intestines, kidneys, livers, and spleens of cold-exposed hyperphagic mice suggest matches between capacities of these organs. Thus, symmorphosis – if regarded as an essential testable hypothesis rather than as a literal truth – may provide a unifying set of three principles for quantitative evolutionary understanding of biological design.

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Pathways for oxidative fuel provision to working muscles: Ecological consequences of maximal supply limitations

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Abstract. The study of metabolic fuel provision and its regulation has reached an exciting stage where specific molecular events can be correlated with parameters of the organism's ecology. This paper examines substrate supply pathways from storage sites to locomotory muscle mitochondria and discusses ecological implications of the limits for maximal flux through these pathways. The relative importance of the different oxidative fuels is shown to depend on aerobic capacity. Very aerobic, endurance-adapted animals such as long distance migrants favor the use of lipids and intramuscular fuels over carbohydrates and circulatory fuels. The hypothesis of functional co-adaptation between oxygen and metabolic fuel supply systems allows us to predict that the capacity of several biochemical processes should be scaled with maximal oxygen consumption. Key enzymes, transmembrane transporter proteins, glucose precursor supply and soluble fatty acid transport proteins must all be geared to support higher maximal glucose and fatty acid fluxes in aerobic than in sedentary species.
Key words. Metabolic substrates; aerobic capacity; regulation; glucose; lactate; fatty acid; migration.

Introduction

Animals show an amazingly wide variety of locomotory adaptations allowing them to occupy otherwise inaccessible ecological niches. These specializations provide unique opportunities to live in particular environments or to support specific lifestyles. Habitat size, food quality and distribution, predator-prey interaction, and reproductive behavior are major ecological parameters that depend very strongly upon a species' aptitude for movement[2, 50]. Both endurance capacity and maximal speed are important determinants of these life-history characteristics. Here, I will focus on sustainable locomotion, a correlate of oxidative metabolism, rather than on maxi-

mal speed that depends mostly on anaerobic ATP production.

The ability to perform prolonged physical activity is closely linked with aerobic capacity defined as mass-specific maximal oxygen consumption ($\dot{V}O_{2\,max}$)[57,58]. In turn, and at any $\dot{V}O_2$ up to $\dot{V}O_{2\,max}$, the organism has to match fluxes of *oxidative fuels* and *oxygen* to mitochondria[26,62]. Therefore, the structural, physiological, and biochemical adaptations associated with metabolic substrate storage, transport, and utilization, play a very important role in determining the ecological options available to each species. For example, long distance migrants operate at the limit of their energy reserves and they typify extreme cases of endurance exercise adaptation. In these animals, efficient metabolic fuel management is absolutely crucial at all levels of organization: from populations to individuals, organs, cells, and perhaps most fundamentally, to all molecular events associated directly or indirectly with the energetics of locomotion.

The pathway for oxygen transport through the respiratory system from the environment to muscle mitochondria has been investigated thoroughly[58,66,67]. Yet, *oxidative fuel provision*, has received very little attention in comparison, probably because it is much more complex to investigate – several fuels can be selected from, and they are stored within *and* outside working muscles.

Co-adaptation is a pervasive concept in biology. The various parts of a complex living system must be co-adapted if this system is to play its biological role economically. The concept of symmorphosis has been proposed to express this notion of economic design[68]. In the present context, maximal rates of oxygen and oxidative fuel supply must be matched, and the different steps of these supply pathways should be functionally co-adapted. Within each organism, building fuel pathways capable of supporting maximal oxidation rates higher than necessary to reach $\dot{V}O_{2\,max}$ would be wasteful. Interspecies comparisons should therefore reveal that maximal fluxes of oxidative substrates to muscle mitochondria are directly correlated with aerobic capacity.

The goal of this paper is to examine metabolic substrate transport from synthesis or storage sites to oxidation sites in locomotory muscle mitochondria, and to discuss ecological implications of the limits for maximal flux through these pathways. First, I will examine what oxidative fuels are used for sustainable locomotion, and where these fuels are stored as a function of aerobic capacity. Then, the 'flux-generating' or 'controlling' steps of the different supply pathways will be identified because they are the most susceptible to adaptive change.

Fuel preference and aerobic capacity

Aerobic locomotion is supported by the catabolism of carbohydrates, lipids and proteins stored within working muscles, *intramuscular sources*; or in other tissues, *circulatory sources*. The role of proteins will not be considered in detail because most of the information presently available pertains to mammalian species only. In this group of vertebrates, protein oxidation accounts for a minor fraction of total energy turnover[17] except during extended starvation[12]. It is responsible for less than 2% of all the ATP generated at rest[45] and the effect of prolonged exercise on protein breakdown remains controversial[70]. Some authors report a net increase in protein catabolism[8], others find that exercise has no effect[9]. In any case, the net contribution of protein oxidation becomes negligible *during aerobic exercise* in post-absorptive mammals.

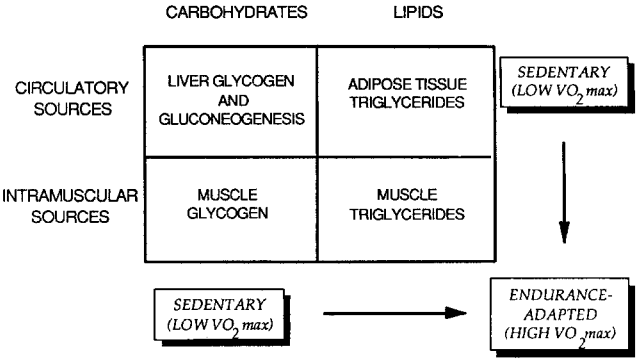

Figure 1. Major oxidative fuels and their storage sites. Arrows indicate that endurance-adapted species favor lipids and intramuscular fuels over carbohydrates and circulatory fuels ($\dot{V}O_{2max}$: maximal mass-specific oxygen consumption).

Unfortunately, amino acid mobilization and supply pathways in non-mammalian species using protein as a significant metabolic fuel have only been investigated superficially and the energetic contribution of such pathways has not been quantified. Several studies in vertebrates (fish[14,39] and birds[33]) and in invertebrates (cephalopods[27] and insects[1]), show that protein can be an important substrate for locomotion. In these animals, the quantitative contribution of protein breakdown to total metabolism should be investigated.

Other sources of oxidative fuel will be considered here in more detail: two circulatory sources, *hepatic glucose* and *adipose tissue triglycerides*; and two intramuscular sources, *muscle glycogen* and *muscle triglycerides* (fig. 1). Examining these substrates suggests basic questions about the co-adaptation of the fuel and oxygen supply pathways: Do the respective contributions of (a) carbohydrates vs lipids, and (b) circulatory vs intramuscular fuels change with aerobic capacity? Species well adapted for prolonged locomotion (high $\dot{V}O_{2\,max}$) would be expected to use proportionately more lipids than low-aerobic, sedentary species (low $\dot{V}O_{2\,max}$) to spare limited carbohydrates and take advantage of large fat reserves. Furthermore, endurance athletes should exploit the fact that, per gram of fuel, lipids can yield almost 10 times more energy than carbohydrates, in large part because glycogen has to be stored in hydrated form[28]. Recent experiments comparing goats with dogs and coyotes

($\dot{V}O_{2\,max}$ 2.5 and 3.5 times higher than goats, respectively) show that the relative proportions of fat and carbohydrates oxidized at equivalent exercise intensities (same percentage of $\dot{V}O_{2\,max}$) are similar for all species[46]; dogs and coyotes consume approximately 2.5 and 3.5 times more fat than goats. In mammals exercising at the same % $\dot{V}O_{2\,max}$, therefore, the relative use of lipids and carbohydrates is independent of aerobic capacity. It is very important to note, however, that highly aerobic animals will rely much more on lipid oxidation than sedentary species at *any given speed* because they operate at a much lower percentage of their $\dot{V}O_{2\,max}$. This adaptation may exist in all aerobic animals but it is premature to generalize beyond mammals.

Should substrate reserves be deposited preferentially in locomotory muscle cells or in other tissues? This choice is particularly relevant for species that must store large amounts of oxidative fuel in preparation for prolonged locomotion. Loading intramuscular reserves provides a major advantage because fuels can be readily available for mitochondrial oxidation. However, storing excessive amounts in myocytes may impair muscle function by reducing space available for the contractile machinery itself. In comparison, the size of non-muscular stores is practically not restricted, yet maximal circulatory fuel provision to muscle mitochondria is constrained by significant membrane and convective transport limitations[62]. The strategy used by endurance champions should therefore reflect a compromise between maximal use of intramuscular stores to avoid these limitations, and safeguard against an impairment of acto-myosin function. Preliminary data in mammals suggest that aerobic species rely more on intramuscular fuel stores than sedentary species, and that rates of circulatory and intramuscular fuel oxidation are consequently not scaled with $\dot{V}O_{2\,max}$[31,62]. For example, dogs and goats running at the same % $\dot{V}O_{2\,max}$ only show a 1.6-fold difference in circulatory glucose utilization even though their actual $\dot{V}O_2$ is 2.5-fold different ($\dot{V}O_{2\,max}$ dog/$\dot{V}O_{2\,max}$ goat = 2.5) (Weber, Roberts and Taylor, in preparation). The above analysis suggests that, overall, highly aerobic organisms will favor lipids over carbohydrates and intramuscular over circulatory fuels when compared with sedentary species (fig. 1). Let us now examine what may limit maximal carbohydrate and lipid fluxes.

Muscle glycogen and role of lactate export

The rate of muscle glycogen mobilization should never limit maximal carbohydrate oxidation because, during anaerobic exercise, the supply of pyruvate from this source can be increased by more than 20-fold above maximal aerobic rates[48]. However, because muscle glycogen stores are rather small, their size will limit the duration of high intensity aerobic exercise[13,37]. Consequently, in endurance-adapted animals, supply systems from the other three sources identified in figure 1 should be designed to minimize intramuscular glycogen use.

Traditionally, it is believed that energy stored as muscle glycogen cannot be transferred to the circulation because myocytes lack glucose-6-phosphatase[13]. However, such reasoning is only valid if glycogen is exported as glucose. In fact, a very large fraction of the energy stored as glycogen in glycolytic muscle fibers can be provided to oxidative tissues in the form of lactate via the circulation[4,37]. Thus, muscle glycogen cannot be viewed strictly as an endogenous fuel. A significant fraction of the carbon energy available in intramuscular glycogen is channeled through circulatory transport before oxidation takes place. Aerobic animals do not exploit this lactate exchange mechanism to a larger extent than sedentary species of equivalent size[65], possibly because of the constraints associated with lactate transfer across cell membranes[62]. However, it is interesting to note that lactate, unlike glucose[64], is a much more important oxidative fuel for small than for large animals[63]. In light of the crucial ecological role played by body size[43], a more systematic investigation of the molecular basis for this role should provide fascinating insights.

The glucose pathway: from hepatocytes to muscle mitochondria

The steps susceptible to limit maximal flux through the pathway providing glucose to working muscles fall in three categories: 1) key enzymatic reactions in hepatic gluconeogenesis and muscle glucose oxidation, 2) transmembrane glucose transport, and 3) supply of glucogenic precursors to the liver. The glucose pathway is summarized in figure 2 where the steps potentially affected by selective pressure for high endurance ability are emphasized.

Key enzymes

Gluconeogenesis has been discussed in detail in a number of reviews[7,40,56]. It shares several near equilibrium reactions with glycolysis but is distinct at three non-equilibrium points where specific gluconeogenic reactions are necessary to bypass irreversible glycolytic enzymes[40]. These points are: 1) pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK) to bypass pyruvate kinase (PK), 2) fructose 1,6-bisphosphatase (FBPase) to bypass phosphofructokinase (PFK), and 3) glucose-6-phosphatase (G6Pase) to bypass hexokinase (HK). In the vertebrate species investigated to date, activities of these four gluconeogenic enzymes are well correlated with rates of glucose production at the whole organism level or in isolated hepatocytes[38].

Hepatic glucose production is regulated by reciprocal control of glycolysis and gluconeogenesis at several levels. Recent studies using control theory to determine which steps of the pathway are involved in determining flux conclude that the PEP/pyruvate substrate cycle plays the most important role[24]. During exercise, gluconeogenic rate is increased via PC activation (mediated by

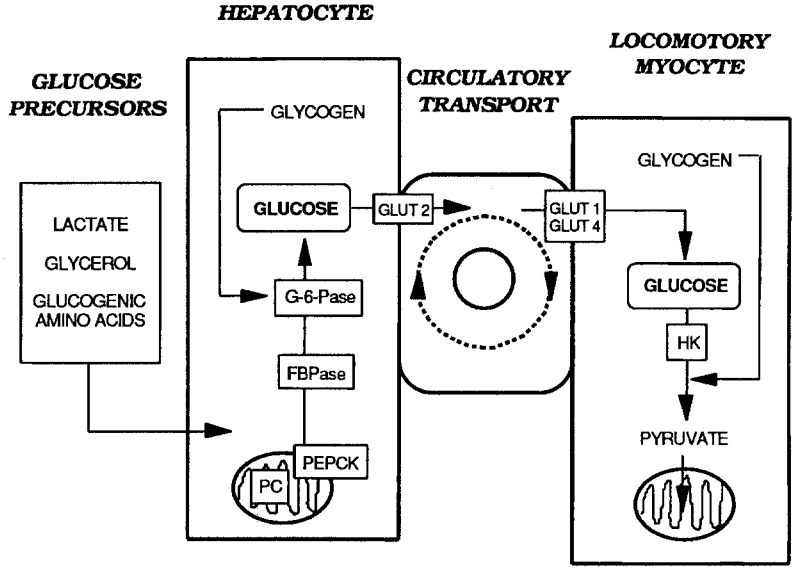

Figure 2. Pathway for glucose supply to muscle mitochondria. FBPase: fructose bisphosphatase; G-6-Pase: glucose-6-phosphatase; GLUT2: hepatocyte glucose transporter; GLUT1 and GLUT4: skeletal muscle glucose transporters; HK: hexokinase; PC: pyruvate carboxylase; PEPCK: phosphoenolpyruvate carboxykinase.

acetyl CoA) and PK inhibition (allosteric modulation and cyclic AMP-dependent phosphorylation)[35]. The last enzyme involved in the PEP/pyruvate cycle, PEPCK, is another likely candidate as a control site for gluconeogenesis. No allosteric effector has been found for PEPCK, but its activity appears to be controlled by simple changes in enzyme concentration[24, 35]. Similarly, reciprocal control of gluconeogenesis and glycolysis occurs at the level of the fructose-6-phosphate/fructose bisphosphate where FBPase plays a key regulating role[29, 44]. Before being released in the circulation, glucose-6-phosphate is converted to glucose by glucose-6-phosphatase and this enzyme may also limit maximal flux through the pathway[42].

In addition to the hepatic reactions mentioned above, the initial step for glucose utilization in muscle should also be considered. The phosphorylation of glucose in skeletal myocytes by hexokinase may restrict maximal glucose oxidation. In fact, hexokinase is the only glycolytic enzyme whose activity is increased by endurance training[30].

Glucose translocation across cell membranes

There is strong evidence to support the hypothesis that metabolic fuel transport across cell membranes can limit the supply of substrates to working muscles from the circulation[62]. In the last few years, protein carriers for transmembrane glucose transport have attracted a lot of attention. Early work on the kinetics of the transporter found in erythrocytes (GLUT 1) have been reviewed previously[3]. Several recent technical advances have allowed determination of the nature, location, density, and functional properties of the transporter in a variety of cell types[10, 22]. It is tissue specific and some cells, like adipocytes, possess more than one type of glucose transporter[32]. In the present context, glucose has to be carried from liver cells to locomotory myocytes and must therefore move across several cell membranes before it can be used to generate ATP for contraction (fig. 2). The specific transmembrane transporters involved in the glucose pathway are GLUT 2, in the hepatocyte membrane, and GLUT 1/GLUT 4 in the skeletal muscle membrane[22]. Also, it has been reported that glucose transport through the endothelial cells of muscle capillaries may play a role in limiting glucose uptake by this tissue[60]. Recent studies have clearly shown that the transporter protein has the ability to move from a perinuclear location to the cell membrane in response to stress[16, 69]. The higher muscle glucose uptake observed during exercise is achieved by increasing transporter density in the membrane (structural change) *and* by augmenting the intrinsic glucose translocation rate of each transporter (functional change)[20, 21, 25].

Per unit mass, aerobic species are capable of sustaining much higher glucose fluxes than sedentary species (Weber, Roberts and Taylor, in preparation). Transporter structure, density, intrinsic rate, and ability to move within the cell are all parameters potentially susceptible to endurance adaptation. The specific aspects of the glucose translocation system allowing more rapid movements across cell membranes in aerobic species remains to be established.

Glucose precursor supply

Circulating glucose originates from two sources: liver glycogen and gluconeogenesis. At the onset of locomo-

tion, the contribution of hepatic glycogen breakdown accounts for a large fraction of total glucose release[61]. When activity is prolonged, however, liver glycogen stores can become depleted, and the relative contribution of gluconeogenesis must increase to maintain glucose production[30].

The main gluconeogenic substrates are lactate, glycerol, and several amino acids[61]. The availability of all these precursors increases during prolonged locomotion: 1) lactate production is strongly stimulated in a variety of vertebrates including mammals[65], fish[63], and reptiles[19], 2) lipolytic rate keeps augmenting throughout exercise, providing more and more glycerol for gluconeogenesis[71], and 3) the relative contribution of amino acids (alanine, glutamine, glutamate, glycine, serine, and threonine) to total glucose output rises[61]. Despite these adjustments, hypoglycemia is often observed in marathon runners because high rates of glucose production cannot be maintained throughout the race[41]. Aerobic animal athletes should be able to provide more gluconeogenic substrates to their liver than sedentary species, thereby permitting high maximal rates of glucose production even when hepatic glycogen is totally depleted.

In summary, the hypothesis of functional co-adaptation between the oxygen and glucose pathways allows to predict that: 1) the key enzymes PC, PEPCK, FBPase, G6Pase, and muscle HK, 2) the glucose transporters GLUT 1, GLUT 2, and GLUT 4, and 3) the supply systems for gluconeogenic precursors are all designed to support much higher maximal glucose fluxes in very aerobic, endurance-adapted species than in sedentary, low-aerobic species.

The fatty acid pathway: from adipocyte to muscle mitochondria

In mammals and many other vertebrates, most of the lipid fuel is provided to muscle mitochondria as fatty acid. Nevertheless, it is important to remember that some animals use alternative metabolites such as triglycerides (e.g. birds)[33] and diglycerides (e.g. insects)[59] to supply lipid energy to their working muscles. In these species, the regulation of fat oxidation during locomotion has not been characterized and, for that reason, the following discussion will only deal with fatty acid metabolism.

The relevant parameters to consider in the limitation of flux through the fatty acid pathway fall into three categories: 1) key enzyme reactions in the mobilization and oxidation process, 2) translocation across cell membranes, and 3) soluble cytoplasmic and plasma proteins for fatty acid transport. The steps potentially modified through the evolution of high aerobic capacity are summarized in figure 3. Adaptive changes of the flux-generating steps in the fatty acid pathway should be particularly pronounced because the relative contribution of lipid oxidation to total metabolism is increased in highly aerobic animals (see section on fuel preference).

Key enzymes

Lipolysis always occurs at higher rates than necessary to keep up with FFA oxidation and a large fraction of the fatty acids released are reesterified, even during exercise[5]. The triglyceride-fatty acid cycle observed in adipocytes and myocytes is used to regulate FFA availability for locomotory muscles[71]. This cycle is under hormonal control[36] and its operation could limit maximal FFA oxidation.

In myocytes, fatty acids are converted to fatty acyl CoA by fatty acyl CoA synthetase, and the activity of this enzyme together with total availability of coenzyme A may restrict maximal oxidation. Following this activation step, the fatty acyl CoA has to be translocated across the mitochondrial membrane. This transfer is achieved by the carnitine shuttle system involving a group of enzymes: carnitine acyl-transferase I and II, including carnitine palmitoyl transferase (CPT). The catalytic activity of these shuttle enzymes could limit fatty acid uptake by muscle mitochondria, but the availability of carnitine itself does not[51]. It is also interesting to note that CPT may be closely involved in the behavioral control of food intake because it regulates the partitioning between storage and oxidation pathways for fatty acids[15]. This enzyme may be one of the most important metabolic links governing the extent of fat deposition before long distance migration.

The oxidation of fatty acyl CoA occurs through a series of enzymatic steps within the mitochondrial matrix. The main steps of β-oxidation are catalyzed by fatty acyl-CoA dehydrogenase, enoyl-CoA hydratase, β-hydroxyacyl-CoA dehydrogenase (HOAD), and acetyl-CoA acetyltransferase (ketothiolase). The exact steps responsible for the regulation of β-oxidation have yet to be identified[35]. HOAD has been used often as an index of cellular potential for fatty acid oxidation, and its activity increases drastically with endurance training[23]. Acetyl CoA concentration and $NADH/NAD^+$ ratio are both modulators of HOAD activity, but this enzyme is unlikely to represent the only controlling step because it is located towards the end of the pathway[35]. Some or all other β-oxidation enzymes may therefore also play a role in the regulation of the whole pathway.

Fatty acid transport across cell membranes

Unlike glucose, fatty acids are readily soluble in lipid membranes and they can diffuse freely across them. Free diffusion by itself would be slow, however, and a specific carrier protein for fatty acids (FFA T) resides in the plasma membrane to accelerate fatty acid translocation (fig. 3). Albumin seems to play a very important role in the regulation of the membrane transporter itself and greatly facilitates the translocation process[6]. The fatty acid carrier has been characterized in hepatocytes[54,55] and cardiomyocytes[53] but its exact position in the membrane, presence in other cell types, density, and kinetics have not been investigated in much detail. In particular,

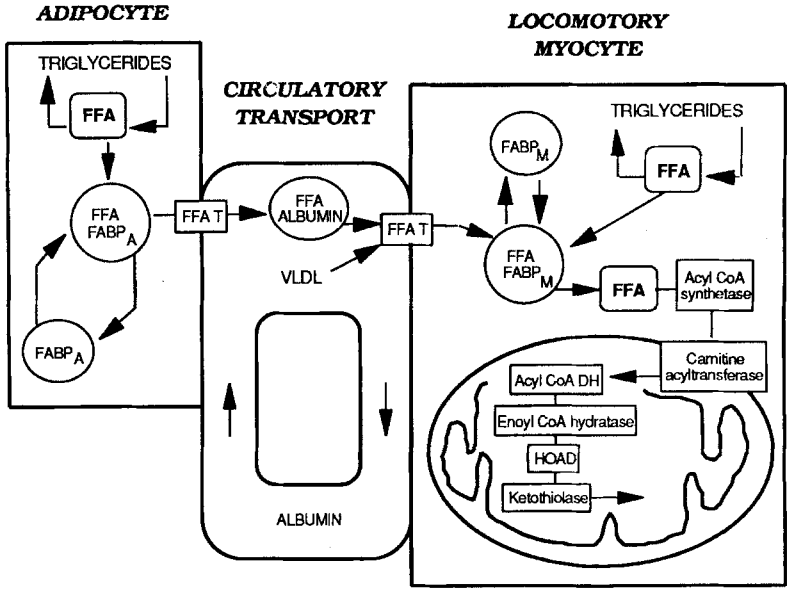

Figure 3. Pathway for fatty acid supply to muscle mitochondria. Acyl CoA DH: acyl CoA dehydrogenase; $FABP_A$: adipocyte fatty acid binding protein; $FABP_M$: skeletal muscle fatty acid binding protein; FFA: free fatty acids; FFA T: membrane bound fatty acid transporter; HOAD: β-hydroxyacylCoA dehydrogenase; Ketothiolase = acetyl CoA acetyltransferase; VLDL: very low density lipoproteins.

it is not known whether the fatty acid transporter can move between subcellular locations in response to exercise, and its relationship with the cytosolic fatty acid binding proteins (see next section) has not been established. Furthermore, muscle capillary endothelium may also represent a major barrier for fatty acid uptake as suggested in heart myocytes[47].

Extra- and intracellular fatty acid binding proteins

Because fatty acids are not soluble in aqueous solutions, their convective transport through the circulation and the cytoplasm has to rely on albumin and intracellular protein binding, respectively. The rate of circulatory fatty acid oxidation may therefore be limited by maximal flux of albumin binding sites between adipocyte and locomotory muscle capillaries[62]. Similarly, the transport of fatty acids, fatty acyl-CoA, and acylcarnitine esters through the cytoplasm may be limited by the availability of smaller cell-specific carrier proteins found in several vertebrates (FABP = fatty acid binding proteins)[11,18,49,52]. Two observations are highly relevant to this analysis: 1) the ability of different tissues to oxidize fatty acids is strongly correlated with the fatty acid binding capacity of their respective cytosolic proteins[18], and 2) muscle FABP concentration increases after electrical stimulation[34].

The hypothesis of functional co-adaptation between the oxygen and the fatty acid pathways allows us to generate a number of testable predictions: 1) fatty acyl CoA synthetase, carnitine shuttle components, and β-oxidation enzymes, 2) the density or intrinsic translocation rate by the membrane-bound FFA transporter of adipocyte, myocyte, and capillary endothelium, 3) the supply of albumin binding sites to muscle capillaries, and 4) the abundance and binding capacity of adipocyte and muscle FABP are key parameters geared to support higher maximal fatty acid fluxes in aerobic than in sedentary species.

Concluding comments

The study of metabolic fuel pathways and their regulation has reached an exciting stage where specific molecular events can be directly linked with important parameters of the organism's ecology. This paper has examined the hypothesis of co-adaptation between oxygen and oxidative substrate supply to locomotory muscle mitochondria. Strategic biochemical targets for adaptation to highly aerobic, endurance locomotion have been identified along the glucose and fatty acid supply pathways. It is predicted that the functional capacity of all these targets should be scaled directly with endurance ability of each organism measured as its maximal oxygen consumption. I hope that this analysis will contribute to the weakening of the artificial boundaries historically set between biochemistry, physiology and ecology.

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