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Mitochondrial respiration in hummingbird flight muscles

(energy metabolism/exercise/mitochondrial volume density/mitochondrial ultrastructure)

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ABSTRACT **Respiration rates of muscle mitochondria in flying hummingbirds range from 7 to 10 ml of O_2 per cm^3 of mitochondria per min, which is about 2 times higher than the range obtained in the locomotory muscles of mammals running at their maximum aerobic capacities ($\dot{V}o_{2max}$). Capillary volume density is higher in hummingbird flight muscles than in mammalian skeletal muscles. Mitochondria occupy ≈35% of fiber volume in hummingbird flight muscles and cluster beneath the sarcolemmal membrane adjacent to capillaries to a greater extent than in mammalian muscles. Measurements of protein content, citrate synthase activity, and respiratory rates *in vitro* per unit mitochondrial volume reveal no significant differences between hummingbird and mammalian skeletal muscle mitochondria. However, inner membrane surface areas per unit mitochondrial volume [S_V(im,m)] are higher than those in mammalian muscle. We propose that both mitochondrial volume densities and S_V(im,m) are near their maximum theoretical limits in hummingbirds and that higher rates of mitochondrial respiration than those observed in mammals are achieved *in vivo* as a result of higher capacities for O_2 delivery and substrate catabolism.**

The mass-specific aerobic metabolic rates (O_2 consumption per unit body mass; $\dot{V}o_2/M_b$) of many species of birds and mammals increase by 10-fold or more during the transition from rest to maximal aerobic exercise. Maximal O_2 consumption during exercise ($\dot{V}o_{2max}$) is due mainly to mitochondrial respiration in locomotory muscles. Although much is known regarding the respiration of muscle mitochondria *in vitro*, little is known about mitochondrial function in exercising muscles *in vivo*. In particular, it is not known at what rate muscle mitochondria respire when animals exercise at $\dot{V}o_{2max}$. In a series of studies, Taylor, Weibel, and their collaborators (1) measured the $\dot{V}o_{2max}$ of mammals differing in body mass by more than 5 orders of magnitude. Subsequent measurement of the mass of locomotory muscles in these animals and estimation of their mitochondrial volumes allowed calculation of rates of mitochondrial respiration *in vivo*. This yielded a range of between 3 and 5 ml of O_2 per cm^3 of mitochondria per min, which was considered to represent the maximal rate of mitochondrial respiration (2). In addition, it was concluded that muscle mitochondria do not differ in their maximal capacities for respiration; different species of animals capable of achieving widely different $\dot{V}o_2/M_b$ simply possess widely different volumes of muscle mitochondria (2, 3).

However, running at $\dot{V}o_{2max}$ does not necessarily result in maximal recruitment of all of the muscles involved in locomotion. In addition, mammalian locomotory muscles typically consist of different fiber types; not every fiber type may be maximally recruited in each locomotory muscle as animals run at $\dot{V}o_{2max}$. Thus, muscle mitochondria may not all function at their maximal rates *in vivo* when animals exercise at $\dot{V}o_{2max}$. Indeed, it has been suggested that the range of 3–5 ml of $O_2/(cm^3 \times min)$ represents the lower limit of mitochondrial oxygen consumption under these conditions (3).

To obtain better estimates of mitochondrial respiration rates *in vivo*, we measured $\dot{V}o_2/M_b$ during hovering flight in rufous hummingbirds (*Selasphorus rufus*). The mass of locomotory muscles and their mitochondrial volume densities were measured to allow estimation of mitochondrial respiration rates *in vivo*. Mitochondrial respiration rates *in vitro* are compared with rates estimated *in vivo*; these results are compared with those obtained from cat muscles by Schwerzmann *et al.* (4). We report that rates of mitochondrial respiration during hovering flight in hummingbirds are in excess of those estimated in mammals running at $\dot{V}o_{2max}$. Evidence is presented indicating that these higher rates are not simply due to differences in the properties of muscle mitochondria of hummingbirds and mammals.

MATERIALS AND METHODS

Respirometry. Hummingbirds (*S. rufus*) of both sexes, weighing 3–4 g, were caught and maintained as described (5). Respiration rates ($\dot{V}o_2$) during hovering flight were measured using a mask respirometer attached to a feeder as described (6).

Electron Microscopy. Birds were anesthetized and perfusion fixed as in ref. 7. Morphometric measurements and estimation of volume density of mitochondria and surface density of mitochondrial membranes were done as described (8, 9). Two randomly chosen blocks were analyzed from pectoralis and supracoracoideus muscles from each animal. A total of 32 micrographs of each muscle examined at a final magnification of ×24,000 were used to estimate mitochondrial volume density, V_V(mt,f). The surface density of inner mitochondrial membranes, S_V(im,m), was estimated from pictures of 20 interfibrillar and 20 subsarcolemmal mitochondria taken at random from 5–10 fibers from each muscle at a final magnification of ×220,000. Capillary volume density, V_V(c,f), was estimated as described (7).

Mitochondrial Respiration *in Vitro*. Mitochondria were isolated from flight muscles and respiration rates were measured in a previous study (5). Mitochondrial suspensions were stored at −80°C for less than a week. These were freeze-thawed three times, diluted 1:10 with cold 50 mM Tris·HCl (pH 7.6) containing 1 mM EDTA and 0.1% Triton X-100, and sonicated for 10 sec three times. Citrate synthase (CS) activity was measured using 10-μl aliquots as described (5). CS activities in our samples are stable to freezing and to the extraction procedures described above (5, 6). Respiration rates per unit mitochondrial volume *in vitro* and *in vivo* were estimated from CS activity per mg of mitochondrial protein, CS activity per g of muscle, mitochondrial volume density, $\dot{V}o_2$ per mg of mitochondrial protein *in vitro*, and $\dot{V}o_2$ per g of muscle *in vivo* (see *Results*).

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Abbreviation: CS, citrate synthase.
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Table 1. Volume density of mitochondria [total, $V_V(mt,f)$; subsarcolemmal, $V_V(ms,f)$], volume density of capillaries, $V_V(c,f)$, and surface density of mitochondrial inner membrane, $S_V(im,m)$, in hummingbird flight muscles

	V_V(mt,f), %	V_V(ms,f), %	V_V(c,f), %	S_V(im,m), cm²cm³
Pectoralis	33.0 ± 0.4 (4)	12.8 ± 1.0 (4)	9.2 ± 2.3 (4)	569,416 ± 24,707 (4)
Supracoracoideus	36.6 ± 1.3 (3)	16.1 ± 1.5 (3)	8.9 ± 0.7 (3)	597,365 ± 6,540 (3)

Values are means ± SEM; sample size (number of animals) is in parentheses.

RESULTS

Morphometric Measurements. The pectoralis and supracoracoideus muscles account for 30% ± 1% (mean ± SEM; $n = 5$) of total body mass, which is within the range previously reported for various species of hummingbirds (10). Capillary volume density, $V_V(c,f)$, is ≈9% (Table 1), which is 2–6 times greater than that measured in mammalian hindlimb muscles (7). Since fiber volume as a fraction of total muscle volume in mammals is >90% in skeletal muscles (11) and 85% in the heart (12), we may conservatively estimate

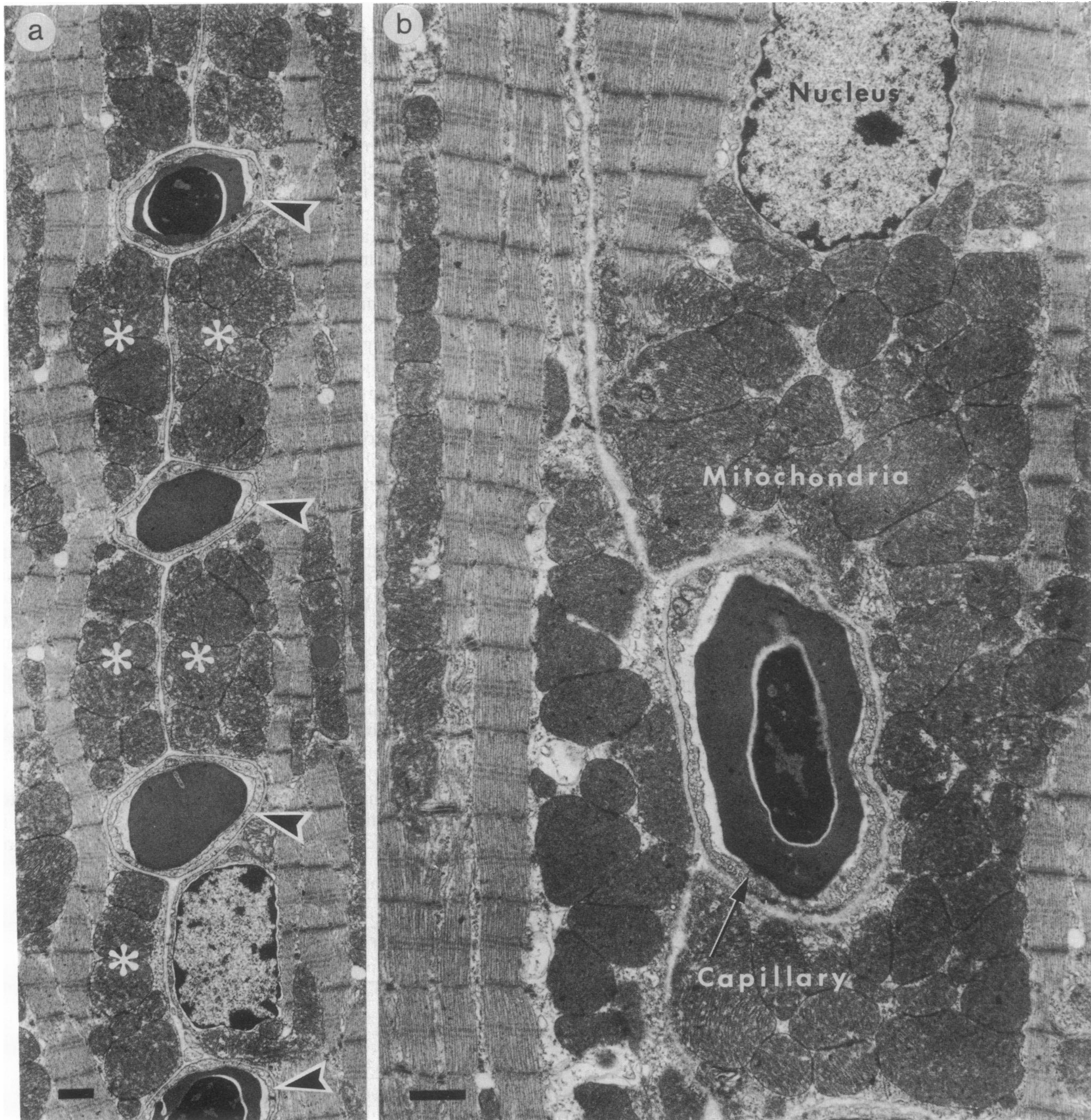

FIG. 1. Transmission electron micrographs of longitudinally sectioned hummingbird pectoralis muscle. (*a*) Numerous capillaries (arrowheads) are found in the space between two adjacent muscle fibers. Subsarcolemmal mitochondria (asterisks) form aggregates in association with the capillaries. This is especially evident in *b*, which shows a capillary completely surrounded by the mitochondria of two adjacent fibers. Micrographs were prepared by Wayne Vogl (Department of Anatomy, University of British Columbia). (Bars = 1 μm.)

this to be ≈85% of muscle volume in hummingbirds. Mitochondrial volume density, V_V(mt,f), is ≈35% (Table 1), a figure considerably lower than the value of 50% estimated previously by Lasiewski *et al.* (13) using unspecified morphometric techniques. About 40% of the mitochondria are localized in the subsarcolemmal regions of the muscle fibers, while the rest (60%) are interfibrillar (Table 1). The subsarcolemmal mitochondria are often found in close association with the numerous capillaries found in the tissue (Fig. 1). Mitochondrial inner membrane surface area per unit mitochondrial volume, S_V(im,m), is ≈580,000 cm^2/cm^3 (Table 1), which is greater than those measured in mammalian skeletal (4, 8) and cardiac (14) muscles.

Mitochondrial Respiration Rates *in Vivo* and *in Vitro*. Hovering rufous hummingbirds respire at a rate of 38.3 ± 1.1 ml of O_2/(g × hr) (mean ± SEM of 10 measurements on four birds) (6). Since the flight muscles account for 30% of total body mass and probably account for most of the O_2 consumption during hovering flight, it can be calculated that the rate of respiration of the flight muscles is ≈2.1 ml of O_2/(g × min) or 82 μmol of O_2/(g × min) (6). Given the above morphometric data and assuming that 1 cm^3 of muscle weighs ≈1.06 g (15), it can be calculated that 1 g of flight muscle tissue occupies a volume of 0.94 cm^3 and that this consists of 0.80 cm^3 of fibers containing 0.28 cm^3 of mitochondria. Based on these, the rate of mitochondrial respiration *in vivo* is 7.1 ml of O_2 per cm^3 of mitochondria per min, which is equivalent to 276 μmol of O_2 per cm^3 of mitochondria per min at 40°C. Expressed as a function of inner membrane surface area, these rates are equivalent to 122 μl of $O_2/(m^2$ × min) or 4.8 μmol of $O_2/(m^2$ × min).

CS (an exclusively mitochondrial enzyme) occurs at a maximum activity of ≈450 units/g (1 unit = 1 μmol of substrate converted to product per min) in the flight muscles (6), while mitochondria isolated from this tissue possess 2.33 ± 0.20 units per mg of mitochondria protein (mean ± SEM; n = 5). Thus, 1 g of flight muscle possesses (450 units/g)/(2.33 units/mg) or 193 mg of mitochondrial protein, and 1 mg of mitochondrial protein is equivalent to 280 μl per 193 mg or 1.45 μl of mitochondrial volume. The mitochondrial volume of 1.45 μl per mg of protein is almost identical to that estimated by Schwerzmann *et al.* (4) in cat muscles and allows expression of respiration rates obtained *in vitro* per unit mitochondrial volume (Table 2). The highest rates of state III respiration achieved by isolated flight muscle mitochondria are obtained when pyruvate plus malate are provided as substrates. Slightly lower rates are obtained when palmitoyl-CoA plus L-carnitine plus malate are used. The rates obtained with pyruvate plus malate (110 μmol of O_2 per cm^3 of mitochondria per min or 2.8 ml of O_2 per cm^3 of mitochondria per min) are ≈39% of the rates estimated *in vivo* during hovering flight. In comparison, Schwerzmann *et al.* (4) obtained a rate of 3.1 ml of O_2 per cm^3 of mitochondria per min (with pyruvate plus malate as substrates) using mitochondria isolated from cat muscles, which is 62% of the highest rates of mitochondrial respiration obtained in mammals *in vivo* (5 ml of O_2 per cm^3 of mitochondria per min).

When expressed as a function of inner membrane surface area (Table 2), the rates obtained *in vitro* with pyruvate plus malate as substrates are ≈56% of those estimated using mitochondria from cat skeletal muscles (4).

DISCUSSION

Hummingbirds are excellent experimental models for the measurement of mitochondrial respiration rates in locomotory muscles *in vivo*. Their flight muscles are anatomically well defined, consist exclusively of type II fibers (13, 16), perform mechanical work at well-established rates (17), and probably account for most of the O_2 consumed during hovering. The mitochondrial respiration rates we estimate in hummingbird flight muscles of 7 ml of O_2 per cm^3 of mitochondria per min are in excess of the highest rates reported in mammals (2, 3). However, estimation of the O_2 consumption rate per unit inner membrane surface area yields a value of 122 μl/(m^2 × min), which is close to the rate estimated in mammalian muscle (4). While the higher respiration rate per cm^3 may be due to the higher S_V(im,m) values, we suggest that this cannot totally account for the higher mitochondrial respiration rates achieved *in vivo*. First, rates of mitochondrial respiration in hummingbirds higher than those we estimate in the present study may be possible. Epting (18) observed that hummingbirds with increased wing disc loading resulting from loss of some wing feathers during molting display up to 50% higher V_{O_2}/M_b than those previously measured using the same animals. This implies that V_{O_2max} was not achieved during the hovering flight involved in our studies and that mitochondrial respiration rates exceeding 10 ml of O_2 per cm^3 of mitochondria per min or 183 μl of $O_2/(m^2$ × min) are possible. Under these circumstances, the rates expressed per cm^3 of mitochondria and per m^2 of inner membrane are both in excess of the rates estimated in mammals running at V_{O_2max} (4). Second, the data obtained *in vitro* using hummingbird mitochondria reveal nothing unusual in comparison with mitochondria from mammalian muscles. CS activities per mg of mitochondrial protein are similar to values obtained using mammalian muscle mitochondria (19). Protein content per unit mitochondrial volume is exactly as reported by Schwerzmann *et al.* (4) using cat skeletal muscle mitochondria. State III rates of respiration (expressed per mg of mitochondrial protein or per cm^3 of mitochondria) obtained *in vitro* using physiological substrates are similar to values obtained using mammalian mitochondria (4, 19).

How do hummingbirds achieve such high rates of mitochondrial respiration *in vivo*? The answer cannot be found in the data available from *in vitro* studies since both we and

Table 2. Respiratory rates (V_{O_2}) of mitochondria isolated from hummingbird flight muscles

Substrates	State III respiration rates: nmol of O_2 per mg of protein	μmol of O_2 per cm^3 per min	ml of O_2 per cm^3 per min	μl of O_2 per m^2 per min	n
Pyruvate + malate	159.3 ± 9.7	109.9 ± 6.7	2.8 ± 0.2	48.7 ± 3.0	4
Palmitoyl-CoA + L-carnitine + malate	138.1 ± 16	95.2 ± 11	2.5 ± 0.3	42.2 ± 4.9	4
Malate	14.6 ± 1.8	10.2 ± 1.3	0.3 ± 0.04	4.5 ± 0.6	3

Values are means ± SEM; n, number of mitochondrial preparations. The highest rates per mg of mitochondrial protein (obtained after first pulse of ADP when pyruvate plus malate are substrates, second pulse when palmitoyl-CoA plus carnitine plus malate are substrates) are obtained from previously reported data (5). Rates per cm^3 are calculated given 1.45 μl of mitochondrial volume per mg of mitochondrial protein and 25.7 liters per mol of O_2 at 40°C. Rates per m^2 of mitochondrial inner membrane are calculated given 580,000 cm^2/cm^3 (see Table 1). Respiratory control ratios (state III rate/state IV rate) were 5.4 ± 0.5 with pyruvate plus malate and 2.7 ± 0.1 with palmitoyl-CoA plus L-carnitine plus malate as substrates. Substrate concentrations were as follows: pyruvate, 5 mM; palmitoyl-CoA, 0.09 mM; L-carnitine, 5 mM; malate, 0.1 mM.

Schwerzmann *et al.* (4) significantly underestimate the respiration rates achieved *in vivo* in our attempts to measure coupled rates of respiration using physiological substrates *in vitro*. The reason for this is not clear. Rates of enzyme-catalyzed reactions under optimal conditions *in vitro* typically exceed rates of metabolic flux *in vivo* by large factors. It is possible that true maximal rates were not obtained because of suboptimal assay conditions in our studies. Schwerzmann *et al.* (4) observed that cat skeletal muscle mitochondria swelled as a result of isolation. It has been found that swelling results in inhibition of the oxidation of pyruvate plus malate but not in inhibition of the oxidation of succinate by mitochondria from rat liver (20).

An attractive explanation is that hummingbird flight muscle mitochondria may function under conditions that allow attainment of a higher fraction of maximal respiratory capacity than possible in mammalian muscles. Hummingbirds possess lungs with almost 10 times higher O_2 diffusion capacities than similar-sized mammals (21) and hearts that are twice as large as predicted on the basis of scaling considerations (22). Heart rates are ≈1400 per min, cardiac output is ≈5 times total body mass per min, and whole body circulation times are ≈1 sec during hovering flight (17). These, as well as high capillary densities (Table 1) and high myoglobin contents (23), may allow higher rates of flux of O_2 and substrates to working muscles than in mammals. In addition, it has been suggested that a high degree of clustering of subsarcolemmal mitochondria adjacent to capillaries as is seen in hummingbird flight muscles makes possible higher rates of O_2 flux than would be possible if mitochondria were uniformly distributed within muscle fibers (24). In contrast, mammalian muscle mitochondria consuming oxygen at a rate of 5 ml of $O_2/(cm^3 \times min)$ are probably not respiring at maximal capacity. This suggestion is consistent with the observation that single limb exercise in humans occurs with higher rates of O_2 consumption per unit mass of muscle than is possible in whole body exercise to Vo_{2max} (25) and mathematical models which predict that the delivery of O_2 is limiting to mitochondrial respiration in mammalian locomotory muscles during high-intensity aerobic exercise (26).

The idea that hummingbird flight muscle mitochondria are able to achieve a greater fraction of their maximum respiratory capacity as a result of higher capacities for delivery of O_2 and/or metabolic substrates is also consistent with recent proposals regarding the factors determining the upper limits to mitochondrial volume density in muscles and inner membrane surface area per unit mitochondrial volume. First, it has been pointed out that there may be a theoretical upper limit to mitochondrial volume density in locomotory muscles beyond which contractile function would be impaired (27, 28). The highest mitochondrial volume densities known in locomotory muscles [≈40% in dipteran flight muscles (29)] are not much higher than those we find in hummingbirds. Second, there may be an upper limit to inner membrane surface area (the site of the electron transport chain enzymes) per unit mitochondrial volume, $S_V(im,m)$, since mitochondria with more cristae have less room for matrix (and, therefore, Krebs cycle enzymes) (30). $S_V(im,m)$ in highly aerobic insect flight muscles [500,000–600,000 cm^2/cm^3 in diptera (29)] is within the range measured in hummingbirds (Table 1). These ranges of $S_V(im,m)$ have been estimated to allow room for only three or four average sized Krebs cycle enzymes between the inner surfaces of the cristae (30).

Hummingbird evolution has resulted in near-maximal mitochondrial content and inner membrane surface area per unit mitochondrial volume. Matched with increased capacities for the delivery of O_2 and substrates, as well as increased enzymatic capacities for substrate catabolism (5), such adaptations allow the achievement of the highest known mass-specific metabolic rates in the vertebrate world.

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