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DESIGN OF THE OXYGEN AND SUBSTRATE PATHWAYS

VII. DIFFERENT STRUCTURAL LIMITS FOR OXYGEN AND SUBSTRATE SUPPLY TO MUSCLE MITOCHONDRIA

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Summary

This paper integrates the results of a series of studies on the supply of O_2 and substrates for oxidative muscle metabolism and draws conclusions on the role of structural design in partitioning and limiting substrate supply. The studies compared dogs and goats exercising at different intensities and combined physiological, biochemical and morphometric investigations. In both species, the rate of fatty acid oxidation reached an upper limit at low exercise intensities, and only glucose consumption was increased at higher exercise intensities. The supply of both glucose and fatty acids from the capillaries reached maximal rates at low exercise intensities; this limitation is related to the design of the sarcolemma as calculations suggest that the endothelium introduces only a small resistance to substrate flux. From these findings, it appears that the capillaries are designed to satisfy O_2 supply up to maximal O_2 demand. The increase in substrate supply to the mitochondria at higher exercise intensities is achieved by drawing on intracellular stores of glycogen and lipids. The size of these stores is larger in dogs than in goats, providing the athletic species with twice the fuel reserves. These findings are interpreted on the basis of a network model with fluxes partitioned between direct and indirect pathways and with some structures shared by more than one function. Whereas O_2 is supplied through a direct pathway, the supply of both substrates is split temporally to allow, during exercise, immediate fuel supply to the mitochondria from intracellular stores; these are replaced from the vasculature, during periods of rest, to a size commensurate with high rates of combustion. Considering this complexity, we conclude that the results are compatible with the principle of symmorphosis applied to a network structure and that the adjustment of design to functional demand involves different structures for O_2 and for substrates.

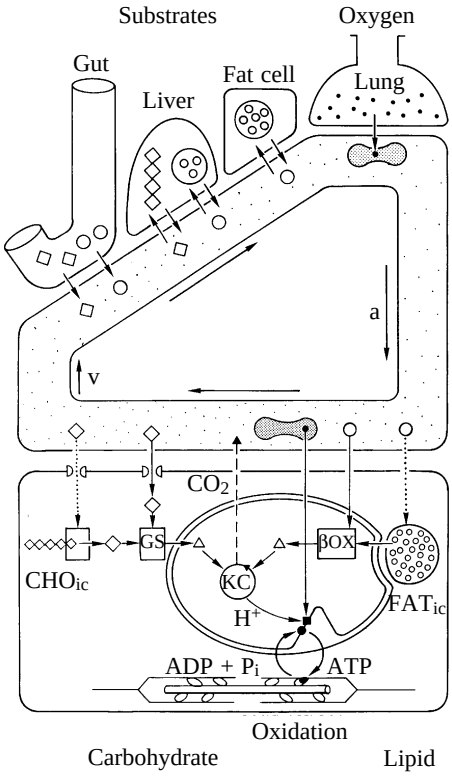
Key words: muscle oxidative metabolism, substrate pathways, carbohydrates, glucose, fatty acids, capillaries, sarcolemma, mitochondria, symmorphosis, dog, goat.

Introduction

It is generally accepted that the upper limit of aerobic muscle metabolism is set by the maximal rate of oxygen consumption, $\dot{M}_{O_2max}$. The progressive increase in the rate of O_2 consumption with increasing exercise intensity is matched by a proportional increase in the consumption of substrates, mostly glucose and fatty acids. In this series of studies, we investigated how substrate supply changes when dogs and goats increase their demand for oxidative metabolism, whether and when substrate supply reaches an upper limit, and how this could be related to differences in the design of the pathways for substrate supply to the muscle cells and their mitochondria.

In our original studies, we found that the structures supporting the pathway for oxygen are, to a large extent, co-adjusted to the overall functional capacity of the system and we have interpreted this as supporting the hypothesis of symmorphosis, which postulates that no more structure is built into a functional system than is needed (Weibel *et al.* 1991). This conclusion was reached by comparing (1) athletic with sedentary species, where $\dot{M}_{O_2max}$ varies by 2.5-fold (adaptive variation; Taylor *et al.* 1987), and (2) animals of varying body size, where mass-specific $\dot{M}_{O_2max}$ varies by sixfold (allometric variation; Weibel and Taylor, 1981). We concluded that the differences in $\dot{M}_{O_2max}$ were matched at the cellular level by proportional differences in mitochondrial volume and at the level of the microvasculature by proportional differences in the capillary red cell volume (Weibel *et al.* 1992).

Here we ask whether similar relationships exist for the structures supporting the pathways for the supply of substrates



O_2 flux	Substrate flux	Compartments	Surfaces
$\dot{M}_{O_2}$		V(ec)	S(c) − S(sl)
	$\dot{M}_{CHO}$(iv)	V(p) × C_{gluc}(p)	S(c) − S(sl)
	$\dot{M}_{FFA}$(iv)	V(p) × C_{FFA}(p)	S(c) − S(sl)
	$\dot{M}_{CHO}$(ic)	$C_{glycogen}$(f)	S(om)
	$\dot{M}_{FFA}$(ic)	V(li)	S(li-om)
$\dot{M}_{O_2}$		V(mt)	S(imi)

Fig. 1. Network model of the structure–function relationships of the oxygen and substrate pathways from the supply organs (lung, gut, liver, fat cells) through the circulation (a, arterial; v, venous) to the muscle cells (bottom) with mitochondrion, intracellular fat droplet (FAT_{ic}), glycogen granules (CHO_{ic}) and actomyosin complex. Substrates are oxidized at the terminal oxidase in the inner mitochondrial membrane (black square), generating the energy used to phosphorylate, at the associated F_1-ATPase, ADP to ATP. The oxygen pathway is marked with black dots, the fatty acid pathway with circles and the glucose pathway with squares. Arrows indicate transport processes between compartments. Oxygen is supplied to the blood in the lung; substrates are taken up in the gut, and the liver and adipose tissue (fat cells) serve as buffers for glucose and fatty acid concentration in the blood. The glucose uptake by the muscle cell is mediated by GLUT transporters (paired hemicircles) in the sarcolemma. Note that the substrate pathways from the microcirculation to the mitochondria are split into a direct and an indirect pathway. The direct pathways (solid arrows) lead directly to the sites of glycolysis (GS) in the cytosol and of β-oxidation (βOX) in the mitochondria. The indirect pathways lead through intracellular stores of glycogen (row of squares, CHO_{ic}) and of lipid droplets (FAT_{ic}); the dotted arrows indicate that substrate supply from the blood to the stores is temporally offset from utilisation. The breakdown product of glycolysis and β-oxidation, acetyl-CoA (triangles), enters the Krebs tricarboxylic acid cycle (KC) to generate reducing equivalents (H^+) for oxidation, releasing CO_2 that diffuses to the capillaries (broken arrow) for discharge through the lung. The physiological parameters, molar flux rates $\dot{M}$, for O_2, carbohydrates (CHO) and fatty acids (FFA) from intravascular (iv) and intracellular (ic) sources are listed together with the correlated morphometric parameters that characterize compartment volumes (V) and surface areas (S) of capillaries (c), plasma (p), erythrocytes (ec), sarcolemma (sl), mitochondria (mt), outer and inner mitochondrial membranes (om, imi), lipid (li) and muscle fibre (f). C_{gluc}, $C_{glycogen}$, C_{FFA}, concentrations of glucose, glycogen and fatty acids, respectively.

for oxidative metabolism (Taylor *et al.* 1996). However, the situation is not as simple. The model of Fig. 1 outlines the network design of the combined O_2 and substrate pathways as they converge at the mitochondrial respiratory chain, in particular the four branches that direct the supply of carbohydrate and fatty acids to the mitochondrial matrix and the various structures that are involved. From the original concept of symmorphosis, we would predict that the morphometric parameters that characterize the design of these structures (Vock *et al.* 1996*a*,*b*) will be matched to the maximal flow rates in the different branches of the network (Roberts *et al.* 1996; Weber *et al.* 1996*a*,*b*). However, to establish this similarity is not as simple as with the linear O_2 pathway. For fatty acids as well as carbohydrates, the flux from blood to mitochondria can be direct, as for O_2, and will therefore be determined largely by the conductances of the endothelium and sarcolemma. Alternatively, these fluxes can occur indirectly in two steps separated in time, the first leading from blood plasma to intracellular stores (lipid droplets and glycogen granules) and the second from these stores to the mitochondria. In the two-step process, the critical flux of substrates from the stores to the mitochondria during exercise depends only on the relationships between the mitochondria and the intracellular stores and is independent of the flow limitations imposed by the endothelium and the sarcolemma.

The general question we ask in this series of papers is how the network design of these pathways is quantitatively adjusted to serve the delivery of both O_2 and substrates efficiently, making the best use of the different options available, and whether the structures supporting these pathways are matched to the specific functional needs. Finally, we will ask whether the concept of symmorphosis is applicable to partitioned functions related to network structures in which some of the branches are shared by different functional fluxes.

However, the general hypothesis of symmorphosis cannot be tested, in this context, with the simple question: how much structure is enough? Where parallel transport options exist, we must ask whether the partitioning of fluxes is related to design constraints in the different branches of the network. The principle of symmorphosis predicts, in the sense of a general

hypothesis, that the best use is made of the options offered by the design of the system and that the design of each step is matched to the specific functional demand. However, it will not suffice to test specific hypotheses on structure–function relationships in individual branches; in addition, we will need to consider the functional balance achieved over the entire network by the co-adjusted design of its parts.

Results of the physiological studies summarized

The most important results of the physiological studies can be summarized as follows. Indirect calorimetry revealed that, as exercise intensity increases from resting values, the rate of fat oxidation increases by two- to fourfold, reaching a maximal rate at approximately 40 % $\dot{M}_{O_2max}$ in both species. Further increases in intensity are fuelled entirely by carbohydrates (Roberts *et al.* 1996). Three-quarters of the energy used is supplied by fat oxidation at 40 % $\dot{M}_{O_2max}$, but the relative importance of fats as a fuel decreases as exercise intensity increases, until carbohydrates supply 80 % of the energy used at 85 % $\dot{M}_{O_2max}$. Although there is no difference in these proportions between the two species, the dogs have higher absolute rates owing to their higher aerobic capacity (Fig. 2).

The rates at which both glucose and free fatty acids flow directly from intravascular sources to the mitochondria increase with exercise intensity until an upper limit is reached (Fig. 3). Then, at higher intensities, the additional energy is supplied entirely from intracellular stores (Weber *et al.* 1996*a*,*b*). The upper limits suggest that a structural limitation exists in these pathways, and we return to this later.

Results of the morphometric studies summarized

The morphometric analyses of random samples of the whole musculature (Vock *et al.* 1996*a*,*b*) confirmed our previous findings of structure–function relationships in the oxygen pathway (Weibel *et al.* 1992). Mitochondrial volume was greater in dogs than in goats, in direct proportion to their differences in aerobic capacity. Thus, at $\dot{M}_{O_2max}$, each mitochondrion consumed oxygen at about the same rate whether it resided in the muscles of a dog or a goat. Likewise, capillary red cell volume, the structural parameter determining the oxygen-transporting capacity of the blood, was also proportional to aerobic capacity. A greater capillary volume and red cell volume contributed equally to the greater oxygen-transporting capacity of the capillary bed of the dog.

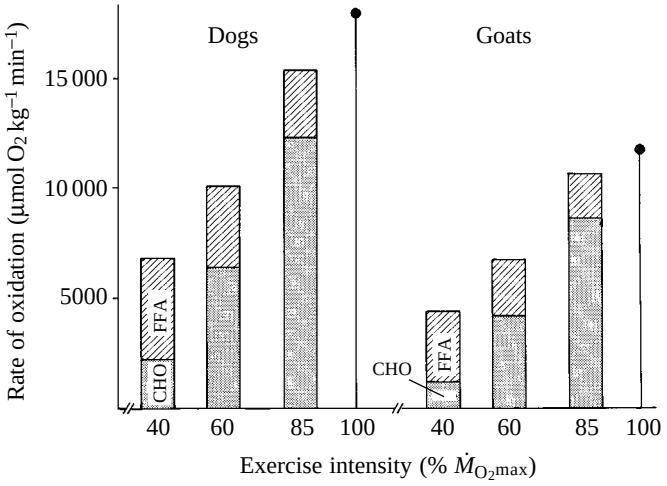


Fig. 2. Rates of oxidation of glucose (CHO) and fatty acids (FFA) at different exercise intensities in dogs and goats. The black dot marks maximal oxidation rate. The data, given in O_2 equivalents, are from Roberts *et al.* (1996), but values here are expressed per kilogram muscle mass.

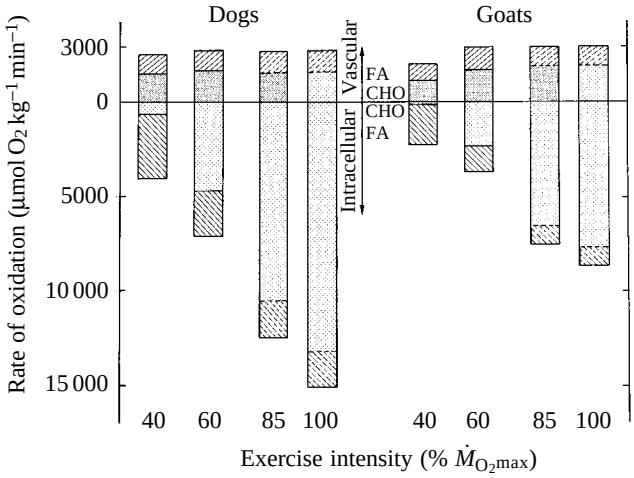


Fig. 3. Rates of oxidation of glucose (CHO) and fatty acids (FFA) at different exercise intensities partitioned with respect to the supply pathways from vascular and intracellular substrate pools. Oxidation rate of vascular substrates is plotted above the baseline, that of intracellular substrates below the baseline. Calculated after data from Roberts *et al.* (1996) and Weber *et al.* (1996*a*,*b*). The partitioning of fatty acid supply at 85 % and 100 % $\dot{M}_{O_2max}$ is extrapolated and is shown with a broken line and different shading.

As a consequence of the differences in haematocrit, the plasma volume was the same in dogs and goats despite a 1.5-fold difference in capillary blood volume (Figs 4, 5). One might expect that the concentrations of fatty acids and glucose in the blood of exercising dogs would be lower than in the goats because they are carried mainly in the plasma, but they were not. Most of the fatty acids are transported bound to albumin, and the reduction of plasma volume due to the higher haematocrit in dogs was compensated almost exactly by an adaptation of the dogs' albumin for binding 1.5 times as much fatty acids (McClelland *et al.* 1994).

The intracellular stores of both glycogen and lipids were much greater in the dog, remarkably by factors larger than those for $\dot{M}_{O_2max}$ (Figs 4, 5). Some interesting details about the distribution of substrate deposits should be noted. Glycogen granules appear to be dispersed throughout the muscle cell, some even occurring within the myofibrils, with seemingly no preferential association with mitochondria. In contrast, lipid droplets were always found tightly associated with

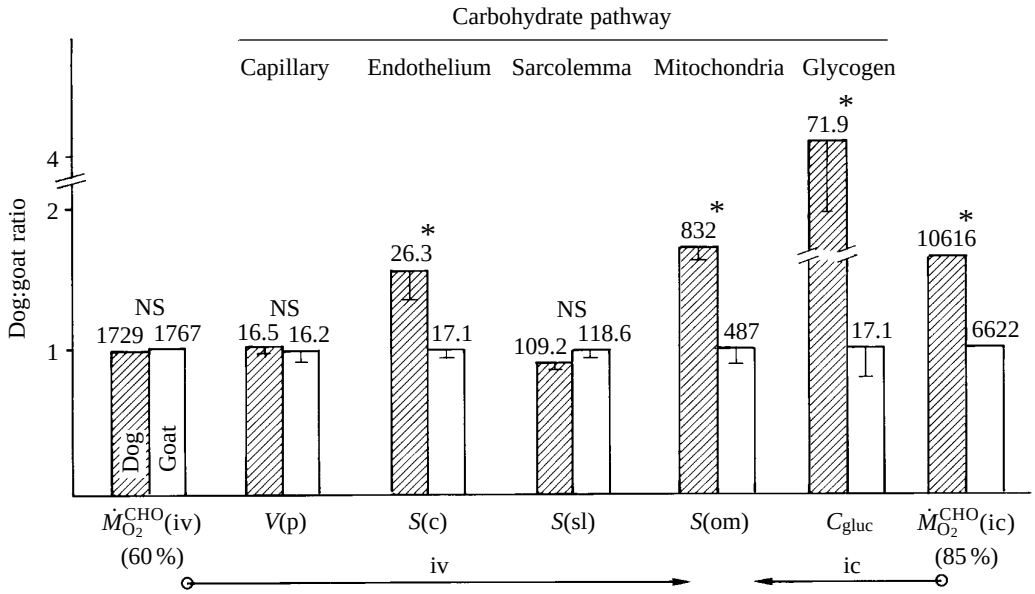


Fig. 4. Comparison of physiological and morphometric data for the carbohydrate pathways from intravascular (iv: left to right, exercise intensity 60 % $\dot{M}_{O_2max}$) and intracellular (ic: right to left, exercise intensity 85 % $\dot{M}_{O_2max}$) pools. All values are expressed per kilogram muscle mass and are plotted as dog:goat ratios; absolute values are given above each column. *N*=3 animals per group. Asterisks mark significant differences between species; NS, not significant. $\dot{M}_{O_2}^{CHO}$, carbohydrate flux in O_2 equivalents ($\mu mol\,kg^{-1}\,min^{-1}$); *V*(p), plasma volume ($ml\,kg^{-1}$); *S*(c), capillary surface area; *S*(sl), sarcolemmal surface area; *S*(om), outer mitochondrial membrane surface area ($m^2\,kg^{-1}$); C_{gluc}, molar content of glycogen glucosyl units ($mmol\,kg^{-1}$). Physiological data are taken from Weber *et al.* (1996*b*), morphometric data from Vock *et al.* (1996*a*,*b*). Values are means + S.E.M.

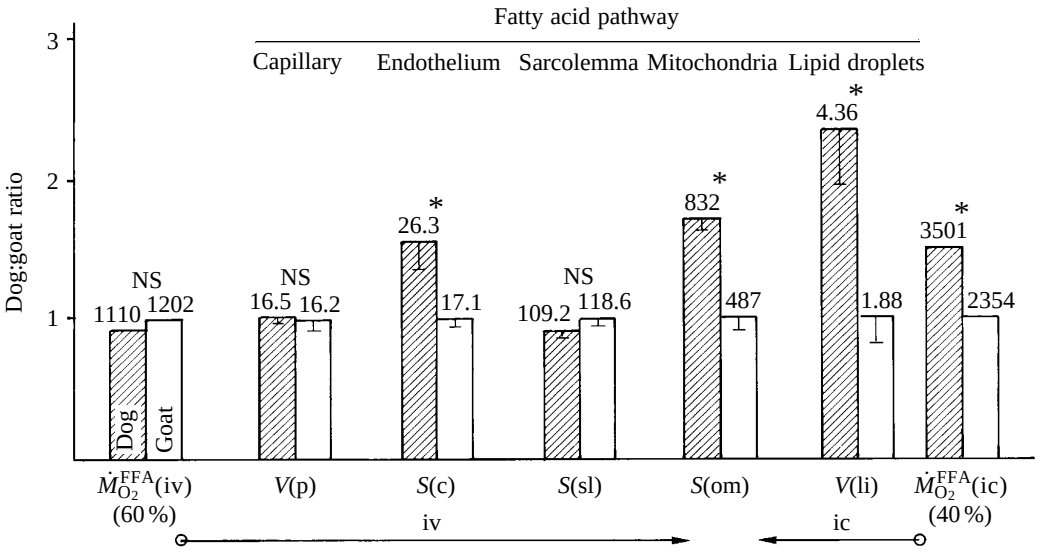


Fig. 5. Comparison of physiological and morphometric data for the fatty acid pathways from intravascular (iv) and intracellular (ic) pools. All values are expressed per kilogram muscle mass and are plotted as dog:goat ratios; absolute values are given above each column. *N*=3 animals per group. Asterisks mark significant differences between species. $\dot{M}_{O_2}^{FFA}$, fatty acid flux in O_2 equivalents ($\mu mol\,kg^{-1}\,min^{-1}$); *V*(li), volume of lipid droplets ($ml\,kg^{-1}$); other symbols and units as in Fig. 4. Physiological data are taken from Weber *et al.* (1996*a*), morphometric data from Vock *et al.* (1996*a*,*b*). Values are means + S.E.M.

mitochondria (Vock *et al.* 1996*a*) such that 40 % and 23 % of the droplet surface is in direct contact with outer mitochondrial membranes in dogs and goats, respectively.

Role of design in adjusting to quantitative differences in aerobic metabolism

The pathways for O_2 and substrates converge at the respiratory enzymes of the inner mitochondrial membrane (Fig. 1). The O_2 pathway is unbranched: O_2 is loaded into erythrocytes in the lung and transported to the muscle capillaries, from where it diffuses to the site of combustion in the mitochondria. The mechanism is diffusion, and therefore the role of the design is to establish adequate diffusion surfaces and sufficiently short diffusion paths to maintain a steady flow of O_2 matched to the needs for oxidative catabolism of fuels. O_2 supply is critical in that there is no substantial capacity to store O_2 so that it must be supplied in a steady flow from the ambient store in environmental air through the lung and the blood circulation. The process has a limit beyond which additional energy can only be produced anaerobically by glycolysis.

In contrast, the substrate pathways for carbohydrate and fatty acids are more complex. They converge in the mitochondria at the tricarboxylic acid cycle, which delivers reducing equivalents to the respiratory chain, where they are

oxidized to water and the energy liberated is used to rephosphorylate ADP to ATP (Fig. 1). These reducing equivalents are obtained by dehydrogenation of acetyl-CoA, which is derived either from glycolysis or from β-oxidation of fatty acids. Proximal to this final common path, the pathways for carbohydrate and fatty acids are separate, involving different enzyme systems. However, they have one quality in common: both substrates can be stored in appreciable quantities within the muscle cells, carbohydrates in the form of glycogen granules and fatty acids in the form of triglyceride droplets. The most direct pathway for substrate supply to the mitochondria is therefore from these intracellular stores since substrate stores and mitochondria are within the same compartment with no barriers interposed.

Eventually substrates must be supplied to the muscle cell from the circulation, a pathway partly shared with that for oxygen and a process regulated by varying blood flow according to the metabolic needs of the muscles. It is therefore interesting to ask whether the rate of vascular substrate supply also benefits from this up-regulation of blood flow. The transfer of glucose and fatty acids from the capillaries to the cell is not a diffusive process as simple as that for O_2. Glucose is soluble in aqueous media such as plasma, interstitial fluid and cytosol, but it is insoluble in the lipid layers that constitute the membranes. Accordingly, glucose transfer will depend on the design of an adequate pore system in the capillary endothelial wall as well as in the sarcolemma, where specific transporter molecules are involved. For fatty acids, the situation is reversed: they can, in principle, diffuse across the lipoproteinaceous plasma membranes but must be solubilized by binding to albumin in the plasma and interstitial space and to fatty-acid-binding proteins in the cytosol. The overall driving force for substrate supply to the muscle cells is their concentration in plasma, which is maintained under hormonal regulation from special storage organs, liver and adipose tissue, in addition to a possible direct supply from the gut.

The two substrate pathways therefore have multiple points at which the rate of supply can be regulated and where variations in design can be expected to exert a significant influence. In terms of functional regulation, we can ask whether the regulation of capillary perfusion, which is so important for adjusting O_2 delivery to demand, is also an important mechanism in regulating substrate supply. In terms of structural design, two major barriers appear to be important (the capillary endothelium and the sarcolemma), and we must ask whether their morphometric properties are adjusted to differences in substrate demand.

In order to establish quantitative structure–function relationships, we must first consider the stoichiometry of the molar flux rates through these pathways for O_2, $\dot{M}_{O_2}(\mathrm{mt})$, and the two substrates, $\dot{M}_{O_2}^{CHO}(\mathrm{mt})$ and $\dot{M}_{O_2}^{FFA}(\mathrm{mt})$. For the purposes of integration of the pathways, these are best expressed in terms of O_2 equivalents:

$$\dot{M}_{O_2}(\mathrm{mt}) = \dot{M}_{O_2}^{CHO}(\mathrm{mt}) + \dot{M}_{O_2}^{FFA}(\mathrm{mt}), \quad (1)$$

whereby the ratio between carbohydrate and fat oxidation can be estimated by indirect calorimetry (Roberts *et al.* 1996). This is related to the substrate flux rates as:

$$\dot{M}_{O_2}(\mathrm{mt}) = 6\dot{M}_{CHO}(\mathrm{mt}) + 23\dot{M}_{FFA}(\mathrm{mt}), \quad (2)$$

since the oxidation of 1 mol of carbohydrate and fatty acids consumes 6 and 23 mol of O_2, respectively. Finally, the substrate flux rates are partitioned between those from intravascular pools (iv) and those from intracellular stores (ic), so that the overall stoichiometric relationship is:

$$\dot{M}_{O_2}(\mathrm{mt}) = 6[\dot{M}_{CHO}(\mathrm{iv}) + \dot{M}_{CHO}(\mathrm{ic})] + 23[\dot{M}_{FFA}(\mathrm{iv}) + \dot{M}_{FFA}(\mathrm{ic})]. \quad (3)$$

With reference to Fig. 1, it is apparent that each of these partial flux rates will use different routes from the source to the mitochondria. What is the most efficient combination of these four flux rates and how is it affected by variations in fuel requirement between rest and increasing exercise intensity?

In view of the general hypothesis of symmorphosis, the crucial question is how structural design is related to the partitioning of substrate flux to the four main branches of the pathways. Do we find limitations in any of these branches and are they related to quantitative design parameters? Are there differences in design that ensure higher fuel supply in the dog compared with the goat?

Mitochondrial fuel sink and partitioning of substrate supply

Total fuel consumption by the mitochondria obviously follows O_2 consumption and thus increases linearly with exercise intensity up to $\dot{M}_{O_2max}$ (Fig. 2). In both species, the oxidation of fatty acids supplies most of the fuel until their oxidation rate reaches its upper limit. Then glucosyl units supply all of the additional energy required for higher exercise intensities (Roberts *et al.* 1996). The maximal rate of mitochondrial fatty acid oxidation is about 40 % higher in the dog than in the goat. It is not increased when fuel demand increases at higher exercise intensities; on the contrary, it may even fall to some extent as carbohydrates increase their share in fuel supply (Fig. 2).

The total flux of carbohydrate-derived acetyl-CoA into the mitochondrial oxidation system is about two times greater in the dog than in the goat (Roberts *et al.* 1996), and this correlates well with the twofold greater mitochondrial volume in the dog and reasonably well with the surface area of the outer mitochondrial membrane, which is 1.7 times larger in the dog. When we calculate the maximal flux density of pyruvate, generated by glycolysis, across the outer mitochondrial membrane this amounts to 29 and 34 $\mu mol\,m^{-2}\,min^{-1}$ in dogs and goats, respectively, at 85 % $\dot{M}_{O_2max}$, values that are quite similar. The pyruvate carrier that mediates transfer into the matrix is in the inner mitochondrial membrane, whose surface area is about three times that of the outer membrane, with no observed species differences, so that the maximal flux densities across this membrane are about 9 and 11 $\mu mol\,m^{-2}\,min^{-1}$ in dogs and goats, respectively.

Relationship between flux rates and structural design parameters

The glucose pathways

Intracellular carbohydrate pathway

$\dot{M}_{CHO}$(ic). The flux of glucose from intracellular stores, $\dot{M}_{CHO}$(ic), begins at the glycogen stores and extends to the outer mitochondrial membrane (Fig. 1). Glycogen granules are broken down phosphorolytically by means of glycogen phosphorylase into glucose 1-phosphate which, after conversion to glucose 6-phosphate, enters glycolysis. This generates pyruvate and some ATP. When O_2 is available, pyruvate enters the mitochondria, mediated by a pyruvate carrier in the outer mitochondrial membrane, and is converted to acetyl-CoA for entry into the Krebs cycle. An essential design feature of this system is the size of the glycogen pool, which is much larger in the dog than the goat (Fig. 4). The branched glycogen chains are hydrated and associated with several enzymes, notably phosphorylase, which clips off glycosyl units as glucose 1-phosphate. In muscle, the glycogen forms small rather uniform granules of about 10 nm in diameter, giving the glycogen stores a large surface area. Glycogen granules may accumulate in packets around the mitochondria, but they are also found within the myofibrils and around the sarcoplasmic reticulum, where they may contribute to anaerobic ATP production.

We have found that increasing exercise intensity results in an increased utilization of intracellular carbohydrates (Fig. 3). At high exercise intensities, about 80 % of the glucose shuttled into the oxidation pathway stems from intracellular glycogen stores (Weber *et al.* 1996*b*). This rate is 1.6 times greater in the dog than in the goat, while the amount of glycogen in the stores is four times greater, as shown in Fig. 4 (Vock *et al.* 1996*a*). Accordingly, the dog uses a smaller fraction of its muscle stores during exercise and this should allow this athletic animal to run for longer than a goat. Indeed, we estimated that, at an exercise intensity of 85 % $\dot{M}_{O_2max}$, the dog's intracellular glycogen stores can fuel aerobic metabolism for 40 min compared with no more than 15 min in the goat; at 60 % $\dot{M}_{O_2max}$, the reserves last for 90 and 45 min, respectively (Vock *et al.* 1996*a*). We conclude that the dog, as an endurance athlete, builds intracellular carbohydrate reserves that last more than twice as long as those in the goat.

Glucose transfer from the capillary

$\dot{M}_{CHO}$(iv). Glucose molecules are drawn from the pool of dissolved glucose in the plasma whose concentration is regulated by mobilizing liver glycogen. Plasma glucose is transferred to the muscle cell by diffusion and meets two major barriers, the endothelial cell and the sarcolemma, which are separated by the interstitial space. In the sarcoplasm, glucose diffuses freely to reach the sites of glycolysis.

In reviewing our physiological findings (Weber *et al.* 1996*b*), we first note that the glucose supply to muscle cells from intravascular sources occurs at similar rates in dogs and goats, and that it does not increase as exercise intensity is increased above 40 % $\dot{M}_{O_2max}$ in the dog and 60 % $\dot{M}_{O_2max}$ in the goat (Fig. 3). The plasma volume per unit muscle mass is the same in the two species (Fig. 4), but the plasma glucose concentration is 1.8 times higher in goats (Weber *et al.* 1996*b*). Thus, the plasma glucose pool available for extraction is smaller in the dogs: 87 and 150 μmol kg^{-1} muscle mass in dogs and goats, respectively. We estimate that dogs and goats draw glucose from the blood at rates of 4.5 and 5.5 μmol kg^{-1} s^{-1}, extracting 4–5 % of the plasma glucose pool every second.

Considering the mechanisms of glucose delivery to the muscle tissue, we must ask what role is played by the increased rate of perfusion with increasing exercise. An important parameter is capillary transit time, t_c, which can be calculated as the ratio of capillary blood volume to cardiac output from the data collected by Vock *et al.* (1996*b*) and McClelland *et al.* (1994). At 85 % $\dot{M}_{O_2max}$, we estimate t_c to be 0.87 s in the dog and 0.65 s in the goat, with the result that the dog and goat extract the same amount of glucose from the blood during one transit time, namely 3.9 μmol kg^{-1} in the dog compared with 3.6 μmol kg^{-1} in the goat.

Flux rate across the endothelium. Fig. 4 shows that the capillary endothelial surface area is 1.5 times greater in the dog than in the goat (Vock *et al.* 1996*b*). Since the flux rate of glucose from plasma is similar or even slightly higher in the goat, the flux density across the endothelial surface is 1.9 times greater in the goat. However, this is not very relevant because glucose transfer occurs not through the entire capillary surface but rather through the pore system in the endothelial junctions (Pappenheimer, 1953; Crone and Levitt, 1984). We found that the length of endothelial junction lines per unit capillary surface was similar in both species (Vock *et al.* 1996*b*) so that the flux density across the unit length of junction is some 60 % higher in the goat. In order to interpret this further, we must turn to estimates of the permeability characteristics of muscle capillaries (Crone and Levitt, 1984). Unfortunately, no information is available for the comparison between dogs and goats in this respect. Because the permeability is largely determined by the endothelial junctions and a qualitative study of the junction structure did not show any differences, we assume, as first approximation, that the permeability characteristics are similar in the two species.

The net flux rate across the endothelium is:

$$\dot{M}_{CHO}(iv) = p \times S(c) \times \Delta\overline{M}_{gluc}, \quad (4)$$

where the permeability coefficient p is the product of the diffusion coefficient D and the restricted pore area per unit path length expressed per unit surface area. Taking data on these two factors from the literature (Pappenheimer, 1953; Crone and Levitt, 1984; Curry, 1984), we have estimated the molar transendothelial glucose concentration difference, $\Delta\overline{M}_{gluc}$, needed to drive the flux rate to be about 1–2 mmol l^{-1} in the dog and 1.5–3 mmol l^{-1} in the goat (Vock *et al.* 1996*b*). We have further found that, at 85 % $\dot{M}_{O_2max}$, the glucose concentration in plasma was 5.4 mmol l^{-1} in the dog and 11.5 mmol l^{-1} in the goat (Weber *et al.* 1996*b*), so that the transendothelial gradient is approximately one-quarter of the

plasma concentration in both species. This suggests that the capillary endothelium offers only a weak resistance to the diffusion of glucose from the plasma to the interstitial space.

Glucose transfer through the interstitium. The driving force for glucose transfer across the sarcolemma by facilitated diffusion is the perisarcolemmal glucose concentration. This is determined by the pericapillary glucose concentration and the capillary spacing. Subtracting the transendothelial concentration difference from the plasma concentration, we estimate the pericapillary concentration to be 4 and 9 mmol l^{-1} in the dog and goat, respectively (Vock *et al.* 1996*b*). The capillary spacing is, on average, about 66 μm in the dog *versus* 100 μm in the goat (Vock *et al.* 1996*b*). Although diffusion through the interstitial space is rapid, we must consider that most interstitial spaces are rather narrow, no more than 1–2 μm wide, and that glucose is extracted at a fairly high rate by the two sarcolemmal membranes bounding this space. We estimate that each μm^2 of sarcolemmal membrane extracts about 1 % of the interstitial glucose content every second. Since the half-distance between capillaries is about 50 % longer in the goat than in the dog, this may well result in similar average glucose concentrations at the sarcolemmal surface. Consequently, the driving force for facilitated glucose diffusion across the sarcolemma will be similar in both species.

Glucose transfer across the sarcolemma. We found the surface area of the sarcolemma per unit muscle mass to be invariant between dogs and goats (Fig. 4). The sarcolemmal conductivity for glucose is largely determined by the glucose transporters GLUT-1 and GLUT-4 (see Mueckler, 1994, for a review); their density and activity are not known in these two species because most studies have been performed on humans or small rodents. But it was generally found that GLUT-4 transporters are recruited to the sarcolemmal surface even at moderate exercise intensities (Barnard and Youngren, 1992). Since our measurements of glucose flux were made at high exercise levels, it is likely that all the available reserves have been recruited. Fig. 3 shows that, in the goat, $\dot{M}_{CHO}$(iv) increases between 40 % $\dot{M}_{O_2max}$ and 60 % $\dot{M}_{O_2max}$ and does not increase further beyond this exercise intensity; in the dog, $\dot{M}_{CHO}$(iv) does not increase beyond 40 % $\dot{M}_{O_2max}$. From this, we estimate the maximal flux density of glucose per unit surface area of sarcolemmal membrane, $\dot{M}_{CHO}(iv)/S(sl)$, to be about 2.5 μmol m^{-2} min^{-1} in both goats and dogs. This maximal flux density is achieved at 40 % $\dot{M}_{O_2max}$ in the dog and at 60 % $\dot{M}_{O_2max}$ in the goat. This suggests that the transfer capacity of the sarcolemmal membrane, or rather that of its transporters, has become saturated at these exercise intensities and that, as a result, the sarcolemmal conductivity limits glucose uptake by the muscle cell.

Overview of the carbohydrate pathway

In conclusion, we note that, of the two limbs of the glucose pathway, only the recruitment of glucose from intracellular glycogen stores can be increased to cover the higher fuel needs at higher exercise intensities. In contrast, the supply from intravascular sources is limited, probably by the relatively low conductivity of the sarcolemma; it may be taken as a further argument for these barriers being limiting that the flux rate increases when the driving force is larger, as in the goats.

It is therefore advantageous to load the muscle cells with glycogen during periods of rest, thus building up good conditions for efficient recruitment of carbohydrate fuel during exercise when ATP demands are high. It is interesting that one of the well-known consequences of endurance exercise training is indeed an enlargement of the intracellular fuel stores.

The fatty acid pathways

As in the case of carbohydrates, the pathway for fatty acid oxidation has two branches: the vascular pathway is supplemented by local recruitment of fatty acids from the cytoplasmic triglyceride stores (Fig. 1). Fatty acids are fuels for low-intensity work. By the time exercise intensity reaches 60 % $\dot{M}_{O_2max}$, fatty acids contribute only about one-third of the fuel oxidized, and oxidation rates are about 30 % higher in the dog than in the goat.

Intracellular fatty acid pathway

$\dot{M}_{FFA}$(ic). Muscle cells store fatty acids as triacylglycerols in the form of lipid droplets. These are highly concentrated stores of energy, about twice as energy-dense as glucose; also, they are free of water so that the energy yield from 1 g of lipid stores is six times as high as that from 1 g of glycogen (which is hydrated). Lipid droplets are tightly associated with mitochondrial outer membranes with about one-third (23 % in goats, 40 % in dogs) of the droplet surface area in direct contact (Vock *et al.* 1996*a*). At the surface of the lipid droplets (possibly at the contact surface with the outer mitochondrial membrane), fatty acids are clipped off from triacylglycerols by a lipase and shuttled across the mitochondrial membranes as acyl-carnitine by means of a translocase, to reach the mitochondrial matrix. Here, they are broken down by β-oxidation into acetyl-CoA, which then enters the Krebs cycle. It seems likely that some free fatty acids are also supplied directly from the cytoplasmic pool associated with fatty-acid-binding proteins. The design parameters that determine the flux rate from intracellular stores to mitochondria appear to be the number or total volume of lipid droplets and the contact surface area with mitochondria.

We found that two-thirds of the fatty acids burned are drawn from intracellular deposits, maximal rates being 50 % higher in the dogs than in the goats (Fig. 5). The size of the intracellular lipid deposits is therefore most important and these are 2.3 times larger in the dog. As a result, dogs extract a smaller fraction of their lipid stores per unit time, namely 22 μmol ml^{-1} min^{-1} compared with 33 μmol ml^{-1} min^{-1} in the goat. We estimate that, at an exercise intensity of approximately 40 % $\dot{M}_{O_2max}$, the intracellular lipid stores can provide fuel for 3 h in the dog and for only 2 h in the goat. Furthermore, they last twice as long as the carbohydrate stores. All lipid droplets are tightly associated with mitochondria, and we have estimated that the contact surface area covers some

40 % of the lipid droplet surface in the dog compared with 23 % in the goat, and 2 % and 1 % of the mitochondrial surface, respectively (Vock *et al.* 1996*a*). The lipid–mitochondria contact surface area is therefore nearly four times larger in the dog, which is much greater than the 50 % higher relative fatty acid extraction rate. We cannot interpret this finding until we learn more about the mechanisms of interaction between the outer mitochondrial membrane and the lipid droplets. It seems, however, that the dog's intracellular lipid reserves are provided with a considerable degree of redundancy. This may well be an advantage in this high-performance endurance athlete.

Fatty acid transfer from the capillary

$\dot{M}_{FFA}$(iv). In the blood plasma, the hydrophobic fatty acids are solubilized by binding to albumin. There are species differences in that the fatty acid binding capacity of albumin is 1.5 times as high in the dog as in the goat (McClelland *et al.* 1994). In the interstitial fluid, fatty acids are also bound to albumin. The transfer of fatty acids across the two barriers, the endothelium and the sarcolemma, is not entirely understood. As they can freely cross lipoproteinaceous cell membranes, fatty acids can diffuse through the endothelial membrane, but it is also possible that some fatty acids pass through the endothelial pore system bound to albumin. In the sarcolemma, free diffusion across the membrane is a possible pathway, but it is still disputed whether special fatty acid transporter proteins exist in the sarcolemma (Higgins, 1994). In the sarcoplasm, fatty acids are bound to fatty-acid-binding proteins (Glatz *et al.* 1988), which allow them to diffuse from the sarcolemma to the mitochondria. The design features determining the fatty acid flux rate from the plasma are the surface areas of the endothelium and sarcolemma, and possibly the density of pores passable by albumin in the endothelium.

As with carbohydrates, we found that dogs extract somewhat more fatty acids from the circulating blood than do goats, but that this is again not increased at exercise intensities above 40 % $\dot{M}_{O_2max}$ (Fig. 3). It is interesting that the concentration of free fatty acids in blood plasma is twice as high in the dog as in the goat and that this is related to a 1.5-fold higher fatty acid binding capacity of albumin in the dog (McClelland *et al.* 1994). From the data collected in this study (McClelland *et al.* 1994; Weber *et al.* 1996*a*; Vock *et al.* 1996*b*), we can calculate that capillary plasma contains 31 and 16 μmol of free fatty acid per kilogram muscle mass in dogs and goats, respectively. At the respective measured fatty acid flux rates from blood, $\dot{M}_{FFA}$(iv)/M_m, of 0.78 and 0.63 μmol kg^{-1} s^{-1}, we estimate that dogs extract 2.5 % of their plasma fatty acid pool every second compared with 3.9 % in the goat. However, from the data collected by McClelland *et al.* (1994) and Vock *et al.* (1996*b*), we estimate that the capillary transit time for both whole blood and plasma at approximately 60 % $\dot{M}_{O_2max}$ is 1.4 s in the dog compared with 0.8 s in the goat. This means that during each transit time both species extract approximately 3.5 % of their plasma fatty acid pool. It is interesting that the extraction fraction for fatty acids is very similar to that for glucose and that both are relatively small, a further indication that the transfer of substrates from the capillaries may be limited by design features.

Dogs have a larger capillary surface area per unit muscle mass than goats, whereas the sarcolemmal surface area is the same in both species (Fig. 5). Accordingly, the flux density for fatty acids across the endothelial barrier is somewhat lower in the dogs, whereas the flux density across the sarcolemmal membrane is invariant between the two species. Little is known for certain about the transfer mechanisms across these membranes or about how the free fatty acid concentration acts as a driving force because the fatty acids are bound to proteins in the aqueous spaces; these differences in the transmembrane flux densities cannot therefore be further interpreted.

In conclusion we note that the flux density through the two branches of the fatty acid pathway is not increased with increasing exercise intensity. The supply of fatty acids from intravascular sources is limited, as is the case for carbohydrates. Since recruitment of fatty acids from intracellular stores is also limited, it appears that this limit is set either by the capacity for transfer of fatty acids into the mitochondria or by their capacity for fatty acid β-oxidation.

General conclusions

Summary of the main findings

The combined physiological and morphometric study of oxidative metabolism presented in this series of papers leads to the following conclusions.

(1) Oxidative metabolism is fuelled by both carbohydrate and fatty acids at all exercise levels, but only carbohydrate metabolism is up-regulated to cover the higher fuel demand at higher work loads.

(2) When exercising at the same intensity (same percentage $\dot{M}_{O_2max}$), the relative contributions of carbohydrate and lipid oxidation are the same for dogs and goats and, in fact, for animals of all aerobic capacities.

(3) The supply rate of glucose and fatty acids from intravascular sources is not increased above an exercise intensity of 60 % $\dot{M}_{O_2max}$ and it is approximately the same in dogs and goats.

(4) The sarcolemma represents the major limiting factor to the supply of substrates from plasma, whereas the endothelium offers a low diffusion resistance.

(5) Whereas regulation of O_2 supply at higher workloads is achieved by up-regulating vascular perfusion, the exercise-induced increase in substrate supply depends on intensifying the utilization of intracellular substrate deposits.

(6) The rates of fatty acid and glucose recruitment from intracellular stores are greater in the dog than in the goat.

(7) The intracellular stores of lipid and glycogen are more than twice as large in the dog, so that oxidative metabolism can be sustained from these sources for nearly twice as long in dogs as in goats.

(8) Lipid droplets are in direct and intimate contact with the mitochondrial outer membrane, and the contact surface area is

greater in dogs, supporting their higher fatty acid flux into the mitochondria.

(9) The structure of the supply pathway from the blood to the mitochondria is designed primarily for the steady supply of O_2 up to maximal rates of oxidation. It is inadequate to supply glucose and fatty acids from vascular pools at the high rates required during strenuous exercise.

(10) Capillary substrate supply through this pathway occurs at low rates, predominantly during periods of rest or low activity, thus building up intracellular stocks of carbohydrate and lipids that can be exploited during exercise.

Conclusions on symmorphosis

The concept of symmorphosis was developed in conjunction with an investigation into the pathway for O_2 which comprises a linear sequence of steps, from the lung and circulation to the capillaries and mitochondria. This is a *simple pathway* for which a *chain* is an equivalent design model. In this type of structural system, symmorphosis, formulated as a hypothesis, predicts that the functional capacity of each step (or chain link) is co-adjusted to the maximal overall load on the system, which is the same for all links. When this hypothesis was tested for the pathway for O_2, by working out quantitative relationships between structural parameters and maximal rates of O_2 consumption, it was found to be supported for all steps except at the lung (Weibel *et al.* 1992).

The pathways for substrate supply are not as simple: we have seen that sequential steps from the blood to the mitochondria branch into parallel or alternative pathways. These are *complex pathways* for which a *network* design with multiple inlets is a more appropriate structural equivalent. It has two essential properties: (1) structural links are *shared* by different functions, and (2) the functional substrate fluxes can be *partitioned* to take different routes using different structural entities at different times. Shared steps and partitioned functions introduce a considerable degree of complexity into our model for structure–function relationships, and the concept of symmorphosis does not appear to be testable in a simple linear fashion.

The feature of *shared structures* introduces particular constraints on the functions served. For example, the sarcolemma serves a number of functions, such as excitation–contraction coupling through ion channels, glucose uptake, fatty acid uptake and O_2 diffusion; the capillaries serve to supply O_2 from erythrocytes and substrates from plasma, but also to remove CO_2, lactate and waste products as well as to dissipate heat. In order to assess symmorphosis, we will have to determine whether there is a dominant function among those that share a step and how this will affect the quantitative design properties. We might expect shared structures to be quantitatively matched only to their primary function. For example, the ratio of sarcolemmal surface area to muscle cell volume cannot be varied at will because it relates to the contractile properties of the particular muscle fibres. This may well be a dominant determinant of muscle fibre size and thus of sarcolemmal surface area.

With respect to the *partitioning* of functional fluxes between branches of a pathway, an important consideration will be how the fluxes through one particular path (or branch) are constrained. The functional requirements of one pathway may impose limitations on independent pathways with which it shares structures. For example, if sarcolemmal surface area is determined by certain contractile properties of the muscle and the density of GLUT transporters in the membrane is limited, then glucose uptake will be limited independently of substrate need. Another consideration will be whether the different pathways or branches offer particular advantages or disadvantages. For instance, intracellular substrate stores are close to mitochondria and can thus be accessed directly, but they are quite rapidly depleted if they are not replenished.

This study does not provide quantitative answers to all the questions raised above, and this may limit our ability to draw conclusions on whether the combined pathways for O_2 and substrate supply to muscle cells are designed according to the principles of symmorphosis. We can nevertheless attempt to answer this question by examining structure–function correlations for the different steps of each pathway. The hypothesis of symmorphosis predicts that no more structure is built at each step in a pathway than is required to meet functional demand. To what extent is this prediction fulfilled when comparing dogs and goats with their different overall functional demands?

Considering first the capillaries, we find that the volume of erythrocytes is proportional to the maximal O_2 supply, as shown previously (Weibel *et al.* 1991, 1992), whereas the plasma volume is proportional to the substrate supply from vascular sources (Fig. 4). The capillary endothelial surface area, however, is not related to the substrate fluxes: it appears to be over large in the dog. Here the hypothesis of symmorphosis appears not to be supported for the substrate pathway, but it does hold for O_2 supply when haematocrit is taken into account (Weibel *et al.* 1992).

The sarcolemmal surface area is the same in both species and is therefore not proportional to the differences in maximal O_2 flux rate, but the sarcolemmal membrane is not a significant resistance to O_2 diffusion. The sarcolemmal surface area is, however, strictly proportional to the maximal flux rates from circulatory sources for both substrates (Figs 4, 5). The observations that both glucose and fatty acid supply from the capillaries becomes limited at low exercise intensities and at equal levels in both species (Fig. 3), and that all additional energy must be drawn from intracellular stores, strongly suggest that this limitation may occur when all of the conductance capacity of the sarcolemma is utilized. From this, we could conclude that the hypothesis of symmorphosis is supported at this step because structure and function are tightly related. However, the match of structure and function at this step may not reflect the design of the sarcolemma to match substrate flow, but rather that substrate flow is limited by a sarcolemma designed primarily for contraction control. For this reason, the sarcolemma may limit substrate supply at rates not related to

the total substrate needs of the cell. This is possible because the cell has alternative ways of obtaining substrates for oxidation at high work rates, namely from intracellular stores.

We find that the rate of substrate supply from intracellular stores at higher work rates is higher in the dog than in the goat (Fig. 3). The hypothesis of symmorphosis predicts that the size of the glycogen and lipid stores should be higher in the dog than in the goat, and this is the case (Figs 4, 5). The hypothesis is therefore supported, at least as a trend. However, we find that the sizes of both the glycogen and the lipid stores are over large in the dog, which has a larger intracellular substrate reserve than the goat. This is mainly the case for glycogen. Does this refute the hypothesis of symmorphosis? Not necessarily, for two reasons. First, because there is no continuous replenishment of these stores during exercise (we assume that all substrates drawn from intravascular sources are directed to the mitochondria), drawing on these stores causes their gradual depletion. The flux *rate* from intracellular stores is therefore not the correct functional parameter to be considered: it should rather be total substrate flux, which is flux rate multiplied by endurance time. Thus, the larger stores in the dog suggest that it should have greater endurance, i.e. be able to run a greater distance. Although the present experiments did not measure this parameter, it is well known that the dog has a high endurance capacity. Second, we must note that glycogen stores serve not only oxidative metabolism but also the glycolytic generation of ATP during anaerobic burst activity, and this demands higher glycogen reserves.

As a general conclusion, we find that the pathways for O_2 and for the two substrates are designed to comply to different constraints, and that this is compatible with the principle of symmorphosis applied to network structures. Although individual pathways may have steps in which structure and function are not quantitatively matched, when both the substrate and O_2 pathways are considered, we find that no excess structure is maintained. Thus, capillaries are designed to supply O_2 at the highest required rates, while intracellular substrate stores are designed to supply substrates at these rates. The substrate supply pathways thus show a temporal split in the sense that the transfer of both glucose and fatty acids from the circulation to the muscle cells occurs predominantly during periods of rest (and is therefore not rate-limited), whereas the recruitment of fuels for oxidation at high rates during exercise occurs from the intracellular substrates stocked up during that time.

The design of these pathways accounts for the very different nature of O_2 and the two substrates. The whole organism and the muscle cells cannot store any appreciable amount of O_2 so that it must be steadily and nearly instantaneously supplied from ambient air through the respiratory system. This is possible because haemoglobin transfers O_2 at high concentration from the lung to the muscle capillaries, and O_2 diffuses freely through membranes and aqueous spaces. In contrast, loading cells with substrates is a complex and much slower process as it involves transporter molecules. But substrates can be stored in condensed form within the cytoplasm, so that equipping the cell with enzyme systems for their efficient recruitment offers the possibility of the rapid and direct supply of substrates to the mitochondria without the need to involve the complex pathways from the gut or from storage organs through the various transfer barriers.

More generally, we note that food is only intermittently available to animals and cannot be imported easily when energy is required, whereas O_2 is always available and can be absorbed from the atmosphere even during intensive work, with the additional benefits of discharging the large amounts of CO_2 and heat generated by the working muscle cells. The strategies to set up properly designed pathways for O_2 and substrates must therefore be different, and this is reflected in the solutions found by the body and observed in this study.

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