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Étude du métabolisme musculaire équin et canin en conditions physiologiques et pathologiques : focus sur la fonction mitochondriale et les profils acylcarnitine

Study of muscle metabolism in horses and dogs in physiologic and pathologic conditions: focusing on mitochondrial function and acylcarnitines profile

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THESE PRESENTEE EN VUE DE L'OBTENTION DU GRADE DE
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Se qualcosa ti si presenta difficile da realizzare, non pensare che sia impossibile per l'uomo; piuttosto, se qualcosa è possibile e appropriato all'uomo, consideralo raggiungibile anche per te.

Marco Aurelio, « Pensieri ».

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Abbreviations

ACC	ACC
ACh	Acetylcholine
ACS	Acyl-CoA Synthase
ADP	Adenosine Diphosphate
AGL	Gene encoding the Glycogen Debranching Enzyme
AM	Atypical Myopathy
AMP	Adenosine Monophosphate
AP	Action Potential
AST	Aspartate Aminotransferase
ATL	Adipose Triglyceride Lipase
ATP	Adenosine triphosphate
cAMP	Cyclic Adenosine Monophosphate
C0	Free Carnitine
CI	Complex I (NADH dehydrogenase)
CII	Complex II (succinate dehydrogenase)
CIII	Complex III (ubiquinone:cytochrome *c*-oxidoreductase)
CIV	Complex IV (cytochrome *c* oxidase)
CV	Complex V (ATP synthase)
C2	Acetylcarnitine
CACT	Carnitine-Acylcarnitine Translocase
CAT	Carnitine Acyltransferase
CD36	Cluster of Differentiation 36
CHO	Carbohydrates
CK	Creatine Kinase
CO_2	Carbon Dioxide
CoA/CoASH	Coenzyme A
CPT	Carnitine Palmitoyltransferase
Cr	Creatine
EPSM	Equine Polysaccharide Storage Myopathy
ER	Endoplasmic Reticulum
ET-pathway	Electron Transfer-pathway
ETS	Electron Transfer System
FA/FFA/NEFA	Fatty Acids/Free Fatty Acids/Non-Esterified Fatty Acids

FABP	Fatty Acid Binding Protein
$FADH_2$	Reduced Form of Flavin Adenine Dinucleotide
FAO	Fatty Acid Oxidation
FAODs	Fatty Acid Oxidation Disorders
FAT/CD36	Fatty Acid Translocase/Cluster of Differentiation 36
FATP	Fatty Acid Transport Protein
FCCP	Carbonyl Cyanide-4-(trifluoromethoxy)-Phenylhydrazone
FCR	Flux Control Ratio
G-1-P	Glucose-1-Phosphate
G-6-P	Glucose-6-Phosphate
G-6-Pase	Glucose-6-Phosphatase
GAA	Acid α-Glucosidase
GBE	Glycogen Branching Enzyme
GBE1	Glycogen Branching Enzyme gene 1
GBED	Glycogen Branching Enzyme Deficiency
GDE	Glycogen Debranching Enzyme
GLUT-1	Glucose Transporter type 1
GLUT-4	Glucose Transporter type 4
gm	Gluteus Medius
GP	Glycogen Phospohorylase
GS	Glycogen Synthase
GSD	Glycogen Storage Disorder
GYS1	Glycogen Synthase 1 gene
HRR	High-Resolution Respirometry
HSL	Hormone-Sensitive Lipase
IMP	Inosine Monophosphate
IMTG	Intra-Muscular Triglycerides
IVGTT	Intra-Venous Glucose Tolerance Test
LCACs	Long-Chain Acylcarnitines
LCAS	Long-Chain Acyl-CoA esters
LCFA	Long-Chain Fatty Acids
LDH	Lactate Dehydrogenase
MADD	Multiple Acyl-CoA Dehydrogenase Deficiency
MCACs	Medium-Chain Acylcarnitines
MCFA	Medium Chain Fatty Acids

MG	Muscle Glycogen
MFO	Maximal Fat Oxidation
MHC	Myosin Heavy Chain
mRNA	Messenger Ribonucleic Acid
NAD	Oxidized form of Nicotinamide Adenine Dinucleotide
NADH+H$^+$	Reduced form of Nicotinamide Adenine Dinucleotide
NEFA	Non-Esterified Fatty Acid
NSC	Non-structural Carbohydrates
OXPHOS	Oxidative Phosphorylation
P/O Ratio	Phosphate/Oxygen Ratio
PAS	Periodic Acid-Schiff
PCR	Polymerase Chain Reaction
PCr	Phosphocreatine
PDH	Pyruvate Dehydrogenase Complex
PFK	Phosphofructokinase
PHOS	Glycogen Phosphorylase
Pi	Inorganic Phosphate
PSSM	Polysaccharide Storage Myopathy
RER	Respiratory Exchange Ratio
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCACs	Short-Chain Acylcarnitines
SCFA	Short Chain Fatty Acids
SUIT	Substrate-Uncoupler-Inhibitor-Titration
TCA	Tricarboxylic Acid Cycle
tb	Triceps brachii
TG	Triglycerides
UDP-glucose	Uridine Diphosphate Glucose
VO_{2max}	Maximal Oxygen Consumption

Résumé - Abstract

Résumé

Les processus physiologiques qui régulent la gestion des substrats énergétiques chez les athlètes de différentes espèces (homme, cheval et chien) représentent le fil conducteur de ce manuscrit. La typologie des fibres musculaires, leur distribution, le contenu de substrat énergétique dans le muscle et le choix de la source énergétique en fonction de l'exercice et de l'entrainement et de l'exercice varient d'une espèce à l'autre. Par exemple, les chevaux, comparés au chien et à l'homme, semblent être plus dépendants du glycogène musculaire pendant l'effort, probablement dû à une composition différente des fibres musculaires. D'un autre côté, les chiens de traineau d'Alaska développent une stratégie métabolique particulière lors d'un exercice prolongé, qui ne le rend pas moins dépendant des hydrates de carbone et du glucose que les deux autres espèces.

Le glycogène représente la forme principale de stockage des hydrates de carbone dans le muscle squelettique et dans le foie mais il est également présent, en quantité moins significative, dans d'autres tissus. Les troubles du stockage et du métabolisme du glycogène, appelés aussi glycogénoses, sont décrits chez l'homme, le cheval et le chien, avec des présentations cliniques et causes subjacentes différentes. La Myopathie à Stockage de Polysaccharides (PSSM) représente un modèle animal unique de dysfonction du métabolisme du glycogène musculaire, étant caractérisée par une accumulation de glycogène anormal dans les fibres musculaires, un métabolisme oxydatif déficitaire, une augmentation de la sensibilité à l'insuline, et une assimilation du glucose pendant l'effort. Cependant, le lien entre la mutation génétique qui cause cette maladie et les symptômes cliniques est encore obscur.

Des nouvelles technologies, tests fonctionnels et biomarqueurs, sont constamment recherchés dans le domaine de la physiologie de l'effort pour faciliter la compréhension et la discrimination des mécanismes physiologiques et pathologiques, dans le but ultime d'améliorer le bien-être et la performance.

Le but de notre recherche était d'utiliser deux outils différents, d'une part, la respirométrie à haute résolution (HRR) pour évaluer la fonction mitochondriale, et d'autre part, le dosage des acylcarnitines dans le sérum ou plasma chez les chevaux souffrant de PSSM comparés aux chevaux sains, et chez les chiens de traineaux pendant un exercice sous-maximal de plusieurs jours. Nous présumions que ce type d'approche puisse nous fournir des informations utiles concernant la gestion des substrats énergétiques chez les deux espèces et dans les deux types de conditions.

Dans notre première étude, nous avons détecté une déficience bioénergétique significative au niveau des mitochondries des chevaux atteints de PSSM de type-1, suggérant que la dysfonction mitochondriale pourrait participer à l'apparition des symptômes cliniques de la maladie et aider à la compréhension de sa pathophysiologie.

Dans notre deuxième étude, nous avons observé que l'effort sous-maximal aérobie affecte la cinétique des acylcarnitines d'une manière qui semble spécifique selon la longueur de leur chaine et selon le groupe de chevaux considérés, soit les chevaux PSSM ou les contrôles. Nous suggérons que les profils acylcarnitines interprétés à la lumière du statut métabolique du sujet seraient un indicateur des différentes capacités oxydatives. De plus, les profils des acylcarnitines au repos et leur cinétique à l'effort chez les chevaux PSSM pourraient être des biomarqueurs de stress et surcharge mitochondriale.

Notre troisième étude portait sur le dosage des profils des acylcarnitines lors d'un exercice sous-maximal de plusieurs jours chez les chiens de traineau d'Alaska, donc dans un contexte physiologique. Un exercice d'endurance a influencé les profiles des acylcarnitines d'une manière spécifique selon la longueur du profil considéré. Cette étude représente à notre avis une partie du puzzle de la recherche scientifique sur le métabolisme à l'exercice de ces athlètes très résistants à la fatigue. Grâce à notre étude, nous avons mis en évidence que le métabolisme à l'effort de ces animaux est particulièrement flexible d'un point de vue métabolique, qu'il dépend du glucose plus que des lipides, et que le foie y joue un rôle central.

Abstract

The physiological processes regulating energy substrate management in different athletic species (humans, horses, and dogs) represent the principal topic of this manuscript. Muscle fiber types and distribution, energy substrate content in muscle and fuel selection shift in function of exercise and training, and vary from one species to the other. For instance, horses, compared to dogs and humans, seem to be more dependent on muscle glycogen during exercise, probably due to a different muscle composition. On the other hand, Alaskan sled dogs develop a particular metabolic strategy during prolonged exercise, rendering them at least as dependent on carbohydrates and glucose as the other two species.

Glycogen represents the major carbohydrate storage form in skeletal muscle and liver although being also present, in less significant amounts, in other tissues. Disturbances of glycogen storage and metabolism, also called glycogenoses, are described in humans, horses and dogs, with different clinical presentations and underlying causes. Polysaccharide Storage Myopathy (PSSM) represents a unique animal model of impaired muscle glycogen metabolism, characterized by abnormal glycogen accumulation in myofibers, impaired oxidative metabolism, enhanced insulin sensitivity and glucose uptake during exercise. Nonetheless, the link between the genetic mutation causing this disease and clinical signs of rhabdomyolysis is still blurry.

New technologies, functional tests and biomarkers are constantly sought in the field of exercise physiology to help the comprehension and discrimination of physiologic and pathologic processes, with the ultimate goal to improve health and predict performance.

The aim of our research was to employ two different tools, represented by high-resolution respirometry (HRR), to assess mitochondrial function, and by the dosage of serum/plasma acylcarnitines in horses affected by PSSM compared to healthy controls, and in Alaskan sled dogs during submaximal multiday exercise. We assumed that these approaches would provide valuable information on energy substrate management in both types of conditions and species.

In our first study, we detected significant bioenergetical impairment in mitochondria of type-1 PSSM-affected horses, suggesting that mitochondrial dysfunction can participate to the genesis of clinical symptoms of this disease and help to better understand its pathophysiology.

In our second study, we observed that submaximal aerobic exercise affects acylcarnitine kinetics in a manner that seems chain-specific depending on the group of horses that is considered, either PSSM-affected horses or controls. We suggest that acylcarnitine profiles interpreted in the light of the metabolic status of the subject could be an indicator of different oxidizing capacities.

Moreover, acylcarnitine resting values and kinetics with exercise in PSSM horses could be biomarkers of mitochondrial stress and overload.

Our third study was focused on the assessment of plasma acylcarnitine profiles during submaximal multiday exercise in Alaskan sled dogs, thus in a physiologic condition. Endurance exercise impacted acylcarnitine profiles in sled dogs in a chain-length dependent manner. Our research represents in our opinion a piece fitting in a larger puzzle of scientific investigation on exercise metabolism of these highly fatigue-resistant athletes. With this study, we further highlight the extremely metabolically flexible, glucose- more than fat-dependent and likely liver-centric exercise metabolism of these animals.

Introduction

1. SKELETAL MUSCLE PHYSIOLOGY: EXCITATION-CONTRACTION COUPLING AND ENERGY PROVISION

Skeletal muscle contraction is the results of a physiological phenomenon called excitation-contraction coupling, originating at the neuromuscular junction, i.e. the synapse between a motor neuron and a muscle fiber. Propagation of action potentials (APs) along the motor neuron and the resulting depolarization induces the opening of voltage-gated calcium (Ca2+) channels of the presynaptic membrane. Inward Ca2+ flow causes the release of acetylcholine (ACh) at the neuromuscular junction, which diffuses to the postsynaptic membrane, also called the motor endplate. When ACh binds to the receptors located at the motor endplate, its depolarization initiates AP propagation into the membrane of the muscle fiber. Excitation-contraction coupling refers to the mechanism that converts AP diffusion in the muscle fibers membrane into muscle fiber contraction. The AP disseminates into the T-tubules, which are responsible for conducting the AP from the surface to the interior of the muscle fiber. T-tubules contain dihydropyridine receptors that are adjacent to the terminal cisternae of the sarcoplasmic reticulum. When T-tubules become depolarized, their dihydropyridine receptors undergo a conformational change that mechanically interacts with the ryanodine receptors on the sarcoplasmic reticulum. This interaction opens the ryanodine receptors causing Ca2+ release from the sarcoplasmic reticulum into the sarcoplasm. The intracellular Ca2+ consequently increases, and Ca2+ ions bind to troponin C complex on the thin filaments. The interaction between Ca2+ and troponin C exhibits cooperativity, which means that each Ca2+ that binds troponin C increases the affinity of troponin C binding for the next Ca2+ molecule, up to a total of four Ca2+ ions per troponin C. As a result of Ca2+ binding, the troponin complex undergoes a conformational change causing displacement of tropomyosin from the myosin-binding sites. The cross-bridge cycle is the mechanism by which the thick and thin filaments slide over each other to generate a muscle shortening. Adenosine triphosphate (ATP) binds to myosin head, inducing a conformational change in myosin that decreases its affinity for actin. Consequently, myosin dissociates from actin. Then, ATP bound to myosin is hydrolyzed to adenosine diphosphate (ADP) and one inorganic phosphate molecule, both remaining linked to myosin. As long as Ca^{2+} concentration in the sarcoplasm remains higher than 10-6M, myosin continues to bind to a new site on actin, creating a power stroke that pulls the actin filaments. Each cross-bridge cycling event results in the myosin head progressing up the actin filament under the condition that Ca2+ remains bound to troponin C. Finally, ADP is released, and myosin returns to its original state of rigor where it is bound to actin in the absence of ATP. After contraction, muscle relaxation occurs when Ca2+ reaccumulates in the sarcoplasmic reticulum via the active Ca2+ ATPase pump located into the sarcoplasmic reticulum membrane which maintains low intracellular Ca2+ and induces relaxation of the muscle. In conclusions, muscles cannot contract without a biochemical source of energy, and that energy is provided by the cleavage of high-energy phosphate bonds within ATP. Exercise consequently depends on the constant availability of ATP (necessary for the relaxation processes), but its intracellular stores

are limited rapidly depleted. In order to avoid ATP depletion, two different energy pathways, the aerobic and anaerobic pathways must be both activated. The aerobic metabolism is relatively slow but highly efficient, as it yields theoretically 38 but effectively 30 molecules of ATP approximately per molecule of glucose (or 31 molecules if glucose derives from glycogenolysis). Anaerobic metabolism is faster in releasing energy but it is relatively inefficient as it yields two molecules of ATP per molecule of glucose. The aerobic pathway predominates during prolonged submaximal exercise and the anaerobic pathway dominates during high-intensity exercise (Westerblad et al., 2010). Both pathways are active during exercise but their relative contribution is primarily determined by exercise intensity and duration, and further modulated by many factors, including the type of substrate, oxygen availability, muscle fiber-type composition, gender, diet, training status, etc. (Hargreaves and Spriet, 2018).

1.1 Anaerobic energy metabolism

The anaerobic energy metabolism includes the lactic and the alactic pathways. The alactic pathway involves degradation of phosphocreatine (PCr) via creatine kinase (CK) which catalyzes the following reaction: PCr + ADP ↔ Cr + ATP (ADP stands for adenosine diphosphate). A smaller contribution to the alactic pathway is given by the myokinase, or adenylate kinase, or adenosine monophosphate (AMP) kinase reaction, which catalyzes the reaction ADP + ADP ↔ ATP + AMP. An increase in AMP results in the activation of AMP deaminase, thus increasing muscle inosine monophosphate (IMP) concentration (AMP + H_2O ↔ IMP + NH_3). These reactions occur at top-speed exercise but they produce a small amount of ATP for few seconds. AMP kinase can also be switched on by cellular stress (hypoxia, ischemia, glucose deprivation) that interferes with ATP production or that increases its consumption (Hardie, 2003).

The lactic pathway involves anaerobic glycolysis, independently of oxidative pathways (*i.e*. in absence of oxygen). Anaerobic glycolysis uses glucose-6-phosphate (G-6-P) as substrate that can be issued from glucose phosphorylation by hexokinase or from glycogen stores via glycogenolysis. The end- product of this pathway is pyruvate (2 molecules) that is converted to lactate by lactate dehydrogenase (LDH).

1.2 Aerobic energy metabolism

Aerobic energy metabolism requires oxygen and takes place in mitochondria, where free fatty acids (FFA, or FA, or NEFA) ß-oxidation, tricarboxylic acid cycle (TCA, Krebs Cycle or Citric Acid Cycle) and the oxidative phosphorylation system (OXPHOS) work in combination to produce ATP (Fig. 1). This pathway is the most efficient method for energy production, but it requires a continuous and adequate oxygen supply. The major substrate-contributors during prolonged submaximal exercise are muscle glycogen and FFA, while extracellular glucose contribution increases with exercise duration

(Katz et al., 1991). Amino acids represent an additional substrate but their contribution is limited even in absence of carbohydrate availability (5-10%) (Lemon and Mullin, 1980).

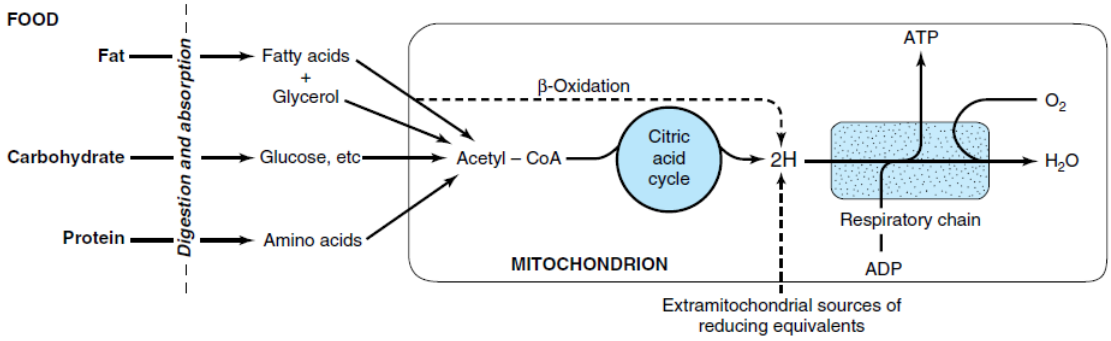

Figure 1. Schematic representation of major nutrients transforming food energy in ATP through conversion to acetyl-CoA. Acetyl-CoA enters the tricarboxylic acid cycle (TCA) or TCA cycle that leads to the generation of reducing equivalents, which are oxidized by the mitochondrial electron transfer system to produce ATP (Mayes and Botham, 2003).

1.3 Mitochondria, TCA cycle and oxidative phosphorylation

Glucose (product of glycolysis or glycogenolysis), FFA (originating from lipolysis) and most amino acids (originating from protein catabolism) converge to TCA cycle, which has a pivotal role in aerobic metabolism (Kay and Weitzman, 1987). To be accepted by the TCA cycle, carbon skeletons of energy substrates are usually converted to acetyl-CoA into mitochondria. Pyruvate derived from glycolysis undergoes oxidative decarboxylation in the mitochondria to yield acetyl-CoA by pyruvate dehydrogenase complex (PDH). This reaction links glycolysis and the TCA cycle in every cell containing mitochondria (Harris et al., 2002). Acetyl-CoA is also derived from ß-oxidation of FFA, issued from triglycerides (TG) stored in muscle or in adipose tissue. Complete oxidation of FFA yields up to 146 molecules of ATP, while complete glucose breakdown produces approximately 30 molecules of ATP. During the TCA cycle, acetyl-CoA is oxidized to CO_2. The energy released by this oxidation is conserved by the coenzymes nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) that are reduced to NADH and $FADH_2$, thus becoming electrons carriers (Phillips et al., 1981). Subsequently, these cofactors are themselves oxidized giving protons (H^+) and electrons. Electrons are then transported through some protein complexes located in the inner mitochondrial membrane to oxygen that is reduced to H_2O. These complexes constitute the electron transport system (ETS): complex I (NADH dehydrogenase; CI), complex II (succinate dehydrogenase; CII), CoQ10 (ubiquinone), complex III (ubiquinone:cytochrome *c*-oxidoreductase; CIII), cytochrome *c* and complex IV (cytochrome c oxidase; CIV). This electron transfer system is coupled to a proton pumping gradient across the inner mitochondrial membrane, thus creating an electrochemical gradient (proton gradient)

that is finally used by the turbine-like ATP synthase (complex V; CV) to generate ATP molecules from ADP. The process in which ATP is synthetized by the mean of the electron transport system is called OXPHOS. Conversion of energy substrates in acetyl-CoA, acetyl-CoA oxidation through the TCA cycle and electron transfer through the ETS with OXPHOS constitute the different stages of "cellular respiration" (Nelson and Cox, 2013).

1.4 Muscle fiber type heterogeneity

Skeletal muscle is able to cope with different physical activities, including standing, prolonged low-intensity exercise and short, explosive movements. Such versatility is primarily due to muscle heterogeneity in terms of fiber composition. Mammalian skeletal muscles fibers are broadly divided into slow-twitch (type I) and fast-twitch (type II) fibers. Type II fibers are further classified into IIa, IIx and IIb (the latter are not expressed in most large mammals) fibers depending on the myosin heavy chain (MHC) gene isoform that they express (Talbot and Maves, 2016). Furthermore, skeletal muscle versatility is also due to the ability to adjust its phenotypic properties depending on the functional demand (Pette and Staron, 2000). This transition between isoforms is regulated by neuromuscular, hormonal or mechanical stimuli as well as by aging. Body size has also an important role in determining the functional demand on skeletal muscle. In fact, muscles from small mammals have predominantly IIb and IIx fibers while large mammals like humans have muscles mainly composed of type I and IIa fibers (Schiaffino and Reggiani, 2011); humans and other large mammals as horses do not express or minimally express the MHC-IIb (Rivero et al., 1999; Chikuni et al., 2004; Pette and Staron, 2000). However, MHC-IIb is widely expressed in guinea pig, llama rabbit and pig and in different marsupial species independently of body size, but not in cat and dog muscles, thus this relation between MHC-IIb and body size is not absolute (Schiaffino and Reggiani, 2011). Muscle fibers are classified as "pure" or "hybrid" depending on the expression of one or more than one MHC isoform. In terms of contraction speed, *i.e.* cross-bridge cycling rates, the MHC-I isoform is the slowest, while MHC-IIa and MHC-IIxb are the intermediate and the fastest isoforms, respectively. Thus, type II fibers create fast cross-bridge cycling so they develop force rapidly and when activated they hydrolyze ATP faster than type I fibers do. Metabolically, slow type I fibers have high number of capillaries, high oxidative but low glycolytic capacity and low glycogen content, while fast type II fibers are efficient power-generators but they have lower oxidative capacity and limited oxygen availability (due to low capillary supply) than type I fibers. Type IIa fibers rely on glycolytic and oxidative metabolism, they have a considerable number of mitochondria and capillaries, rendering them able to sustain high power outputs for longer periods than IIx fibers. Hybrid IIax fibers have intermediate properties (Quiroz-Rothe and Rivero, 2001). Nonetheless, variations exist and it is not possible to strictly predict fiber types depending solely on energy usage. Furthermore, the expression of other structural proteins, trans-sarcolemmal transporters and pumps differs among fibers, thus contributing to their specific identities. Therefore, it has been

suggested that the simple classification of muscle fibers based on myosin composition and levels of oxidative enzymes is convenient for communication, until more complete profiles of gene expression at the transcript and protein level will become available (Schaffino and Reggiani, 2011).

1.4.1 In horses

In horses, there is a large variation in fiber type composition and distribution between and within muscles, largely depending on functional demand (Snow and Guy, 1980). In general, the deeper regions of equine locomotory muscles have a predominance of oxidative type I and type IIa fibers, being more suited for posture and low-intensity, long-lasting muscular activity. On the other hand, superficial regions have a higher content in type IIx fibers, being more adapted for rapid and propulsive movements (Lopez-Rivero et al., 1992; Serrano et al., 1996). Similarly, forelimb muscles have a greater proportion of I and IIa fibers than IIx fibers compared to propulsive hind limbs muscles (Kawai et al., 2009). As stated before, the MHC-IIb isoform is minimally expressed (<1%) in horses (Chinuki et al., 2004). A combination of different myogenic and non-myogenic factors contribute to the determination of muscle fiber phenotype. These factors regulate not only the expression of characteristics specific to each muscle fiber but also the individual fiber type percentage within each muscle. Myogenic factors are represented by genetic lineage and inheritance of fiber type ratios (type I : type II) that are responsible for the wide variations in muscle fibers composition between breeds (Fig. 2). Non-myogenic factors include neuronal input, extracellular factors (*e.g*. drugs, hormones, growth factors, substrate availability) and neuromuscular activity that is determined by exercise and training. Effectively, endurance training induces a typical sequential "fast-to-slow" transition of muscle fibers, *i.e*. IIx → IIax-IIa → IIa-I → I, with a first transition from IIx to IIa fibers during the early phase of training and with a second transition IIa-I when training is sufficiently long. This adaptation is reversible as the return to a sedentary status induces a transition from IIa to IIx fibers (Serrano et al., 2000).

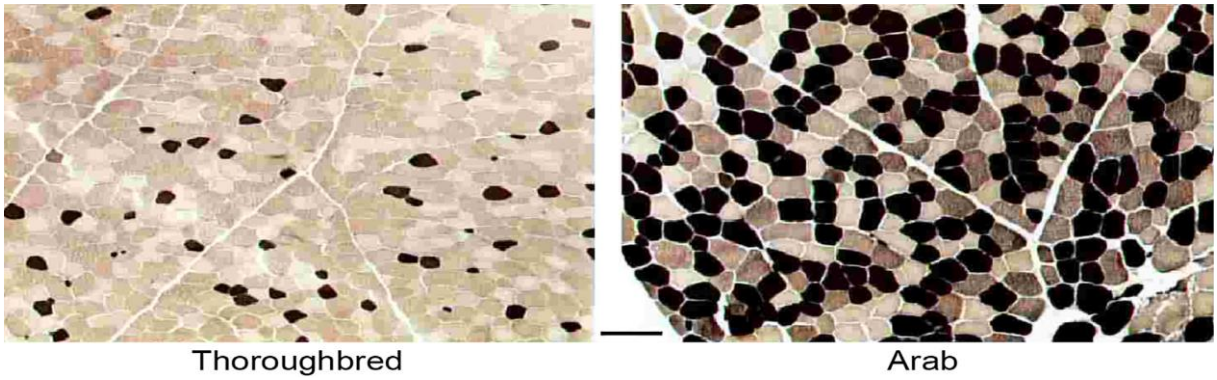

Figure 2. Muscle biopsies obtained from the *gluteus medius* muscle of two horses of different breeds, showing the distinct fiber composition depending on the functional demand of exercise. On the left, the Thoroughbred (racehorse) has a higher proportion of fast fibers (weakly stained). On the right, the Arab (endurance horse) has a higher proportion of slow fibers (darkly stained) (Rivero and Hill, 2016).

1.4.2 In dogs

Muscle physiology of dogs has been raising particular scientific interest in the last decades, not only because they are "man's best friends" but also for their increasing involvement in various sports activities. Early studies have highlighted the high oxidative metabolism of dogs that is responsible for their striking endurance capacity (Snow et al., 1982; Rivero et al., 1994). Concerning muscle fibers characterization, initial investigations, essentially based on ATPase staining, described the presence of type I and II fibers and underlined at the same time the difficulties in differentiating fast fibers (Braund et al., 1978; Snow et al., 1982; Cardinet et al., 1983). Thus, canine fast fibers were initially classified as IIa and as "IIdog" fibers, these latter being peculiar to the dog and not corresponding to classical IIb fibers observed in other species (Snow et al., 1982; Latorre et al., 1993). Highly glycolytic-low oxidative fibers were considered absent (Snow et al., 1982; Latorre et al., 1993). Further studies (Strbenc et al., 2004; Smerdu et al., 2005) suggested that the IIdog fibers actually corresponded to hybrid IIa/IIx fibers and that functional type IIb fibers were absent in canine trunk and limb as observed in other mammals such as humans and horses. Another interesting feature reported in dogs, as in other carnivores and primates (except humans), was the presence of the rare 2m (or MHC-m, or mastricatory isoform) fibers as the predominant fiber type in the jaw-closer muscles (*masseter*, *temporalis* and *pterygoideus medialis*). Thanks to the presence of this myosin isoform, fibers reach a high power level during contraction due to high force development and moderately fast speed shortening (Toniolo et al., 2008; Schaffino and Reggiani, 2011); thus this contractile behaviour would be associated with the aggressive bite of carnivores required for predation (Rowlerson et al., 1983). More recent studies (Acevedo and Rivero, 2006; Toniolo et al 2007) combining histochemistry, immunohistochemistry and even reverse transcription polymerase chain reaction (RT-PCR) confirmed the presence in dogs of IIb fibers but restricted to laryngeal and extra-ocular muscles. They revealed also the occasional presence of IIb messenger ribonucleic acid (mRNA) in absence of the corresponding protein in the semi-membranous muscle in a minority of dogs of large body size (Toniolo et al., 2007). Furthermore, they confirmed the presence of IIx fibers either in a pure form, as frequently found in semi-membranous and *tibialis cranialis* muscles, or more commonly in the hybrid form IIax. From all these literary sources, it can be retained that canine limb and trunk muscles express pure fibers MHC-I, IIa, IIx and hybrid I-IIa and IIax (corresponding to the previously called IIdog) fibers. Type IIa are the most abundant fibers in dog trunk and limb muscles (Toniolo et al., 2007). Moreover, canine muscles show peculiar variations in the phenotypic expression of their myofibers in comparison to other mammals. Dogs have an unusually high number of hybrid fibers, which enables muscle to fine-tune its efficiency to respond more effectively to functional demands (Stephenson, 2001). Dogs possess an unusually high oxidative capacity especially at the level of IIx fibers in comparison to that of equivalent fiber types in other species, thus rendering them more fatigue-resistant than other mammals. Furthermore, from the

morphological, metabolic and contractile point of view, IIx fibers are intermediate between I and IIa fibers, which is again unusual among mammalian species (Acevedo and Rivero, 2006).

1.5 Energy substrates

Carbohydrates (CHO) and lipids represent the main fuel sources for mitochondrial ATP production in the working skeletal muscle. The interaction and reciprocal shifts between these two fuel sources will depend on the extra- and intra-cellular environments that are in turn affected essentially by substrate availability and exercise intensity (Spriet, 2014). Generally speaking, at least in humans and horses, fat metabolism predominates at low to moderate intensity (submaximal) exercise, while CHO metabolism predominates at moderate to high intensity (maximal and supramaximal) exercise. Nonetheless, some species-specificities may exist, as in endurance dogs, as describer later in this chapter, substrate selection does not seem to follow the same pattern. Furthermore, it is important to underline that, both at rest and during exercise, the oxidation of any fuel does not occur in isolation, but many aspects of metabolism are simultaneously active at a given point in time (Spriet, 2014).

1.5.1 Lipids

Lipids are a group of compounds encompassing different chemical structures and biological functions: storage of energy, contribution to the structure of biological membranes and to signalling pathways. Fat represents the largest energy reserve in the body, as the adipose tissue of a lean adult man contains an average of 17500 mmol of TG (Horowitz and Klein, 2000). Intramuscular fat storage is smaller (~300 mmol) and for the majority in the form of lipid droplets in the myocytes; fat is also present in plasma as TG (~ 0.5 mmol) (Hoppeler et al., 1973; Horowitz and Klein, 2000) and FFA, issue of peripheral lipolysis. Intramuscular triglycerides (IMTG) are stored primarily in oxidative type I fibers in close proximity with mitochondria (Moro et al., 2008). There is a differential distribution of IMTG depending on the muscle fiber type. In general, type I-highly oxidative fibers have a higher content than IIa fibers, while the fast-twitch low-oxidative IIx fibers have a negligible IMTG content. The reverse happens for muscle glycogen. However, it has been reported that dogs seem to a higher quantity of IMTG stored in their hindlimb locomotory muscles than horses, due to a higher quantity of lipid droplets in oxidative fibers (Hinchcliff and Geor, 2008). Lipids are mobilized from adipose and/or muscle TG stores via lipolysis that is under regulation of adipose triglyceride lipase (ATL) and hormone-sensitive lipase (HSL). These lipases are sensitive to epinephrine (ATL>HSL) which concentration depends on exercise intensity and/or duration (Zouhal et al., 2008). The hydrolysis of TG releases FFA that are transported into circulation, and glycerol that is mainly used by liver in glycolytic or gluconeogenetic pathways (Zechner et al., 2009). The uptake of FFA by muscle and their transport throughout the plasmalemma are still matter of debate. Different models are proposed: a passive diffusion/“flip-flop” mechanism,

transporter-independent, has been suggested (Hamilton, 1998). However, it is now believed that 90% of FFA uptake is protein-mediated. Proteins involved comprise the cluster of differentiation plasma membrane FAT/CD36, alone or helped by plasma membrane-associated fatty acid binding proteins (FABP) and fatty acid transport protein (FATP) isoforms (Schwenk et al., 2010). Once into the cytosol, FFA are trapped by FABP or converted into fatty acyl-CoA by acyl-CoA synthase (ACS) to prevent FFA efflux and to maintain a concentration gradient between blood and cytosol (McClelland, 2004). Short chain fatty acids (SCFA, <6 carbon atoms) and medium chain fatty acids (MCFA, 6-12 carbon atoms) can enter the mitochondria by simple passive diffusion while long chain fatty acids (LCFA, >12 carbon atoms) need to be esterified into long chain acyl-carnitines by carnitine palmitoyltransferase 1 (CPT1) (Fig. 3). Carnitine palmitoly-transferase 1 is located within the outer mitochondrial membrane; it is expressed in heart, muscle and adipose tissue (Warfel et al., 2017). Then, FAT/CD36 located in the outer mitochondrial membrane transfers acylcarnitines from CPT1 to carnitine-acylcarnitine translocase (CACT), located in the inner mitochondrial membrane (Bezaire et al., 2006). Carnitine-acylcarnitine translocase transfers acylcarnitines in a 1:1 exchange rate with intramitochondrial carnitine (Stephens et al., 2007). Once into the mitochondrial matrix, carnitine palmytolitransferase 2 (CPT2), which is located on the matrix side of the inner mitochondrial membrane, catalyzes the transesterification of long chain acyl-carnitine back to free carnitine (C0) and fatty acyl-CoA that will enter the ß-oxidation pathway. Fatty acids oxidation provides more ATP per molecule than glucose (up to 147 molecules *versus* 30 molecules of ATP) but complete fatty acid oxidation (FAO) needs more oxygen than CHO oxidation does (Hawley, 2001). In terms of available amount, fat is not limiting to endurance exercise performance (Stephens, 2018). In human athletes, fat can virtually sustain exercise for approximately 120 hours of continuous and moderate-intensity exercise, that means from 25% to 65% of maximal oxygen uptake (VO_{2max}) (Hawley, 2001; Horowitz and Klein, 2000). While CHO oxidation is strictly controlled by the energy demand of the working muscle, the availability and utilisation of fatty acids are not tightly matched to the rate of energy expenditure (Hawley, 2001). As consequence, FAO capacity during exercise is limited and mainly determined by CHO utilization and FFA availability, but the exact mechanisms regulating the relative contribution of each energy substrate (CHO and fat) during exercise are still unclear and matter of debate. Again, regulation of fat metabolism is complex and resides in several potential key-regulatory sites controlling FFA availability and the rate of FAO during exercise: 1) regulation of lipolysis within muscle and adipose tissue; 2) transport and delivery of circulating FFA from adipose tissue to muscle; 3) their transport across the sarcolemma and then within cell sarcoplasm (mainly protein-dependent); 4) transport of FFA across mitochondrial membranes via the CPT system; 5) regulation within the ß-oxidation pathway; 6) regulation within the TCA cycle. The relative importance of each site depends on several external factors such as the training status of the individual, habitual nutrition, ingestion of substrates before and during exercise, gender, exercise duration and intensity (Hawley, 2001; Purdom et al., 2018). Thus, each of these steps represents also a potential site of plastic response to endurance training or environmental stress (McClelland, 2004).

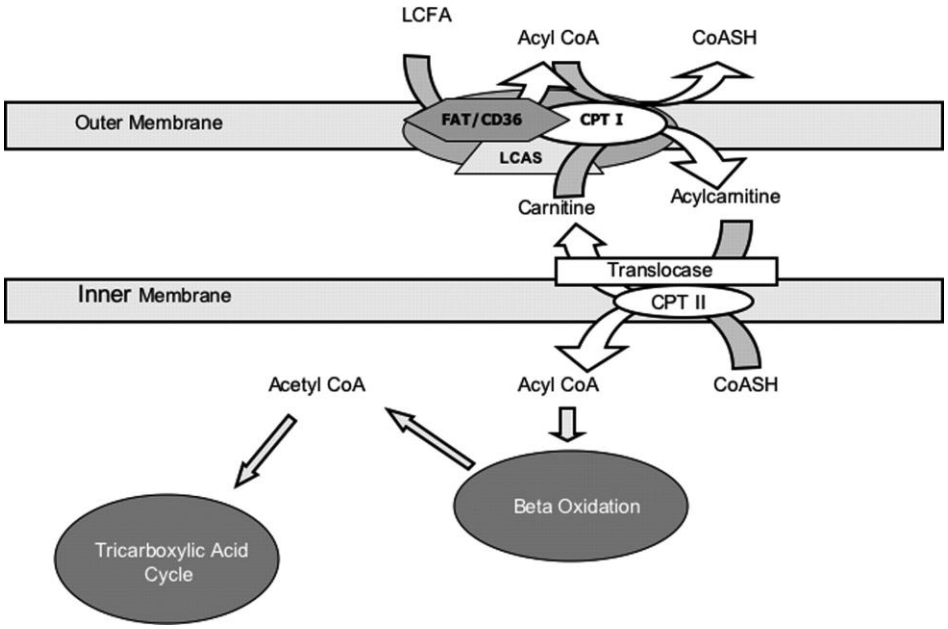

Figure 3. Representation of long chain fatty acids through the outer and inner mitochondrial membrane in skeletal muscle through FAT/CD36 and carnitine shuttle. LCFA, long-chain fatty acid; CPT I, carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II; CoASH, Coenzyme A; LCAS, long-chain acyl-CoA esters (Campbell et al., 2004).

1.5.2. Carbohydrates

Endogenous carbohydrates are mainly stored as glycogen in skeletal muscle and liver but they can also circulate as glucose in circulating blood. In liver, glycogen serves mainly to provide glucose to extra-hepatic tissues, where in muscle it represents a ready source of fuel. Liver glycogen is almost fivefold higher in concentration than muscle glycogen in untrained humans (Adeva-Andany et al., 2016). Nonetheless, glycogen is more than a simple store, as it plays a role in the regulation of signaling events occurring in response to exercise, in insulin sensitivity, in contractile and autophagic processes (Philp et al., 2012). Glycogen stores, in comparison to fat, are limited and their depletion coincides with fatigue during moderate to intense and prolonged physical activity, meaning at >65% of VO_{2max}. In the same manner, liver glycogen depletion is also a cause of fatigue during endurance exercise (Hearris et al., 2018). As stated above, muscle glycogen storage is fiber type-specific, being more abundant in fast-twitch than in slow fibers. Muscle glycogen can be depleted during exercise depending on the recruitment pattern of muscle fibers, thus depending on the type of exercise. During prolonged submaximal exercise type I fibers are preferentially depleted while during maximal or supramaximal exercise, depletion occurs mostly in type II fibers (Gollnick et al., 1974). Glycogen content is also quite different among species of our interest, as horses have a higher content of muscle glycogen (130-150 mmol/kg/wet weight or ww) than humans (80-140 mmol/kg ww) and dogs (70-80 mmol/kg/ww). Early in exercise and when exercise intensity increases from moderate to high (65%-85% VO2max),

carbohydrate metabolism predominates, meaning an increase in muscle and liver glycogenolysis and glucose uptake by muscle. Glycogen breakdown is catalyzed by glycogen phosphorylase (PHOS) (Fig. 4). When exercise intensity increases, ATP hydrolysis increases, creating an accumulation of inorganic phosphate (Pi), ADP and AMP: Pi is a substrate for PHOS and AMP as well as ADP are allosteric regulators of PHOS, activating the enzyme (Howlett et al., 1998). Exogenous glucose uptake (from blood) in muscle is also increased in case of increased exercise intensity to partially compensate for the progressive decrease of muscle glycogen. On the other hand, in case of plasma FFA or muscle glycogen concentrations are high, plasma glucose utilization decreases. Circulating glucose also represents an important energy fuel as it supplies 20-50% of total oxidative energy production (Coggan, 1991). Exogenous glucose (taken up into muscle) and endogenous glucose (issue of glycogenolysis) enter glycolysis which rate-limiting enzyme is phosphofructokinase (PFK) that is also activated by ADP, AMP and Pi. Glucose delivery to muscle is regulated by the glucose-transporters GLUT-1 and GLUT-4 (Adeva-Andany et al., 2016) and it is reflective of an increased liver glycogenolysis. This process is of fundamental importance in maintaining glucose homeostasis. As an example, during initial periods of fasting in humans, liver glycogen contributes to approximately 45% of total glucose production to assure euglycemia (Petersen et al., 1996). Moreover, hepatic glycogen synthesis, or gluconeogenesis (by precursors as glycerol, lactate and amino acids) and glycogenolysis occur simultaneously, thus allowing rapid changes in glucose flux (Mangusson et al., 1991) and restoration of depleted glycogen stores in presence of CHO intake (Nilsson et al., 1974). Liver is also able to autoregulate, as high liver glycogen concentration may stimulate liver glycogenolysis and inhibit its synthesis (Gonzalez et al., 2016). Interestingly, there is also an intricate connection between liver and adipose tissue in maintaining glucose homeostasis. Indeed, liver glycogen can regulate lipolysis during fasting via neural regulation, thus rendering FFA available for muscle and other organs and reducing glucose use (Izumida et al., 2013). In the same manner, FFA and glycerol can suppress hepatic glycogenolysis (Stingl et al., 2001). Of course, liver glycogen regulation is also under control of insulin, glucagon and catecholamines, thus being regulated by exercise and training (Dufour at al., 2009). Even though the role of liver during prolonged exercise is often overlooked because of the difficulties in obtaining tissue samples, it definitely needs further attention.

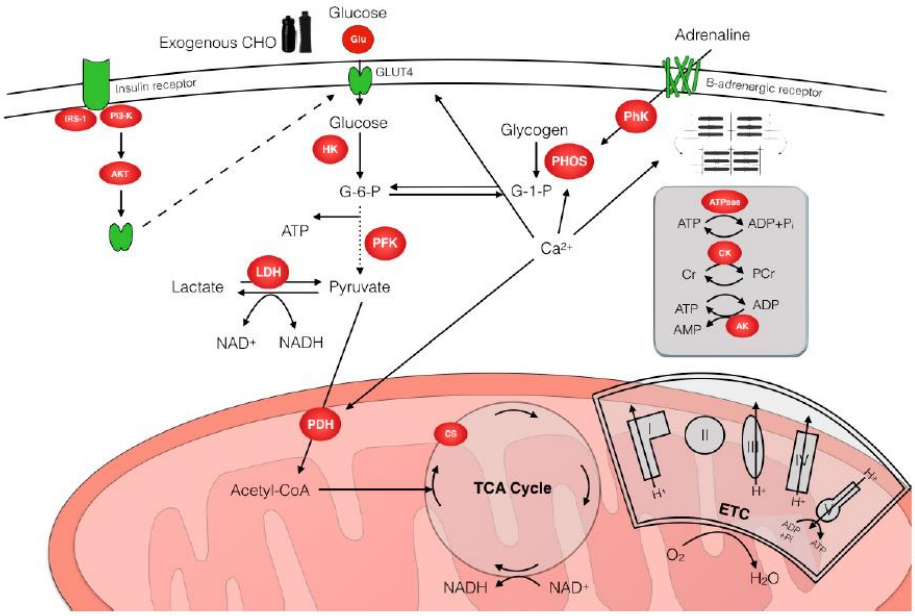

Figure 4. Representation of carbohydrates (CHO) metabolism and its regulation points. PHOS, glycogen phosphorylase; G-6-P, glucose-6-phosphate; PFK, phosphofructokinase; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase (Hearris et al., 2018).

1.5.3. Fuel regulation in function of exercise and training

At rest and in the non-contracting skeletal muscle, lipid is the major fuel source, as it supplies approximately 60% of energy needs. The amount of FFA released in blood by lipolysis of TG (stored in muscle or adipose tissue) exceeds the amount of fat that is being oxidized. Thus, FFA are re-esterified into TG principally by liver (Horowitz and Klein, 2000). At the onset of muscle contraction, there is an immediate need for ATP due to its hydrolysis by ATPases as the sodium/potassium, calcium, and myosin ATPases. During the first 8-10 seconds of exercise, PCr degradation generates about the half of ATP needed. Anaerobic glycolysis is also activated few (4-6) seconds after the onset of exercise and provides the majority of ATP from 8 to 60 seconds of exercise (Gaitanos et al., 1985). After 2 minutes, aerobic glycolysis/glycogenolysis and FAO become the major sources of ATP in presence of molecular oxygen, their relative proportion is at this point determined by exercise duration and intensity. At 25%VO_{2max}, FAO covers more than 90% of the total energy expenditure mainly sustained by plasma FFA and with little contribution of muscle glycogen and IMTG (Purdom et al., 2018). In the transition from mild- to moderate-intensity (submaximal) exercise, meaning at 25-65% VO_{2max}, both muscle glycogen and IMTG oxidation increase (Fig. 5) but FAO remains the predominant fuel source, reaching the point of maximal fat oxidation (MFO) at approximately 50% VO_{2max} (Brooks, 1998).

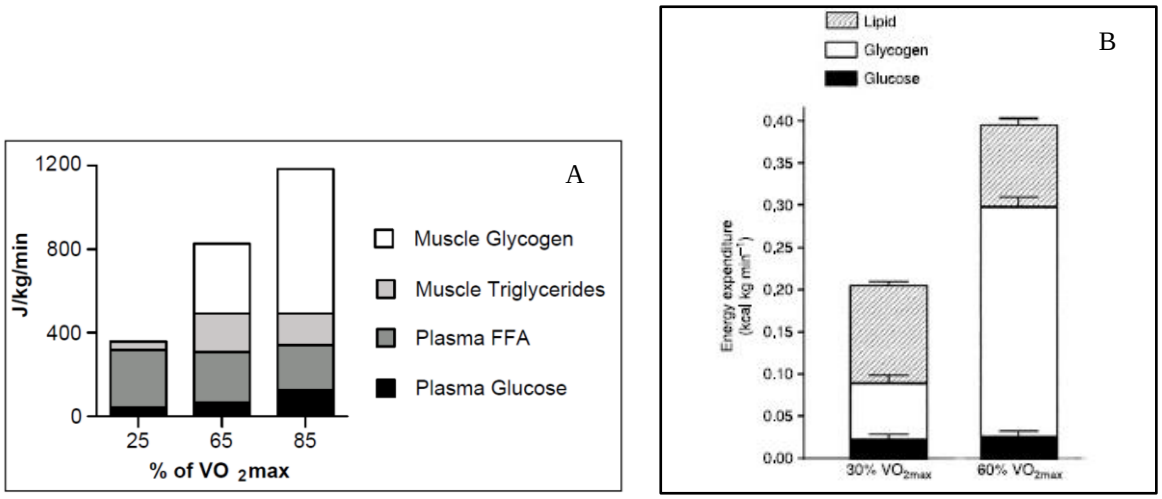

Figure 5. Contribution of different fuel sources to energy expenditure during exercise in humans (A), at 25%, 65% and 85% maximal oxygen uptake (VO_{2max}) and in horses (B), at 30 and 60% VO_{2max} (Hawley, 2001; Votion et al., 2007).

Nonetheless, MFO is highly species-, individual- and training-dependent. In humans, it has been reported to occur between 47 and 75 of VO_{2max} (Achten et al., 2002; Achten and Jeukendrup 2003; Valizadeh et al., 2011). Increasing exercise intensity beyond 65% VO_{2max}, or more specifically beyond MFO, will induce a shift in energy contribution favoring CHO oxidation while reliance on FAO will decrease. This is partly due to the fact that, in mammals, power output sustained by lipids as the sole fuel source is lower than with CHO, while in birds and insects, high power outputs can be sustained by lipids only (McClelland, 2004). In other words, the ratio between ATP produced and oxygen consumed (P/O ratio) is greater when CHO is the substrate (Spriet, 2014). Exercise duration has also an important role in determining energy substrate as it affects the origin of FFA for oxidative purposes. At the initiation of exercise, endogenous IMTG and glycogen represent the main fuel source. As exercise duration increases, in particular after 90 minutes of submaximal exercise, the relative contribution of IMTG metabolization to total FAO decreases probably due to the inhibitory effect of epinephrine and LCFA on HSL (Watt et al., 2002; Moro et al., 2008). In parallel, the relative contribution of plasma FFA to total fat oxidation increases, and after 120 minutes of submaximal exercise, IMTG oxidation returns to resting values (Watt et al., 2002). At this point, after 2 hours of moderate exercise, despite no further decrease in IMTG, the rate of plasma FFA uptake becomes greater than the rate of FAO, meaning that FFA supplied by adipose tissue cover all the fatty acids used by muscle (Horowitz and Klein, 2000). Training status has little influence on the origin of fatty acids during the first 120 minutes of submaximal exercise while during prolonged exercise (>2 hours) trained subjects are able to maintain FAO for a longer duration than untrained subjects, thus sparing CHO usage (Turcotte et al., 1992; Watt et al., 2002). Indeed, on one hand, endurance training in humans increases FFA transport and oxidation

capacity (Purdom, 2018). On the other hand, it leads to a chronic up-regulation of muscle glycogen availability by 20-66%, thus postponing the moment in which glycogen depletion will contribute to fatigue (Bergman et al., 1999; Gonzalez et al., 2015). Glycogen liver availability does not seem to be modified by training but training attenuates liver glycogenolysis rate during moderate to intense exercise (60-85% VO_{2max}), probably due to a blunted hormonal response of trained individuals to exercise (Coggan et al., 1995; Gonzalez et al., 2016).

1.5.4. The "crossover concept" in humans

As stated above, when exercise intensity exceeds MFO (>65-75% VO_{2max}), FAO is inhibited to values below those observed during moderate-intensity exercise. This reduction in FAO has been initially attributed to a reduction of adipose tissue blood flow (and not to a decline in lipolysis) that decreases FFA release and delivery to the contracting muscle. Nonetheless, further research has underlined the interference of muscle glycogen metabolism with FAO (Horowitz and Klein, 2000; Spriet, 2014). Brooks and Mercier (1994) introduced the term "crossover" in human exercise physiology literature to illustrate this shift (crossing over) of fuel selection from lipids to CHO in function of exercise intensity and training (Fig. 6). The crossover concept helps to understand how endurance training induces biochemical adaptations in muscle to increase FAO during mild to moderate intensity exercise while intense exercise (exceeding MFO) increases the contraction-induced muscle glycogenolysis (Brooks and Mercier, 1994).

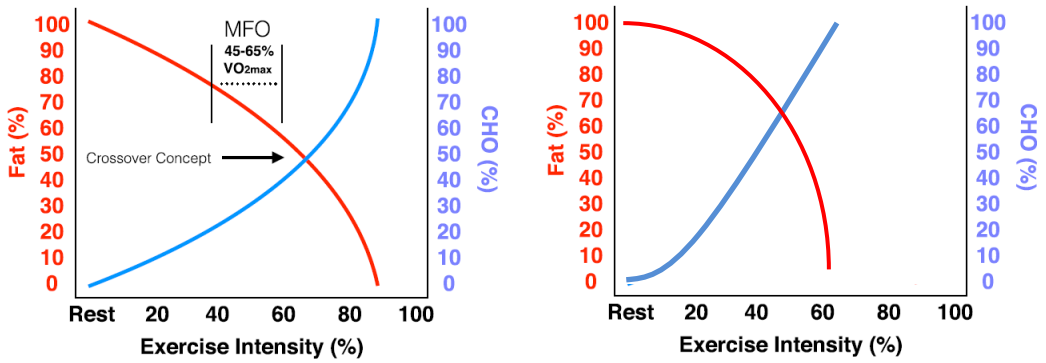

Figure 6. The crossover concept in humans (on the left): the relative decrease in energy provided from fat is accompanied by an increased dependence on carbohydrates (CHO) in function of the increasing exercise intensity. MFO, maximal fat oxidation (Purdom et al., 2018). The image on the right is a personal adaptation to represent the same concept in horses (see below). In dogs, the cross-over concept is more complex.

To explain it more precisely, during high-intensity exercise (>75%VO_{2max}) fast glycolytic flux inhibits the transport of FFA through the sarcolemma but also into the mitochondria. Indeed, the high glycolytic rate occurring during intense exercise induces the production of a high amount of acetyl-CoA that exceeds the rate of utilization by the TCA cycle. Thus, C0 acts as an acceptor of glycolysis-derived

acetyl-CoA to avoid their accumulation that can be deleterious to cellular function, but also to maintain a viable pool of free CoA to permit continuation of pyruvate oxidation (Fig. 7) (Stephens, 2018). This reaction is reversible and catalyzed by the mitochondrial enzyme carnitine acyltransferase (CAT) and it is important to maintain metabolic flexibility (Stephens, 2018). Indeed, acetylcarnitine acts as a metabolic regulator, as high levels of acetyl-CoA may inhibit PDH complex activity and subsequent CHO oxidation (Schooneman et al., 2014) and represent potential membrane-destabilizing agents (Arenas et al., 1991).

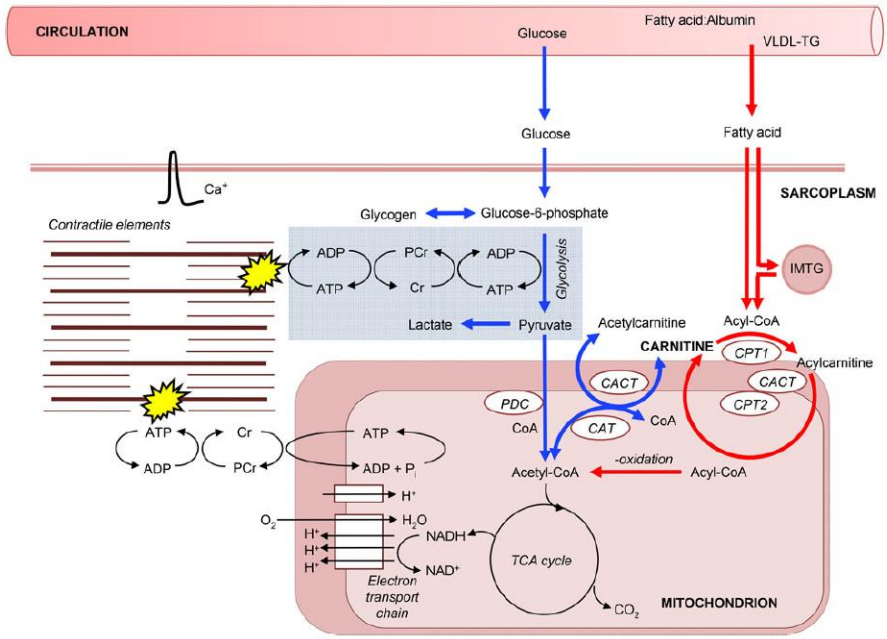

Figure 7. Schematic representation of substrates fate in the context of muscle metabolism during exercise. Lipid-based metabolic path is highlighted on red while glucose-based metabolic path is highlighted in blue. Carnitine plays a major role not only in fat metabolism for translocation of long chain fatty acids (LCFA) into the mitochondria but also as a buffer of potential noxious acetyl-CoA groups and reducing CoA trapping, rendering it available for other metabolic functions. PDC, pyruvate dehydrogenase complex; CAT, carnitine acetyltransferase; CACT, carnitine acylcarnitine translocase; CPT, carnitine palmitoyltransferase; IMTG, intramuscular triglycerides (Stephens, 2018).

Nonetheless, this "buffer" role for acetyl-CoA in situations of high glycolytic flux (such as during intense exercise) monopolizes available muscle C0, thus limiting transport of LCFA into the mitochondria, as CPT1 is a product of C0 (Purdom et al., 2018). This has been observed by studies in which, at rest as during exercise, promoting glucose availability increased glycolytic flux and reduced the uptake and oxidation of LCFA which transport is CPT1-dependent, while the protein-independent MCFA transport was not affected (Sidossis and Wolfe, 1996; Colye et al., 1997). Indeed, the limited availability of FC is a rate-limiting step for (both blood and IMTG-derived) FA transport and oxidation. Moreover, even small changes in muscle pH during intense exercise can largely reduce CPT1 activity

and contribute to FAO down-regulation (Starritt et al., 2000). Malonyl-CoA is a potent inhibitor of CPT1 activity *in vitro* and it regulates LCFA oxidation in human resting skeletal muscle. In fact, during intense exercise, the high glycolytic flux increases concentrations of acetyl-CoA and citrate (an intermediate of the TCA cycle), that activates the enzyme responsible for malonyl-CoA synthesis, thus inhibiting LCFA oxidation (Stephens, 2018). However, research has found out that muscle malonyl-CoA does not seem to regulate LCFA oxidation during exercise, as malonyl-CoA content does not change with exercise at different exercise intensities (Roepstorff et al., 2005). Fatty acid translocase FAT/CD36 plays also a regulatory role on FAO: in human skeletal muscle both acute moderate exercise and endurance training increase content of FAT/CD36, thus increasing FFA intra-cellular transport for oxidation (Schenk and Horowitz, 2006). In the same way, dietary strategies as low-CHO and high-fat diets induce an increase in whole-body and muscle FAO attenuating glycogenolysis rate thus sparing glycogen during submaximal exercise (Yeo et al., 2011). In conclusion, the inhibitory effect of increased CHO availability on lipid metabolism resides more at the level of FAO rather than at the level of FFA availability. In fact, restoring FFA concentration during exercise (by lipid and heparin infusion) did not seem to restore fully FAO rates (Romijn et al., 1995), and increasing FAO did not change FFA uptake or FAT/CD36 protein levels (Roepstorff et al., 2005). In the other sense, when exercise is prolonged over several hours, a shift towards lipid oxidation and a reduction in reliance on muscle glycogen and IMTG take place. The reduction of CHO oxidation is partly due a down-regulation of the PDH activity (Watt et al., 2002). This in turn is due on one side to the decrease in substrate (glycogen and pyruvate as a consequence) and on the other side to the up-regulation, with exercise duration, of PDH kinase that further inhibits PDH activity (Watt et al., 2004). Moreover, increased circulating FFA stimulate lipid oxidation producing acetyl-CoA that further inhibits PDH, and citrate, that inhibits PFK ("The Randle Effect", Randle, 1964). To conclude, many factors as substrate availability, training status, exercise duration and intensity, sex, nutrition, they all influence energy substrate transport and oxidation; however, exercise intensity dictates acutely substrate utilization regardless of diet and training status (Purdom, 2018).

1.5.5. The "crossover concept" in horses

In horses as in humans, exercise intensity is the main factor determining substrate utilization during exercise, but again, both CHO and fat are used, although in different proportions, at any given time during exercise to produce ATP. As stated above, other factors such as exercise duration, diet, feeding state and training will influence the relative contribution of CHO and fat to energy provision. Horses, compared to dogs and humans, seem to be more dependent on muscle glycogen, probably due to a different muscle composition. Glycogen is the primary fuel source employed by horses in both short-term intense exercise (race) and long-term submaximal exercise (endurance), and significant glycogen depletion is associated with the occurrence of fatigue. Where in dogs and humans, at 60-65% VO_{2max},

CHO and fat account respectively for approximately 60% and 40% of total energy expenditure, they account for 75% and 25% at the same exercise intensity in horses exercising on a treadmill (Romijn et al., 1993; Roberts et al., 1996; Geor et al., 2000). During racing (800-2000 m), overall glycogen use is limited, since the rate of depletion accounts for 20-35% of total glycogen. Conversely, in endurance competitions (80-160 km) glycogen depletion rates are more significant, around 50-75%, despite a lower rate of glycogen use in comparison to racehorses (Lindholm et al., 1974; Snow et al., 1981). However, inter-breed differences in substrate selection have been reported probably due to muscle fiber type differences (Prince et al., 2002). Arabian horses dominate, especially at an elite level, endurance events, which are extremely metabolically demanding compared to other equestrian disciplines. As demonstrated by several researches Arabians have a higher proportion of type I and IIa fibers than racehorses and this predominance correlates with performance success (Rivero et al., 1993; Lopez-Rivero et al., 1989). This difference in muscle fiber types and therefore in enzymatic content/activity between breeds can partly explain the finding of a higher fat adaptation in Arabians, as demonstrated by their higher FA concentration and lower respiratory exchange ratio, when compared to Thoroughbreds (Prince et al., 2002). Interestingly, even within endurance races, which can be of variable distance, some degree of metabolic adaptation has been observed in relation to total race distance. Recent publications highlighted the presence of a more glycolytic profile in 6-year-old horses racing over 90 km than in older horses competing over 160 km (Luck et al., 2015) and a metabolic switch towards lipid metabolism progressing from the shortest distance (90 km) to the longest (160 km) (Le Moyec et al., 2019). Another study assessing acylcarnitine profiles in endurance horses covering 160 km showed an eight-fold increase in mitochondrial ß-oxidation and a 17-fold increase in lipolysis in comparison with rest values (Van der Kolk et al., 2020). Further research needs to be done to better understand these shifts, as to determine the work intensity at which peak of fat oxidation (MFO) is attained, which is still unknown in horses. Research performed on a treadmill (Geor et al., 2000) demonstrated that increasing exercise intensity from 30% to 60% VO_{2max} almost did not change the rate of fat oxidation, thus indicating that MFO was reached at a similar or lower work intensity.

1.5.6. The “crossover concept” in dogs

Canids’ survival originally depended on their ability to cover long distances to search for a pray, and their endurance capacity and adaptation is even more developed than in human marathon runners (Roberts et al., 1996; McClelland et al., 1994). Roberts and colleagues (1996) made a first description of the maximal lipid oxidation on Labrador retrievers by analysis of their respiratory exchange ratio (RER). In those dogs, MFO was occurring at 40% VO_{2max} and at this intensity, fat was providing 77% of energy fuel; at higher exercise intensities, the additional energy needed was provided by CHO oxidation. At 85% VO_{2max}, fat was supplying only 19-22% of the energy needed. Even if the relative rate of fat oxidation of dogs was not different from less aerobic species (*i.e.* goats), due to their higher

aerobic capacity (VO_{2max}), dogs' speed at which fat oxidation could supply the highest rate of energy was higher for them than in goats. Nonetheless, as demonstrated in horses, relative contribution of each fuel source to energy expenditure is also partly influenced by the differential recruitment of muscle fibers. Muscle fibers distribution can vary among different canine breeds but in general, as stated above, there is a great proportion of highly oxidative fibers (type I and IIa) in canine skeletal muscle, with the exception of Greyhounds (Gunn, 1978; Guy and Snow, 1981; Rosenblatt et al., 1988). Type I fibers, fueled primarily by fat, are recruited at low speeds and when speed increases, additional faster type II fibers are recruited and additional energy is furnished by CHO oxidation. Furthermore, as observed in humans, a specific low-CHO, high-fat diet and endurance training can enhance fat mobilization and oxidation (Reynolds et al., 1994; Schenk and Horowitz, 2006; Yeo et al., 2011; Volek et al., 2015) while a high-CHO diet is less palatable and negatively affects performances (Downey et al., 1980). Alaskan sled dogs represent an example of the elite endurance canine athlete, covering up to 200 km per day on 8-10 days races as the Iditarod or the Yukon Quest. Their VO_{2max}, even if difficult to measure, is estimated to be around 240 $ml \cdot min^{-1} \cdot kg^{-1}$, which is highly above the VO_{2max} of other studied mammals as human athletes (69-85 $ml \cdot min^{-1} \cdot kg^{-1}$) and racehorses (160-200 $ml \cdot min^{-1} \cdot kg^{-1}$) (Noakes, 1988; Rose et al., 1988; Poole and Erickson, 2011; Miller et al., 2017). These dogs can show an impressive fatigue-resistance on multi-day events where they exercise at approximately 40-50% of their maximal capacity (McKenzie et al., 2008; Davis et al., 2020) and they require a high metabolic intake corresponding to about 12,000 kcal per day (Hinchcliff et al., 1997). Concerning muscle fiber type distribution, Alaskan sled dogs have a higher proportion of type I fibers and a lower proportion of type II fibers in comparison to other breeds (Trevino et al., 1973; McKenzie et al., 2005). Their diet is typically high-fat and low-CHO, as >50%, >25% and <20% of the energy content of their meal is represented by fat, proteins and CHO, respectively (McKenzie et al., 2005). Furthermore, it has been described that a diet rich in fat and proteins may preserve dogs from musculo-skeletal injuries and spare glycogen stores (Reynolds et al., 1985; Reynolds et al., 1999). With these premises, and considering that glycogen depletion is a factor limiting performance in both short-term intensive and prolonged submaximal physical activity, it is presumable that fat is their predominant energy fuel. However, substrate shift in Alaskan sled dogs has been shown to be more complex. Scientific research has demonstrated that Alaskan huskies running 160 km per day during 5 days depleted considerably their muscle glycogen during the first bout of exercise but, after that, a gradual glycogen replenishment occurred, despite a limited CHO intake (McKenzie et al., 2005). Another study assessing muscle glycogen and muscle triglyceride concentrations during a multiday exercise (140 km per day over 4 days) confirmed this attenuation of glycogen use, as 64% of stored muscle glycogen was used during the first 140 km and only 5% during the last 140 km. Furthermore, significant more muscle triglyceride was utilized during the first 140 km compared to the final bout of exercise (McKenzie et al., 2008). These results together suggested that prolonged submaximal exercise in Alaskan sled dogs would induce metabolic adjustments aiming at attenuating muscle glycogen use and enhancing the oxidation of extra-muscular, non-CHO, likely blood-borne

substrates. This concept was further supported by other studies describing an increase in plasma FFA, ketones and urea after a similar exercise (McKenzie et al., 2007) and underlining the importance of fatty acids during prolonged exercise that could supply 70-90% of energy requirements (Paul and Issekutz, 1967; Issekutz, 1979). However, recent studies have challenged this idea: by the mean of respirometry and isotopes infusion, it has been shown that Alaskan sled dogs running a 1,600 km race were actually dependent on CHO metabolism, as demonstrated by an increased capacity to oxidize CHO combined with a decreased capacity to oxidize MCFA (Miller et al., 2015; Miller et al., 2017). Carbohydrates use appeared to be sustained for a high proportion by glycerol and lactate as gluconeogenic substrates, even if they cannot fully account for the total CHO oxidized (Miller et al., 2017). Further research on sarcolemmal transporters and on serum hormones and metabolic substrates supported the idea of a CHO-reliance in sled dogs. These works described an increased basal and exercise-induced glucose-transport activity (Davis et al., 2014) in conditioned sled dogs and a progressive increase in the stimulus for hepatic glucose output during multiday exercise (Davis et al., 2020). The increased glucose output through gluconeogenesis and possibly hepatic glycogenolysis would both fuel submaximal exercise and be at the origin of the replenishment of muscle glycogen transiently depleted, underlining liver importance in energy metabolism during prolonged exercise.

2. ENERGY DISTURBANCES IN SKELETAL MUSCLE

Disturbances of skeletal muscle metabolism or metabolic myopathies include a variety of defects that impair the capacity of skeletal muscle to produce energy; they can be primary (inherited) or secondary (acquired). Primary metabolic myopathies include three main groups of entities: glycogen storage disorders (GSD) or glycogenosis, fatty acid oxidation defects and mitochondrial disorders (Berardo et al., 2010). Clinical presentations can vary from sudden death in infants, to slowly progressive forms or to recurrent, exercise-induced, manifestations. In veterinary medicine, research on this topic is more limited and often based on a comparison to human pathology. Fatty acid oxidation defects and mitochondrial disorders will not be treated in this chapter, which will mainly focus on glycogenoses.

2.1. Glycogen synthesis and breakdown

In humans, glycogen represents the major CHO storage form in skeletal muscle and liver although being also present in less significant amounts in other tissues as brain, kidney, heart, adipose tissue and erythrocytes (Adeva-Andany et al., 2016). Glycogen is stored as a granule-like structure under the sarcolemma and between actin-myosin elements (Tarnopolsky, 2018). Glycogen is a branched polysaccharide consisting of glucose units that are connected in a linear chain by α-1,4-glycosidic bonds while α-1,6-glycosidic linkages create the branch points at even intervals (Adeva-Andany et al., 2016). Glycogen can be synthetized either through a direct pathway from exogenous glucose resulting from the digestion and absorption of dietary CHO, or through an indirect pathway from gluconeogenic precursors (lactate, alanine, glycerol). Free glucose enters muscle cells via glucose transporters GLUT-1 and GLUT-4. The transporter GLUT-1 is constitutive and does not migrate under stimulation by insulin or contraction. The transporter GLUT-4 is situated partially in the sarcolemma and partially within intracellular vesicles that translocate to the sarcolemma during periods of insulin stimulation and muscle contraction (Hayashi et al., 1997). Once in the cell, glucose is phosphorylated to G-6-P by hexokinase isoenzymes (hexokinases in muscle and glucokinase in liver) and by ATP that is the phosphate donor. Depending on the metabolic and hormonal context (in particular epinephrine and insulin), G-6-P can enter glycolysis or glycogen synthesis processes. After a meal, a high insulin concentration promotes glycogen synthesis and inhibits glycogen breakdown, and the contrary happens in case of energy demand. In the latter case, G-6-P is converted to glucose-1-phosphate (G-1-P) by phosphoglucomutase and G-1-P is converted to UDP-glucose, a sugar nucleotide, by the action of UDP-glucose pyrophosphorylase. UDP-glucose is the immediate donor of glucose residues in the formation of glycogen. Glycogen synthesis is mediated by a primer protein, glycogenin, which starts adding glucose units from UDP-glucose to itself (autoglycosylation), and by the enzyme glycogen synthase (GS) which can only extend existent glucose chains of minimum 4 glucose residues (Lehninger et al., 1993). Once that glycogenin has formed a linear chain of 10-20 moieties, GS catalyzes the elongation of this sequence

transferring glucose units (via UDP-glucose) to the growing glycogen strand through α-1,4-linkages. On the other hand, the glycogen-branching enzyme (GBE) catalyzes the formation of α-1,6-glycosydic bonds at regular intervals, thus establishing branch points. Branches continue to grow by addition of further α-1,4-glycosidic bonds and further branching (Mayes and Bender, 2003). The biological purpose of branching is to render glycogen more soluble and more reactive to glycogen phosphorylase and synthase by increasing the number of non-reducing ends (Lehninger et al., 1993). Interestingly, recent literature (Testoni et al., 2017) has demonstrated that, contrary to previous statements, glycogenin is not strictly necessary for glycogen synthesis *in vivo*, as indicated by the fact that glycogenin-deficient mice display over accumulation of glycogen. Thus, glycogenin would regulate the amount and the particle size of glycogen synthetized by acting on GS (Skurat et al., 2006). In case of energy need, as during exercise when epinephrine increases or during fasting when insulin decreases, glycogen phosphorylase (GP) promotes the cleavage of α-1,4-linkages of the glycogen strand, releasing G-1-P. The enzyme glucan transferase exposes the α-1,6-branch points that are hydrolyzed by the glycogen debranching enzyme (GDE). Thus, the combined action of these enzymes leads to complete glycogen breakdown. At this point, G-1-P can be converted to G-6-P by phosphoglucomutase (reversible reaction) and enter glycolysis or it can be converted to free glucose by the enzyme glucose-6-phosphatase and leave the cell via glucose transporters to reach the bloodstream to metabolically support other tissues. A synthetic representation of these pathways is given with Fig. 8. It is important to specify that only liver and kidney can transport glucose back in blood as muscle lacks glucose-6-phosphatase. Glycogen breakdown can also take place in the lysosomes via the enzyme α-glucosidase (GAA). This enzyme is transported via the Golgi from the endoplasmic reticulum (ER) to lysosomes where it degrades glycogen through an autophagy-mediated pathway or “glycophagy” (Kaur and Debnath, 2015). Glycogenosis or GSD is the term used to describe a subset of inherited disorders affecting glycogen metabolism (synthesis or breakdown).

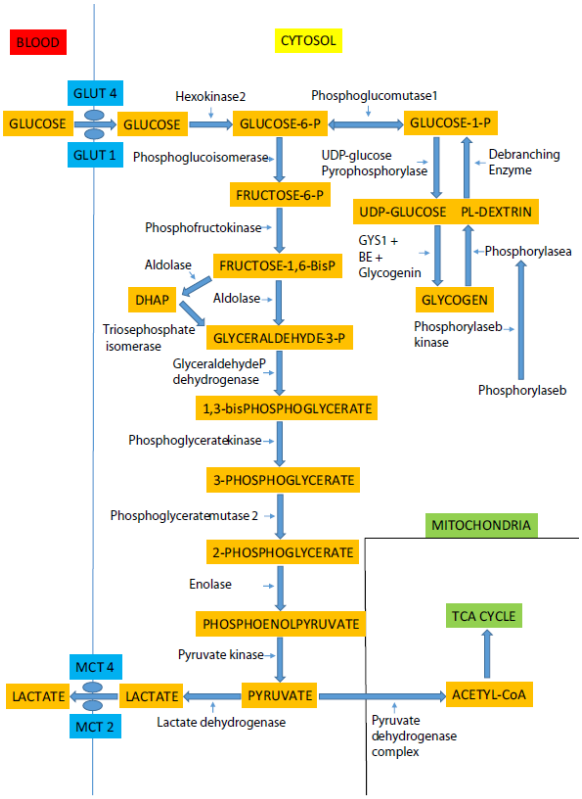

Figure 8. Schematic representation of glycogen and glucose metabolism in skeletal muscle. GLUT, glucose transporter; DHAP; dihydroxyacetone phosphate; GYS1, glycogen synthase, BE, branching enzyme; MCT, monocarboxylate transporter; TCA, tricarboxylic acid cycle (Tarnopolsky, 2018).

2.2. Glycogen storage disorders in humans

Glycogen storage disorders are a subset of genetic disorders due to mutations affecting any of the enzymes implicated in CHO metabolism. They are rare diseases in humans, with an overall approximate incidence of one case per 20,000-43,000 live births (Özen, 2007). Skeletal muscle, liver and heart are primarily affected but kidney and central nervous system can also be involved (Hicks et al., 2011). Glycogenoses are inherited and generally autosomal recessive apart from GSD type IX and Danon disease (X-linked). They can manifest under a variety of phenotypical expressions, from premature death in infants to exercise intolerance in adults; asymptomatic forms are also possible (Hicks et al., 2011). Early diagnosis of GSD is mandatory to reduce systemic damages induced by glycogen accumulation; nonetheless, it remains difficult, especially in mild forms, due to the lack of specificity and heterogenicity of the symptoms. The main classification of GSD is based on the tissue and on the specific mutation involved, which will also dictate the prognosis. A further clinical classification can be used depending on symptoms, whether they are fixed (*e.g.* weakness), with or without systemic involvement, or dynamic (exercise-induced) (Haller and DiMauro, 2012).

When suspecting a glycogenosis, some history details, as the type of exercise causing symptoms (high intensity sprints or prolonged exercise), and the presence of other triggering factors (*e.g.* fasting), are crucial. The type of symptoms will also help to establish the diagnosis as muscle cramps, weakness, fatigue and exercise intolerance are generally suggestive of skeletal muscle involvement while hypoglycaemia and liver enlargement are indicators of hepatic GSD (Özen, 2007). The "second wind" phenomenon, consisting in a rapid increase in exertional heart rate and minute ventilation followed by a decrease when exercise intensity decreases, is another useful clinical feature that is pathognomonic of McArdle disease (GSD type V) (Vissing and Haller, 2003). Other diagnostic tools include muscle and liver biopsy, assessment of blood markers of muscle damage as creatine kinase (CK), aspartate aminotransferase (AST), alanine amino transferase (ALT), lactate dehydrogenase, (LDH) and myoglobin, and of course molecular testing. The list of human GSD is given in table 1 and their intervention on glycogen metabolism is presented with Fig. 9.

Table 1: List of the classical glycogenolytic, glycolytic and glycogen synthesis disorders in humans, or glycogenoses (Adeva-Andany et al., 2016; Ellingwood and Cheng, 2018).

Type	Name or subtype	Affected enzyme	Main feature
0	0a	Liver glycogen synthase	Reduction in liver glycogen synthesis
	0b	Muscle glycogen synthase	Reduction in muscle glycogen synthesis
I	Ia; von Gierke	Glucose-6-phosphatase α	Defect in glucose dephosphorylation, glycogen accumulation in liver and kidney
	Ib; von Gierke	Glucose-6-phosphate transporter	Defect in glucose dephosphorylation, glycogen accumulation in liver and kidney
II	Pompe	Acid α-glucosidase	Defect in lisosomal glycogenolysis with glycogen accumulation
III	Cori/Forbes	Glycogen debranching enzyme	Defect in glycogenolysis, accumulation of glycogen in liver, heart, skeletal muscle
IV	Andersen	Glycogen branching enzyme	Defect in glycogen synthesis
V	McArdle	Muscle glycogen phosphorylase	Defect in skeletal muscle glycogenolysis
VI	Hers	Liver glycogen phosphorylase	Defect in liver glycogenolysis
VII	Tarui	Skeletal muscle phosphofructokinase	Defect in glycolysis
VIII		Glycogen phosphorylase kinase - skeletal muscle isoform	Defect in skeletal muscle glycogenolysis
IX	IXa	Glycogen phosphorylase kinase – liver isoform	Defect in liver glycogenolysis
	IXb	Glycogen phosphorylase kinase (ß subunit)	Defect in liver and muscle glycogenolysis
	IXc	Glycogen phosphorylase kinase (γ subunit) – liver isoform	Defect in liver glycogenolysis
X		Skeletal muscle phosphoglycerate mutase	Defect in skeletal muscle glycolysis
XI	Fanconi-Bickel	Glucose transporter 2	Altered glucose transport
XI		LDH – isoenzyme A	Altered glycolysis
XII		Aldolase A	Defect in skeletal muscle and red blood cells glycolysis
XIII		ß-Enolase	Defect in skeletal muscle glycolysis
XV		Glycogenin-I	Reduction in muscle glycogen synthesis

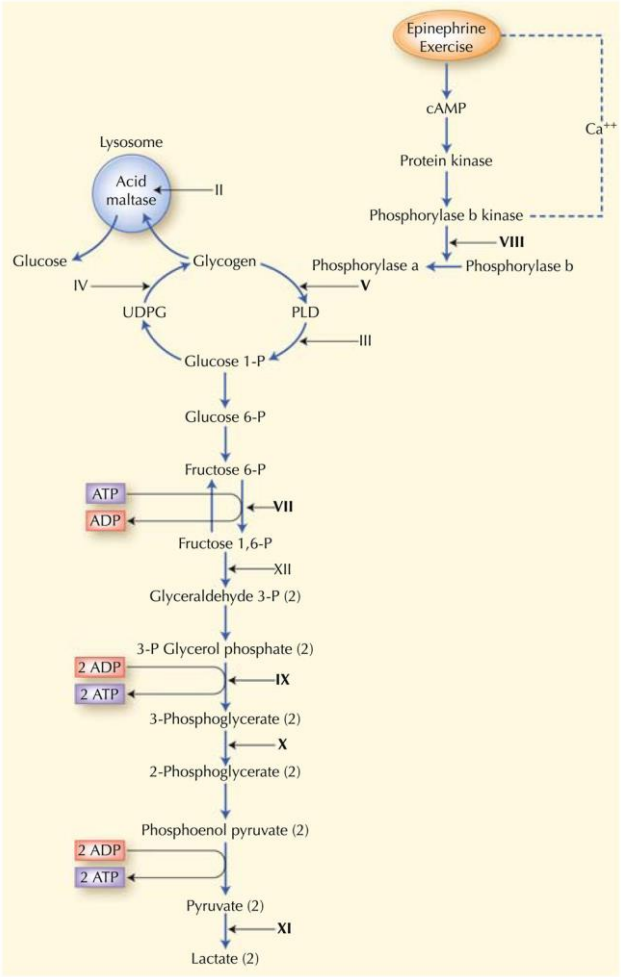

Figure 9. Glycogen and glucose catalytic pathways. Roman numbers indicate glycogen storage disorders (as listed in Table 1) and arrows indicate the affected enzyme. Bold numbers correspond to glycogenoses with dynamic (exercise-induced) symptoms; non-bold numbers label glycogenoses with fixed symptoms. ADP, adenosine diphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PLD, phospholipase D; UDPG, uridine diphosphoglucose (Berardo et al., 2010).

2.3. Glycogen storage disorders in horses

There are only two GSD known in horses so far, both involving glycogen synthesis: type-1 polysaccharide storage myopathy (PSSM) and glycogen branching enzyme deficiency (GBED). Another form of myopathy with abnormal glycogen storage in myofibres is type-2 PSSM that resembles histologically to type-1 PSSM without sharing its genetic basis.

2.3.1. Glycogen Branching Enzyme Deficiency

Equine GBED is a fatal neonatal disease that closely resembles human GSD IV, or Andersen's disease. In humans, GSD IV originates from point mutations in the *GBED1* gene that encodes GBE (Bao et al., 1996), resulting in a reduced activity of this enzyme thus causing an accumulation of abnormally branched glycogen, or "polyglucosan", in multiple tissues. In horses GBED affects mainly Quarter Horses and Paint Horses and it is due to a nonsense mutation in exon 1 of the equine *GBE1* gene that knocks out GBE activity (Ward et al., 2004). Clinically this disease cause stillbirth or death in newborns within few weeks of age, due to cardiac muscle, skeletal muscle and liver dysfunction (Render et al., 1999).

In affected foals, GBE activity is poorly or not detectable in any tissue, and there are cytoplasmic, globular and crystalline inclusions of abnormal (poorly branched) glycogen in numerous tissues as heart, brain, liver and skeletal muscle (Valberg et al., 2001).

2.3.2. Polysaccharide Storage Myopathy

2.3.2.1. Terminology, diagnostic criteria and genetics

Polysaccharide storage myopathy (PSSM), also previously referred to as "azoturia", "Monday morning disease" or equine polysaccharide storage myopathy (EPSM), is widely recognized as a cause of recurrent rhabdomyolysis. The identification of PSSM as a specific myopathy dates back to 1992, based on the observation of excessive polysaccharide inclusions, positive to periodic-acid Schiff (PAS) stain, in type IIa and IIb muscle fibers of Quarter Horses with exertional rhabomyolysis (Valberg et al., 1992). These abnormal sarcoplasmic and/or subsarcolemmal polysaccharide accumulations were either resistant or sensitive to enzymatic digestion with amylase (Valberg et al. 1992; McCue et al. 2006; Firshman et al., 2006). Thus, horses diagnosed with PSSM based on muscle biopsy were further divised in 2 groups, depending on whether the histological evidence of myopathy was combined with excessive amylase-sensitive (grade 1 PSSM) or amylase-resistant (grade 2 PSSM) polysaccharide (McCue et al., 2006) (Fig. 10).

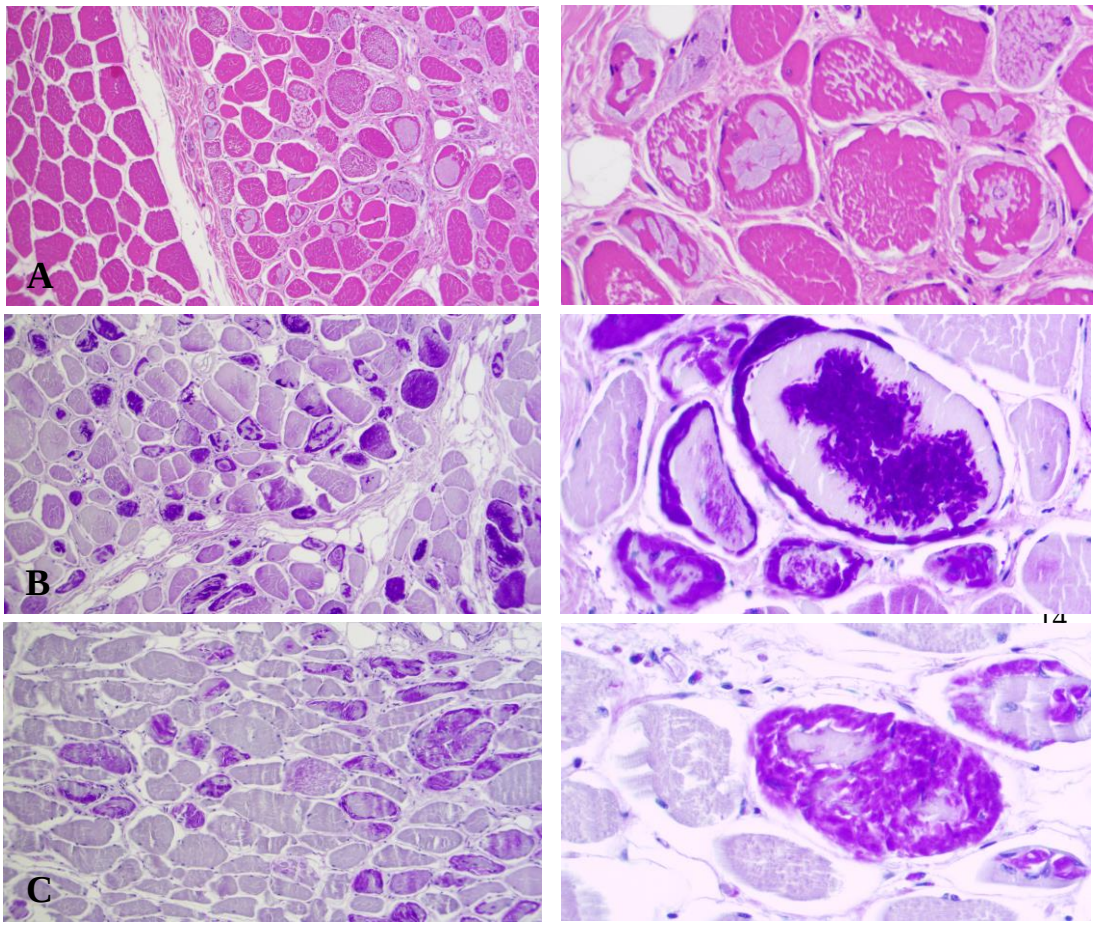

Figure 10. Cross sectional images of *gluteus medius* muscle from a horse affected by PSSM stained with hematoxylin and eosin (HE) (A), periodic-acid Schiff (PAS) (b), and PAS staining after amylase digestion (x20 on the left, x40 on the right). These images show both sarcoplasmic and subsarcolemmal inclusions of PAS-positive, amylase-resistant polyglucosan bodies. *Photo courtesy of Dr. Dominique Cassart and Mélanie Melnik, University of Liège.*

In 2008, McCue and colleagues identified an autosomal dominant gain-of-function mutation in the glycogen synthase 1 gene (*GYS1*) coding for GS enzyme (MCue et al., 2008a).They observed that a single base pair substitution was causing an amino acid change, from normal arginine (R) to histidine (H) at codon 309 in PSSM-affected Quarter Horses. This mutation (Arg309His, or R309H) occurs in a highly conserved, potentially critical region of the GS enzyme and it is associated with a conformational change hindering GS activity in skeletal muscle. Specifically, the R309H mutation disrupts a salt bridge that is important to stabilize the conformation of the GS enzyme basal state This change in conformation renders the enzyme constitutively active (Maile et al., 2017) and leads to an abnormal accumulation in myofibers of polysaccharide with a higher ratio of straight chains of glucose, relative to branched chains, because GBE has a normal activity (McCue et al., 2008b, Stanley et al., 2009). However, the precise mechanisms by which the R309H mutation results in increased GS activity is unknown. It is interesting to notice that the equine *GYS1* gain-of-function mutation contrasts with the aforementioned human GSD type 0b in which the activity of muscle glycogen synthase is deficient, leading to a profound lack of glycogen in skeletal and cardiac muscle fibers, thus provoking weakness, cardiomyopathy and even cardiac arrest (Kollberg et al., 2007). After the discovery of the genetic mutation, comparative histopathological and ultrastructural analyses were performed in horses with and without the *GYS1* mutation. It was observed that the *GYS1* mutation was found in approximately 50% of all biopsy samples diagnosed with PSSM (MCue et al., 2008b). The excessive polysaccharide inclusions in *GYS1*-positive horses were often amylase-resistant, coarse granular in appearance and located in the cytoplasm whereas in *GYS1*-negative horses these were often amylase-sensitive, homogeneous in appearance and subsarcolemmal in location (McCue et al., 2009a). However, several horses classified with either grade 1 or grade 2 PSSM did not carry the *GYS1* mutation and others with grade 1 PSSM possessed the mutation (McCue et al., 2008b; Stanley et al., 2009). Therefore, a new nomenclature was proposed to distinguish horses carrying the *GYS1* mutation and horses without the mutation but diagnosed with PSSM by histopathologic means, thus designating type-1 PSSM the *GYS1*-positive horses and type-2 PSSM the *GYS1*-negative horses (McCue et al., 2008b; McCue et al., 2009a). However, type-2 PSSM is not a specific identity, because it can regroup horses with different muscle disorders, all resulting in the production of glycogen with abnormal histological appearance. Thus, DNA analysis of blood, hair roots, fresh or frozen tissue samples to test for the GYS1 mutation became the gold standard for diagnosis of type-1 PSSM. It is necessary to underline that, in the following sections of this manuscript, a distinction has been made between type-1 and type-2 PSSM when possible. Nonetheless, when citing

literary sources dating prior to 2008, this distinction has not been done, due to the fact that the genetic test was not availale yet. At that time, the diagnosis was based on the sole histopathologic criteria.

2.3.2.2. Prevalence and clinical presentation

Polysaccharide Storage Myopathy (PSSM) was initially described in Quarter Horses and Quarter Horse-related breeds (Valberg et al., 1992; Valberg et al., 1996) as well as in Belgian and Percheron draft horses (Valentine et al., 1997). In these studies, the diagnostic criteria was the presence of PAS-positive amylase-resistant abnormal polysaccharide inclusions (or grade 2 PSSM) in skeletal muscle fibers. When alternate histopathologic criteria as the presence of PAS-positive sarcoplasmic masses, excessive amylase-sensitive cytoplasmic or subsarcolemmal glycogen (or grade 1 PSSM) were used, both the overall and the within breed prevalence of PSSM increased and more than 30 breeds were identified (Valentine and Cooper, 2005; McCue et al., 2006). After its discovery, *GYS1* mutation was found in more than 20 breeds from North America, Europe and the United Kingdom (Stanley et al., 2009; McCue et al., 2008b; Baird et al., 2010). The mutation frequency was identified as particularly high in North American and Belgian Draft breeds and rare to non-existent in athletic light breeds as Thoroughbreds (McCue et al., 2010). Concerning the clinical presentation, some horses affected by PSSM can remain asymptomatic, others may appear normal at rest and show signs of exercise intolerance and muscle pain when exercised (Mickelson and Valberg, 2015). Clinical signs can vary from acute forms as reluctance to move forward, weakness, stiffness, sweating, muscle contractures and even recumbency, to chronic, low-grade clinical signs, as lack of energy under the saddle, gait abnormalities, back pain and poor performance (Valentine et al., 2001; Quiroz-Rothe et al., 2002; Firshman et al., 2003; Firshman et al., 2005; Hunt et al., 2008). Affected horses commonly develop clinical signs of rhabdomyolysis during the first 20 minutes of submaximal aerobic exercise, when blood lactate concentration is unchanged from resting value (<1 mmol/l) (Valberg et al., 1999a). The hindquarters are more frequently affected, but back, abdominal and forelimb muscles can also be involved (Reed et al., 2017). The reason for the variability of clinical signs is unknown, but it seems to be influenced by environmental factors (stress, management), exercise (type, intensity and duration), diet, genotype and interaction with other genes rather than by gender, body size and temperament (Firshman et al., 2003; McCue et al., 2006; Mickelson and Valberg, 2015). Concerning the impact of diet on phenotypic variability, it has been observed that PSSM horses fed a high non-structural CHO diet are at a higher risk of developing muscle pain with exercise than when fed a low-CHO and fat-supplemented diet (Valentine et al., 2001; Ribeiro et al., 2004; Borgia et al., 2010). This seems to be related to the fact that a high CHO-diet increases circulating glucose and insulin, which are activators of GS. Concerning the influence of genotype, it has been shown that homozygotes show more severe clinical signs, muscle histopathological changes and higher muscle enzymes (CK, AST) activity (indicative of muscle damage) when compared to heterozygotes (Naylor et al., 2012). Regarding genetic interactions, Quarter Horses (or related horse breeds) affected at the

same time by the *GYS1* mutation and the *RYR1* or the *SCNA4* mutation, responsible respectively for the Malignant Hyperthermia (MH) and for the Hyperkalemic Periodic Paralysis (HYPP), may suffer from more severe exercise intolerance and even life-threatening rhabomyolysis (McCue et al., 2009b; Tryon et al., 2009).

2.3.2.3. Impairment of energy metabolism

Despite the evidence of a genetic dysfunction at the basis of this glycogenosis, the overall metabolic puzzle underlying type-1 PSSM phenotype is more complex. Indeed, horses affected by PSSM suffer from an impaired energy generation and they have a reduced VO_{2max} when compared to healthy controls (Valberg et al. 1995; Annandale et al. 2005). Indeed, they struggle to achieve maximal speed but muscle damage, in terms of CK elevation, is more severe during submaximal than maximal exercise (Valberg et al., 1999a). This energetic imbalance was originally thought to result from a limitation in glycolysis and not from a limitation in oxygen transport (Valberg et al. 1995). Nonetheless, in contrast to human glycogen storage diseases (GSD), PSSM-affected horses did not show any deficiency in glycolytic or glycogenolytic enzymes and they demonstrated to be able to use glycogen and produce lactate with anaerobic exercise (Valberg et al., 1998; Valberg et al., 1999a). It was also observed that type-1 PSSM horses have a higher resting muscle glycogen concentration (from 1.5 to 4 times) than controls (Valberg et al., 1999a; De La Corte et al., 1999a). Glycoge content is also significantly higher in horses with type-1 PSSM compared to horses with type-2 PSSM, while glycogen content in horses with type-2 PSSM does not differ from controls (Lewis et al., 2017). When the *GYS1* mutation was discovered, this high glycogen concentration typical of type-1 PSSM cases could be attributed the gain-of-function mutation that shifts the conformational equilibrium of the GS enzyme to the active state thus increasing its activity (Maile et al., 2017). Nevertheless, the link between genetics and cell damage, and thus the PSSM phenotype, is still unclear. Abnormal polysaccharide accumulation is an indicator of PSSM, but not the direct cause of rhabdomyolysis. In fact, clinical signs of rhabdomyolysis can appear as early as 6 months of age while abnormal glycogen accumulations can take up to 3 years to become evident in muscle biopsies (De la Corte et al, 2002). Neither glycogen depletion nor lactic acid accumulation are the cause of rhabdomyolysis. Indeed, PSSM horses undergo considerable metabolic stress even when performing short-term aerobic exercise, thus when blood lactate concentration is low and muscle glycogen concentration is still adequate (Valberg et al., 1993; Annandale et al., 2005). Moreover, PSSM horses do not show abnormal blood lactate accumulation compared to controls when performing submaximal exercise (Valberg et al., 1999a); compared to controls, they deplete muscle glycogen to similar extents during exercise and they replete it more quickly after exercise (Mickelson and Valberg, 2015). From a metabolic point of view, type-1 PSSM horses do not show ATP depletion when performing submaximal exercise, but they experience purine nucleotide degradation in their muscle fibers, indicated by the accumulation of IMP. This accumulation is more marked at the level of single muscle fibers than in

whole-muscle analyses (Annandale et al., 2005). An increase in IMP concentration can derive, as previously stated, from the degradation of AMP due to AMP deaminase activation ($AMP + H_2O \leftrightarrow IMP + NH_3$). Activation of AMP deaminase and IMP accumulation generally occur during intense -and not light and submaximal- exercise or in case of metabolic stress. Moreover, shifts in IMP/AMP concentration in skeletal muscle can affect the activity of AMP kinase which is a key sensor of cellular energy status and a regulator of energy supply in skeletal muscle (Hardie, 2003). Altering AMP kinase activity would mean shifting the cellular status from energy-generating pathways (oxidative metabolism of fatty acid and CHO oxidation) to energy-consuming pathways. This disturbance of substrate flux in aerobic metabolism despite adequate glycogen concentration would be consistent with the 2-fold higher muscle lactate concentrations observed in exercising PSSM horses compared to controls. Indeed, it would signify that pyruvate has been converted anaerobically to lactate rather than oxidized to acetyl-CoA in mitochondria (Valberg et al., 1999a). This inability or disability to switch from one substrate to another could partly explain metabolic stress found in PSSM horses; nonetheless, the link between the genetic defect and the energy deficit remains unclear. A potential explanation that has been suggested is that cellular nutrient sensors, as AMP kinase, could interprete the excessive stimulation of GS stimulated by insulin (as in horses fed a high-CHO diet) as an indication not to activate glycogenolysis and lipolysis (Borgia et al., 2010). Considering that acetyl-CoA can also be supplied by FAO and not only by PDC, the fact that PSSM horses experience rhabdomyolysis when exercising at submaximal intensity, thus when both substrates are employed, would mean that their use of CHO and fat to fuel muscle contraction during exercise is equally altered. Activation of AMP kinase in normal conditions inhibits acetyl-CoA carboxylase (ACC) which converts acetyl-CoA to malonyl-CoA, thereby driving the entry of LCFA into mitochondria for FAO (Long and Zierath, 2006), because malonyl-CoA is, as previously stated, an inhibitor of CPT1 activity. Thus, AMP kinase dysregulation would affect FAO via malonyl-CoA accumulation. Moreover, citrate concentration increases in muscles exposed to high-glucose load, thus faster glucose uptake in PSSM horses when fed high-CHO diet would increase citrate, which further activates ACC, increasing malonyl-CoA concentration (Saha et al., 1999; Borgia et al., 2010). Further evidence of an impaired energy metabolism has been provided by a genomic approach describing the down-regulation of many genes involved in mitochondrial activity as well as signs of mitochondrial degeneration in horses affected by type-1 PSSM (Barrey et al., 2009). More precisely, this down-regulation involved most of the mitochondrial tRNA, genes coding for the respiratory chain sub-units, nuclear genes involved in the aerobic metabolism of mitochondria, glucose transporter GLUT2 and mitochondrial carrier ornithine transporter. An up-regulation of pro-inflammatory genes, indicating chronic muscle inflammation, and mitochondrial and muscle ultrastructural changes in mitochondria and in muscle fibers were also evident. In conclusion, it is obvious that type 1-PSSM horses are energetically compromised, even if the precise site of dysfunction and its link to cell damage is blurry.

2.3.2.4. Insulin sensitivity and glucose uptake

Diet has an important influence on type-1 PSSM phenotypic variability. As previously stated, a diet rich in non-structural CHO can precipitate clinical signs of rhabdomyolysis because insulin has a stimulatory effect on glycogen synthase (GS) (Ribero et al., 2004; Borgia et al., 2010). Horses with PSSM have an increased insulin sensitivity compared to controls, meaning that they secrete relatively less insulin in response to the same glucose load (De La Corte et al., 1999b; Valberg et al., 1999a; Annandale et al., 2005). Insulin sensitivity in PSSM horses has been indicated by a lower insulin response, compared to controls, when PSSM horses were administered sweet feed, oral and intravenous glucose and high-CHO hay, as well as when using the euglycemic hyperinsulinemic clamp technique (De La Corte et al., 1999a,b; Annandale et al., 2004; Borgia et al., 2011). A faster rate of glucose uptake by skeletal muscle has also been observed in PSSM-affected horses using both the euglycemic hyperinsulinemic clamp technique and the intravenous glucose tolerance test (IVGTT), probably due in part to increased insulin sensitivity (Annandale et al., 2004; De La Corte et al., 1999b). Interestingly, the 1.5-2 fold faster rate of glucose uptake pointed out in PSSM horses by both the euglycemic hyperinsulinemic clamp and the IVGTT corresponds with the 1.8-2.4 fold higher glycogen content observed in these horses (Valberg et al., 1999a; De La Corte et al., 1999a). Moreover, PSSM horses have a higher post-prandial glycemic and insulinemic response when fed high-CHO hay compared to low-CHO hay and a glycemic response that is also higher than controls when fed high-CHO, even if the latter observation is controversial as the contrary is also reported (De La Corte et al., 1999a,b). Interestingly, enhanced glucose uptake and insulin sensitivity in skeletal muscle of PSSM horses is not related to GLUT-4 content nor to insulin receptor (IR) quantity, which do not differ from control horses (Annandale et al., 2004). Only GLUT-1 has been found in lower concentration in PSSM compared to controls, probably indicating a compensatory response to higher muscle glycogen concentration and insulin sensitivity (Annandale et al., 2004).

2.3.2.5. Management of type-1 PSSM

In conclusion, type-1 PSSM is a pathology with a genetic basis, characterized clinically by episodes of exertional rhabdomyolysis and metabolically by an impaired oxidative metabolism within individual contracting muscle fibers, inability to generate or to use acetyl-CoA, enhanced insulin sensitivity and blood glucose uptake with submaximal exercise. The treatment for this condition aims at decreasing glucose availability and insulin secretion by replacing high-CHO diets with low-CHO diets (hay with 12% or less of non-structural carbohydrates or NSC) supplemented with fatty acids to increase their availability for oxidation in skeletal muscle. Dietary fat sources generally employed are vegetable-based or animal-based oils, ideally containing long-chain fats (*i.e*. corn or soy oil), because oils with shorter chain fats (*e.g*. triheptanoin, a 7-carbon fat) can have detrimental effects due to increased stimulation of

GS via insulin and glucose and to further inhibition of lipid oxidation (Borgia et al. 2010). Finally yet importantly, when managing horses affected by type-1 PSSM, it is crucial to increase daily exercise to train oxidative metabolism or substrate flux, or both (this is not elucidated yet). Interestingly, when diet and exercise recommendations described for PSSM-1 horses are applied in type-2 PSSM cases, the majority of PSSM-2 cases improve clinically, whereas some of them are still affected by the residual effects of this muscle disorder based on owners' viewpoint (Williams et al., 2018). Concerning supplementation, there are no other supplements apart from dietary fats described in literature benefiting horses suffering from type-1 or type-2 PSSM. Carnitine supplementation has been advocated by some authors (Kranenburg et al., 2014) but its use in PSSM horses has not be described so far. A deficiency in plasma carnitine has not be identified in PSSM horses, their concentrations at rest are not significantly different from normal horses (Valberg, *unpublished data*).

2.4. Glycogen storage disorders in dogs

Glycogen storage disorders are described in companion animals and in dogs in particular, thus offering animal models for clinical, pathogenetical and therapeutic investigations (*e.g.* gene therapy) (Brooks and Koeberl, 2015). History, clinical signs, pathological and ultrastructural findings can be suggestive of a glycogenosis; confirmation of the diagnosis is generally based on enzyme activity assays on tissue samples, glycogen quantitation and structure analysis. These analyses can be further supported by amplification of the target gene by PCR when the specific genetic mutation is known. In dogs, four breed-specific types of GSD are reported: GSDIa (Von Gierke disease), GSDII (Pompe disease), GSDIII (Cori disease) and GSDVII (Tarui disease). Von Gierke disease or GSDIa has been reported in Maltese puppies affected by mental depression, growth failure and early death, thus resembling the severe neonatal onset of human GSDIa (Brix et al., 1995). Historical and histopathologic findings, in particular hepatomegaly and nephromegaly due to glycogen accumulation, were similar to those observed in children with the same diseases. The diagnosis was confirmed by the decreased hepatic and renal levels of glucose-6-phosphatase (G-6-Pase), similar to those found in humans with GSDIa, coupled with the increased PAS-positive glycogen in hepatocytes. Further studies revealed the genetic basis of the disease as a missense mutation in the gene encoding the catalytic α-subunit of G-6-Pase (Kishnani et al., 1997). Pompe disease, or GSDII, caused by a deficiency of acid α-glucosidase, has been identified in Swedish and Finnish Lapphund dogs presenting progressive muscular weakness, loss of condition, vomiting due to oesophageal dilation and clinical heart disease (Walvoort, 1985; Seppala et al., 2013). Membrane-bound vacuoles containing abnormal glycogen were found in skeletal, cardiac, oesophageal and smooth muscle (Walvoort et al., 1985). As for GSDIa, the canine form of GSDII closely parallels the infantine form of human GSDII apart from megaoesophagus and consequent vomiting and regurgitation symptoms that seems unique in dogs (Seppala et al., 2013). The cause of Pompe disease has been identified as a nonsense mutation in the acid α-glucosidase gene (Seppala et al., 2013).

Cori disease or GSDIII has been described in curly coated retrievers presenting lethargy and exercise intolerance (Gregory et al., 2007). Biochemical analyses revealed increased serum concentration of liver transaminases and of muscle enzymes; histopathology revealed hepatomegaly with severe PAS-positive glycogen accumulation in hepatocytes. Deficiency of glycogen debranching enzyme activity in both liver and muscle confirmed the disease together with DNA-testing for *AGL* mutation (gene symbol for the glycogen debranching enzyme). Once again, various similarities between dogs and humans affected by Cori disease were identified, as clinical findings, biochemical abnormalities and multiple tissue involvement (Brooks et al., 2016). Tarui disease or GSDVII, due to a deficiency of muscle type PFK, has been reported in English Springer Spaniels exhibiting hemolytic disorders and exertional myopathy as observed in human patients (Giger et al., 1988). A nonsense mutation in the PFK gene has been identified as the underlying cause of this disease, leading to rapid degradation of an unstable truncated PFK-protein (Smith et al., 1996).

3. ASSESSING THE PHYSIOLOGIC AND PATHOLOGIC ENERGY METABOLISM

Exercise physiology research is constantly seeking new technologies, functional tests and biomarkers to identify normal biological processes, to discriminate them from pathogenic processes and ultimately to improve health and predict performance (Lee et al., 2017). This manuscript focuses on two specific tools that can be used to investigate energy metabolism in physiologic and/or pathologic conditions in two different species. These approaches are represented by the dosage of plasma/serum acylcarnitines profile and by the assessment of mitochondrial function by high-resolution respirometry (HRR).

3.1. Carnitine and acylcarnitines as metabolic regulators

Carnitine plays an essential role in energy metabolism as it transports long chain fatty acids (LCFAs) into the mitochondria for ß-oxidation after esterification into LCACs due to inability of LCFAs to penetrate mitochondrial membranes. Inversely, short chain and medium chain fatty acids (SCFAs and MCFAs) cross membranes by passive diffusion (Schönfeld and Wojtczak, 2016). Once in the mitochondrial matrix, acylcarnitines are reconverted into carnitine and acyl-CoA, the latter entering the β-oxidation cycle. As previously stated, these processes of generation and transport of LCACs are catalyzed by CPT1 and CPT2, with CACT facilitating the exchange of LCFAs and carnitine across the inner mitochondrial membrane (McCoin et al., 2015). It is important to notice that this process is bidirectional, as CPT2 and CACT can catalyze both the forward and the reverse reaction (Fig. 11). In fact, in case of incomplete FAO or of excess lipid availability overcoming the capacity of the TCA cycle, LCFA-CoAs accumulate and their respective LCACs can be formed back in the mitochondrial matrix and exported to plasma (Van Hove et al., 1993). Consequently, in physiologic conditions plasma/serum acylcarnitine profiles provide a snapshot of *in vivo* energy substrate flux through steps of FA and amino acids metabolism, depending on the specific acylcarnitine profile that is modified (Koves et al., 2008). In particular, even species of acylcarnitines from C6 to C22 arise from incomplete FAO whereas odd-chain acylcarnitines as C3 and C5 derive from amino acids catabolism, whereas C4 can derive from both FA and amino acid metabolism (Koves et al., 2008).

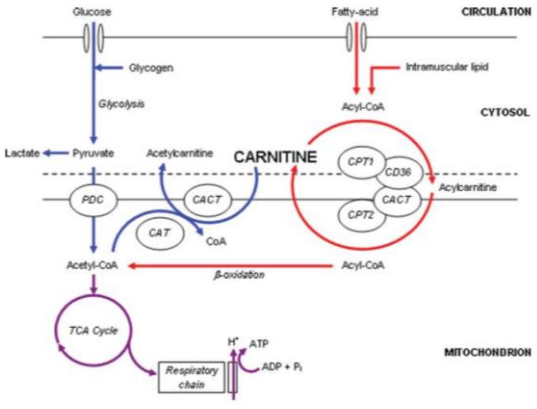

Figure 11. Diagram representing the role of carnitine in both lipid and CHO metabolism in skeletal muscle. Buffering role of carnitine in binding excess acyl-CoA groups is highlighted in blue while its role in transporting long chain fatty acids is indicated in red. PDC, pyruvate dehydrogenase complex; TCA, tricarboxylic acid cycle; CAT, carnitine acetyltransferase;

CACT, carnitine acylcarnitine translocase; CPT, carnitine palmitoyltransferase; CD36, fatty acid translocase (Stephens et al., 2007).

In pathologic conditions, acylcarnitine accumulation in biofluids as plasma and urine can be used as a biomarker for the diagnosis of oxidative disorders influencing mitochondrial and cellular acylcarnitine pool. Indeed, acylcarnitine profile determination is currently used in human medicine as a routine screening method for inborn metabolic errors (Van Hove et al., 1993; Chace et al., 2003). Excess acyl-CoA moieties that accumulate in mitochondria in case of increased FAO or of high glycolytic fluxes are known to be deleterious to cellular function (Brass, 2000; Stephens, 2018). By allowing the efflux of excess acyl groups, carnitine assumes a "buffer role", detoxifying mitochondria and avoiding their overload that could otherwise induce oxidative stress (Koves et al., 2008). Moreover, by tagging acyl-CoA carnitine preserves a viable pool of free CoA to permit continuation of pyruvate oxidation, thus guaranteeing a better matching of pyruvate dehydrogenase activity to glycolytic flux (Stephens 2018). Considering these premises, it is obvious how carnitine and acylcarnitine function extends to both lipid and CHO metabolism where they act as regulators to maintain metabolic flexibility.

3.1.1. Physiologic conditions affecting acylcarnitines profile

In humans and in physiologic conditions, the acylcarnitines profile is known to be influenced by metabolic status such as fasting and exercise (Frohlich et al., 1978; Hiatt et al., 1989). Research exists describing the effect of exercise on plasma and muscle acylcarnitines during exercise in humans (Hiatt et al., 1989; Lennon et al., 1983; Brass and Hiatt, 1994; Arenas et al., 1991) undergoing different exercise protocols. In early publications (1980s-1990s) the most common observation was that high-intensity exercise, and not low-intensity exercise, had an effect on acylcarntine kinetics in humans (Carlin et al., 1985; Decombaz et al., 1985; Hiatt et al., 1989; Sahlin, 1990; Constantin-Teodosiu et al., 1991; Spriet et al., 1992). These changes were more prominent in muscle than in plasma, where they were absent or poorly correlated to muscle kinetics (Hiatt et al., 1989; Brass and Hiatt, 1994). Nonetheless, controversial results exist, as some authors identified an increase in circulating MCACs and LCACs in response to exercise (Arenas et al., 1991; Lehmann et al., 2010) and others suggested that circulating acylcarnitines could be the result of an exchange with other organs and tissues such as the hepatic carnitine pool (Carlin et al., 1985; Soop et al., 1985). This poor correlation between plasma and tissues in terms of acylcarntine kinetics has been further strengthened by recent studies based on tandem mass spectrometry (MS/MS) and on multiorgan fluxes in different species and in different metabolic conditions (fasting, feeding, exercising) (Soeters et al., 2009; Schooneman et al., 2014; Schooneman et al., 2015). Nonetheless, these studies have underlined the flexibility of the circulating carnitine pool due to the contribution of other organs and tissues, as liver and heart in particular, and not only of skeletal muscle (Makrecka et al., 2014; Schooneman et al., 2014; Schooneman et al., 2015; Xu

et al., 2016). Thus, it can be concluded that changes in carnitine metabolism are tissue-specific, chain-length specific, and highly dependent on different factors such as exercise workload and metabolic status (feeding, fasting) of the individual. In horses, few publications exist describing the effect of exercise on acylcarnitine profiles. Once again, results vary depending on the compartment that is assessed (plasma or muscle) and on the type of exercise that is performed. Some authors have focused on muscle carnitine pool modifications induced by sprint exercise, reporting a fall in carnitine matched by an almost equal increase in acetylcarnitine (C2), thus highlighting the role of carnitine as acyl-groups buffer (Foster and Roger, 1987). Other authors have assessed plasma acylcarnitine profile in Standardbreds during exercise and training, observing that intense exercise induced an increase in circulating C2 and a decrease in the SCACs C3 and C4 while training had no effect on the carnitine pool. The authors concluded that C2, C3 and C4 kinetics in plasma were reflecting muscle kinetics, since other potential sources of these SCACs (*e.g*. ketogenesis and/or bacterial fermentation in colon) were unlikely (Westermann et al., 2008a). More recent research employed the arteriovenous difference technique to measure acylcarnitines utilization by the hindlimb of warmblood horses undergoing low-intensity exercise and after carnitine supplementation (Peters et al., 2015). In this study C3 showed, as previously demonstrated (Westermann et al., 2008a), the largest average extraction by the hindlimb musculature both at rest and after exercise, thus suggesting an important role of this acylcarnitine ester in equine hindlimb musculature. Other acylcarnitines as C6 and C14:1 showed also an increased extraction by the hindlimb at rest (C6, C14:1) and others after low-intensity exercise (C5:1, C16). Free carnitine and other acylcarnitines were released into circulation rather than extracted by the hindlimb or their extraction decreased with exercise. Nonetheless, extraction values of various acylcarnitines at rest compared to post-exercise were not significantly different, probably due to the low intensity of exercise. Thus, it can be argued that if the type of effort, in terms of intensity and duration, dictates the choice of the energy substrate, exercise should be sufficiently metabolically challenging to determine perturbations in acylcarnitine profile. For this reason, a recent study has analyzed the response of acylcarnitine profile to a highly metabolically demanding exercise represented by an endurance race of 160 km (van der Kolk et al., 2020). In fact, this type of effort requires substantial energy production for a prolonged period of time and in conditions of presumed glycogen depletion, thus when the equine organism rely almost entirely on β-oxidation of FA to produce ATP (Snow et al., 1981). This study described that a strenuous endurance effort of 160 km increased significantly mitochondrial β-oxidation by 8-fold, fueled by a 17-fold increase in lipolysis, as demonstrated respectively by an increase in serum acetylcarnitine concentration and in serum NEFA concentration. In particular, the circulating (and normalized) C6, C8, C10 among MCACs and C14:2 among LCACs, increased substantially, thus indicating that LCACs and SCACSs were likely oxidated more efficiently than medium-chain acetyl-CoAs (van der Kolk et al., 2020). In endurance dogs, as previously shown, several studies (McKenzie et al., 2005; McKenzie et al., 2007; McKenzie et al., 2008; Miller et al., 2015a, b; Miller et al., 2017; Davis et al., 2018; Davis et al., 2020) have been conducted to investigate their particular metabolic strategy allowing them to sustain

prolonged multiday exercise, but no information exists concerning acylcarnitine profile in these athletes and their response to exercise.

3.1.2. Pathologic conditions affecting acylcarnitine profiles

Acylcarnitine profiles can be influenced by pathologic conditions as diabetes, obesity, insulin resistance and cardiovascular diseases (Mihalik et al., 2010; Ramos-Roman et al., 2021; Kalim et al., 2013; Aguer et al., 2015; Xu et al., 2016). As previously stated, aceylcarnitines are widely used as biomarkers to screen for inherited metabolic diseases in humans as fatty acid and amino acids oxidation disorders (FAODs and organic acidemias) that can perturb acylcarnitine tissue and plasma concentration (Merritt et al., 2018). Their assessment in plasma has been also performed in horses suffering from rhabdomyolysis due to multiple acyl-CoA dehydrogenase deficiency (MADD) to help the understanding of its pathophysiology (Westermann et al., 2007; Westermann et al., 2008b; Lemieux et al., 2016). Moreover, there is also a growing body of literature investigating the effect of their accumulation and thus their involvement in diverse aspects of cellular pathophysiology, such as inflammation, insulin signaling in muscle, cellular stress and cardiac electrophysiology among others (McCoin et al., 2015). In particular, their role in major pathologies of our health medical system as diabetes, obesity and insulin resistance, has been advocated by several authors (Koves et al., 2008; Mihalik et al., 2010; Ramos-Roman et al., 2012; Schooneman et al., 2013; Aguer et al., 2015; Sun et al., 2016; Nowak et al., 2018). In fact, plasma LCACs concentrations are higher in obese and insulin-resistant individuals compared to lean controls (Mihalik et al., 2010). Upon infusion of insulin, that has an inhibitory effect on lipolysis, LCACs in diabetic and insulin-resistant subjects decrease to a lesser degree than in obese (and not diabetic) controls (Mihalik et al., 2010). Thus, elevated LCACs in diabetic and insulin-resistant patients reflect increased lipid flux and demonstrates the close connection between FAO and acylcarnitines. Concerning the origin of IR, previous models have proposed that muscle IR arises from impaired or decreased mitochondrial uptake and FAO, due to defects in mitochondrial OXPHOS activity, leading to an increased cytosolic accumulation of lipids (Petersen et al., 2004; Morino et al., 2006; Muoio et al., 2012). As a consequence, LCACs derived from circulating lipids or from IMTG would be diverted away from CPT1 and directed towards the synthesis of lipid intermediates such as ceramides and diacylglycerol (DAG). These metabolites have been held responsible for the development of IR (Ruderman et al., 1999; Morino et al., 2006; Holland et al., 2007). In fact, they are signaling molecules and building blocks of cell membranes, where the insulin receptor is located (Schooneman et al., 2013). Likewise, dysregulation of malonyl-CoA fuel-sensing and signaling mechanisms, resulting in an abnormally high concentration of malonyl-CoA that is, as previously described, a potent inhibitor of CPT1, has also been advocated (Ruderman et al., 1999). Malonyl-CoA would block LCFAs mitochondrial entrance and thus decrease FAO and increase cytosolic lipid accumulation.

Alternatively, recent works have proposed a different model, suggesting that excessive rather than reduced β-oxidation would induce IR. In this case, IR would be linked to an intra-mitochondrial rather than to a cytosolic disturbance and overload (Koves et al., 2008). According to this theory, lipid overload would increase FAO flux in muscle, rendering it in relative excess to oxidation in TCA and to respiratory complex activity. This mismatch between FAO and TCA cycle would lead to depletion of TCA intermediates and to the development of an imbalanced environment, further exacerbating incomplete β-oxidation. This latter process would lead ultimately to the accumulation of noxious acyl-CoAs, of their respective acylcarnitines and other metabolites contributing to mitochondrial overload, stress, and failure (Koves et al., 2008). Another potential scenario is a direct implication of acylcarnitines in insulin signaling. In fact, acylcarnitines are amphipathic molecules, as such they can reside in cell membranes; moreover, increasing chain length favors partitioning into the membrane phase, especially for acylcarnitines with 16-18 C atoms (Ho et al., 2002; Schooneman et al., 2013). Thus, acylcarnitine efflux in plasma may reflect a failed attempt to alleviate stress caused by mitochondrial overload (Koves et al., 2008). Even if this theory is attractive, it has to be reminded that the role of acylcarnitine in IR remains a theorical speculation and no scientific proof exists to date. Nonetheless, the tight connection of acylcarnitines and FAO strongly suggests an association between IR and acylcarnitine accumulation that may either inflict or simply reflect IR (Schooneman et al., 2013). Indeed, recent research has shown that a panel of plasma LCACs were significantly associated with future risk of type 2 diabetes, suggesting their utility as early predictors in diabetes risk assessment (Sun et al., 2016).

3.2. Assessment of mitochondrial function by high resolution respirometry

Mitochondria play a crucial role in eukaryotic cellular metabolism. One of their main roles is ATP production via OXPHOS. Energy substrates provide electrons to the electron transport system (ETS), allowing the formation of a proton gradient in the mitochondrial intermembrane space. Reduced forms of nicotinamide adenine dinucleotide ($NADH + H^+$) and of flavine adenine dinucleotide ($FADH_2$) originating primarily from glycolysis, TCA and ß-oxidation cycles, deliver their electrons at different levels of the ETS. The ETS, that is composed of four complexes (CI to IV) and two mobile carriers, an ubiquinone pool (Q-junction) which serves to transfer electrons to CIII and a peripheral protein (cytochrome *c*) that transfers electrons from CIII to CIV. The flow of electrons through CI, CIII and CIV is coupled to the release of protons from the mitochondrial matrix to the intermembrane space thus creating an electrochemical gradient that is finally used by the turbine-like ATP synthase to generate ATP molecules while reducing oxygen into H_2O. Thus, oxygen is consumed while ATP is synthetized. Oxygen acts as an essential component being the final electron acceptor in the ETS (Fig. 12).

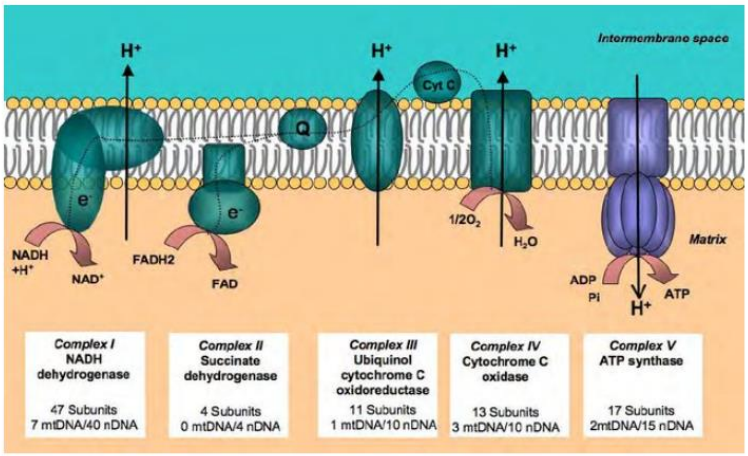

Figure 12. Mitochondrial respiratory system in mammals, consisting of five enzyme complexes (complexes I-V) and two intermediary substrates (Q-junction and cytochrome *c*). Oxidation of NADH+H$^+$ and FADH$_2$, produced by intermediate metabolism, produces a vertical proton pumping coupled to the horizontal electron transport through the complexes. This electrochemical gradient is ultimately used by ATP synthase (complex V) to produce ATP (Bellance et al., 2009).

The principle of HRR, an advanced diagnostic tool assessing mitochondrial function, consists in the application of multiple substrate-uncoupler-inhibitor titration (SUIT) protocols either to permeabilized muscle fibers obtained by muscle biopsy or to isolated mitochondria or to cultured cells. These protocols differ in the type of substrate that is used and/or their sequence. In horses, two protocols have been mainly applied on muscle samples taken by microbiopsy and mechanically as well as chemically permeabilized (Votion et al., 2010; Votion et al., 2012; Lemieux et al., 2016; Houben et al., 2015). Glutamate is the first substrate in SUIT1, pyruvate in SUIT2 using malate as cosubstrate in both protocols. To these substrates ADP is added in conditions of sufficient oxygen provision, electron flow through CI of the respiratory system is sustained. In both SUIT protocols, cytochrome *c* is used to test the integrity of the outer mitochondrial membrane following the permeabilization procedure. Injury of the outer mitochondrial membrane leads to loss of cytochrome *c* from the mitochondria, and to significant stimulation of respiration following addition of exogenous cytochrome *c* to the respiration medium. Absence of significant increase of mitochondrial respiration confirms a good preservation of mitochondrial respiratory function.Then succinate is used in both protocols to support electron flow through CI and CII into the Q-junction, thus reaching maximal OXPHOS capacity (CI+CII linked respiration). At this point, a specific uncoupler (carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone, or FCCP) is gradually added to collapse the proton gradient across the inner mitochondrial membrane to estimate the maximal capacity of ETS that is now uncoupled from ATP synthesis. Rotenone is then added to inhibit CI so as to obtain CII linked respiration alone, whereas antimycin A blocks electron transfer to CIII to obtain residual oxygen consumption (ROX) that is due

to residual oxidative side reactions (Fig. 13). This substrate control with electrons entering separately through CI and CII (via substrates and uncouplers) restricts ETS capacity giving diagnostic information on some of its pathway branches (Pesta and Gnaiger, 2012).

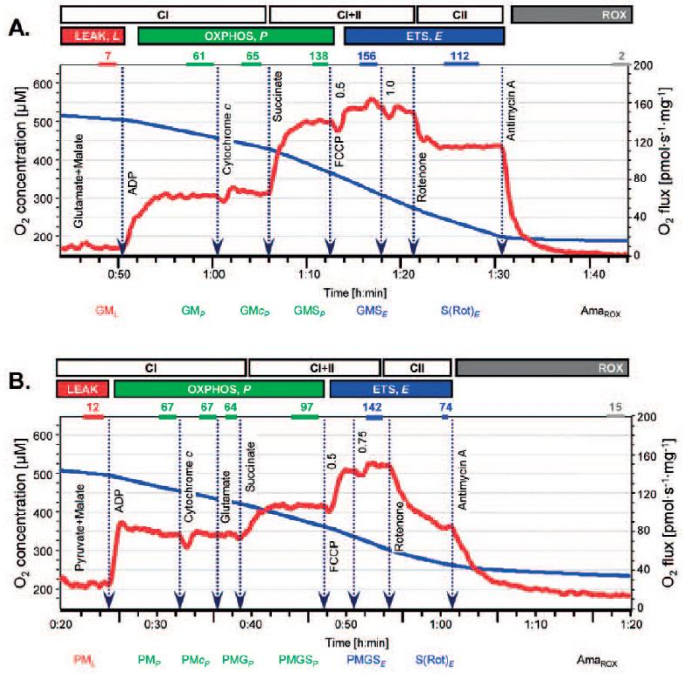

Figure 13. Schematic representation of the two multiple substrate-uncoupler-inhibitor titration (SUIT) protocols applied to permeabilized muscle fibers. Image A represents SUIT1, where electron flow through CI is supported by glutamate and malate; image B represents SUIT2 where electron flow through CI is supported by pyruvate, malate and glutamate. Blue and red lines represent oxygen concentration (µM) and muscle mass-specific oxygen flux (pmol $O2 \cdot s^{-1} \cdot mg^{-1}$ wet weight), respectively. Marked sections correspond to steady-state fluxes at different steady-states and substrate states (Votion et al., 2010).

3.2.1. Application of righ-resolution respirometry to physiologic conditions

Mitochondria are characterized by an impressive plasticity, as their functional properties can adjust to increased metabolic demand and to changing environmental conditions. An increase in mitochondrial density and efficiency in mammalian skeletal muscle is a well-known adaptation to active lifestyle and endurance training (Holloszy, 1967; Hoppeler et al., 1985; Turner et al., 1997; Hoppeler and Fluck, 2003; Meinild Lundby et al., 2018). As a matter of fact, HRR has been employed to further investigate this adaptation in both human and equine species. Respirometric analyses in humans demonstrate that 6 to 10-weeks endurance training programs result not only in quantitative changes of skeletal muscle respiration, but also in qualitative modifications. These improvements consist in an increased maximal mitochondrial oxidative power and lipid oxidation capacity, as observed in both isolated mitochondria and permeabilized muscle fibers (Tonkonogi et al., 2000; Walsh et al., 2001; Pesta et al., 2011). On the contrary, acute high-intensity exercise seems to compromise skeletal muscle oxidative phosphorylation capacity in permeabilized muscle fibers (Layec et al., 2018). Aerobically trained individuals exhibit in their skeletal muscle a better coupling between oxidation and phosphorylation and improved regulatory properties of mitochondrial respiration when compared to sedentary subjects. Thus, muscle aerobic performance is not only set by an increased mitochondrial density but also by qualitative changes, that

are linked to the level of physical activity of the individual (Zoll et al., 2002). High-resolution respirometry has also been applied in horses to investigate the effect of fitness status, of endurance training and racing on mitochondrial function. As observed in humans, respirometric parameters are higher in athletic horses compared to untrained and overweight horses (Votion et al., 2012); 10 weeks of endurance training further improve muscle mitochondrial respiration, even if modifications seem to be muscle-specific (more pronounced in the *triceps brachii, tb,* muscle than in the *gluteus medius, gm*) (Votion et al., 2010). The effect of endurance racing has also been evaluated revealing a decrease in OXPHOS and ETS with associated exercise-induced muscle damage (Votion et al., 2010). If in aerobically trained humans and horses' main adaptations to exercise are represented by an increased use of fat as energy substrate (Yeo et al., 2011; Le Moyec et al., 2019) and by an increase in mitochondrial biogenesis, in endurance dogs these adaptations are different. Indeed, sled dogs do not experience an increase in the rate of mitochondrial protein synthesis during a training program but, as other canids, they have already a very high rate of skeletal muscle protein synthesis in an untrained state compared to humans (Miller et al., 2015). This high basal rate of protein synthesis likely allows rapid remodeling during exercise without the necessity to further increase protein synthesis. Indeed, HRR has pointed out that mitochondrial remodeling in sled dogs undergoing prolonged exercise results in a significant upregulation of substrate-driven coupled and uncoupled respiratory fluxes (OXPHOS and ETS). Moreover, it has shown that sled dogs have the highest peak respiratory capacities ever reported for mammalian skeletal muscle, exceeding those of highly fit humans and competitive endurance horses (Miller et al., 2017). In terms of substrate use, HRR has further supported the hypothesis that the main adaptation of sled dogs is the increased capacity to oxidize CHO instead of FA during prolonged exercise, as indicated by an enhancement of NADH-linked (primarily CHO) substrate utilization in racing dogs and by a decrease in coupled respiration with only fat as substrate. Thus, HRR has revealed to be a useful tool to assess mitochondrial adaptations in sled dogs to explain their quick adjustment to the environmental and metabolic challenge represented by prolonged multiday exercise in extreme cold (Miller et al., 2017).

3.2.2. Application of high-resolution respirometry to pathologic conditions

Even if they are best known as "ATP-producers", mitochondria play also a fundamental role as metabolic signaling centers. Indeed, they intervene in apoptosis signaling; they control, through the production of reactive oxygen species (ROS), cell proliferation and differentiation; they regulate cellular levels of metabolites, amino acids and cofactors for various regulatory enzymes; they play an essential role in metal metabolism (by the synthesis of heme and Fe-S clusters); they participate in Ca^{2+} homeostasis; they act as energy sensors adapting their biogenesis as a function of increasing metabolic needs (reviewed in Nunnari and Suomalainen, 2012). Thus, the variety of roles that mitochondria play in tissues and cells implicates also diverse mitochondrial diseases. An impairment of mitochondrial

activity has been underlined as a key factor in a variety of human neurodegenerative and metabolic pathologies, cardiomyopathies, cancer and aging; in this context, high resolution respirometry has been employed in several studies to assess the effect of pathologic processes on mitochondrial functionality (Sperl et al., 1997; Hütter et al., 2006; Scheiber et al., 2019; Evinova et al., 2020; Risiglione et al., 2020). Changes in mitochondrial activity, alterations in respiratory control ratios and modifications of the effect of inhibitors on cellular respiration can reflect significant mitochondrial damage (Hütter et al., 2006). Of particular interest for our research, some human GSD have also revealed features of mitochondrial dysfunction depending on the GSD type (De Stefano et al., 1996; Selak et al., 2000; Kurbatova et al., 2014; Lim et al., 2015; Farah et al., 2017; Rossi et al., 2018) suggesting its likely contribution to exercise-related symptoms, even if underlying mechanisms still remain poorly understood. Indeed, many factors have been cited as potential contributors in the pathophysiology of mitochondrial dysfunction: decreased mitochondrial content and/or impaired biogenesis; altered substrate delivery; hypoxia; muscle fibers inflammation; morphological distortion of mitochondria due to glycogen cytoplasmic accumulation; mitochondrial damage induced by glucotoxicity and lipotoxicity secondary to excessive intracellular CHO or lipids; activation of mitochondrial apoptosis cascade; down-regulation of nuclear and mitochondrial genes (Selak et al., 2000; Lim et al., 2015; Farah et al., 2017). Despite these findings, application of HRR in GSD research is still scarce. Regarding HRR and equine muscular diseases, there is only one study assessing by HRR the mitochondrial function of subjects affected by atypical myopathy (AM), describing a severe depression of mitochondrial respiration (Lemieux et al., 2016). Another study realized in French Standardbred racehorses reports an association between altered mitochondrial energy metabolism and the predisposition to develop exertional rhabdomyolysis (Houben et al., 2015). Once again, several and diverse pathologic processes can underlie these observations (decreased mitochondrial mass, ultrastructural skeletal muscle changes disturbing mitochondrial morphology, and the inhibitory effect of accumulated toxic acyl-CoAs in AM). To date, there is no study assessing mitochondrial function in horses suffering from PSSM. In dogs, there is no study to date employing HRR in pathologic metabolic conditions.

Objectives

High-resolution respirometry and acylcarnitines profile are useful tools that can help the understanding of both physiologic and pathologic metabolic processes.

Our first aim was to perform specific functional assessment of mitochondrial respiration in horses affected by type-1 PSSM. We wanted to contribute to the understanding of the link between abnormal glycogen storage and impaired energy metabolism characterizing this disease, based on the hypothesis that mitochondrial function would be impaired in muscles of horses with type-1 PSSM. We wanted to compare mitochondrial respiration of PSSM horses with that of healthy controls and of horses suffering from exertional rhabdomyolysis of unknown origin.

Then, in a second time, we wanted to measure plasma concentration of acylcarnitines in type-1 PSSM-affected horses at rest and after submaximal exercise, and to compare their kinetics to those of healthy horses performing the same type of exercise. It is commonly recognized that horses affected by type-1 PSSM suffer from a deficit in energy metabolism. Acylcarnitine profiles are useful biomarkers of oxidative disorders and indicators of metabolic flexibility. For this reason, we wanted to determine if plasma acylcarnitine profiles in horses with type-1 PSSM were significantly affected by exercise and could reflect an attempt of mitochondria to avoid overload by clearing out in the systemic circulation excessive acyl-CoA moieties.

Furthermore, we were interested in employing blood acylcarnitines measurement in healthy endurance Alaskan sled dogs, with the hypothesis that these biomarkers could provide further information on the particular metabolic strategy developed by these highly-aerobic, fatigue-resistant athletes during prolonged multiday exercise.

Experimental section

Experimental section

Study 1 :

Altered mitochondrial oxidative phosphorylation capacity in horses suffering from polysaccharide storage myopathy

Introduction to study 1

Type-1 PSSM is a cause of exertional rhabdomyolysis in horses. Genetically, this disease is caused by an autosomal dominant gain-of-function mutation of the glycogen synthase 1 gene (*GYS1*) coding for GS enzyme (MCue et al., 2008a), rendering the enzyme constitutively active (Maile et al., 2017) and leading to abnormal accumulation in myofibers of polysaccharide.

Metabolically, type-1 PSSM is characterized by impaired oxidative metabolism, by inability to generate or to use acetyl-CoA, by enhanced insulin sensitivity and blood glucose uptake with submaximal exercise (Valberg et al., 1995; De La Corte et al., 1999b, Valberg et al., 1999a; Annandale et al., 2004; Annandale et al., 2005). Down-regulation of several genes involved in mitochondrial activity as well as signs of mitochondrial degeneration have also been described in horses affected by type-1 PSSM (Barrey et al., 2009). Thus, PSSM-affected horses suffer from a genetic defect and from an energy deficit, but the link between these two aspects, and thus between genotype and phenotype, remains unclear. Mitochondria play a central role in cellular energetics and their involvement has been pointed out in human glycogen storage disorders (De Stefano et al., 1996; Selak et al., 2000; Kurbatova et al., 2014; Lim et al., 2015; Farah et al., 2017).

Our first study aimed at assessing mitochondrial function in PSSM-horses by the mean of HRR, with the hypothesis that mitochondrial function would be impaired in myofibers of affected horses. We compared respirometric paramaters of PSSM-horses with those of healthy controls and of horses affected by rhabdomyolysis of unknown origin. We detected significant bioenergetical impairment in mitochondria of type-1 PSSM-affected horses, these changes varied depending on the muscle analyzed and the energetic substrate employed to feed the electron transfer system.

With this study, we proposed the application of an innovative tool, represented by HRR, to investigate the metabolic profile of type-1 PSSM-affected horses. We suggested that modifications in mitochondrial function of PSSM horses can participate to the genesis of clinical symptoms and can help to better understand its pathophysiology. We also advocated the employment of HRR in other pathologies characterized by energetic imbalance and mitochondrial dysfunction in veterinary species.

Experimental section

Study 1

Altered mitochondrial oxidative phosphorylation capacity in horses suffering from polysaccharide storage myopathy

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Altered mitochondrial oxidative phosphorylation capacity in horses suffering from polysaccharide storage myopathy

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Abstract
Polysaccharide storage myopathy (PSSM) is a widely described cause of exertional rhabdomyolysis in horses. Mitochondria play a central role in cellular energetics and are involved in human glycogen storage diseases but their role has been overlooked in equine PSSM. We hypothesized that the mitochondrial function is impaired in the myofibers of PSSM-affected horses. Nine horses with a history of recurrent exercise-associated rhabdomyolysis were tested for the glycogen synthase 1 gene (*GYS1*) mutation: 5 were tested positive (PSSM group) and 4 were tested negative (horses suffering from rhabdomyolysis of unknown origin, RUO group). Microbiopsies were collected from the *gluteus medius* (*gm*) and *triceps brachii* (*tb*) muscles of PSSM, RUO and healthy controls (HC) horses and used for histological analysis and for assessment of oxidative phosphorylation (OXPHOS) using high-resolution respirometry. The modification of mitochondrial respiration between HC, PSSM and RUO horses varied according to the muscle and to substrates feeding OXPHOS. In particular, compared to HC horses, the *gm* muscle of PSSM horses showed decreased OXPHOS- and electron transfer (ET)-capacities in presence of glutamate&malate&succinate. RUO horses showed a higher OXPHOS-capacity (with glutamate&malate) and ET-capacity (with glutamate&malate&succinate) in both muscles in comparison to the PSSM group. When expressed as ratios, our results highlighted a higher contribution of the NADH pathway (feeding electrons into Complex I) to maximal OXPHOS or ET-capacity in both rhabdomyolysis groups compared to the HC. Specific modifications in mitochondrial function might contribute to the pathogenesis of PSSM and of other types of exertional rhabdomyolyses.

Keywords Polysaccharide storage myopathy · Exertional rhabdomyolysis · Microbiopsy · High-resolution respirometry

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Introduction

Muscular disorders represent a common cause of disability and of poor athletic performance in horses (Aleman 2008; Martin et al. 2000). Among them, polysaccharide storage myopathy (PSSM) is widely recognized as a cause of recurrent episodes of muscle disease. Clinical symptoms and results of traditional complementary exams such as muscle enzymes measurement and urinalysis are generally not very useful for the specific diagnosis of PSSM, while muscle biopsy is considered an essential tool for this purpose. Characteristic features of PSSM on histological examination are the presence of abnormal accumulations of glycogen under the cell membrane of myofibers or in the cytoplasma. This glycogen is typically stained ("positive") by the periodic-acid Schiff (PAS) staining and it can be resistant or sensitive to enzymatic digestion with amylase (Valberg et al. 1992; McCue et al. 2006). In 2008, a major advance was made with the discovery of a dominant gain-of-function mutation in the glycogen synthase 1 gene (*GYS1*) coding for glycogen synthase (*GS*) enzyme (McCue et al. 2008a, b). This mutation is associated to a conformational change perturbing the *GS* activity in skeletal muscle, rendering the enzyme constitutively active (Maile et al. 2017), thus leading to abnormal polysaccharide inclusions in myofibers (McCue et al. 2008a, b; Stanley et al. 2009). The discovery of the mutation in the (*GYS1* gene coding for the *GS* enzyme allowed the development of a sensitive and specific genetic test. Based on the genetic testing, PSSM mutation has been observed histologically in 16% of horses with amylase-sensitive polysaccharides and in 70% in horses with amylase-resistant polysaccharides (McCue et al. 2008a). Indeed, it has been suggested that two forms of PSSM exist, even if they share the same histological features: a type-I PSSM, positive to *GYS1* mutation and a type-II PSSM, lacking *GYS1* mutation (McCue et al. 2009; Stanley et al. 2009). Besides recent evidence of a genetic mutation at the origin of this glycogenosis, the whole metabolic puzzle underlying the PSSM phenotype is still incomplete. Impaired energy transformation in PSSM-affected muscles and reduced maximal oxygen consumption per body mass (V_{O2max}) in these horses, in comparison to healthy controls, have been documented (Valberg et al. 1995; Annandale et al. 2005). This energetic imbalance was originally thought to result from a limitation in glycolysis and not from a limitation in oxygen transport (Valberg et al. 1995). In contrast to human glycogen storage diseases (GSD), however, PSSM-affected horses showed no deficiencies in glycolytic or glycogenolytic enzymes (Valberg et al. 1998). It has been reported that muscles of affected horses show no change in post-exercise levels of pyruvate and ATP, but measures were realized on whole muscle homogenates and not on individual fibers (Annandale et al. 2005; Borgia et al. 2010). These researches have suggested that PSSM muscle could be energetically compromised, but the precise site of dysfunction is still unclear. Studies of individual muscle fibers in animals affected by metabolic myopathies have been advocated so as to better localize the metabolic failure (Annandale et al. 2005; Borgia et al. 2010; Valberg et al. 1998).

Mitochondria play a crucial role in eukaryotic cellular metabolism, producing ATP through the oxidative phosphorylation (OXPHOS) process. In human medicine, impairment of mitochondrial activity has been underlined as a key factor in a variety of neurodegenerative and metabolic diseases, cardiomyopathies and cancer (Nunnari and Suomalainen 2012). Of particular interest, some human GSD have also revealed features of mitochondrial dysfunction suggesting its likely contribution to exercise-related symptoms. These abnormalities have been observed in both mitochondrial morphology and bioactivity; specific causes and features of mitochondrial impairment vary depending on the specific GSD type (De Stefano et al. 1996; Selak et al. 2000; Kurbatova et al. 2014; Lim et al. 2015; Melis et al. 2016; Rossi et al. 2018). Concerning specifically equine PSSM, in one study (Barrey et al. 2009) a down-regulation of many genes involved in mitochondrial activity as well as signs of mitochondrial degeneration have been observed in horses affected by type-I PSSM. More precisely, this down-regulation involved most of the mitochondrial tRNA, genes coding for the respiratory chain sub-units (ND2, ND3, ND5, ND6, COX2 and COX3), nuclear genes involved in the aerobic metabolism of mitochondria (GNAS, SLC2a2, ATP5L, ATP5J, ATP5D and ATP5H), glucose transporter GLUT2 and mitochondrial carrier ornithine transporter (SLC25A15). An up-regulation of pro-inflammatory genes (as IL-18 among others) was observed, thus indicating chronic muscle inflammation. Moreover, ultrastructural changes were also detected in mitochondria and muscle fibers: decreased cristae number, mitochondrial swelling, formation of myelinic bodies and severe mitochondrial and myofibrillar loss due to abnormal glycogen accumulation. A down-regulation of VEGFα, involved in capillarization and oxygen distribution of regenerated muscle fibers was also observed.

Up to now, specific functional assessment of mitochondrial respiration in muscles of PSSM-affected horses has not been performed. This study uses high resolution respirometry (HRR), an advanced diagnostic tool already employed to detect mitochondrial dysfunctions in human (Sperl et al. 1997; Gehrig et al. 2016) and horse diseases (Votion et al. 2010; Votion et al. 2012; Houben et al. 2015). The aim is to determine the functional changes in muscle of horses suffering from PSSM horses by comparing them with healthy controls (HC) and with horses suffering from exertional rhabdomyolysis of unknown origin (RUO). We hypothesized that the mitochondrial function is impaired in PSSM-affected horses. On the basis of what is described in literature, we expected to find

a decreased mitochondrial respiration in horses affected by PSSM when compared to healthy horses.

Materials and methods

Horses and complementary exams

Nine horses of different breeds (2 French Saddle, 1 Paint Horse, 2 light draft horses, 1 Quarter Horse, 1 Belgian Warmblood, 1 Holstein, and 1 crossbred horse, mean age 9.1 ± 5.1 years old, mean weight 537 ± 76.1 kg) were referred between October 2011 and October 2014 to the Faculty of Veterinary Medicine at the University of Liège with a history of exercise-induced recurrent rhabdomyolysis. Inclusion criteria for the study were the presence of one or more of clinical symptoms as exercise intolerance, stiffness, sweating, muscle trembling, reluctance to move and gait abnormalities and when possible a blood sample collected within 24 h after the beginning of symptoms revealing an abnormal increase of serum creatinine kinase (CK) activity (Aleman 2008). In our study in 8 cases out of 9 a blood sample had been done by the treating veterinarian. Eight horses out of 9 were referred for a poor performance and/or suspicion of rhabdomyolysis, several days or even weeks after the last episode of myopathy. One horse (horse 9) was referred for acute rhabdomyolysis in an emergency setting. A complete clinical examination was performed so as to exclude other potential non-muscular pathologies. Blood was collected by jugular venipuncture for hematological and biochemical analyses and to measure vitamin E and blood electrolytes, as vitamin E deficits as well as abnormal electrolyte clearances can predispose to recurrent rhabdomyolysis. Urine was collected to detect pigmenturia (using commercial urine dipstick tests Krulab, Kruuse, Denmark) and to perform a fractional electrolyte excretion test so as to evaluate horses' electrolyte balance (for Na^+, K^+ and Cl^-). Clinical data relative to the history of each horse are summarized in Table 1. A blood sample on EDTA was sent for genotyping at the Comparative Neuromuscular Disease Laboratory of the Royal Veterinary College in London. Muscle biopsies were taken from the *gm* and *tb* muscles by means of the microbiopsy technique. These muscles were chosen because of their different function in locomotion and their different fiber composition (Van den Hoven et al. 1985). Moreover, *gm* muscle is one of the primarily and most severely affected muscles in cases of PSSM (Valentine 2003). If the horse was considered clinically sound the day of the consultation, a 12-min exercise test of moderate intensity (trot and canter) on the high-speed treadmill or on a lunge line was performed in order to measure post-exercise CK, 4 h after the end of exercise. Owners gave their consent to use muscle biopsies of their horses for HRR and to use clinical data for this study.

Muscle tissue sampling

Biopsy sampling procedure was approved by the Animal Ethic Commission of the University of Liege (agreement number 07–629). An average of 20 mg of muscle tissue was collected at a 50 mm depth from the *tb* and *gm* muscles using a 14 G biopsy needle mounted on an automatic instrument (Pro-Mag™ Ultra Biopsy Instrument, Angiotech, Gainesville, Fl, USA). The sampling site was shaved (1 cm^2). Skin was desensitized with 0.5 ml of mepivacaine (Scandicaine® 2%, AstraZeneca, Brussels, Belgium), strictly injected under the skin not to interfere with muscle mitochondrial energetics (Nouette-Gaulaina et al. 2011). The zone was aseptically prepared with povidone iodine and alcohol. After a skin incision with a scalpel blade n. 11, muscle samples were taken in the long head of the *tb* muscle determined as the intersection

Table 1 Breed, age, weight, sex, body condition score (BCS) according to the Henneke system, history details and muscle enzymes activity of the 9 horses included in the study

Horse	Breed	Age (y.o.)	Weight (kg)	Sex	BCS	Activity (level)	CK values (UI/l)	Frequency of episodes
1	French Saddle	7	560	F	5/9	Jumping (intermediate)	3400	1/month
2	Paint Horse	6	430	F	6/9	Jumping and dressage (intermediate)	1600	1/week
3	Quarter Horse	7	520	M	7/9	Reproduction and pleasure (intermediate)	162,720	1 over 1 year
4	Light draft horse	4	590	F	7/9	Pleasure (light)	500	2 over 1 year
5	Light draft horse	4	622	F	7/9	Pleasure (light)	9672	2 over 1 year
6	Holstein	14	606	G	5/9	Jumping (high)	3000	2 over 1 year
7	French Saddle	7	430	F	4/9	Jumping and dressage (intermediate)	2520	2 over 1 year
8	BWP	16	600	F	5/9	Eventing (intermediate)	nm	2 over 3 years
9	Cross-bred	17	475	G	4/9	Pleasure (light)	8577	3 over 2 years

BWP, Belgisch Warmbloedpaard (Belgian Warmblood); *F*, female; *G*, gelding; *M*, male; *nm*, not measured; *CK*, creatine kinase (reference values: 50–200 UI/l)

between a vertical line raised from the *tricipital line* and a line running from the point of the shoulder to the elbow, as previously described (Votion et al. 2010; Votion et al. 2012). Biopsy on the *gm* muscle was performed one-third the distance along a line running from the *tuber coxae* to the root of the tail (Lindholm and Piehl 1974). The small skin incision was not closed but only disinfected after the procedure. Biopsies were well tolerated and sedation was not required apart from one particularly reactive horse that received dose of 0.01 mg/kg of detomidine (Domidine®, Eurovet Animal Health, Belgium).

Muscle samples were immediately transferred into 10 ml of ice-cold relaxing solution BIOPS, a special solution for preservation of muscle biopsy for the assessment of mitochondrial function (Letellier et al. 1992; Veksler et al. 1987), or into buffered 10% formalin for histological analysis. Fibers were transported on ice to the laboratory, then stored at 4 °C until further preparation and analyzed within 24 h from sampling.

Permeabilized muscle fiber preparation

Muscle samples kept into ice-cold BIOPS were separated mechanically using two pairs of forceps and connective tissue was removed. Permeabilization of plasma membrane was ensured by agitation during 30 min at 4 °C in 2 ml of BIOPS solution containing 50 μg/ml saponin. Fiber bundles were rinsed by gentle agitation for 10 min in ice-cold mitochondrial respiration medium MiR05 (Gnaiger et al. 2000). Permeabilized muscle fibers were immediately used for HRR.

High-resolution respirometry

Two to 3 mg wet weight (W_w; microbalance; Mettler Toledo, Zaventem, Belgium) of permeabilized muscle fibers were added to respiration chambers (Oroboros Oxygraph-2 k, Innsbruck, Austria) containing 2 ml of MiR05 at 37.0 °C. Mitochondrial OXPHOS- and electron transfer (ET)-capacities were determined in permeabilized fibers using two substrate-uncoupler-inhibitor-titration (SUIT 1 and SUIT 2) protocols as previously described (Pesta and Gnaiger 2012; Votion et al. 2012). These two protocols differed basically by the first substrates added to chambers to initiate the mitochondrial respiration. In SUIT 1, glutamate&malate (GM; 10 and 2 mM) were first added, while in SUIT 2 pyruvate&malate (PM; 5 and 2 mM) were initially used. When adding ADP (2.5 mM) in presence of these NADH-linked substrates (N), electrons flow through Complex I (N-OXPHOS capacity: GM and PM in SUIT 1 and SUIT 2, respectively). Whereas both SUIT 1 and 2 measure the capacity of Complex I, they involve different dehydrogenase and transporters. N-OXPHOS capacity relates on glutamate dehydrogenase for SUIT 1 and on pyruvate dehydrogenase complex (PDC) and pyruvate transporter for SUIT 2. Then, it was checked that the measured oxygen consumption remained at the same level after addition of cytochrome *c* to ensure that the preservation or permeabilization procedures had not damaged the outer mitochondrial membrane. In SUIT 2, OXPHOS was subsequently stimulated by addition of another NADH-linked substrate, i.e. glutamate (G; 10 mM) thus obtaining N-OXPHOS capacity with PMG. The following steps were common to both SUIT protocols. The addition of succinate (S; 10 mM), a $FADH_2$-linked substrate, allowed the measurement of OXPHOS when electron transfer in the NADH- and succinate pathways (NS pathway) converged at the Q-junction (NS pathway OXPHOS-capacity): GMS and PMGS in SUIT 1 and 2, respectively. At this step, the ETS is coupled to the phosphorylation system and proton pumps generate an electrochemical potential that drives phosphorylation of ADP to ATP (coupled flow). A stepwise addition of protonophore, FCCP (0.05 μM followed by 0.025 μM steps until reaching maximal oxygen flux), was then performed to uncouple the ETS from ATP synthesis to measure the capacity of the ET (NS pathway ET-capacity; GMS in SUIT 1 and PMGS in SUIT 2). The NS pathway ET-capacity is not limited by the substrate or the phosphorylation system and thus, gives an estimate of maximal capacity for electron transfer. Then rotenone (Rot; 0.5 μM) was added to inhibit Complex I so as to obtain S-sustained respiration alone (S pathway ET-capacity). Finally, antimycin A (Ama; 2.4 μM) was added to block the ETS at Complex III thus obtaining residual oxygen consumption (ROX) due to oxidative side reactions. Oxygen concentration (μM) and oxygen fluxes per muscle mass (pmol $O_2 \cdot s^{-} \cdot mg^{-1}$ W_w) were recorded and analyzed using Datlab software (Oroboros Instruments, Innsbruck, Austria). During the SUIT protocols, oxygen levels were maintained above air saturation (between 200 and 500 μM O_2) to avoid experimental oxygen limitation (Gnaiger 2014). Oxygen fluxes were corrected for the flux of oxygen due to instrumental background and for ROX.

Steady-state oxygen fluxes were expressed as flux control ratios, *FCR*, with internal normalization for ET-capacity with convergent electron input into the NADH and succinate pathways (NS pathway OXPHOS-capacity). The *FCR* give punctual information about coupling and substrate control independent of mitochondria content of tissue. Substrate control ratios (*SCR*) were also calculated in each SUIT protocol; while *FCR* are calculated at a constant substrate state, *SCR* are flux ratios at constant coupling state (LEAK, OXPHOS, ET). The phosphorylation system control ratio (OXPHOS/ET) was also calculated as it represents an expression of the gap existing between OXPHOS and ET-capacity (Votion et al. 2012). Respirometric measurements of each specimen were performed in duplicate for each protocol. Data were compared with values previously obtained from healthy, fit horses (HC), but not in competitive training. Ten horses were used as HC for SUIT 2 and 8 were available for SUIT 1, with 2 to 4

measurements for each horse. Unfortunately, we had no available data for the *gm* muscle of HC in SUIT 2.

Muscle histopathology

Muscle biopsies fixed in 10% buffered formalin were trimmed and then embedded in paraffin wax according to standard laboratory methods. Tissue sections of 5 μm were stained with hematoxylin and eosin (HE), PAS, amylase-PAS, and then examined by light microscopy. The HE stains were used for evaluating the presence of pathological changes such as: fiber swelling, Zencker degeneration/necrosis, internalized nuclei, atrophy and subsarcolemmal or intracytoplasmic vacuoles. For the amylase-PAS stain, sections were digested with α-amylase following the protocol furnished by the producer (Sigma-Aldrich Steinheim, Germany, procedure n°395).

Genotyping

Genotyping was performed at the Comparative Neuromuscular Diseases Laboratory of the Royal Veterinary College in London. A DNA sample from EDTA blood was tested for *GYS1* mutation (R309H) associated to type-I polysaccharide storage myopathy (McCue et al. 2008a, b). Horses were identified as homozygous (H/H), heterozygous (H/R) or normal (R/R).

Statistical analysis

A general linear mixed model (SAS, Cary, NC, USA) was used for data analysis. The response variables were the respirometric parameters, while the predictive variables (fixed effects) were the muscle sampled and the group of animals (HC, PSSM or RUO) as well as the interaction between the two. Horse was considered a random effect. In this multifactorial design results were reported as least square means and significance was set at $p < 0.05$.

Results

Genotype confirmation of the pathology

Five horses out of nine (cases 1, 2, 3, 4, 7) were diagnosed affected by type-I PSSM because tested positive for the *GYS1* mutation (R309H). These five horses were all heterozygous (R/H). Horses were then divided in two groups depending on results of the genetic test, and classified as type-I PSSM group (i.e. with a positive genetic test) or as horses suffering from RUO (negative to the genetic test).

Clinical examination, blood and urine tests

Hematology and electrolyte status were normal in all horses apart from horse 9 that showed a neutrophilic leukocytosis, mild hypocalcaemia and hyperphosphatemia. Serum CK and AST concentrations measured at rest were outside reference ranges in 6/9 and in 7/9 horses, respectively. Vitamin E status was lower than normal limits (<2 mg/l) in 2/9 horses. Pigmenturia was present in horse 9 only and fractional electrolyte excretion was normal in all cases apart from horse 9 where the analysis was not performed due to isotonic fluid therapy that had been administered at home by the treating veterinarian before referral. Detailed results of biochemical analyses are summarized in Table 2.

Effort test

The 12-min effort test of moderate intensity (walk, trot and canter either on a lung line or on the treadmill) was well tolerated by each horse without triggering an episode of rhabdomyolysis. Horse 1 showed a significant increase in serum CK concentration after exercise (more than two folds the rest value). Only horse 9 did not perform an exercise test because still painful at the moment of examination.

Muscle histopathology

In six horses (cases 1, 2, 3, 4, 6, 7, 9), muscle histopathology revealed non-specific, degenerative lesions mainly consisting in swollen, rounded and asymmetric fibers, internal nuclei and rimmed cytoplasmic vacuoles (Fig. 1). The extent and the degree of the degenerative process varied between cases and muscles but without any apparent correlation with age, severity of clinical signs or activity of muscles enzymes. Two horses (case 5 and 8) had very mild histological lesion that were not considered significant. Abnormal subsarcolemmal and cytoplasmic glycogen was PAS-positive in all confirmed type-I PSSM horses and amylase-sensible in all cases except for case 7 where it was resistant to amylase digestion.

Mitochondrial function

Figure 2 shows respiration in *tb* and *gm* muscles in type-I PSSM, RUO and HC groups. Within each group, no significant differences were observed between *tb* and *gm* muscles for all measured parameters except for succinate pathway capacity in the type-I PSSM group that was significantly lower in the *gm* than in *tb* muscle in both protocols ($p = 0.005$ for SUIT 1 and $p = 0.038$ for SUIT 2).

Table 2 Results of complementary exams: biochemistry and urine tests results for each horse

Group	Horse	CK rest (UI/l)	CK post-effort (UI/l)	AST rest (UI/l)	Vitamin E (mg/l)	Selenium (µg/l)	FEE	Pigmenturia (yes/no)	Serum creatinine (µmol/l)
	Ref	50–200	<2× CK rest	100–400	>2 mg/l	60–120			44–176
PSSM	1	1344	2397	2184	2.3	67	wnl	No	wnl
PSSM	2	251	386	403	14.9	137	wnl	No	wnl
PSSM	3	515	559	964	1.6	100	wnl	No	182
PSSM	4	270	332	595	nm	nm	wnl	No	wnl
PSSM	7	251	321	579	1.9	128	wnl	No	wnl
RUO	5	419	486	495	3.4	nm	wnl	No	wnl
RUO	6	193	183	319	4.2	128	wnl	No	188
RUO	8	152	195	223	4.9	142	wnl	No	wnl
RUO	9	8577	nm	1333	nm	nm	nm	Yes	304

PSSM (polysaccharide storage myopathy) and RUO (rhabdomyolysis of unknown origin) indicate if the horse was tested positive (PSSM) or negative (RU) for the *GYS1* mutation. Values outside normal ranges are highlighted in bold characters. FEE, fractional electrolyte excretion; wnl, within normal limits; nm, not measured

Polysaccharide storage myopathy horses versus healthy controls

The *tb* (Fig. 2a) and *gm* muscles (Fig. 2b) were compared between HC, RUO and PSSM for respiratory capacity expressed as flux per mass of tissue. The differences between the HC and the PSSM groups in SUIT1 were muscle specific. In PSSM horses compared to controls, the *tb* muscle (Fig. 2a) showed a reduced NS pathway ET-capacity (PMGS; $p = 0.019$), whereas the *gm* muscle (Fig. 2b) showed reduced NS-OXPHOS capacity (GMS; $p = 0.01$) and reduced S pathway ET-capacity ($p = 0.02$). Furthermore, in the *gm* muscle, the difference in NS pathway ET- capacity (GMS) between PSSM and HC approached significance ($p = 0.065$).

The respirometry data were expressed as *FCR* over maximal ET-capacity (Fig. 3 and Table 3). In the *tb* muscle, *FCR* differed significantly between HC and PSSM horses (Fig. 3). Generally speaking, all the *FCR* changes indicated a greater contribution of NADH pathway to maximal ET-capacity (NS pathway ET-capacity) in PSSM horses compared to HC. In the *gm* muscle, the *FCR* did not show any significant differences between HC and PSSM horses (Table 3). Both muscle showed changes in the *SCR* (Fig. 4). When compared to HC, PSSM horses showed a greater contribution of NADH pathway (Complex-I linked substrates) to both maximal OXPHOS-capacity and S pathway ET-capacity.

Observations in horses suffering from exertional rhabdomyolysis of unknown origin

In the *gm* muscle, all fluxes in SUIT 1 protocol were significantly higher in the RUO group compared to the PSSM group (Fig. 2b). In contrast, in the *tb* muscle, only N pathway OXPHOS-capacity (GM) and NS pathway OXPHOS-capacity (GMS) differed significantly between PSSM and RUO horses ($p = 0.013$ and $p = 0.029$ respectively). No significant differences were found in SUIT 2 between PSSM and RUO groups in *gm* or in *tb* muscle biopsies.

The only significant changes observed in *FCR* and *SCR* parameters in RUO versus PSSM horses concerned the *tb* muscle with a lower relative contribution in the RUO group of succinate pathway to NS pathway ET-capacity in SUIT 1 ($p = 0.041$). A similar difference was measured in SUIT1 between RUO horses and HC, a lower contribution of succinate pathway to NS pathway ET-capacity ($p = 0.006$) and to maximal OXPHOS-capacity ($p = 0.046$) in RUO horses.

The N pathway OXPHOS-capacity in the presence of glutamate&malate was significantly higher in RUO horses compared to HC for both muscles ($p = 0.0005$ and $p = 0.046$ respectively in *tb* and *gm*; Fig. 2). No other significant difference in fluxes per mass was found between RUO and HC groups.

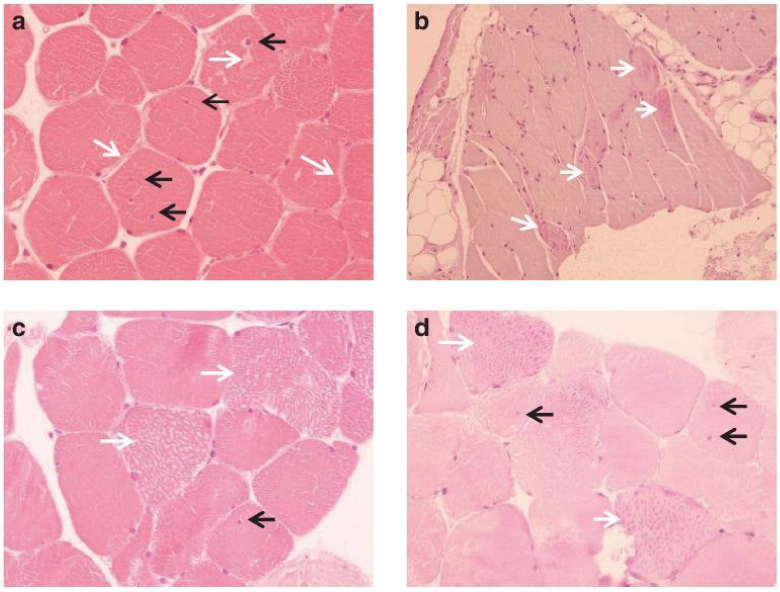

Fig. 1 Histopathologic images of muscle biopsies obtained from 2 subjects. Subsarcolemmal and cytoplasmic accumulation of complex polysaccharides (white arrows) are already visible at the hematoxylin and eosin staining (a, c) and positive at the periodic acid-Schiff (PAS) staining (b, d). Muscle fibers appear swollen, rounded and asymmetric and central nuclei (black arrows) are visible (a, c, d). Images a, c and d belong to horse 3 while image b comes from case 7. In image b abnormal glycogen is resistant to amylase digestion

Significant changes in *FCR* and *SCR* measures were observed in RUO horses compared to HC in both muscles and with the same trend of PSSM horses (Table 3a, b), these differences concerned mostly SUIT 1. Again, these changes indicated a greater contribution of Complex I to NS pathway OXPHOS-capacity in RUO horses versus HC.

Alterations in individual horse

It has to be noticed that one type-I PSSM horse (horse 7) presented a different tendency in comparison to the other horses of the group. At each step of SUIT 1 and SUIT 2 protocols oxygen fluxes were higher than the other 4 PSSM horses but also higher than HC, while respiratory ratios (*FCR* and *SCR*) were not different from other type-I PSSM cases for both SUIT protocols.

Discussion

Mitochondria play a central role in cellular energetic metabolism and mitochondrial damage is associated with a variety of human neurodegenerative disorders. In particular, patterns of mitochondrial dysfunction have been observed in some human GSD such as McArdle disease (GSD V) and Pompe disease (GSD II) (De Stefano et al. 1996; Kurbatova et al. 2014; Lim et al. 2015; Selak et al. 2000; Melis et al. 2016; Rossi et al. 2018). Equine PSSM represents a common cause of exertional rhabdomyolysis in many equine breeds (Valberg et al. 1992). Despite the recent discovery of a point mutation of the *GYS1* gene as responsible of the glycogen storage trouble in type-I PSSM, the metabolic phenomena behind this pathology have not been totally elucidated. We investigated PSSM-affected equine muscle fibers using an innovative diagnostic tool, the HRR. This tool is aimed at taking an important place in the future as a diagnostic routine in the context of equine muscle disorders, where the complexity of underlying metabolic troubles overcomes the limits imposed by basic ancillary complementary exams.

Results of our study reveal for the first time changes in mitochondrial function in horses diagnosed with type-I PSSM in contrast to both HC and horses affected by RUO. Differences emerged depending on the muscle studied, most probably due to different fibers composition and metabolic properties among muscles (Van den Hoven et al. 1985). Our study shows that quantitative changes in mitochondrial respiration (i.e. per mg of tissue) are more readily observable in the *gm* than in the *tb* but in contrast, the qualitative changes (i.e. modifications in *ratios, FCR* and *SCR*) are more prominent in the *tb*. In a previous study investigating the mitochondrial function of French Standardbred racehorses, it was also found that *FCR* and *SCR* calculated from fluxes recorded in *tb* enabled the discrimination of horses at risk of exertional rhabdomyolysis versus unaffected horses while absolute OXPHOS and ET-capacities were not informative (Houben et al. 2015). In the aforementioned study involving racehorses, *gm* muscle was not sampled. Both studies suggest that qualitative changes more efficiently reflect the pathological process.

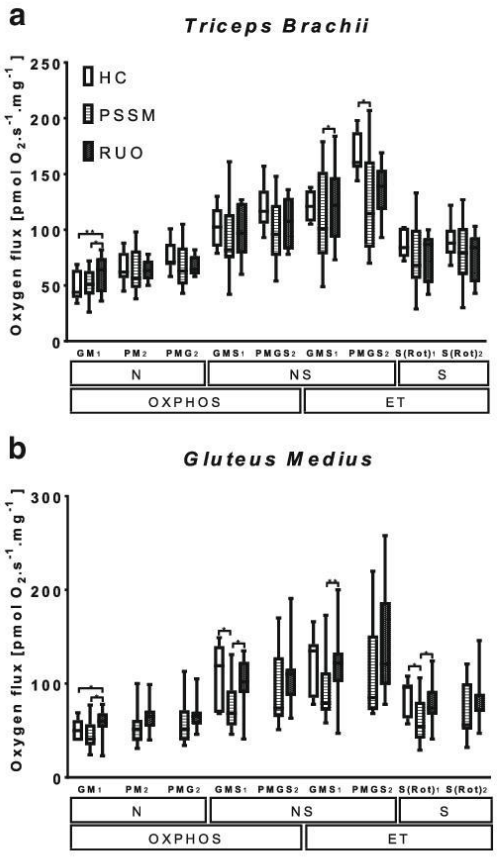

Fig. 2 Tissue-oxidative phosphorylation capacity (OXPHOS-capacity, coupled) and electron transport system (ET-capacity, noncoupled) capacities obtained by high-resolution respirometry. The number of horses per group is indicated by *N* and the number of myofiber incubations by *n*. Two muscles are analyzed, *triceps brachii* (*tb,* a) and *gluteus medius* (*gm,* b). Three groups are compared: healthy controls (HC, $n = 8$ in for *tb* and *gm* in SUIT 1 and $n = 10$ only for *tb* in SUIT 2, $N = 8$ and 10 horses respectively), horses affected by polysaccharide storage myopathy (PSSM, $n = 11$ for *tb* and $n = 13$ for *gm* in SUIT 1, $n = 10$ for *tb* and $n = 14$ for *gm* in SUIT 2, $N = 5$), and horses affected by rhabdomyolysis of unknown origin (RUO, $n = 9$ for *tb*, $n = 11$ for *gm* in SUIT 1, $n = 10$ for *tb* and $n = 11$ for *gm* in SUIT 2, $N = 4$). Two states (OXPHOS and ET-capacities) and three pathways (N for NADH pathway, S for succinate pathway and NS pahway for NADH and succinate pathway) are included. Substrates employed are GM glutalte&malate (GM), glutamate&malate&succinate (GMS), pyruvate&malate (PM), pyruvate&malate&glutamate (PMG), pyruvate&malate&glutamate&succinate (PMGS), and succinate in presence of rotenone (S (Rot)). Subscript 1 and 2 indicates the data coming from SUIT 1 and 2 respectively. Data in (a) are for the *tb* muscle and data in (b) are for the *gm* muscle. Box plots indicate the minimum, 25th percentile, median, 75th percentile, and maximum. Significant differences between groups are identified with * for *p* values <0.05 and with ** for *p* values <0.01. Values from HC were not available from our biobank for *gm* muscle in SUIT 2

We are aware that the limited number of horses in the PSSM and in the RUO groups represents a not negligible limit in our study. Nonetheless, it has to be taken into account that muscle problems are an important but not a major cause of poor-performance in equine veterinary medicine, and PSSM is only one among other potential muscle disorders. Thus, recruiting PSSM- or RUO-affected horses represented and still represents a challenge. Moreover, the lack of data from the *gm* muscle of HC in SUIT2 limits the conclusions that can be drawn from our results. Further sampling to obtain data from the *gm* muscle of sound horses is advocated.

In this study we did not assess the mitochondrial content using a marker as citrate synthase activity, for example. It could be hypothesized that mitochondrial density might be impaired in RUO and PSSM affected horses, thus helping to interpret some of our results. Unfortunately we were limited in our muscle biopsies to about 30 mg of tissue and thus, we were only able to perform SUIT 1 and SUIT2.

Type-I polysaccharide storage myopathy horses versus healthy controls

When comparing PSSM to HC horses, we observed that the most apparent decrease in mitochondrial respiration concerned NS pathway OXPHOS-capacity and S pathway ET-capacity in *gm* in the absence of pyruvate (SUIT 1), while only NS-ET was decreased in *tb* in the presence of pyruvate (SUIT 2). It has been demonstrated that PSSM-affected horses have a lower V_{O2max} than healthy animals as well as a lower maximum total power (aerobic and anaerobic) (Valberg et al. 1995) even if not as severe as those measured in a case of an Arabian filly suffering from Complex I deficiency (Valberg et al. 1994). Thus, the hypothesis that PSSM horses have a dysregulation in the muscle oxidative capacity and in the energy generation pathway has been formulated (Valberg et al. 1995). Different studies have been conducted so as to identify the key-point of metabolic dysfunction, if it lies upstream or downstream the Embden-Meyerhof pathway. It does not appear in literature that the limitation of OXPHOS is induced by an impaired substrate delivery, as it happens in some human glycogenoses (i.e. phosphofructokinase or myophosphorylase deficit). In different studies PSSM horses did not show significantly different muscle concentrations of pyruvate, G-6-P, lactate or ATP depletion with exercise in comparison to controls (Annandale et al. 2005; Borgia et al. 2011; Valberg et al. 1999) but an accumulation of inosine monophosphate (IMP) concentration revealing premature adenine nucleotide degradation (Annandale et al. 2005), so it has been suggested that the limitation of oxidative metabolism would lie after the

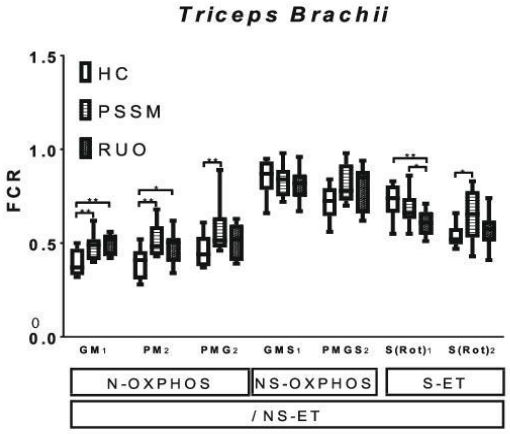

Fig. 3 Respiratory parameters in the *triceps brachii* (*tb*) expressed as flux control ratios (*FCR*), over the maximal ET-capacity with substrates feeding electrons into the NADH and the succinate pathways simultaneously (NS). *N* and *n* are defined as for Fig.2. Three groups are compared: healthy controls (HC), horses affected by polysaccharide storage myopathy (PSSM), and horses affected by rhabdomyolysis of unknown origin (RUO). Two states (OXPHOS and ET-capacities) and three pathways (N for NADH pathway, S for succinate pathway and NS for NADH and succinate pathway) are included. Substrates employed are GM glutalte&malate (GM), glutamate&malate&succinate (GMS) in SUIT 1, pyruvate&malate (PM), pyruvate&malate&glutamate (PMG), pyruvate&malate&glutamate&succinate (PMGS) in SUIT 2. Succinate in presence of rotenone (S (Rot)) is common to both protocols. Subscript 1 and 2 indicates the data coming from SUIT 1 and 2 respectively. Box plots indicate the minimum, 25th percentile, median, 75th percentile, and maximum. Significant differences between groups are identified with * for p values <0.05 and with ** for p values <0.01. Values from HC were not available from our biobank for *gm* muscle in SUIT 2

conversion of glycogen to pyruvate (Borgia et al. 2010). In our study, an altered mitochondrial activity appeared despite the provision of exogenous energetic substrates. In addition, because there was no change in NADH-OXPHOS-capacity involving the PDH complex (SUIT 2, with pyruvate&malate) our results are not suggestive of a defect at the level of this enzymatic complex or at the pyruvate transporter. Features of mitochondrial dysfunction have already been described in PSSM equine muscles, as demonstrated by the down-regulation of many genes involved in mitochondrial activity and by the observation of ultrastructural changes (Barrey et al. 2009). Here we used another method to measure the functional changes in mitochondrial OXPHOS PSSM-affected horses. Our results show that despite the provision of energetic substrates, the capacity of some mitochondrial components is modified. As suggested by human and equine literature, many factors can participate to the pathophysiology of the altered mitochondrial function: hypoxia, muscle fibers inflammation, morphological distortion of mitochondria due to glycogen cytoplasmic accumulation and down-regulation of nuclear and mitochondrial genes. The exact link between all these features in the complex metabolic puzzle of equine PSSM has yet to be determined.

It has been suggested that caution must be taken when analyzing mitochondrial activity in muscle storage diseases (by lipids or glycogen) as results can be misinterpreted if not properly normalized (Selak et al. 2000). The *FCR* used in the present study represent the steady-state oxygen flux with internal normalization for ET-capacity with convergent electron input into the NADH and succinate pathways (NS pathway ET-capacity). This internal normalization helps distinguishing changes of mitochondrial quality versus mitochondrial density, and it yields higher statistical resolution compared to OXPHOS analysis based on external mitochondrial markers (Nunnari and Suomalainen 2012). The OXPHOS/ET ratios with NS pathway obtained in both protocols were equal in the three groups of horses studied, indicating no change in limitation on OXPHOS-capacity by the phosphorylation

Table 3 Respiratory parameters in the *gluteus medius* (*gm*) muscle expressed as flux control ratios (*FCR*), over the maximal ET-capacity with substrates feeding electrons into the NADH and the succinate pathways simultaneously

Muscle	Substrates	HC	PSSM	RUO
gm	GM_1/NS-ET	0.44 (0.29–0.51)	0.51 (0.41–0.55)	0.50 (0.38–0.57)
	PM_2/NS-ET	–	0.49 (0.39–0.65)	0.52 (0.36–0.58)
	PMG_2/NS-ET	–	0.52 (0.43–0.68)	0.53 (0.35–0.61)
	GMS_1/NS-ET	0.89 (0.80–0.98)	0.82 (0.73–0.90)	0.85 (0.66–0.98)
	$PMGS_2$/NS-ET	–	0.81 (0.71–0.97)	0.88 (0.60–0.93)
	S $(Rot)_1$/NS-ET	0.70 (0.65–0.74)	0.66 (0.51–0.84)	0.64 (0.52–0.88)
	S $(Rot)_2$/NS-ET	–	0.67 (0.45–0.76)	0.62 (0.48–0.71)

N and *n* are defined as for Fig.2. Three groups are compared: healthy controls (HC, n and $N = 8$ in SUIT1), horses affected by polysaccharide storage myopathy (PSSM, $n = 13$ in SUIT1 and $n = 14$ in SUIT2, $N = 5$), and horses affected by rhabdomyolysis of unknown origin (RUO, $n = 11$ in SUIT1 and 2, $N = 4$). Two states (OXPHOS and ET-capacities) and three pathways (N for NADH pathway, S for succinate pathway and NS for NADH and succinate pathway) are included. Subscript 1 and 2 indicates the data coming from SUIT 1 and 2 respectively. Values are presented as the median (min-max) for each group. Values from HC were not available from our biobank for *gm* muscle in SUIT2. No significant differences were found between the three groups

system. Nonetheless, in both protocols, the increase in the *FCR* for N pathway OXPHOS-capacity in the *tb* of PSSM horses in comparison to HC implies a higher contribution of NADH-linked substrates to maximal ET-capacity; this could indicate an enhanced capacity of Complex I in PSSM horses. In contrast to *FCR*, *SCR* values are obtained at the same mitochondrial coupling state (Lemieux et al. 2016; Votion et al. 2012) and also show a greater contribution of NADH-linked pathway over both NADH and succinate pathways (NS pathway) and succinate pathway (S pathway) in type I-PSSM compared to HC.

Observations in horses suffering from exertional rhabdomyolysis of unknown origin

Surprisingly, compared to control and PSSM groups, RUO horses showed a higher N-OXPHOS-capacity in the presence of glutamate&malate (SUIT 1) in both *tb* and *gm* muscles. Again, the change was not observed when pyruvate&malate were used as substrate to feed the NADH pathway. This observation could support the hypothesis that RUO horses, but not type-I PSSM, may show specific changes in NADH-sustained OXPHOS independent of the PDH complex activity. This specific modification of the NADH pathway also impacted maximal ET-capacity in the presence of glutamate&malate&succinate (NS pathway ET-capacity) and was accompanied with a lower contribution of succinate-linked pathways (S pathway) to maximal OXPHOS and ET-capacity in *tb* muscle of RUO horses in comparison to the other two groups. Similar to the results in PSSM horses, *FCR* and *SCR* also showed a higher contribution of Complex I-linked substrates (NADH pathway) to maximal ET-capacity in RUO when compared to HC. These results suggest that some specific changes at the level of the ET take place in muscle of horses suffering from myopathy, and these changes differ depending on the primary pathology. Furthermore, in horses affected by RUO a final diagnosis was not obtained, so the possibility of an involvement of pathologies linked to type-I PSSM (as PSSM type-II) that could potentially share similar metabolic features (thus rendering differences between type-I PSSM and RUO results less definite) cannot be excluded. Obviously, another important limit of this study is represented by the small number of subjects that could have influenced and reduced the statistical relevance of some results. Furthermore, the use in the future of other energy substrates such as fatty acids could help the differentiation between different types of myopathy.

Observations in individual horse

One mare (case 7) belonging to the type-I PSSM group showed abnormally high oxygen flux levels in comparison to the mean of other PSSM horses and to HC while respiratory ratios were equivalent to those of other PSSM cases. It has to be underlined that this mare was the only one in the PSSM group that was already correctly managed (in nutrition and physical activity, as generally indicated in case of PSSM diagnosis) since months by her owners, after the last episode of muscle pain. Indeed, at the time of consultation, the mare was performing well. It is difficult to discuss these results, but it could be speculated that nutritional and sports measures

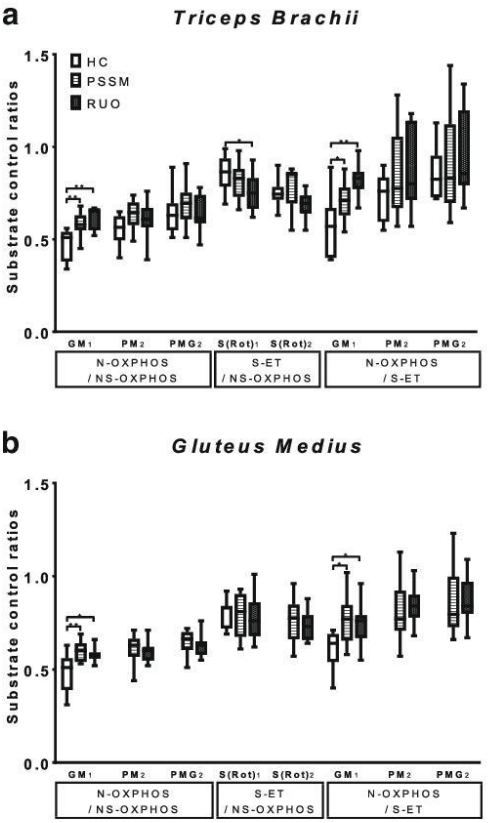

Fig. 4 Respiratory parameters expressed as substrate control ratios (*SCR*). *N* and *n* are defined as for Fig. 2. Two muscles are analyzed, *triceps brachii* (*tb*, a) and *gluteus medius* (*gm*, b). Three groups are compared: healthy controls (HC), horses affected by polysaccharide storage myopathy (PSSM), and horses affected by rhabdomyolysis of unknown origin (RUO). Substrates employed are GM glutalte&malate (GM), glutamate&malate&succinate (GMS) in SUIT1, pyruvate&malate (PM), pyruvate&malate&glutamate (PMG), pyruvate&malate&glutamate&succinate (PMGS) in SUIT2. Succinate in presence of rotenone (S (Rot)) is common to both protocols. Subscript 1 and 2 indicates the data coming from SUIT1 and 2 respectively. Box plots indicate the minimum, 25th percentile, median, 75th percentile, and maximum. Significant differences between groups are identified with * for p values <0.05 and with ** for p values <0.01. Values from HC were not available from our biobank for *gm* muscle in SUIT 2

already applied in this case could have contributed to mitochondrial recovery, or that a compensatory up-regulation of mitochondrial number or activity could lay beyond these findings. It is known that mitochondrial genetic disorders in human medicine are generally irreversible apart from few entities (i.e. reversible infantile respiratory chain deficiency) (Boczonadi et al. 2015) but little information exists about the reversibility of mitochondrial dysfunction associated to storage myopathies in humans in response of specific treatments (Nunnari and Suomalainen 2012). Moreover, type-I PSSM represents a unique animal model of abnormal glycogen metabolism different from human glycogenoses and the recent suggestion of a compromised mitochondrial function in affected horses it is yet to be completely elucidated and linked to the primary *GYS1* mutation.

Conclusion

This study suggests that investigation of mitochondrial function using HRR on muscle fibers obtained by non-invasive muscle microbiopsy offers a useful tool for the assessment of mitochondrial dysfunction in horses suffering from muscular disorders. We detected significant bioenergetical impairments in mitochondria of type-I PSSM-affected horses, changes that are likely participating to the genesis of the observed clinical symptoms. Our results also show that some results are specific to one muscle emphasizing the need for sampling both muscles (*tb* and *gm*) when investigating equine muscle disorders. HRR is an innovating tool that allows the assessment of mitochondrial function in different pathologies involving an energetic imbalance in human and veterinary medicine, its usefulness in different equine muscle disorders needs to be advocated.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Experimental section

Study 2 :

Serum acylcarnitine profiles assessment in horses affected by type-1 polysaccharide storage myopathy before and after submaximal aerobic exercise

Introduction to study 2

In our first study we observed an impairement of mitochondrial function in horses suffering from type-1 PSSM. The subsequent step in our investigation of the metabolic profile of this disease intended to assess plasma acylcarnitine profiles in type-1 PSSM horses. There results have not been submitted yet for publication, but they offer in our opinion a better comprehension of the impaired energy metabolism characterizing horses affected by type-1 PSSM.

As previously mentioned, the most common trigger for clinical symptoms in PSSM horses is less than 20 minutes of submaximal aerobic exercise (Valberg et al., 1999a), so at <50% VO_{2max}, when both glycogen and fat are recruited as energy substrates to fuel muscle contraction. As already stated, type-1 PSSM horses do not exhibit a deficit in glycolytic/glycogenolytic enzymes (Valberg et al., 1998) nor a limitation in substrate availability (Borgia et al., 2010; Valberg et al., 1999a; Annandale et al., 2005; Borgia et al., 2010). Despite this apparent substrate availability, horses affected by type-I PSSM suffer from an impaired energy generation to fuel muscle contraction during submaximal exercise, from either CHO or fat metabolism. It has been observed that type-1 PSSM, when fed grains, have low plasma NEFA (Borgia et al., 2010), possibly due to an insulin-driven suppression of lipolysis (Coyle et al., 1997, Spriet, 2014), that could result in a lower rate of FAO. Otherwise, considered that PSSM horses have a notably faster glucose uptake, especially when fed high-CHO diet (De la Corte et al., 1999b; Annandale et al., 2004), high glucose uptake in their muscle may increase muscle citrate concentration (Saha et al., 1999). As a consequence, high muscle citrate concentration may further reduce FAO. Citrate increases, via ACC activation, the concentration of malonyl-CoA that is an inhibitor of CPT1 activity (Stephens, 2018), and that is probably further activated by AMP kinase dysregulation (Annandale et al., 2005). However, in humans, muscle malonyl-CoA does not seem to regulate LCFA oxidation during exercise, as malonyl-CoA content does not change with exercise at different exercise intensities (Roepstorff et al., 2005).

Carnitine and acylcarnitine are, as previously decribed, metabolic regulators involved in both glucose and lipid metabolism, that can in physiologic conditions provide a snapshot of energy substrate flux, and in pathologic conditions, they can reveale oxidative disorders influencing mitochondrial and cellular acylcarnitine pool. Our hypothesis was that if excess acyl-CoA moiteties were cumulating in mitochondria of type-1 PSSM-affected horses because of an inability to use them for oxidative purposes, their efflux in plasma to detoxify muscle cells and preserve their function and relief oxidative stress could be detected as a modification of these profiles at rest and/or after submaximal exercise.

Up to date, there is no study reporting values of plasma acylcarnitine profile on horses suffering from type-1 PSSM.

The only information available on this subject reports that a deficiency in plasma carnitine in PSSM horses has not been detected (Valberg, *upublished data*). Another study assessed plasma free carnitine, acetylcarnitine (C2), some SCACs (C3, C5, C7) and some LCACs (C16, C18:1 and C18:2) by mass spectrometry in PSSM horses before and after exercise but horses were receiveing a specific diet (corn oil diet and triheptanoin supplementation) (Borgia et al., 2010).

Thus, our aim with this complementary data was to assess plasma acylcarnitine profiles in horses affected by type-1 PSSM at rest and after mild submaximal aerobic exercise, and to compare these profiles to those of healthy horses performing the same type of exercise.

Material and methods

For this study we used seven horses (mean age 7.4 ± 3.1 y.o.) of different breeds and different physical fitness level, all being referred to the Equine clinic of the Faculty of Veterinary Medicine at the University of Liège with a history of chronic exertional exertional rhabdomyolysis. All these horses had been tested positive for the *GYS1* and identified as heterozygous (H/R). Four of these horses were the same animals recruited in the first study, whereas the other three were new cases diagnosed after completion of the first study. We included in these analyses only horses being clinically symptomatic in the period prior to their referral to our clinic.

As four horses were recruited retrospectively, we used their blood that had been originally collected (and then stored) at rest and 1 (4 horses) to 2 hours (3 horses) after submaximal aerobic exercise to measure resting and post-exercise CK. For this reason, we respected the same sampling schedule in new cases, in order to have a greater number of horses. Exercise test was represented by a 12 minutes effort test on the track at each gait (4 min walking, 4 min trotting, 2 min galopping, 2 min recovery). This exercise protocol was decided on the basis of literary sources, indicating that if horses have normal serum muscle enzymes at rest, an exercise test consisting of a maximum of 15 minutes lunging at a walk and trot may help to determine if subclinical exercise rhabomyolysis is present, without necessarly triggering clinical symptoms (Valberg et al., 1999b; Reed et al., 2018). Nine healthy mares (mean age 16 ± 6.1 y.o.) of different breeds (3 standardbreds, 4 warmbloods, 2 cross-breds) were used as controls. They were housed in different stables, thay had different fitness levels and no history of rhabdomyolysis, they were fed with different diets, mainly based on hay or haylage and concentrate feed, without any specific supplement apart from salt blocks available ad libitum. Their body condition score was comprised between 4 and 6 (out of a score of 9). These mares underwent the same exercise protocol, blood was collected at rest and one hour after exercise. Concerning healthy horses, blood specimen collection procedure was not subjected to the Ethics Committee due to its non-invasive nature. Concerning type-1 PSSM horses, blood was collected for clinical purposes, thus the remainder serum was used for acylcarnitine profiles assessment.

Serum was then separated from whole blood and sent to the University of Liège's Biochemical Genetics Laboratory, Belgium, for acylcarnitine analysis by MS/MS. Practically, serum proteins were precipitated with a methanol solution containing labelled internal standards. Supernatants were evaporated under nitrogen stream and derivatized with butanolic-HCl. Butylated samples were then re constituted with water/acetonitrile/formic acid (20/80/0.025) and analyzed with a TQ5500 mass spectrometer (Sciex, Framingham, MA, USA). Acylcarnitine profile analysis included C0, short-chain (SCACs, < 6 carbon atoms), medium chain (MCACs, 6 to 10 carbon atoms), long-chain acylcarnitines (LCACs, > 10 carbon atoms) and hydroxyl- and dicarboxyl-species.

Statistical analysis was performed using a commercial software statistical software (JMP® Pro 15.2.0, SAS, Cary, NC). Data were transformed into their natural logarithm (ln) prior to analysis, then an ANOVA test on repeated measures (rest vs post-exercise) was realized. In all measures, $P<0.05$ was considered significant.

Results

In horses affected by type-1 PSSM, there was a significant increase of some SCACs and MCACs with aerobic submaximal exercise, specifically of C3-DC (P = 0.049) C5:1 (P = 0.017), C6 (P = 0.024), C10:2 (P = 0.005). Another profile, C8:1, showed a statistical trend towards significance (P = 0.066). Free carnitine (C0) and acetylcarnitine plasma concentration were not significantly affected by exercise (Fig. 1). There was no significant effect of exercise on other acylcarnitine profiles (SCACs and LCACs) in PSSM horses, except from the long-chain profile C18 (P = 0.015). In healthy controls, submaximal aerobic exercise had a significant effect on C2 (P = 0.0025) (Fig. 1) and on some LCACs (Fig. 3), as C14:1 (P = 0.016), C16 (P = 0.0308), C18 (P = 0.012), but no effect was detected on SCACs and MCACs. When comparing PSSM horses and healthy controls at rest, C0, C2 and SCACs were not significantly different between the two groups (Fig. 1). Regarding MCACs and LCACs (Fig. 2 and Fig. 3), PSSM had a significant higher resting concentration, compared to controls, of C8 (P= 0.011), C10 (P = 0.0002), C10:1 (P = 0.0002) among MCACs; of C12:1 (P = <0.0001) C14:1 (P = 0.021), C16:1 (P = 0.007), C16:1-OH (P = 0.017), C18 (P = 0.005) C18:2 (P = 0.009), C18:1 (P = 0.004), C18:1-OH (P = 0.0007) among LCACs. As expected, most of these profiles were also significantly different between PSSM horses and controls after submaximal exercise, precisely C8 (P = 0.013), C10:1 (P = 0.0002), C10 (P < 0.0001), C12:1 (P = 0.005), C16:1-OH (P = 0.018), C18:2 (P = 0.005), C18:1 (P = 0.009), C18 (P = 0.003), C18:1-OH (P = 0.001). Few other profiles being not significantly different at rest between the two groups, revealed a significant difference after exercise: C5:1 (P = 0.012), C6 (P = 0.032), C6-DC (P = 0.006), C8:1 (P = 0.001), C10:2 (P = 0.013), C12 (P = 0.016), C14:2 (P = 0.039), C14 (P = 0.028), C18:2-OH (P = 0.0007). The contrary happened only for C16:1 and for C14:1, being significantly higher in PSSM compared to controls at rest but nor after exercise.

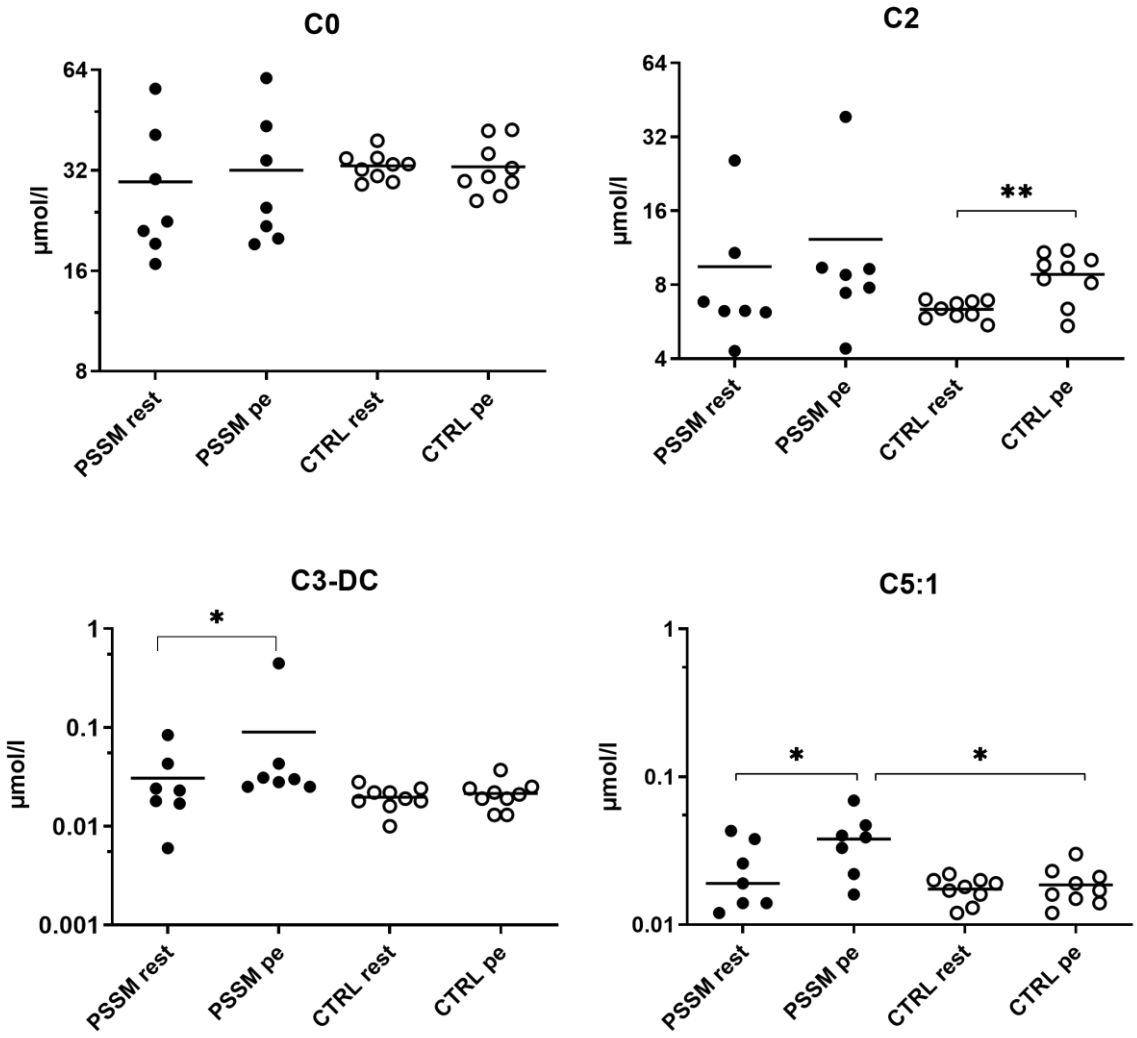
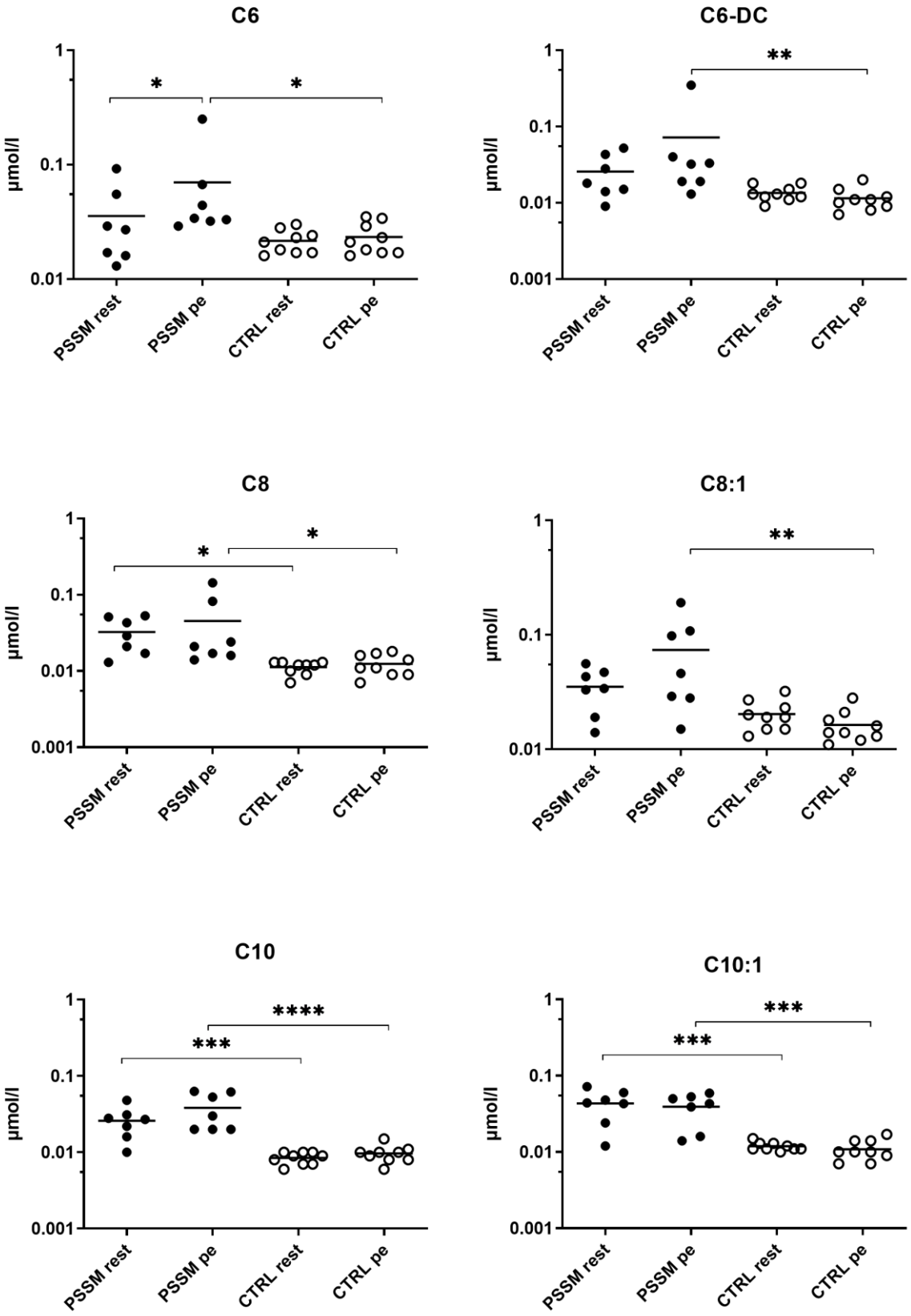


Fig 1. Serum free carnitine (C0), acetylcarntine (C2) and short-chain acylcarnitine C3-DC and C5:1 (SCAC) in horses affected by type-1 Polysaccharide Strorage Myopathy (type-1 PSSM) (n = 7) at rest and after a 12 minutes submaximal aerobic exercise (*pe* for post exercise), compared to healthy controls (n = 9), sampled before and after the same type of exercise. Data are presented as individual raw data (with the mean) on a logarithmic axis. Significant differences between groups are displayed with * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, **** for $P \leq 0.0001$. Other short-chain profiles that were not significantly different among after exercise or between groups are not represented.


C6
C6-DC
C8
C8:1
C10
C10:1
µmol/l
PSSM rest
PSSM pe
CTRL rest
CTRL pe

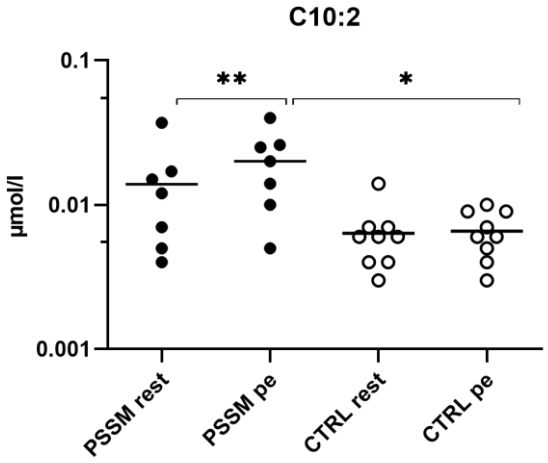
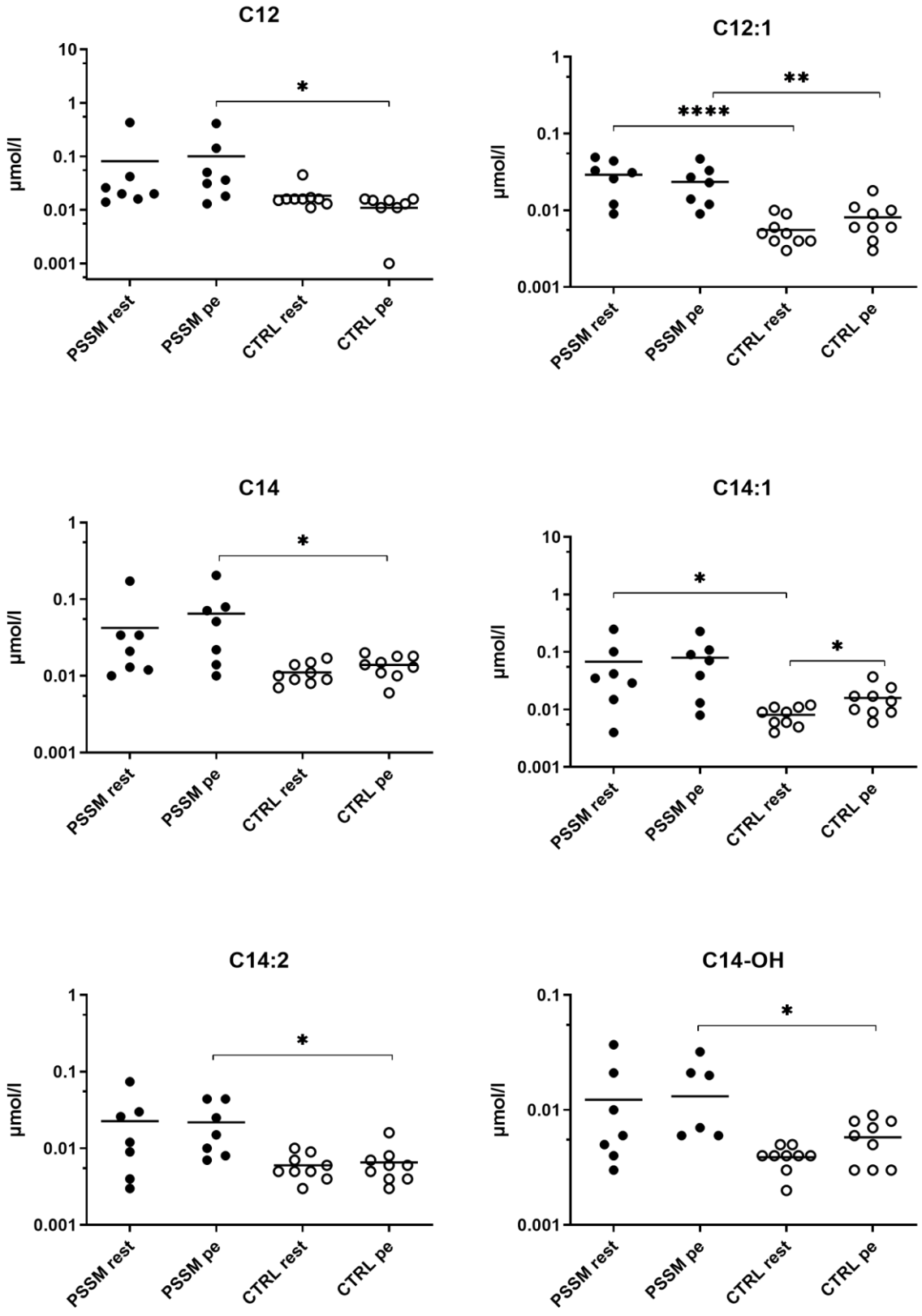


Fig 2. Serum medium-chain acylcarnitines (MCACs) in horses affected by type-1 Polysaccharide Strorage Myopathy (type-1 PSSM) (n = 7) at rest and after a 12 minutes submaximal aerobic exercise (*pe* for post exercise), compared to healthy controls (n = 9), sampled before and after the same type of exercise. Data are presented as individual raw data (with the mean) on a logarithmic axis. Significant differences between groups are displayed with * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, **** for $P \leq 0.0001$. Other medium-chain profiles that were not significantly different after exercise or between groups are not represented.


C12
µmol/l
10
1
0.1
0.01
0.001
*
PSSM rest
PSSM pe
CTRL rest
CTRL pe
C12:1
µmol/l
1
0.1
0.01
0.001
****
**
PSSM rest
PSSM pe
CTRL rest
CTRL pe
C14
µmol/l
1
0.1
0.01
0.001
*
PSSM rest
PSSM pe
CTRL rest
CTRL pe
C14:1
µmol/l
10
1
0.1
0.01
0.001
*
*
PSSM rest
PSSM pe
CTRL rest
CTRL pe
C14:2
µmol/l
1
0.1
0.01
0.001
*
PSSM rest
PSSM pe
CTRL rest
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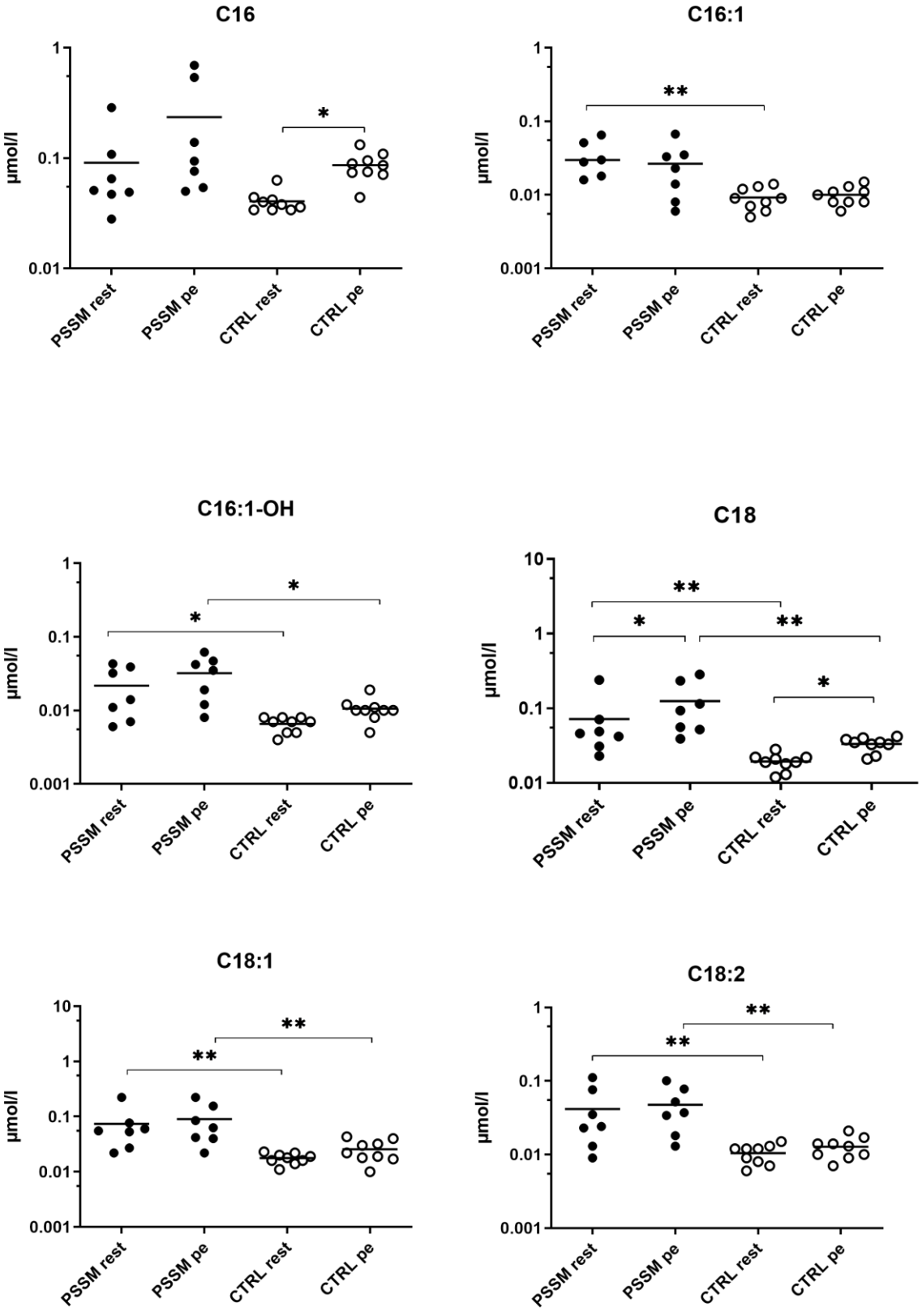
C14-OH
µmol/l
0.1
0.01
0.001
*
PSSM rest
PSSM pe
CTRL rest
CTRL pe


C16
µmol/l
1
0.1
0.01
*
PSSM rest
PSSM pe
CTRL rest
CTRL pe
C16:1
µmol/l
1
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PSSM rest
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CTRL rest
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C16:1-OH
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PSSM rest
PSSM pe
CTRL rest
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C18
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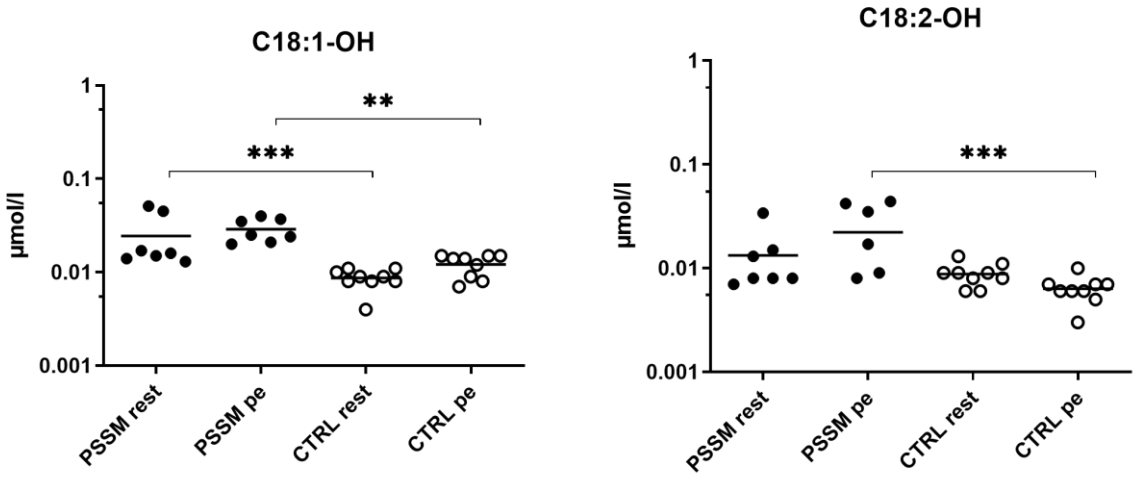

Fig 3. Serum long-chain acylcarnitines (LCACs) in horses affected by type-1 Polysaccharide Strorage Myopathy (type-1 PSSM) (n = 7) at rest and after a 12 minutes submaximal aerobic exercise (*pe* for post exercise), compared to healthy controls (n = 9), sampled before and after the same type of exercise. Data are presented as individual raw data (with the mean) on a logarithmic axis. Significant differences between groups are displayed with * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, **** for $P \leq 0.0001$.

Experimental section

Study 3 :

Acylcarnitine profile in Alaskan sled dogs during submaximal multiday exercise points out metabolic flexibility and liver role in energy metabolism

Introduction to study 3

Alaskan sled dogs are elite endurance athletes, covering up to 200 km/day on multiday races without showing overt signs of fatigue. During prolonged submaximal exercise, they develop a particular metabolic strategy switching their reliance from intra- to extra-muscular energy sources (McKenzie et al., 2005; McKenzie et al., 2007; McKenzie et al., 2008). Specifically, they show an increased stimulus for hepatic glucose output, likely sustained by gluconeogenesis and glycogenolysis, aiming both at fueling exercise and replenishing the initially depleted muscle glycogen (Davis et al., 2020).

Carnitine and acylcarnitines (AC) play an essential role as metabolic regulators in both fat and glucose metabolism; they serve as biomarkers in both physiological and pathological conditions. The objective of the following study was to assess the effect of multiday exercise on plasma acylcarnitine profiles, measured by tandem mass spectrometry (MS/MS), in conditioned sled dogs.

Our results show a chain-specific modifications of ACs profile. We speculated that changes in circulating acylcarnitine pool would reflect different aspects of sled dog metabolism during prolonged submaximal exercise: increased protein catabolism, hepatic uptake for energy-generating purposes to sustain gluconeogenesis, muscle attempt to preserve glucose oxidation and insulin-sensitivity of these dogs.

These findings help, in our opinion, understanding the complex and unique energy strategy developed by sled dogs to sustain prolonged multiday exercise and they further suggest that the metabolism of these dogs during endurance exercise is carbohydrates- rather than fat-dependent and liver-centric.

Experimental section

Study 3

Acylcarnitine profile in Alaskan sled dogs during submaximal multiday exercise points out metabolic flexibility and liver role in energy metabolism

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Tosi I, Art T, Boemer F, Votion DM, Davis MS

PLOS ONE

RESEARCH ARTICLE

Acylcarnitine profile in Alaskan sled dogs during submaximal multiday exercise points out metabolic flexibility and liver role in energy metabolism

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Abstract

Alaskan sled dogs develop a particular metabolic strategy during multiday submaximal exercise, allowing them to switch from intra-muscular to extra-muscular energy substrates thus postponing fatigue. Specifically, a progressively increasing stimulus for hepatic glycogenolysis and gluconeogenesis provides glucose for both fueling exercise and replenishing the depleted muscle glycogen. Moreover, recent studies have shown that with continuation of exercise sled dogs increase their insulin-sensitivity and their capacity to transport and oxidize glucose and carbohydrates rather than oxidizing fatty acids. Carnitine and acylcarnitines (AC) play an essential role as metabolic regulators in both fat and glucose metabolism; they serve as biomarkers in different species in both physiologic and pathologic conditions. We assessed the effect of multiday exercise in conditioned sled dogs on plasma short (SC), medium (MC) and long (LC) chain AC by tandem mass spectrometry (MS/MS). Our results show chain-specific modification of AC profiles during the exercise challenge: LCACs maintained a steady increase throughout exercise, some SCACs increased during the last phase of exercise and acetylcarnitine (C2) initially increased before decreasing during the later phase of exercise. We speculated that SCACs kinetics could reflect an increased protein catabolism and C2 pattern could reflect its hepatic uptake for energy-generating purposes to sustain gluconeogenesis. LCACs may be exported by muscle to avoid their accumulation to preserve glucose oxidation and insulin-sensitivity or they could be distributed by liver as energy substrates. These findings, although representing a "snapshot" of blood as a crossing point between different organs, shed further light on sled dogs metabolism that is liver-centric and more carbohydrate-dependent than fat-dependent and during prolonged submaximal exercise.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Alaskan sled dogs are highly aerobic mammals and elite endurance athletes. They are able to sustain a prolonged effort over consecutive days, running at approximately 50% of their maximal oxygen consumption (VO_{2max}) [1]. The value of their VO_{2max}, even if still inaccurate as difficult to measure, has been estimated at 198 $ml \cdot min^{-1} \cdot kg^{-1}$ in moderately trained but unraced yearling sled dogs [2], a value that is among the highest in aerobic mammals [3]. Moreover, as with most canids except for Greyhounds, sled dogs have a predominance of slow-twitch highly oxidative (type I and IIa) muscle fibers [4–6] which store more intramuscular triglycerides (IMTG) and less glycogen than fast fibers. Energy expenditure in sled dogs reaches 12,000 kcal/day [7]; their diet is typically low in carbohydrates (CHO) but high in lipids and proteins. This diet composition could reduce the incidence of musculoskeletal injuries and spare their muscle glycogen (MG) stores, thus postponing fatigue [8, 9].

With these premises, it seems consistent to consider lipids as the main fuel of energy in running sled dogs. This belief has been further supported by scientific research demonstrating a transient but not cumulative MG depletion in sled dogs during multiday exercise, followed by its gradual replenishment despite a limited CHO intake [6, 10]. In parallel, significant IMTG depletion, mainly occurring after the first 140 km of a multiday run, as well as increased post-exercise plasma non-esterified fatty acids (NEFA), ketones and urea, was observed [10, 11]. These findings have strengthened the idea that prolonged submaximal exercise in Alaskan sled dogs would induce metabolic adjustments aiming at attenuating MG use and enhancing the oxidation of non-CHO, extra-muscular substrates. However, substrate shift in Alaskan sled dogs has been shown to be more complex. In fact, recent works have shown an increased capacity to oxidize CHO in parallel to a decreased capacity to oxidize medium-chain fatty acids (MCFA) in sled dogs running a 1,600 km race [12, 13]. Furthermore, conditioned sled dogs show an increased basal and exercise-induced glucose-transport activity [14] as well as a progressive increase in the stimulus for hepatic glucose output during multiday exercise. In fact, hormonal and substrate kinetics, and more specifically an increase in glucagon to insulin ratio, support the idea that in these dogs glucose output would be sustained by gluconeogenesis and possibly by hepatic glycogenolysis [1]. Thus, it has been suggested that increased glucose output would on one side fuel submaximal exercise and on the other be at the origin of the replenishment of MG that has been transiently depleted. These findings have shed new light on exercise metabolism of sled dogs that seems to be CHO-dependent and likely liver-centric during multiday submaximal exercise. Liver function as a source of energy substrates during exercise is still poorly described in humans due to the difficult access to tissue samples. The main role of liver especially during prolonged fasting and exercise is to maintain glucose homeostasis via gluconeogenesis and glycogenolysis. In case of prolonged fasting, prolonged exercise and limited CHO intake, liver uses gluconeogenic substrates as glycerol, lactate and amino acids, generated in the liver itself or delivered to the liver by extrahepatic tissues, to synthetize glucose through gluconeogenesis [15].

L-carnitine is an amino acid derivative that can be obtained by diet and by biosynthesis in mammals [16]. After synthesis, it is released into circulation mainly as free carnitine (C0) and acetylcarnitine (C2, two C atoms). Although physiologically present in all biological fluids, carnitine is most abundant in high energy demanding tissues as liver, skeletal and cardiac muscle [17]. Some tissues such as skeletal muscle cannot synthetize carnitine, so they acquire it from the circulation [16], thus ester patterns found in plasma depend on the uptake and release from peripheral tissues. Carnitine plays an essential role in energy metabolism as it transports long-chain fatty acids (LCFAs) into the mitochondria for ß-oxidation after esterification into long-chain acylcarnitines (LCACs). In fact, LCFAs cannot penetrate mitochondrial membranes

whereas short and medium chain fatty acids (SCFAs and MCFAs) cross them by passive diffusion [18]. Once in the mitochondrial matrix, acylcarnitines are reconverted into acyl-CoA and carnitine, but this process is bidirectional, so acylcarnitines can be formed back in the mitochondrial matrix and exported to plasma [19]. Furthermore, carnitine acts as a metabolic regulator by buffering excess acyl-CoA moieties which accumulate in cases of increased fatty acid oxidation (FAO) or of high glycolytic fluxes, becoming deleterious to cellular functions [20, 21]. This "buffer role" preserves a viable pool of free CoA to permit continuation of pyruvate oxidation and a better matching of pyruvate dehydrogenase activity and glycolytic flux [21]. Thus it is clear that carnitine and acylcarnitine function extends to both lipid and CHO metabolism where they maintain metabolic flexibility. Acylcarnitine profile is known in humans to be influenced by metabolic status such as fasting and exercise [22, 23] and by pathologic conditions such as diabetes, obesity, insulin resistance and cardiovascular diseases [24–28].

Acylcarnitine profile determination is currently used in human medicine as a routine screening method for inborn metabolic errors [19, 29]. Research exists describing the effect of exercise on plasma and muscle acylcarnitines during exercise in humans [23, 30–32] and in horses [33–36] undergoing different exercise protocols. Early publications (1980s-1990s) on acylcarnitine kinetics in exercising humans reported that high-intensity exercise, and not low-intensity exercise, was able to alter muscle carnitine and acylcarnitine redistribution [23, 37–41]. On the contrary, plasma changes in carnitine homeostasis were either small, absent or not correlated to muscle changes [23, 31]. However, controversy exists as some investigators identified an increase in circulating MCACs and LCACs in response to exercise [32, 42] and others suggested that circulating acylcarnitines could be the result of an exchange with other organs and tissues such as the hepatic carnitine pool [41, 43]. Recent works assessing acylcarnitine metabolism based on tandem mass spectrometry (MS/MS) and on multiorgan fluxes in different species and in different metabolic conditions (fasting, feeding, exercising) have further underlined how muscle poorly interacts with plasma and other compartments [44–46]. On the other hand, these studies have underlined that other organs/tissues, such as liver and heart, contribute to the circulating levels of acylcarnitines [28, 45–47]. Specifically, liver may distribute acylcarnitines as energy substrates or spill them over from its FAO activity to avoid their accumulation [46]. Thus, it seems consistent to infer from this body of literature that changes in carnitine metabolism are compartmentalized (tissue-specific) and highly dependent on different factors such as exercise workload, metabolic status (feeding, fasting) of the individual and on acylcarnitine chain length. Moreover, plasma acylcarnitine profile would be more dependent on the intervention of other tissues and organs, in particular liver, than on muscle carnitine kinetics, thus further highlighting the role of liver as "metabolic hub" during prolonged exercise. To our knowledge, no publication exists assessing acylcarnitines in endurance dogs. Our aim was to assess the effect of multiday exercise on plasma acylcarnitine profile in conditioned sled dogs in order to contribute to the understanding of their unique exercise metabolism.

Materials and methods

All procedures were approved by the Oklahoma State University Institutional Animal Care and Use Committee according to the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals. Study design is based on a retrospective analysis of blood samples recovered from 9 conditioned sled dogs recruited for other research protocols [1, 6, 11, 48]. These dogs had completed a 5-day, 800 km run and had been sampled at rest and within 60 min after each 160 km run. Whole biochemistry, as described in another publication [1], had been performed on serum, at rest and after exercise. Main results of biochemistry indicated a

lower concentration of insulin and a higher concentration of glucagon after completion of exercise compared to resting samples. Non esterified fatty acids (NEFA), β-hydroxybutyrate (BHB) and glycerol concentrations increased after the first day of exercise in comparison to resting values but they gradually decreased returning to baseline with the progression of exercise. Serum glucose remained stable throughout the trial while lactate decreased significantly.

Heparin plasma samples stored at -80˚C were sent to the University of Liège's Biochemical Genetics Laboratory, Belgium, for acylcarnitine analysis by MS/MS [49]. Practically, plasma proteins were precipitated with a methanol solution containing labelled internal standards. Supernatants were evaporated under nitrogen stream and derivatized with butanolic-HCl. Butylated samples were then reconstituted with water/acetonitrile/formic acid (20/80/0.025) and analyzed with a TQ5500 mass spectrometer (Sciex, Framingham, MA, USA). Acylcarnitine profile analysis included C0, short-chain (SCACs, < 6 carbon atoms), medium chain (MCACs, 6 to 10 carbon atoms), long-chain acylcarnitines (LCACs, > 10 carbon atoms) and hydroxyl- and dicarboxyl-species.

Statistical analysis was performed using a commercial software statistical software (Graphpad Prism 6.0, San Diego, CA, USA). Data were transformed into their natural logarithm (ln) prior to analysis and then an ANOVA test on repeated measures was performed. Given the repeated nature of measures, a Mauchly's test was run to test the sphericity of data related to each acylcarnitine profile; for acylcarnitine profiles that did not pass the Mauchly's test of sphericity, a Greenhouse-Geisser correction was applied. A Bonferroni's post-hoc test was used to realize a multiple comparison between different time points. In all measures, $P<0.05$ was considered significant.

Results

Acetylcarnitine (C2) increased significantly in comparison to baseline (prior to exercise) after the first bout of exercise (160 km), then it decreased progressively over the subsequent bouts of exercise (Fig 1B). Indeed, after 800 km, C2 was not significantly different from pre-exercise value. Free carnitine (C0) was significantly higher than baseline at 160 and 640 km, but not at other time points (Fig 1A).

There was no effect of exercise on C3 and on C3-DC, while C4 was significantly different from baseline after the initial bout of 160 km (Fig 2A). Concerning C4-DC, this profile was significantly higher than prior to exercise after 640 and 800 km (Fig 2B). Similarly, C5 and C5:1 increased significantly compared to baseline after 800 km (Fig 2C and 2D) while there was no significant change for C5-DC and for C5-OH (Table 1).

Most MCACs and their hydroxyl- and dicarboxyl-derivatives (C6, C8, C8:1, C6-DC, C8-DC, C10) did not show any significant increase at any time point (Table 1). Some exceptions (Fig 3) were represented by C10:1 and C10-DC (Fig 3A and 3C) that increased significantly after the first 160 km then returning to values not significantly different from baseline. Concerning C10:2, its value was significantly higher than prior to exercise, but only after 480 km (Fig 3B).

Multiday exercise induced a significant increase in nearly all LCACs (C12, C12:1, C14, C14-OH, C14:1, 14:2, C16:1-OH, C16-OH, C18, C18:1, C18:2, C18:1-OH, C18:2-OH) (Fig 4), except for C16 (Table 1). More precisely, each LCACs profile increased significantly after the first bout of exercise compared to the corresponding pre-exercise values. This increase remained constant throughout the rest of the study, thus being significantly different from pre-exercise values but not different from other time points. It is important to underline that the magnitude of plasmatic increase of acylcarnitine profile with exercise was not homogeneous among different profiles. As an example, variation of SCACs with exercise, even if

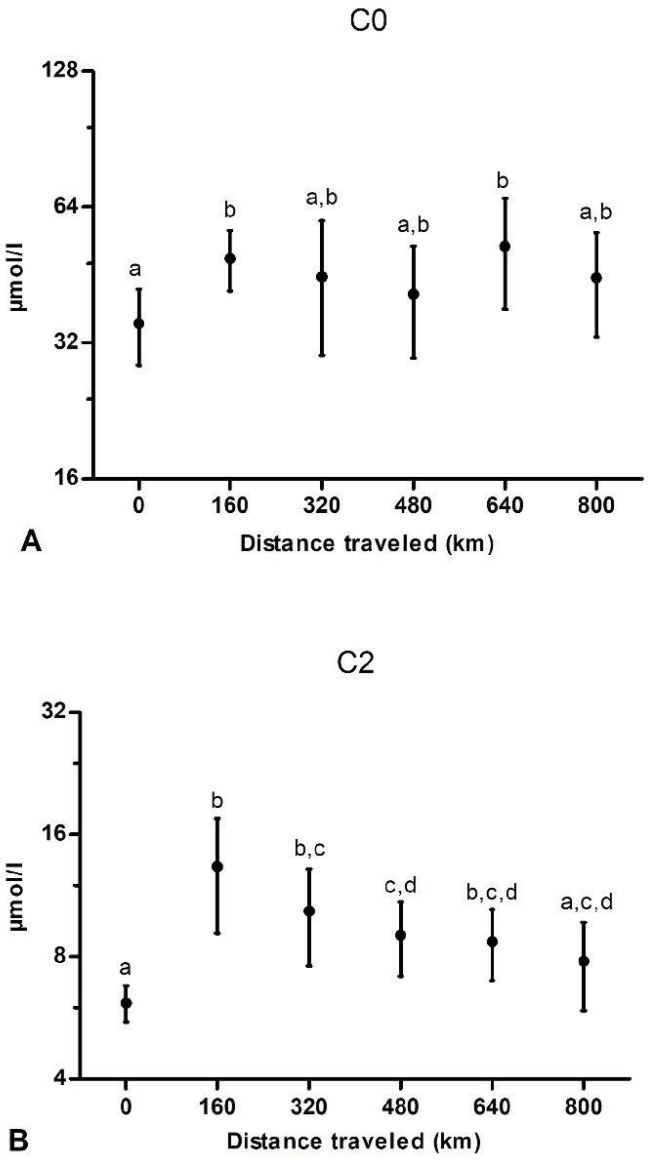

Fig 1. Effect of multiday exercise on plasma free carnitine, or C0 (A) and acetylcarnitine, or C2 (B). Samples were obtained from 9 dogs at 0, 160, 320, 480, 640 and 800 km. Data are displayed as raw data (mean ± SD) on a logarithmic axis. Columns with different superscripts (a, b, c, d) are significantly different between them ($P<0.05$) and not significantly different from columns with the same superscript.

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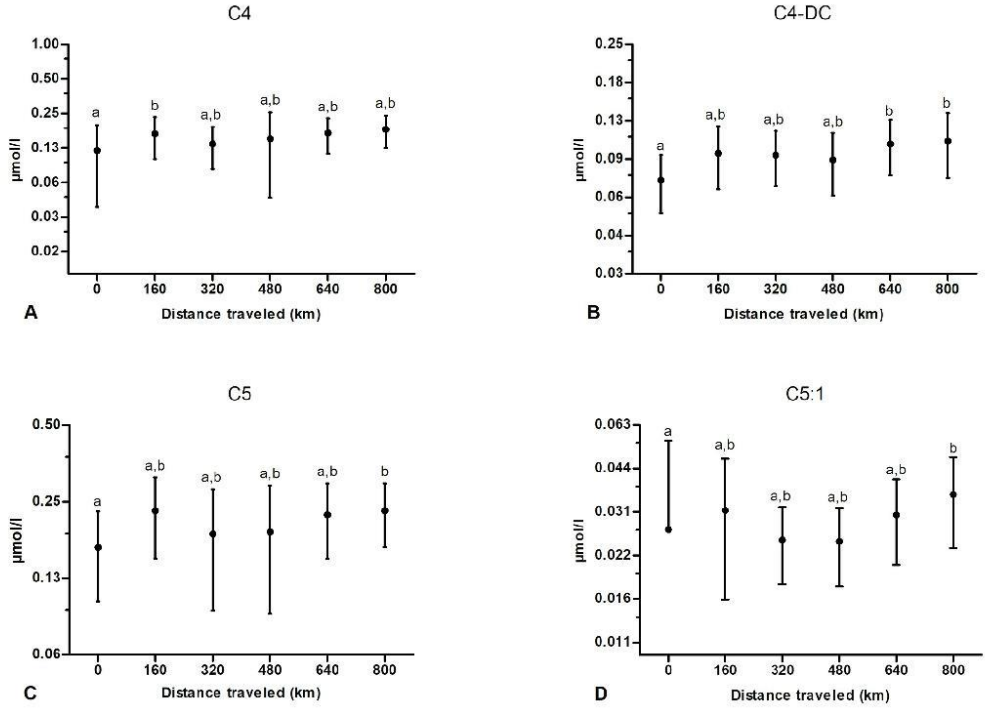

Fig 2. Effect of multiday exercise on some plasma short-chain acylcarnitines (SCACs). Samples were obtained from 9 dogs at 0, 160, 320, 480, 640 and 800 km. Data are displayed as raw data (mean ± SD) on a logarithmic axis. Columns with different superscripts (a, b) are significantly different between them ($P<0.05$) and not significantly different from columns with the same superscript. Missing low error bars correspond to negative values that cannot be displayed on a logarithmic axis.

https://doi.org/10.1371/journal.pone.0256009.g002

Table 1. Plasma concentrations (µmol/l) of short (SC), medium (MC) and long-chain (LC) acylcarnitines not significantly affected by multiday exercise.

Profile[a]		Rest	160 km	320 km	480 km	640 km	800 km
SC	C3	0.383±0.194	0.532±0.232	0.455±0.164	0.444±0.271	0.530±0.234	0.525±0.237
	C3-DC	0.063±0.052	0.065±0.037	0.050±0.011	0.053±0.010	0.056±0.013	0.069±0.033
	C5-DC	0.144±0.091	0.169±0.113	0.129±0.031	0.133±0.056	0.128±0.044	0.158±0.083
	C5-OH	0.104±0.047	0.105±0.034	0.083±0.027	0.082±0.013	0.094±0.034	0.100±0.028
MC	C6	0.043±0.036	0.049±0.026	0.046±0.021	0.049±0.027	0.053±0.030	0.047±0.023
	C6-DC	0.053±0.047	0.063±0.036	0.049±0.012	0.047±0.007	0.049±0.010	0.055±0.018
	C8	0.053±0.044	0.065±0.036	0.056±0.019	0.047±0.024	0.050±0.018	0.056±0.025
	C8:1	0.060±0.052	0.083±0.082	0.067±0.021	0.062±0.020	0.080±0.059	0.084±0.046
	C8-DC	0.049±0.034	0.052±0.020	0.057±0.010	0.045±0.007	0.049±0.009	0.046±0.010
	C10	0.032±0.024	0.044±0.026	0.039±0.014	0.036±0.018	0.041±0.021	0.042±0.019
LC	C16	0.412±0.148	0.481±0.165	0.485±0.164	0.471±0.180	0.492±0.147	0.467±0.175

Table legend: [a]Data are obtained from 9 dogs and reported as mean ± SD. No significant effect of exercise was found at any time point.

https://doi.org/10.1371/journal.pone.0256009.t001

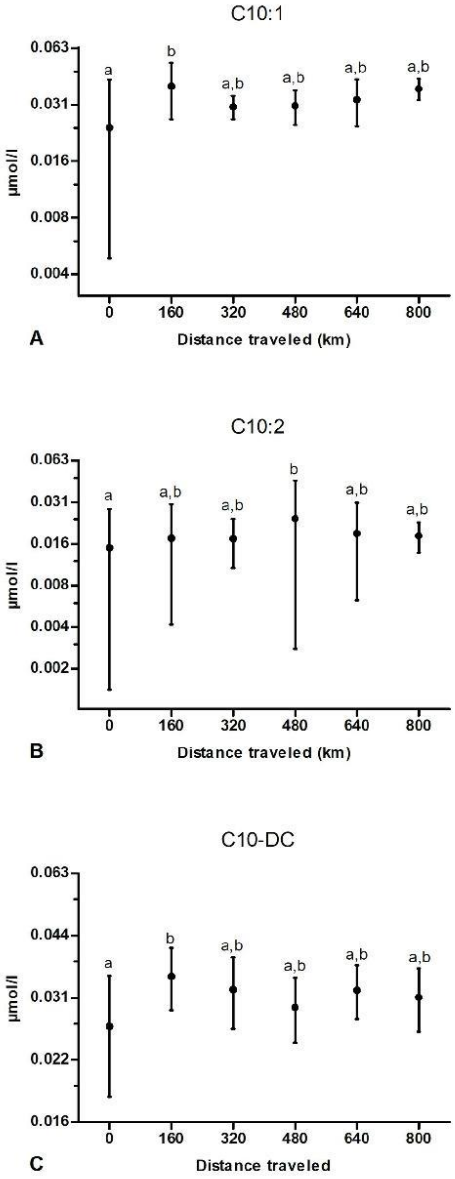

Fig 3. Effect of multiday exercise on some plasma medium-chain acylcarnitines (MCACs). Samples were obtained from 9 dogs at 0, 160, 320, 480, 640 and 800 km. Data are displayed as raw data (mean ± SD) on a logarithmic axis. Columns with different superscripts (a, b) are significantly different between them ($P<0.05$) and not significantly different from columns with the same superscript.

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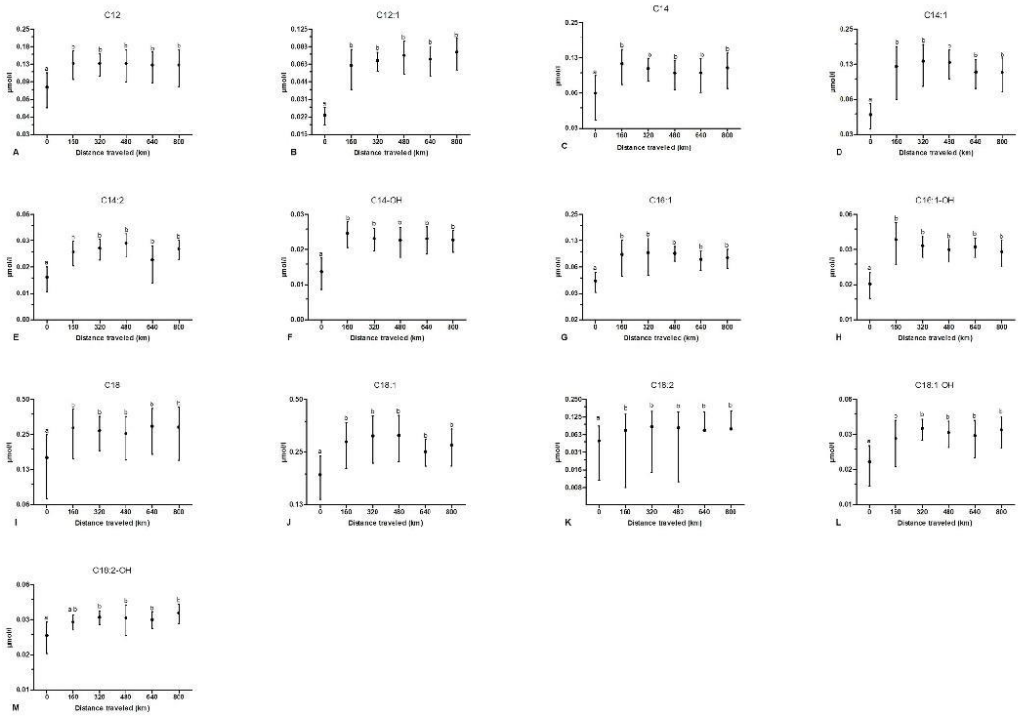

Fig 4. Effect of multiple consecutive days of exercise on plasma long-chain acylcarnitines (LCACs). Samples were obtained from 9 dogs at 0, 160, 320, 480, 640 and 800 km. Data are displayed as raw data (mean ± SD) on a logarithmic axis. Columns with different superscripts (a, b) are significantly different between them ($P<0.05$) and not significantly different from columns with the same superscript. Missing low error bars correspond to negative values that cannot be displayed on a logarithmic axis.

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statistically significant, was less striking (increasing by less than 50% its resting value) compared to the change of C2 that increased of more than 100% of its resting value after 160 km. In the same sense, some LCACs doubled their resting concentration in plasma while other increased (significantly) but to a lesser extent. The biological/energetic impact of these differential variations on the metabolic balance of dogs is unknown.

Discussion

Contrary to previous hypotheses, there is recent scientific evidence that endurance sled dogs, despite their diet (low-CHO, high-fat and high-protein), the composition of their muscle fibers (predominantly slow and highly oxidative) and the type of exercise they perform (submaximal and prolonged), may actually not be using lipids as their main energy substrate. Indeed, their reliance on CHO as energy source does not decrease but increases with increased fitness [12, 13] in concomitance with an increase in sarcolemmal transport activity of glucose [14]. Moreover, after initial reliance on IMTG and MG during the first day of a multiday exercise challenge, sled dogs experience a metabolic shift away from intramuscular towards blood-borne

substrates, and a strong stimulus for glucose output [1]. This metabolic strategy is suggested, as previously described, by an increase of urea production [11], a slight increase in serum glucose and an increase in glucagon/insulin ratio [1].

Our aim was to contribute to the comprehension of this particular substrate management in endurance sled dogs, using plasma acylcarnitines as biomarkers. To our knowledge, this is the first time that acylcarnitines profile has been determined not only in endurance dogs but also in response to a particular metabolic challenge represented by multiday and prolonged exercise.

The most notable result in our study was an increase in almost all LCACs (Fig 4); this increase was significantly different from baseline after the first 160 km and remained steadily constant until completion of the 800 km. Even-chain species from C6 to C22 (MCACs and LCACs) are known to arise from incomplete β-oxidation of fatty acids [50]. Their increase during prolonged exercise and short-term fasting in humans [22, 51] has been related to an increase in NEFA availability provided by lipolysis [52, 53]. Thus their presence in plasma has been suggested to be due to surplus acylcarnitines cleared by muscle to prevent acyl-CoA accumulation in case of increased FAO, to lipids mobilized from lipolysis in form of acylcarnitines or to surplus or newly synthetized acylcarnitines from the liver or other fat oxidizing compartments [45].

Alaskan sled dogs undergoing prolonged submaximal exercise are known from other studies to be in negative caloric balance [1, 7, 10] and to experience active lipolysis as indicated by their glycerol turnover [12]. In another piece of research performed on Alaskan sled dogs exercising over subsequent days, serum NEFA concentration showed, as in our dogs, an initial increase after the first 160 km followed by a progressive decrease despite a progressively increasing glucagon/insulin ratio [1]. Therefore, authors suggested that either a reduction in lipolysis due to body mass reduction (and not insulin-driven) or an increased contraction-mediated extraction of NEFA by skeletal muscle could be at the basis of such NEFA kinetics [1]. However, in our study, LCACs rise observed in plasma was paralleled only after the first 160 km by the rise in circulating NEFA, which afterwards, contrary to LCACs, decreased with continuation of exercise. Thus, it is difficult to relate LCACs rise to an increased availability of NEFA due to lipolysis.

Contracting muscle has often been considered as the main source of plasma acylcarnitines change during exercise because of its increased glucose and lipid metabolism during exercise [23, 30]. Muscle carnitine efflux is likely to be more significant than uptake as muscle has the largest carnitine depot of the body (75% of the total carnitine pool) for FAO, so it necessitates little, even if constant, influx [46]. Skeletal muscle can release acylcarnitines into blood with the aim to remove acyl-CoA moieties that are potentially harmful to cellular functions [54], especially when the provision of energy substrates exceeds the oxidative capacity of the tricarboxylic acid (TCA) cycle. This seems, at a first glance, in contrast with the concept that Alaskan sled dogs are in negative caloric balance [1] and with their switch, due to MG depletion, to extra-muscular non-CHO substrates to sustain energy demand [6, 10]. Nonetheless, it has been demonstrated by recent works that sled dogs exhibit an increased capacity for basal and contraction-mediated sarcolemmal transport of LCFAs and glucose [14] as well as a conditioning-induced clearance of circulating glucose, which result in an enhanced availability of energy substrates in muscle cells. On one hand, these observations underline a discrepancy, or rather a compartmentalized metabolic response, between a systemic negative caloric balance of sled dogs on one side and an "overfed" status of individual muscle cells on the other. On the other hand, they would fit with the idea of muscle "ejecting" surplus acyl-CoA moieties, because of high fluxes of energy substrates massively mobilized during the first bout of 160 km. Moreover, sled dogs have a remarkably high basal insulin sensitivity that is even impressively higher after

endurance conditioning [55, 56]. In human medicine, the interrelation between obesity, lipotoxicity and insulin resistance is generally accepted, and the direct or indirect role of acylcarnitines in insulin-resistance has been recently highlighted [24, 50, 57, 58]. Muscle mitochondria are particularly vulnerable to energy overload and serve as the principal lipid sensors in this tissue [50]. Accumulation of intermediates of β-oxidation impair glucose metabolism (by CoA trapping) and mitochondrial performance (by provoking oxidative stress); moreover, it interferes with insulin signaling. Indeed, it has been recently suggested that in human subjects excess LCACs can be converted to metabolites as diacylglycerol and ceramides and activate stress kinases, thus disturbing insulin signaling and contributing to insulin resistance [58, 59]. Looking back at sled dogs, they increase their capacity to transport and to oxidize glucose rather than fatty acids [13, 14], exhibit high values of mitochondrial respiratory capacity [13] and further enhance their insulin-sensitivity [56] during multiday exercise. Therefore, if muscle is the main source of circulating LCACs, we suggest that, even if the initial plasmatic rise in LCACs could still be related to increased FAO rates, their subsequent steady increase would reflect the carnitine-detoxifying role. This function would aim at sustaining CHO metabolism by avoiding CoA trapping, necessary to sustain pyruvate dehydrogenase and thus glucose oxidation, guarantee glucose uptake, conductance to mitochondria and mitochondrial performance.

As previously stated, liver has been shown to play a central role in whole body carnitine metabolism, by distributing it as energy substrate or by spilling it over from its FAO activity [46]. In a recent work [28] assessing the contribution of liver and of skeletal muscle to plasma acylcarnitines in humans, exercise resulted in a systemic increase of LCACs, which were not released (but some even taken up) by skeletal muscle nor by liver. The authors suggested that other oxidizing compartments could be responsible for the elevated circulating levels of LCACs during exercise, and this also could be applied to our findings.

Kidneys can also synthetize carnitine and oxidize acylcarnitines as energy substrate thus regulating the whole body carnitine pool [16]. Nonetheless, a recent study using a porcine transorgan model showed that kidney predominantly clears acylcarnitines up from circulation rather than synthetizing them [46], so its contribution in terms of release may be negligible. Working heart is also known to contribute to changes in MCACs and LCACs in plasma as it uses preferentially fatty acids for ATP production [28, 60].

Exposure to cold is known to trigger in humans and mice systemic changes in lipid metabolism by stimulation of brown adipose tissue metabolic activity, NEFA release by white adipose tissue and hepatic NEFA oxidation [53, 61]. Once released into circulation, NEFA can be directly internalized by brown adipose tissue or indirectly taken up after hepatic esterification in LCACs. In mice, LCACs can be taken up by brown adipocytes as energy fuel for thermogenesis, but they can also improve thermoregulation through the metabolic flux in the liver, producing heat as a byproduct of acylcarnitine synthesis [53]. Sled dogs are exposed to cold temperatures so it could be tempting to hypothesize that LCACs are released constantly into circulation with the aim to favor thermogenesis. Nonetheless, overheating, more than hypothermia, is a common cause of poor performance in sled dogs and it has already been demonstrated that normal working conditions increase dramatically their rectal temperature [62]. Moreover, sled dogs participating in this protocol were spending nearly 50% of the time per day in an exercising state (approximately 10 hours) rather than resting in the cold (approximately 7–8 hours) [11]. Furthermore, blood samples were taken within one hour after the end of exercise, and rectal temperature in running dogs can take more than 30 minutes to drop significantly after exercise [63]. Thus it is unlikely in this context, as in normal racing conditions in which running time largely oversteps resting time, that sled dogs were adopting metabolic strategies to enhance heat production.

In our study, the shorter MCACs as C6, C8, C8:1, C6-DC, C8-DC and C10 (Fig 3) did not show any significant change with exercise. Human literature describes MCACs as the dominating biomarkers of moderate-intensity exercise having the potential biological function to support lipid oxidation during exercise [42]. In a transorgan human model, only MCACs were released by exercising muscles [28] and several of them showed an uptake by the hepato-splanchnic bed. An increase in hydroxyl- and dicarboxyl-acylcarnitines such as C6-DC and C8-DC can reflect an increase in ω-oxidation [64], that is a minor route for FAO taking place in the endoplasmic reticulum of the liver [65]. In humans, omega-oxidation seem to act as a scavenger pathway, to reduce the availability of acyl-CoA metabolites when their intra-cellular level is high [66] due to substrate oversupply. Indeed, these metabolites could be used for the synthesis of potentially lipotoxic species (ceramides and diacyl-glycerols) that could impair insulin signaling [66]. The fact that in our study these profiles did not increase, together with the high insulin sensitivity of these dogs as previously mentioned, may confirm an absence of accumulation of noxious acyl-CoA originating from incomplete β-oxidation. It could be that MCACs in our study were not significantly released by muscle, or that they were promptly picked up from circulation by other organs as energy substrates. This conclusion underlines again the likely predominance of the anabolic function of acylcarnitines in our study, as they represent a pool of C-atoms backbones, available into circulation for the biosynthesis of cellular function and as a potential energy substrate [67]. The catabolic, "detoxifying" function of acylcarnitines allowing efflux of excess acyl groups to alleviate mitochondrial stress [50] seems less likely in our context. A minority of MCACs increased with exercise only at specific time points and depending on the profile. In fact, species as C10:1 and C10-DC increased significantly compared to resting values after the first bout of exercise, then returning to baseline (Fig 3A and 3C), C10:2 increased significantly from baseline only at 480 km (Fig 3B). These punctual significant differences are difficult to explain biologically and need probably a larger number of individuals and further assessment to be confirmed and better understood. In this regard, acylcarnitines classification still lacks consensus and often differs from one publication to another, especially concerning the cutoff between MC and LCACs. The absence of a uniform classification, together with the observation that the kinetics of C12 and C12:1 profiles was more analogous to other LCACs than to MCACs, explains our choice to consider C12 as an LCAC, contrary to other works.

Concerning SCACs (Fig 2), odd-chain carnitine as C3 and C5 derive from amino acids catabolism [50]. In our study, we did not observe any significant change in C3 profile, despite a subjective observation of a tendency to increase, whereas C5 and C5:1 carnitine were significantly higher than baseline but only after 800 km and not at other time points (Fig 2C and 2D); C4-DC followed a similar pattern (Fig 2B). Concerning C4, deriving from both FA and amino acids catabolism, it increased significantly compared to baseline after the first exercise bout (Fig 2A). Previous research performed on the same sled dogs than as those of our study described a decrease in serum protein and an increase in serum urea nitrogen with multiday exercise, which may suggest an enhanced protein catabolism [11]. In that study, serum globulin in particular decreased progressively in a linear fashion with continued exercise. Its concentration was significantly lower than baseline after 480 and 800 km compared with pre-exercise value. Similarly, serum albumin decreased significantly after 320, 480, 640, and 800 km in comparison to its value prior to exercise. Even if our observation remains speculative, the kinetics of SCACs could reflect an increase in protein catabolism as SCACs increase in plasma is simultaneous to the decrease in serum globulins previously observed [11]. It has already been observed that dogs have a high gluconeogenic capacity from precursors as glycerol and lactate (the latter to a small extent) [12, 68, 69], and, probably more importantly, from amino acids [70]. Circulating amino acids derive either from dietary proteins or from endogenous

protein catabolism. Commercial diets for sled dogs have a high protein content (>25–30%) [6, 70]; the increase in serum urea nitrogen concentration observed in sled dogs during multiday exercise [11] sustains the idea that an important fraction of these proteins is used for gluconeogenesis [70]. Moreover, a decrease in serum globulin concentration [11], a loss of body mass and an increase in circulating cortisol have been reported in sled dogs during prolonged multiday exercise [1, 11]. Cortisol, among its effects, stimulates proteolysis, thus increasing amino acids availability for gluconeogenesis. Further amino acid availability is induced by glucagon that increases amino acid extraction by the liver [71]. This extraction can be potentially high if proportional to serum glucagon rise observed in exercising sled dogs [70]. In humans, it has been suggested that despite the fact that protein contribution to energy expenditure is minor, exercise induces an increase in amino acids catabolism due to metabolic processes such as hepatic gluconeogenesis and TCA cycle. This phenomenon would partly explain the continuous rise in blood ammonia of humans during prolonged exercise [72]. The recognized depression of protein synthesis in human skeletal muscle during exercise would leave amino acids available for catabolic processes [73]. Recent works demonstrated that Alaskan dogs have a resting mitochondrial protein synthesis rate four times higher than that of resting humans and they maintain this rate during a training program [74]. Nonetheless, this translation of mitochondrial proteins appears to be selective as during exercise training non-mitochondrial (cytosolic and myofibrillar) fractions decrease in Alaskan sled dogs [74]. All these observations, taken together, reflect an increased availability, and subsequent catabolism, of exogenous (dietary) and endogenous (mainly circulating and skeletal-muscle derived) amino acids, as demonstrated by the SCACs profiles of our study, thus matching with the concept of the high gluconeogenic potential and precursor demand of sled dogs.

In our study, C2 (Fig 1B) showed an interesting kinetics, as it increased significantly after the first 160 km in comparison to pre-exercise value, then it decreased progressively with continuation of exercise, returning to a value after 800 km that was not significantly different from baseline. Acetylcarnitine is together with C0 the main circulating form of carnitine released by liver after its synthesis. Cellular enzymes can readily convert carnitine to C2 and back depending on the metabolic needs of the cell, thus these compounds are easily interchangeable [75]. Acetylcarnitine derives from acetyl-CoA, which is the universal product of degradation of different energy substrates converging into their respective catabolic pathway (β-oxidation of fatty acids, catabolism of some amino acids and pyruvate oxidation). Thus C2 can reflect a prolonged and/or massive acetyl-CoA production leading to formation of ketone bodies, thereby becoming a marker of ketosis. In fact, in the mitochondria, when production of acetyl-CoA production oversteps TCA capacity, the acetyl group is transferred to carnitine via the enzyme carnitine acetyltransferase dependent on the equilibrium constant of the enzyme [76]. Therefore, C2 synthesis serves both to maintain a constant pool of free CoA to permit other cellular functions and to buffer excessive and noxious acyl/acetyl groups. Initial rise in C2 observed after 160 km could reflect an acute flow of substrates mobilized from different sources and directed to muscle (muscle glycogen, muscle TG, NEFA released from adipose tissue). Given the similarity between C2, NEFA, BHB and glycerol kinetics and considering the role, among others, of C2 as maker of ketosis, it could be suggested that the decrease of C2 in a linear fashion with continuation of exercise simply mirrors the decreased availability of NEFA and of ketones as a consequence [1]. Nonetheless, C2 carnitine serves also to disseminate energy via acetyl-CoA and represents a 2 C-atoms backbone that can be easily taken up from circulation for energy-generating purposes, especially when, as in this case, stored energy and caloric intake of dogs can no longer meet exercise demand for fuel. Acetyl-CoA in liver activates pyruvate decarboxylase that catalyzes the first step of gluconeogenesis. Submaximal prolonged exercise demands an increase in glucose disposal, so gluconeogenesis has a crucial role in

maintaining glucose homeostasis during prolonged exercise as during fasting [77]. A high acetyl-CoA content in hepatic cells together with the rise in serum glucagon of Alaskan sled dogs, as observed by Davis and colleagues [1], both represent powerful stimuli for hepatic gluconeogenesis. Hepatic gluconeogenesis, and possibly glycolysis, would result in sustained glucose output to fuel sustained exercise and to allow MG spare and replenishment [1]. Thus, the constant decrease, after an initial increase, of C2 during multiday exercise can be interpreted as a sign of increasing hepatic uptake of C2 from circulation to stimulate gluconeogenesis rather than as the consequence of a decreased production of ketones.

Free carnitine, C0 (Fig 1A), increased significantly after 160 km and after 640 km in comparison to baseline, but not at other time points. The initial rise in C0 after 160 km seems in accordance with the acute mobilization of energy substrates (IMTG and MG) induced by the first bout of exercise and with the rise in circulating LCACs and C2, thus indicating either an increased rate of (hepatic) synthesis due to increasing demand or to an increased (contracting muscle) release. The following decrease can indicate that C0 release is blunted or that it is being acylated in organs other than contracting muscle (*i.e.* liver) as suggested elsewhere [43]. However, it is difficult to explain the significant increase of C0 after 640 km, but it could be related to the observed increased protein catabolism.

We did not compare carnitine/acylcarnitine values of sled dogs to those of non-athletic or sedentary dogs. Plasma carnitine and AC reference values in non-athletic dogs have been reported elsewhere [78–80]. In these works, circulating carnitine has been classified into free and esterified fractions. Interestingly, resting free carnitine concentration, or C0, in the dogs of our study (24–48 μmol/l) seems comparable or slightly higher than reference values obtained from sedentary dogs (12–38 nmol/ml, 9–45 μmol/l) [78–80] and to another population of sled dogs we sampled in an untrained state (12–28 μmol/l)(data not shown). The esterified carnitine fraction generally refers to the sum of C2 and all other short, medium and long-chain profiles; its reference values in non-athletic dogs ranges from 0 to 7 nmol/ml [78, 79] and from 4 to 5 μmol/l in athletic untrained sled dogs (data not show). In our study we presented C2 and other esterified profiles separately. The former ranges from 5 to 7 μmol/l, while all other profiles summed together account only for 1 to 2 μmol/l maximum. Taken into account this calculation, the esterified carnitine fraction in our study seems also quite similar to the corresponding values observed in non-athletic and in untrained dogs. In humans, different metabolic and dietary circumstances, as fasting and long-chain triglycerides load in particular, can affect plasma acylcarnitine levels [51]. Thus, the different composition of diet for sled dogs compared to the diet for sedentary dogs may potentially induce different baseline values but our comparison is descriptive and not sufficiently rigorous to highlight potential differences between the two groups.

Considering the high glucose and CHO-dependence of endurance sled dogs during multiday exercise, as suggested by previous publications and further highlighted by this study, the question could be raised whether if a high-CHO diet would be more beneficial for these dogs than a high-fat diet. A diet rich in fat and proteins, compared to a high CHO-diet, preserves sled dogs from musculo-skeletal injuries and spares glycogen stores [9, 81]. Since dog domestication, humans have imposed a selective pressure that has impacted dog biology, by inducing metabolic and dietary adaptations reflecting co-evolutionary traits of these two species. In both humans and dogs, a copy-number expansion of the pancreatic amylase (*AMY2B*) gene has accompanied the rise and the worldwide spread of agriculture [82], enabling more effective processing of complex carbohydrates. Indeed, pancreatic amylase (AMY2B) catalyzes the breakdown of starch into oligosaccharides, and a high amylase activity is this associated with high copy numbers of the *AMY2B* gene [83]. It has been observed that *AMY2B* copy number distribution in canine breeds follows a pattern matching the geographic spread of prehistoric

agriculture. Moreover, few copy numbers have been found in Greenland dogs and Siberian Huskies, populating Arctic regions with no or only recent agricultural practices [84]. Nonetheless, the *AMY2B* copy number can also vary within the same breed, as observed in Siberian Huskies and Alaskan Malamutes [85]. This has been attributed to different hypotheses: to an introgression of alleles from wolves or from other indigenous breeds with low copy number; to a relaxation or to a reinforcement of selection pressure. This relaxation or reinforcement could be the respective results of a switch to a low starch-diet on one side, and of the conservation of a high-starch diet on the other, depending on the local dietary habits of humans after dog breeds migration across the world [85]. Thus, local dietary human habits and/or the introgressive hybridization from wolves may have negatively influenced the ability to process CHO in sled dogs.

Conclusions

The functional role of plasma acylcarnitines is still unclear. Plasma acylcarnitines provide a snapshot of *in vivo* flux of energy substrates through specific steps of fat, CHO and amino acids catabolism [50], thus the significance of their blood kinetics has to be interpreted with caution. Alaskan sled dogs are impressive fatigue-resistant athletes when submitted to a particular metabolic challenge represented by prolonged multiday exercise in conditions of limited caloric intake. Plasma acylcarnitine profile in sled dogs has shown to be impacted by prolonged multiday exercise in a chain-length dependent manner. Our research represents a piece fitting in a larger puzzle of scientific investigation on exercise metabolism of Alaskan sled dogs. Indeed, our study further highlights the recently underlined key-points of the unique energetic strategy of these dogs, that is 1) extremely metabolically flexible 2) CHO- and glucose-, and not fat-, dependent 3) likely liver-centric. Our study is limited principally by the small number of dogs sampled and by the fact that, due to the retrospective nature of the study, only plasma acylcarnitines could be assessed, and no other analysis on tissues (*i.e.* muscle) or fluids (*i.e.* urine) could be done. Blood is a "crossing point" where organs (skeletal muscle, liver, heart and kidney) release, take up and exchange acylcarnitines depending on their physiological status, on their respective carnitine turnover rate, and on their response to a given metabolic challenge. Nonetheless, our conclusions remain descriptive and are intended to encourage further investigation specifically of liver role in prolonged submaximal exercise metabolism. Liver seems to cover a role of "metabolic hub" in sustaining prolonged exercise and in maintaining glucose homeostasis and in orchestrating metabolites transit into blood to maintain substrate availability. Even if this is still speculative, an increased hepatic functionality (higher metabolic rates, changes in the hormonal response to exercise or in the hepatic gene expression) may occur in endurance sled dogs as an adaptive response to the metabolic demand dictated by prolonged exercise, and thus privileged and transmitted throughout years of selective breeding.

Supporting information

S1 File. Raw and transformed acylcarnitine serum concentrations obtained from Alaskan sled dogs exercising for 5 days at 160 km/day.
(XLS)

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Discussion - Perspectives

Exercise physiology, and in particular the comparative aspect of energy substrate management during exercise in the equine and canine athlete, constitutes the core of our research.

In humans, muscle glycogen is the preferential energy substrate early in exercise and at intensities higher than 65% of VO_{2max}, the depletion of its limited stores is one of the factors initiating fatigue. As stated above, in terms of glycogen dependence and content, inter-species differences exist, as horses have a higher content of muscle glycogen and they are more glycogen-dependent than humans and dogs. In fact, horses recruit glycogen as the primary fuel source during both short-term intense (race) and long-term submaximal exercise (endurance), so glycogen premature depletion and disturbances of its metabolism can be dramatically constraining for any physical activity. In this regard, type-1 PSSM represented the initial subject of interest of our studies. Equine type-1 PSSM is a common cause of exertional rhabdomyolysis in many equine breeds (Valberg et al., 1992) and a unique animal model of glycogen storage disorder. This pathology is caused by a dominant gain-of-function mutation in the *GYS1* gene (McCue et al., 2008b) rendering the GS enzyme constitutively active (Maile et al., 2017) and leading to an accumulation of polyglucosam bodies in myofibers. Indeed, as previously described, unlike other glycogenoses described in humans and domestic animals, type-1 PSSM-affected horses have no deficit in glycolytic and glycogenolytic enzyme activity and they are able to use glycogen to produce lactate anaerobically (Valberg et al., 1992; Valberg et al., 1998). Neither abnormal polysaccharide accumulation nor ATP nor glycogen depletion nor lactic acid accumulation are the cause of rhabdomyolysis (Valberg et al., 1993; De la Corte et al., 2002; Annandale et al., 2005). Horses affected by type-1 PSSM suffer from a disturbance in oxidative metabolism and from mitochondrial dysfunction, as demonstrated by the down-regulation of many genes involved in mitochondrial activity and by the observation of ultrastructural changes of mitochondria (Barrey et al. 2009). We were intrigued by the complex metabolic profile of this disease and the blurry link between genetic mutation, oxidative impairement and clinical symptoms. Patterns of mitochondrial dysfunction have also been observed in human glycogenoses depending on the GSD type (De Stefano et al., 1996; Selak et al., 2000; Kurbatova et al., 2014; Lim et al., 2015; Farah et al., 2017; Rossi et al., 2018) suggesting their likely contribution to exercise-related symptoms, but the application of HRR is still overlooked in this pathologies. Thus, our first aim was to apply for the first time an innovative diagnostic approach, represented by HRR, to investigate type-1 PSSM from the mitochondrial point of view.

With our first study, we compared mitochondrial function in horses affected by type-1 PSSM to healthy horses and to horses suffering from other unspecific exertional rhabdomyolyses. With our study, we observed alterations in mitochondrial function compared to the other two groups of horses. We found out quantitative and qualitative changes in mitochondrial respiration that were muscle-dependent, most probably due to different fibers composition and metabolic properties among muscles (Van den Hoven et al., 1985).

In fact, quantitative changes in mitochondrial respiration were more prominent in the *gm* than in the *tb* whereas qualitative changes were more pronounced in the *tb,* thus highlighting the interest of sampling the two muscles for HRR. Mitochondrial activity in type-1 PSSM horses was altered despite the provision of exogenous substrates, and our results were not suggestive of a defect at the level of the PDH complex or at the level of the pyruvate transporter (no change in NADH-OXPHOS-capacity in SUIT2 with pyruvate and malate). These results confirm that type-1 PSSM horses are not substrate-limited in contrast with other human GSD and that the key-point of metabolic dysfunction in these horses lies after the conversion of glycogen to pyruvate. They further suggest that altered mitochondrial functionality may contribute to the pathogenesis of this disease and to the apparition of clinical symptoms. In humans, the role of mitochondrial damage in the pathogenesis of some GSD is a novel finding. Scientific literature suggests that many factors could participate to the pathophysiology of mitochondrial dysfunction: hypoxia, muscle fibers inflammation, defects in mitochondrial membrane structure, morphological distortion of mitochondria due to glycogen cytoplasmic accumulation and down-regulation of nuclear and mitochondrial genes (Selak et al., 2000; Barrey et al., 2009; Lim et al., 2015; Farah et al., 2017). The exact link between all these features in the complex metabolic puzzle of equine PSSM has yet to be determined but we believe that our study brought a valuable contribution.

The main limitations of this study were represented by the limited number of horses being type-1 PSSM-positive (n = 5). Moreover, we did not assess the mitochondrial content, because being limited in our muscle biopsies to 30 mg of tissue. It could be hypothesized that mitochondrial density might be impaired in type-1 PSSM affected horses, thus helping to interpret some of our results. Mitochondrial content could be assessed by citrate synthase, even if this is an enzymatic biomarker that could be influenced by the pathologic status of the individual and not be sufficiently reliable. Other approaches such as the measure of mitochondrial density relative to whole cell area by transmission electron microscopy imaging, or the employment of antibodies against OXPHOS complexes by Western Blot, could potentially be employed to objectivate mitochondrial content. We did not search for a correlation between respirometric parameters and CK activity to suggest a potential relationship between exercise-induced muscle damage and impaired mitochondrial function, as proposed elsewhere (Votion et al., 2010). It is also true that only one horse out of type-1 PSSM cases exhibited significantly increased CK after 12 minutes of submaximal exercise, while in other cases this was not observed. Ultimately, we did not determine muscle fibers types of our samples used for HRR, despite the diversity of breeds and training status of our type-1 PSSM group. Metabolic profiles of muscle fibers can dramatically change with depth (Van den Hoven et al., 1985; Serrano et al., 1996) so our results could not be interpreted depending on the functional characteristics of the fibers we sampled. Nonetheless, we standardized the sampling depth for both muscles at 50 mm, where in horses there is a general predominance of oxidative type I and IIa fibers. At this depth, we sampled muscle for both HRR and histopathology. Considered that all our type-1 PSSM cases exhibited histological evidence of abnormal PAS-positive glycogen

accumulation, we suggest that the fibers we sampled were in the same metabolic status of those showing histopathologic abnormalities, both being contiguous to each other. As future projects, we wish to systematically identify muscle fiber types when performing HRR to contextualize our results. Moreover, as we presented in our first study, we are interested in following-up by HRR the mitochondrial function of PSSM horses after they have been managed in terms of physical activity and nutrition. In fact, daily exercise, combined to a suitable diet, has a beneficial effect on the oxidative capacity of type-1 PSSM horses. We believe that HRR could provide a useful tool for the follow-up of these horses.

In the second part of our study, we wanted to assess serum concentration of acylcarnitines in type-1 PSSM-affected horses at rest and after exercise, and to compare their kinetics to those of healthy horses realizing the same type of exercise. In fact, horses with type-1 PSSM are unable to generate sufficient energy for muscle contraction during submaximal exercise, either from CHO or fat metabolism. As previously stated, circulating acylcarnitines can be composed of: a surplus of acylcarnitines cleared by muscle to prevent their accumulation when acetyl-coA is in excess relative to the flux into the TCA; mobilized lipids from lipolysis in adipose tissue in form of acetylcarnitine; surplus of newly synthetized carnitine and acylcarnitines from the liver; acylcarnitines distributed into circulation by other smaller fat oxidizing compartments (Ribel-Madsen et al., 2016; Schooneman et al., 2014). Our aim was to determine if acylcarnitine profiles in horses with type-1 PSSM were significantly affected by submaximal aerobic exercise; this type of exercise is supposed to trigger clinical symptoms or in any case to stimulate the defective energy metabolism. Free carnitine (C0) was not significantly different in type-1 PSSM horses at rest compared to healthy horses, so we can state that type-I PSSM-horses do not show a deficit in circulating carnitine, as suggested elsewhere (Valberg, *unpublished data*) and its concentration was not affected by exercise in any of the two groups. Acetylcarnitine, or C2, significantly increased with exercise in control horses but not in PSSM horses. Acetylcarnitine is the universal product of degradation of all metabolic substrates: β-oxidation of fatty acids, catabolism of some amino acids, pyruvate oxidation. It is also a marker of ketosis as it derives from acetyl-CoA; it is the most abundant carnitine species in both tissues and blood (Koves et al., 2008). A significant increase in C2 plasma concentration has been described in Standardbreds sampled 1h after intense exercise (Westermann et al., 2008a). Authors suggested that plasma C2 increase was likely reflecting muscle C2 increase, because hepatic and intestinal sources of C2 were less probable. The fact that in type-1 PSSM horses there was no change in serum C2 concentration after exercise compared to controls might reflect the inability of PSSM horses to oxidize properly energy substrates and to generate acetyl-CoA. Interestingly, in PSSM-1 horses, submaximal aerobic exercise determined a significant increase in one short chain profile, C5:1, and in two medium-chain profiles, C6 and C10:2. Even chain acylcarnitine species (C6-C22) arise from incomplete ß-oxidation of FA; odd chain SCACs such as C3, C4, C5 and intermediates as C3-DC, C5-OH ad C5:1 originate from the degradation of branched-chain amino acids (BCAA), with the exception of C4 that can also derive from FA metabolism (Roe et al., 2000; Kirchberg

et al., 2015). Branched chain amino acids are oxidized in skeletal muscle while other essential amino acids are catabolized mainly in the liver (Rennie, 1996). Exercise stimulates BCAA oxidation for energy provision and for expansion of the pool of TCA cycle intermediates (anaplerosis) (Rennie, 1996; Shimomura et al., 2004). Our results show a significant increase in C3-DC and C5:1 with submaximal exercise in type-1 PSSM horses; the post-effort value of C5:1 was significantly higher than the corresponding value (*i.e.* post exercise) in healthy horses. The fact that in type-1 PSSM horses C5:1 and C3-DC increased significantly could be interpreted as a recruitment of BCAA to be oxidized or to furnish CAC intermediates, because of their potential premature depletion, as proposed elsewhere (Borgia et al., 2010). The fact that other SCAC profiles were not significantly affected by exercise may be due to a sub-optimal sampling timing hiding other potential significant differences or to the limited number of horses. Other striking findings concerned MCACs and LCACs. In fact, in type-1 PSSM horses medium-chain profiles as C6 and C10:2 increased significantly with exercise, while no effect of exercise was noticed in MCACs of controls; other medium-chain profiles (C8, C10, C10:1) were significantly higher both at rest and after exercise in PSSM horses compared to the respective values in controls; some others were significantly higher than controls after exercise, despite not being different between each other at rest (C6, C6-DC, C8:1, C10:2). Concerning LCACs, type-1 PSSM horses had significant higher resting concentration, compared to controls, of C12:1, C14:1, C16:1, C16:1-OH, C18, C18:1, C18:2, C18:1-OH and the post-exercise values of these profiles in PSSM horses were significantly higher than those observed in controls (except for C14:1 and C16:1). In humans, as previously stated, reports on the effect of exercise on circulating acylcarnitine pool are controversial, depending on exercise intensity (low-intensity versus high-intensity exercise), duration (sprinting versus long distance) and on the analyzed compartment (blood versus muscle) (Hiatt et al., 1989; Shalik 1990; Constantin-Teodosiu et al., 1991; Decombaz et al., 1985; Spriet et al. 1992; Carlin et al., 1985; Soop et al., 1985). Main conclusions from these studies suggest that muscle poorly interacts with plasma and other compartments, and that high-intensity more than low-intensity exercise and endurance exercise more than sprinting seems to affect muscle and/or plasma carnitine and acylcarnitine pool. Nonetheless, early studies (80-90s) on this topic were suffering from methodological limitations. Recent literature based on tandem mass spectrometry (MS/MS) and on multiorgan fluxes in exercising men has pointed out that other organs/tissues, such as liver and heart, contribute to the circulating levels of acylcarnitines (Xu et al., 2016; Schooneman et al., 2014; Schooneman et al., 2015; Makrecka et al., 2014). In fact, liver may distribute acylcarnitines as energy substrates or spill them over from its FAO activity to avoid their accumulation (Schooneman et al., 2015). It has also been observed that in healthy humans exercising realizing a moderate-intensity aerobic exercise MCACs and LCACs are the predominant biomarkers in the immediate post-exercise phase and that they decrease very rapidly in the recovery phase (Lehmann et al., 2010). In horses, as previously stated, high-intensity exercise did not affect plasma MCACs and LCACs but it decreased SCACs in Standardbreds, samples being collected 1h after exercise (Westermann et al., 2008a). In other study assessing the effect of low-intensity exercise on

acylcarnitine concentrations assessed by arteriovenous difference, only C3, C5:1 and C16 showed the largest extraction by the hindlimb (Peters et al., 2015). On the other hand, strenuous endurance exercise (160 km endurance race) induced a significant increase in circulating C6, C8, C10 among MCACs and C14:2 among LCACs, thus indicating an increase in mitochondrial β-oxidation and in lipolysis and a more efficient oxidation of LCACs and SCACSs in comparison to medium-chain acetyl-CoAs (van der Kolk et al., 2020). In our study, submaximal aerobic exercise induced an effect on acylcarnitine kinetics that was chain-specific depending on the group of horses that was considered. Indeed, exercise increased mainly MCACs in type-1 PSSM horses and LCACs in controls; this could be an indicator of the different oxidizing capacity of the two groups of horses during exercise and it could be differently interpreted depending on the metabolic status of the horse. In fact, as previously described, efflux in plasma of acylcarnitines can reflect the physiologic increase in substrates availability for ß-oxidation (due to lipolysis or to hepatic release among others) associated with exercise but it can also indicate an excess of mitochondrial acetyl-CoA that oversteps the oxidation capacity of the TCA cycle. Thus, significant increase in serum MCACs of type-1 PSSM horses with submaximal exercise as well as their significantly higher resting and/or post-exercise LCACs concentration compared to healthy horses may reflect the attempt of mitochondria to avoid their own overload. In fact, muscle are particularly vulnerable to energy overload (Koves et al., 2008). Mitochondria in type-1 PSSM horses could be actually clearing out in the systemic circulation excessive and noxious acyl-CoA moieties derived from incomplete FAO using carnitine as a buffer. In fact, circulating acylcarnitines, beyond the acute effect of exercise that can modify their kinetics, reflect a complex sum of past metabolic changes rather than the current metabolic status of the individual (Schooneman et al., 2014), thus likely explaining resting different values between type-1 PSSM-affected and healthy horses. It is also to be underlined that the difference in the mean age of the type-1 PSSM group compared to the control group was undeniable (7.4 versus 16), and with aging, mammalian skeletal muscle shifts from a more glycolytic to a more oxidative profile. As stated above, IMTG are stored primarily in oxidative type I fibers, where the glycogen content is lower than in glycolytic fibers, the latter being caractherized by a negligible IMTG content. Thus, the difference between the two groups in terms of muscle fiber type composition and proportion in function of their age could have influenced the choice for energy substrate flux and thus acylcarnitine profiles. Moreover, as metabolic and dietary circumstances can influence plasma acylcarnitine profiles, the fact that the two groups of horses were differently housed and fed, could have influenced our results. Nonetheless, the control group was quite heterogeneous in its composition, in terms of diet, housing conditions and fitess level, and despite this, the variability of its population distribution was not as striking (subjectively) as for the type-1 PSSM group. It is interesting to notice that acylcarnitines accumulation has been suggested to be a potential responsible of mitochondrial stress and impaired mitochondrial enzyme activity (Sauer et al., 2008), particularly in humans suffering from GSDI (Rossi et al., 2018). Moreover, as acylcarnitines can reside in cell membranes they can interfere and inhibit insulin signaling (through their conversion to diacylglycerols and ceramides), thus their

potential role in the pathogenesis of IR has been suggested in diabetic and obese individuals (Schooneman et al., 2013) as well as in humans with some GSD (Rossi et al., 2018). Horses with type-1 PSSM have an increased insulin sensitivity compared to controls, meaning that they secrete relatively less insulin in response to the same glucose load (De La Corte et al., 1999b; Valberg et al., 1999a; Annandale et al., 2005). This insulin-sensitivity in type-1 PSSM horses does not seem to be related to GLUT-4 content nor to insulin receptor (IR) quantity, which do not differ from control horses (Annandale et al., 2004). Even if type-1 PSSM horses exhibit increased insulin-sensitivity rather than IR as observed in humans with GSDI or diabetes, it is interesting to speculate on a potential role of acylcarnitines in PSSM horses in insulin reception dysregulation.

The main limitation of this study is once again the limited number of type-1 PSSM horses and the fact that their pathologic status was not "standardized", as the duration of the illness, the type and severity of clinical symptoms were variable from one horse to another. On the other hand, it is true that all horses were heterozygotes, so we did not have the bias of homozygotes, generally more severely affected (Naylor et al., 2012). In the same manner, the fitness and training status as well as the diet of these horses was not homogeneous within the group, factor that could have influenced acylcarnitine resting values. Moreover, as suggested by human literature, the effect of exercise on plasma acylcarnitine kinetics can rapidly vanish during the recovery phase (Hiatt et al., 1989; Lehmann et al., 2010), thus sampling horses 1h after exercise could have hidden other potential significant differences or more meaningful kinetics. We were limited by the fact that acylcarnitine measurement had been done retrospectively on frozen serum collected 1h after exercise in type-1 PSSM horses to measure post-exercise muscle enzymes (CK). Furthermore, 5 horses out of 7 had been collected 1h after exercise, whereas 2 horses had been collected 2h after exercise, thus inducing a further bias and reducing the standardization of our analyses. Since then, we decided for the new type-1 PSSM cases to collect blood immediately at rest, immediately after exercise and 1h after exercise, to follow more strictly and precisely acylcarnitines kinetics. Unfortunately, we have collected up to now only 3 horses with a complete kinetics, so did not perform any statistical analysis due to the limited number of horses, but this definitely represents a goal for future studies. Finally, we only measured at specific time points serum acylcarnitines in type-1 PSSM and healthy horses, and as previously stated circulating acylcarnitines provide a snapshot of *in vivo* flux of energy substrates through specific steps of fat, CHO and amino acids catabolism (Koves et al., 2008), thus the significance of their blood kinetics has to be interpreted with caution. The assessment of acylcarnitines concentration in muscle or by arteriovenous difference would provide more information on trans-organ fluxes of these metabolites.

Being interested in the comparative aspect of energy substrate management between different athletic species, we conducted our third study on plasma acylcarnitine measurement in healthy endurance Alaskan sled dogs that, to our knowledge, has never been performed up to now. We expected that

acylcarnitine profiles could provide valuable information on the particular metabolic strategy developed by these extremely fatigue-resistant athletes during prolonged multiday exercise and in conditions of limited caloric intake. Their muscle fibers composition (mainly slow-twitch), their diet (fat-based) and their activity (submaximal, prolonged exercise) would point out fat as predominant energy fuel. However, after an initial depletion of glycogen and triglycerides, with continuation of exercise, sled dogs switch their reliance from intra- to extra-muscular sources. Sled dogs running over 1,600 km increase their capacity to oxidize CHO while their capacity to oxidize MCFA decrease (Miller et al., 2015a; Miller et al., 2017). Moreover, they show an increased stimulus for hepatic glucose output, likely sustained by gluconeogenesis and glycogenolysis, aiming both at fueling exercise and replenishing the depleted muscle glycogen (Davis et al., 2020). Thus, contrary to previous hypotheses, sled dogs metabolism during prolonged submaximal exercise seems to be more CHO-dependent rather than fat-dependent and likely liver-centric. Our study showed a chain-specific modification of acylcarnitine profiles: LCACs increases significantly after the first 160 km and maintained a steady increase throughout the whole exercise period; some SCACs increased during the last phase of the race; C2 initially increased to decrease constantly afterwards. Once again, LCACs release in humans and horses has been related to increased NEFA availability provided by lipolysis (Wolf et al., 2013; Simcox et al., 2017), but in our study, LCACs plasmatic rise was paralleled only after the first 160 km by a rise in circulating NEFA. Thus, it was difficult to link LCACs rise to increased NEFA availability. As stated in the introduction and in our second study, skeletal muscle can release acylcarnitines into circulation with the aim to "eject" surplus acyl-CoA moieties, fact that would seem at a first glance in contrast with the fact that Alaskan sled dogs are in negative caloric balance during multiday exercise (Davis et al., 2020). Nonetheless, it could be argued that the initial plasmatic rise in LCACs could be related to increased FAO due to high fluxes of energy substrates mobilized during the first 160 km (IMTG and MG). Their subsequent steady increase could reflect the previously described detoxifying role of carnitine, to avoid CoA trapping, in order to sustain PDH activity and glucose oxidation, thus guaranteeing glucose uptake and mitochondrial performance. This interpretation of LCACs kinetics would explain the fact that sled dogs, with continuation of exercise over multiple days, increase their capacity to transport and oxidize glucose rather than FA, that they exhibit high values of mitochondrial respiratory capacity and that they further enhance their insulin-sensitivity (Davis et al., 2014; Miller et al., 2017; Davis et al., 2018). It is also true that, even if contracting muscle has often been considered as the main source of plasma acylcarnitines during exercise because of its increased substrate turnover (Hiatt et al., 1989; Lennon et al., 1983), liver and other oxidizing compartments may be responsible of LCACs efflux, distributing them as energy substrates or spilling them over from FAO activity (Schooneman et al., 2015; Xu et al., 2016). Concerning medium-chain profiles, the fact that the majority of MCACs did not show any significant change with exercise may confirm an absence of accumulation of noxious acyl-CoA originating from incomplete FAO, or that these metabolites were more efficiently oxidized, thus preserving the high insulin-sensitivity in these dogs. Concerning SCACs, we suggested

that their significant increase compared to baseline towards the end of the exercise period would reflect an increase in protein catabolism. This has also been suggested by a decrease in serum globulins and by an increase in serum urea nitrogen, observed in the same dogs, at the same time points of multiday exercise, and described in a previous study (McKenzie et al., 2007). Moreover, an increase in circulating cortisol has been observed in sled dogs during prolonged multiday exercise (McKenzie et al., 2007; Davis et al., 2020) and cortisol, among its effects, stimulates proteolysis, thus increasing amino acids availability for gluconeogenesis. An increased amino acid catabolism would also agree with to observation of a decrease in non-mitochondrial protein fractions observed in Alaskan sled dogs during exercise training (Miller et al., 2015b). Amino acids, together with glycerol and lactate, would partly fuel hepatic gluconeogenesis, thus matching with the high gluconeogenic potential and precursor demand of sled dogs (Shaw et al., 1976; Miller et al., 2015a, Davis et al., 2020). Kinetics of acetylcarnitine, increasing significantly after the first 160 km then decreasing progressively with continuation of exercise, up to a return to baseline after 800 km, could also be interpreted in several manners. In fact, C2 increase is a marker of ketosis, thus reflecting prolonged and/or massive acetyl-CoA production. The initial rise in C2 after 160 km could reflect an acute flow of substrates mobilized from different sources and directed to muscle (MG, IMTG, NEFA). The decrease of C2 in a linear fashion with continuation of exercise could mirror the decrease availability of NEFA and ketones (Davis et al., 2020), but it could also be interpreted as an increasing hepatic uptake of C2 from circulation to stimulate gluconeogenesis. In fact, C2 represents a 2 C-atoms backbone disseminating energy via acetyl-CoA, an in liver, a high acetyl-CoA content, together with a rise in glucagon, represents a powerful stimulus for hepatic gluconeogenesis (Davis et al., 2020). Indeed, acetyl-CoA in liver activates pyruvate decarboxylase that catalyzes the first step of gluconeogenesis. Our study was mainly limited by its retrospective nature, rendering difficult to realize other analyses on tissues or organic fluids. Blood remains a “crossing point” where organs release, take up and exchange acylcarnitines, thus analyses of transorgan fluxes and arteriovenous differences provide more precise information. Moreover, we did not compare the measures in our dogs with acylcarnitine values in sedentary or non-exercising dogs. Plasma carnitine and AC reference values in non-athletic dogs have been reported elsewhere (Keene et al., 1991; Neumann et al., 2007): at a first glance, purely descriptive and not statistical, the esterified carnitine fraction in our study (C2 and all other acylcarnitine profiles taken together) seems quite similar to the corresponding values observed in non-athletic and in untrained dogs. Nonetheless, it is recognized in humans that different metabolic and dietary circumstances can affect plasma acylcarnitine levels, thus, the different composition of diet for sled dogs compared to the diet for sedentary dogs may potentially induce different baseline values.

Nonetheless, we have demonstrated that plasma acylcarnitine profile in sled dogs is impacted by prolonged multiday exercise in a chain-length dependent manner.

Our research represents a piece fitting in a larger puzzle of scientific investigation on exercise metabolism of Alaskan sled dogs, that is extremely metabolically flexible, CHO- and glucose-dependent and likely liver-centric.

After introducing general premises on exercise physiology and on inter-species differences, between humans, horses and dogs, in terms of substrate selection during exercise, we have focused our attention on the application of HRR and acylcarnitine profiles measurement in horses and dogs, in pathologic and physiologic conditions.

Type-1 Polysaccharide Storage Myopathy represents a unique model of glycogenosis, as this disease is originally characterized by abnormal glycogen storage, but PSSM horses exhibit also an impaired oxidative metabolism and they are unable to generate or to use acetyl-CoA from either CHO or fat sources. Our research based on HRR and acylcarnitine profiles measurement has provided useful information allowing a better comprehension of the complex metabolic profile characterizing horses with PSSM. Features of mitochondrial dysfunction and overload perturbing cellular bioenergetics have been identified and proposed in the light of our results; these findings could partly explain impaired energy metabolism, metabolic inflexibility and insulin dysregulation in type-1 PSSM horses.

Contrary to horses, exercise metabolism in endurance dogs has been considered for years to be more fat- than CHO-dependent, due to their diet, muscle fiber composition, and due to the type of exercise they realize. However, recent literature has demonstrated that energy substrate shift in Alaskan sled dogs is more complex. Our study focusing on plasma acylcarnitine profiles assessment has improved, in our opinion, the comprehension of the unique metabolic strategy developed by these highly fatigue-resistant dogs during multiday submaximal exercise. In particular, acylcarnitine kinetics have highlighted features of metabolic flexibility, CHO- and glucose-dependence and the central role of liver as a metabolic hub during endurance exercise in these canids.

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