Skeletal Muscle Fuel Selection Occurs at the Mitochondrial Level

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Abbreviations:

21 2-OG: 2-oxoglutarate
22 A: aspartate
23 AcCoA: Acetyl-CoA
24 ANT: adenine nucleotide translocase
25 Asp: aspartate
26 ATP synth: F1-ATP synthase
27 CK: creatine kinase
28 Cr: creatine
29 CPT I: carnitine palmitoyltransferase I
30 DH: substrate dehydrogenase enzymes
31 ETC: electron transport chain
32 G: glutamate
33 G3P: glycerol-3-phosphate
34 IDH: NAD+-linked isocitrate dehydrogenase
35 J0: rate of mitochondrial oxygen consumption
36 Je: electron current
37 Keq: equilibrium constant
38 KmADP: kinetic control of Jo by [ADP]0 alone
39 M: malate
40 MDH: malate dehydrogenase
41 mS: milliSiemens
42 OAA: oxaloacetate
43 OGDH: 2-oxoglutarate dehydrogenase
44 P: pyruvate
45 P1: inorganic phosphate
46 PC: palmitoyl-l-carnitine
47 PCr: phosphocreatine
48 PDH: pyruvate dehydrogenase
49 PDH_A: active (dephosphorylated) pyruvate dehydrogenase
50 R: ideal gas constant
51 RER: respiratory exchange ratio
52 RQ: respiratory quotient
53 RM: respiration medium
54 T: temperature in Kelvin
55 VO2: O2 consumption rate
56 VO2max: maximum O2 consumption rate
57 ΔEh: redox potential difference
58 ΔGATP: ATP free energy
59 ΔGredox: redox free energy difference down the ETC
60 Δp: protonmotive force across the inner mitochondrial membrane
61 Γ: mass action ratio
SUMMARY

Mammals exponentially increase the rate of carbohydrate oxidation as exercise intensity rises, while birds combust lipid almost exclusively while flying at high percentages of aerobic capacity. The fuel oxidized by contracting muscle depends on many factors: whole body fuel storage masses, mobilization, blood transport, cellular uptake, and substrate selection at the level of the mitochondrion. We examined the fuel preferences of mitochondria isolated from mammalian and avian locomotory muscles using two approaches. First, the influence of substrates on the kinetics of respiration ($K_{M\text{ADP}}$ and $V_{\text{max}}$) was evaluated. For all substrates and combinations, $K_{M\text{ADP}}$ was generally two-fold higher in avian mitochondria. Second, fuel competition between pyruvate, glutamate, and/or palmitoyl-l-carnitine at three levels of ATP free energy was determined using the principle of mass balance and the measured rates of $O_2$ consumption and metabolite accumulation/utilization. Avian mitochondria strongly spared pyruvate from oxidation when another substrate was available and fatty acid was the dominant substrate, regardless of energy state. Mammalian mitochondria exhibited some preference for fatty acid over pyruvate at lower flux (higher energy state), but exhibited much greater tendency to select pyruvate and glutamate when available. Studies in sonicated mitochondria revealed two-fold higher electron transport chain electron conductance in avian mitochondria. We conclude that substantial fuel selection occurs at the level of the mitochondrial matrix and that avian flight muscle mitochondria are particularly biased toward the selection of fatty acid, possibly by facilitating high $\beta$-oxidation flux by maintaining a more oxidized matrix.
INTRODUCTION

Post-absorptive mammals rest at a whole body respiratory quotient (RQ) near 0.80, with fat providing ~⅔ of the energy substrates (Brooks and Donovan, 1983; Willis et al., 2005). Skeletal muscle arterio-venous RQ is yet lower, showing an even stronger bias toward fat oxidation in resting muscle (Dagenais et al., 1976). Fasted birds generally rest at a lower whole body RQ. For example, 2.8 g hummingbirds rest at RQ of 0.72 (Suarez et al., 1990), 23 g sparrows at 0.71 (Walsberg and Wolf, 1995), and 480 g ravens at 0.80 (Hudson and Bernstein, 1983). These observations taken together support the concept that, in the post-absorptive state, resting mammalian and avian skeletal muscle cells preferentially select lipid as their oxidative substrate.

Most mammalian and avian skeletal muscle cells rest at very low fractions of their aerobic capacity, probably less than 1%. Studies in several mammalian species have clearly demonstrated the enormous aerobic scope of mammalian skeletal muscle. For example, human muscle, which has been shown to rest at about 2 ml O₂·min⁻¹·kg⁻¹ (Dagenais et al., 1976; Zurlo et al., 1990), may achieve maximum O₂ consumption rates in excess of 300 ml·min⁻¹·kg⁻¹ (Andersen and Saltin, 1985; Richardson et al., 1993). The metabolic control signals underlying this expansive dynamic range, while not definitively understood, have been well characterized in many mammalian species using several methodologies (Chance et al., 1985; Connett, 1987; Jeneson et al., 1995). Contractile activity elevates the rate of ATP hydrolysis and results in the cytosolic accumulation of the ATP hydrolysis products ADP and inorganic phosphate (Pᵢ), and thus decreased (less negative) ATP free energy (ΔGₐₜₚ). A nearly linear relation between O₂ consumption rate and ΔGₐₜₚ, consistent with simple feedback control, has been repeatedly demonstrated both in vivo (Kushmerick et al., 1992; Jeneson et al., 1995) and in isolated mitochondria in vitro (Rottenberg, 1973; Davis and Davis-Van Thienen, 1989; Willis and Jackman, 1994; Messer et al., 2004; Glancy et al., 2008; Glancy et al., 2013). Due to the near-equilibrium maintained by the creatine kinase and adenylate kinase reactions, in vivo Pᵢ rises nearly linearly with muscle VO₂, while ADP, and particularly AMP, rise more abruptly as high fractions of the aerobic capacity are approached (Funk et al., 1989; Foley et al., 1991). All three of these energy phosphates exert strong and independent positive modulation on important rate-controlling enzymes of the glycogenolytic pathway.
As both ADP and AMP rise exponentially as whole body maximum O$_2$ consumption rate (VO$_2$max) is approached, this energy phosphate control of mitochondrial oxidative phosphorylation appears consistent with the observation that mammalian muscle exponentially increases the recruitment of carbohydrate into the fuel supply as exercise intensity rises toward VO$_2$max (Brooks and Mercier, 1994; Roberts et al., 1996). At and above the moderate intensity, mammalian muscle remains critically dependent on carbohydrate even in the absence of high blood lactate or glucose concentrations (O'Brien et al., 1993) and actively suppresses fat oxidation at high ATP turnover rates (Romijn et al., 1995; Sidossis and Wolfe, 1996). Indeed, glycogen depletion precipitates the failure of mammalian muscle contractile output (Wahren et al., 1971; Baldwin et al., 1973; Fitts et al., 1975), despite the presence of a vast lipid energy reserve.

In profound contrast, birds fly at high percentages of VO$_2$max combusting fat at nearly the complete exclusion of carbohydrate (Rothe, 1987; Suarez et al., 1990), which indicates that the fuel selection of highly active avian muscle categorically differs from that of mammals. Moreover, avian skeletal muscle must achieve extremely high aerobic energy turnover to support flight and is equipped with a mitochondrial density 2-5x that of mammalian skeletal muscle to do so. Pigeon pectoralis muscle provides an informative example: 300 g pigeons fly at a whole body VO$_2$ of roughly 200 ml·min$^{-1}$·kg body$^{-1}$ (Rothe, 1987) and the pectoralis, with a mass of about 10% body (30 g), must account for well over half of this. This conservative estimation gives a muscle tissue VO$_2$ of at least 3000 ml·min$^{-1}$·kg muscle$^{-1}$, almost all of which goes toward the combustion of lipid, because whole body RQ is ~ 0.72 (Rothe, 1987). Thus, while mammalian muscle transitions from a lipid-based fuel metabolism at rest to near complete reliance on carbohydrate as an aerobic maximum perhaps 100-fold higher is approached, avian muscle VO$_2$ rising far higher continues to select lipid as its oxidative fuel.

The mechanisms underlying this dramatically different fuel metabolism remain poorly understood. The State 3 (maximum) rate of mitochondrial oxygen consumption, $J_0$, of pigeon pectoralis mitochondria oxidizing pyruvate is roughly equal to that of palmitoyl-l-carnitine (Rasmussen et al., 2004) and the pioneering work of Suarez et al. showed similar trends in hummingbird pectoralis (Suarez et al., 1986). Pectoralis
mitochondria of the house sparrow further confirm this avian pattern, as recently reported by our laboratory (Kuzmiak et al., 2012). As both pyruvate (carbohydrate) and β-oxidation (fatty acid) pathways elicit equal rates of oxygen consumption, the selection of fatty acid fuel during avian flight cannot be simply explained by a higher catalytic potential for fat oxidation. Moreover, in vivo studies on pigeon and hummingbird clearly show that avian muscle exhibits substantial metabolic flexibility under certain conditions. From the complementary pigeon studies of Rothe et al. (Rothe, 1987) and Butler et al. (Butler et al., 1977) emerges the concept that a large glycogenolysis initiated by the onset of flight mobilizes carbohydrate carbon as lactate into the blood, as whole body respiratory exchange ratio (RER) rises toward 1.00 (Butler et al., 1977; Rothe, 1987). Continued steady rate flight over the next 30 min or so results in a progressively falling RER toward a steady state value near 0.7 (Rothe, 1987). Another example of avian metabolic flexibility is the hovering hummingbird, in which whole body RER promptly rises from near 0.7 to 1.0 when nectar feeding is allowed (Suarez et al., 1990).

Mammalian skeletal muscle also exhibits metabolic flexibility as it adjusts fuel selection toward the oxidation of lactate (Mazzeo et al., 1986; Stanley et al., 1986) and glucose (Horowitz et al., 1999; Koutsari and Sidossis, 2003) when these carbohydrate fuels are made available.

The present study focused on the metabolism of mitochondria isolated from the locomotory muscle of a mammal (rat mixed hindlimb muscle) and a bird (house sparrow pectoralis). Three fundamental characteristics were studied: 1) In avian muscle, very little is known about the sensitivity of respiratory control (e.g., the relationship between ATP free energy and VO₂ or the KMADP for respiration) or the extent to which the fuel supply available to mitochondria might influence this control sensitivity. We therefore determined this relationship between energy phosphate levels and mitochondrial O₂ consumption rate (Jo) in mitochondria provided saturating levels of three oxidative substrates: pyruvate, glutamate, and palmitoyl-l-carnitine, as well as combinations of these fuels. 2) With these same fuels, we assessed the extent to which fuels competed for oxidation (fuel competition) or simply added together to a higher flux (fuel additivity) at three experimentally fixed levels of ATP free energy (ΔGATP), -14.0, -13.5, and -13.0 kcal·mol⁻¹. 3) Finally, it has been shown that a reduced (high NADH/NAD⁺ ratio)
mitochondrial matrix strongly inhibits the β-oxidation of fatty acids (Lumeng et al., 1976). Our previous work has shown NADH cytochrome c reductase activity is 80% higher in sparrow vs. rat skeletal muscle, indicating a greater maximal rate of electron transport chain (ETC) flux in birds compared to mammals (Kuzmiak et al., 2012). Because an ETC capable of achieving a given VO_{2} at a low matrix NADH/NAD^{+} should provide a competitive advantage for fatty acid to contribute to the oxidative fuel supply, we predicted higher conductance for electron flow down the ETC in avian compared to mammalian mitochondria. We therefore evaluated the kinetics of NADH oxidation by the ETC in sonicated mitochondria.

The results show that, with every fuel and fuel combination tested, avian muscle mitochondria control respiration across a wider range of ATP free energy than mammals or, stated in familiar kinetic terms, the avian K_{MADP} for respiration was significantly higher. Further, it is clearly shown in both mammalian and avian mitochondria, but especially in the bird, that muscle mitochondria provided fatty acid suppress pyruvate oxidation more strongly than can be explained by the relative catalytic potentials of the two competing pathways. The findings also demonstrate that glutamate conversion to aspartate (the mitochondrial steps of the malate-aspartate shuttle) suppresses the oxidation of fatty acid in both types of mitochondria, but do so to a much greater extent in mammalian muscle. Finally, as predicted, the electron conductance of avian ETC exceeded that of the mammal, by over 50%.

RESULTS

Isolated mitochondria were of good functional integrity as indicated by high maximal respiration rates and energy coupling. With pyruvate (P) + malate (M) as substrates, State 3 J_{O} was 753±81 nmol·mg^{-1}·min^{-1} in rat and 548±9 nmol·mg^{-1}·min^{-1} in sparrow mitochondria (Supplemental Table I). The corresponding ADP/O ratios were 2.83±0.15 and 3.00±0.04, and respiratory control ratios were 5.5±0.8 and 5.4±1.0 (Supplemental Table I).

**Thermodynamic Assessment of Respiratory Control Sensitivity**

Typically, the relationship between ΔG_{ATP} and J_{O} is nearly linear. The slope of this relation is one index of respiratory control sensitivity and can be described as the elasticity of mitochondrial J_{O} to ΔG_{ATP}. Near-linear relationships were observed in
mitochondria oxidizing all fuel combinations in the present study (Fig. 1), and the slopes, or elasticities, often differed depending on species and substrate (Table 1). For example, rat mitochondria exhibited similar slopes with all substrates with the notable exceptions of palmitoyl-l-carnitine (PC) + M and glutamate (G) + M + aspartate (A) (Table 1). Specifically, elasticity with P+M was over 7 and 5 times greater than PC+M and G+M+A, respectively (Table 1). Sparrow mitochondria exhibited similar force:flow slopes for all substrates with two exceptions: The addition of glutamate to P+M significantly increased the slope by 72% and the addition of arsenite to G+M significantly reduced the slope by about 64% (Fig. 1 & Table 1). Indeed, the addition of the 2-oxoglutarate (2-OG) dehydrogenase (OGDH) inhibitor arsenite to mitochondria oxidizing G+M decreased respiration in both species by approximately 54% at the calculated \( V_{\text{max}} J_0 \) (Table 2). Sparrow mitochondria, however, were slightly less affected by this OGDH inhibition; at \( \Delta G_{\text{ATP}} \) of -13.0 kcal·mol\(^{-1}\) the G+M+A/G+M ratio was significantly higher in sparrow compared to rat (0.66 ± 0.03 and 0.53 ± 0.03, respectively).

**Kinetic Assessment of Respiratory Control Sensitivity**

Using the data set above, the \( K_{\text{MADP}} \) and \( V_{\text{max}} \) for respiration for each fuel combination were determined from Eadie-Hofstee analysis; these are reported in Table 2. In rat mitochondria P+M \( V_{\text{max}} \) was 2.5 times higher than PC+M and 2.3 times higher than G+M+A (Table 2). In contrast, in sparrow P+M \( V_{\text{max}} \) was similar to PC+M, and also to all other substrate combinations with one exception. Specifically, the addition of glutamate to P+M almost doubled \( V_{\text{max}} \) and similarly doubled the \( K_{\text{MADP}} \) (Table 2). In contrast, this addition of glutamate to P+M did not significantly increase \( V_{\text{max}} \) over P+M alone in rat mitochondria, with the average increase a modest 13% compared to the 96% increase in sparrow mitochondria (Table 2). Importantly, the \( V_{\text{max}} \) values reported in Table 2, determined using Eadie-Hofstee analysis of steady state conditions established with the creatine kinase energy clamp, are in excellent agreement with State 3 rates determined conventionally with saturating addition of ADP as seen in Supplemental Figure II, and also as recently reported by our laboratory (Kuzmiak et al., 2012).

In sparrow mitochondria, no fuel combination \( K_{\text{MADP}} \) was significantly different than the P+M value, with the exception of the much higher \( K_{\text{MADP}} \) observed with
P+G+M noted above (Table 2). For all fuel combinations except P+PC+M, sparrow KMADP values were significantly higher than rat (Table 2). In rat mitochondria very low KMADP were observed for fat oxidation (PC+M) and for G+M+A, the mitochondrial steps of the malate-aspartate shuttle (Table 2).

Fuel $J_O$ values across all energy states are reported as a percentage of $V_{max}$ in Table 3. These values represent the mean for all fuel combinations at a given $\Delta G_{ATP}$. At the three clamped energy states ($\Delta G_{ATP} = -14.0$, -13.5, and -13.0 kcal·mol$^{-1}$), average rat $J_O$ were 60, 75, and 86% $V_{max}$, respectively, while corresponding sparrow values were 36, 53, and 69% $V_{max}$. $J_O/V_{max}$ values for each fuel combination can be found in Supplemental Table III.

**Fuel Utilization**

**Pyruvate Sparing**

In sparrow mitochondria under any energetic condition, pyruvate metabolism was strongly suppressed by the addition of any other fuel (Fig. 2). The addition of glutamate, fatty acid, or their combination markedly decreased pyruvate utilization (Fig. 2B) and this suppression was even more pronounced when expressed relative to the oxygen consumption rate (Fig. 2D). In marked contrast, in the rat, suppression of pyruvate utilization was only observed at the highest energy state ($\Delta G_{ATP} = -14.0$ kcal·mol$^{-1}$), which is the energetic condition most similar to the low flux of rest, or when both G and PC were added (Fig. 2C).

The percent decrease in pyruvate utilization resulting from the addition of palmitoyl-l-carnitine and/or glutamate is shown in Figure 3, panels A and B. The figure clearly shows that sparrow mitochondria reduce the pyruvate utilized, by roughly 70-80%, when either $\beta$-oxidation, the malate-aspartate shuttle, or their combination are fueled, regardless of energy state. Rat mitochondria also spare pyruvate from oxidation when other fuels are available, but, unlike the sparrow, the extent of pyruvate sparing is highly dependent upon both the energy state (Fig. 3B) and what alternative fuels are available (Fig. 3A). For examples, at an energy state closer to that of resting muscle ($\Delta G_{ATP} = -14.0$ kcal·mol$^{-1}$), pyruvate utilization decreases 67±4% when both palmitoyl-l-carnitine and glutamate are also available. But at an energy state associated with
moderate intensity locomotion ($\Delta G_{\text{ATP}} = -13.0 \text{ kcal mol}^{-1}$), the same additional fuel supply decreased pyruvate utilization 50±3%.

*Contributions of Pyruvate, Glutamate, and Palmitoyl-l-Carnitine to Substrate Oxidation*

The average relative contribution of each fuel to oxygen consumption are presented in Figs 4A-D and 5A-D. In sparrow mitochondria fatty acid consistently provided the majority of the fuel regardless of energy state, while pyruvate contributed very little (Fig. 5B-D). Glutamate competed slightly more successfully with fatty acids than pyruvate, but nevertheless failed to make more than a minor contribution under any energetic condition (Fig. 5B-D). In dramatic contrast, rat mitochondrial fuel metabolism was dominated by pyruvate and glutamate (Fig. 5A-D). While, as noted above, fatty acid outcompeted pyruvate under high ATP energy (low flux) conditions, it is much less successful against glutamate, especially as ATP free energy was relaxed and flux rose (Fig. 5C). The delivery of “glycolytic pathway products” (the combination of pyruvate and glutamate) to rat muscle mitochondria nearly abolished fatty acid metabolism under all energetic conditions (Fig. 5D).

*Electron Transport Chain Conductance*

A linear thermodynamic force:metabolic flow ($\Delta E_{\text{h}}:J_O$) relationship was observed for NADH oxidation in sonicated mitochondria from both rat and sparrow (Fig. 6A). A slight loss of linearity, suggesting kinetic saturation, was seen at the highest $\Delta E_{\text{h}}$ and $J_O$ values. Eliminating that data improved the fit of the regression and did not affect the differences between the species. The slope of the $\Delta E_{\text{h}}:J_O$ relationship was 1.7-fold higher in sparrow compared to rat mitochondria (14589 ± 1811 vs. 8797 ± 1322, respectively); these slopes converted to proportional conductance units gave values of 94 ± 12 vs 57 ± 9 mS/mg, respectively (Fig. 6A,C). Additionally, the $V_{\text{max}}$ of NADH oxidation was over 2 times greater in sparrow mitochondria (1074 ± 118 vs 516 ± 42 nmol O$_2$ min$^{-1}$ mg$^{-1}$, respectively) (Fig. 6B).

**DISCUSSION**

Three fundamental functional differences between avian and mammalian skeletal muscle mitochondria were revealed by these studies: 1) Mammalian mitochondria control respiration over a more negative range of ATP free energy. In more familiar kinetic terms, the $K_M$ADP for respiration is lower in rat compared to sparrow muscle
mitochondria. This is particularly the case when fatty acid was the respiratory substrate.

2) Avian mitochondria primarily oxidize fatty acids and strongly spare pyruvate from oxidation across all energy states. The ability of a fuel to outcompete another for oxidation was not the simple consequence of a higher V_{max}. 3) Avian mitochondria possess much greater conductance for electron flow down the ETC, suggesting that a given VO_{2} can be achieved with a more oxidized matrix i.e., higher NAD^{+}/NADH ratio (at any given level of downstream backpressure exerted by the protonmotive force and ATP free energy).

Substrate additivity vs competition

Previous in vitro studies of mitochondrial fuel selection (Ashour and Hansford, 1983) established a constant ATP turnover rate by subjecting mitochondria to an ATP-splitting enzyme system (e.g., hexokinase + glucose). The mitochondrial response to elevated ATP demand therefore included not only fuel selection but also concentration control of the ATP/ADP ratio. Our objective was to instead assess the effect of fuel availability on fuel oxidation rates at experimentally clamped energy states (ATP/ADP ratios). In this approach, because the energy clamp has complete concentration control, the mitochondria have complete control of flux (Cornish-Bowden, 1999; Messer et al., 2004). The clamp controlled the free energy of ATP at one of three levels, -14.0, -13.5, or -13.0 kcal·mol^{-1}; a less negative value means less “backpressure” from downstream ATP opposing oxidative flux (Davis and Davis-Van Thienen, 1989). According to the chemiosmotic theory (see Fig. 7B), flux reflects the disequilibrium between this fixed downstream backpressure vs. the upstream “input pressure” developed by the oxidation of added substrate(s) (Koretsky and Balaban, 1987; Messer et al., 2004). Fuel “B” added to an incubation in which mitochondria are oxidizing fuel “A” will thus either simply add J_{0} flux, or “B” will compete with “A,” depending on the interplay between the fuels and their interactions with the citric acid cycle. Measurements of the rates of O_{2} consumption and fuel utilization thus provide a clear evaluation of the extents to which substrates either add together (“additivity”) or compete with one another to develop the matrix redox pressure. Two cases provide illustrative examples: 1) In rat muscle mitochondria, fatty acid (PC) added to pyruvate + malate (P+M) does not add flux (Fig. 1C, Table 2). This suggests that PC simply competes with pyruvate to develop the matrix redox
developed by P+M (Fig. 7A). These two pathways merge at Acetyl-CoA (AcCoA), which has long been considered an important locus of control in the selection of carbohydrate vs. fat (see below). 2) Flux rises when glutamate is added to P+M (Fig. 1C, Table 2). This additivity is observed at all energy states in both species, but additivity is particularly prominent in sparrow (Fig. 1C,D). Indeed, the addition of glutamate to P+M increased the calculated $V_{\text{max}}$ 96% in sparrow mitochondria, but only 13% in rat mitochondria (Table 2). The oxidative pathways of glutamate and pyruvate might compete because they share a dependence on oxaloacetate (OAA) availability; pyruvate oxidation requires the citrate synthase reaction and glutamate oxidation in muscle requires the aspartate aminotransferase reaction (Fig. 7A). In turn, the matrix [OAA] depends on two distinctive features of the malate dehydrogenase (MDH) reaction: A very large positive standard free energy in the direction of OAA formation and an extremely high MDH $V_{\text{max}}$, which maintains near equilibrium in the reaction (Williamson, 1979). As a result, matrix [OAA] is maintained at very low micromolar levels and varies according to the matrix redox state:

$$[\text{OAA}] = K_{eq} \times [M] \times \frac{\text{NAD}^+/\text{NADH}}$$

in which the $K_{eq}$ is in the vicinity of $10^{-5}$. Thus, if small decreases in matrix NAD$^+/\text{NADH}$ elicit large increases in electron flux toward Complex IV, in other words if the ETC has high electron conductance, then glutamate addition to P+M would more likely increase flux, rather than compete with pyruvate by diminishing matrix [OAA].

The observation made here that avian mitochondria exhibit marked additivity of flux when glutamate is added to P+M is therefore consistent with the observed much higher avian ETC conductance for electron transport. Indeed, additivity of multiple substrates was generally evident in sparrow mitochondrial incubations (Fig. 2D, Table 2).

**Control of Respiration**

For almost all fuels and combinations, the $K_{M,\text{ADP}}$ was higher in sparrow compared to rat mitochondria (Table 2), indicating the sparrow controls respiration over a greater range of $\Delta G_{\text{ATP}}$. At the 3 values of $\Delta G_{\text{ATP}}$ investigated in the present study, sparrow mitochondria were operating at a lower percentage of $V_{\text{max}}$ (Table 3). It is therefore likely that $\Delta G_{\text{ATP}}$ can fall lower in sparrow compared to rat mitochondria and still maintain useful contractile function. It is also likely that sparrow mitochondria
energy production is activated by both [ADP] and [Pi]. The experiments in the present study employ a [Pi] of 10 mM. In unpublished experiments from our lab, we found that NAD-linked isocitrate dehydrogenase (IDH), which is activated by ADP in mammalian mitochondria, is dependent on [Pi] in sparrow mitochondria over a range of 0-25 mM. In fact, in the absence of Pi, IDH activity was undetectable, corroborating very low rates reported for whole muscle (Alp et al., 1976). This suggests an activation of NADH production by rising [Pi] before $\Delta G_{ATP}$ falls significantly.

It is also important to point out that compared to mammalian limb muscle that supports locomotion, avian pectoralis muscle has a much higher mass-specific ATP turnover rate. The maximal (State 3) mitochondrial oxygen consumption rates expressed per mg of mitochondrial protein is not very different between these species (Table 2 & Supplemental Figure I) (Kuzmiak et al., 2012). As such, the energy requirements of flight can only be met due to the higher mitochondrial volume and protein density of avian myocytes. As pioneered by Wiebel and examined for avian species by our lab, a linear relationship exists between mitochondrial protein density and VO$_{2\text{max}}$, demonstrating mammals and birds exhibit similar respiratory capacities per mitochondrial unit (Weibel et al., 1991; Weibel et al., 2004; Kuzmiak et al., 2012).

Avian mitochondria strongly spare pyruvate

The mass-specific rate of energy demand required to support avian flight is enormous (Schmidt-Nielsen, 1984) and the energy density of carbohydrate in its stored, hydrated form is roughly a mere 1/10 that of lipid (Flatt, 1995; Jenni and Jenni-Eiermann, 1998). Many avian species are able to fly long distances without dietary energy replacement due to an almost exclusive reliance on fat oxidation (Rothe, 1987). Sparrow mitochondria provided fatty acid strongly suppressed pyruvate utilization, by 69-78% (Fig. 2B&D), across the entire range of energy states tested. In rat mitochondria pyruvate sparing was much more flux/energy state dependent: At low ATP turnover ($\Delta G_{ATP} = -14.0$ kcal·mol$^{-1}$) fatty acids inhibited pyruvate oxidation by 60%, consistent with the dominance of fatty acid oxidation in mammalian muscle at rest and during low intensity exercise (Fig. 2A&C) (Romijn et al., 1993; Roberts et al., 1996; Kelley and Mandarino, 2000; Willis et al., 2005). But as energy state was relaxed and the fuel oxidation rate rose, fatty acid addition spared only 30% of the pyruvate oxidized in its
absence (Fig. 2A&C). Thus, in the mammalian mitochondria, as the ATP turnover rate increased, pyruvate combustion markedly rose in both absolute and relative terms. Viewed in the other direction, PC accounted for 16% of oxygen consumption at $\Delta G_{ATP} = -13.0$ kcal·mol$^{-1}$, while it accounted for 59% at -14.0 kcal·mol$^{-1}$. These findings are similar to the PDH flux results of Ashour and Hansford, who used an ATP-utilizing reaction (hexokinase + glucose) to experimentally establish the in vitro rate of ATP turnover (Ashour and Hansford, 1983).

Our results clearly support the contention that fuel competition outcomes are not predictable on the basis of the relative oxidation rates of the individual fuels. For example, in sparrow mitochondria respiring at $\Delta G_{ATP} = -13.0$ kcal·mol$^{-1}$, $J_O$ with P+M, PC+M, and P+PC+M were the same (274, 313, and 315 nmol·mg$^{-1}$·min$^{-1}$, respectively) (Fig. 1D). It is tempting to predict that since all three values were similar, then pyruvate and palmitoyl-l-carnitine made essentially equal contributions when the fuels were combined (along with malate as priming carbon). According to this approach, the fraction contributed by pyruvate is predicted to be 0.47:

$$J_O \text{ from } P = \frac{P \cdot J_O}{(P \cdot J_O + PC \cdot J_O)} = \frac{274}{(274 + 313)} = 0.47 \quad (2)$$

where “$J_O$ from P” is the $O_2$ consumed by electrons due to pyruvate oxidation. However, the data show that pyruvate oxidation accounted for only 25% of the $O_2$ consumed under these conditions (Fig. 5B). Apparently, products of fatty acid oxidation inhibit pyruvate oxidation more strongly than they feed back to inhibit their own production. Indeed, it is well known that $\beta$-oxidation products, AcCoA and NADH, activate pyruvate dehydrogenase kinase, which phosphorylates and inactivates PDH (Sugden and Holness, 1994). AcCoA, and not NADH, seems to be the particularly effective inhibitor; Ashour and Hansford concluded that the PDH$A$ decrease attending palmitoyl-l-carnitine addition tracked the AcCoA/CoA ratio and not the NADH/NAD$^+$ ratio (Ashour and Hansford, 1983).

**Suppression of Fat Oxidation by Matrix Redox**

In mammals fat oxidation rate rises with exercise intensity, as long as the intensity remains within the mild to moderate range (Romijn et al., 1993; Willis et al., 2005) i.e., at intensities below the lactate threshold. Across this range myocyte NAD$^+/NADH$ also rises (Sahlin et al., 1987). At intensities exceeding roughly 60-70% VO$_2$max i.e., the
lactate threshold, however, myocyte NAD$^+/NADH$ falls (Sahlin et al., 1987), as does the
fat oxidation rate (Jones et al., 1980; Romijn et al., 1993). There is evidence that
mitochondrial fatty acid oxidation is actively suppressed at these high intensities (Romijn
et al., 1995), but the mechanism proposed, that of malonyl-CoA inhibition at carnitine
palmitoyl transferase I (CPT-I), is not consistent with the observation that malonyl-CoA
levels fail to change in the predicted direction (Winder et al., 1989; Odland et al., 1996).

An alternative mechanism is, however, supported by available evidence in a
compelling fashion. The falling myocyte NAD$^+/NADH$ levels, associated with over 10-
fold increases in muscle lactate/pyruvate ratios (Sahlin et al., 1987), reflect a fundamental
requirement for aerobic glycolysis: the shuttling of cytosolic NADH electrons into
mitochondria. Over 30 years ago Lumeng et al. (Lumeng et al., 1976) demonstrated in rat
liver mitochondria incubated with saturating levels of palmitoyl-l-carnitine that the
reconstruction of the malate-aspartate electron shuttle abolished fatty acid oxidation. Fat
oxidation was also completely inhibited when the glycerol-3-phosphate (G3P) electron
shuttle was fueled in liver mitochondria isolated from rats pretreated with thyroid
hormone, which upregulates hepatic mitochondrial G3P dehydrogenase. In both cases, β-
oxidation pathway flux decreased as the matrix NADH/NAD$^+$ ratio increased. Sahlin’s
laboratory has also suggested the possibility of control of β-oxidation flux by
NADH/NAD$^+$ (Mogensen and Sahlin, 2005). In the present study, as in the study by
Lumeng et al. (Lumeng et al., 1976), fatty acid was provided to mitochondria as PC,
which bypasses CPT-I entirely. Corroborating Lumeng et al., glutamate addition to fuel
the mitochondrial steps of the malate-aspartate shuttle (Bookelman et al., 1979),
suppressed fatty acid utilization concomitant with markedly increased $J_O$ flux, which is
consistent with an elevated matrix redox pressure. This pattern was dramatically the case
in mammalian mitochondria, while it occurred to a minor extent in avian mitochondria.
The findings above strongly suggest that fatty acid oxidation may be a more competitive
substrate in sparrow mitochondria due to their very high ETC electron conductance.

In both species, electron flow down the ETC is a linear function of the driving force
($\Delta E_m$) (Fig. 6), providing a compelling example of a pathway flux conforming to a linear
thermodynamic force:metabolic flow relationship (Rottenberg, 1973; Van der Meer et al.,
1980; Kushmerick et al., 1992; Glancy et al., 2013). $V_{max} J_O$ in disrupted mitochondria
oxidizing NADH was higher in sparrow compared to rat in agreement with previous avian mammalian comparisons (Rasmussen et al., 2004; Kuzmiak et al., 2012). This greater catalytic potential in avian ETC might facilitate at least two functional outcomes. First, during routine flight, sparrow pectoralis mitochondria could generate a high ETC flux at a low matrix redox potential, which would minimize inhibition of β-oxidation, allowing fatty acids to produce AcCoA and inhibit pyruvate oxidation. Secondly, under conditions of rising lactate availability due, for example, to a rapid glycogenolysis proceeding in the same or another cell (Rothe, 1987), glycolytic products (pyruvate and the malate-aspartate shuttle) would additively fuel a yet higher rate of oxidative phosphorylation. Our data show that bird mitochondria oxidizing a single substrate increase $V_{\text{max}} J_0$ by 50% when multiple substrates are added, in comparison with an 11% increase in rat mitochondria (Table 2).

SUMMARY

Mitochondrial fuel oxidation and selection mirrored that of the whole body: in rat mitochondria the reliance on carbohydrate increased and the relative contribution of fat decreased as the rate of oxygen consumption increased, whereas fat dominated under all conditions in the sparrow. This indicates that fuel selection, at least in part, can be modulated at the level of the mitochondrial matrix when multiple substrates are present at saturating levels. As an increase in matrix redox potential has been linked to a suppression of palmitoyl-L-carnitine (Lumeng et al., 1976), we suggest a high electron transport chain conductance relative to dehydrogenase activity in avian compared to rat mitochondria observed by our lab and others (Rothe, 1987) may result in a selective oxidation of fatty acids for energy in avian compared to mammalian mitochondria.

MATERIALS & METHODS

Animals

All procedures are in accordance with the guiding principles in the care and use of animals at Arizona State University. House sparrows (*Passer domesticus*) were captured at a Livestock Auction (Phoenix East Valley, AZ, USA) by mist net the morning of each experiment. The sparrows, weighing 21-28 g, were fasted 2-3 hours during transport to the laboratory. Sprague-Dawley rats (*Rattus norvegicus*), weighing 300-400 g, and sparrows were euthanized with an overdose of CO$_2$. Pectoralis muscle was extracted and...
removed from the keel of a sparrow and the quadriceps femoris and triceps surae groups 
were extracted from the rats. These species were chosen as 1) a characterization of the 
oxygen consumption and ROS production in these species was previously conducted 
(Kuzmiak et al., 2012), 2) rat hindlimb muscle provided adequate tissue for 
mitochondrial isolation and functional assessment, 3) the fuel mixture oxidized by the rat 
is representative of mammalian patterns, 4) the glucose and fatty acid uptake by sparrow 
muscle has been previously examined (Sweazea and Braun, 2005, 2006) and 5) there is 
no apparent correlation between body mass and mitochondrial oxygen consumption rate 
(Rasmussen et al., 2004). Extracted muscles were immediately placed in an ice cold 
solution of (in mM) 100 KCl, 40 TrisHCl, 10 Tris base, 5 MgCl2, 1 EDTA, and 1 ATP, 
pH = 7.4 (solution I) (Makenin and Lee, 1968) and mitochondria were isolated as 
described previously (Kuzmiak et al., 2012). Protein concentrations of the final 
mitochondrial suspensions were 15.0±1.3 and 8.4 ± 0.9 mg mitochondrial protein· ml⁻¹ 
for sparrows and rats, respectively.

**Mitochondrial oxygen consumption and fuel utilization**

Respiration studies were performed in ~2.0 ml of a respiration medium (RM) 
containing (in mM) 100 KCl, 50 MOPS, 20 Glucose, 10 KPO₄, 10 MgCl₂, 1 EDTA, and 0.2% BSA, pH = 7.0. A Clark type O₂ electrode was used to measure mitochondrial O₂ 
consumption (Jₒ) at 37°C (Rank Brothers, Cambridge, UK) as described by Messer et al. 
(Messer et al., 2004). Other assay conditions and substrates additions are as noted in the 
legends of figures and tables.

**Creatine kinase energy clamp**

Steady state respiratory rates (Jₒ) intermediate to the maximum rate (State 3) and 
resting rate (State 4) were established by incubating mitochondria over a range of 
eneric states using a modification of the creatine kinase (CK) energetic clamp 
described by Messer et al. (Messer et al., 2004). Incubations were primed with 5 mM 
creatine (Cr), 5 mM ATP, 10 mM Pi, and excess CK. Separate incubations contained 
2.5, 5, or 10 mM phosphocreatine (PCr) to obtain PCr/Cr ratios of 0.5, 1.0, or 2.0. 
Because incubations were buffered at pH 7.0, the equilibrium constant of the CK reaction 
(K_ck),
\[ K_{\text{ck}} = \frac{[\text{Cr}][\text{ATP}]}{[\text{PCr}][\text{ADP}][\text{H}^+]} \]  

(3)

In which \( K_{\text{ck}} \) equals \( 1.77 \times 10^9 \) (Teague et al., 1996), reduces to a value of 177 with \([\text{H}^+]\) maintained at \( 10^{-7} \) M. Thus, the ATP/ADP ratio was experimentally established (clamped) with appropriate additions of PCr and Cr as:

\[ \frac{[\text{ATP}]}{[\text{ADP}]} = 177 \frac{[\text{PCr}]}{[\text{Cr}]} \]  

(4)

At an experimentally clamped ATP/ADP ratio, the Gibbs free energy of ATP \( (\Delta G_{\text{ATP}}) \) is calculated as:

\[ \Delta G_{\text{ATP}} = RT \ln \frac{\Gamma}{K_{\text{ATP}}} \]  

(5)

In which the mass action ratio, \( \Gamma \), of the ATP hydrolysis reaction is:

\[ \Gamma = \frac{[\text{ADP}][P_i]}{[\text{ATP}]} \]  

(6)

And \( K_{\text{ATP}} \), the equilibrium constant of the ATP hydrolysis reaction, equals 225,000 at pH 7.0 (Teague et al., 1996). \( R \) is the gas constant (1.987 cal·deg\(^{-1}\)·mol\(^{-1}\)), and \( T \) is the temperature in Kelvin (310 K). Thus PCr/Cr ratios of 0.5, 1.0, and 2.0 resulted in ATP/ADP ratios of 75, 150, and 300, respectively, with corresponding final [ADP] of 66.7, 33.3, and 16.7 \( \mu \)M. At the incubation [Pi] of 10 mM, these energy phosphate conditions resulted in \( \Delta G_{\text{ATP}} \) values of -13.0, -13.5, -14.0 kcal·mol\(^{-1}\), respectively.

**Oxidative substrates**

Substrates provided to respiring mitochondria, either alone or in combinations, included 0.5 mM pyruvate (P), 5.0 mM glutamate (G), and 10 \( \mu \)M palmitoyl-L-carnitine (PC). Importantly, PC is translocated directly into the mitochondrial matrix in exchange for carnitine i.e., fat metabolism in these studies did not involve carnitine palmitoyltransferase I (CPTI). In all incubations, the citrate cycle was primed with 0.5 mM malate (M). After malate priming, fuels were provided alone as P+M, G+M, or PC+M, as well as in combinations as P+G+M, P+PC+M, G+PC+M or P+G+PC+M. Additionally, G+M was added with 2.0 mM arsenite (A), which specifically reconstructs
the mitochondrial steps of the malate-aspartate shuttle. Arsenite inhibition of 2-oxoglutarate (2-OG) dehydrogenase (OGDH) ensures that the malate dehydrogenase (MDH) reaction (Mal + NAD$^+$ $\rightarrow$ oxaloacetate (OAA) + NADH) is the sole site generating reducing power, as glutamate transaminates with oxaloacetate to form 2-OG and aspartate (Asp) and the formed 2-OG and aspartate then exchange with extramitochondrial malate and glutamate, respectively. The glycerol-3-phosphate (G3P) shuttle was not assessed as previous work indicates it does not operate in sparrow mitochondria (Kuzmiak et al., 2012).

**Substrate competition assays**

Timed incubations were carried out to determine fuel oxidation rates. To the incubation medium the energy clamp components and substrates were added and, after a few minutes equilibration period, the incubation was initiated by the addition of ~200 µg mitochondrial protein. A "pre-incubation" sample (500 µL) was immediately withdrawn and quenched in 150 µL 25% HClO$_4$ and $J_O$ was followed continuously until a second "post-incubation" aliquot was similarly sampled and quenched. Incubations were approximately 10 min in duration; all durations were precisely measured and recorded times were used to calculate substrate fluxes. Acidified samples were promptly centrifuged for 1.0 min at 14,000 g and supernatants were neutralized with 2N KOH + 0.5 M MOPS. An aliquot of this acid/neutralized extract was stored on ice to fluorometrically assay for pyruvate on the same day; the remainder was stored at -80°C for subsequent metabolite analyses.

For each fuel combination, $J_O$ was plotted against $\Delta G_{ATP}$ to obtain the "thermodynamic force:metabolic flow" relation, a measure of mitochondrial sensitivity to a respiratory signal. Using the same data set, $K_{M,ADP}$ and $V_{max}$ $J_O$ were determined using Eadie-Hofstee analysis, plotting $J_O$ against $J_O/[ADP]$.

**Metabolite Assays**

All metabolite assays were carried out by enzymatically linking substrate metabolism to the oxidation/reduction of NAD$^+/NADH$ by fluorescence changes (excitation $\lambda$ 360 nm, emission $\lambda$ 465 nm) in a 96 well microplate reader (TECAN GENios, Durham, NC). Assays were run in duplicate using 10-40 µL of acidified/neutralized extract in a final assay volume of 200 µL. Standard curves were run.
daily. Pyruvate was assayed by a method adapted from (Trautschold et al., 1985). Malate, 2-OG, and Asp assays were adapted from Williamson & Corkey (Williamson and Corkey, 1969). Preliminary experimental incubations were assayed for OAA, citrate, isocitrate, lactate, and alanine and these metabolites did not show measurable accumulation with any substrate or substrate combination used (data not shown). Net metabolite disappearance and appearance rates were expressed per mg of mitochondrial protein in the incubation.

**Fuel utilization calculations**

The net contributions of pyruvate, glutamate, and palmitoyl-L-carnitine to the mitochondrial fuel supply were based on the principle of mass balance and the measured rates of O₂ consumption, pyruvate utilization, malate utilization, and aspartate formation (see Fig. 1). For these calculations, O₂ consumption rates and substrate utilization and formation rates were averaged for each condition so the data represents the mean for all experiments. In these fuel utilization studies 100-200 µg of mitochondrial protein were incubated in a 2 ml incubation system sealed from the environment. This quantity of mitochondria carries with it essentially no stored oxidizable substrates (Messer et al., 2004). All reducing equivalents available to the mitochondria were therefore experimentally controlled as fuel added in the form of malate, pyruvate, glutamate, and/or palmitoyl-L-carnitine. In this highly controlled environment, the total O₂ consumed provides the total number of electron pairs extracted from added fuels. As seen in Fig. 8, the pyruvate and palmitoyl-L-carnitine pathways converge at acetyl-CoA (AcCoA), thus both require OAA to advance to citrate via the citrate synthase reaction. In turn, OAA is derived from malate, which was added to prime the citrate cycle at 0.50 mM. Glutamate metabolism also utilizes malate priming carbon when it transaminates with OAA to form Asp and 2-OG. Thus, the metabolism of all three substrates involves 2-OG formation, which can either be oxidized at OGDH or exchanged with extramitochondrial malate via the dicarboxylate carrier into a vast volume of respiration medium where it would accumulate. Net 2-OG accumulation was measured to assess the extent to which carbon either exited the mitochondrial matrix or advanced through OGDH into the second span of the citrate cycle. Further details on fuel utilization calculations are provided in supplemental materials. Briefly, the approach and
assumptions on which these calculations are based can be fairly described as a process which rigorously assesses the contributions of pyruvate and glutamate oxidation to fuel metabolism, and thus uses O₂ consumption and mass balance to estimate the reducing equivalents generated by the beta oxidation of fatty acids.

**NADH oxidation in disrupted mitochondria**

NADH oxidation by sonicated mitochondria assessed the electron conductance of the ETC from Complex I → Complex IV. Frozen mitochondria were diluted 50% with 10 mM KPO₄, pH 7.0, and sonicated in three 10 sec bouts with a Branson Sonifier at 40% power, resting on ice 5 minutes between each bout. The assay began with 2.0 mL RM to which was added ~100 µg of sonicated mitochondrial protein followed by a 750 nmol bolus of NADH. The O₂ content of the RM was monitored continuously as it fell exponentially toward a zero slope plateau, indicating the complete oxidation of added NADH. Due to the 1:1 stoichiometry between NADH oxidation to NAD⁺ and atomic oxygen reduction to water, the O₂ electrode signal could be used to calculate J₀ (hence Jₑ⁻, the electron current), as well as the ratios of NAD⁺/NADH and ½O₂/H₂O at any point along the progress curve. The total oxygen consumed was determined and the top and bottom 10% of the oxidation curve were excluded. The remaining field was divided into 5 equally-spaced regions and in each both flux (J₀ or Jₑ⁻) and the driving force (the voltage drop) down the ETC were analyzed.

Electron flux, J₀, of a region was determined as the tangential slope at the midpoint O₂ signal of that region. The voltage drop (the redox potential difference, ΔEₚ) down the ETC was determined from the NAD⁺/NADH and ½O₂/H₂O ratios at the midpoint. The redox potential difference (ΔEₚ) is:

\[
\Delta E_p = E_{h(acceptor)} - E_{h(donor)}
\]  

In turn, the redox potential of the electron acceptor couple at Complex IV (Eₚ(acceptor)), which depends on the ½O₂/H₂O ratio, and that of the electron donor at Complex I (Eₚ(donor)), which depends on the NAD⁺/NADH ratio, were calculated according to:

\[
E_h = E_m + \frac{RT}{nF} \ln \frac{[oxidized]}{[reduced]}
\]

where Eₘ is the midpoint potential (mV), R is the gas constant (1.987 cal·deg⁻¹·mol⁻¹), T is the temperature in Kelvin (310°K), n is the number of electrons transferred (2 in this
case), and $F$ is the Faraday constant (23.062 cal·mV⁻¹·mol⁻¹). The $E_m$ values used were -320 mV for the NAD⁺/NADH couple and +820 mV for the ½O₂/H₂O couple. $J_O$ was then plotted against $\Delta E_h$ to produce a graph of thermodynamic force: metabolic flow, which was found to be consistently linear (Ohmic). After conversion of $J_O$ to $J_e^-$, the slope of this $\Delta E_h:J_e^-$ relationship, the apparent conductance of the ETC for electron transfer, was expressed in units of milliSiemens/mg mitochondrial protein.

**Statistics**

Differences in the $\Delta G_{ATP}:J_O$ slope relationship, $V_{max}$ $J_O$, $K_M$ADP, and pyruvate utilization within a species were determined using a one-way ANOVA with a Tukey post-hoc test with $P<0.05$. Independent t-tests were used to determined differences in $V_{max}$, $K_M$ADP, percent decreases in pyruvate utilization, as well as ETC conductance between the species with $P<0.05$.

**Funding:** This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.
References


Table 1
Average slopes ± s.e.m. for ∆G<sub>ATP</sub>:J<sub>O</sub> relationships shown in Fig. 2. † significantly different than P+M at same ∆G<sub>ATP</sub>.

<table>
<thead>
<tr>
<th>Fuels</th>
<th>Rat</th>
<th>Sparrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>P + M</td>
<td>△</td>
<td>245 ± 31</td>
</tr>
<tr>
<td>G + M</td>
<td>□</td>
<td>280 ± 69</td>
</tr>
<tr>
<td>PC + M</td>
<td>○</td>
<td>32 ± 11†</td>
</tr>
<tr>
<td>G + M + A</td>
<td>‡</td>
<td>44 ± 14†</td>
</tr>
<tr>
<td>P + G + M</td>
<td>■</td>
<td>286 ± 18</td>
</tr>
<tr>
<td>P + PC + M</td>
<td>●</td>
<td>167 ± 55</td>
</tr>
<tr>
<td>G + PC + M</td>
<td>✗</td>
<td>159 ± 23</td>
</tr>
<tr>
<td>P + G + PC + M</td>
<td>—</td>
<td>229 ± 24</td>
</tr>
</tbody>
</table>

**Table 2**
V<sub>max</sub> and K<sub>M</sub>ADP values determined from Eadie-Hofstee plots of J<sub>O</sub>/[ADP] vs. J<sub>O</sub>. Values represent mean ± s.e.m. † significantly different than P+M in same species, *significantly different than rat. n = 4 rats, 3 sparrows.

<table>
<thead>
<tr>
<th>Fuels</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (nmol O&lt;sub&gt;2&lt;/sub&gt;·mg&lt;sup&gt;-1&lt;/sup&gt;·min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;M&lt;/sub&gt;ADP (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat</td>
<td>Sparrow</td>
</tr>
<tr>
<td>P+M</td>
<td>810 ± 87</td>
<td>408 ± 50*</td>
</tr>
<tr>
<td>G+M</td>
<td>825 ± 104</td>
<td>527 ± 38</td>
</tr>
<tr>
<td>PC+M</td>
<td>321 ± 51†</td>
<td>414 ± 4</td>
</tr>
<tr>
<td>G + M + A</td>
<td>357 ± 35†</td>
<td>260 ± 26</td>
</tr>
<tr>
<td>P+G+M</td>
<td>916 ± 54</td>
<td>801 ± 70†</td>
</tr>
<tr>
<td>P+PC+M</td>
<td>640 ± 131</td>
<td>465 ± 66</td>
</tr>
<tr>
<td>G+PC+M</td>
<td>626 ± 44</td>
<td>506 ± 79</td>
</tr>
<tr>
<td>P+G+PC+M</td>
<td>731 ± 107</td>
<td>503 ± 75</td>
</tr>
</tbody>
</table>
Table 3
Ratio of average steady state $J_0$ at a given value of $\Delta G_{\text{ATP}}$ to the $V_{\text{max}}$ $J_0$ determined from Eadie-Hofstee analysis. Values represent mean of all fuel combinations at a given $\Delta G_{\text{ATP}} \pm$ s.e.m. Data for individual fuel combinations can be found in Supplemental Table III.

<table>
<thead>
<tr>
<th>$\Delta G_{\text{ATP}}$ (kcal·mol⁻¹)</th>
<th>Rat</th>
<th>Sparrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>-13.0</td>
<td>86 ± 2%</td>
<td>69 ± 4%</td>
</tr>
<tr>
<td>-13.5</td>
<td>75 ± 4%</td>
<td>53 ± 4%</td>
</tr>
<tr>
<td>-14.0</td>
<td>60 ± 6%</td>
<td>36 ± 5%</td>
</tr>
</tbody>
</table>

Figure 1. Oxygen consumption rate ($J_0$) as a function of $\Delta G_{\text{ATP}}$. Data for single fuel combinations for rat (A) and sparrow (B) are presented in the top panels. Data for incubations with multiple substrates for rat (C) and sparrow (D) are presented in the lower panels. P+M is included in both as a reference. Fuel combinations are $\triangle P+M$, $\Box G+M$, $\bigcirc PC+M$, $\triangleleft G+M+A$, $\blacksquare P+G+M$, $\bullet P+PC+M$, $\times G+PC+M$, $\neg P+G+PC+M$. Pyruvate (P), malate (M), glutamate (G), palmitoyl-l-carnitine (PC), and arsenite (A). n = 4 rats, 3 sparrows.

Figure 2: Pyruvate utilization rates. Absolute rate of pyruvate utilization for rat (A) and sparrow (B) and pyruvate utilization rate normalized to oxygen consumption rate (P:O) for rat (C) and sparrow (D) during steady state respiration at three values of $\Delta G_{\text{ATP}}$. Fuel combinations are P+M (open bars), P+G+M (black bars), P+PC+M (checkered bars), & P+G+PC+M (grey bars). Values represent mean $\pm$ s.e.m. † significantly different than P+M at same $\Delta G_{\text{ATP}}$. n = 4 rats, 3 sparrows.

Figure 3: Change in pyruvate utilization with additional fuel. A) Average decrease in pyruvate utilization at all energy states by the addition of G, PC, or G + PC to P + M oxidation. B) Average decrease in pyruvate utilization with any additional fuel across $\Delta G_{\text{ATP}}$. Data for rat (light grey bars) and sparrow (dark grey bars) mitochondria. Values represent mean $\pm$ s.e.m. *significantly greater decrease in pyruvate utilization vs. rat. n = 4 rats, 3 sparrows.

Figure 4: Fuel contribution to $J_0$. Relative contributions of fuels to oxygen consumption rate at three values of $\Delta G_{\text{ATP}}$ in rat (left panels) and sparrow (right panels). Fuel combinations: A) P+M B) G+M C) PC+M and D) G+M+A. Fuel contributors to $J_0$
are P (grey), G (checked), PC (white), and unknown (black). Fuel contribution calculations as described in Methods. n = 4 rats, 3 sparrows.

**Figure 5: Fuel contribution to J_O.** Relative contributions of fuels to oxygen consumption rate at three values of ΔG_ATP in rat (left panels) and sparrow (right panels). Fuel combinations: A) P+G+M B) P+PC+M C) G+PC+M and D) P+G+PC+M. Fuel contributors to J_O are P (grey), G (checked), PC (white), and unknown (black). Fuel contribution calculations as described in Methods. n = 4 rats, 3 sparrows.

**Figure 6: ETC conductance.** A) Linear relationship between J_O and ΔE_h down the ETC was observed in both rat (□) and sparrow (×) mitochondria. The slopes were significantly different: 14589 ± 1811 for sparrow and 8797 ± 1322 for rat mitochondria. B) V_max J_O fueled by NADH oxidation. C) Apparent electron conductance from Complex I→IV in rat and sparrow mitochondria. Values represent means ±s.e.m. *significantly greater than rat. n = 6 rats, n = 8 sparrows.

**Figure 7: Schematic representations of the citric acid cycle and chemiosmotic energy transduction.** A) Entry points of carbon and enzymatic reactions of the citric acid cycle and related enzymes. Fuels were provided as pyruvate, glutamate, or palmitoyl-l-carnitine, which is converted to palmitoyl-CoA by carnitine palmitoyl transferase II before undergoing β-oxidation. Electron harvesting reactions are highlighted with grey circles: pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (IDH) 2-oxoglutarate dehydrogenase (OGDH), succinate dehydrogenase (SDH), and malate dehydrogenase (MDH). B) Model of the chemiosmotic energy transduction pathway (Mitchell, 1961). The modules represent 1) the substrate dehydrogenase enzymes (DH), 2) the electron transport chain (ETC), and 3) the combined actions of F1-ATP synthase (ATP synth), the phosphate transporter, and the adenine nucleotide translocase (ANT). As fuel is broken down, the chemical potential energy is transformed into a redox potential, or ΔG_redox. This energy is further transduced into the protonmotive force (Δp) via H⁺ pumping as electrons travel down the ETC.
The figures show the results of experiments on the coenzyme activities of rat and sparrow.

**Figure A (Rat):**
- The y-axis represents nmol.mg⁻¹.min⁻¹.
- The x-axis shows different energy levels (-14.0, -13.5, -13.0 kcal.mol⁻¹).
- Different bars represent various conditions: P+M, P+G+M, P+PC+M, P+G+PC+M.
- Significant differences are indicated by †.

**Figure B (Sparrow):**
- Similar to Figure A, showing coenzyme activities for sparrow under different energy levels.

**Figure C (Rat) and D (Sparrow):**
- The y-axis represents P:O ratio.
- The x-axis shows ΔGₜₚ (kcal.mol⁻¹).
- Significant differences are indicated by †.

The data suggest that there are significant differences in coenzyme activities and P:O ratios between rat and sparrow across different energy levels.
The figure shows the distribution of different metabolic compounds in Rat and Sparrow under various conditions.

- **A**: Comparison of Pyruvate (P) and Glutamate (M) in Rat and Sparrow.
- **B**: Comparison of Glutamate (G) and Palmitoyl-l-carnitine (P) in Rat and Sparrow.
- **C**: Comparison of Palmitoyl-l-carnitine (PC) and Glutamate (M) in Rat and Sparrow.
- **D**: Comparison of Glutamate (G) and Palmitoyl-l-carnitine (M) in Rat and Sparrow.

The ΔG_{ATP} (kcal·mol⁻¹) values are shown for each condition, with different colors and patterns representing different compounds.

- **Pyruvate**: Gray squares
- **Glutamate**: Black squares
- **Palmitoyl-l-carnitine**: White squares
- **Unknown**: Checkered pattern