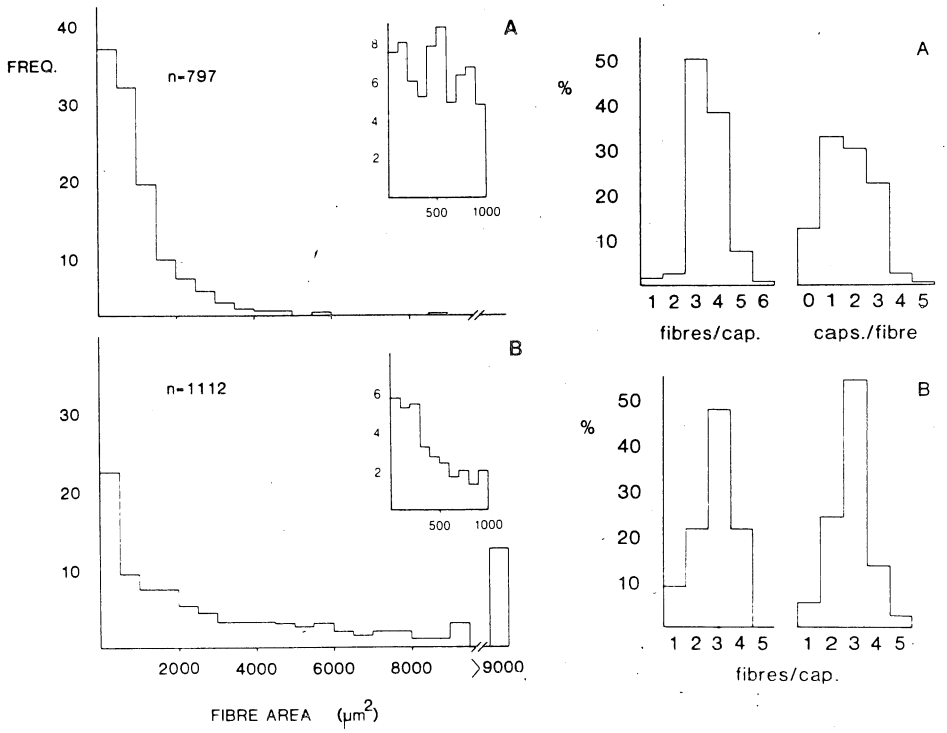

Fig. 2. Frequency distribution histograms for conger slow (A) and fast (B) muscle fibre size. Insets: expanded view of the distribution across the 0-1000 μm^2 range.

Fig. 3. Histograms showing the distribution of values for the average number of fibres around a capillary (sharing factor), $\overline{n}(f,c)$, and average number of capillaries around a fibre, $\overline{n}(c,f)$. A) conger slow muscle; B) conger fast muscle, two examples from different fish.

Stereological analysis of capillary surface and volume densities requires the accurate determination of capillary density, $N_A(c,f)$. As N(f) increases, Type 1 (sampling) error tends to zero and any variation in the estimate of population mean will be due to Type 2 (methodological) error and natural variability, only. Such a value will not then change with further increase in N(f). The optimal, or minimum, value for N(f) is different for each index, muscle type and species investigated. This can be derived by simple graphical estimation (Fig. 4). It is recommended that this procedure be adopted for each new study; for example, a surprisingly high N(f) is necessary in order to obtain

reliable estimates of $N_A(c,f)$ in conger slow muscle, where a 10% variation in mean values occurs between N(c) = 200 and 300. Carp slow muscle, however, reaches a steady value at N(f) = 100 (Fig. 5) and tench slow muscle N(f) = 200. In addition to N(f), the variation in population mean is determined by individual variation of the samples (animals) used in the analyses. It can be shown that N(f) = 100 is usually sufficient to obtain a reproducible estimate of both $\bar{a}(f)$ and $N_A(c,f)$ population means for any individual fish, but 4-5 times this figure is necessary to obtain a representative value for the species mean. With conger, then, a sample of 75-100 fibres from each of 5 fish would seem to be appropriate.

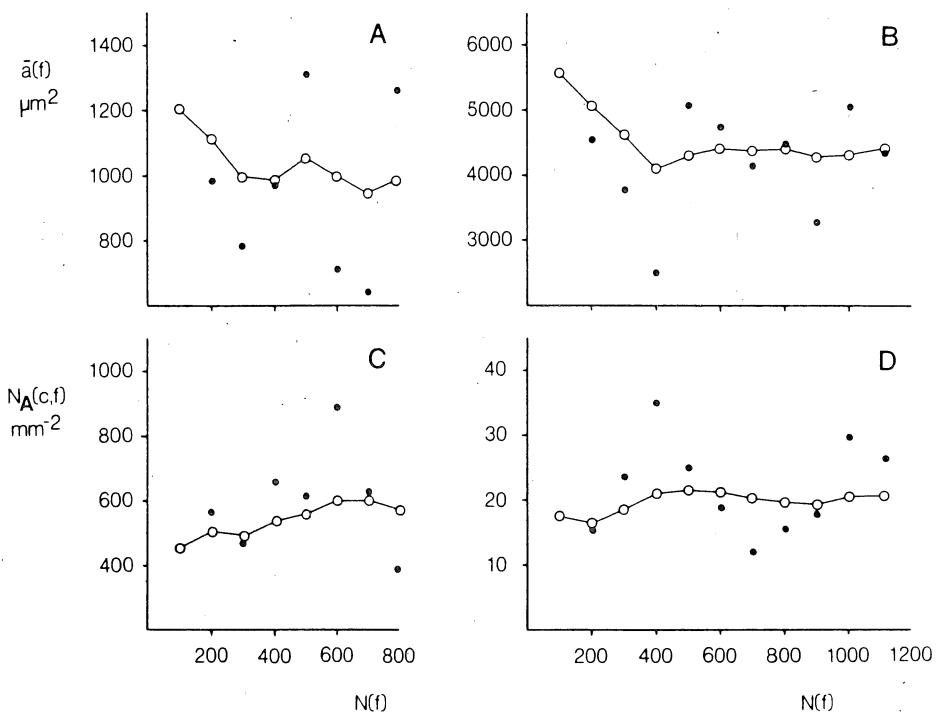

Fig. 4. Plots of cumulative (large circles) and sub-sample (small circles) mean values as a function of increase in sample size, denoted by the number of fibres included in the analysis, N(f). A,B: mean fibre size, $\bar{a}(f)$, for conger slow and fast muscle, respectively. C,D: capillary density, $N_A(c,f)$, for slow and fast muscle.

There are major differences in the functional design

and maximum capacities of the respiratory systems between fishes and mammals. Maximum rates of oxygen consumption are around 10-15 times lower in ectotherms than homeotherms, and are mirrored by a reduction in oxygen-binding capacity of the blood and lowered systemic blood pressure.

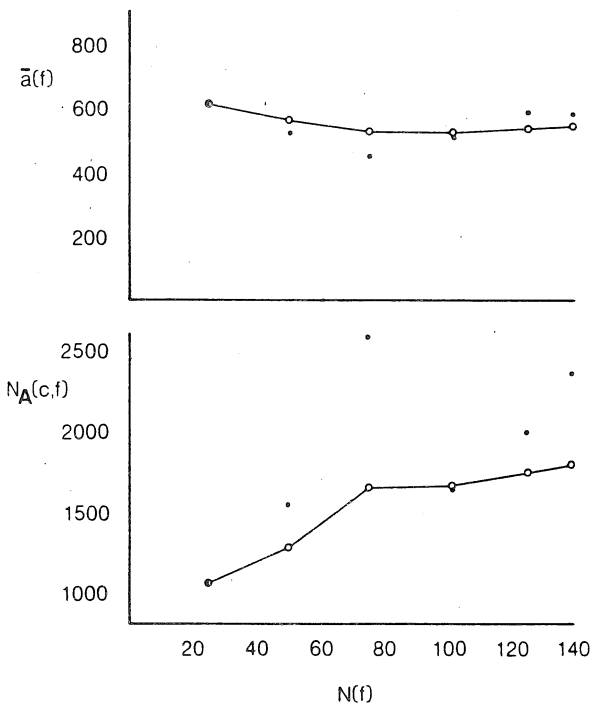

Fig. 5. Similar plot to Fig. 4, for carp slow muscle. Note the higher value of $N_A(c,f)$ and corresponding lower value of $N(f)$ required for consistent estimate of mean values.

A lowered blood pressure requires a decrease in peripheral resistance to maintain efficient perfusion and oxygen supply to the muscle; this may be achieved by an increase in $\bar{a}(c)$ and/or $N_A(c,f)$. From the limited data available, it seems likely that capillary dimensions in fish are similar to mammals of similar body weight, e.g. rat heart 40 μm^2, catfish slow 20 μm^2. A degree of adjustment may be possible, as seen in the haemoglobinless fish, Chaenocephalus aceratus, where $\bar{a}(c)$ = 54 μm^2. Capillary surface density, $S_V(c,f)$, and volume density,

TABLE 1: Comparative data on capillary supply to fish and mammalian aerobic muscles.

SPECIES	MUSCLE	$\bar{a}(f)$ μm^2	$\bar{n}(c,f)$	$N_A(c,f)$ mm^{-2}	$V_V(mt,f)$	REF.
Elver	slow	190.5	0.98	2364	0.214	1
Catfish	slow	660.0	1.90	1899	0.160	2
Conger	slow	950.5	1.71	615	-	3
Icefish	slow	3772	2.40	625	0.304	4
Rat	soleus	-	2.05	396	-	5
Man	quadriceps	4150	1.36	329	-	6
Genet Cat	VM	-	-	731	0.069	7
Dik Dik	VM	1234	-	923	0.036	7
Wildebeest	VM	1248	-	716	0.032	7
Eland	VM	-	-	332	0.035	7

Note: VM = M. vastus medialis; $\bar{n}(c,f)$ = average number of capillaries per fibre. Data taken from: 1, Egginton and Johnston, 1982; 2,4, unpublished data; 3, this study; 5, Plyley and Groom, 1975; 6, Andersen and Henriksson, 1977; 7, Hoppeler et al., 1981.

TABLE 2: Quantitative analyses of the capillary bed in three species of fish, illustrating the variability of homologous muscles.

SPECIES	$\bar{a}(c)$ μm^2	$\bar{b}(c)$ μm	$V_V(mt,f)$	$N_A(c,f)$ mm^{-2}	$S_V(c,f)$ cm^{-1}	$V_V(c,f)$	REF.
Slow muscle:							
Anguilla anguilla	13.5	14.3	0.214	2364	379	0.036	1
Clarias mossambicus	20.3	18.7	0.160	1899	398	0.043	2
Tinca tinca	21.8	19.4	0.230	5092	1106	0.120	3
Fast muscle:							
Anguilla anguilla	10.9	12.8	0.0012	229	32.0	0.0027	1
Clarias mossambicus	20.3	18.7	0.0025	188	39.3	0.0038	2
Tinca tinca	16.3	14.5	0.0025	893	175	0.016	3

Note: $S_V(c,f) = \bar{b}(c).J_V(c,f)$ and $V_V(c,f) = \bar{a}(c).J_V(c,f)$, where $J_V(c,f)$ is the length of capillary per unit volume of muscle and has the dimensions cm^{-2} (Hoppeler et al., 1981). This parameter is derived from capillary density, $N_A(c,f)$, and a multiplication factor, $\chi 1\ (K,O)^{-1} = 1.12$ (Weibel, 1980) to allow for the anisotropic arrangement of the capillary bed.

Sources: 1, Egginton and Johnston, 1982; 2 and 3, Johnston and Bernard, unpublished data.

$V_V(c,f)$, are proportional to the available surface area for gas exchange and volume of capillary blood, respectively. The higher estimates of these parameters in fish slow relative to mammalian red muscles, reflects the higher $V_V(mt,f)$ (Table 2). Quantification of $S_V(c,f)$ and $V_V(c,f)$ may provide a better estimate of capillary supply than $\overline{n}(c,f)$ or $N_A(c,f)$ (Table 1), since the volume of blood delivered to Icefish slow muscle during exercise will be much greater than may be assumed from its capillary density, as the capillary dimensions are some 2-3 times larger than other teleosts. These parameters require a greater knowledge of the physical dimensions of the capillary network before detailed models can be derived. There has been no systematic study of capillary anisotropy, and the values derived for cat (Hoppeler et al. 1981; see Table 2) may not be appropriate for fish muscle. Work is in progress to determine the concentration parameter, K, for different fish muscle. However, the value is likely to be similar to that proposed for mammals since, in Conger slow muscle, only 2% of capillaries were orentated transversely, and around 3% obliquely, to the longitudinal fibre axis.

In conclusion, we consider the best approach for the quantification of the indices mentioned above to be the combination of data from two resolution levels. Semi-thin sections provide a large sample size for determining capillary density by point-counting, whereas electron micrographs provide the necessary resolution for measuring capillary dimensions from a smaller sample area.

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