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Relationships between enzymatic flux capacities and metabolic flux rates: Nonequilibrium reactions in muscle glycolysis

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ABSTRACT **The rules that govern the relationships between enzymatic flux capacities (V_{max}) and maximum physiological flux rates (v) at enzyme-catalyzed steps in pathways are poorly understood. We relate *in vitro* V_{max} values with *in vivo* flux rates for glycogen phosphorylase, hexokinase, and phosphofructokinase, enzymes catalyzing nonequilibrium reactions, from a variety of muscle types in fishes, insects, birds, and mammals. Flux capacities are in large excess over physiological flux rates in low-flux muscles, resulting in low fractional velocities ($\%V_{max} = v/V_{max} \times 100$) *in vivo*. In high-flux muscles, close matches between flux capacities and flux rates (resulting in fractional velocities approaching 100% *in vivo*) are observed. These empirical observations are reconciled with current concepts concerning enzyme function and regulation. We suggest that in high-flux muscles, close matches between enzymatic flux capacities and metabolic flux rates (i.e., the lack of excess capacities) may result from space constraints in the sarcoplasm.**

Studies of structural and functional design in animals have led to valuable insights into the relationships between functional capacities and maximum physiological requirements or loads (1–3). At the biochemical level, biological design has been studied in terms of relationships between protein structure and function (4), pathway stoichiometry (5, 6), and mechanisms of regulation (5, 7). However, the synthesis and turnover of the thousands of metabolic enzymes possessed by each cell type is a costly enterprise (8), and the various compartments in cells appear to be highly crowded (9, 10). Current evidence suggests that there are probably upper limits to the design of biochemical capacities (11, 12). Despite this, the rules that govern the design of functional capacities at the biochemical level are poorly understood. What are the relationships between enzymatic capacities for flux and maximum physiological flux rates through pathways? How much enzyme, in Diamond's words (13), is "enough but not too much?"

Locomotory and cardiac muscles are ideally suited for the examination of capacity/load relationships at the biochemical level because the maximum rate at which they do mechanical work defines the maximum rate at which ATP is hydrolyzed and, therefore, the maximum rate at which ATP resynthesizing pathways must operate. Herein, we present patterns of relationships, thus far unrecognized, between enzymatic flux capacities at nonequilibrium reactions in glycolysis and rates of glycolytic flux in muscles during exercise.

Sources and Analysis of Data

Data concerning flux rates (v) and flux capacities (enzyme V_{max} values) at three nonequilibrium steps in the glycolytic pathway, the hexokinase, glycogen phosphorylase, and phosphofructokinase reactions, are considered. These are taken from our own studies and published work from other laboratories. Most of the reactions in glycolysis lie close to equilibrium *in vivo* and are catalyzed by enzymes whose V_{max} values may exceed pathway flux rates by up to three orders of magnitude (14). [We deal with the match between flux capacities and physiological flux rates at a near-equilibrium reaction, phosphoglucoisomerase, elsewhere (15).] In contrast, the reactions we consider herein are held far from equilibrium (16). It is important to note that we make no *a priori* assumptions concerning the *degree* to which the hexokinase, glycogen phosphorylase, and phosphofructokinase reactions contribute to the regulation of flux (i.e., their flux control coefficients) and that our analysis does not require that such assumptions be made. The enzymes catalyzing these reactions have been well studied and are known to be subject to complex mechanisms of regulation. Much evidence indicates that they are involved in the regulation of glucose-based (16–18) or glycogen-based (19, 20) glycolytic flux under various conditions. However, the estimation of their flux control coefficients under various physiological conditions is a formidable undertaking, accomplished in only one published study, thus far (16).

Measurement of Flux Rates. *Hexokinase.* In our own studies, rates of glucose uptake and phosphorylation were estimated in cardiac and skeletal muscles of exercising rainbow trout and in isolated perfused rainbow trout hearts by using radiolabeled 2-deoxyglucose (21). This technique was also used in the estimation of glucose phosphorylation rates in exercising rats (22). We also estimated the oxidation rates of hexose sugars in flying hummingbirds (23) and honeybees (24) by respirometry. That exogenous sugars are oxidized as the main energy source by exercising muscles in these animals is indicated by respiratory quotients ($RQ = VCO_2/VO_2$) equal to 1.0 in both animals (23, 25) under the conditions of these measurements and high capacities for hexose sugar utilization by flight muscles (23, 24). In honeybees, capacities for glycogenolysis (26) and fatty acid oxidation (27) are too low to support flight, whereas utilization of hexose sugars derived directly from dietary sucrose has been demonstrated (28). In hummingbirds, foraging flights (involving ingestion of sucrose) after a period of fasting occur with a rapid shift from fatty acid to carbohydrate oxidation (23). Under captive conditions at low temperature, foraging bouts may occur at rates of up to 1,200 h^{-1} (C. L. Gass, personal communication). As in honeybees, this is consistent with a high degree of reliance upon dietary sugar as a metabolic fuel.

Glycogen Phosphorylase. Many types of muscles possess higher capacities for glycogenolysis than for glucose uptake and phosphorylation. Under certain circumstances (e.g., short-term burst exercise), glycogenolysis supplies most of the carbon going through the glycolytic pathway, and glycolytic rates greatly exceed the capacity for flux at the hexokinase step. (Under such conditions, measured flux rates through

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hexokinase obviously do not represent glycolytic flux rates). Rates of glycogenolysis *in vivo* during exercise were obtained from differences in glycogen contents of muscles in exercised animals or muscles electrically stimulated to contract *in situ* and muscles of control, resting or unstimulated animals. Alternatively, rates of lactate or lactate plus hexose phosphate accumulation were used (29, 30).

Phosphofructokinase. In fish and mammals, the highest glycolytic rates estimated *in vivo* (obtained from rates of glycogenolysis or lactate accumulation) were used for determination of maximum flux rates through phosphofructokinase. Although rates of glycogenolysis during burst exercise may transiently exceed glycolytic rates, resulting in accumulation of hexose phosphates (29, 30), differences between these are usually small relative to glycolytic flux rates. In highly aerobic hummingbird and honeybee flight muscles, phosphofructokinase flux rates during flight are equal to the carbohydrate oxidation rates estimated by respirometry (23, 24).

V_{max} Values as Measures of Flux Capacities. Enzymatic flux capacities in tissues have been defined in the literature as either V_{max}/k_m (e.g., refs. 31 and 32) or as V_{max} (33, 34). V_{max} values obtained *in vitro* can be expressed in the same units as flux rates *in vivo*, making direct comparisons between these parameters possible. Thus, in assessing capacity/load relationships at the biochemical level, flux capacities are best represented by V_{max} values (equal to [E] $\times$ k_{cat}, where [E] and k_{cat} denote enzyme concentration and catalytic efficiency or turnover number, respectively) at each of the individual steps in pathways. The practice of measuring V_{max} values under optimal conditions *in vitro* and relating these to *in vivo* flux rates is based on a sound theoretical framework (33, 34). In combination with other information (e.g., intracellular substrate and product concentrations), V_{max} values and physiological flux rates provide insights into enzyme function and regulation (e.g., refs. 16 and 35) *in vivo*. V_{max} values serve as measures of upper limits to flux at specific steps in biochemical pathways (33, 34). As such, they can be used as measures of functional capacities in analyses of biochemical design (36).

Flux Rates and Flux Capacities

Data (from refs. 19, 21–24, 29, 30, and 37–53) concerning flux rates (v), flux capacities (V_{max}), and fractional velocities (v/V_{max}) are presented in tabular form elsewhere [Tables 1–3 are available through the internet at http://lifesci.ucsb.edu/~suarez/pnas1997tables.html or from the senior author (R.K.S.)]. These are used to establish the relationships between fractional velocities [expressed as $\%V_{max} = (v/V_{max}) \times 100$] and flux rates shown graphically in Figs. 1–3.

Hexokinase. The V_{max} values for hexokinase in our data set vary over a 100-fold range across species at their respective physiological temperatures, while the flux rates during exercise at this step differ by as much as 80,000-fold. Fig. 1 reveals a striking trend indicating increasing enzyme fractional velocities (v/V_{max}) as a function of increasing pathway flux rates at this step. Hexokinase functions at extremely low fractional velocities (0.07 to 2.5% of V_{max}) during exercise in fish skeletal and cardiac muscles but operates close to V_{max} in flying hummingbirds and honeybees, animals with high mass-specific metabolic rates and nectarivorous diets. Mammalian muscle hexokinases function over a range of intermediate fractional velocities (6–28%) during exercise.

Glycogen Phosphorylase. With the exception of rat quadriceps (where glycogen phosphorylase operates at 0.2–0.4% of V_{max}) during exercise, rates of glycogenolysis account for substantial fractions of total enzymatic capacities in vertebrate skeletal muscles, clustering between 12 and 58% of V_{max} (Fig. 2). This range of fractional velocities results from a variety of factors that include exercise intensities, fiber type differences among the muscles, and patterns of muscle fiber recruitment.

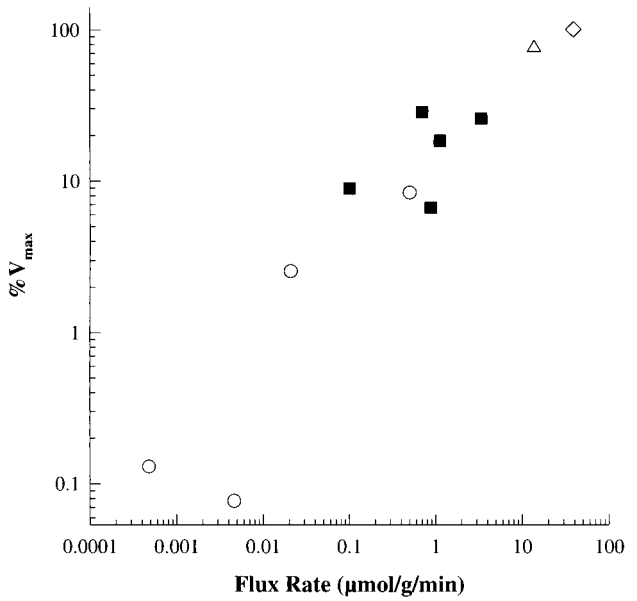

FIG. 1. Hexokinase fractional velocities, $\%V_{max}$ ($= v/V_{max} \times 100$), plotted against glucose phosphorylation rates, v, *in vivo*. Data are taken from Table 1 (available at http://lifesci.ucsb.edu/~suarez/pnas1997tables.html). ■, data obtained from rats; ○, △, and ◇, data from trout (*Oncorhynchus mykiss*), hummingbird (*Selasphorus rufus*), and honeybee (*Apis mellifera*), respectively.

Higher exercise intensities (i.e., higher running or swimming speeds) are likely to yield higher v/V_{max} values in at least some of the muscles included in our data set. This suggestion is supported by work done on human quadriceps muscles in voluntary isometric contraction, showing that glycogenolytic rates can occur at 78% of V_{max} (19). Similar fractional velocities are estimated in hagfish longitudinal retractor ("tongue") muscles during feeding (44). Thus, at least in some skeletal muscles, glycogen phosphorylase can operate close to V_{max} under physiological circumstances *in vivo*.

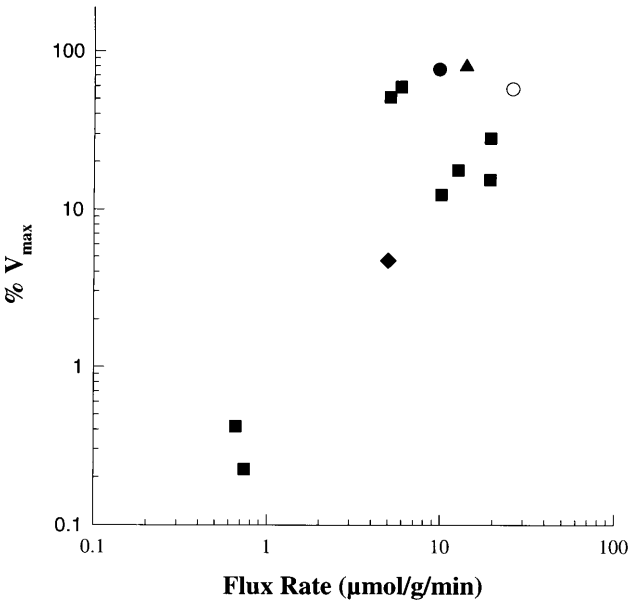

FIG. 2. Glycogen phosphorylase fractional velocities, $\%V_{max}$ ($= v/V_{max} \times 100$), plotted against rates of glycogenolysis, v, *in vivo*. Data are taken from Table 2 (available at http://lifesci.ucsb.edu/~suarez/pnas1997tables.html). ■, ◆, ●, and ▲, data from rat, tuna (*Katsuwonus pelamis*), hagfish (*Eptatretus cirrhatus*), and human, respectively; ○, trout.

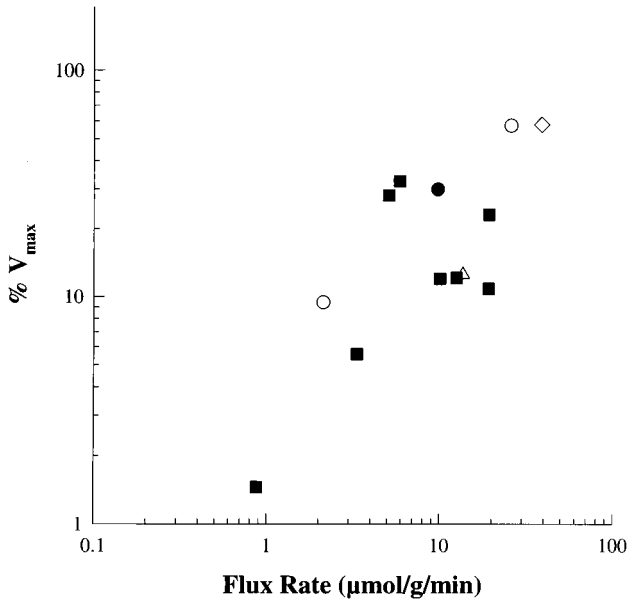

FIG. 3. Phosphofructokinase fractional velocities, $\%V_{max}$ ($= v/V_{max} \times 100$), plotted against glycolytic rates, v, *in vivo*. Data are taken from Table 3 (available at http://lifesci.ucsb.edu/~suarez/pnas1997tables.html). ■ and ●, data from rat and hagfish; ○, △, and ◇ trout, hummingbird, and honeybee, respectively.

Phosphofructokinase. At the respective physiological temperatures, the muscles in our data set differ over a 10-fold range in their capacities for flux at the phosphofructokinase step. Flux rates are such that the enzyme functions in various muscle types over a wide range of fractional velocities under physiological circumstances. Again, a trend indicating increasing fractional velocities with increasing flux rates is evident (Fig. 3). However, an important distinction between phosphofructokinase and the previous two enzymes is that it appears not to function close to V_{max} in exercising muscles.

Potential Sources of Error. The two potential sources of error in this study involve possible underestimation either of *in vitro* flux capacities or *in vivo* flux rates. In our experience, the most significant source of error in obtaining V_{max} values is insufficient extraction of the enzymes of interest. This is primarily due to enzyme binding to particulate material (mitochondrial membranes or myofibrils) and can be prevented, or at least minimized, through various means. For example, in our studies on hummingbirds and honeybees, we included detergent in the homogenization medium and sonicated the homogenates (23, 24). However, underestimation of V_{max} values would have made our estimates of v/V_{max} artificially high. It is apparent from Figs. 1–3 that this is not the case. For hexokinase and phosphofructokinase, most v/V_{max} values are low, while values approaching 100% are clearly not the outcome of underestimation of V_{max} (23, 24). Many of the v/V_{max} values for glycogen phosphorylase are significant fractions of the maximal capacities for glycogenolysis for reasons that are well understood (19, 20) and unlikely to be due to underestimation of the V_{max} values for this enzyme. Indeed, it has been known for 33 years that rates of glycogenolysis in electrically stimulated muscles can approach the V_{max} values for glycogen phosphorylase (54).

Glycolytic flux rates would be expected to increase with greater muscle power outputs. Thus, the possibility of underestimation of flux rates through each of the reactions considered herein merits consideration. In the case of hexokinase, it is unlikely that higher exercise intensities would substantially increase glucose phosphorylation rates (therefore, fractional velocities) beyond the values listed. Higher exercise intensities, particularly the transition from aerobic steady-state exercise to burst exercise, would result in the activation of glycogenolysis. This would increase intracellular concentrations of glucose 6-phosphate and inhibit glucose phosphorylation (48, 55, 56). In vertebrate hearts, extreme (e.g., anoxic) conditions result in fractional velocities that remain relatively low (8–25% of V_{max}) despite activation of rates of glucose uptake and phosphorylation (21, 43, 57). In contrast, glycogenolytic rates would be expected to increase with power output. Therefore, as discussed previously, higher v/V_{max} values than those shown in Fig. 2 are not unexpected. In the case of phosphofructokinase, most V_{max} values greatly exceed *in vivo* flux rates. This means that further increases in v (with greater power output) would not be expected to greatly alter the relationships between v/V_{max} and v shown in Fig. 3. At the highest glycolytic flux rates reported herein [39 μmol per g of thorax per min in flying honeybees (24)], fractional velocities remain well below V_{max}. We have suggested previously that such an arrangement allows the enzyme to remain poised for regulation by allosteric modulators even at maximum glycolytic rates (24).

Lastly, an issue that must be considered is the possibility that we may have underestimated *in vivo* flux rates (v) because of high rates of substrate cycling (58), particularly between glucose and glucose 6-phosphate and between fructose 6-phosphate and fructose 1,6-bisphosphate. Thus, because of glucose 6-phosphatase and fructose 1,6-bisphosphatase activities, our values of v may actually represent net (forward minus reverse) flux rates, rather than true rates of hexokinase and phosphofructokinase catalysis *in vivo*. That such cycling rates would be large enough in the muscles included in our study to cause significant errors in our estimates of v, and therefore v/V_{max}, is unlikely. With very few known exceptions (and these are not included in our sample set), glucose 6-phosphatase and fructose 1,6-bisphosphatase activities are much lower than hexokinase and phosphofructokinase activities in muscles (59). Apart from such exceptions [e.g., hawk moth (60)], mechanisms of reciprocal regulation ensure that substrate cycling rates are minimal *in vivo*, particularly during exercise when glycolytic flux rates are high (61, 62).

Mechanistic and Evolutionary Implications

Paradigms and Patterns. Because of crowded conditions in the cell (9, 10) and because protein synthesis costs 5 ATP equivalents per peptide bond (when the cost of amino acid transport is included in the estimate) (8), one might imagine that economic design at the level of biochemical pathways should involve 1:1 matches between enzyme V_{max} values and maximum pathway flux rates. In other words, there might be just enough enzyme present to catalyze each reaction, such that $v = V_{max}$ and fractional velocities, v/V_{max}, equal 1.0 at each step. This would minimize [E], given $V_{max} = [E] \times k_{cat}$. Such an arrangement might be considered to conform to Weibel and Taylor's concept (1, 63) of "symmorphosis," an influential (2, 36) but controversial (64–66) optimality hypothesis that predicts that structures and functional capacities should match but not exceed maximum physiological requirements or loads.

In physiological systems, capacities in excess of maximum loads may be found for various reasons including the need for safety margins (67). However, such safety margins can be reconciled with a less restrictive concept of economic design that says animals should be designed to possess "enough but not too much" structure and functional capacity (68). How much is enough but not too much enzyme? In the case of metabolic pathways, biochemists have understood for decades why enzymatic capacities for flux do not simply match but instead greatly exceed pathway flux rates. At reversible, near-equilibrium reactions, rates of net flux represent the difference between forward and reverse flux rates. It can be calculated with the Haldane equation (15, 69, 70) that the V_{max} values required to achieve a given rate of net physiological flux

increase exponentially as equilibrium is approached at such steps in pathways. This explains why V_{max} values may exceed maximum flux rates by up to three orders of magnitude (14, 35). Fersht (71) has proposed that pathway enzymes have evolved high k_{cat} values partly as a result of decreased affinities (higher k_m values) for substrates, such that $k_m >$ [substrate]. At thermodynamically irreversible, nonequilibrium steps such as those considered herein, *in vivo* flux rates equal to, or less than $\frac{1}{2}V_{max}$ make possible the regulation of rates of enzyme-catalyzed reactions through changes in their affinities for substrates (5). Thus, the V_{max} values in excess of v observed in many muscles in our data set are not unexpected. However, the ability of glycogen phosphorylase to operate at, or close to, V_{max} in some muscles is also not surprising in light of the need for sudden, "explosive" rates of glycogenolysis in "fight or flight" responses, and on the basis of current understanding of the regulation of this process (20). It is therefore reasonable to conclude that at both near-equilibrium and nonequilibrium steps in metabolic pathways, V_{max} values in excess over v do not necessarily represent superfluous or unnecessary capacities.

Glycolysis Under Crowded Intracellular Conditions. Since the comparative studies of pathways of energy metabolism in muscles conducted by Crabtree and Newsholme (26, 27) more than 20 years ago, it has been recognized that high flux rates *in vivo* are made possible by high enzymatic flux capacities (measured *in vitro*). Indeed, such correlations led Newsholme and colleagues (33, 34) to suggest that V_{max} values at nonequilibrium steps serve as measures (i.e., predictors) of maximum flux rates *in vivo*. The relationships between v and V_{max} presented herein clearly show that this generalization is not universally correct. Although the "excess capacities" at nonequilibrium reactions in glycolysis can be reconciled, at least qualitatively, with established biochemical concepts, the decline in such excess capacities (resulting in fractional velocities approaching 100%) with increasing metabolic rates is less easily explained.

Contractile function in the skeletal and cardiac muscles of vertebrates and in the flight muscles in insects requires that a large fraction of fiber volume be occupied by myofibrils. In rabbit skeletal muscle, Yates and Greaser (72) found that actin and myosin make up 43 and 22% of myofibrillar protein mass. Concentrations of actin and myosin heavy and light chains were estimated to be about 610, 188, and 184 μM, respectively. Given the molecular weights of these proteins (72), it can be calculated that actin and myosin contribute about 81 g, and the rest of the myofibrillar proteins contribute about 44 g of protein per liter of muscle volume. Albe *et al.* (73) compiled data concerning the glycolytic enzymes in rabbit fast twitch skeletal muscles. From the concentrations of the enzymes and their molecular weights, it can also be calculated that there are about 330 g of glycolytic enzyme protein per liter of muscle volume. If the partial specific volume for proteins (74) is about 0.725 cm^3/g, then about a third of the intracellular volume in fast twitch glycolytic muscles is occupied by myofibrils and glycolytic enzymes.

Mitochondria occupy about 35% of fiber volume in hummingbird flight muscles (75). Casey *et al.* (76) estimated mitochondrial volume densities of about 43% in the flight muscles of 0.1-g Euglossine bees, insects similar to honeybees in their mass-specific metabolic rates during flight. In these insects, myofibrillar volume densities estimated by electron microscopy ranged from about 53% in 0.1-g bees to 61% in 1-g bees (76). Given the need to allocate space for the contractile apparatus, it has been suggested (11, 12, 77) that evolution toward higher aerobic capacities in locomotory muscles may be constrained, at least partly, by upper limits to mitochondrial volume densities. However, in more highly aerobic muscles, large mitochondrial volume densities would impose severe restrictions on the mount of space available in the sarcoplasm for the other components required for muscle function. In vertebrate muscles and synchronous insect muscles, the fraction of cell volume occupied by sarcoplasmic reticulum would be expected to increase as maximum contraction frequencies become greater (78, 79), further limiting the amount of space available for sarcoplasmic enzymes. In toadfish sonic muscles, the sarcoplasmic reticulum accounts for an estimated 30% of muscle volume (79).

Biological structures and functional capacities take time, energy and materials to build and maintain and are the outcome of trade-offs between various costs and benefits (36, 67). We suggest, in addition to these, that the availability of space might set upper limits to glycolytic capacities in high-flux muscles. Such space limitations may be due to large fractional volumes occupied by myofibrils, mitochondria, sarcoplasmic reticulum, and the glycolytic enzymes themselves. In the case of nonequilibrium reactions such as hexokinase and phosphofructokinase, space constraints may be such that the degrees to which V_{max} values exceed v decline with increasing v when muscles are compared across species. Because V_{max} values must necessarily exceed v by large factors at near-equilibrium steps according to the Haldane relationship (69), space constraints may result in more exact matches between predicted enzyme V_{max} values and those actually measured in high-metabolic rate muscles (15) than in slower muscles, where greater apparent excess capacities are possible. It is tempting to speculate that the excess enzymatic capacities in lower-metabolic rate muscles make possible their wide range of contraction frequencies or power outputs. In contrast, flight using high-metabolic rate muscles in insects and hummingbirds occurs with larger more-abrupt metabolic transitions from rest to exercise, with less residual capacity for achieving higher metabolic rates (24, 75).

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