REVIEW

"Alternative" fuels contributing to mitochondrial electron transport: importance of non-classical pathways in the diversity of animal metabolism.

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Running title: Bioenergetic diversity in animals

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Abstract:

The study of glycolysis, the TCA cycle, and oxidative phosphorylation in animals has yielded a wealth of information about bioenergetics. Less is known about how animals use fuels other than glucose and less characterized enzymes that are also used to provide electrons to the electron transport system. It has become clear that bioenergetic flexibility is employed by a wide variety of animals in order to successfully grow, maintain cells, and reproduce, and has contributed to the exploitation of new environments and ecological niches through evolution. In most cases, the discovery of these “alternative” fuels and non-classical pathways is relatively recent, but is starting to call into question long believed paradigms about the diversity of animal bioenergetics. We present several specific examples of these “alternatives” and the animals that use them and present some implications for animal mitochondrial physiology research.

Keywords: proline, proline dehydrogenase, insects, energetics, mitochondria, electron transport system, glycerol-3-phosphate dehydrogenase

Introduction:

It is highly likely that life arose on Earth under anoxic conditions (Lyons et al., 2014). Initially, only two potential energy sources were available to early life on Earth; solar radiation and molecules produced via geochemical processes (Judson, 2017). Existing processes that are able to transduce these energy sources into usable chemical energy to do work (ATP) involve reduction-oxidation reactions (Judson, 2017). When it comes to the use of solar radiation as an energy source, various lines of evidence indicate that anoxygenic photosynthesis utilizing sulfur and iron containing substrates developed first

(Allen and Williams, 2011, Judson, 2017). However, it was the appearance of oxygenic photosynthesis in cyanobacteria that changed the face of the planet to an oxygen containing atmosphere over billions of years (Lyons et al., 2014). Initially this oxygen would have been toxic to most organisms, due to the reactivity of oxygen and its propensity for attacking other molecules (e.g. DNA, membrane lipids, proteins), and species which survived this insult possessed metabolisms capable of scavenging oxygen (Gomes et al., 2001). Over evolutionary time, prokaryotes began to use oxygen in redox reactions involved in energy metabolism due to the amounts of energy that can be generated by using it as a final electron acceptor (Judson, 2017).

The presence of appreciable amounts of oxygen in the atmosphere likely precipitated the most significant evolutionary innovations that have occurred on our planet to date; namely the endosymbiotic events that led to the generation of the mitochondrion and the chloroplast (Judson, 2017). The event that gave rise to the mitochondrion likely occurred first and is proposed to be the union of an archaea and a proteobacterium (Gray et al., 1999). The proteobacterial partner was likely energetically complex and capable of both anaerobic and aerobic respiration given the array of different mitochondria and organelles of mitochondrial origin present on the planet today (Martin and Muller, 1998). The second primary endosymbiotic event occurred between a cyanobacterium and a eukaryotic cell and resulted in the chloroplast (McFadden, 2001). Based on these timelines, it is likely that oxidative phosphorylation (OXPHOS) originated in prokaryotes prior to the development of the eukaryotic cell (Pfeiffer et al., 2001), conferring the ability to transduce large amounts of ATP from various nutrients which in turn contributed to the capacity of animals to increase their sensory abilities,

move at increasing speeds using new methods of locomotion, and to prey on non-microbial organisms for the first time (Smith and Harper, 2013; Sperling et al., 2013).

OXPHOS yields much more energy per substrate molecule than any known anaerobic process (Pfeiffer et al., 2001), and can therefore be considered as more energetically efficient. However, it is worth thinking about whether real life trade-offs are made by animals between the competing strategies of energetic efficiency vs. energetic flexibility. For example, animals that are sessile (i.e. do not move or do so at slow speeds) may have different energetic demands for ATP than highly mobile animals. Different organisms may exhibit different rates of ATP production in addition to the differences in ATP yield that can occur (Pfeiffer et al., 2001). For example, in very active muscle cells, high rates of ATP are required, and metabolism shifts to fermentation from OXPHOS (Pfeiffer et al., 2001). Similarly, during mitotic events which require large amounts of ATP and/or the production of metabolic intermediates, fermentation may be preferred (e.g. during pollen development) (Tadege and Kuhlemeier, 1997). Moreover, recent modelling indicates that organisms that need to maintain a higher metabolic rate may be forced to switch from OXPHOS to a mixture of OXPHOS and fermentation due to macromolecular density constraints (i.e. the proteins in OXPHOS take up more physical space than those used in fermentation) (Vazquez and Oltvai, 2016).

This switching flexibility between OXPHOS and fermentation reflects that the life history and selective pressures experienced by each animal species must be considered. One bioenergetic strategy is not inherently better than another as long as the animal can successfully grow, perform cellular maintenance, and reproduce by matching ATP supply to ATP demand. The focus of this review is the capacity of different organisms to

use alternate fuels via several enzymatic pathways leading to the transduction of energy in the form of ATP. While the demands for and consumption of ATP are equally important, we will not be addressing them here.

As noted above, the transduction of energy into ATP requires a fuel source. Unlike microbes and plants, no animals are truly autotrophic throughout their entire lifecycle, although several are autotrophic during some stages due to kleptoplasty (Rumpho et al., 2000). Animals have many different fuel sources available, but they must ingest other organisms or macronutrients in order to obtain them. In many animals, the fuels are processed through some form of digestion and often involves symbiotic microbes (Michl et al., 2017). The collection and conversion of fuels can therefore be considered the first step of energy metabolism. The fuel (and resulting forms obtained via digestion) must then circulate or be directed to the locations in the animal's body where it is needed (Yan, 2017). Some tissues or organs in the animals' body will require higher amounts of fuel in order to support a higher rate of ATP biosynthesis which in turn will enable energetically expensive activities to take place (Broxterman et al., 2017). Transportation of substrates derived from fuels could therefore serve as a bottleneck in energy metabolism in animals. The transduction of substrate molecules into usable ATP is the last step of energy metabolism in animals that we will consider in this review. This is the step where most of our focus as animal biologists has resided. Unfortunately, we are often fixated on classical mitochondrial metabolism (i.e. the use of glucose as fuel, and the biosynthesis of ATP using glycolysis and oxidative phosphorylation) to the extent that we ignore or are ignorant of other processes used by animals to transduce ATP. This review provides examples of different fuels used by animals and alternative

means of extracting energy from these molecules. Such energy metabolism pathways used by large groups of animals, highlight that our knowledge of such metabolic diversity is incomplete, and demonstrate that much remains to be explored.

The “Gold Standard” of Energy Transduction

There are several different metabolic pathways in animals that can be used to transduce the energy present in fuel molecules into the energetic currency of ATP. One of the most common and universal means of transducing the energy in molecules into ATP is via substrate level phosphorylation (Martin and Thauer, 2017). This process does not require the presence of oxygen and is of ancient origin (Martin and Thauer, 2017). The role of this process in energy metabolism within individuals has likely been vastly underestimated and overshadowed by our fixation on oxidative phosphorylation. Indeed, glycolysis produces ATP in this way, as does fermentation.

The process of glycolysis can then be followed by aerobic respiration, the combination of both representing the central pathway for harvesting the energy contained in carbohydrates. After glucose is catabolized into pyruvate in the cytosol of the cell, this metabolite can then be transported inside the mitochondria where it is further processed by the tricarboxylic acid (TCA) cycle (Fig. 1). During both glycolysis and the TCA cycle, NADH is produced in the cytosol and in the mitochondria, respectively (Ying, 2006). The mitochondrial NADH is oxidized at the level of complex I, which constitutes the main entry point of electrons into the electron transport system (ETS), participating in the generation of most of the cell’s ATP. Several other metabolic pathways converge at the TCA cycle. Several amino-acids can be converted to pyruvate or TCA cycle intermediates and thereafter fuel OXPHOS (Brown, 1992). Moreover, the fatty acids derived from lipids can

146 form fatty acyl-CoA in the cytosol and are transported inside the mitochondria (Wanders
147 et al., 1999). Once in the mitochondria, the fatty acyl-CoA is converted by the β-oxidation
148 to acetyl-CoA, which can enter the TCA cycle (Brown, 1992). β-oxidation also produces
149 mitochondrial NADH, and enables the transport of some electrons to the electron-
150 transferring-flavoprotein of the ETS, thereby further participating in OXPHOS (Ruzicka
151 and Beinert, 1977).

152 In contrast to the NADH produced inside the mitochondria, the cytosolic NADH cannot
153 cross the mitochondrial membranes and its energy must be passed to other molecules
154 in order to enter the mitochondria and maintain redox balance between the
155 mitochondrial and cytosolic compartments (Dawson, 1979).

156 NADH recycling during fermentation

157 During anaerobic conditions that favor fermentation, animals can produce various end
158 products, thereby converting NADH to NAD^+ in the cell's cytosol. The most common
159 reaction is catalyzed by lactate dehydrogenase transforming pyruvate to lactate (Fig. 1).
160 This enzyme is particularly active during intense exercise, hypoxia, or during some
161 pathophysiological conditions such as some types of cancer. This reaction takes place
162 at the expense of the mitochondrial oxidation of pyruvate, therefore decreasing the
163 amount of ATP produced per glucose, with no NADH utilized for mitochondrial
164 respiration. An NAD^+-dependent mitochondrial lactate oxidation has however been
165 demonstrated to occur in brain and skeletal muscle (Brooks et al., 1999; Chen et al.,
166 2016; Kane, 2014). It is suggested that this oxidation is performed via a lactate
167 dehydrogenase that acts in the mitochondrial intermembrane space, and considering
168 that NADH cannot cross the inner mitochondrial membrane, the produced NADH should

not participate to mitochondrial respiration (Chen et al., 2016; Kane, 2014). The role of the mitochondrial lactate oxidation in the recycling of cytosolic NADH and in the contribution of mitochondrial oxygen consumption is still far from understood and will therefore not be further discussed in this review. Besides this process, the transfer of the reducing power of NADH into the mitochondria is achieved by two different shuttles, the malate-aspartate (MAS) shuttle and the glycerophosphate (GP) shuttle (Fig. 1) (Dawson, 1979).

The NADH shuttles: coupling glycolysis and oxidative phosphorylation

The MAS shuttle is believed to be the main NADH shuttle in different oxidative tissues and is comprised of different carriers, as well as cytosolic and mitochondrial isoforms of malate dehydrogenase and aspartate amino-transferase (Dawson, 1979). This shuttle interconverts and transports amino-acids (glutamate and aspartate) and dicarboxylic acids (malate and α-ketoglutarate), transferring the reducing power of NADH from the cytosol to the mitochondria at the level of the TCA cycle (Dawson, 1979). This NADH can then be directly oxidized by the complex I of the ETS (Fig. 1).

The GP shuttle is comprised of two enzymes: the soluble NAD^+-linked cytosolic glycerol-3-phosphate dehydrogenase (cG3PDH), and the membrane bound FAD-linked mitochondrial glycerol-3-phosphate dehydrogenase (mG3PDH). The cG3PDH converts the dihydroxyacetone phosphate, a triose phosphate generated by the glycolysis, to glycerol-3-phosphate (G3P) with the oxidation of NADH to NAD^+ (Dawson, 1979). The G3P is then transported to the mG3PDH anchored to the inner mitochondrial membrane where it is oxidized back to dihydroxyacetone phosphate, with simultaneous reduction of the flavin adenine dinucleotide (FAD) prosthetic group of the mG3PDH (Mráček et al.,

2013). The electrons of the resulting $FADH_2$ are then directly transferred to the ubiquinone pool inside the inner mitochondrial membrane and participate to increase the electron flux in the ETS (Fig. 1).

These two shuttles can act independently as they are not relying on the oxidation of the same metabolites (except for NADH). However, it has been shown in several cases that each shuttle can compensate for a functional deficiency of the other one. Indeed, a knock-out mice model for mG3PDH has been shown to have no gross abnormalities except for a small impairment in body weight gain (Eto et al., 1999). However, when an inhibitor of the MAS shuttle (aminooxyacetate) was administered, ATP content of pancreatic islets, as well as mitochondrial membrane potential were reduced (Eto et al., 1999). Similarly, knock-out mice for the Slc25a13 gene encoding the mitochondrial aspartate-glutamate carrier, an essential component of the MAS shuttle (Fig. 1), displayed marked reduction of MAS shuttle activity *in vitro* with no apparent changes in any phenotype *in vivo* (Saheki et al., 2007). However, when mG3PDH was concurrently knocked-out, a 30% decreased survival by 20-40 days of age, was observed and the double KO mice presented metabolite perturbations, citrullinemia, hypoglycemia, growth retardation and hyperammonemia (Saheki, 2014; Saheki et al., 2012, 2011, 2007). Therefore, it seems that the MAS and GP shuttles are important in the recycling of cytosolic NADH and in the maintenance of cellular homeostasis. Most research efforts have however been focused on the MAS shuttle (Mráček et al., 2013), and it is thus even more important to consider and characterize the GP shuttle during different metabolic conditions.

"Alternative" Fuels used by Animals

Most animals ingest a variety of foods that are species dependent and may include archaea, bacteria, protists, algae, plants, fungi, other animals, and macronutrients found in nature; this provides them with flexibility in the fuels that they obtain from their environment. In contrast, some animals are constrained by past evolutionary processes or specialized as a result of ecological opportunities and subsist on a single food and these represent excellent systems in which to ask questions about energy metabolism. Some alternative fuels used by animals require physiological and biochemical adaptations upstream of the mitochondrial ETS.

Fructose

In most vertebrates, high rates of muscle activity are fueled using a combination of circulating sugars in the blood and intracellular fuels (Weber, 2011; Welch Jr. and Chen, 2014). These animals use glucose to transport fuel between different tissues, organs, and cells. They often employ reactions that modify the molecule after import into the cell to prevent its transport back to the circulatory system (e.g. phosphorylating glucose to trap it in the cell). In migratory birds, flight is primarily fueled by fatty acid oxidation (Weber, 2009). In many vertebrates, a combination of these fuels are used because the enzymatic breakdown of sucrose is not particularly efficient and the uptake of glucose and fructose across the intestinal epithelia is limited. Such systems are therefore limited by the animal's ability to deliver glucose or fructose to the muscles via the circulatory system, the ability and rate of transport into the muscles, and the "trapping" of sugars, therefore preventing their return to the blood through phosphorylation (Welch Jr. and

Chen, 2014). As such, a high contribution of stored fuels is used to support energetically expensive muscle activity in most vertebrates.

Perhaps surprisingly, some animals that must support extraordinary muscle activity can do so by utilizing only nectar as a fuel source. In vertebrates, the best studied of these animals are hummingbirds which fuel their energetically costly flight exclusively with sucrose, glucose, and/or fructose (Chen and Welch Jr., 2014). This requires the hummingbirds to employ a “sugar oxidation cascade” that involves several adaptations to allow for the rapid delivery and use of dietary sugars to highly active muscles (Welch Jr. and Chen, 2014). The first of these is the high expression of the enzyme sucrase in the hummingbird intestine in order to support the production of glucose and fructose (Suarez and Welch Jr., 2017). The second is the efficient uptake of glucose and fructose across the intestinal wall which has been determined to require active transport as well as a paracellular transport mechanism, and the third is the presence of fructokinase in muscles (Suarez and Welch Jr., 2017). Glucose is the energy molecule that has received the most attention in terms of research, but recently the role of fructose as a source of energy started to be fully appreciated. This has been best studied in several species of hummingbirds (Welch Jr. and Chen, 2014) and to a lesser extent in some bat species. It has been hypothesized that the oxidation of glucose or fructose yields a higher phosphate/oxygen ratio compared to using fatty acids as a fuel and that avoiding the conversion of sugar to fat for use later also represents a substantial energy savings for the animals (Suarez and Welch Jr., 2017).

A second example in which fructose has emerged as an important fuel is in the naked mole rat (*Heterocephalus glaber*). These animals live in communal burrows that

commonly contain a low O_2 and high CO_2 environment and are hypoxic (Park et al., 2017). Naked mole rats can tolerate up to 5 hours of exposure to 5% O_2, while mice experience 100% mortality after 15 minutes of exposure (Park et al., 2017). Naked mole rats can recover from 18 minutes of 0% O_2 exposure, while mice died within 50 seconds of anoxia (Park et al., 2017). The naked mole rat was found to use fructose as a fuel during low oxygen conditions in order to maintain brain function (Park et al., 2017). This occurs by using an increase in fructose transporter expression and increased flux through ketohexokinase in order to by-pass phosphofructokinase and avoid feedback inhibition by ATP, low pH, and downstream products (Park et al., 2017).

Proline

Among amino acids, proline serves many functions in animals, but also in organisms of all kingdoms. Proline catabolism consists of the integration of proline derived carbon in the TCA cycle through a four-step pathway, three being enzymatically catalyzed, leading to α-ketoglutarate (Fig. 2). Among its diverse functions, proline is well known as a compatible solute that can be accumulated when facing environmental osmotic challenges such as water limitation in plants (Verslues and Sharma, 2010) and overwintering insects (Storey et al., 1981; Morgan and Chippendale, 1983). In addition, proline metabolism is associated with fundamental physiological processes such as programmed cell death in animals and plants (Phang et al., 2008, Verslues and Sharma, 2010, Cecchini et al., 2011, Servet et al., 2012). Proline, however, is also known to serve as an energy substrate in organisms including bacteria, protists, plants and animals (reviewed by Servet et al. 2012).

In mammals, proline's role as an energy substrate is associated with acute starvation where protein degradation and transformation by liver cells become significant. In this context, the regulation of proline's contribution to the TCA cycle intermediates has been a topic of interest for some time (e.g. Kawaloff et al., 1977). Since then, proline metabolism in mammals has been associated with not only nutrient stress, but with several other central physiological properties such as cellular redox control, apoptosis, autophagy, and cancer suppression (Phang et al., 2008).

In contrast with vertebrates, some invertebrates exhibit exceptional reliance on proline as energy substrate. Pioneering work on the blood-feeding insect *Glossina*, the tsetse fly, laid out the framework for subsequent studies (Bursell, 1963; Bursell and Slack, 1976; Sacktor, 1975). This species is able to power the high energy demand associated with flight using proline as a single fuel. The use of proline requires its partial oxidation using a shunted TCA cycle that involves alanine aminotransferase and malic enzyme (Fig.2). Such an arrangement is required to take full advantage of proline as a sustainable substrate and avoid nitrogen waste management (Gäde and Auerswald, 2002). Further work on other dipterans and coleopterans showed that many insect species can utilize proline in combination with other substrates, or as an anaplerotic carbon source. The Colorado beetle uses a combination of carbohydrates and proline using the same partial oxidation of proline as described in the tsetse fly (Weeda et al., 1980). Some species can completely oxidize proline in combination with carbohydrate derived substrate by using glutamate dehydrogenase to form α-ketoglutarate instead of alanine aminotransferase (Hansford and Johnson, 1975). Other insect species, however, use proline to a lesser extent by providing intermediates to the TCA cycle and therefore proline has been

referred to as a sparker substrate that enhances the oxidation of acetyl-CoA (Sacktor and Childress, 1967).

This metabolic arrangement of insects is also found in cephalopods. Squid species contain high intracellular proline concentrations, which are depleted during aerobic activity and followed by a stoichiometric increase of alanine (Storey and Storey, 1978). Further characterization of squid metabolic properties support the high rate of proline use during aerobic activity (Fields and Hochachka, 1982, Mommsen and Hochachka, 1981), although this property was further shown to be diverse among squid species (Pörtner et al., 1993). It is proposed that the partial oxidation of proline augments TCA cycle intermediates thereby enhancing the rate of acetyl-CoA oxidation (Hochachka and Fields, 1982; Moyes et al., 1990). The significance of this metabolic adaptation in squids was associated with an increased P/O ratio (Hochachka, 1995).

Several invertebrate groups rely on proline as a major energy substrate, but the ultimate and proximate causes of the observed metabolic diversity remain unresolved. The case of hymenopterans illustrates some of the remaining challenges. Literature on proline metabolism often cites the honeybee as a species oxidizing proline as energy substrate (Phang et al., 2008, Servet et al., 2012), but evidence does not indicate that proline is a substantial fuel in this species (Barker and Lehner 1972; Micheu et al. 2000; Berger et al., 1997; Teulier et al., 2016). Recent literature further contributes to this perception as studies on isolated mitochondria from honeybee flight muscle suggests that proline elicits maximal mitochondrial respiration (Campbell et al., 2016). However, when comparing hymenopteran species including a wasp, a bumblebee, and a honeybee species, striking differences in metabolic properties are found (Teulier et al., 2016). The permeabilized

muscle fibre, a model known to be more physiologically relevant than isolated mitochondria (Kuznetsov et al., 2008), of all three species show similar capacities to oxidize carbohydrate derived substrates, but proline can be oxidized readily by wasp and bumblebee species, and not the closely related honeybee. These observations demonstrate how diverse this phenotype is in a group of related species, highlighting that we still do not grasp how and why this energetic property evolved in animals.

Alternative Entry of Electrons into the Electron Transport System

Studies investigating bioenergetics in living organisms have revealed that there are three ways that electrons can enter an electron transport system: via dehydrogenases or oxidoreductases, via quinones, or via mobile cytochrome carriers. In animal systems, the familiar entry points for electrons into the electron transport system are Complex I (NADH: ubiquinone oxidoreductase) which catalyzes the transfer of electrons from NADH to ubiquinone; and Complex II (succinate dehydrogenase) which catalyzes the oxidation of succinate to fumarate with the reduction of ubiquinone. Additional proteins can serve as direct or indirect electron providers to the ETS, participating in mitochondrial respiration (Lemieux et al., 2017) including: i) electron-transferring-flavoprotein dehydrogenase (ETF DH) which accepts electrons from the acyl-CoA derived from the oxidation of fatty acids and transfers them to ubiquinone; ii) the dihydro-orotate dehydrogenase which transfers electrons from the pyrimidine synthesis to ubiquinone by converting dihydro-orotate to orotate (Fang et al., 2013); iii) the sulfide-ubiquinone oxidoreductase which oxidizes amino-acid derived sulfides with the concurrent transfer of electrons to ubiquinone (Bouillaud and Blachier, 2011); iv) the choline dehydrogenase – betaine-aldehyde dehydrogenase system which converts

choline to betaine, producing mitochondrial NADH, as well as the dimethylglycine dehydrogenase which oxidizes dimethylglycine to sarcosine and transfers the electrons yielded to the ubiquinone (Mailloux et al., 2016); v) the proline dehydrogenase (ProDH); and vi) the mG3PDH. In addition, several alternative NAD(P)H dehydrogenases have been identified in animals (Matus-Ortega et al., 2011).We will focus on and provide examples of two of these electron providers and their importance in the ETS; mG3PDH and ProDH.

Mitochondrial glycerol-3-phosphate dehydrogenase

Despite the fact that the enzymatic reactions happening at the level of the GP shuttle are well-known, there is still a clear gap of knowledge concerning the structure, function, and regulation of this system. This is particularly true for the mG3PDH which is the rate limiting enzyme for the GP shuttle (Mráček et al., 2013), and is involved in other metabolic pathways such as fatty acid metabolism. The mG3PDH is a simple component of the ETS, comprised of a single subunit of 74 kDa (Mráček et al., 2013). It is believed to be encoded by a single slow evolving gene, *GPD2* (also known as *glpD* in bacteria and *GPO-1* in *Drosophila*), as it presents high degree of homology from yeast to mammals, contrasting with the cytoplasmic membrane protein ortholog *GPD1* (Mráček et al., 2013, Yeh et al., 2008, Shen et al., 2006, Shen et al., 2003). In bacteria, this is one of the key flavin-linked primary dehydrogenases of the ETS, constituting as much as 10% of the inner cytoplasmic membrane protein in *E. coli* (Robinson and Weiner, 1980; Schryvers et al., 1978), and serves an essential function for aerobic growth on glycerol (Austin and Larson, 1991; Weissenborn et al., 1992). Based on the solved structure of the cytoplasmic isoform (Yeh et al., 2008) it is proposed that

mG3PDH is associated with the inner mitochondrial membrane as a peripheral protein, similarly to the ETF DH (Mráček et al., 2013; Zhang et al., 2006).

The main function of mG3PDH is likely the re-oxidation of cytosolic NADH produced by the glycolysis (see section above), however, considering its wide distribution in organisms and its high variation in tissue expression levels (Koza et al., 1996; Chowdhury et al., 2005; Taleux et al., 2009), it may serve other metabolic purposes. Additionally to its synthesis from the dihydroxyacetone phosphate formed by the glycolysis, G3P can also be formed via phosphorylation of the glycerol derived from triglyceride catabolism by the glycerol kinase and is also used as a substrate for the synthesis of phospholipids and triglycerides. Its function as a regulator of G3P concentration has been shown to influence lipid synthesis (Bell and Coleman, 1980). The G3P acyltransferase, the first and rate-limiting step of triglyceride synthesis, competes with the mG3PDH for G3P (Bell and Coleman, 1980). Therefore, high activity of mG3PDH will decrease the availability of G3P and hence the rate of triglyceride synthesis, which by extension decreases phospholipid synthesis via diglycerides (Bell and Coleman, 1980; Mráček et al., 2013). High levels of free fatty acids and acyl-CoA esters are known to suppress G3P oxidation via the mG3PDH and regulate GP shuttle activity and the lipid synthesis (Houštěk and Drahota, 1975).

Therefore, mG3PDH is at the crossroads of glycolysis, oxidative phosphorylation, and fatty acid metabolism (Mráček et al., 2013; Orr et al., 2014), and should logically underlie important physiological and metabolic functions. In addition to the recycling of cytosolic NADH, it has been suggested that mG3PDH is involved in thermogenesis, as its activity is the highest in mouse brown adipose tissue (BAT) (Koza et al., 1996; Ohkawa et al.,

1969; Silva, 2006). Mice exposed to cold temperatures displayed higher activity and mRNA levels of mG3PDH in this tissue (Koza et al., 1996). In rodents, mG3PDh is highly expressed in BAT but also in skeletal muscle (Silva, 2006); tissues respectively expressing the uncoupling proteins 1 and 3 (UCP1 and UCP3) essential for the thermogenesis process (Ricquier and Bouillaud, 2000). Moreover, mG3PDH knock out mice have been shown to have reduced in energy turnover (food intake and oxygen consumption), increased thyroidal secretion (both thyroxine and triiodothyronine), BAT atrophy when animals were exposed to 32°C, and increased uncoupling protein 3 (UCP3), suggesting an attempt to compensate for a thermogenic deficiency (DosSantos et al. 2003; Silva, 2006).

mG3PDH has also been shown to be an important site of superoxide production (Miwa and Brand, 2003, 2005; Vrbacký et al., 2007) on both sides of the inner mitochondrial membrane, mainly at the level of the ubiquinone-binding site (Mráček et al., 2014; Orr et al., 2012). Specifically, Orr et al. (2012) elegantly measured the H_2O_2 production rate derived from superoxide of several sites in different tissues and found that mG3PDH was responsible for around 43%, 63%, 23% and 28% of the total H_2O_2 production measured in the brain, the BAT, the heart and the muscle, respectively (Orr et al., 2012). Moreover, this production follows the activity patterns, with the highest production in BAT and the lowest in heart (Orr et al., 2012). Therefore, mG3PDH may contribute to cellular dysfunctions via oxidative damage generated by ROS. Alternatively, it may participate in signaling the cell's energetic status, as it is now accepted that ROS play a fundamental role in mitochondrial quality control (Finkel, 2012; Reczek and Chandel, 2015). It is however not known what could be the relevance and the precise physiological

circumstances of ROS production by mG3PDH (Mráček et al., 2014; Orr et al., 2012; Tretter et al., 2007). Considering that UCPs can control the ROS production by mitochondria (Brand and Esteves, 2005) and the role of mG3PDH and UCPs in thermogenesis, an interesting research avenue would be to investigate the link between these proteins in the context of ROS production during thermogenesis.

As discussed above, several animals are capable of using fuels other than carbohydrates to power flight. Although all cytoplasmic and mitochondrial enzymes necessary for the MAS shuttle exist in insect flight muscle (Yaginuma and Yamashita, 1986), the GP shuttle is considered to be the dominant redox shuttle (Sacktor, 1975; Sacktor and Dick, 1962), and the activity of mG3PDH in this tissue is probably the highest of all the animal kingdom (Kubišta, 1957; Lennie and Birt, 1967; Sacktor and Cochran, 1958; Soares et al., 2015; Zebe and McShan, 1957). This high activity of mG3PDH combined with a very low activity of lactate dehydrogenase represents a specialization in insect flight muscle glycolysis, as neither G3P nor lactate accumulate (Kubišta, 1957; Zebe and McShan, 1957). This allows a tight coupling between glycolysis and mitochondrial respiration which enables a sustained rate of both cytosolic and mitochondrial ATP production for flight, as long as glycogen or trehalose are available. However, not all insects have the capacity to oxidize G3P at a high rate.

The capacity of insects to use G3P as a substrate for mitochondrial respiration has been studied from the late fifties' by the pioneering work of B. Sacktor (Chance and Sacktor, 1958; Estabrook and Sacktor, 1958). Since then, different species of diptera, hymenoptera, othoptera, and coleoptera have been tested, and their mitochondria have been shown to oxidize G3P at very different rates. Notably, Soares et al. (2015) have

443 reported that it could be the major substrate for mitochondrial respiration when compared
444 to mitochondrial NADH-dependent substrates in some insects, while it triggers a minor
445 increase in others. For example, mitochondrial respiration with G3P is 2.7 times higher in
446 mitochondria isolated from housefly (*Musca domestica*) than with NADH-dependent
447 substrates (pyruvate and malate) (Van Den Bergh and Slater, 1962). Moreover, after
448 addition of NADH-dependent substrates (pyruvate, malate), G3P increases the
449 mitochondrial respiration of bumblebees (*Bombus impatiens*) similarly to proline
450 (unpublished results), but when added after proline, it cannot further increase
451 mitochondrial respiration rates (Teulier et al., 2016). In *Drosophila*, G3P increases
452 mitochondrial respiration from 47 to 90%, depending on the strain and the rearing
453 conditions (Pichaud et al., 2013, 2012, 2011). In this same species, it is quite clear that
454 mG3PDH is essential for flight: null mutants drosophila for mG3PDH are unable to fly and
455 exhibit shortened lifespan, and mutants with enzyme deficiency have a dramatic
456 decrease of flight ability (Carmon et al., 2010; Carmon and MacIntyre, 2010). However,
457 such reliance on G3P oxidation by mG3Pdh is not evident in other flying insects, such as
458 coleoptera (*Tenebrio molitor* and *Dermestes maculatus*) (Newell et al., 2016).

459 These differences in the oxidation capacity of the mG3PDH flight muscle between
460 different species of insects highly suggest that this enzyme can be modulated via the
461 *modus vivendi* of the species. Specifically, G3P can be derived both from carbohydrates
462 during glycolysis and from the glycerol backbone of triglycerides and diglycerides, thus
463 representing a node of different metabolic pathways. Considering the ability of insects to
464 cope with different environmental challenges such as seasonal changes in dietary

resources and temperature, it is therefore likely that mG3PDH could have played a key role in the evolution and adaptation of these organisms.

Recently, a study of geographic variation in 127 single nucleotide polymorphism frequencies in natural populations of *Drosophila* showed an increasing expression of both the cG3PDH and the mG3PDH genes with latitude (Lavington et al., 2014). This increase was suggested to be linked to the availability of nutrients along a latitudinal tropical-temperate gradient that varies seasonally and locally (Lavington et al., 2014). Considering the role of G3P in glycolysis, fatty acid metabolism, and mitochondrial respiration, diet should have the potential to influence the mG3PDH capacity. As a matter of fact, recent work in the laboratory of N. Pichaud showed in *Drosophila melanogaster* that G3P oxidation capacities were significantly increased after an exposure of four days to either a high sucrose or a high fat diet (31% and 40% increase in mitochondrial oxygen consumption with G3P, respectively, unpublished results).

Considering the involvement of G3P in glycerol metabolism and potentially in thermogenesis, it is tempting to suggest that temperature is also a modulator of mG3PDH. In some cold-hardening insects, glycerol is a major cryoprotective substance enabling overwintering (Teets and Denlinger, 2013). This increased glycerol accumulation has been shown to be correlated to mitochondrial degradation and reduced oxidative metabolism (Kukal et al., 1989). It has been observed that cessation of glycerol synthesis in the overwintering gall fly larva (*Eurosta solidaginis*) coincided with accumulation of G3P (Storey et al., 1981), suggesting the involvement of a suppressed mG3PDH activity. Moreover, in the same larva, increased activity of the GP shuttle upon warming in the

spring has been suggested to be the key to removal and oxidation of reducing equivalents generated from cryoprotectant catabolism (Joanisse and Storey, 1994).

In light of these results, the evaluation of G3P dependent mitochondrial respiration in insects either facing fluctuations in dietary resources or able to cope with the cold temperatures of winter through different strategies may bring new understanding of the different metabolic roles of the mG3PDH. The retention of this enzyme through evolution highly suggests its importance as a metabolic cornerstone, and its study in different organisms under several environmental and pathophysiological conditions is sure to provide significant insights on the fundamentals of metabolism, as well as on species' adaptations.

Proline dehydrogenase

ProDH is a flavoenzyme best characterized in bacteria and shown to use FAD cofactor as an electron acceptor in bacteria and eukaryotes (Adams and Frank, 1980; White et al., 2007; Paes et al. 2013), which is the current model thought to apply in plants and humans (Verslues and Sharma, 2010; Natarajan and Becker, 2012). Many current efforts are dedicated to the characterization of ProDH O_2 reactivity (White et al., 2007; Natarajan and Becker, 2012; Goncalves et al., 2014), its kinetics properties and cofactor specificity (Huijbers et al., 2017), its relationship in the regulation of the ETS (Hancock et al., 2016), and its regulation (Schertl et al., 2014), in diverse organisms.

Animal physiology literature emphasizes how proline mainly enhances acetyl-CoA oxidation and contributes as a major NADH linked substrate, but the actual contribution of the flavoenzyme ProDH as an electron donor to the ETS is seldom mentioned. Work

conducted on bacteria (Abrahamson et al., 1983; Moxley et al., 2011), yeast (Wanduragala et al., 2010), and plants (Elthon and Stewart, 1982; Rasmusson et al., 2008; Schertl et al., 2014), indicates that ProDH may donate electrons directly to ubiquinone, in addition to generating succinate and NADH in the downstream reactions leading to α-ketoglutarate and the partial or complete TCA cycle (Fig. 2). Studies such as Teulier et al. (2016) indicate that much of the proline induced mitochondrial respiration is independent of complexes I and II of the ETS, suggesting that ProDH is an important electron source for the ETS of certain insect species and many studies refer to such a mechanism (Goncalves et al., 2014; Soares et al., 2015). This hypothesis is supported by recent work conducted on mouse liver mitochondria and cancer cell lines (Hancock et al., 2016). ProDH utilizes ubiquinone as electron acceptor and it supports respiration even though the activity of complexes I and II were inhibited. These findings are not only relevant to the role of ProDH and its interaction with the ETS in the context of tumorigenesis (Hancock et al., 2016), but also reinvigorate our view of proline metabolism diversity in animals.

Role of ProDH and mG3PDH in pathologies

Mitochondrial dysfunctions are at the center of many diseases and aging, and evaluation of each ETS contributor is of paramount importance to have a cohesive picture of the mitochondrial physiology. Both ProDH and mG3PDH are key players contributing to mitochondrial metabolism. In this context, their characterization during mitochondrial dysfunctions would be the first step to determine their involvement in the expression, development, and underlying mechanisms of several diseases.

As mentioned above, it is now clear that proline metabolism is involved in several cancers (Phang et al., 2015). It has been shown that ProDH is one of the genes induced by p53 expression, which partly controls programmed cell death and the cell cycle (Shangary et al., 2008; Vousden and Lu, 2002). Proline metabolism and ProDH are therefore viewed as important stress modulated molecules capable of mediating reactive oxygen species production and initiating apoptosis (Phang et al., 2008). Studies have shown that ProDH overexpression reduces tumour formation and is thus directly associated with p53-induced tumour suppression (Liu et al., 2009). Yet, some cancer cells thrive using proline as energy substrate (Pandhare et al., 2009; Liu et al., 2012). Given the central role of proline in regulating cell death and its apparent central role in the metabolic phenotype of cancer cells, mechanisms linking proline oxidation and ROS production have been recently investigated in mammalian cancer cell lines (Gonvalves et al., 2014). The metabolism of proline is now known to not only serve as a source of energy during stress, but also provides signaling ROS for epigenetic reprogramming and regulates redox homeostasis (Phang et al., 2015). Moreover, it has recently been demonstrated that the effect of the regulatory protein PARK7 in Parkinson's disease is mainly due to the modulation of proline metabolism, causing redox dysregulation (Yasuda et al., 2013; Phang et al., 2015).

Differential expression of mG3PDH has also been linked to several pathologies in mammals, including neurological conditions (Tretter et al., 2007), metabolic diseases (Eto et al., 1999; Saheki et al., 2011; Taleux et al., 2009) and cancers (Singh, 2014). The mG3PDH has been suggested to be involved in insulin signaling, as mG3Pdh has a relatively high activity in pancreatic β-cells. In several models of diabetes such as db/db

554 mice and streptozotocin injected neonatal rats, the mG3PDH activity is decreased,
555 suggesting an important role for glucose-induced insulin release (Giroix et al., 1991;
556 Sener et al., 1993). Moreover, in Lou/C rats (an inbred strain derived from Wistar rats), a
557 pronounced resistance to aging and diet-induced obesity has been related to an
558 increased mG3PDH expression (Taleux et al., 2009). This increase was linked to elevated
559 mitochondrial respiration, enhanced fatty acid oxidation, as well as with increased
560 cytosolic ATP/ADP and NAD^+/NADH ratios (Taleux et al., 2009). In prostate cancer cell
561 lines, mG3PDH displays high activity and mRNA levels (Chowdhury et al., 2005). This
562 increased activity is thought to be partly responsible for the higher ROS production and
563 innate oxidative stress observed in prostate cancer. Therefore, it has recently been
564 suggested that inhibitors of mG3PDH activity can elicit anti-proliferative effects on cancer
565 cells (Orr et al., 2014; Singh, 2014).

566 Implications of mitochondrial diversity in animals

567 Mitochondrial physiology experiments in non-model animal species require careful
568 planning to encompass the diverse possibilities in mitochondrial metabolism. The
569 apparently simple case of proline metabolism can help illustrate some of the challenges.
570 Oxidation of pyruvate at its full potential requires the assistance of sparker substrates,
571 which is thought to be a core function of proline in many species of insects or cephalopods
572 (Saktor and Childress, 1967; Hochachka and Fields, 1982, Moyes et al., 1990). Simple
573 measurements where rates of proline and pyruvate oxidation are greater than rates
574 obtained from pyruvate alone does not indicate how proline contributes to the ETS.
575 Proline can serve as sparking substrate to the same extent as malate (e.g. Syromyatnikov
576 et al., 2013), therefore conclusions that proline is a substrate providing electrons to the

ETS cannot be reached (see discussion in Teulier et al., 2016). Comparison of studies conducted on mosquitoes and bumblebees highlight that the nature of proline oxidation can be scrutinized by further breakdown of metabolic properties using mitochondrial inhibitors. In *A. aegypti*, complex I inhibition using rotenone following stimulation of respiration using pyruvate and proline results in approximately 10% of ProDH contribution to ETS that is complex I independent (Soares et al. 2015), while in *B. impatiens*, 50% of ProDH contribution is complex I independent (Teulier et al., 2016). How ProDH contributes to electron flow of the ETS in species using proline as central fuel, co-substrate, or simply as a sparker metabolite is likely diverse across species. Now that we recognize this possibility, mitochondrial physiology experiments in animals should incorporate a broader view of the ETS as exemplified by proline metabolism. Studies reporting proline supported respiration in other insect species often lack the necessary information to assess the contribution of proline and other substrates to the diverse entry points of the ETS (e.g. Campbell et al., 2016; Newell et al., 2016).

Not only should the range of possible substrates be considered by comparative mitochondrial physiologists, but also the various means by which these substrates can contribute to the ETS are of crucial importance. A better understanding of evolutionary mitochondrial physiology in animals will occur from an appreciation of the broader metabolic possibilities found in microorganisms, plants, and cancer cells. One size doesn't fit all; assumptions about energy metabolism are dangerous and researchers should attempt to identify the enzymes present in their study organisms using bioinformatics, molecular biology, and/or careful biochemistry and respirometry. We are

hopeful that the emergence of new technologies and techniques will help to understand the implication and the role of these enzymes in mitochondrial metabolism.

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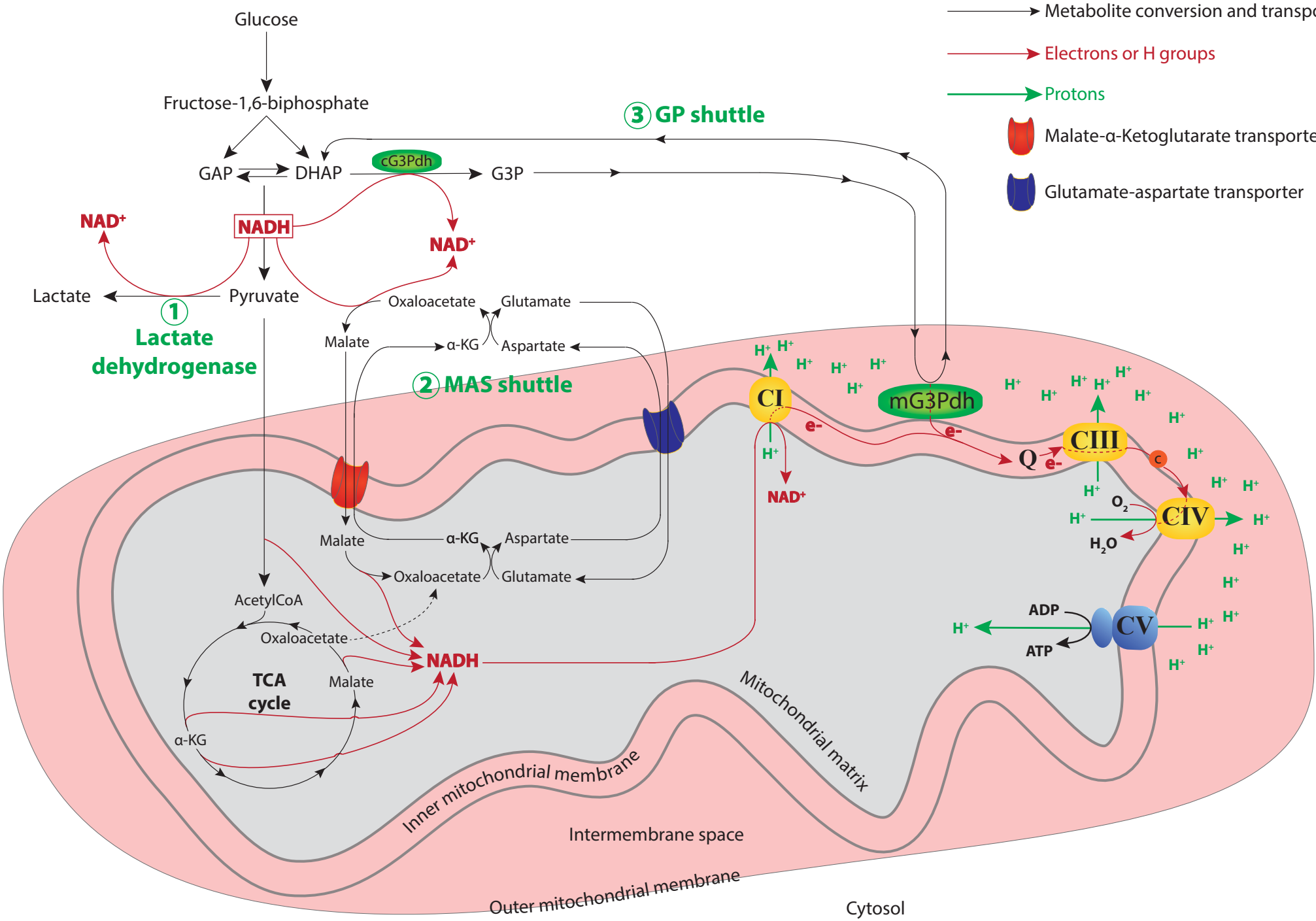
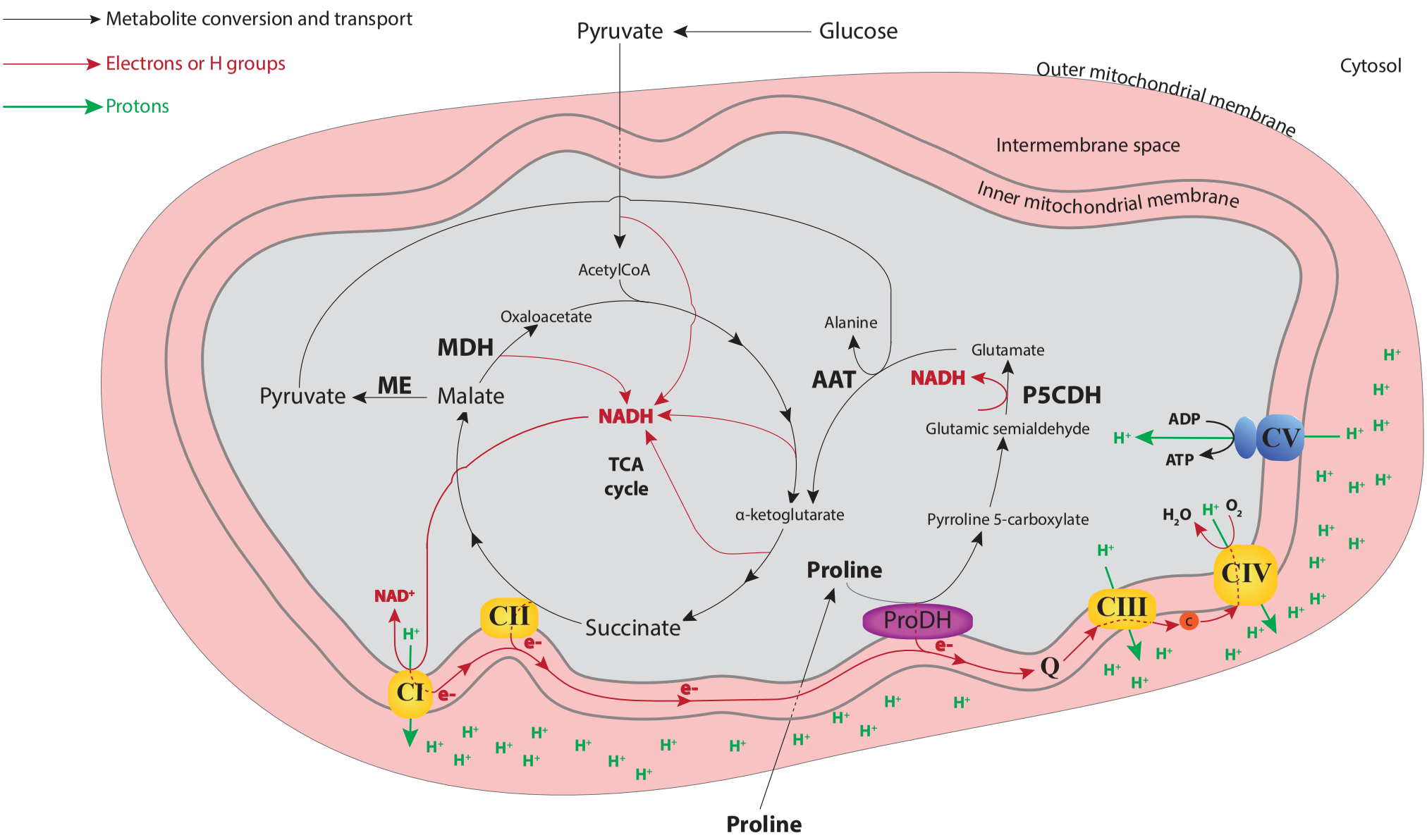
Legends

Figure 1. The cytosolic NADH recycling systems. During glycolysis, glucose is converted to pyruvate through different steps, producing cytosolic NADH at the level of the glyceraldehyde-3-phosphate dehydrogenase. The NADH has to be recycled to NAD^+ mainly to not limit glycolysis, via different systems: 1) The lactate dehydrogenase which converts pyruvate to lactate; 2) the MAS shuttle which is comprised of cytosolic and mitochondrial malate dehydrogenases and aspartate aminotransferases, as well as of malate-α-ketoglutarate and glutamate-aspartate transporters. The cytosolic NADH is oxidized to NAD^+ by the cytosolic malate dehydrogenase which converts cytosolic oxaloacetate to malate. After being transported inside the mitochondrion, the malate is converted back to oxaloacetate, allowing the regeneration of NADH. This NADH can then be used by complex I to generate an electron flux and participate to mitochondrial respiration; 3) the GP shuttle is composed of the cG3Pdh and the mG3Pdh. The dihydroxyacetone phosphate generated by glycolysis can be converted to G3P by the cG3Pdh with concomitant oxidation of the cytosolic NADH. G3P is then transported to the mG3Pdh associated to the inner mitochondrial membrane, where it is converted back to dihydroxyacetone phosphate. This conversion allows the transfer of electrons inside the inner mitochondrial membrane to the ubiquinone pool, which increases the electron flux and participates in the mitochondrial respiration. α-KG: α-ketoglutarate; AcetylCoA: acetylcoenzyme A; c: cytochrome c; CI: complex I; CIII: complex III; CIV: complex IV; CV: complex V-ATPsynthase; cG3Pdh: cytosolic glycerol-3-phosphate dehydrogenase; DHAP: dihydroxyacetone phosphate; e^-: electrons; GP shuttle: glycerophosphate shuttle; GAP: glyceraldehyde-3-phosphate; G3P: glycerol-3-phosphate; H^+: protons; MAS shuttle: malate-aspartate shuttle; Q: ubiquinone pool; TCA: tricarboxylic acid.

Figure 2. Proline as a metabolic fuel. Proline is used as metabolic fuel source in many animals. Its catabolism is initiated by proline dehydrogenase (ProDH) that can directly provide electrons to the electron transport system (ETS) at the ubiquinone (Q) pool. Species using proline as the main metabolic fuel, or in combination with carbohydrate derived substrates, partially oxidize it using the enzymes pyrroline-5-carboxylate dehydrogenase (P5CDH) and alanine aminotransferase (AAT) to produce the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate, thereby further producing reducing equivalents for complexes I and II of the ETS. Malic enzyme (ME) is required to regenerate substrate for AAT by the partial oxidation of proline. Species can also use proline as a minor fuel source and anaplerotic reaction by completely oxidizing proline using the enzyme glutamate dehydrogenase instead of AAT.


Metabolite conversion and transport
Electrons or H groups
Protons
Malate-α-Ketoglutarate transporter
Glutamate-aspartate transporter
Glucose
Fructose-1,6-biphosphate
GAP
DHAP
cG3Pdh
G3P
3 GP shuttle
NAD+
NADH
NAD+
Lactate
Pyruvate
1
Lactate dehydrogenase
Oxaloacetate
Glutamate
Malate
α-KG
Aspartate
2 MAS shuttle
CI
mG3Pdh
CIII
CIV
CV
Q
e-
c
NAD+
O2
H2O
ADP
ATP
Malate
α-KG
Aspartate
Oxaloacetate
Glutamate
AcetylCoA
Oxaloacetate
TCA cycle
Malate
NADH
α-KG
Mitochondrial matrix
Inner mitochondrial membrane
Intermembrane space
Outer mitochondrial membrane
Cytosol


Metabolite conversion and transport
Electrons or H groups
Protons
Pyruvate
Glucose
Cytosol
Outer mitochondrial membrane
Intermembrane space
Inner mitochondrial membrane
AcetylCoA
Oxaloacetate
MDH
Malate
ME
Pyruvate
NADH
TCA cycle
α-ketoglutarate
AAT
Alanine
Glutamate
NADH
P5CDH
Glutamic semialdehyde
Pyrroline 5-carboxylate
Proline
ProDH
Succinate
NAD+
CI
CII
CIII
CIV
CV
Q
c
e-
ADP
ATP
H2O
O2
H+
Proline